FUNCTIONAL TRAITS AS INDICATORS OF PAST ENVIRONMENTAL CHANGES

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FUNCTIONAL TRAITS AS INDICATORS OF PAST ENVIRONMENTAL CHANGES

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EDITORIAL
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Editorial: Functional Traits as Indicators of Past Environmental Changes

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Editorial on the Research Topic

Functional Traits as Indicators of Past Environmental Changes

Paleoecology uses the biological remains in lake and bog sediments to reconstruct past environmental changes and to provide a valuable historical perspective on climatic and ecosystem changes occurring in the present day. Most paleoecological reconstructions are based on the analysis of the relative abundance of sub-fossils of plant and animal remains (e.g., testate amoebae, diatoms, fossil pollen), which are regarded as a proxy for past environmental conditions. Calibration data sets can be used to infer quantitative reconstructions of past environmental variables such as water-table depth, pH, and temperature. One emerging sub-discipline in paleoecology aims to reconstruct past functional diversity patterns of plants (e.g., using pollen and plant macro-remains) and other organisms (e.g., testate amoebae, diatoms, and chironomids) through a focus on functional traits. Such an approach has been argued to offer an alternative perspective from paleoecological archives, especially for understanding how past climate changes and human impacts influenced species' functional diversity and then ecosystem functions over long timescales.

The papers in this Research Topic are based around the theme of using functional traits for better understanding past environmental changes from sediments. Across the fossil record, species may appear and disappear through environmental filtering, but certain traits might remain regardless of which species carries the trait (Lamentowicz et al., 2019). Thus, if the aim is to understand how current climate changes influence species and their functions over long-time scales, focusing on functional traits is of paramount importance for gaining insight. Such an approach will allow us to (1) build hypotheses for past and/or future patterns and processers based on different traits and (2) use the fossil record to test the strengths and weaknesses of different modeling approaches in predicting biodiversity patterns in response to current and future climate changes. The papers in this Research Topic demonstrate the potential breadth and scope of functional paleoecology, with contributions related to ecosystem ecology, forest management, peatland conservation, paleoclimatology, paleolimnology, and biogeography.

THE USE OF PLANT TRAITS IN UNDERSTANDING PAST VEGETATION AND ECOSYSTEM PROCESSES

Several papers published in this Research Topic evaluate the use and potential of plant traits in paleoecological studies using pollen-based proxies. For example, Blaus et al. investigate modern plant-pollen relationships from moss pollsters sampled in Estonian fens. They investigate the correlations between several modern pollen and plant diversity estimates, including taxonomic

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richness, functional diversity (FD), phylogenetic diversity (PD), in addition to ecological attributes such as Ellenberg indicator values (EIVs). They find that the modern relationships between pollen and plant diversity indicators are both variable and scale-dependent, and that separation of herbaceous and woody plant taxa is a useful approach if such relationships are to be applied to reconstructions. After applying their metrics to a sediment core spanning the past 9,000 years, they argue that an integration of functional diversity, phylogenetic diversity and Ellenberg Indicator Values provide new opportunities for studying the long-term patterns of community assembly which are not usually observed in traditional pollen diagrams.

Similarly, Brussel and Brewer used The Neotoma Paleoecology Database to link surface pollen samples to three functional traits (leaf area, plant height and seed mass) that reflect plant ecological strategies from the BIEN database (Enquist et al., 2016). Further, they show that continental functional diversity patterns inferred from modern pollen datasets generally match those patterns inferred from tree assemblages (Lamanna et al., 2014). Their results suggest that, despite challenges in taxonomic precision, pollen percentages can be used to infer past patterns of functional diversity change in North American Quaternary pollen sequences. Such pollen-plant trait relationships might then be used to provide explanations of evolutionary, ecological, and biodiversity dynamics that can contribute to sustainable conservations and management.

The studies above demonstrate a range of perspectives that can be introduced when paleoecological records can be viewed through the lens of plant traits. However, the review provided by Birks in this special issue introduces some important caveats. Firstly, Birks highlights the differences between ecological attributes (or ecological characteristics) and plant traits. Whilst an ecological attribute is a general feature of a taxon describing its overall ecology, a plant trait is a measurable property of an organism measured individually and which is comparable across species. But whilst a plant trait might be considered intrinsic to its genome, an attribute may not be considered a plant trait since it might be dependent on other factors (e.g., the geographic distribution of a species is dependent on historical chance events) (Birks). In his paper, Birks argues that traits and ecological attribute analyses have played a vital role for reconstructing ecological and environmental changes for the last 120 years in paleoecological studies, and that such analyses are complementary to current interest in the use of plant traits in macroecology (McGill et al., 2006). However, he suggests that the challenges related to taxonomic precision in Quaternary assemblages might limit the development of a "new" Quaternary botany based solely on plant traits.

As an alternative to estimating functional ecological changes based on a reinterpretation of palynological data, Loisel and Bunsen explored general ecosystem functional properties related to peatland trophic state. Through the analysis of 90 peat profiles from southernmost South America, they demonstrated that a synchronous, regional scale transition from fen to bog occurred 4,200 yr BP, with allogenic drivers most likely being responsible for this change. This functional critical shift appeared to be related to peatland-carbon accumulation that was much greater and stable for bogs than for a fenland ecological state. The authors built conceptual models for non-linear fen-bog transition scenarios that allow for improved understanding of the dynamics of the terrestrial carbon sink in South America.

MICROBIAL TRAITS AS INDICATORS OF PAST ENVIRONMENTAL CONDITIONS

Protist functional traits have been used increasingly to reconstruct past environmental conditions and ecosystem functions. Many studies from the Research Topic focused on testate amoebae, which have been the focus of paleoecologists for several years. Marcisz et al. provide a review of this topic, showing the increasing potential of testate amoebae morphological traits in the exploration of past ecosystems. They describe and comment on the most common testate amoebae morphological traits when studied in the context of environmental gradients in lakes and peatlands, with examples from *Sphagnum* peatlands, fens, tropical peatlands and lacustrine habitats. The paper provides the first of its kind look at the current knowledge about testate amoebae traits as proxies of past changes in the environment e.g., climate change, deforestation, hydrological dynamics, eutrophication, dust deposition, carbon accumulation, and food web transformation. It also underlines the importance of testate amoebae as a robust proxy with well-identified functional trait potential and stresses the need for a better understanding of testate amoebae trait-environment relationships.

Given the need for a better understanding of trait-environment relationships, Macumber et al. addressed questions related to testate amoebae morphological traits variability in lakes using unprecedented geometric morphometric analyses. They explored test size and shape along a temporal gradient of eutrophication in Loch Leven, Scotland (United Kingdom) and found that decreasing test size might be related to disturbance connected with eutrophication. This contribution shows a strong agreement in the stratigraphic comparison between testate amoebae morphological traits, testate amoebae taxonomic composition and plant macrofossils in the lake sediment core. It further shows that using only the shape and test size of testate amoebae provides complimentary summaries of ecological function not captured by taxonomy. They finally suggest that such approaches might be further developed in paleoecological studies on lake ecosystems.

In another contribution, Krashevska et al. studied testate amoebae taxonomy and trait-based transfer functions. They used surface samples from the tropical peatland in Sumatra to build a data set that will be used for quantitative paleoenvironmental reconstructions. The best performance of the model with the depth to the water table (describing peatland hydrology) was obtained with five morphological traits: shell width, aperture shape, aperture invagination, shell shape, and shell compression. This work brings a novel data set from an undersampled area of the world with the potential to improve understanding of the paleohydrology of SW Asian tropical peatlands.

Comparison of four testate amoeba functional traits (mixotrophy, biovolume, aperture size, and aperture position) to C accumulation, hydrological, and vegetation changes in 12 peat profiles in Northwestern Québec, Canada is provided by Zhang et al. This study is a rare example of the study exploring multiple peat sections to compare testate amoebae functional traits with vegetation changes and carbon accumulation rates. Results show a different sensitivity of various traits to environmental changes. The authors of this paper also try to predict future peatland ecological functions and adaptation of testate amoebae communities with the different trait types. Furthermore, the contribution of mixotrophic testate amoebae as important drivers of peatland C assimilation is discussed in the perspective of the future drier peatlands.

Besides testate amoebae, two other contributions focused on microbes. Kearns et al. showed the potential of the individual trait-based analysis to explore past changes in the ocean. They explored the potential of planktonic foraminifera in the genus *Subbotina* by morphological measurements of the tests to understand trait changes through the Middle Eocene Climatic Optimum [MECO: ∼40 Myr ago (mega annum, Ma)]. They investigated functional trait changes through climatic perturbations along with the MECO. However, no relationship was found between measured traits in terms of size also concerning stable isotopes of oxygen and carbon. The authors suggest that the study could be extended to other genera, to explore changes in the deep using novel imaging techniques.

The final contribution in our Research Topic explores morphological traits of Chironomidae, another organism commonly used as a quantitative proxy in paleoecology. Remains of chironomid larvae found in lake sediments are used mainly for air temperature reconstructions. In this study, the authors identified several features in the mouthparts of larvae that might be promising indicators of the habitat changes including physicochemical conditions and vegetation. This assumption was tested using the sediment cores that were taken from a sequence that originates from a Late Medieval moat, and from a second core which covers the Late Weichselian paleochannel history. The study provides an alternative perspective on Chironomida paleoecology, suggesting that Chironomida traits provide complementary information about the local changes in the sedimentary environment.

CONCLUSIONS AND FUTURE DIRECTIONS

The case studies and syntheses published in this Research Topic provide additional insights into the application of functional traits in paleoecological research. Whilst a taxonomic perspective has been used as the standard in paleoecology and remains as the basis of the quantitative paleoenvironmental reconstructions focused on past climate or human impact, the papers in this special issue demonstrate that the integration of trait-based approaches may lead to reconstructions of ecosystem functions that are often not possible to be explored in a taxonomic context. However, an important distinction should be made between those studies which rely on indirect estimation of traits through translation tables (e.g., Brussel and Brewer), and those studies which allow for direct measurements of phenotypic features that may be related to ecosystem functions (e.g., Macumber et al.). If morphological traits can be directly linked to the functional performance under different environmental conditions, it is possible that this may lead to more refined proxy reconstructions of different environmental parameters across different terrestrial, freshwater, and oceanic habitats. Thus, a priority for future studies, particularly in relation to morphological analyses of microorganisms is to establish better links between organismal traits and ecosystem functions.

It has also been stressed in several papers in this special issue that functional traits analysis cannot be applied alone, and it should be complementary to standard paleoecological work. Nevertheless, the papers in this Research Topic demonstrate that techniques related to functional paleoecological research are likely to continue to play an increasingly important role in contributing to our understanding of the environmental changes that have occurred when interpreting sediment records. Such an approach can provide novel proxies of the past human-related disturbances like deforestation, pollution, in addition to hydrological disturbance related to increasing drainage. Such information is also useful for studies into ecosystem management and for assessing results of restoration efforts.

AUTHOR CONTRIBUTIONS

VJ, ML, and AS proposed the Research Topic. ML wrote the first draft. All co-authors edited the text and provided the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Reflections on the Use of Ecological Attributes and Traits in Quaternary Botany

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There has been an upsurge of interest and research activity in trait-based approaches in ecology, biogeography, and macroecology. I discuss if this upsurge has impacted Quaternary botany (the study of plant remains preserved in sediments). I show that ecological attributes (including traits) have played and continue to play an integral part in the interpretation of Quaternary botanical data in terms of reconstructing past environments and interpreting long-term changes in plant assemblages. This use started over 120 years ago and continues to the present. It is unclear if a "new" Quaternary botany based on traits will develop because of the taxonomic limitations of much Quaternary botanical data.

Keywords: climate, ecological indicator values, environmental reconstruction, functional diversity, plant functional types, plant macrofossils, pollen analysis

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INTRODUCTION

In modern plant ecology there has been a great upsurge of interest and research effort in trait-based ecology in the last 20 years (e.g., Shipley, 2010; Garnier et al., 2016) since the pioneering efforts of ecologists in the early 20th century (e.g., Raunkiaer, 1907; Blackman, 1920; Raunkiaer, 1934; Iversen, 1936) and late 20th century (e.g., Grime, 1979; Bloom et al., 1985; Jurado et al., 1991; Leishman and Westoby, 1992; Grime, 2001; Westoby et al., 2002). The trait-based approach extends back at least to 300 BCE (Theophrastus, 1916; Weiher et al., 1999). This recent upsurge in trait-based ecology proposes "Rebuilding community ecology from functional traits" (McGill et al., 2006), "... using plant functional traits to understand ecological processes" (Funk et al., 2017), "predicting communities from functional traits" (Cadotte et al., 2015), and "using functional traits to predict ecosystem change" (Suding and Goldstein, 2008). Using functional traits to generalize complex community assembly and dynamics and to predict effects of environmental change on ecosystems is referred to as a "Holy Grail" in ecology (e.g., Lavorel and Garnier, 2002; Suding and Goldstein, 2008; Funk et al., 2017). Recent publications have spectacular titles such as "Predictive traits to the rescue" (Guisan, 2014), "Trait-based assessment of climate-change impacts on interacting species" (Schleuning et al., 2020), "Functional traits explain the Hutchinsonian niches of plant species" (Treurnicht et al., 2020), and "Elevation gradient of successful plant traits for colonizing alpine summits under climate change" (Matteodo et al., 2013). Trait-based approaches have also been proposed to create a "functional biogeography" that "bridges species-based biogeography and earth science to provide ideas and tools to explain gradients in multifaceted diversity (including species, functional, and phylogenetic diversities), predict ecosystem functioning and services worldwide, and infuse regional and global conservation programs with a functional basis" (Violle et al., 2014, p. 13690).

Given the considerable interest and activities in trait-based approaches in contemporary plant-community ecology, ecosystem ecology (Díaz and Cabido, 2001), biogeography (Violle et al., 2014), and macroecology (He et al., 2019), are these current interests and activities impacting Quaternary botany? In this article, I discuss the use of ecological attributes and traits in Quaternary botany over the last 120 years and show that such use has been and remains an integral part of the subject. I first present some essential definitions. I then discuss the use of ecological attributes and then review the use of ecological traits in Quaternary botany. I conclude by discussing if a trait-based approach can contribute further to Quaternary botany and outline what the major limitations of developing this approach further are in Quaternary botany.

DEFINITIONS

Quaternary (last 2.6 million years) botany involves the study of plant megafossils (e.g., tree stumps, logs, and wood remains), plant macrofossils (e.g., seeds, fruits, leaves, and bryophyte remains), and plant microfossils (e.g., pollen, spores, phytoliths, charcoal, and non-pollen palynomorphs) preserved in peat and in lake and ocean sediments. Although megafossils and macrofossils have been studied since the late 18th century, Quaternary botany today is dominated by pollen analysis (Birks, 2019).

Ecological attributes (=ecological characteristics) are features of a taxon such as its range size, life history, geographical distribution, climatic tolerances, habitat types, environmental indicator values, edaphic preferences, and so on, that describe the overall ecology of the taxon (Hill et al., 2004; Hill et al., 2007).

Ecological traits have been defined in several ways (e.g., Semenova and Van Der Maarel, 2000; Violle et al., 2007; Shipley, 2010; Garnier et al., 2016; Körner, 2018). A definition that is widely adopted is that a trait is "a well-defined, measurable property of organisms, usually measured at the individual level and used comparatively across species. A functional trait is one that strongly influences organismal performance" (McGill et al., 2006, p. 178). There are other types of traits used in ecology such as vital attributes and ecophysiological, stress tolerance, life-history, demography, biology, response, performance, effect, neutral, and *a posteriori* traits (Violle et al., 2007; Garnier et al., 2016; Körner, 2018). Traits are always attributes, but many attributes such as geographical distribution are not traits because they may depend on historical accidents. A trait is thus an attribute that can, in theory, be observed in an individual plant and is intrinsic to its genome, whereas an attribute is a property of a taxon considered as a whole (Hill et al., 2019, pers. comm.). Deciding whether a trait is 'functional' in the sense of being "important for performance" is not always clear (see Weiher et al., 1999; Diaz et al., 2004; Bernhardt-Römermann et al., 2008; Shipley et al., 2016). Körner (2018) noted that "it appears that often certain traits are thought to be important because they have been measured because tools have been available. There are many traits that are potentially of great value; however, they have not been accounted for because their study is very laborious, or they have not yet been thought of." Here I use the general term "trait" except when the type of trait being discussed is clear.

This review only considers Quaternary botany. Testate amoebae are not considered even though they are often studied in conjunction with botanical studies on peat sequences (e.g., Fournier et al., 2015; Lamentowicz et al., 2015; Marcisz et al., 2016; Marcisz et al., 2020).

ECOLOGICAL ATTRIBUTES

The first use of ecological attributes, in this case a species distribution and its inferred climatic limits and tolerances, to reconstruct past climate change is probably the classic work by Andersson (1902, 1903, 1909). He mapped the occurrence of fossil *Corylus avellana* (hazel) nuts in Swedish and Norwegian peats. He showed that the present-day northern limit of hazel coincided closely with the mean July temperature isotherm of 12°C but fossil nuts occurred as far north as today's July isotherm of 9.5°C (see Birks, 2008). Andersson proposed that mean July temperature in the early- and mid-Holocene was 2–2.5°C warmer than today. Following this, Samuelsson (1916) analyzed hazel's present-day northern limit in greater detail. He showed that summer temperature was not uniform along today's limit and that a lower summer temperature was compensated for by a longer growing season (Birks, 2008). He modeled the climatic demands of hazel in terms of both summer temperature and growing-season duration and proposed that both summer and winter temperature, and hence growing-season length, had changed during the Holocene (Birks, 2008). He emphasized that the occurrence of fossil hazel nuts does not give a direct single paleoclimatic estimate but a range of possible values, each corresponding to a given growing-season duration (see also Faegri, 1950; Birks, 2008). These pioneering studies led to the unique study by Iversen (1944) on the climatic tolerances of *Viscum album, Hedera helix,* and *Ilex aquifolium* in Denmark.

On the basis of detailed field observations over several years, Iversen (1944) delimited the "thermal limits" or "bioclimatic envelopes" within which these three shrubs flowered and produced seed [the Grinnellian (Grinnell, 1917) and regeneration niches (Grubb, 1977)] within Denmark. Iversen (1944) showed that *Ilex* is intolerant of cold winters but tolerant of cold summers. *Hedera* is intolerant of winters with mean temperatures less than −1.5°C but requires warmer summers than *Ilex*. *Viscum* is tolerant of cold winters but requires warmer summers than either *Ilex* or *Hedera*. These taxa are ideal "indicator species" because their pollen is readily identifiable to species level and is not blown great distances so "false positives" are unlikely, and their fruits are rapidly dispersed by birds (Iversen, 1944). Their distributions, at least at the scale of Denmark, are likely to be in equilibrium with climate (Birks et al., 2010). The validity of this critical assumption is elegantly demonstrated by Walther et al. (2005) who showed that the northern limit of *Ilex* has shifted northwards in Scandinavia in the last 50 years, presumably in response to milder winters in recent decades

(Birks, 2008). From fossil pollen occurrences, Iversen (1944) applied his modern climate envelopes (attributes) to infer that mid-Holocene summers were 2–3°C warmer and winters were 1–2°C warmer than today in Denmark.

The general "bioclimatic envelope" approach of Andersson, Samuelsson, and Iversen has been extended to consider the climatic limits based on the current distributions of several taxa simultaneously to identify areas of climatic overlap for pollen, plant macrofossils, and other organisms, the so-called Mutual Climate Range (MCR) approach (Atkinson et al., 1986; Birks et al., 2010). Attempts have been made to narrow the climate range inferred by MCR (e.g., Marra et al., 2004; Marra et al., 2006) with varying success (see Birks et al., 2010 for details).

Kühl et al. (2002) and Kühl (2003) combined the various approaches outlined above to develop a rigorous means of inferring past climate based on presence/absence data (e.g., plant macrofossils) involving probability density functions (pdfs). It combines modern distributions for individual taxa with gridded modern climate data and summarizes the modern climate limits of the taxa as conditional pdfs (climate| taxon present) rather than as ranges or thresholds (Birks et al., 2010). A normal distribution is used to estimate the pdf. By assuming statistical independence, a joint pdf is calculated for a fossil assemblage as a product of the pdfs of the individual taxa in the assemblage. Each taxon is weighted by the dispersion of the modern climate range, so "narrow" indicators carry "high" weight. The pdf product is the most likely past climate and its mean and confidence intervals are inferred to be the most probable climate and its uncertainty (Birks et al., 2010). The reconstructed climate is based on the complete distribution and climate data of all taxa considered and because it is a multiplicative technique, no single taxon is decisive (Kühl and Litt, 2003). The pdf approach has been used in infer Holocene and last interglacial climate from pollen assemblages (expressed as presence/absence; e.g., Kühl, 2003; Kühl and Litt, 2003; Litt et al., 2009). The approach has been extended to become a Bayesian indicator taxon model (Neumann et al., 2006). This development combines the conditional pdfs with the prior probability density of climate to estimate Bayesian posterior pdfs. Two-dimensional normal distributions are used as parametric functions to describe the conditional pdfs of mean July and January temperature, thereby allowing the influence of these two climatic variables to be considered together and hence for both to be inferred simultaneously (Kühl and Gobet, 2010). Recent developments in the pdf approach (Chevalier et al., 2014; Chevalier and Chase, 2015; Chevalier, 2019) have extended it to use pollen percentages and to consider the dimensionality and modality of the fitted pdfs.

This correlative approach of comparing modern distributions with contemporary climatic variables has proliferated in biogeography and global-change ecology in the last 20 years (e.g., Franklin, 2010). It is variously called bioclimatic-envelope, species-distribution, or species-niche modeling, and involves a range of spatial regression techniques. The assumptions common to all these models to infer past or future climate or taxon distributions are (i) that contemporary distributions are controlled by climate, (ii) that such distributions are in equilibrium with climate today, (iii) that the distributional and climatic data are reliable, and (iv) that the observed realized distribution area is close to the potential and fundamental distribution areas (e.g., Pearson and Dawson, 2003; Dormann, 2007; Beale et al., 2008; Birks et al., 2010; Svenning and Sandel, 2013; Birks, 2019). The greatest uncertainties are whether climate limits the observed distribution and the whether the distribution is in equilibrium with climate. These assumptions are critical in all bioclimatic-envelope models used to infer past or future climate or taxon distributions from contemporary climatic and distributional data (Svenning et al., 2011).

Prior to 1916, Quaternary botany only considered megafossils (logs, tree stumps, etc.) and macrofossils (leaves, seeds, and fruits, etc.). It underwent a major change in 1916 as a result of von Post's seminal lecture in Kristiania (Oslo) in July (Von Post, 1916, 1918) in which he presented the principles and methodology of pollen analysis, discussed problems of interpretation, and demonstrated its potential as a tool for studying temporal and spatial changes in pollen assemblages and hence in vegetation. At first, pollen analysis was confined to trees and used to reconstruct forest history and climate change and to provide a relative dating technique. The taxonomic resolution of pollen-stratigraphical data greatly improved in the 1930s and 1940s through the work of, *inter alia*, Franz Firbas, Gunnar Erdtman, Jørgen Troels-Smith, and Johannes Iversen (Birks and Berglund, 2018). This culminated in the publication of the *Text-book of Modern Pollen Analysis* (Faegri and Iversen, 1950). As a result, pollen analysis began to shift from being primarily a tool in Quaternary geology to a tool in Quaternary botany and paleoecology. Aspects of the ecology of taxa found as fossils (e.g., light, moisture, and soil preferences, geographical distribution, climatic tolerances–"ecological attributes" *sensu* Hill et al. (2004)] were increasingly used in the interpretation of pollen-stratigraphical data. Examples of this approach include Iversen's demonstration of the influence of prehistoric people on vegetation and the interpretation of the ecological changes resulting from forest clearance (Iversen, 1941, 1949, 1973), and of the unique nature of the flora, vegetation, and environment of the late-glacial interval in Denmark (Iversen, 1954, 1973).

At about the same time, ecologists began to use modern plant assemblages as a basis for assessing modern environmental conditions, co-called bio-indication, by means of ecological indicator values (EIVs; e.g., Ellenberg, 1948). Ordinal EIVs ("optima") have been estimated for the central European flora on the basis of field, laboratory, and phytosociological studies for light, moisture, soil reaction, nitrogen, salt tolerance, temperature, and continentality (Ellenberg et al., 1992). Given a modern plant assemblage, the environmental values for its site is inferred to be the average of the indicator value (if presence/absence data only are available), or the abundance-weighted average (Holtland et al., 2010) of the indicator values if quantitative or semi-quantitative data are available. EIVs may vary between large geographical areas (e.g., Lawesson et al., 2003; Godefroid and Dana, 2007), and Hill et al. (2000) developed an iterative numerical procedure for extending Ellenberg's EIVs into new areas (e.g., Hill et al., 2004; Hill et al., 2007). Diekmann (2003) and Kollmann and Fischer (2003) provide a thorough review of EIVs as a tool in applied ecology. Excellent examples of their use in modern ecology include (Hawkes et al., 1997; Hill and Carey, 1997; Dzwonko, 2001; Cornwell and Grubb, 2003; Bennie et al., 2006; Holtland et al., 2010; Hellegers et al., 2020). The underlying mathematical basis for the use of EIVs is outlined by Ter Braak and Barendregt (1986); Ter Braak and Gremmen (1987), and Holtland et al. (2010).

EIVs have been partially criticized for being subjective and semi-quantitative, not being based on field measurements, and being based, in part, on central European phytosociological classes. However, the current general consensus about their robustness was well summarized by Körner (2018; an ecophysiologist) as follows. "While such semi-quantitative estimates may not allow us to identify accurately physiologically based preferences within an individual plant species (for instance between an indicator value of 3 or 4), it is surprising how well mean indicator values reflect site characteristics across several species in a given community. As such, indicator values may reflect habitat conditions more accurately than physico-chemical analyses of soil because they represent a long-term response of species to habitat conditions. . . . Indicator values are a reliable tool for making predictions about plant community changes in relation to changes in environmental conditions. Community-level indicator values represent a trait syndrome that can be validated." Körner (2018) considered EIVs to be an *a posteriori* trait concept, namely traits that reflect "observed responses to the environment, rather than responses predicted by theory." Van Der Maarel (1993) viewed EIVs from a phytosociological and field ecological viewpoint and concluded that "ecological indicator values have a valuable element of synthesis and integration which incidental measurements may miss."

Körner (2018) proposed that EIVs are similar to the plant life-strategies proposed by Grime (1979, 2001) in both being *a posteriori* traits. Interestingly, Thompson et al. (1993) showed close relationships between EIVs and quantitative measurements from both the field and from laboratory screening experiments (e.g., rooting depth, foliar Ca values, foliar N concentrations, and iron tolerance). Thompson et al. (1993) noted that "We have attempted to marry the Ellenberg tradition with the screening approach of UCPE [Unit of Comparative Plant Ecology], Paul Keddy, Mark Westoby, and others. [Our results] suggest that the two are complementary and that each has much to gain from an interaction with the other." This view, presented over 25 years ago, was rejuvenated by Bartelheimer and Poschlod (2016) in their review of functional characterizations of Ellenberg values in relation to ecophysiological variables such as maximum frost resistance, time of leaf unfolding, maximum relative growth rate, embryo + endosperm weight, and time to 50% germination. They concluded (p. 506) "Functional characterizations of the different EIVs can thus be deduced which help to understand the mechanisms and processes driving the ecological niche of a plant."

Despite the many demonstrations of the robustness of EIVs in environmental inferences (e.g., Van Der Maarel, 1993), the increasing discoveries of the links between EIVs and functional characteristics of species (e.g., Thompson et al., 1993), and the increasing use of EIVs in ecology from 20 studies in 1985–1989 to 277 studies in 2010–2014 (Bartelheimer and Poschlod, 2016), EIVs are surprisingly rarely used in Quaternary botany despite their numerical simplicity and robustness. They have been used primarily with macrofossil assemblages where species-level identifications are often possible (Birks, 2001, 2013, 2014). EIVs have been used to infer local-scale conditions of light, soil reaction (=calcium and hence pH: Schaffers and Sýkora, 2000), soil nitrogen (=soil fertility or aboveground productivity: Hill and Carey, 1997), and soil moisture from late-glacial macrofossil assemblages (e.g., Mortensen et al., 2011; Felde and Birks, 2019), from assemblages associated with archeological sequences (e.g., Wasylikowa, 1978; Wasylikowa et al., 1985; Wasylikowa, 1989; Cappers, 1993/1994), and from fossil bryophyte assemblages (e.g., Odgaard, 1981; Jonsgard and Birks, 1995; Lamentowicz et al., 2015; Zechmeister et al., 2019).

The use of EIVs is more problematic with pollen-stratigraphical data as most pollen types can currently only be identified to genus or family level. Naturally there are very few uses of EIVs with pollen assemblages (e.g., Birks, 1990; Kuneš et al., 2011; Reitalu et al., 2015; Enevold et al., 2019). Genus or family level pollen identifications necessitate deriving a mean or median EIV for taxa growing in the relevant study area today (e.g., Birks, 1990; Kuneš et al., 2011; Reitalu et al., 2015). Enevold et al. (2019) derived EIVs for light, moisture, soil fertility, and pH for a terrestrial pollen type as the mean of the EIV for the regionally dominant or common species today, if five or more species contribute to that pollen type or, if four or fewer species are included in that type, a mean EIV for all those species is used. The same approach was used to derive EIVs for wetland macrofossil taxa if a taxon represented more than one species (Enevold et al., 2019). Given mean or median EIVs for the pollen types present, an assemblage-weighted mean (=community-weighted mean: Diekmann, 2003) can be used to infer past environmental conditions.

EIVs have considerable potential value in inferring past environments from Quaternary botanical data; one factor that limits their use is the importance of having taxonomically detailed assemblage data. There is a current marked decline in macrofossil studies globally and this may render EIV approaches very rare or impossible in the future.

ECOLOGICAL TRAITS

Turning to the use in Quaternary botany of what modern plant ecologists may term traits, perhaps the first use is the simple functional division of pollen taxa into arboreal and non-arboreal pollen (Firbas and Preuss, 1934; Reitalu et al., 2015). The division has been almost universally used by palynologists worldwide for over 90 years. An extension of this division has been the use of plant functional types (PFTs) to assign pollen assemblages into biomes as a means of reconstructing major broadscale distributions of inferred vegetation (e.g., continents) and of using past vegetation data in Earth system models (e.g., Prentice et al., 1996; Fyfe et al., 2010; Collins et al., 2012; Davis et al., 2015; Zanon et al., 2018). They have also been used to summarize modern and fossil pollen assemblages in the quantitative reconstruction of past climate (e.g., Davis et al., 2003;

Brewer et al., 2008; Mauri et al., 2015) and past forest cover (e.g., Zanon et al., 2018). Gachet et al. (2003) applied a probabilistic approach to characterize modern pollen taxa using one or more modes of several attributes or traits (e.g., climatic preferences, stature, phenology, and life-form). Conditional probabilities were defined to represent the probability that a given attribute mode occurs in a given pollen sample when the pollen assemblage is known. They compared their approach using modern pollen samples with existing biome classifications and remote-sensing data. They then used their approach to reconstruct biomes for 6000 years ago from European fossil data. This approach does not appear to have been used since the original study.

Using 302 modern pollen assemblages from the Mediterranean region, Barboni et al. (2004) identified "plausible trends" in the frequency of traits of 12 plant morphological and phenological characteristics. Combinations of traits appeared to be more climatically diagnostic than individual traits. The major patterns in trait frequencies were correlated with available moisture, winter temperature, or a combination of both. Traits such as leaf size, texture, thickness, pubescence, thorniness, aphylly, and aromatic leaves also varied, not surprisingly, with life-form, leaf-type, and phenology (Barboni et al., 2004).

Iversen (1960) used data from the pioneering work of Vaupell (1863) on the different shade tolerances, longevities, reproduction behavior, and edaphic preferences of the major forest trees in Denmark to present a null hypothesis for the early- and mid-Holocene forest development solely in terms of endogenous biotic interactions (Birks, 1986, 2019). Vaupell (1863) showed the importance of light in woodland successional dynamics in which light-demanding taxa are replaced over time by shade-tolerant trees, provided that conditions for growth are favorable for the latter (see also Jensen, 1910, 1949; Iversen, 1941). Following Iversen (1960), Birks (1986) compiled 20 general traits of trees characteristic of the protocratic, mesocratic, and oligocratic + telocratic phases of an interglacial cycle in northwest Europe (Iversen, 1958; Birks and Birks, 2004; Birks, 2019). These traits include crown geometry, seedling light tolerance and mortality, growth rate, life-history features, longevity, and age and frequency of seed set (Birks, 1986).

Attributes such as longevity, seed size, vegetative reproduction, habitat preference, and geographical distribution, termed biogeographical traits by Bhagwat and Willis (2008), were used as presence/absence variables to assess differences in the attributes of taxa that persisted in the last glacial maximum only in refugia in southern Europe ("macrorefugia" Birks, 2019) or taxa that also grew in refugia in both southern and northern Europe ("microrefugia" or "cryptic refugia" Bhagwat and Willis, 2008; Birks and Willis, 2008; Birks, 2019). Taxa confined to southern refugia only are mainly large-seeded angiosperms, whereas taxa that also grew in more northerly microrefugia are mostly wind-dispersed and often, but not always, show vegetative reproduction (Bhagwat and Willis, 2008).

Lacourse (2009) used life-history and stress-tolerance traits such as minimum seed-bearing age, waterlogging, shade, and drought tolerances, size longevity, seed mass, and relative growth rate (RGR) for 11 taxa, ten of whose pollen can be assigned to species level in the context of coastal British Columbia. The one exception not assignable to species level was *Populus* pollen which could be derived from *P. trichocarpa* or *P. balsamifera*. The traits used for *Populus* were the means for the two species. Lacourse compiled independent paleoenvironmental data for her pollen sequence such as summer insolation, mean annual precipitation, coastal sea-surface temperatures, and biomass. Both RLQ (Dolédec et al., 1996) and fourth-corner (Legendre et al., 1997) analyses were used to link the traits to past environment over the last 15,000 years through the fossil pollen record from Misty Lake, British Columbia and hence to help interpret the ecological patterns and their underlying processes at the site. RLQ analysis showed that relative growth rate and temperature were most strongly correlated with the major long-term variation in pollen stratigraphy. Both numerical analyses showed statistically significant correlations between many of the other traits and insolation and temperature changes. Following deglaciation climate change appears to have favored taxa with high RGR, short life-span, and low shade tolerance (e.g., *Alnus sinuata, Pinus contorta*), whereas in the last 8000 years, climatic stability favored taxa such as *Tsuga heterophylla* with its low RGR, long life-span, and high shade tolerance. Fourth-corner analysis modified for abundance data (Dray and Legendre, 2008) showed correlations between all paleoenvironmental variables and most traits, but the strongest was between temperature and plant height, reflecting the dominant competitive abundance of tree height. Lacourse (2009) concluded that exogenous climate was the ultimate control on early-Holocene forest composition and taxon abundance but in the last 8000 years long-term forest vegetation composition was constrained through endogenous interspecific differences in traits, a conclusion similar to Iversen (1960) in his analysis of forest development in Denmark. An important general conclusion from Lacourse's (2009) pioneering quantitative study is that a suite of traits and trait differences, as opposed to a single trait, were important in mediating changes in the long-term forest composition in the Pacific North West that were primarily driven by climate, just as Iversen (1960) showed in his qualitative analysis that several ecological attributes or traits were important in understanding the Holocene forest development in Denmark.

There has been a small number of recent Quaternary botanical studies where life-history and functional traits have been used to aid interpretation of long-term pollen records (e.g., Brussel et al., 2018; Connor et al., 2018; Carvalho et al., 2019; Van Der Sande et al., 2019). Application of traits to pollen-stratigraphical data is greatly hampered by the fact that many of the abundant pollen taxa can only be identified to genus (e.g., *Quercus, Pinus*) or family level (e.g., Poaceae). Relevant numerical tools for robust and statistically rigorous trait-analysis exist (e.g., Dray and Legendre, 2008; Ter Braak et al., 2012; Brown et al., 2014; Ter Braak, 2017, 2019; Ter Braak et al., 2017, 2018) but appropriate trait information is lacking for many of the fossil pollen taxa. The concept of traits for a large family such as Poaceae is currently unresolved. Van Der Sande et al. (2019) used phylogenetic signals to derive genus-level or even family-level mean trait values, whereas Connor et al. (2018) used traits of a representative plant species for a particular pollen type (e.g., *Phleum phleoides* for Poaceae, *Torilis arvensis* for Apiaceae).

Rather surprisingly, traits have not been widely studied for plant-macrofossil assemblages (Birks, 2014) except for Bhagwat and Willis (2008; see above) and Jabłońska et al. (2019). The latter study centered on mesotrophic rich-fens and tested the hypothesis that fen stability is controlled by plant stress induced by waterlogging with calcium-rich but nutrient-poor groundwater. Thirteen traits or attributes for vascular plants and bryophytes identified from 32 European Holocene rich-fen macrofossil profiles were compiled. Traits and attributes considered included plant height, clonal spread, diaspore mass, specific leaf area, leaf dry matter, Ellenberg moisture values, and mycorrhizal status. Jabłońska et al. (2019) showed using their trait and attribute data that fens undergo a gradual autogenic process that may reduce fen resistance and enhance shifts to other mire types, explaining why long-lasting rich fens are a rare habitat type today (cf. Rybnícek, 1973). A study of *Sphagnum*-dominated mires in Poland involved macrofossils, testate amoebae, and mosses preserved in the peat (Lamentowicz et al., 2019) to detect a possible tipping-point in mire water-level. In this study, 15 functional traits for *Sphagnum* were compiled but, surprisingly, *Sphagnum* functional diversity (FD) remained very stable despite hydrological changes within the peatlands (Lamentowicz et al., 2019).

Various biochemical, morphological, and physiological "traits" have been used to reconstruct different aspects of the past environment based on Quaternary botanical fossils (Lomax and Fraser, 2015). Fossil pollen and spores can be used to quantify ultraviolet-B (UV-B) radiation flux through time using the concentration of UV-B-absorbing compounds preserved in fossil sporopollenin (Magri, 2011). This is a complex biopolymer and consists, in part, of *para*-coumaric acid (*p*CA) and ferulic acid. These compounds absorb UV-B (280–315 nm) and their abundance in pollen and spores increases in response to increasing UV-B flux (Rozema et al., 2001; Rozema et al., 2002; Blokker et al., 2006; Willis et al., 2011). The concentration of *p*CA can be consistently estimated by a modified version of Thermally Assisted Hydrolysis and Methylation with pyrolysis Gas-Chromatography Mass-Spectroscopy (Seddon et al., 2017). Several attempts have been made to reconstruct changes in UV-B flux through historic and Holocene time (e.g., Rozema et al., 2001; Lomax et al., 2008; Willis et al., 2011; Jardine et al., 2016, 2020).

A second "trait" that is widely used to reconstruct past atmospheric CO_2 concentrations is leaf stomatal density. It has long been known from experiments that many plants respond to increased CO_2 by an increase in photosynthetic rate and a decrease in stomatal opening (Woodward, 1988). By examining herbarium specimens of leaves of eight temperate trees collected over the last 200 years, Woodward (1987) showed a 40% decrease in stomatal density (stomata per unit leaf area) in response to increases in CO_2 values from pre-industrial levels. Körner et al. (1979) and Woodward (1986) found that stomatal density often increased with elevation, In light of this work on stomatal numbers and atmospheric CO_2 concentrations, Quaternary botanists measured stomatal density (SD numbers mm^{-2}) or stomatal index [=SD/(SD + ED) × 100 where ED is the epidermal cell density (mm^{-2}; Salisbury, 1927)] on fossil leaves of, for example, *Salix herbacea, Betula nana, B. pendula, B. pubescens,* and *Quercus* spp. To reconstruct past CO_2 concentrations from these stomatal frequencies, stomatal density or index was measured for modern leaves collected at or grown under different CO_2 levels to derive calibration functions between stomatal density or index and atmospheric CO_2 concentration (e.g., Beerling et al., 1995; Rundgren and Beerling, 1999; Rundgren et al., 2005). These calibration functions have been used to estimate past CO_2 concentrations in the last interglacial (e.g., Rundgren and Bennike, 2002; Rundgren et al., 2005), the last glacial maximum (e.g., Beerling and Chaloner, 1994), the late-glacial (e.g., Beerling et al., 1995; Rundgren and Björck, 2003), and the Holocene (e.g., Rundgren and Beerling, 1999; Wagner et al., 1999; Beerling and Rundgren, 2000; Rundgren and Björck, 2003; Jessen et al., 2007). The taxa used in these reconstructions in Europe have mainly been *Salix herbacea* (e.g., Beerling et al., 1995; Rundgren and Beerling, 1999; Rundgren and Bennike, 2002), *Betula nana* (e.g., Beerling, 1993; Rundgren and Björck, 2003; Finsinger and Wagner-Cremer, 2009; Steinthorsdottir et al., 2013), *B. pubescens* or *B. pendula* (e.g., Wagner et al., 1999; Wagner et al., 2002; Wagner et al., 2004; García-Amorena et al., 2008), *Quercus robur* or *Q. petraea* (e.g., Wagner et al., 2004; Van Hoof et al., 2006), *S. cinerea* (e.g., McElwain et al., 1995), or *Buxus balearica* and *B. sempervirens* (e.g., Rivera et al., 2014). In North America, needles of *Pinus flexilis* (e.g., Van Der Water et al., 1994), *Larix laricina*, *Picea glauca*, and *P. mariana*, and leaves of *Dryas integrifolia* (e.g., McElwain et al., 2002) have been used.

A recent study in eastern Australia (Hill et al., 2019) on leaves of *Malaleuca quinquenervia* did not find any relationship between stomatal density or index and CO_2 concentration, temperature, or rainfall. Hill et al. (2019) concluded that stomatal numbers are a highly plastic "trait" in this flood-plain and lake-side tree and that stomatal changes may not reflect functional changes in the leaves.

An additional leaf "trait" that has been used in environmental reconstructions is the epidermal cell undulation index, which is a quantitative estimate of the degree of sinuosity of epidermal cell walls (Wagner-Cremer et al., 2010). It was developed as a means of reconstructing past changes in growing degree-days using fossil *Betula nana* leaves in northern Fennoscandia. The approach has recently been extended to mountain birch, *B. pubescens* ssp. *czerepanovii* (Ercan et al., 2020), which permits the use of the approach over much of Fennoscandia. It has not, as far as I know, been applied to fossil tree-birch leaves.

Relationships between leaf "traits" and their environment, particularly climate, have been discussed for over 100 years (e.g., Billings, 1905; Bailey and Sinnott, 1915, 1916). These relationships have been extensively explored and exploited in pre-Quaternary botany (see Peppe et al., 2018). Interest has recently focused on the leaf economic spectrum (LES) that provides a framework for leaf functional ecology (cf. Osnas et al., 2013). Vein networks are an important component of the LES. Many LES variables can easily be measured from modern leaves (e.g., Wang et al., 2020 but see Osnas et al., 2013), but they cannot be directly measured on fossil leaves (Peppe et al., 2018). Variables that can be measured on fossil leaves such as leaf area, petiole width, and vein density have been used to estimate critical LES variables such as leaf life-span, leaf mass per area, and carbon assimilation and respiration rates (Peppe et al., 2018). Although they are, in theory, applicable across many plant groups throughout the geological record, they do not appear to have been used in Quaternary botany.

A different set of leaf "traits" are organic biomarkers derived from leaf waxes. Such biomarkers are increasingly used as tools for reconstructing past environments (Berke, 2018). Organic geochemistry is a rapidly developing field and largely falls outside the scope of this paper. Within a leaf cuticle there is a matrix of cutin, a polyester biopolymer consisting of hydroxy fatty acids and waxes. The epicuticular wax layer contains *n*-alkanes and free and ester-bound long-chain *n*-alkyl wax lipids (Diefendorf et al., 2011). Leaf-wax composition and abundance can vary with ontogeny, environment, and among plant families, genera, species, and individuals. The isotopic composition of leaf-wax hydrogen (δD) and of the stable carbon isotope composition of leaf-wax ($\delta^{13}C$) are increasingly used as proxies for changes in vegetation composition and density (C_3/C_4 plants – e.g., Huang et al., 2006; Uno et al., 2016), precipitation (e.g., Huang et al., 1999; Sauer et al., 2001; Douglas et al., 2012; Garcin et al., 2012), and Arctic and temperate temperatures (e.g., Shuman et al., 2004; Thomas et al., 2012). Organic and isotope geochemistry are rapidly developing and complex research areas. A major challenge is to derive past environmental reconstructions from lake sediments whose organic material is derived from multiple sources including plankton, aquatic and terrestrial plants, as well as microbes (Huang et al., 2002). Bacterial cell membrane lipids such as branched glycerol dialkyl glycerol tetraethers appear to be nearly ubiquitous in lakes (e.g., Pearson et al., 2011). They have been used as an independent temperature proxy in Arctic Canada to evaluate the local colonization of dwarf *Betula*, as evidenced by sedimentary ancient DNA in relation to climate change (Crump et al., 2019). This study shows the potential of a multiproxy approach utilizing biogeochemical "traits" and molecular techniques such as ancient DNA. Further exciting advances in this type of study bridging biogeochemistry, Quaternary botany, and ancient DNA analyses can be expected in the next five to ten years.

Functional diversity (FD) is an important component of biodiversity that considers the range of functions that organisms perform in ecosystems (Hooper et al., 2002). It not only serves as a descriptor of an assemblage, but it can also be an indicator of ecosystem function. As FD is the diversity of taxon traits in an assemblage, it may capture information about traits that may be missing in measures of taxonomic richness or diversity (Birks et al., 2016). To date, very few Quaternary botanical studies have considered FD other than PFT diversity estimated from palynological data (e.g., Davis et al., 2015). Reitalu et al. (2015) explored in detail temporal patterns in FD as well as in taxonomic richness and evenness and phylogenetic diversity (PD) estimated from late-glacial and Holocene pollen data from 20 sites in Estonia and Latvia. They showed that shifts in FD and PD of the pollen data were closely related to climate change (as recorded in the Greenland NGRIP ice-core) and suggested that trait differences may have played an important role in long-term biotic responses to climate change. Human impact in the last 2000 years had a negative influence on FD and PD due to the decline of plant taxa with certain traits leading to functional convergence and the expansion of some taxa from particular phylogenetic lineages. In their study, Reitalu et al. (2015) faced the problem of assigning trait values to pollen taxa representing several plant species. For such taxa, they used a mean trait value for the species present in their study area for pollen taxa representing up to ten plant species. For pollen taxa with more than ten plant species, they used a random subset of species so that the number was approximately proportional to the total number of species in the Estonian flora belonging to that pollen taxon.

Clearly there is a need for critical comparisons of different estimation procedures for traits for pollen types to ascertain which procedures are robust and ecologically realistic. Given such procedures, there are exciting challenges in exploring simultaneously taxonomic, functional, and PD of modern and fossil pollen assemblages at a range of spatial and temporal scales.

DISCUSSION AND CONCLUSION

As documented above, there is a long history of ecological attributes (including traits) being used in Quaternary botany extending over nearly 120 years. The main use of attributes has been to aid in the reconstruction of past environments such as climate (e.g., Kühl, 2003), soil conditions (e.g., Felde and Birks, 2019), or vegetation (e.g., Zanon et al., 2018). Traits have also been used to investigate taxon responses to past environmental changes (e.g., Lacourse, 2009) or to generate hypotheses about the underlying drivers of past vegetation dynamics (e.g., Iversen, 1960).

The major limitations in using attributes or traits in Quaternary botany is the taxonomic level at which many Quaternary plant fossils, particularly pollen, and can be identified to (e.g., Reitalu et al., 2015). Identifications at the family or genus levels pose problems in the assignment of attribute or trait values to such taxa. Plant macrofossils have the advantage over pollen in that fossil seeds and fruits can often be identified to species level (Birks and Birks, 2000; Birks, 2001, 2003, 2014), thereby allowing an effective use of attributes such as indicator values with macrofossil assemblages (e.g., Felde and Birks, 2019). Despite detailed macrofossil identifications, traits have rarely been used in macrofossil studies (Birks, 2014), possibly because of some important limitations of macrofossil data, such as the abundance of zero values, mixed (nominal, qualitative, and quantitative) data types, and problems of false absences (=false negatives; Birks, 2014).

Enhancing interpretation of Quaternary botanical assemblages in terms of vegetational or ecosystem dynamics by means of attributes or traits demands that environmental reconstructions are available that are independent of the botanical assemblages (e.g., Birks and Birks, 2008; Lacourse, 2009; Reitalu et al., 2015). This demand avoids dangers of circular reasoning where changes in an assemblage are "explained" in terms of the traits of the taxa in the assemblage, just as assemblages should not be "explained" on the basis of the past environment inferred from the assemblage itself. Environmental reconstructions based on taxa, attributes, or traits are certainly valuable in some topics within paleoenvironmental research, but they should not be used to interpret or "explain" changes in the biological assemblages.

Reconstruction is a form of description in paleoecology ("descriptive and narrative approaches"–Birks and Berglund, 2018) whereas the testing of explicit hypotheses about the underlying "drivers" or processes behind the observed patterns in assemblages ("analytical approach"–Birks and Berglund, 2018) often requires not only assemblage data ("responses") but also independent environmental data ("predictors"; e.g., Birks and Birks, 2006; Birks, 2008; Crump et al., 2019). Major methodological advances are being made in other branches of Quaternary science to reconstruct different aspects of the past environment using, for example, biomarkers (e.g., Pearson et al., 2011; Crump et al., 2019), and stable isotopes (e.g., Colombaroli et al., 2016). Such independent environmental reconstructions will allow ecological attributes and traits to be used to further the interpretation of past biotic changes, along the lines pioneered by Iversen (1960), Lacourse (2009), and Reitalu et al. (2015). Novel approaches for defining ecological traits at broad spatial scales are also being developed (e.g., Ustin and Gamon, 2010; He et al., 2019) that are directly relevant to the spatial scale of regional pollen stratigraphies (Birks, 2019).

Ecological attributes and traits are potentially useful tools in Quaternary botany but they are no substitute for a good ecological knowledge of the taxa found in assemblages. Faegri (1966) proposed in his discussion of the interpretation of pollen assemblages "it presumes a very intimate knowledge of the ecology and sociology of the vegetation types concerned. ... pollen analysis of any vegetation types without such knowledge is bound to become at its best a lifeless stratigraphical tool, at its worst useless altogether," The role of species identity is one of the biggest challenges for any understanding of plant responses, past, present, or future to the environment (Clark, 2008). Clark (2016) showed, using forest data from eastern North America, that an "analysis of species better explains and predicts traits than does direct analysis of the traits themselves; trait data contain less, not more, information than species on environmental responses."

Despite the great upsurge of interest in trait-based ecology in recent decades, in Quaternary botany the use of attributes and traits has been and remains an integral part of the interpretation of botanical assemblages in terms of past vegetation or environmental change or in the reconstruction of past environments. There seems little prospect of (or need for) a "new" Quaternary botany or paleoecology based on traits. Quaternary botany, by necessity, follows what McGill et al. (2006) called "a nomenclatural approach by focusing on species identities," which they proposed "has resulted in a loss of ecological generality." They argued that "statements about traits give generality and predictability, whereas nomenclatural ecology tends toward contingent rules and special cases." Only time will tell if this search for the "Holy Grail" in ecology will fulfill the exciting proposals of trait-based ecology and "clean up the 'mess"' of community ecology (McGill et al., 2006) and if this approach will contribute to more refined interpretations of Quaternary botanical data and improve our understanding of long-term vegetation and ecosystem dynamics.

Bradshaw (1987) emphasized in his discussion of different approaches to plant ecology that "at any point of time, any discipline in science tends to be seized by a particular methodology or enthusiasm and other approaches get 'dumped'. So it is in ecology–although because of obstinate individualism, nothing is ever completely forgotten." The contrast between the rapid upsurge of interest in trait-based ecology in the last 15–20 years and the use of attributes and traits in Quaternary botany over the last 120 years reinforce Bradshaw's perceptive comment.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Modern Pollen–Plant Diversity Relationships Inform Palaeoecological Reconstructions of Functional and Phylogenetic Diversity in Calcareous Fens

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Predicting the trajectory of ongoing diversity loss requires knowledge of historical development of community assemblages. Long-term data from palaeoecological investigations combined with key biodiversity measures in ecology such as taxonomic richness, functional diversity (FD), phylogenetic diversity (PD) and environmental factors expressed as Ellenberg indicator values (EIVs) could provide that knowledge. We explored the modern pollen–plant (moss polster pollen vs. surrounding vegetation) diversity relationships for herbaceous and woody taxa in calcareous fens from two different regions in Estonia, NE Europe. Associations of taxonomic richness, vegetation composition, FD (including functional alpha diversity and trait composition), PD and EIVs in modern pollen vs. plant data were studied with correlation analysis, Procrustes analysis and linear regression models. To test their potential use in palaeoreconstructions, diversity measures were applied on pollen data from Kanna spring fen reflecting fen vegetation development over the last nine millennia and diversity changes through time were studied using generalized additive models. Results showed significant pollen–plant richness correlations for herbaceous taxa at vegetation estimate scales up to 6 m radius and Procrustes analysis showed significant compositional associations at all plant estimate scales (up to 100 m). Woody taxa had no significant pollen–plant richness correlations but composition relationships were significant at plant estimate scales of 6–100 m. Traits that were best reflected by pollen data (both in terms of trait composition and functional alpha diversity) among woody and herbaceous taxa were seed number, clonality, SLA and LDMC. PD of herbaceous species was reflected by pollen data. Among the EIVs, Ellenberg L and T were significantly reflected by pollen data for both woody and herbaceous communities. Palaeoreconstruction from Kanna fen indicates that trends of woody taxa are mostly related to long-term changes in climate while diversity variables of herbaceous taxa closely follow autogenic processes within the fen. We suggest that pollen-based diversity estimates should be calculated separately for woody and herbaceous taxa as they clearly represent different spatial scales. Present study suggests that linking sedimentary pollen data with FD, PD and EIVs provides possibilities to examine long-term trends in community assembly and ecosystem processes that would be undetectable from traditional pollen diagrams.

Keywords: pollen–plant relationships, calcareous spring fens, functional diversity, phylogenetic diversity, Ellenberg indicators, Holocene, palynological diversity

INTRODUCTION

Identifying the drivers underlying biodiversity changes is among the key research questions both in ecology (Vellend et al., 2017) and palaeoecology (Birks et al., 2016b). Biodiversity can be measured at different organizational levels – from genetic diversity to taxonomic (species) diversity, to landscape diversity. Coupling these measures with community functional diversity (FD), which reflects the type, scope, and abundance of functional traits in communities provides a link between species diversity and ecosystem functioning (Tilman, 2001; Lavorel and Garnier, 2002; Díaz et al., 2007). In addition, phylogenetic diversity, which estimates the phylogenetic relatedness among the coexisting species (Faith, 1992) connects local community assembly and evolutionary processes (Gerhold et al., 2015, 2018). The concepts of functional and phylogenetic diversity are of central interest in a large number of contemporary ecological studies of land plants (e.g., Cadotte et al., 2009; Cavender-Bares et al., 2009; Purschke et al., 2013; Díaz et al., 2016). In palaeoecology, plant functional types have been used for several decades to enable the transformation of pollen records to biomes or land-cover types for comparison with climate models (e.g., Prentice et al., 1996; Fyfe et al., 2010; Davis et al., 2015). The more detailed studies of functional and phylogenetic aspects of palaeo communities are relatively rare (e.g., Lacourse, 2009; Reitalu et al., 2015; Brussel et al., 2018; Carvalho et al., 2019; Jabłońska et al., 2019) and have demonstrated the great potential of functional and phylogenetic palaeodiversity to contribute to a better understanding of processes underlying long-term patterns of community assembly. For example, Brussel et al. (2018) showed that long-term fire frequency variations may drive directional selection for fire–adapted plant community attributes.

In palaeoecology, sedimentary pollen analysis is one of the main tools for reconstructing changes in past vegetation and plant diversity in the late Quaternary (Berglund and Ralska-Jasiewiczowa, 1986; Birks and Birks, 2006; Gaillard et al., 2008; Reitalu et al., 2015). However, interpreting pollen data in terms of past plant diversity is not straightforward because of the low taxonomic resolution of pollen data and interspecific variation in pollen production and dispersal (Odgaard, 1999; Liu et al., 2014; Birks et al., 2016a; Carvalho et al., 2019). To clarify the pollen–plant relationships and to aid the pollen-based biodiversity reconstructions, modern pollen assemblages acquired from moss polsters, pollen traps, and lake surface sediments have been compared with detailed information of the surrounding vegetation (Bunting and Hjelle, 2010; Meltsov et al., 2011, 2012; Matthias et al., 2015; Felde et al., 2016; Julier et al., 2018; Reitalu et al., 2019). Several studies have demonstrated a positive association between modern pollen richness and plant richness (Meltsov et al., 2011; Matthias et al., 2015; Felde et al., 2016; Reitalu et al., 2019). Pollen of woody taxa is expected to be produced in greater quantities, to travel larger distances and to reflect broader spatial scales than the pollen of herbaceous and/or insect-pollinated taxa (Meltsov et al., 2011; Felde et al., 2016; Reitalu et al., 2019). In the present study, we will, therefore, examine the woody and herbaceous species/pollen taxa separately.

Carvalho et al. (2019) have highlighted the importance of using modern pollen–plant relationships also for functional palaeodiversity. They showed in modern pollen–plant study from fen peatland that some leaf traits (C and N content) were better reconstructed from pollen data than others (specific leaf area and leaf dry matter content). The choice of traits for analyzing functional diversity is often debated and depends on the research system, hypotheses and data availability (Lavorel and Garnier, 2002). In the current study, we have focused on the commonly used traits in plant functional ecology that could be assumed to be relevant for shedding light to the past plant communities. The chosen traits reflect different aspects of plant life history related to vegetative growth [clonality, plant height, specific leaf area (SLA), leaf dry matter content (LDMC)], reproduction (seed size and number) and biotic interactions (mycorrhizal type). In palaeoecological context, plant height, SLA, LDMC, seed size and mycorrhizal type can be assumed to be related to long-term climate variability and indicative of niche differentiation during favorable climatic conditions (Lacourse, 2009; Reitalu et al., 2015; Knight et al., 2020). Clonality, plant height, seed number and mycorrhizal type can be assumed to be associated with plant establishment in the harsh conditions of novel habitats (Ye et al., 2014; Pither et al., 2018) and with waterlogging in wetlands (Jabłońska et al., 2019).

There is a wide array of measures and several conceptual frameworks for estimating the functional and phylogenetic aspects of community assembly (see overviews in Vellend et al., 2011; Garnier et al., 2016). The functional structure of the community (functional diversity *sensu lato*, FD) can be characterized by average trait values and by measures estimating the variation in trait values (functional diversity *sensu stricto* or functional alpha diversity) (Garnier et al., 2016). Community weighted mean (CWM) trait values are widely used for evaluating links between community dynamics and ecosystem processes (Lavorel et al., 2008; Muscarella and Uriarte, 2016; Duarte et al., 2017). Functional alpha diversity (FDa) reflects the variability of traits among the coexisting species and allows to differentiate between functional divergence and convergence (Mason et al., 2005; de Bello et al., 2010; Laliberté and Legendre, 2010).

While stochastic effects also influence patterns of functional trait variation, selection and adaptation to changing environment will lead to non-random patterns. Environmental changes are assumed to select for or against species with certain traits and result in functional convergence (species being more similar in their traits than expected by random) (Pillar et al., 2009). Competition can be assumed to lead to functional divergence due to niche differentiation (species being less similar in their traits than expected by random) (Purschke et al., 2013). Phylogenetic diversity (PD) reflects the variability of phylogenetic distances among the coexisting species and helps to differentiate between phylogenetic divergence (coexisting species are phylogenetically more distant than expected by random) and phylogenetic convergence (coexisting species are phylogenetically more similar than expected by random) (Grime, 2006; Vellend et al., 2011). In the case of phylogenetically conserved traits, FDa and PD should show similar patterns but trait variation could also be large within phylogenetic lineages (Prinzing et al., 2008). In the current study, we follow the example of Reitalu et al. (2015) and use three measures to characterize the functional and phylogenetic structure of community assembly: (1) functional composition representing the community weighted mean trait values (CWMs), (2) functional alpha diversity (FDa) reflecting the variability of traits among the coexisting species, (3) phylogenetic diversity (PD) reflecting the variability in distances between species/taxa in a phylogenetic tree. We test the applicability of these measures for pollen-based biodiversity reconstructions.

In addition to CWM, FDa, and PD, we will use Ellenberg indicator values (EIVs) (Ellenberg et al., 1991) to characterize plant/pollen assemblages in terms of their environmental tolerances. EIVs provide an understanding of environmental differences between sites where environmental conditions cannot be directly measured, such as in palaeoecological reconstructions (Kuneš et al., 2011; Reitalu et al., 2015; Felde and Birks, 2019).

To study palaeoecological changes in biodiversity, calcareous spring fens are suitable environments – they are recognized as hotspots of biodiversity (Bedford and Godwin, 2003; Hájková et al., 2015) and provide continuous sediment archives that preserve proxy records for biodiversity and environmental reconstructions. Calcareous fens are of ecological interest to both palaeo- and contemporary ecologists as they provide data of recent and long-term vegetation changes (Almendinger and Leete, 1998; Hájková and Hájek, 2003; Grootjans et al., 2006; Blaus et al., 2019; Carvalho et al., 2019; Jabłońska et al., 2019). Human activities such as artificial drainage, agricultural practices, and eutrophication have negatively influenced the directional development of calcareous spring fens leading to a gradual disappearance of characteristic species or habitats (Stammel et al., 2003; Topiæ and Stanèiæ, 2006; Osadowski et al., 2018). There are several palaeoecological studies of calcareous fens in Europe (e.g., Pidek et al., 2012; Hájková et al., 2012, 2015; Gałka et al., 2018; Jamrichová et al., 2018; Blaus et al., 2019; Jabłońska et al., 2019) but none of them have investigated modern pollen-plant diversity relationships. The current study of modern pollen-plant diversity from spring fens provides valuable information for palaeo reconstructions of fen vegetation by examining the functional and phylogenetic aspects of fen biodiversity offering a new angle on the development of these unique environments.

We focus on modern pollen–plant diversity relationships in spring fens from two different regions in Estonia: southern Estonia and Saaremaa Island in western Estonia. Pollen data is derived from moss polsters and plant cover data is estimated from the surrounding vegetation. We compare taxonomic richness, vegetation composition, FDa, trait CWM, PD and EIVs in modern pollen and plant data (**Supplementary Figure S1**). We aim to test the following hypotheses:

1. Pollen-based richness and composition estimates of woody and herbaceous taxa are positively associated with corresponding plant-based estimates but at different spatial scales;
2. Pollen- and plant-based CWM, FDa, PD and EIV estimates are positively correlated both in woody and herbaceous taxa;
3. Pollen–plant diversity relationships do not differ between study areas (southern Estonia and western Estonia).

Further, we will use the information from the modern pollen–plant diversity relationships and apply the CWM, FDa, PD, and EIVs that have significant modern pollen–plant correlations to sedimentary pollen data (**Supplementary Figure S1**). Using existing well-dated pollen dataset from Kanna spring fen in western Estonia (Blaus et al., 2019), we will reconstruct the changes in different diversity components over the 9000-year history of the Kanna fen. Kanna fen developed first as a small bog and a shift to minerotrophic fen started ca 7000 years ago (Blaus et al., 2019). We expect to find high values of CWM of traits related to survival in nutrient-poor habitats (clonality, LDMC, mycorrhizal type) and tendency toward functional convergence due to environmental filtering in the initial bog phase of the mire development. In the minerotrophic fen phase during the mid-Holocene warm period, we expect to find high values of CWM of traits related to competitive abilities (SLA, plant height, seed size) and a tendency toward functional divergence due to niche differentiation. Among the Ellenberg indicator values, we expect the reaction (R), nutrient availability (N) and temperature (T) values to show the highest rate of change through the fen development.

MATERIALS AND METHODS

Study Area

Pollen and plant data were collected from 34 calcareous fens in Estonia. Of the 34 sites, seven open and seven forested sites are located in southern Estonia (South region), with ten open and eight forested sites located in Saaremaa Island (West region) (**Figure 1**). The sites distributed between the two regions were located at least 2.5 km apart but the open and forested fens were sampled relatively close to each other (60 to 300 m apart). The terrain in the South region is predominantly dome-shaped consisting of undulating hillocky dead-ice landforms covered with forest and grasslands. Between hillocks, there are wet meadows, lakes and patches of mires

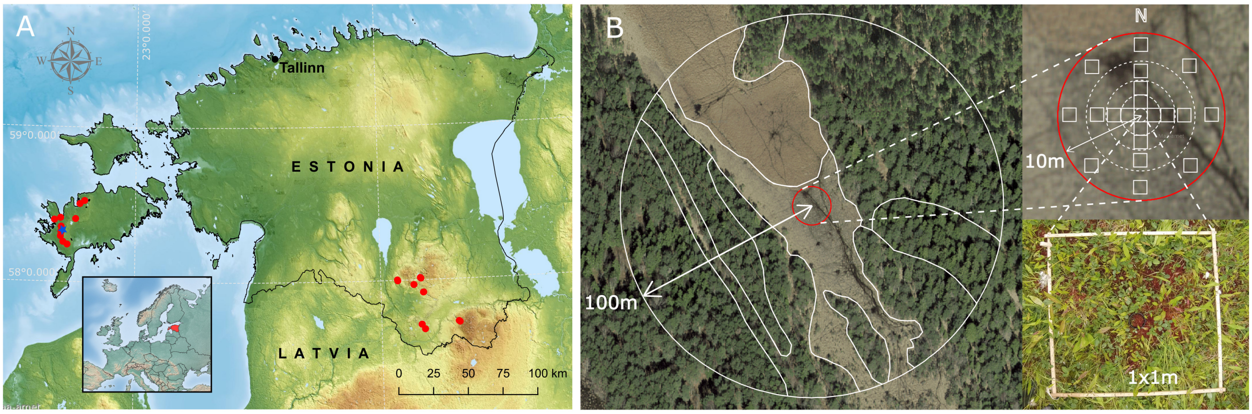

FIGURE 1 | Study area and sampling scheme. **(A)** Red dots indicate the sampled spring fens in southern Estonia and Saaremaa Island. Blue star marks the location of a Kanna spring fen used for sediment pollen based reconstructions. **(B)** The schematic overview of vegetation survey strategy modified from Bunting et al. (2013).

(transitional mires, bogs, fens, alder fens). About 70% of the region is covered with boreonemoral forest dominated by *Pinus sylvestris, Picea abies, Betula pubescens,* and *Alnus glutinosa*. Saaremaa is the largest island in Estonia, located in the Baltic Sea. The island is composed of Silurian carbonate rocks, primarily limestone, that are covered by glacial sediments reworked by fluvial geomorphology during the development of the Baltic Sea (Saarse et al., 2009). Over 40% of the island is covered with forests, predominantly pine forests (*Pinus sylvestris*), mixed forests and broad-leaved (deciduous) trees (e.g., *Quercus robur*) are found. Sandy soils (Podzols), peatland soils (Histosols), waterlogged soils (Gleysols) and lessive soils (Luvisols) comprise most of the soil cover in southern Estonia, whereas Limestone rendzinas (Rendzic Leptosols) and calcareous soils (Calcaric Regosols) are typical in western Estonia (Reintam et al., 2005). Both study areas are regions with the densest calcareous spring fen network in Estonia. The fens in West region in Saaremaa, have relatively uniform vegetation, dominated by *Schoenus ferruginaeus*, *Carex davalliana* and *Molinia caerulea*, while the fens in South region are more variable in species composition but usually dominated by *Carex* spp., *Molinia caerulea*, *Phragmites australis*.

Pollen Data

Moss polster samples (size of 5 cm in radius) were collected to determine the modern pollen assemblage from each of the fen sites ($n = 34$). The moss samples consisted of a relatively wide range of species, most abundant being *Calliergonella cuspidata*, *Plagiomnium ellipticum, Scorpidium cossonii, Campylium stellatum, Sphagnum subnitens.* In the majority of sites, the mosses did not form tense tussocks but the structure was relatively loose. For pollen analysis, only the green (living) upper part of the mosses was collected. Moss sample collection was synchronized with vegetation inventories. All the sampling and preservation of moss polsters followed the Crackles Bequest Project protocol (Bunting et al., 2013). For diversity reconstructions, we used sediment pollen assemblages from 53 sedimentary samples of Kanna spring fen (Blaus et al., 2019).

Both modern (polster) and historic (sediment) pollen samples were treated with HCl and 10% KOH followed by standard acetolysis method (Berglund and Ralska-Jasiewiczowa, 1986; Faegri and Iversen, 1989). Both moss and sediment samples were examined under a light microscope at magnifications of 250, 400, and 1000x. Approximately 1000 terrestrial and aquatic pollen grains per moss polster and sediment pollen sample were counted (min = 920; max = 1115), spores of vascular plants were also counted.

The potential of pollen data to reconstruct vegetation proportions (and vegetation diversity) is hampered by interspecific differences in pollen production and dispersal causing over-representation of some taxa and under-representation of other taxa in pollen assemblages (Birks et al., 2016a). To reduce the influence of high pollen producers, we used Andersen's correction factors (Andersen, 1970) to downweight the dominant pollen taxa – a method that has been shown to improve the pollen-plant diversity relationships in earlier studies (Felde et al., 2016; Reitalu et al., 2019). All the pollen-based diversity estimates were, therefore, calculated on Andersen-transformed pollen counts.

Pollen data was divided into woody taxa (including trees and shrubs) and herbaceous taxa (including grasses and dwarf shrubs from the Ericaceae family) prior to the diversity calculations. Spores of pteridophytes are included in diversity calculations but for simplicity, we refer to the dataset as "pollen data" in the article.

Vegetation Data

Vegetation survey around each moss sample was carried out at the end of the flowering season during the second half of July and August (2017 and 2018). Survey timing and vegetation recording methodology followed Crackles Bequest Project protocol (Bunting et al., 2013), which is designed to produce vegetation abundance estimates in different distance classes in concentric areas around the pollen sample. Although vegetation mapping was conducted relatively late, only a few spring-flowering ephemerals (e.g., *Anemone*) might have been overlooked. At each site, vegetation was recorded within a 100 m radius around the moss sample (**Figure 1**). Within a 10 m radius, the vegetation was described in detail in five concentric areas at radii of 0.5, 1.5, 3, 6, and 10 m. In each 10 m area, twenty-one 1 × 1 m quadrats were systematically placed: one quadrat on top of the moss sample, four quadrats positioned at each cardinal direction at 1, 2.25, and 4.5 m from the central moss polster, and eight quadrats at 8 m distance from the center (**Figure 1**). In each 1 × 1 m quadrat, the percentage cover of all vascular plants was recorded. Additionally, species not occurring in the quadrats but within the circles were recorded. Between 10 and 100 m, vegetation types were mapped by using orthophoto maps (incl. infrared orthophoto maps) and field-work observations. The vegetation types were delimited keeping in mind the pollen perspective – for example, single trees and shrubs in open fen areas were mapped as separate entities because their role in pollen signal is potentially high (Bunting et al., 2013). Species composition of each vegetation type in 100 m radius was characterized in the field. For woody taxa, the percentage cover of each species was estimated. For herbaceous taxa, the Braun-Blanquet cover-abundance scale was used (Braun-Blanquet, 1964). The abundances were then translated to cover estimates within each vegetation type as follows: ±0.01%; 1 – 5%; 2 – 10%; 3 – 25%; 4 – 50%; 5 – 75%.

Altogether, plant cover estimates were calculated for nine different radii; 0.5, 1.5, 3, 6, 10, 25, 50, 75, and 100 m. For the radiuses up to 10 m, the plant cover estimates were averaged from the quadrats belonging to the particular radius. For the radiuses 25, 50, 75, and 100 m, the cover of each species was calculated from species cover estimates in different vegetation types weighted by the area of the vegetation type within the radius (**Figure 1**).

Similarly to pollen data, plant data was divided into woody taxa (including trees and shrubs) and herbaceous taxa (including grasses and dwarf shrubs from the Ericaceae family) prior to the diversity calculations.

Pollen−Plant Richness and Compositional Relationships in Different Radii

In order to clarify which spatial scale of vegetation is best reflected by modern pollen, we used plant cover estimates of nine different radii (0.5, 1.5, 3, 6, 10, 25, 50, 75, and 100 m) and plant richness of woody and herbaceous species was calculated for each radius. In addition to richness estimates, we used Principal Component Analysis (PCA) with Hellinger-transformed plant cover data (Legendre and Gallagher, 2001) to characterize the vegetation composition. PCA was performed separately for woody and herbaceous plant species in each radius. PCA ordinations of pollen data and plant data for two radii (6 m for herbaceous species and 100 m for woody species) are given in **Supplementary Figure S2**. Pollen richness estimates and PCA ordinations of Hellinger-transformed woody and herbaceous pollen percentages were used for comparison with corresponding plant estimates in different radii. Pollen-plant richness relationships in different radii were studied with the help of Pearson's correlation coefficients. Pollen–plant compositional relationships were analyzed with the help of Procrustes analysis (Gower, 2001) that introduces uniform scaling and rotation to minimize the squared differences between pollen-based and vegetation-based ordinations. The Procrustes symmetric sum of squares was used as "Procrustes correlation" to quantify the pollen–plant compositional relationship. We used the bootstrap method to provide confidence intervals for Pearson's correlation coefficients and Procrustes correlations (Davison and Hinkley, 1997).

The R environment (version 3.4.4) (R Core Team, 2018) was used for the analyses with packages "vegan" (Oksanen et al., 2017) and "boot" (Canty and Ripley, 2019) for ordination analyses and bootstrapping, respectively.

Functional Diversity and Ellenberg Indicators

To calculate the functional trait composition (CWM) and functional alpha diversity (FDa), we used nine traits that reflect different aspects of plant life history: clonality, height, specific leaf area (SLA), leaf dry matter content (LDMC), seed size, seed number, mycorrhizal type. For mycorrhizal type, we used the affiliation of plant species in three plant mycorrhizal types: arbuscular mycorrhiza (AM), ectomycorrhiza (EM) and ericoid mycorrhiza (ERM). Traits, the sources and availability of trait data and the functional significance of different traits are summarized in **Table 1**. Trait availability was the lowest for LDMC (only 40% of the species) and for SLA (62%), however, trait values were available for the most dominant taxa and we chose to include them in functional diversity calculations. Pteridophytes were included in trait assignments (except for seed weight and number).

Assigning trait values to pollen types that have only one species (for example, *Pinus* pollen type only includes *P. sylvestris* in Estonia) was straightforward. To calculate trait values for pollen types that included several different species the average trait values were calculated from the species recorded in our vegetation survey (for example, *Potentilla* pollen type includes *P. anserina*, *P. erecta*, *P. reptans*). Averages were also used for mycorrhizal types. In case one species can form two types of mycorrhizal associations, it is used in both estimates. Averaged trait values for pollen types are given in **Supplementary Table S1**.

Functional alpha diversity and CWM trait values were used to express FD. Species cover estimates and Andersen-transformed pollen percentages were used as weights for CWM calculations in plant and pollen data, respectively. "Functcomp" function in the "FD" R package (Laliberté et al., 2014) was used to calculate CWMs. FDa was calculated as an abundance weighted standardized effect size of mean pairwise distance (SESmpd) according to de Bello et al. (2016) for individual traits as well as all traits combined. Negative SESmpd indicates functional convergence and positive SESmpd indicates functional divergence; values ≤ -2 and ≥ 2 indicate significant ($P < 0.05$) convergence and divergence, respectively (Gotelli and Graves, 1996). All diversity measures were calculated separately for herbaceous and woody taxa.

TABLE 1 | Plant traits used to examine functional composition (CWM) and alpha diversity (FDa) of pollen and plant communities.

Trait	Source	Definition and functional significance	Trait availability (% of species/pollen)
Seed weight (g)	Kühn et al., 2004; Liu et al., 2008	Related to dispersal distances and seed nutrient provision strategy, larger seeds have a better chance to establish as seedlings but generally shorter dispersal distances (Kleyer et al., 2008).	78/87
Height (m) (AH)	Kleyer et al., 2008	Correlated with life span and time to maturity, reflecting species' ability to compete for light (Westoby et al., 2002).	86/99
Leaf dry matter content (mg/g) (LDMC)	Kleyer et al., 2008	The ratio of leaf dry mass to fresh mass, an indicator of plant resource use strategy. High LDMC reflects conservation of resources usually in unproductive environments (Garnier et al., 2001)	40/45
Specific leaf area (mm^2/mg) (SLA)	Kleyer et al., 2008	The ratio of leaf area to leaf dry mass. Responsive to light and moisture levels, related to drought stress (Cornelissen et al., 2003).	62/73
Seed number	Kleyer et al., 2008	Related to regeneration strategies, usually inversely associated with seed size (Saatkamp et al., 2018).	70/84
Clonality	Kühn et al., 2004	Derived from "plant reproduction type" with the original qualitative trait coded as follows: "mostly vegetatively, rarely by seed" = 0.9, "by seed and vegetatively" = 0.5, "mostly by seed, rarely vegetatively" = 0.1, "by seed" = 0. Reflects plant ability to spread vegetatively. Clonal structures can also serve as storage organs (Cornelissen et al., 2014).	100/100
Arbuscular mycorrhiza (AM)	Hempel et al., 2013; Chaudhary et al., 2016; Bueno et al., 2019	Association between plants and fungi defined by the presence of arbuscules in root cortex cells (Brundrett and Tedersoo, 2018). Most widespread among herbaceous plants. Related to acquisition of soil resources, mainly phosphorus (Brundrett, 2009).	98/100
Ectomycorrhiza (EM)	Hempel et al., 2013; Chaudhary et al., 2016; Bueno et al., 2019	Association between plants and fungi defined by the presence of a Hartig net and mantle (Brundrett and Tedersoo, 2018). Dominant mycorrhizal type in temperate and boreal forest ecosystems, related to acquisition of soil resources, mostly nitrogen (Brundrett, 2009).	98/100
Ericoid mycorrhiza (ERM)	Hempel et al., 2013; Chaudhary et al., 2016; Bueno et al., 2019	Formed between plants in the family Ericaceae and a diverse group of soil fungi. Represents adaptation to harsh, acidic and nutrient poor soils such as in bogs and heathlands (Cairney and Meharg, 2003).	98/100

In addition to the functional diversity, community weighted EIVs of light (L), temperature (T), continentality (K), moisture (F), pH reaction (R) and nitrogen or nutrient availability (N) were tested with modern pollen-plant relationships. EIVs were calculated as community weighted mean values similarly to CWM trait values described above.

Phylogenetic Diversity

Phylogenetic diversity (PD) is based on the phylogenetic relationship amongst the coexisting species in the community and is represented by a phylogenetic tree (Brocchieri, 2016). To estimate PD, we calculated the phylogenetic tree from the latest megaphylogeny for vascular plants in the "V.PhyloMaker" R package (Jin and Qian, 2019). Similarly to FDa, PD was expressed as the standardized effect size of mean pairwise distance (SESmpd). The mean pairwise distance was estimated across the whole phylogenetic tree by averaging all species pairwise distances (Pavoine and Bonsall, 2010). SESmpd was calculated by comparing the observed community mpd to the null distribution of randomized communities with equal species richness using the null model "taxa.labels," implemented in the "picante" R package (Kembel et al., 2010). SESmpd was calculated separately for woody and herbaceous species, and additionally for only angiosperms in both woody and herbaceous groups. Negative values of SESmpd indicate phylogenetic clustering (i.e., the coexistence of closely related species), whereas positive values indicate over-dispersion (i.e., the coexistence of distantly related species) (Webb et al., 2002). When pollen taxa included more than one species, we randomly chose one representative species from the list of species in our vegetation survey, for example, Primulaceae was represented by *Primula farinosa*.

Pollen-Plant Functional and Phylogenetic Diversity Comparisons

Based on our results of richness and vegetation composition analyses in different radii (**Figure 1**) and published literature on the source area of pollen for the herbaceous (Hjelle, 1998; Bunting, 2003; Bunting and Hjelle, 2010) and woody species (Broström et al., 2005; Mazier et al., 2008), we used two radii – 6 m for herbaceous species and 100 m for woody species – to test the modern pollen–plant functional and phylogenetic diversity and EIV relationships. To test for significant pollen-plant associations of CWM, FDa, PD, and EIV measures we used correlation analyses. Prior to the correlation tests, we tested for the normal distribution of the variables and several variables were log-transformed to achieve normality (**Tables 2–5**). Pearson's correlation coefficient was used when both variables were normally distributed. Alternatively, Kendall's Tau coefficient was used. To control for the effect of multiple tests (52 correlation tests altogether), we used Benjamini and Hochberg (1995) correction approach.

To test the influence of the region (West and South) on the pollen–plant diversity relationships, linear regression models were fitted for all pollen–plant diversity associations (CWM, FDa, PD, and EIV) with pollen diversity (pollenD) as the response variable and corresponding plant diversity (plantD), region (Reg) and their interaction term (plantD:Reg) as explanatory variables. The full model was given as: pollenD $\sim$ plantD + plantD:Reg. Backward selection with the function "stepAIC" [and with $k = 4$ (the multiple of the number of degrees of freedom used for the penalty)] in the "MASS" R package (Venables and Ripley, 2002) was used to clarify which combination of predictors significantly explained pollen richness. Assumptions for using linear models were tested. In some cases, outlier(s) were excluded in order to meet the assumptions for linear modeling (**Supplementary Figure S3**).

Sediment Pollen-Based Reconstructions

Finally, we applied the different diversity metrics (richness, CWM, FDa, PD) and EIVs tested in modern pollen–plant data to Kanna spring fen pollen sequence (53 pollen samples). Kanna spring fen is located in Viidumäe Nature Reserve (22.096721 °E; 58.325031 °N) in the western part of Saaremaa Island (**Figure 1**). The peat accumulation in Kanna began 9.2 ka (calibrated kilo annum before present). The age-depth model is based on 11 AMS radiocarbon dates and dates from spherical fly ash particles, peat accumulation was generally uniform and continuous (Blaus et al., 2019). Blaus et al. (2019) separated three main stages of the Kanna fen development: bog phase (9.2–7.2 ka) characterized by ombrotrophic conditions; fen phase (7.2–0.4 ka) characterized by minerotrophic conditions; and "recent phase" characterized by shifts in dominant fen taxa, possibly caused by human impact. These three development phases were also used in the current study to examine the functional (FDa, CWM), PD and EIV shifts associated with major changes in Kanna fen development. We used the diversity metrics which showed significant correlations between modern pollen and plant data (**Tables 2–5**) for reconstructions. Generalized additive models (GAM) with the 95% confidence intervals were used to fit the relationships of each diversity variable with time using the "mgcv" R package (Wood, 2011).

RESULTS

Pollen–Plant Richness and Compositional Relationships

The results of Pearson's correlations with bootstrapped 95% confidence intervals showed significant positive correlations

TABLE 2 | Correlation test results for pollen- and plant-based community weighted mean (CWM) trait values (functional trait composition).

Trait	Log	Correlation coefficient	*p*-Value/BH *p*-Value	LM results
Woody taxa				
AM		**$r = 0.59$**	**0.000/0.001**	**PlantD + Reg**
EM		$\tau = 0.21$	0.086/0.135	PlantD
Clonality	log(pol), log(pla)	**$r = 0.42$**	**0.013/0.027**	**PlantD + Reg**
Seed weight	log(pol), log(pla)	$r = 0.28$	0.111/0.166	PlantD*Reg
Height		**$r = 0.41$**	**0.017/0.033**	**PlantD**
LDMC		**$\tau = 0.54$**	**0.000/0.000**	**PlantD + Reg**
Seed number	log(pol), log(pla)	**$r = 0.57$**	**0.000/0.002**	**PlantD + Reg**
SLA	log(pol)	**$r = 0.85$**	**0.000/0.000**	**PlantD + Reg**
Herbaceous taxa				
AM		$\tau = -0.33$	0.005/0.013	PlantD*Reg
EM		$\tau = 0.10$	0.443/0.536	–
ERM		**$\tau = 0.51$**	**0.000/0.001**	**–**
Clonality		$r = -0.08$	0.670/0.741	Reg
Seed weight	log(pla)	$r = -0.15$	0.410/0.521	–
Height	log(pla)	$r = 0.36$	0.034/0.061	PlantD
LDMC		**$r = 0.56$**	**0.001/0.002**	**PlantD**
Seed number	log(pol), log(pla)	**$r = 0.40$**	**0.018/0.035**	**PlantD**
SLA		**$r = 0.66$**	**0.000/0.000**	**PlantD*Reg**

*Plant CWMs were calculated at 100 m for woody taxa and at 6 m for herbaceous taxa. Trait abbreviations are clarified in **Table 1**. Column "Log" indicates for which variables plant (pla) and/or pollen (pol) data were log-transformed prior to the correlation test. When the tested variables were normally distributed, Pearson's correlation coefficient (r) was used, alternatively Kendall's Tau (τ) was used. Original p-Value and the Benjamini-Hochberg corrected p-Value (BH p-Value) are given. Results with BH p-Value < 0.05 for correlation are marked bold. Linear models (LM) results show whether corresponding plant diversity (PlantD), region (Reg) and their interaction (PlantD*Reg) were significant ($p < 0.05$) predictors of pollen diversity.*

TABLE 3 | Correlation test results for pollen- and plant-based functional alpha diversity FDa estimates.

Trait	Log	Correlation coefficient	*p*-Value/BH *p*-Value	LM results
Woody taxa				
AM		**$\tau = 0.33$**	**0.006/0.014**	**Reg**
EM		$r = 0.34$	0.046/0.080	PlantD*Reg
Clonality		**$\tau = 0.39$**	**0.001/0.003**	**PlantD**
Seed weight	log(pla)	$r = 0.02$	0.904/0.930	Reg
Height		$\tau = 0.10$	0.426/0.528	–
LDMC		$r = -0.03$	0.870/0.923	–
Seed number		**$r = 0.66$**	**0.000/0.000**	**PlantD**
SLA		**$r = 0.54$**	**0.001/0.003**	**PlantD + Reg**
All traits		**$r = 0.60$**	**0.000/0.001**	**PlantD**
Herbaceous taxa				
AM		$\tau = -0.20$	0.097/0.148	Reg
EM		$\tau = 0.14$	0.262/0.349	–
ERM	log(pol), log(pla)	**$r = 0.56$**	**0.001/0.002**	**PlantD**
Clonality		**$r = 0.43$**	**0.012/0.027**	**PlantD**
Seed weight		$\tau = -0.11$	0.377/0.489	Reg
Height	log(pol), log(pla)	$r = 0.10$	0.575/0.665	–
LDMC		$r = 0.38$	0.028/0.052	PlantD + Reg
Seed number		$\tau = -0.01$	0.930/0.930	–
SLA	log(pol)	**$r = 0.41$**	**0.016/0.032**	**PlantD*Reg**
All traits		$r = -0.02$	0.917/0.930	Reg

*Trait abbreviations are clarified in **Table 1**. Plant FDa was calculated at 100 m for woody taxa and at 6 m for herbaceous taxa. Column "Log" indicates for which variables plant and/or pollen data were log-transformed prior to the correlation test. When the tested variables were normally distributed, Pearson's correlation coefficient (r) was used, alternatively Kendall's Tau (τ) was used. Original p-Value and the Benjamini-Hochberg corrected p-Value (BH p-Value) are given. Results with BH p-Value < 0.05 for correlation are marked bold. Linear models (LM) results show whether corresponding plant diversity (PlantD), region (Reg) and their interaction (PlantD*Reg) were significant (p < 0.05) predictors of pollen diversity.*

TABLE 4 | Correlation test results between pollen and plant based phylogenetic diversity.

Trait	Log	Correlation coefficient	*p*-Value/BH *p*-Value	LM results
Woody taxa				
All		$\tau = 0.23$	0.053/0.089	–
Angiosperms		$\tau = 0.06$	0.638/0.721	–
Herbaceous taxa				
All	log(pol), log(pla)	**$r = 0.63$**	**0.000/0.003**	**PlantD*Reg**
Angiosperms		$r = 0.20$	0.262/0.349	–

*Plant PD was calculated at 100 m for woody taxa and at 6 m for herbaceous taxa. Column "Log" indicates for which variables plant and/or pollen data were log-transformed prior to the correlation test. When the tested variables were normally distributed, Pearson's correlation coefficient (r) was used, alternatively Kendall's Tau (τ) was used. Original p-Value and the Benjamini-Hochberg corrected p-Value (BH p-Value) are given. Results with BH p-Value < 0.05 for correlation are marked bold. Linear models (LM) results show whether corresponding plant diversity (PlantD), region (Reg) and their interaction (PlantD*Reg) were significant (p < 0.05) predictors of pollen diversity.*

for herbaceous taxa at the scales of vegetation estimate up to 6 m ($r = 0.42$–0.47, **Figure 2**). For woody taxa, the pollen-plant richness correlation was not significant at any of the vegetation estimate scales. Compositional relationships assessed by Procrustes' analysis showed positive associations for herbaceous taxa at all vegetation estimate scales. For woody taxa, the Procrustes correlations were significantly positive at the scales of 6 to 100 m.

Linear regression model showed that for herbaceous taxa both plant richness ($t = 4.26$, $p < 0.001$) and region ($t = -5.02$, $p < 0.001$) were significant predictors of pollen richness (determination coefficient $R^2 = 0.5$). Pollen richness increased with increasing plant richness in both regions and region South had significantly higher pollen richness than region West (**Figure 3**). In contrast, for woody taxa, the regression model did not indicate any significant variables describing pollen richness (**Supplementary Figure S1b**).

Pollen-Plant Functional and Phylogenetic Diversity Comparisons

Correlations between pollen–plant CWM traits of woody taxa showed significant positive pollen–plant associations for SLA, seed number, AM, LDMC, clonality and plant height (**Table 2**). Linear regressions for CWMs of woody taxa indicated that both plant-based CWM estimate and region (South vs. West)

TABLE 5 | Results of correlation tests between pollen-plant based Ellenberg indicators (EIVs) of light (L); temperature (T); continentality (K); moisture (F); soil pH reaction (R); nutrient availability (N) for woody and herbaceous plants.

Trait	Correlation coefficient	*p*-Value/BH *p*-Value	LM results
Woody taxa			
L	***r* = 0.72**	**0.000/0.000**	**PlantD + Reg**
T	***r* = 0.59**	**0.000/0.001**	**PlantD**
K	τ = 0.22	0.075/0.122	–
F	**τ = 0.41**	**0.001/0.002**	**PlantD + Reg**
R	***r* = 0.50**	**0.003/0.007**	**Reg**
N	*r* = 0.21	0.224/0.315	PlantD*Reg
Herbaceous taxa			
L	**τ = 0.66**	**0.000/0.000**	**PlantD + Reg**
T	**τ = 0.34**	**0.004/0.010**	**PlantD*Reg**
K	τ = 0.17	0.174/0.251	–
F	τ = −0.03	0.814/0.882	Reg
R	τ = −0.08	0.536/0.634	–
N	**τ = 0.39**	**0.001/0.003**	**PlantD**

*When the tested variables were normally distributed, Pearson's correlation coefficient (r) was used, alternatively Kendall's Tau (τ) was used. Original p-Value and the Benjamini-Hochberg corrected p-Value (BH p-Value) are given. Results with BH p-Value < 0.05 for correlation are marked bold. Linear models (LM) results show whether corresponding plant diversity (PlantD), region (Reg) and their interaction (PlantD*Reg) were significant ($p < 0.05$) predictors of pollen diversity.*

were significant predictors of pollen CWM (**Table 2** and **Supplementary Figure S3**). However, the interaction term of the region and plant CWM was not significant. For herbaceous taxa, pollen and plant CWM traits were significantly positively correlated for four traits: ERM, SLA, LDMC and seed number. For the herbaceous CWM of LDMC and seed number, the plant-based estimate was the only significant predictor of pollen-based CWM estimate (**Table 2**). For SLA, the pollen-plant CWM relationship differed significantly between the regions.

Pollen and plant functional alpha diversity (FDa) of woody taxa was significantly positively correlated for four traits: seed number, SLA, clonality and AM (**Table 3**). Linear models for FDa of woody taxa showed that for seed number and clonality, plant estimate was the only significant predictor of pollen diversity, for SLA and clonality, region was also significant (**Table 3** and **Supplementary Figure S3**). The correlation between pollen and plant FDa calculated across all traits was significantly positive for woody taxa and the association did not depend on the region. Herbaceous taxa showed positive significant FDa correlation for three traits: ERM, clonality and SLA. Linear regressions showed that for ERM and clonality, corresponding plant-based diversity estimate was the only significant predictor, for SLA the pollen-plant relationship differed between the regions (**Table 3**).

Phylogenetic diversity (PD) was significantly positively correlated between pollen and plant data only for herbaceous taxa, the significant correlation disappeared when pterophytes were excluded from the calculation (**Table 4**). Linear regression indicated that the slope of herbaceous pollen-plant PD relationship differed between the regions (**Table 4**). Woody taxa did not show significant PD correlations.

The Ellenberg indicator values (EIVs) based on woody taxa were significantly positively correlated between pollen- and plant-based estimates for the indicators of light (L), temperature (T), soil reaction (R) and moisture (F) (**Table 5**). Linear regressions showed that for woody taxa pollen-based T estimate, plant-based T was the only significant predictor, pollen-based L and F were significantly associated with corresponding plant-based estimate and region (**Table 5**). Ellenberg R estimate based on woody pollen taxa was significantly associated only with the region. EIVs based on herbaceous taxa were significantly positively correlated for L, T and nutrient content (N). Linear regressions showed that for herbaceous pollen-based N estimate, plant-based N was the only significant predictor, for L and T region was also significant (**Table 5** and **Supplementary Figure S3**).

Reconstructions in Time

Pollen richness of both woody and herbaceous taxa was the lowest in the beginning of the mire development (9-8 ka) and increased between 8 and 6 ka (**Figure 4**). Richness of herbaceous taxa was more or less stable until present day but richness of woody taxa decreased during the last 2 ka.

Community weighted mean values of AM, SLA and LDMC showed relatively similar trends for woody and herbaceous taxa through the fen development – AM and SLA had their maximum values between 6 and 2 ka and LDMC was the lowest during the same period (**Figure 5**). Plant height, seed number and clonality had contrasting trends through time for woody and herbaceous taxon groups. For woody taxa, clonality was the highest and height was the lowest between 7 and 2 ka. For herbaceous taxa, clonality was the lowest around 7 ka and the plant height had its maximum between 6 and 2 ka. Herbaceous seed number and ERM were the highest in the beginning of the study period (9-7 ka) (**Figure 5**).

Functional alpha diversity was the highest in the beginning of the study period for several traits: AM and clonality for both woody and herbaceous taxa, seed number and ERM for herbaceous taxa and SLA for woody taxa (**Figure 6**). During the middle of the study period, the FDa values of several traits decreased notably indicating a shift from functional divergence toward functional clustering. FDa values were close to or above 2 indicating significant functional divergence for seed number, AM and ERM in herbaceous taxa and for SLA and AM in woody taxa. The tendency toward functional convergence was detected only for woody taxon clonality.

Phylogenetic diversity showed relatively little change through time (**Figure 7**). The highest PD based on woody taxa was shown for the beginning of the study period (9-7 ka) when PD was close to or above 2 indicating phylogenetic divergence. When gymnosperms were excluded from the PD calculation, the values dropped drastically and indicated phylogenetic convergence throughout the study period (SESmpd < -2). PD of herbaceous angiosperms increased around 7 to 6 ka.

Ellenberg indicator valuess based on woody taxa indicated that moisture (F), light (L), and continentality (K) were higher during the beginning of the study period and close to the present-day and

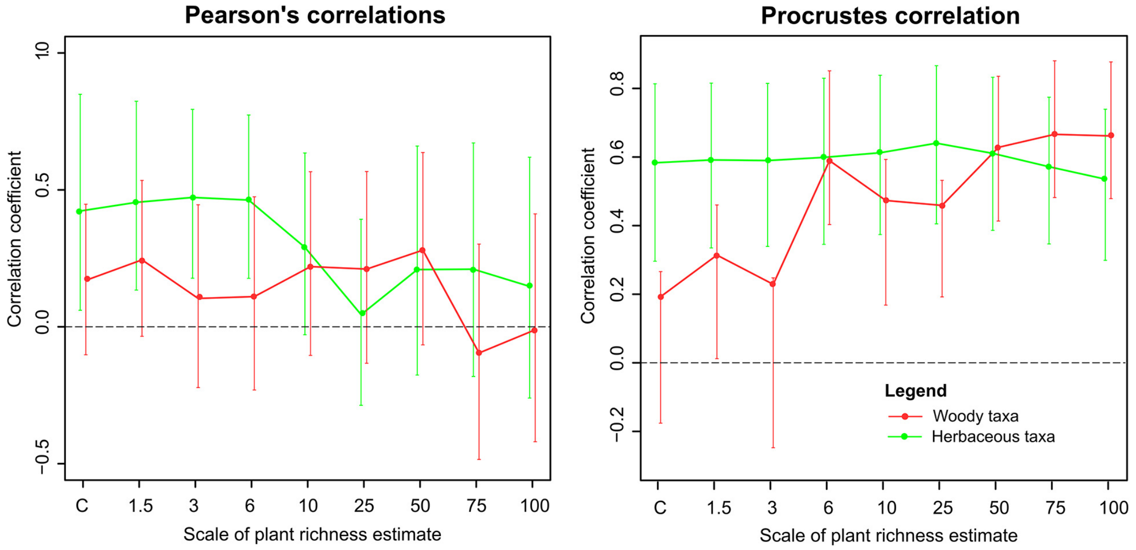

FIGURE 2 | Pollen–plant richness and composition correlations. *Y*-axis: the values of Pearson's correlation coefficients (for richness on the left) and Protest correlations (for vegetation composition on the right). *X*-axis: the radius of plant estimate. Vertical lines indicate bootstrapped 95% confidence intervals for the correlations.

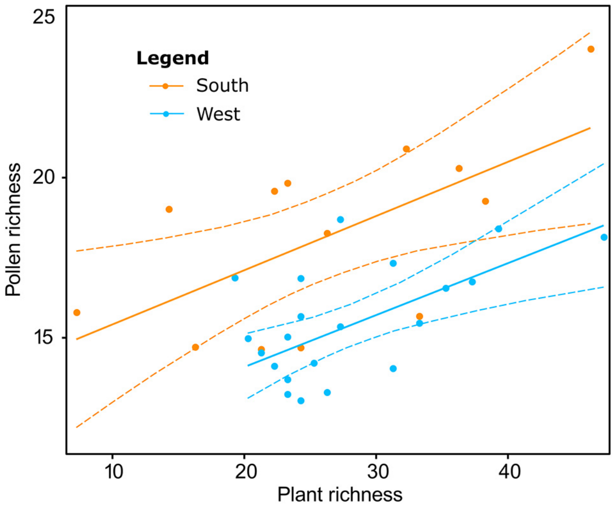

FIGURE 3 | Association between pollen-based and plant-based herbaceous richness showing the influence of a geographical region [South (orange) vs. West (blue)]. Herbaceous plant richness at 6 m scale is used. Linear model with backward selection of variables indicates that plant richness (t = 4.26, p < 0.001) and region (t = –5.02, p < 0.001) are significant predictors of pollen richness (determination coefficient R^2 = 0.5).

had the lowest values between 6-5 ka. For herbaceous taxa, the same indicators show little change or slight increase toward the present-day. The R for herbaceous taxa showed a strong increase from 7 to 5 ka, R for woody taxa increased during the same period but the change was less pronounced. Nutrient availability (N) increased according to both woody and herbaceous taxa between 7 and 5 ka but the N-value based on woody plants decreased and N-value based on herbaceous taxa stayed more-or-less stable from 5 ka until the present.

DISCUSSION

The use of sedimentary pollen to reconstruct functional and phylogenetic diversity is a relatively novel approach with previous studies focusing mainly on functional trait composition (community weighted mean trait values) (e.g., Reitalu et al., 2015; Brussel et al., 2018; Carvalho et al., 2019; van der Sande et al., 2019). In addition, Reitalu et al. (2015) calculated sedimentary pollen based functional alpha diversity and phylogenetic diversity that allow to study convergence and divergence patterns in community assembly. Our study adds to the evidence from modern pollen-plant studies of functional aspects (Carvalho et al., 2019) that both functional composition and alpha diversity can be studied using sedimentary pollen data and have the potential to provide valuable knowledge on palaeo-environments in addition to the conventional methods. However, not all studied variables had significant pollen-plant associations highlighting that uncritical use of different diversity metrics in pollen-based reconstructions may be misleading.

Taxonomic Diversity

Modern Pollen–Plant Richness and Composition

We hypothesized that modern pollen richness and composition reflect vegetation richness and composition, and that this relationship is dependent on plant group (woody vs. herbaceous) and spatial scale. The results partly supported our predictions showing that pollen richness of herbaceous taxa in moss polster samples best reflects herbaceous vegetation richness within 0.5–6 m radius and pollen-plant compositional relationship

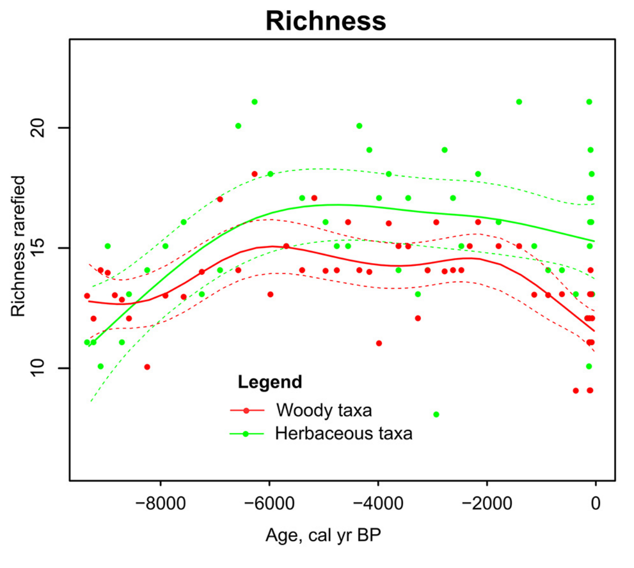

FIGURE 4 | Sedimentary pollen based richness in Kanna spring fen. Generalized additive model of herbaceous taxa (green line) and woody taxa (red line) in time. Dashed lines enclose the 95% confidence intervals of trend lines.

for herbaceous taxa was significant at all scales (**Figure 2**). The positive richness association existed in both study areas (**Figure 3**). This adds to the growing evidence that pollen taxonomic diversity is positively associated with the surrounding plant diversity (Urrego et al., 2011; Meltsov et al., 2012; Jantz et al., 2014; Matthias et al., 2015; Birks et al., 2016a; Mourelle and Prieto, 2016; Reitalu et al., 2019). In pollen analytical studies, the scale of the vegetation reflected in pollen sites is expressed as the relevant source area of pollen (RSAP). The RSAP is the area beyond which the pollen–plant correlation does not improve (Sugita, 1994). Our results for herbaceous taxa agree with existing studies from moss polsters where the RSAP of non-arboreal (non-woody) taxa is in the range of 0.5 – 10 m (Hjelle, 1998; Bunting, 2003; Bunting and Hjelle, 2010). When the RSAP calculation involves woody taxa, the RSAP estimations from the moss polsters are in the range of 300 – 400 m (Broström et al., 2005; Mazier et al., 2008) exceeding the scale of our vegetation survey. The lack of significant richness correlation for woody taxa in our study may be caused by the fact that our study radius was too small. However, the forests in the study regions are relatively uniform and increasing the radius would not add many new woody species. Pollen richness of woody taxa has been shown to be strongly positively associated with woody plant richness in a broad-scale study across entire N Europe (Reitalu et al., 2019) where the number of tree species varied from 3 to 80. In the current study, the richness of woody plant species varied from 11 to 22 and we can surmise that the variation in woody plant richness is too small to be reflected by the pollen data. However, the compositional relationship between pollen-plant data of woody taxa was significant at scales of 6 – 100 m radius indicating that vegetation composition of woody taxa is reflected by the pollen data.

While palaeo-diversity is traditionally calculated for all taxa combined (see overview in Birks et al., 2016a), our results support the studies suggesting to look at the diversity of wind-pollinated and insect-pollinated taxa (Meltsov et al., 2011) or at woody and herbaceous taxa separately (Carvalho et al., 2019; Reitalu et al., 2019). To conclude, dividing woody and herbaceous taxa in pollen-based diversity estimates improves our understanding of spatial scales reflected in palaeo-diversity reconstructions. Woody and herbaceous plants clearly form different functional groups (Díaz et al., 2016), and analyzing their diversity separately allows for better comparisons with contemporary plant ecological studies.

Sediment Pollen Based Richness Reconstruction

The richness of both woody and herbaceous taxa was relatively low in the beginning of the fen development from 9.2 to 7.2 ka and increased to the highest levels by ca 6 ka. Similar trend of increasing palynological richness in the beginning of the Holocene can be observed in other studies from Saaremaa (Poska and Saarse, 2002) and in the entire region (Reitalu et al., 2015). Palaeoecological studies from N Europe often show an increase in pollen richness during the last 2 or even 4 ka, associated with human-induced landscape opening (Birks and Line, 1992; Berglund et al., 2008; Reitalu et al., 2015). In the current study, herbaceous richness was relatively stable and woody richness decreased during the last millennia. Based on the modern pollen-plant correlations, herbaceous pollen richness from the fen samples is most likely to reflect local within-fen richness changes and taxa associated with agricultural activities and landscape opening coming from outside the fen are less likely to contribute. The decline in the richness of woody species is most likely a reflection of climate-driven decrease in broadleaved trees and increase in pine dominance that today has established a relatively homogeneous forest cover in the landscape.

Functional Diversity

Modern Pollen–Plant Relationships

We documented significant positive pollen-plant correlations for six CWM traits for woody taxa and for four CWM traits for herbaceous taxa (**Table 2**). Functional alpha diversity (FDa) had significant positive associations for five traits in woody taxa and for three traits in herbaceous taxa (**Table 3**). Woody taxa might show stronger relationships because fewer species are included in each pollen type, and when trait values for pollen types are calculated by trait averaging more information is lost for herbaceous species. In our dataset, the only woody pollen type including large number of species is *Salix* but for herbaceous taxa many pollen types have comparatively large numbers of species (e.g., Asteraceae $n = 27$; Cyperaceae $n = 51$; Poaceae $n = 41$; Orchidaceae $n = 17$). Similarly to our study, the modern pollen-plant study from fens in England showed that trait averaging for herbaceous pollen types might reduce the pollen-derived trait variability (Carvalho et al., 2019). Trait averaging is likely to have a stronger effect on the FDa (measured as mean trait distance between species/taxa in a community) than on the CWM that is already a result of averaging. FDa is particularly difficult to assess for taxon-trait combinations where the trait variation

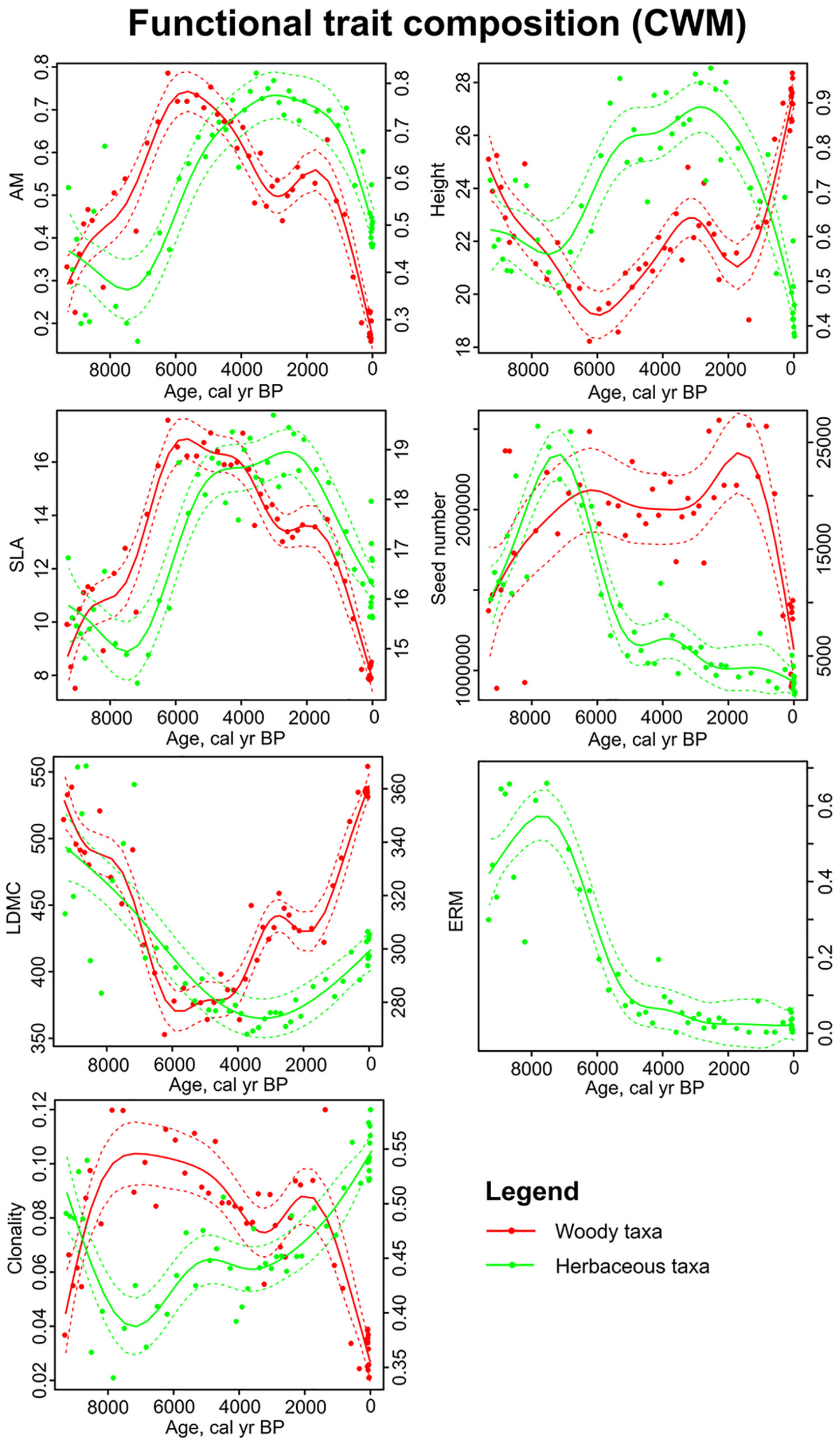

FIGURE 5 | Sedimentary pollen based reconstructions of functional trait composition (CWM) in Kanna spring fen. Generalized additive models in relation to time for woody (red line) and herbaceous taxa (green line). Red and green dashed lines enclose the 95% confidence intervals of trend lines. Traits of herbaceous taxa is plotted on the secondary y-axes. Abbreviations of trait names are explained in **Table 1**.

within pollen taxon is large (for example AM and plant height in Cyperaceae) leading to significant effects of trait averaging in pollen data. Even though mycorrhizal type, plant height and seed weight can be expected to be associated with waterlogging and nutrient stress in fen systems (Jabłońska et al., 2019), our modern pollen-plant analyses indicate that pollen might not be the best

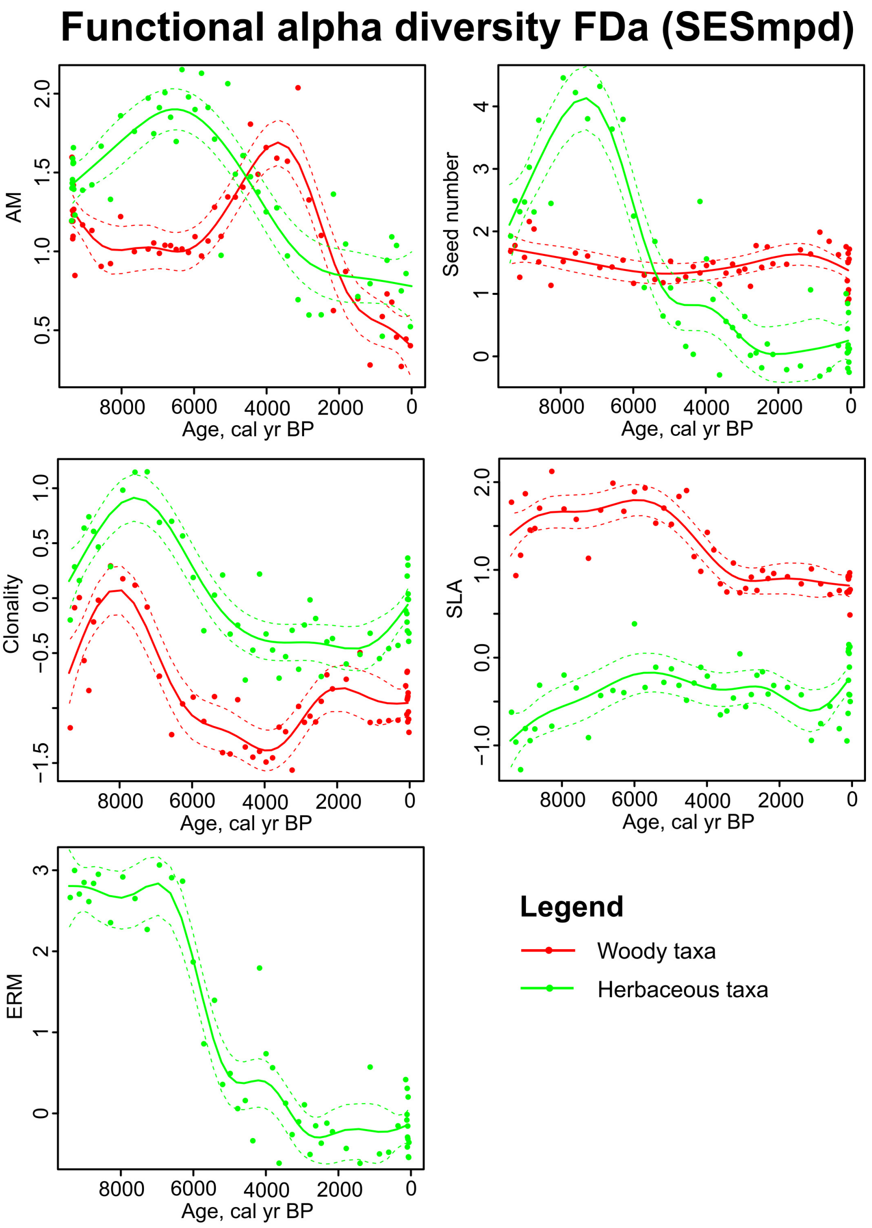

FIGURE 6 | Sedimentary pollen based reconstructions of functional alpha diversity (FDa) in Kanna spring fen. Generalized additive models in relation to time for woody (red line) and herbaceous taxa (green line). FDa is expressed as a standardized effect size of mean pairwise distance (SESmpd). Red and green dashed lines enclose the 95% confidence intervals of trend lines. Abbreviations of the trait names are explained in **Table 1**.

proxy to reconstruct the long-term changes in these traits. Plant macro remains (Felde and Birks, 2019; Jabłońska et al., 2019) or sedaDNA (Parducci et al., 2014) may give better taxonomic resolution. Another possible reason for the lack of significant correlation in modern pollen-plant data is related to short gradients within the study area, for example, EM associations are characteristic to the majority of present-day Estonian woody taxa and the CWM of EM varied between 0.8 and 1. Similarly to the missing woody taxon richness association, we can surmise that the variation is too small to be reflected by the pollen data and modern pollen-plant studies across larger geographic extent and longer diversity gradients would improve our understanding of the pollen plant diversity associations (Davis et al., 2013).

The diversity estimates that showed significant pollen-plant associations for woody taxa can be assumed to be associated with climate change (mycorrhizal type, clonality, height, LDMC, SLA), disturbance (height, clonality, seed number) and competitive strength (mycorrhizal type, height, clonality, SLA) (Cornelissen et al., 2003; Brundrett and Tedersoo, 2018). The woody taxon functional diversity – reflecting the landscape-scale changes – can therefore be used to reconstruct trait responses to climate change and long-term human disturbances and

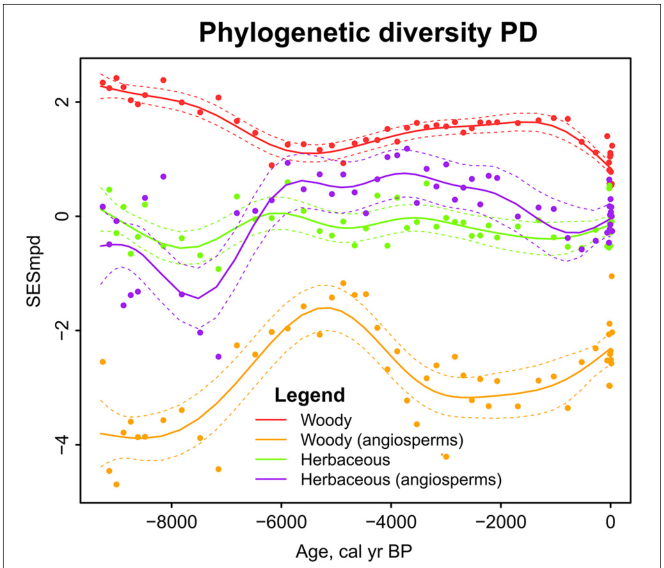

FIGURE 7 | Sedimentary pollen based reconstruction of phylogenetic diversity (PD) in Kanna spring fen. Generalized additive models in relation to time. Dashed lines enclose the 95% confidence intervals of trend lines.

to quantify the role of competition for woody community assembly (Reitalu et al., 2015). As herbaceous pollen diversity mostly reflects change in local fen community, we can expect traits to respond to waterlogging and nutrient stress (SLA, LDMC, clonality) (Jabłońska et al., 2019), changes in soil reaction (ERM), disturbance by drainage (seed number) and to indicate competition within the fen community (clonality, SLA) (Cornelissen et al., 2003).

We hypothesized that the pollen–plant diversity relationships do not differ between the study areas (southern Estonia and western Estonia). This was true for most of the functional diversity estimates – out of the traits that had significant pollen-plant correlations, the interaction with region was only significant for herbaceous SLA (**Tables 2–3**). The region effect on SLA is most likely the result of small variation in SLA in the fens of western region while in the southern region, there is much longer gradient in the SLA (**Supplementary Figures S3k,ab**). For the majority of traits, pollen-plant association was similar in both regions giving assurance that the diversity estimate can be used in palaeoreconstructions.

Sediment Pollen Based Functional Diversity Reconstruction

Woody taxa

The trait composition (CWM) of woody taxa showed low clonality, low proportion of AM associations, low SLA, and high LDMC during the first millennia of Kanna site development (9.2-7 ka) (**Figure 5**). Both SLA and LDMC have been widely used to reflect species' response to soil fertility and climate change (Cornelissen et al., 2003; Laughlin et al., 2010) with SLA being positively and LDMC negatively associated with soil fertility and warmer climate. After the ice retreated, Saaremaa was under the waters of the Baltic Ice Lake and the highest parts of the island emerged after the drainage of the lake at about 10.3 ka (Poska and Saarse, 2002). The peat in Kanna site began to accumulate right after the site emerged from the water due to land uplift (Blaus et al., 2019) and the beginning of vegetation development represents establishment of the pioneer communities. Early site development was dominated by *Pinus sylvestris* (Blaus et al., 2019) that is a good example of species with resource conservation strategy by reducing water loss as an adaptation to nutrient-poor environments through low LDMC (Qin and Shangguan, 2019). Clonality, which is associated with abiotic stress (Klimeš et al., 1997; Ye et al., 2014), reached maximum around 8 ka. However, the variation in clonality of woody plants is relatively small and is likely to be mainly associated with encroachment by clonal shrubs like *Salix* and *Myrica* in the wet depressions.

Most of the trait CWMs showed relatively rapid turnover during 8-6 ka and the period between 6 and 4 ka is characterized by the highest SLA and AM values and the lowest LDMC for the entire period. These responses are likely to be associated with fundamental trade-offs related to nutrient conservation, nutrient acquisition and turnover (Wright et al., 2004), that in turn reflect warmer and drier climatic conditions during the mid-Holocene (Hammarlund et al., 2002) when favorable conditions for expansion of thermophilous tree species existed in Estonia (Saarse and Veski, 2001; Sillasoo et al., 2007). High CWM of AM coincides with immigration of some AM associated species e.g., *Ulmus gabra* (Thomas et al., 2018) and *Fraxinus excelsior* (Seven and Polle, 2014) and the trends are in line with findings that show correlation between warmer temperature and general AM fungal activity (Compant et al., 2010; Hempel et al., 2013). Around 2 ka, CWM of SLA and AM declined rapidly coinciding with climate cooling in the area (Hammarlund et al., 2002) and spruce (*Picea abies*) expansion in Saaremaa (Saarse et al., 1999). Overall, the trait CWM changes of woody taxa in our study area are compatible with reconstructed biome dynamics for Northern Europe based on plant functional types (Davis et al., 2015).

The FDa values of woody plants were above zero for most of the traits except clonality (**Figure 6**). Although most of the values were below significant trait overdispersion, there was a strong overall tendency toward niche differentiation in AM, seed number and SLA, especially in the beginning of the study period. This pattern is most likely associated with the many fundamental functional differences between gymnosperms and angiosperms (Brodribb et al., 2012) allowing for their long-term coexistence.

Herbaceous taxa

Between 9 and 7 ka Kanna fen developed as a small bog with a high abundance of Ericaceae species, presence of Poaceae and Cyperaceae (Blaus et al., 2019) with a shift to minerotrophic fen starting around 7 ka. For the "bog phase" of the mire development, we expected to find trait composition indicative of low productivity and adaptations to acidic conditions. In accordance with the expectations, our results indicated high values for ERM, seed number, LDMC, clonality and low values for SLA and plant height (**Figure 5**). Several of the traits have been hypothesized to mitigate nutrient scarcity in wetlands

(LDMC, mycorrhizal type, clonality) (Moor et al., 2017; Jabłońska et al., 2019) and to be associated with waterlogging gradient (LDMC, SLA) (Violle et al., 2011; Baastrup-Spohr et al., 2015; Jabłońska et al., 2019). The "bog phase" was characterized by high proportion of Ericaceae taxa (that have low SLA, high LDMC and ERM) that are typical for nutrient poor and acidic soils (Cairney and Meharg, 2003).

Substantial changes in fen plant CWM traits during 7-5 ka coincide with the site transition to minerotrophic fen environment caused by the unique combination of climatic, topographic and hydrological conditions (Blaus et al., 2019). The "fen phase" of the mire development is characterized by increased water, calcium carbonate and organic matter content and occurrence of fen habitat specialists (e.g., *Potentilla erecta, Parnassia palustris*) as well as by high palynological richness (Blaus et al., 2019). High SLA and low LDMC during the fen phase are in good accordance with earlier studies showing that high SLA and low LDMC are associated with increase in soil reaction (Bartelheimer and Poschlod, 2015), in water and nutrient content (e.g., Díaz et al., 2004; Ordońez et al., 2010; Moor et al., 2015), anoxia (Klimkowska et al., 2019), organic matter accumulation (Nieder and Benbi, 2008), and species richness (Violle et al., 2011). High values for AM during the fen phase suggest similarly to Ramírez-Viga et al. (2018) that AM associations have an important role in wetlands.

After 2 ka, herbaceous CWM decreased for SLA and seed number but increased for LDMC and clonality. This functional change is related to the increasing proportions of Cyperaceae and the decline in the abundance of forbs that characterizes the "recent phase" of Kanna fen development (Blaus et al., 2019). Decreasing SLA and increasing LDMC have been associated with drier conditions in wetlands (Baastrup-Spohr et al., 2015). Jabłońska et al. (2019) showed that the importance of tussock-forming species with small clonal spread increased in fens prior to the termination of fen phase (and development of bog or mire woodland) and the reported functional changes might be an early "warning" for nature conservation. The actual reasons behind the recent functional changes and increase in Cyperaceae are rather unclear and it is difficult to draw a border between local and larger scale processes. Even if the changes are autogenic or successional these processes are usually following to allogenic disturbances (Bodini and Klotz, 2009). Besides, some studies have shown that climate induced hydrological changes in wetlands might have greater impact on plant community changes compared to autogenic processes (Wilcox, 2004; Dieleman et al., 2014).

Functional alpha diversity of ERM and seed number showed significant functional divergence (**Figure 6**). In grassland communities, functional divergence has been associated with niche differentiation at fine scales (within one square meter and less) where herbaceous species interact (de Bello et al., 2013). At larger spatial scales (within habitat patches), the functional divergence is likely a result of fine-scale within-habitat environmental heterogeneity where species with different traits occur side by side (de Bello et al., 2013; Bergholz et al., 2017). In addition to Ericaceae and other bog indicators, several typical fen species (*Potentilla*, *Parnassia*, *Cladium mariscus*) were present in low numbers in Kanna fen initial phase pollen samples (Blaus et al., 2019) and most probably grew on the edges of the small bog leading to the functional divergence pattern in the pollen signal.

Phylogenetic Diversity

Pteridophytes and gymnosperms, being evolutionarily distant from angiosperms, have a strong impact on the phylogenetic structure of the vegetation (e.g., Massante et al., 2019). Modern pollen-plant PD associations in our study were also strongly influenced by pteridophytes (especially ferns) and gymnosperms. Pollen-plant PD correlation was significant for herbaceous taxa but only when pteridophytes were included in the calculation (**Table 4**). Ferns and their spores were closely related between vegetation and pollen samples, meaning that if ferns were present in vegetation their spores occurred frequently in the moss polster.

Results of PD reconstruction in Kanna fen exhibited significant clustering (phylogenetic convergence) of woody taxa throughout the 9.2 ka when gymnosperms were excluded from the PD calculation but overdispersion (phylogenetic divergence) was evident when gymnosperms were included in the reconstruction (**Figure 7**). This pattern is consistent with the study of Reitalu et al. (2015) who showed that gymnosperms significantly increased the phylogenetic overdispersion. Massante et al. (2019) found that woody taxa are phylogenetically overdispersed at high latitudes compared to low latitudes, because after originating in the tropics, specific and distant lineages of woody taxa, i.e. gymnosperms, were able to adapt and survive extinctions in cold temperate conditions. The slight decrease in overdispersion in woody taxa in the mid-Holocene is probably caused by climate changes that shaped PD by colonization of broad-leaved species (Eiserhardt et al., 2015). Weakening of the clustering of woody angiosperms at the same time supports the studies that identify overdispersion as related to warm and nutrient-rich conditions (Cavender-Bares et al., 2004; Spasojevic and Suding, 2012). These favorable conditions for plant growth most probably weaken the effect of environmental filtering for specific phylogenetic clades with adaptations for extreme environmental conditions, and allow species from distant lineages to coexist.

The PD of angiosperm herbaceous taxa followed the main phases of Kanna fen development (**Figure 7**). During the ombrotrophic stage (ca. 9-7 ka), the PD of herbaceous angiosperms tended toward phylogenetic clustering, most probably influenced by the high abundances of different Ericaceae species (Blaus et al., 2019). Clustering has been observed for species pools of relatively young habitats (Lososová et al., 2015), and could be related to site emergence from the sea and extreme environmental conditions. Clustering has commonly been explained as a result of environmental filtering, since closely related species are usually expected to be ecologically similar (Webb et al., 2002). In particular, low nitrogen and soil reaction 9.2 ka might have led to environmental stress and filtering the lineages not adapted to particular conditions (González-Caro et al., 2014). However, the functional diversity reconstructions showed functional over-dispersion for several traits during the same period, indicating that the functional-phylogenetic diversity relationships are more complex.

The fen phase (ca. 7-2 ka) was characterized by relatively high PD as a result of phylogenetically distinct species co-occurring together, and may depend mechanistically on climate as shown by Svenning et al. (2015). This stage coincides with the increase in summer temperatures (Renssen et al., 2009) and the maximum of palynological richness (**Figure 4**). Empirical studies have justified that PD typically covaries with species richness at different scales (Mace and Purvis, 2008; Mooers et al., 2008; Kluge and Kessler, 2011), reflecting patterns of species migration and diversification (Forest et al., 2007). PDs of both herbaceous and woody taxa showed a slight decrease toward the present day, likely in response to the increase in Cyperaceae within the fen, and decline of broad-leaved taxa in the surrounding landscape, respectively. The decline also coincides with decreasing pollen richness. In contrast, Reitalu et al. (2015) showed a decrease in PD in spite of the increase in pollen-derived richness associated with human impact, particularly, the facilitated increase in ruderal communities. To conclude, PD can be reconstructed from pollen data at least to some extent, it has a different dynamic in time among the plant growth forms and our example shows that period of high taxonomic richness in the natural fen system is also characterized by high PD.

Ellenberg Indicator Values

The applicability of Ellenberg indicator values (EIVs) for different habitat types and in different regions in Europe has evoked considerable discussion in plant ecological literature (e.g., Schaffers and Sýkora, 2000; Diekmann, 2003; Smart and Scott, 2004; Williams et al., 2011; Bartelheimer and Poschlod, 2015). EIVs have been widely used in different mire systems to study a wide range of hypothesis (e.g., Cornwell and Grubb, 2003; Williams et al., 2011; Andersen et al., 2013; Gawenda-Kempczyńska, 2016; Klimkowska et al., 2019) and our findings add to those studies by showing the indicators that could be applied on sediment pollen and therefore could be used to interpret historical environmental gradients.

In current study, we found significant positive pollen-plant correlations for woody taxon EIVs of light (L), temperature (T), humidity (F), and soil reaction (R) (**Table 5**). Diekmann (2003) points out that short environmental gradients and within-sample heterogeneity may lead to unreliable EIV results. Because pollen samples of woody taxa are "sampling" vegetation from a relatively large area, results from heterogeneous study areas may be misleading as they can only provide average values for the entire area.

For herbaceous taxa significant pollen-plant associations were found for EIVs of light (L), temperature (T) and nutrient availability (N) (**Table 5**). Humidity (F) and soil reaction (R) that are recognized as main drivers of plant species diversity and composition of spring fen ecosystems (Hájková and Hájek, 2003; Sekulová et al., 2011) did not show significant pollen-plant associations. Our modern pollen-plant sampling scheme was designed to include open and overgrown fens but did not include large gradients of pH and humidity. The mean pH was 6.5 ± 0.7 and only three sites had pH < 6. Spring fens have relatively constant water availability, therefore moisture levels are high and do not vary much between the fens. The lack of positive pollen-plant associations for F and R may, therefore, be related to the relatively short gradient lengths in these indicators (cf. Diekmann, 2003). Ellenberg R has been shown to be relatively weakly associated with pH in wetlands (Williams et al., 2011) and might be better associated with calcium content (Schaffers and Sýkora, 2000). Our results showing significant pollen-plant correlations for L and N are promising as both indicators have

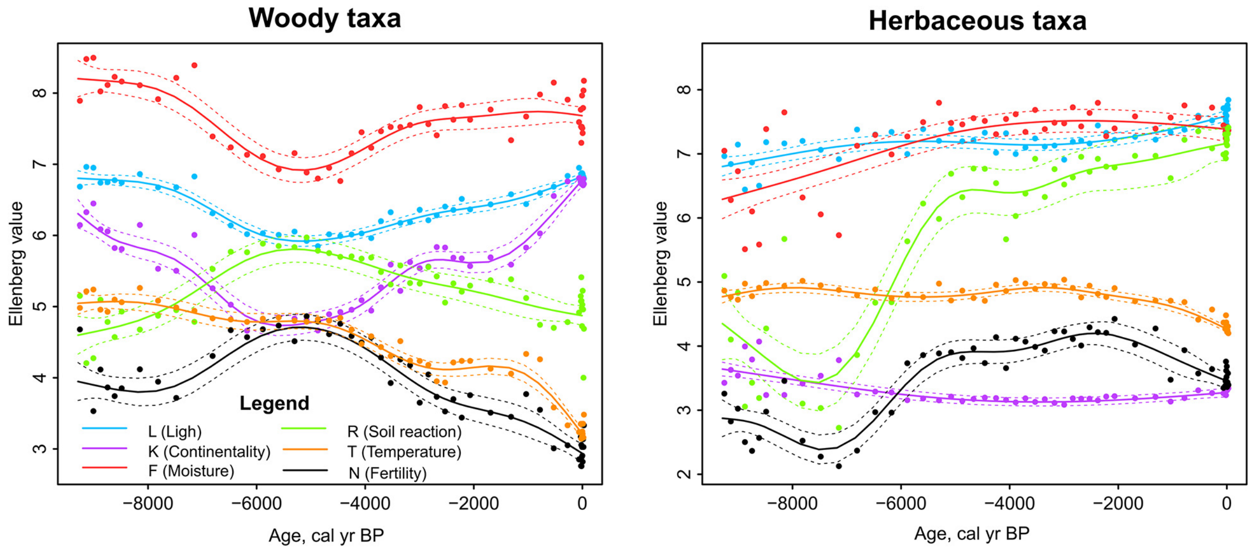

FIGURE 8 | Sedimentary pollen based reconstructions of Ellenberg indicator values (EIVs) in Kanna spring fen. Generalized additive models in relation to time. Dashed lines enclose the 95% confidence intervals of trend lines.

been shown to be significantly associated with major gradients in fen species composition (Kotowski and van Diggelen, 2004; Andersen et al., 2013) and have great potential for pollen-based palaeoreconstructions from fens.

The reconstruction of EIVs from Kanna sediment pollen shows that the beginning of site development is characterized by low Ellenberg values of N and R (**Figure 8**) – which is in line with functional characteristics of both woody and herbaceous taxa and with earlier interpretations of site development history (Blaus et al., 2019). Indicators of L, F and K revealed by woody taxa were relatively low from 7 to 3 ka coinciding with mid-Holocene thermal maximum (Davis et al., 2003) agreeing with the studies reporting dryer climatic conditions during the mid-Holocene (Hammarlund, 2003; Seppä and Poska, 2004). The opposite trends in R and L in the mid-Holocene support the results showing negative R and L correlations in forest, mostly because acido-tolerant but light-demanding species are outshaded by closed forest canopy (Diekmann, 2003).

From the herbaceous EIV reconstructions, R and N showed an increase between 7 and 5 ka (**Figure 8**) corresponding to the period when minerotrophic conditions developed in the fen. Ellenberg R has been shown to be well correlated with fen calcium carbonates (Schaffers and Sýkora, 2000) while Ellenberg N reflects biomass or fertility (Hill et al., 2000; Schaffers and Sýkora, 2000; Diekmann, 2003). Our results agree well with Bartelheimer and Poschlod (2015) who showed that N is positively associated with SLA and plant height among herbaceous species. High N coincides with high pollen richness and with high phylogenetic diversity of herbaceous taxa.

Temperature reconstructions from pollen data based on modern pollen calibration datasets are widely used in Quaternary palaeoecology (e.g., Seppä and Poska, 2004; Salonen et al., 2012). Our results together with earlier studies (e.g., Kuneš et al., 2011; Reitalu et al., 2015; Felde and Birks, 2019) indicate that there is also potential for reconstructing long-term changes in soil reaction (calcium content), fertility, moisture and light.

CONCLUSION

In a modern pollen-plant study from Estonian calcareous fens, our results suggest that while pollen of herbaceous taxa in fens reflects vegetation at local fen scale, the pollen of woody taxa is likely to reflect larger landscape scale forest vegetation. Dividing woody and herbaceous taxa in pollen-based diversity estimates improves our understanding of spatial scales reflected in palaeodiversity reconstructions. Woody and herbaceous plants clearly form different functional groups (Díaz et al., 2016), and analyzing their diversity separately allows for better comparisons with contemporary plant ecological studies.

Correlations between pollen- and plant-based estimates of functional and phylogenetic diversity indicated that pollen data reflected the functional and phylogenetic aspects of plant communities reasonably well. For most of the indicators pollen-plant association did not differ between the two study regions. However, not all tested variables exhibited positive modern pollen-plant associations. The CWM values were better reflected in pollen data than the FDa values based on mean pairwise distances between taxa in a sample. The interpretation of functional variables that have large variation within pollen types may be difficult because information is lost by averaging trait values for pollen types. Testing for various pollen–plant diversity associations in our dataset may have been partly hindered by relatively short gradients in diversity variables and we, therefore, encourage further studies of modern pollen–plant diversity relationships not only from fens but across various environments to help to increase the reliability of interpreting historical processes from sedimentary pollen data.

Reconstructions of different diversity aspects in Kanna spring fen through 9.2 ka of fen development showed that diversity of woody taxa was closely related to the main climate changes with the mid-Holocene warm period having contrasting functional and phylogenetic diversity values compared to both early- and late-Holocene. The largest changes in the FD and PD of herbaceous taxa were related to the bog-fen transition around 7 ka. Our results show that climate and other abiotic processes have considerably influenced community FD and PD through the 9.2 ka. FDa of both woody and herbaceous taxa indicated functional divergence for several periods of community development where coexisting taxa were less similar in their traits than expected by random.

To conclude, pollen-based functional and phylogenetic diversity estimates and EIVs provide valuable knowledge in addition to the conventional pollen analysis. The use of different diversity metrics helps to achieve a better understanding of environmental effects on different strategy mechanisms of plants and to better interpret long-term processes affecting community assembly in various palaeoenvironments.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Dryad data repository the Dryad Digital Repository (doi: 10.5061/dryad.wstqjq2hh).

AUTHOR CONTRIBUTIONS

AB and TR were the principal conceivers of the manuscript with the study design and conceptual idea by TR and the leading of writing by AB, with the input from all authors. AB and TR conducted the fieldworks of modern vegetation and pollen sample data collection and performed the data analysis. JM provided the script for phylogenetic analysis. IH compiled mycorrhizal type data. PG, IH, JM, and SV reviewed the manuscript and contributed significantly with the comments and valuable insights. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00207/full#supplementary-material

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Recent Changes in Peatland Testate Amoeba Functional Traits and Hydrology Within a Replicated Site Network in Northwestern Québec, Canada

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Northern peatlands, which are highly heterogeneous ecosystems, are a globally important carbon (C) store. Understanding the drivers and predicting the future trajectory of the peatland C store requires upscaling from cores and sites to regions and continents, alongside a detailed understanding of the mechanisms governing their C sequestration. Studies incorporating replication are therefore important to quantify how peatland heterogeneity may affect upscaling from local-scale dynamics to models. In addition, we need to better understand the processes driving observed variability, but the interplay between plants, microbes and C cycling in peatlands remains poorly understood. One approach to address both issues is to examine replicated microbiological functional traits within a multi-proxy framework to provide an ecosystem-level perspective on ecological and biogeochemical processes. Peatland testate amoebae are a functionally important group of protists that are well suited to such an approach. Analysing testate amoeba functional traits provides an opportunity to examine processes that may affect key peatland ecosystem services, such as C sequestration. Here, we compared four key testate amoeba functional traits (mixotrophy, biovolume, aperture size and aperture position) to C accumulation, hydrological and vegetation changes in 12 post-Little Ice Age peat records. Samples were collected from high-boreal and low-subarctic regions in northwestern Québec, Canada in an experimental design that includes internal and external replication at both site and regional scales. Our results showed that correspondence between C accumulation, hydrology and testate amoeba functional traits varied, but recent changes in mixotrophy and aperture size, which may affect peatland C sequestration potential and microbial food web structure, respectively, showed tentative links to recent C accumulation increases. Vegetation, especially *Sphagnum* abundance was important in promoting mixotrophy and small aperture size in testate amoeba communities. Future impacts of climate change on peatland vegetation will further influence the functional role of testate amoebae on C sequestration through changing mixotrophic testate amoeba abundance.

Keywords: testate amoebae, functional traits, palaeohydrology, vegetation, carbon accumulation, replicated sites, peatlands, climate change

INTRODUCTION

Peatlands, as an important terrestrial carbon (C) store (Yu, 2012; Loisel et al., 2014; Nichols and Peteet, 2019), are sensitive to climate change due to consequent changes in hydrology, vegetation and microbial communities. Understanding the response of peatland C cycling to climate change requires comprehensive interpretation of ecosystem-climate feedbacks. Several studies focus on the interactions between peatland hydrology and vegetation (Magnan et al., 2018; Van Bellen et al., 2018; Zhang et al., 2020), revealing spatial and temporal variations in their response to climate changes. Testate amoebae, a group of single-celled protists that are abundant and diverse in *Sphagnum*-dominated peatlands, are key constituents of peatland microbial communities, representing a considerable portion of microbial biomass (Gilbert et al., 1998). They are essential parts of peatland microbial food webs and play critical roles in nutrient and C cycling (Jassey et al., 2015). However, studies on such peatland microbial communities and their interactions with climate change, and ultimate influences on peatland C accumulation, are limited, except via their use as hydrological proxies.

Testate amoeba functional traits have been shown to strongly relate to ecosystem processes (Fournier et al., 2015; Jassey et al., 2015; Marcisz et al., 2016). Traditionally, two categories of functional traits can be defined: (1) response traits (i.e., shell biovolume, shell compression and aperture position) that reflect species' response to environmental change and (2) effect traits (i.e., aperture size and mixotrophy) that reveal species' impact on ecosystem processes (Violle et al., 2007). However, some traits can also act as both response and effect traits. For example, mixotrophy responds both to moisture and landscape openness while it also meaningfully contributes to C sequestration in peatlands (Jassey et al., 2015; Herbert et al., 2019; Lamentowicz et al., 2020). Previous studies have shown links between these traits and warming (Jassey et al., 2015), peatland water table (Lamentowicz et al., 2020), fire and dust deposition (Marcisz et al., 2016, 2019) and light penetration (Herbert et al., 2019). However, the derived patterns also suggest a high degree of spatial and temporal variation in these traits (Fournier et al., 2015). Particularly, in terms of understanding peatland C cycling, more studies are needed to understand how mixotrophic testate amoebae vary temporally and spatially in response to external environmental drivers, as they contribute to photosynthetic C fixation (Gilbert et al., 1998). Peatland C fixation is generally estimated by taking into account only *Sphagnum* growth, without considering photosynthetic microorganisms, despite the fact that C intake during photosynthesis by mixotrophic testate amoebae may be significant at the ecosystem level (Jassey et al., 2015; Zhang et al., 2020).

Future climate change, defined by increasing temperatures, spatially variable precipitation trends and more extreme events (Collins et al., 2013) may cause environmental stress and disturbance, leading to shifts in peatland ecosystem structure and function, for example permafrost thawing (Swindles et al., 2015a), increased fire activity (De Groot et al., 2013) and vegetation turn over (Kokkonen et al., 2019). All these might cause changes in microbial communities and functional trait assemblages. Due to the high degree of observed spatial and temporal variability in derived patterns between testate amoeba functional traits and environmental changes, the consequences to microbial communities of these climate change- (or potentially autogenically driven-, see Gałka et al., 2017b) induced switches in peatland vegetation and hydrology are largely unknown, but may have important implications for C and nutrient cycling. The upscaling or modeling of global peatland C cycling under climate change is based on the patterns derived from local studies. In order to constrain the changes that we see in our data and provide better information for global estimates, there is a need to understand the local- and regional-scale impacts of these drivers and how they might vary between site types (e.g., permafrost thawing).

In this study, we examined testate amoeba functional traits, i.e., mixotrophy, biovolume, aperture size and aperture position, and two key environmental factors in peatlands, i.e., hydrology and vegetation, in 12 peat records collected from four peatlands of two regions in northern Québec, Canada. We aim to investigate (1) the spatial and temporal variations of peatland testate amoeba functional traits using replicated peat records at the site and regional scale, (2) the external drivers of variations in functional traits, and (3) the potential impacts of functional trait-environment interactions on peatland C cycling.

MATERIALS AND METHODS

Study sites

The studied regions, Kuujjuarapik and Radisson, are located within the discontinuous and sporadic permafrost zones in northwestern Quebéc, Canada (**Figure 1**). Kuujjuarapik (peatland sites K1P and K2) represents the subarctic, forested tundra ecoregion at the southernmost limit of the discontinuous permafrost zone. K1P peatland is characterized by palsa mounds, whereas K2 is a small fen with peat thickness of ca. 1–2 meters. Radisson (peatland sites LG2 and Rad) represents the northernmost boreal ecoregion. LG2 and Rad are ombrotrophic

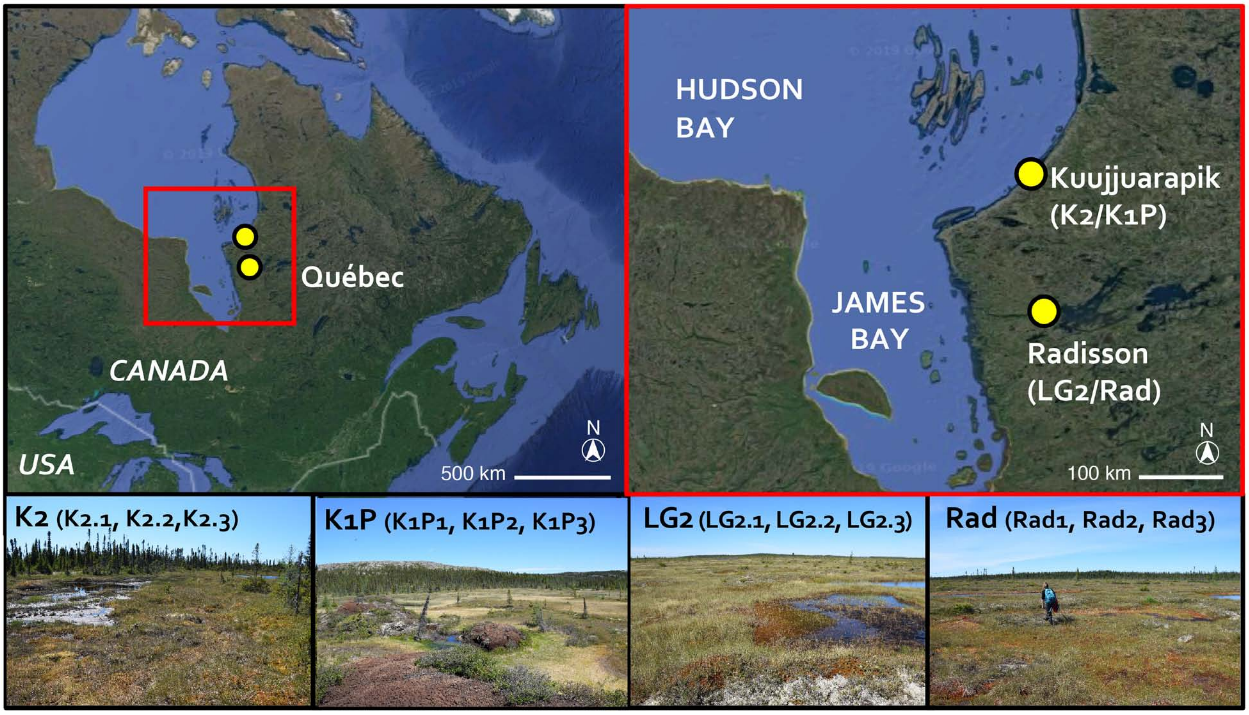

FIGURE 1 | Study site locations marked with yellow dots (base map © 2020 Google). Peatlands K2 and K1P are in Kuujjuarapik; LG2 and Rad are in Radisson. Pictures showing the coring sites (© SP) with the replicated three cores in each site indicated.

with peat thickness of ca. 3–4 meters. For more details of the studied peatlands, please refer to Piilo et al. (2019). In both study regions, mean annual temperatures are below 0°C (**Table 1**). In Kuujjuarapik, mean summer temperature has increased 1°C since 1961, while mean winter temperature has increased 0.5°C since 1971, and the increase in growing degree-days (GDD0) (between 1971–2000 and 1981–2010) is *ca.* 6%. In Radisson, since 1971, the biggest seasonal increase of 0.5°C is in mean autumn temperatures, and the increase in GDD0 is *ca.* 4%. In both regions, autumn rainfall especially has increased, and 35–40% of the precipitation falls as snow (Environment Canada, 2019).

Sampling

The studied 12 peat cores, three from each four peatlands (K2, K1P, LG2, and Rad), were collected in early July 2017 (**Figures 1**, **2** and **Table 1**). The core lengths ranged between 32 and 39 cm. Peat sections were sampled by hand, sawing the layer overlying seasonal frost or permafrost from intermediate lawn microforms (water tables ranged from 13 to 23 cm) inhabited mainly by *Sphagnum fuscum*. Once in the laboratory, the peat cores were cut into contiguous 1 cm slices for further analyses. Plant macrofossil, peat property and carbon accumulation results of these cores were previously published in Piilo et al. (2019).

Testate Amoeba Analysis

Testate amoeba analysis was performed at 2 cm resolution. Processing of testate amoeba samples followed a modified version of the standard method (Booth et al., 2010). Samples were simmered in distilled water for 15 min and stirred occasionally. The samples were then sieved with a 300-μm mesh and back sieved with a 15-μm mesh. Materials retained on the 15-μm sieve were centrifuged at 3000 rpm for 5 min. At least 100 individual shells for each sample were counted and identified to species or "type" level under a light microscope at the magnification of 200–400. Taxonomy followed Charman et al. (2000), supplemented with online sources (Siemensma, 2019).

Testate Amoeba Functional Trait Calculation and Data Analysis

We calculated the community weighted mean values of each standardized trait (**Table 1**; mixotrophy, biovolume, aperture size and aperture position), which is an index of functional composition expressed as the mean trait value of species present in the community, weighted by their relative abundances (Fournier et al., 2015). In addition, we calculated species richness and *Shannon-Wiener* diversity index (SWDI) for each sample. Values for test dimensions used to calculate all functional traits with the exception of mixotrophy were those published in the supplementary information of Fournier et al. (2015) except for species that were not included in that study. In those cases, we used average dimensions as described by Siemensma (2019) and in the case of one taxon (Pyxidicula type), an average of multiple replicates of our own light microscopic measurements. Mixotrophy was defined as a binary 0 (non-mixotroph) or 1 (mixotroph), also based on the definitions in Fournier et al. (2015).

TABLE 1 | Site information and analyzed testate amoeba functional trait description.

Region	MAT	MAP	GDD0	Peatland	Core	Testate amoeba functional trait description (Fournier et al., 2015)
Kuujjuarapik	−4.0	661	1384	K2	K2.1	Mixotrophy: indicates presence or absence of endosymbiotic algae-whether a species is a mixotroph or heterotroph (categorical variable, coded as 0-heterotroph, 1-mixotroph)
					K2.2	
					K2.2	
				K1P	K1P1	
					K1P2	Biovolume: volume of the shell occupied by the living amoeba (μm^3)
					K1P3	
Radisson	−2.9	697	1684	LG2	LG2.1	Aperture size: width of the shell aperture (μm)
					LG2.2	
					LG2.3	Aperture position: semi-continuous variable, with categories from terminal (1) to completely hidden (4)
				Rad	Rad1	
					Rad2	
					Rad3	

The meteorological data [(1981-2010): mean annual temperature (MAT, °C), mean annual precipitation (MAP, mm) and growing degree-days above zero (GDD0, presented as temperature degree sum)] are from stations Kuujjuarapik A in Kuujjuarapik and La Grande Rivière in Radisson (Environment Canada, 2019).

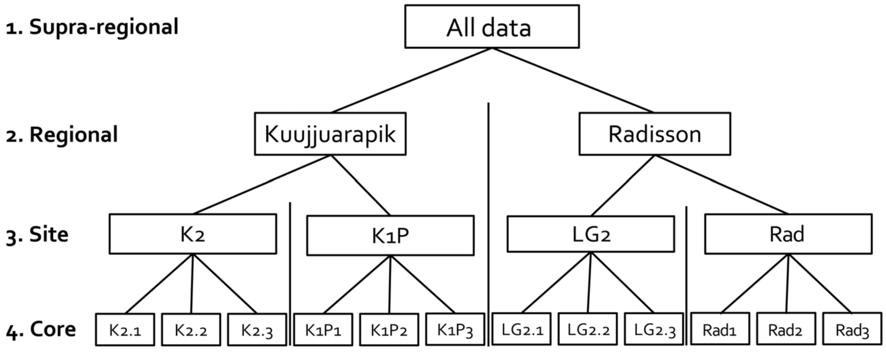

FIGURE 2 | Illustration showing the replicated site network design in this study. Numbered terms represent different spatial scales.

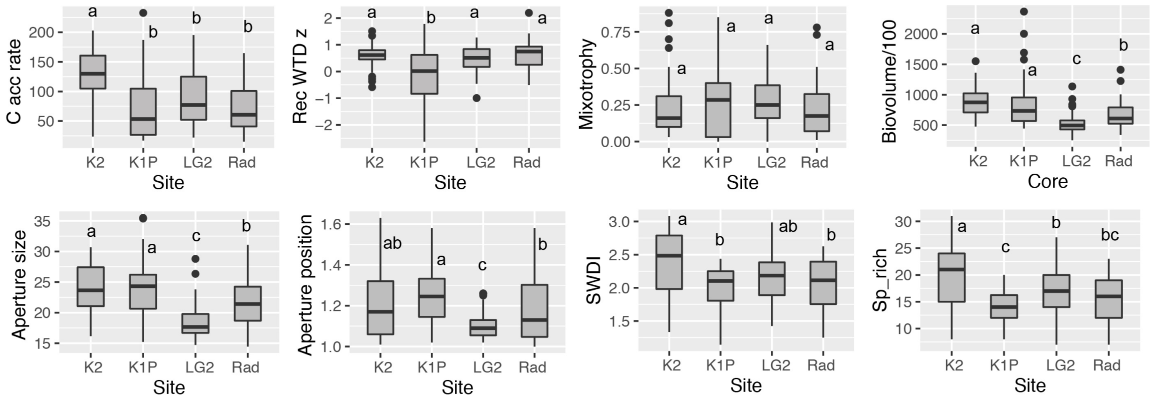

FIGURE 3 | Box plots of carbon accumulation rate (C acc rate; g C m^{-2} yr^{-1}), reconstructed water-table depth *z* score (Rec WTD *z*), mixotrophy, biovolume (μm^3), aperture size (μm), aperture position, *Shannon-Wiener* diversity index (SWDI), species richness (Sp_rich) and at each site. Different letters indicate significant differences ($p < 0.05$) among sites calculated using the Turkey's Honest Significant Difference method. Note the time span of each record included for each site varied and can be found in **Figure 4**.

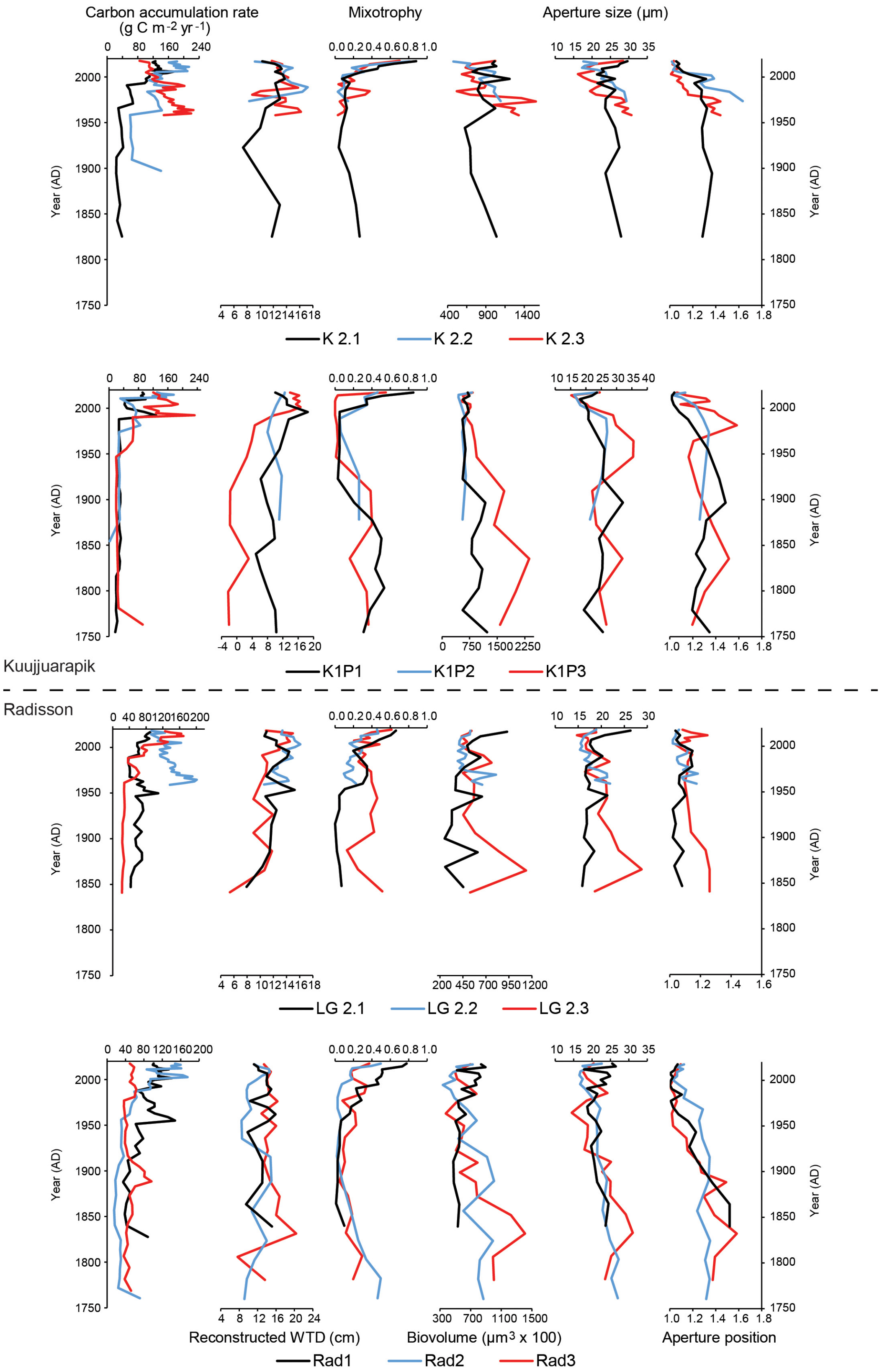

FIGURE 4 | Diagrams of carbon accumulation rates, reconstructed water-table depth (WTD), mixotrophy, biovolume, aperture size and position of testate amoebae for the studied peat records.

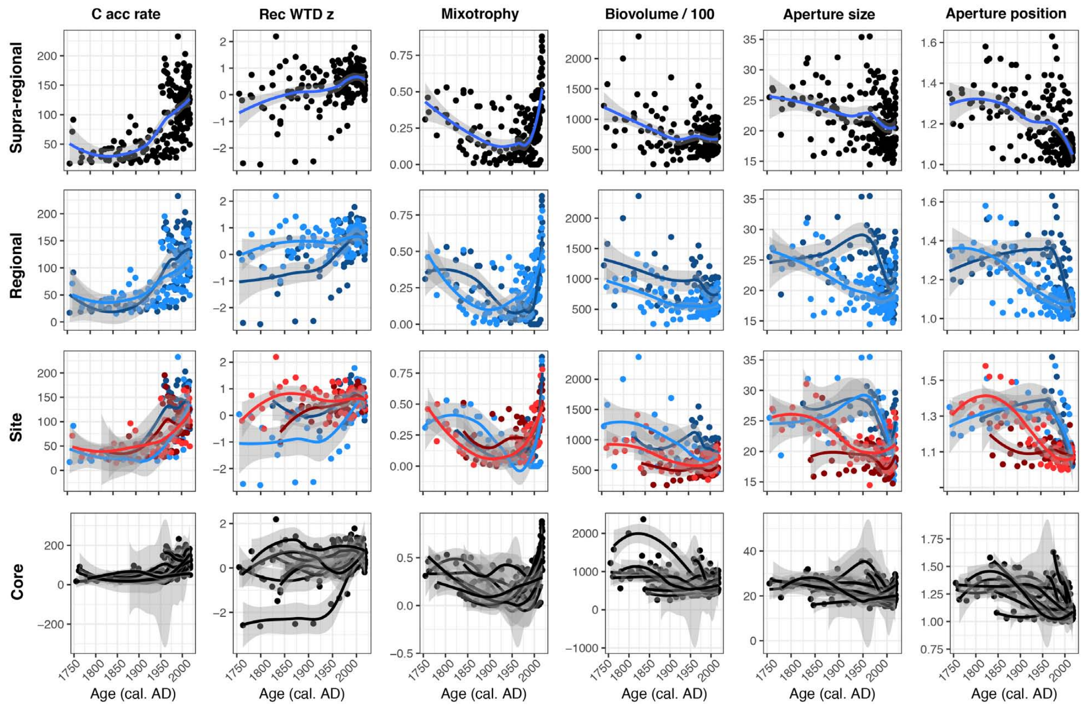

FIGURE 5 | Locally estimated scatterplot smoothing plots showing supra-regional, regional, site and core level variations of carbon accumulation rates (C acc rate; g C m^{-2} yr^{-1}), reconstructed water-table depth z score (Rec WTD *z*), mixotrophy, biovolume/100 (μm^3), aperture size (μm) and aperture position. In the Regional panels, dark blue represents region Kuujjuarapik and light blue represents Radisson. In the Site panels, dark blue represents site K2 and light blue represents K1P, while dark red represents site LG2 and light red represents Rad.

Testate amoeba-based water-table depth (WTD) reconstructions were performed using the transfer function developed by Amesbury et al. (2018). The absolute WTD values were normalised to z scores over the length of cores from each region (Swindles et al., 2015b; Amesbury et al., 2016). $Z > 0$ indicates drier conditions than the region's average, $z < 0$ indicates wetter than average. The analysis was conducted using "rioja" package in R version of 3.6.0 (R Core Team, 2019).

The chronologies for each peat record, developed using the "Bacon" package in R and based on ^{14}C and ^{210}Pb dating, were previously published in Piilo et al. (2019). A locally estimated scatterplot smoothing (LOESS) function with a span-value (degree of smoothing) setting of 0.5 was applied to the supra-regional, regional, site and core level data of C accumulation rate, reconstructed WTD and testate amoeba functional trait to explore the temporal trends and spatial variations. The analysis was conducted using the function loess () in R. Linear regression analysis (95% confidence intervals displayed) was applied to C accumulation and testate amoeba functional trait, and reconstructed WTD data using the function lm() in R.

A non-metric multidimensional scaling (NMDS) ordination analysis was applied to testate amoeba functional traits and reconstructed WTD, vegetation types (Piilo et al., 2019) and testate amoeba richness and diversity data to investigate the spatial variations and drivers of the recorded functional trait features. The analysis was performed using "vegan" package in R.

RESULTS

The age-depth models show that peat accumulation rates varied, and individual core basal ages varied from *ca*. cal. −550 AD to *ca*. cal. 1960 AD. In general, peat accumulation rates increased toward the present, starting from the 1950s, even though peatland-specific variations in accumulation rates existed. However, this recent increase of peat accumulation rates might be partly due to the incomplete decomposition process and lower compaction of the surface peat. In spite of this uncertainty, by using specified focus periods, the data nevertheless enable spatial-temporal comparisons (Piilo et al., 2019). The focused time-period in this study is the past *ca*. 200 years, which covers the post-Little Ice Age warming and recent warming since 1850 AD. The plant macrofossil (Piilo et al., 2019), testate amoeba community, testate amoeba functional trait, and carbon accumulation (Piilo et al., 2019) data show some consistent features, but also clear core-, site- and region-specific variations (**Figures 3–5**).

Testate Amoeba Community and Reconstructed Water-Table Depth

In total, 64 taxa were found in the studied 12 peat records (**Supplementary Figure S1**). In general, the most commonly recorded taxa in all peat cores were *Alabasta militaris*, *Archerella flavum*, *Hyalosphenia elegans*, and *H. papilio*. Also, *Difflugia pulex*

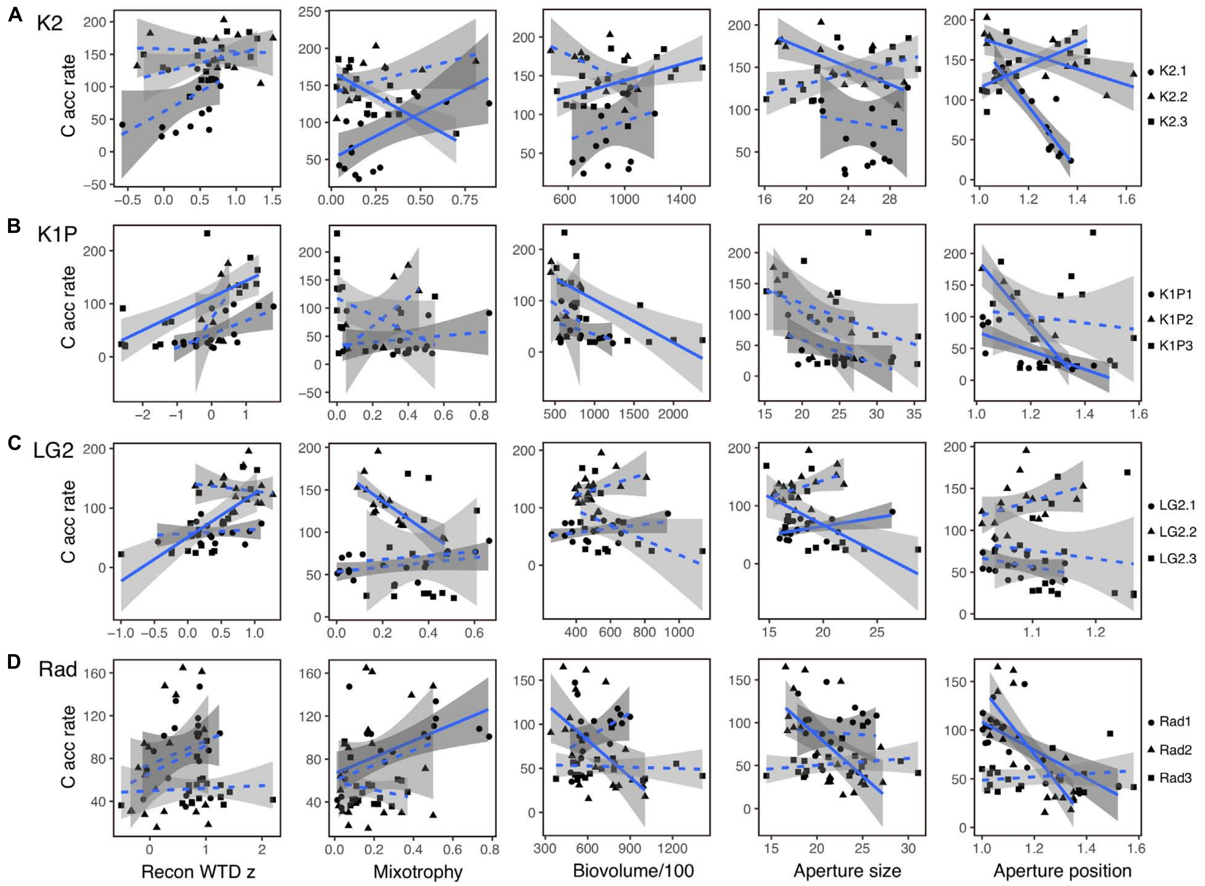

FIGURE 6 | Linear regression analysis of carbon accumulation rate (C acc rate; g C m^{-2} y^{-1}) and reconstructed water-table depth z score (Rec WTD *z*), mixotrophy, biovolume/100 (μm^3), aperture size (μm) and aperture position for each peat record at sites K2 **(A)**, K1P **(B)**, LG2 **(C)** and Rad **(D)**. The gray shading areas represent the 95% confidence intervals. Correlations with $p < 0.05$ were indicated using solid lines and correlations with $p > 0.05$ using dashed lines.

was dominant in Radisson cores. In addition, the Pyxidicula type (**Supplementary Figure S2**), currently unidentifiable to the species level, was found in all the peat records, and in some cases the proportion reached 50%. Site K2 showed higher species richness (average 21) than the other three sites (average around 15) (**Figure 3**). Sample-specific values for the Shannon-Wiener diversity index (SWDI) were higher at site K2 (average 2.5) than other sites (average 2.1). Temporally, both richness and diversity presented a remarkable decrease starting from *ca.* 1980 AD at 11 out of 12 records (**Supplementary Figure S1**). The compositional change of testate amoebae clearly indicated a consistent pattern in all the 12 records, i.e., mixotrophic taxa *Hyalosphenia elegans*, *H. papilio* and for some cores *Heleopera sphagni* became dominant (peak percentage around 50%) in the surface peat sections, even though the timing varied for different cores, ranging from early 1900 to late 1900 AD. Detailed testate amoeba community composition for each peat record can be found in **Supplementary Figure S1**.

Reconstructed water-table depth showed variations within each site and each region. The moisture conditions for the studied records mainly remained lawn features for the last few centuries. K1P site (especially core K1P3) recorded the largest variations and the wettest conditions (**Figures 3**, **4**). Kuujjuarapik region was wetter than Radisson, especially for the period before 1950 AD (**Figure 5**), and Kuujjuarapik showed a clear drying starting from early 1900s, while the moisture conditions at Radisson remained relatively stable.

Testate Amoeba Functional Traits

Mixotrophy did not show significant differences between different sites, but a consistent increasing trend toward recent decades (**Figures 3–5**). The lowest mixotrophy occurred around 1950–2000 and 1900 at regions Kuujjuarapik and Radisson, respectively. Biovolume and aperture size were significantly higher at sites LG2 and Rad than K2 and K1P. Biovolume showed a general decreasing trend toward the present, even though some records showed an increasing trend (LG2.1 and Rad1). Aperture position at site LG2 was lower than other three sites. Regionally, aperture size and position at Radisson showed a decreasing trend toward the present, while at Kuujjuarapik first increased until 1950 AD and then started to decrease. In addition, aperture size and position were much higher at Radisson than Kuujjuarapik starting from 1900 to 2000 AD.

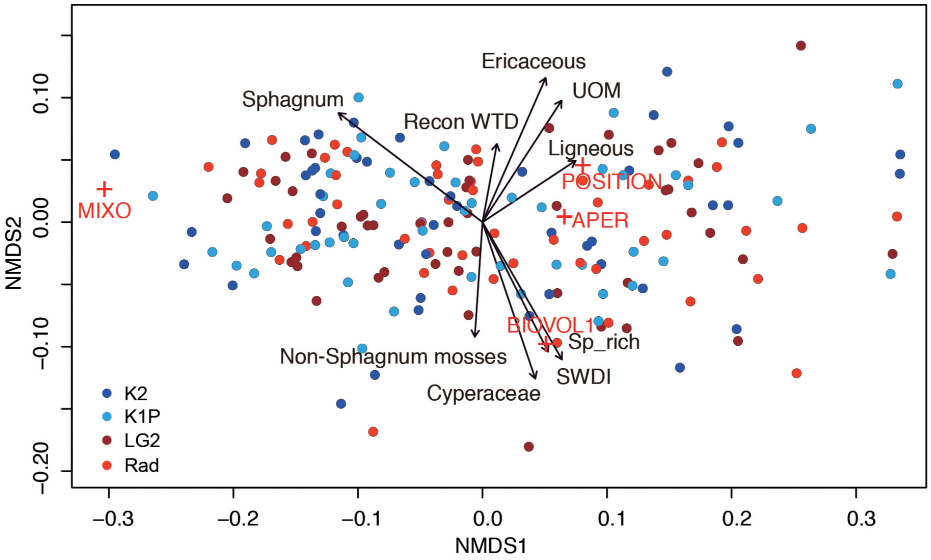

FIGURE 7 | Non-metric multidimensional scaling of testate amoeba functional traits (stress: 0.04; MIXO: mixotrophy; BIOVOL: biovolume; APER: aperture size; POSITION: aperture position) with reconstructed water-table depth (WTD), testate amoeba richness (Sp_rich), diversity (SWDI), and vegetation types as environmental factors (all factors are significant with $p < 0.01$). UOM: Unidentifiable organic matter. Red crosses indicate testate amoeba functional traits and circles with the same color indicate samples from the same site.

Functional trait, C accumulation and reconstructed WTD results at different spatial scales showed differing degrees of variability (**Figure 5**). C accumulation and mixotrophy results were relatively consistent across all spatial scales, showing recent increases. Reconstructed WTD values tended to show a relatively consistent trend toward drier conditions, but the regional level data showed this was a more recent and sharper trend in the Kuujjuarapik region, compared to a more gradual trend in the Radisson region. A similar pattern is evident in the aperture size and aperture position data: both showed consistent decreasing trends using all data, but regional level sub-division showed that the trends were more severe and recent in the Kuujjuarapik region and more gradual in the Radisson region. Biovolume tended to decrease over the study period, but regional level data showed that biovolume was generally higher in the Kuujjuarapik compared to Radisson region and core level data showed that the trend toward lower biovolume was much stronger in some cores, whereas others displayed relatively little change.

There were significant correlations between C accumulation and testate amoeba functional trait data, but the patterns were not consistent (**Figure 6**). For example, despite qualitatively similar recent increases in both C accumulation and mixotrophy across many cores (**Figure 5**), correlations between these factors were significant in both positive (K2.1, Rad1) and negative (K2.3, LG2.2) directions, even at the same site (K2) (**Figure 6**). Correlations between aperture size and aperture position were generally more consistent, with 3 of 4 and 6 of 7 significant correlations, respectively, between C accumulation and these functional traits across all sites being negative (**Figure 6**).

General Patterns of Testate Amoeba Functional Traits and Peatland Conditions

The NMDS results, i.e., no clear clusters of samples from a certain site, showed that the testate amoeba functional traits in each peatland were relatively homogeneous (**Figure 7**). Mixotrophy was highly linked to the first axis and clearly preferred habitats with *Sphagnum* mosses. Aperture size located on the other side of the first axis showed a shorter length, i.e., smaller aperture in *Sphagnum*-dominated habitats. The second axis can be explained by water table conditions, with the upper part indicating dry conditions with abundant woody species and the lower part indicating wetter conditions with sedges and non-*Sphagnum* mosses and higher testate amoeba richness and diversity. Biovolume was closely linked to the second axis, with larger values occurring in wetter conditions. Aperture position with hidden apertures showed a preference to drier conditions with abundant woody plants.

DISCUSSION

The studied peat cores, peatlands and regions differed in the patterns of inferred hydrology, vegetation, testate amoeba functional traits and carbon (C) accumulation. These differences further indicated that variations of peatland conditions affected the testate amoeba communities through their response traits and in the effect of these community changes for functioning of the microbial food web through their effect traits (Fournier et al., 2015 and references therein). In general, temporal trends of C accumulation, hydrology and testate amoeba functional traits observed in southern region Radisson were smoother compared with those in northern region Kuujjuarapik that were typically more recent and distinct. This could be linked to changes in thawing permafrost dynamics in the northern Kuujjuarapik region (Piilo et al., 2019).

Responses and Effects of Testate Amoeba Functional Traits on Peatland Environmental Change and Functioning

Changes in response traits at the community scale are inferred as being driven by changes in environmental conditions, while changes in effect traits may indicate shifts in process at the ecosystem scale. Previous studies have shown that correlations between response traits and effect traits can be used to determine how environmental change will influence ecosystem functioning (Lavorel and Garnier, 2002; Suding et al., 2008; Hillebrand and Matthiessen, 2009).

Biovolume and aperture position are suggested to be correlated with moisture, with low water level promoting smaller biovolume and hidden and protected apertures (Fournier et al., 2015; Marcisz et al., 2016). Our data strongly supported such links, especially for biovolume; that higher biovolume highly correlated with wetter conditions. Unlike biovolume, in addition to water table, aperture position might be influenced by other factors like the abundance of woody plants; for example, Rad1 had protected aperture (higher values of aperture position) in response to drier conditions, while for more records, e.g., LG2.2, Rad2, Rad3, K1P1, K1P2, K2.2, and K2.3, less protected aperture increased under wetter conditions. This suggests that variations in water level may not be a decisive factor for changing aperture position. Also, it may be explained by our field sampling design, i.e., all the peat cores were collected from lawn microforms with relatively good water availability, so the detected variations in water table were not strong enough to drive aperture position composition changes. Previous studies have also shown that larger disturbances, like flooding, fire, very low water tables and deforestation might have stronger impacts on such trait compositions (Marcisz et al., 2016, 2019). While dry conditions that supported woody plant growth was not a driver for aperture position, the altered vegetation composition (i.e., more woody plants) might nevertheless be challenging for testate amoebae to adapt to (Marcisz et al., 2016 and references therein).

Mixotrophy, seem to show relatively consistent increasing trends over recent decades, which are in line with the increased carbon accumulation rates, even though these increases of C accumulation rates might partly due to less decomposition of recently accumulated peats (Piilo et al., 2019). Mixotrophic testate amoebae that acquire most of their C by photosynthesis of their symbionts, essentially functioning as autotrophs (Lara and Gomaa, 2017) are suggested to play an important role in driving peatland C accumulation, with higher abundances promoting C accumulation (Marcisz et al., 2016; Zhang et al., 2020), but the opposite pattern can also be expected depending on the specific condtions. For example, in this study both positive and negative linear links between mixotrophic testate amoebae and C accumulation were derived (**Figure 6**), which might depend on the prey availability, i.e., mixotrophic testate amoebae either favour photosynthesis and form a positive link between C accumulation and their abundance, or predation and constitute a negative link between C accumulation and their abundance (Jassey et al., 2012, 2013; Mieczan et al., 2015). Furthermore, in most of our cases the larger the aperture size, the lower the C accumulated, which supports the latter mechanism. Although it has been shown before that lowering of water table leads to a decrease in the abundance of mixotrophs (Marcisz et al., 2016), this is not visible in our records. This might also be due to the sample collecting habitats, i.e., lawns that supply sufficient water and did not reach the threshold for changes of mixotrophy (Jassey et al., 2018; Koenig et al., 2018; Lamentowicz et al., 2019; Zhang et al., 2020). Instead, we found that increases in mixotrophy were linked to increased *Sphagnum* (mainly *S. fuscum*) abundance (Van Bellen et al., 2018). This suggests that mixotrophs appear to thrive under increasingly acidic and ombrotrophic conditions (Fournier et al., 2015). In addition, higher light intensity at the surface could be another potential reason for increased near surface mixotrophy (Marcisz et al., 2014; Payne et al., 2016; Creevy et al., 2018), even though increase of mixotrophy might also occur under low light intensity conditions (Herbert et al., 2019). Aperture size that indicates the trophic position of testate amoebae in the microbial food web (Jassey et al., 2013) was found to mainly present a negative correlation to C accumulation, however, an adverse pattern was also captured (**Figure 6**). Similar to mixotrophy, aperture size also showed no links to water table, but a negative response to increase of *Sphagnum*, with smaller size observed in *Sphagnum*-dominated habitats.

Implications of Peatland Testate Amoeba Functional Traits on Peatland Carbon Dynamics

As discussed above, mixotrophic testate amoebae might increase or decrease peatland C accumulation depending on different biotic conditions. Therefore, changes in their abundance will have impacts on peatland C dynamics. Our results show that the lowest abundance (close to zero) of mixotrophic testate amoebae occurred when *Sphagnum* dominance remarkably decreased. Post-LIA warming seems to play an important role in driving such vegetation change, followed by increasing of *Sphagnum* (Piilo et al., 2019) and correspondent mixotrophic testate amoebae, thus testate amoebae-peatland functioning. Particularly, the palsa site (K1P) showed synchronous vegetation changes, characterised by decreased *Sphagnum* abundance, for all records starting from *ca.* cal. AD 1950s and lasting until mid-1990s, which could be linked to changes in thawing permafrost dynamics in the site (Piilo et al., 2019). Increased snowfall since the 1950s and warm temperatures in the 1990s triggered rapid permafrost thawing on the eastern coast of Hudson Bay. Hence, permafrost is predicted to disappear from subarctic Québec in the coming decade (Payette et al., 2004), likely causing drying or wetting of the peat surface (Swindles et al., 2015a; Zhang et al., 2018b) and altering the vegetation (Zhang et al., 2018b; Piilo et al., 2019) and mixotrophic testate amoeba abundance, and ultimately changing peatland carbon dynamics.

In addition, boreal peatlands in, for example, western Canada are highly sensitive to fire because of the dry climate and high tree density, which sustains the spread of fire. Enhanced warming and drying during the 21st century will result in more intense regional fire regimes (De Groot et al., 2013). Fires were captured in 11 out of 12 studied records, especially in peatland Rad (Piilo et al., 2019). Atmospheric deposition of mineral dusts, whether they are supplied by natural or anthropogenic sources, may affect testate amoeba communities and their functional traits (Fialkiewicz-Koziel et al., 2015; Lamentowicz et al., 2015). For example, enhanced inputs of atmospheric dust are likely to limit mixotrophic testate amoeba abundance and therefore C uptake. However, the effects of drivers such as dust deposition are likely to have less effects on changes of testate amoeba taxa when compared with vegetation change, which partly depends on the volume of dust (Van Bellen et al., 2018), which seems to be supported also by our records that fires did not trigger clear shifts on testate amoeba composition.

Warming could enhance peatland C uptake due to increased growing season length (Charman et al., 2013; Gallego-Sala et al., 2018), more specifically increased plant photosynthesis. However, in terms of photosynthetic microorganisms, warming and subsequent changes in hydrology and vegetation all have impacts on mixotrophic testate amoebae. Studies have shown a reduction in mixotrophic testate amoebae with rising temperature (Wilken et al., 2013; Jassey et al., 2015) and lowering water tables (Marcisz et al., 2016; Basińska et al., 2020). However, one hypothesis is that global warming will lead into water level drawdown in peatlands, which favours new establishment of *Sphagnum* (Tahvanainen, 2011; Magnan et al., 2018). This *Sphagnum*-related change would likely to promote mixotrophic testate amoeba presence and mitigate the decrease of mixotrophic testate amoebae caused by warming and lowering of water table. On the other hand, drying might also cause shrub expansion (Gałka et al., 2017a; Zhang et al., 2018b) and decrease the openness of the peatland, thus hinder peatland C uptake through decreases in mixotrophic testate amoebae (Payne et al., 2016; Lamentowicz et al., 2020). Nevertheless, future climate change might influence peatland C sequestration through not only directly altering the hydrology, but also vegetation and consequent changes in mixotrophic testate amoebae, even though the magnitude and direction of such changes are not well constrained and might vary from one ecoclimatic region to another.

Future Perspective on Detecting Reliable Peatland-Climate Feedback Signal

The spatial and temporal variations detected within both peatlands and regions highlight the importance of studying multiple peat sections from one study site and preferably the approach should be extended to regional-scales (Loisel and Garneau, 2010; Lamarre et al., 2012; Mathijssen et al., 2017; Zhang et al., 2018a,b; Piilo et al., 2019). To enable a spatial comparison of our records, we collected peat cores consistently from lawn habitats, however, even in a particular site large differences between the three replicated cores were captured. It has been previously reported that different peatland habitats may also undergo different peatland-climate feedback pathways (Zhang et al., 2020). Any one site-based discussion of regional feedbacks is likely to be biased because different peatland types can experience specific successions under similar climate conditions, such as the differences observed between palsa K1P and fen K2 in region Kuujjuarapik in this study, let alone different regions that have different climate (Sim et al., 2019). This problem is exacerbated when within-site data, even taken from a consistent microform, is also variable. As one main aim of small-scale studies is to provide information for global modeling of peatland-climate feedbacks, we need to keep it in mind that any single core is likely not represent the overall site or regional response. Consistent signals detected from replicated records are definitely more convincing in terms of interpretations on peatland-climate feedbacks. However, due to practical issues for particular studies, we do not provide any specific suggestions here about, for example, the number of records that are needed for a reliable signal, but emphasize the need for multiple cores in peatland-climate feedback studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

MV, SP, AG-S, and MG collected the samples. MA analyzed the testate amoeba samples. HZ and MA performed the data analyses. HZ wrote the manuscript with MA. All other authors contributed with discussions and comments on the text.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00228/full#supplementary-material

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Testate Amoeba Species- and Trait-Based Transfer Functions for Reconstruction of Hydrological Regime in Tropical Peatland of Central Sumatra, Indonesia

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Tropical peatlands play an important role in carbon storage and in water regulation on a landscape level. However, our understanding of their ecology and long-term hydrological dynamics remains limited. Transfer functions, constructed on the basis of biological indicators (proxies) with known ecological preferences, allow us to infer past environmental conditions and serve as a basis for prediction of future changes in peatlands. Here, we use testate amoebae to develop the first species- and functional trait-based transfer functions for the Southeast Asia. This provides a valuable tool for future reconstructions of past hydrological changes in tropical peatlands, their development, and climatic changes. Surface samples for testate amoeba analysis were taken in various biotopes along two transects across the Sungai Buluh peatland in Central Sumatra. The following environmental characteristics were measured: water table depth (WTD), light intensity, pH, total C and N concentrations. The analysis of the surface samples revealed 145 morphotypes of testate amoebae belonging to 25 genera. A significant fraction of the variance in testate amoeba morphotypes and functional trait composition was explained by WTD and pH. The wide WTD range (0–120 cm) seems more valuable for reconstruction than the extremely short pH gradient (2.5–3.8). Thus, transfer functions were developed only for WTD, based on weighted averaging model for morphotypes and multiple linear regression for functional traits. Both species- and trait-based model have a predictive ability for WTD reconstruction. For traits, the best performance of the model was reached by including five morphological traits: shell width, aperture shape, aperture invagination, shell shape and shell compression.

We discuss the ecology of several taxa and highlight the traits, which reflect hydrological changes in this system. Though the hydrological preferences of some species are similar to those in high and middle latitude peatlands, we argue that latitudinal differences in morphospecies composition and variations in environmental relationships of species require the development of region-specific transfer functions. Moreover, our results indicate that ecological preferences of morphotypes within morphospecies also need to be considered and included in future studies.

Keywords: water table depth, protists, shell size, training set, *Hylaosphenia*, indicator, peat-swamp forest, hydrology

INTRODUCTION

Tropical peatlands play a crucial role in carbon sequestration, regulating water resources and safeguarding rich biodiversity (Page et al., 2011; Posa et al., 2011; Hapsari et al., 2017). These peatlands, however, are vulnerable to climate changes and threatened by their extensive conversion into agricultural production systems (Page et al., 2011; Miettinen et al., 2012, 2013). About a century ago, Polak (1933) wrote that the study on peatlands in the tropics was still at an early stage. This statement, unfortunately, is still true to this day for the Asian tropics, in particular for palaeoecological research in the region (Biagioni et al., 2015). Facing the current changes, there is the pressing need to study these "palaeoenvironmental archives" (Jackson and Charman, 2010) to evaluate past environmental conditions and to predict future changes of these ecosystems. However, crucial steps in palaeoenvironmental reconstructions are the development of a representative present-day organisms-environment training set and the application of adequate numerical methods to model the relationship between the occurrence and abundance of recent organisms and their environment (Juggins and Birks, 2012). Using these so-called transfer functions allows a quantitative estimation of past environmental conditions. Transfer functions have been frequently developed and applied to predict temperature, precipitation, sea level, pH and water table depth based on species composition of pollen, chironomids, diatoms, and testate amoebae in lake and mire sediments across many regions (Birks et al., 1990; Gehrels, 2000; Wilmshurst et al., 2003; Charman and Blundell, 2007; Klemm et al., 2013; Massaferro and Larocque-Tobler, 2013).

Testate amoebae are common organisms in many mires and, due to their test (shell), they are often preserved in peats (Swindles and Roe, 2007; Mitchell et al., 2008a). Diverse shell morphological characteristics and prominent environmental preferences make testate amoebae valuable ecological indicators. Previous studies showed that species composition of testate amoeba communities are controlled by surface wetness (often estimated as water table depth), pH, temperature and sea-level change (Charman, 2001; Mitchell et al., 2008a).

A number of transfer functions have been developed based on the relationship between testate amoebae and peatland hydrology in the Northern Hemisphere. For summarised studies on transfer functions from North America and Europe see, for example, Amesbury et al. (2016, 2018). However, only few transfer functions were developed for the Southern Hemisphere (Wilmshurst et al., 2003; Swindles et al., 2014, 2018a; van Bellen et al., 2014). In part, this might be due to the fact that information on ecological preferences of testate amoebae in tropical peatlands is extremely limited. However, data obtained from peatlands of temperate and polar regions may not be applicable to low-latitude peatlands (Charman, 1997).

Traditionally, the construction of transfer functions is species-based, which is difficult if identification of the taxa is hampered (Mitchell et al., 2014). Also, the environmental preferences of many species are not well known and this restricts their use as ecological indicators (Charman, 1997). Alternatively, ecologically significant morphological and physiological functional traits may serve as indicators of the environmental conditions the organisms live in Fournier et al. (2015), van Bellen et al. (2017), and Koenig et al. (2018). Thus, functional traits may provide powerful links to ecosystem processes and help to strengthen the transfer functions approach based on taxonomic information only (Fournier et al., 2015; Lamentowicz et al., 2015; van Bellen et al., 2017). In recent years, a new approach for reconstructing past environmental conditions based on testate amoeba functional traits has been developed and successfully applied (for details, see Marcisz et al., 2020, submitted). However, the approach still needs refinement and this applies in particular to tropical peatlands. Therefore, this study aims at (1) extending knowledge on testate amoeba communities in Sumatra, (2) evaluating the relationships among testate amoeba morphospecies, functional traits and environmental variables, and (3) developing species- and trait-based transfer functions for future palaeoecological reconstructions of hydrological regime.

MATERIALS AND METHODS

Study Site

The Sungai Buluh peatland is a protected forested swamp which covers an area of 18,000 ha. It is located approximately 19 km from the coastline and 30 km north-east of the city of Jambi in Central Sumatra with an elevation ranging from 9 to 25 m above sea level (Hapsari et al., 2017). From the geomorphological and hydrological point of view, the peatland of Sungai Buluh is an extensive coastal peat dome, which is delimited by two rivers located to the east and west sides. The mouths of the rivers are located on the coast about 20 km north to the peatland and are influenced by strong diurnal tides. Peat depths at the study site (measured with the Peat Probe to the mineral layer) vary from 216 to 720 cm (mean 430 cm).

The Sungai Buluh area is covered with a secondary peat-swamp forest, which has been regenerating after selective logging in the 1960s and 1970s (Nurjanah et al., 2013). In 1997, *El Niño* related fires reduced the forest canopy (Tata et al., 2016) so the seedlings of the tree species *Shorea pauciflora* and *Dyera polyphylla* were planted in 2003 (Nurjanah et al., 2013). The Sungai Buluh peatland is surrounded by agricultural fields and plantations, for example, pulp wood *Acacia* spp. and oil palm *Elaeis guineensis* (Melati et al., 2015).

The climate of the area is tropical humid. There is little temperature variation throughout the year with mean annual temperature of 26°C. The average annual precipitation in the area is 2400 mm with a slightly drier season from June to September (Aldrian and Susanto, 2003; Karger et al., 2016). The inter-annual variability of rainfall is controlled by the El Niño Southern Oscillation (Aldrian and Susanto, 2003) and the Indian Ocean Dipole (Saji et al., 1999). While the precipitation patterns of the area are influenced by the Asian-Australian monsoon and the Intertropical Convergence Zone (Saji et al., 1999).

Field Work

For the development of the training set, surface samples of testate amoeba communities were collected along two transects (NS and WE) across the peatland on August 3 – 6, 2016 (**Figure 1**). In total, 29 plots were laid out along the NS transect (total length ∼500 m) and 41 plots along the WE transect (total length ∼600 m). The distance between the NS and WE transects was about 12 km. The NS transect was located in the centre of the peatland and represented the conditions typical for the internal part of the dome with

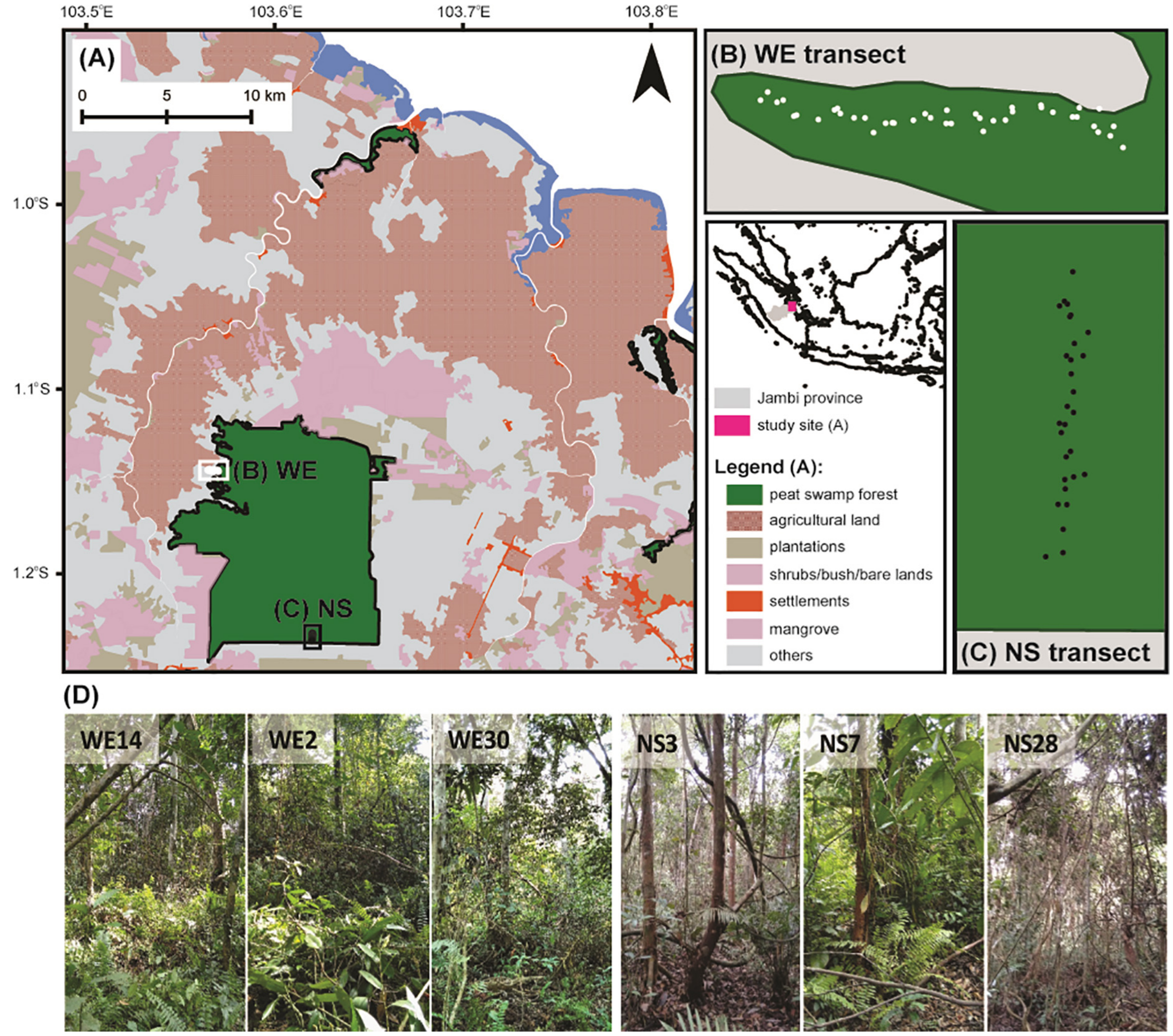

FIGURE 1 | Map of the Sungai Buluh protected peat swamp forest **(A)** with studied WE **(B)** and NS **(C)** transects. Each point on the WE and NS transects indicates the sampling location. **(D)** Examples of some sampling locations.

the deepest peat deposits, however, with a rapid change in land use, this is no longer visible on the map (**Figure 1**). On the other hand, the WE transect was located closer to one of the rivers and represented the peripheral part of the peatland characterised by minerogenic conditions. The most common plant families were Myristicaceae, Dipterocarpaceae, Anacardiaceae, Myrtaceae, Euphorbiaceaea. The most common species were *Myristica lowiana*, *Macaranga* sp., *Shorea rugosa*, *Syzygium attenuatum*, *Dyera polyphylla*, *Gluta aptera* (personal observations). These species widely occur in natural swamp rainforests of Southeast Asia and indicate successful restoration measures at the protected forest (Graham et al., 2017). Both locations were chosen because of their natural conditions and accessibility.

The location of all 70 sampling plots was identified using the GPS of a smart phone with connectivity to GLONASS, Galileo, and Beidou location services (Aquaris X Pro model, BQ; for sampling plot details see **Supplementary Table 1**). At each plot, a sample of the top 50 mm litter/fermentation layer was taken with a corer (diameter 50 mm) and used for testate amoeba analysis and the determination of substrate water content, pH, and concentrations of carbon (C) and nitrogen (N). The extracted substrate of all samples was classified as tree litter, herbal litter, rooted soils or pool sediments. The samples for testate amoeba analysis were air dried (25°C) and stored at 4°C until analysis (Mazei et al., 2015). At each plot, microrelief was classified as pool, hollow, flat or hummock. The water table depths (WTD, cm) was measured in relation to the peatland surface (negative values denote submerged substrates) after augering with a corer. The measurements were repeated several times until values were stable. Light intensity (illuminance, lux) was determined using a lux meter of the camera from the same smart phone used for the GPS location (Gutierrez-Martinez et al., 2017). Despite differences in day time of measurement (from 11 am until 3 pm), light intensity is generally driven by canopy cover and the measurements, therefore, reflect light conditions at the sampling sites. The characteristics of the sampling plots are presented in **Supplementary Table 1**.

Laboratory Analyses

Substrate pH ($CaCl_2$) was measured using a digital pH meter. For measuring concentrations of C and N, aliquots of the substrate were dried at 65°C for 72 h, milled and analysed using an elemental analyser (Carlo Erba, Milan, Italy). Water content of the substrate was determined gravimetrically (wet weight percentage), for details see **Supplementary Table 1**.

Testate amoeba analysis was performed according to the procedure described by Mazei et al. (2011). To facilitate detachment of testate amoeba shells from litter, 4–6 g air-dried material was placed in sterile tap water at 5°C for 24 h. Then, the suspension was stirred for 10 min, filtered through 500 μm mesh and left to settle at 5°C for 24 h. The settled material was analysed at 200–400× magnification using a light microscope (Zeiss Axiostar plus, Germany). A minimum of 150 shells was counted for each sample. Two samples were omitted from further analyses because of low shell numbers, i.e., NS18 (0 shells) and WE30 (17 shells).

Measurements of the Shell and Functional Traits Assignments

Most individuals could be assigned to species (morphospecies), others were listed as morphotypes, based on differences in shell size (Hoogenraad and de Groot, 1940, 1942; Bartos, 1963; Mazei and Tsyganov, 2006); for the taxa list see **Supplementary Table 2**. Shell size measurements were done for each specimen to assign it to a particular size class. If the size range (shell length and shell width) corresponded to the original description (see Authorship in the **Supplementary Table 2**), we assigned this individual to morphospecies level; if the shell length or shell width was variable, we assigned each individual into morphotypes of known morphospecies with a precise indication of the shell size for each of them and named them as "morph," as shown in **Supplementary Table 2**. The total number of each measured individuals of morphotype/morphospecies is shown in **Supplementary Table 2**, see column named "Individuals, found in all samples".

The selection of functional traits of testate amoebae was based on their ecological importance as indicated by previous publications (Fournier et al., 2015; van Bellen et al., 2017; Koenig et al., 2018). The initial list of functional traits included the following characteristics: (1) shell length, shell width, aperture size and biovolume (Gilbert et al., 1998), measured for each found individual and for 150 shells for each sample, see **Supplementary Table 2**; and (2) shell shape, shell compression, shell composition, presence of mineral particles, aperture position, aperture shape, aperture invagination, mixotrophy and order-based phylogenetic group, taken from original descriptions (see **Supplementary Table 2**, column Authorship). All qualitative characteristics were categorised into two to six categories (**Supplementary Table 2**). Briefly, for shell shape six categories were defined: circular, oviform, pyriform, oviform/elongate, oviform/pyriform and flask-shaped. For shell compression-six categories: very compressed, compressed, sub-spherical, hemispherical, spherical/cylinder and spherical. For shell composition-six: proteinaceous, siliceous, agglutinate/mineral, agglutinate/siliceous, agglutinate/mineral/siliceous, and calcareous. For mineral matter presence-two categories: present and absent. For aperture position-three: terminal, sub-terminal and central. For aperture shape-six: oval, oval/circular, circular/irregular, circular, irregular and slit-like. For aperture invagination-three: invaginated, not-invaginated and slightly invaginated. For mixotrophy-two: no and yes. For order-four: Arcellinida, Amphitremida, Euglyphida and Gromiida. Some of the traits, e.g., shell size, shell compression, aperture position were assumed to reflect substrate wetness conditions and represent adaptations to wet or dry conditions (Fournier et al., 2015; van Bellen et al., 2017), whereas shell composition (more siliceous or more proteinaceous) may indicate pH or C/N ratio, i.e., the nutrient status of the environment (Mitchell et al., 2008b). The abovementioned functional traits were determined for each morphotype and combined in data frame for subsequent calculations (**Supplementary Table 2**). For details on the occurrence of each trait in the samples see **Supplementary Table 2**. For previously undescribed morphotypes, measurable (quantitative) characteristics were assessed, non-measurable (qualitative) were taken from the known genus level after visual validation under the microscope.

Data Analyses

All calculations and statistical analyses were performed in R (R Core Team, 2018). Based on the traits, community weighted means (CWMs) were calculated for each sample, reflecting the functional composition, expressed as the mean trait value of species present in the community weighted by their relative abundance for quantitative traits (shell length, shell width, biovolume, aperture size), or the relative abundance of all taxa with the respective trait for qualitative traits (Laliberté et al., 2014). Effects of environmental variables (substrate type, microrelief, WTD, water content, pH, C-to-N ratio, and light intensity) on the morphotype and functional traitcomposition of testate amoeba communities were analysed using redundancy analysis [RDA and CWM-RDA, see Kleyer et al. (2012)] in the *vegan* package (Oksanen et al., 2017). Environmental parameters affecting the community composition were selected using the forward selection procedure with the function "*ordistep*" (package *vegan*). Variation partitioning was performed for the selected environmental parameters in order to estimate the contribution of each variable to the variation in community composition. The traits with the strongest linkage to WTD were selected for building the trait-based transfer functions based on their scores in RDA. A Monte Carlo permutation test (1000 iterations) was used to test the significance of the models. The relationships between quantitative environmental variables were estimated with Spearman's *rho* statistic (rank-based measure of association; function "*cor.test*").

Transfer functions were constructed using the main calibration methods used in palaeoecology: weighted averaging (WA), weighted averaging with tolerance down weighting (WA-Tol), where high-tolerance species are assigned lower weights than low-tolerance species to reduce their contribution to the model, partial least squares (PLS) and multivariate regression (MR) in *rioja* package (Juggins, 2017). Model performance was evaluated using leave-one-out (LOO) and bootstrapping (n = 1000) cross validation techniques. The best models were selected using the coefficient of determination (R^2), the root mean square error of prediction (RMSEP) and maximum bias. The estimation and visualisation of morphotype optima and tolerances for the selected environmental variables was performed with the function "*caterpillar*" in the package *analog* (Simpson, 2007).

RESULTS

General Observations

The summary statistics of the measured environmental variables along the studied transect is presented in **Table 1** and in **Supplementary Figure 1**. The overall range of the WTD values varied from −12 to 120 cm (mean value 61.7 cm, SD = 32.5 cm) and was characterised by a bimodal distribution with peaks at around 25 and 75 cm. The overall correlation (Spearman *rho* value) between WTD and surface water content was −0.60 and was greater along the NS transect (−0.76) as compared to WE transect (−0.33), which was due to the fact that the WE transect was generally drier and WTD therefore less affected surface water content. Both variables were included in further analyses to evaluate their effects on testate amoeba communities. The soils were generally very acid with the pH varying from 2.5 to 3.8. Most of the sites were located under dense canopy cover with a light intensity <5000 lux. The C-to-N ratio of the substrate varied from 19.5 to 35.5.

TABLE 1 | Summary statistics of the measured environmental variables along NS and WE transects.

	Mean		SD		Min		Max	
Transect	**NS**	**WE**	**NS**	**WE**	**NS**	**WE**	**NS**	**WE**
WTD, cm	24	86	11	13	-12	66	48	120
Water content, %	72.5	66.6	5.4	6.2	59.2	53.3	82.6	77.2
pH value	2.9	3.1	0.3	0.2	2.5	2.8	3.8	3.6
C-to-N ratio	27.7	24.0	2.9	2.3	23.1	19.5	35.5	29.5
Light intensity, lux	1964	2972	3175	3043	120	248	13785	11193

In total, 10,217 shells were counted and assigned to 145 morphotypes of testate amoebae belonging to 65 morphospecies and 24 genera (**Supplementary Table 2**). The most abundant morphospecies were *Hyalosphenia subflava* (morph 2 – 15.6% of total counts, morph 1 – 11.6%, morph 4 – 9.1%, morph 5 – 3.1%), *Hyalosphenia minuta* (9.6%), cf. *Cyclopyxis eurystoma* v. *parvula* morph 2 (9.0%), cf. *C. eurystoma* v. *parvula* morph 1 (5.9%). These morphospecies were the most common and were observed in more than 85% of the samples. Thirty-two morphospecies were present in one sample only with the maximum relative abundance per sample rarely exceeding 2.0% except for *Trinema complanatum* v. *globulosa* (maximum relative abundance per sample 13.0%).

Effects of Environmental Variables on Morphotype and Functional Trait Composition

Redundancy analysis of morphotypes composition indicated that the environmental variables (substrate type, microrelief, WTD, substrate water content, pH, C-to-N ratio and light intensity) explained 29.6% (R^2) of the total variance (*pseudo-F* = 2.4, p < 0.001, df = 10,56). Forward selection procedure identified WTD and pH as the most important environmental variables explaining 14.0% (*pseudo-F* = 11.1, p < 0.001, df = 1,64) and 3.4% (*pseudo-F* = 2.7, p = 0.02, df = 1,64) of the total variation (**Figure 2**). Most of the taxa were generally associated with wet acidic biotopes. Surprisingly, the morphotypes of *H. subflava* showed opposite relations to WTD, i.e., morph 1 and 2 were positively related to WTD, whereas morph 3, 5, 7, and 10 were negatively related to WTD. *H. subflava* morph 4 showed little correlation to WTD in general. Morphotypes *Cyclopyxis*

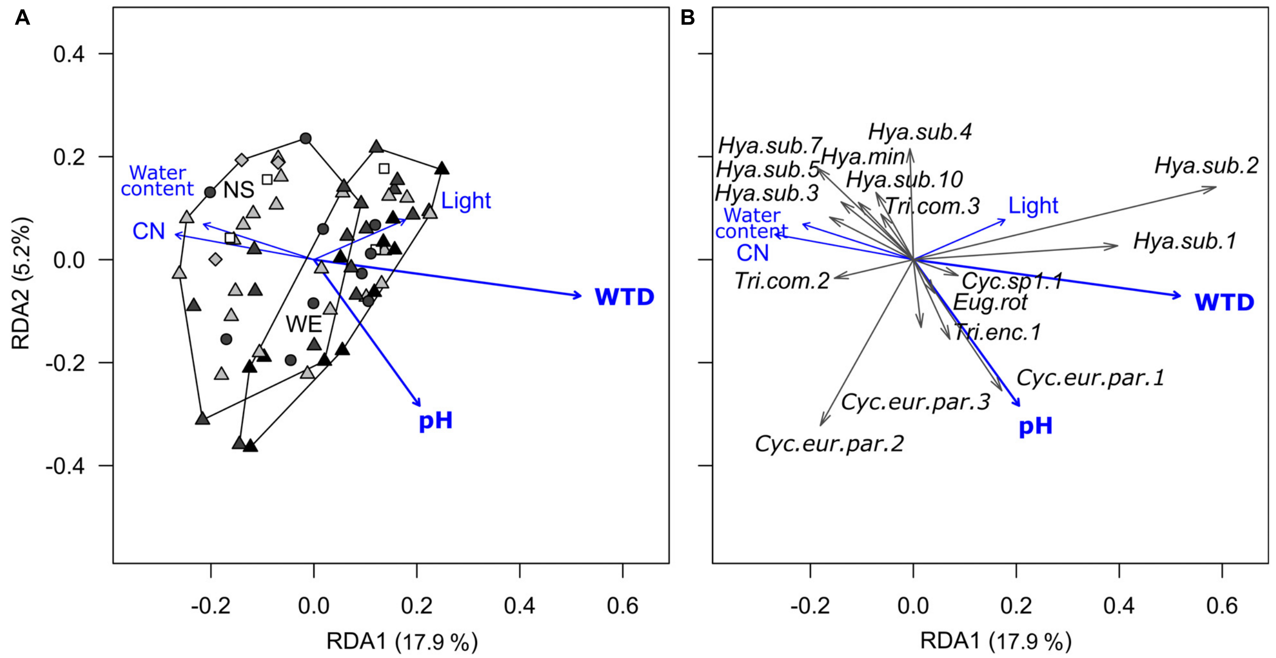

FIGURE 2 | Ordination diagrams based on the result of RDA showing the variation in morphotype composition (unscaled data) of testate amoeba communities in surface samples from the Sungai Buluh peatland in response to environmental variables. **(A)** Sample scores: the polygons encircle samples from the same transect (NS or WE); symbol colour denotes the microrelief (black – hummock, dark grey – flat, light grey – hollow, white – pool), symbol shape denotes the substrate type (circle – herbal litter, square – pool sediments, diamond – rooted soil, triangles – tree litter). **(B)** Species scores (only values ≥ |0.04| with at least one of the axes are shown). The number in parentheses is the proportion of the total variance explained by the axis. Significant environmental variables (WTD and pH) are shown in bold. WTD, for water table depth; CN, for C-to-N ratio; Light, for light intensity. For species abbreviations, see **Supplementary Table 2**.

eurystoma vs. *parvula* morph 1, *Trinema enchelys* morph 1 preferred biotopes with the upper range of pH value.

For functional traits, CWM-RDA showed similar results. The full model constrained by all environmental variables (**Figure 3**) explained 20% (R^2) of the total variance in functional traits composition (*pseudo-F* = 1.41, *df* = 10, 56, *p* = 0.041). Forward selection showed that both pH and WTD should be retained for the parsimonious model, but the WTD (R^2 = 2.8%, *pseudo-F* = 1.93, *df* = 1, 64, *p* = 0.048) explained less than pH (R^2 = 5.3% *pseudo-F* = 3.71, *df* = 1, 64, *p* = 0.009). Based on the functional trait scores in the RDA, the strongest association with the WTD were detected for biovolume (−0.55), shell length (−0.48), oval/circular aperture (−0.45), shell width (−0.44) and sub-spherical shell compression (−0.26), i.e., in drier locations shells were smaller, sub-spherical, with oval/circular apertures (**Supplementary Table 3**). In addition, positive relationships with WTD were determined for oviform/elongate shells (0.35) and slightly compressed shells (0.32), i.e., they were dominating in drier locations. A number of functional traits (shell length, shell width, biovolume, oviform and elongated shell shapes and oval/circular aperture shells) were related to pH, even though it varied in a small range along the transects (for details, see **Supplementary Table 3**). No significant correlations were detected between any of the functional traits and light intensity, presumably because the abundance of mixotrophic species was very low (single occurrences in few samples), see **Figure 3**. All the above-mentioned traits related to WTD were selected for the transfer function development. The relationships between the selected functional traits and WTD were generally linear, except for shells with slightly invaginated aperture (**Supplementary Figure 2**). Shell width, shell length and biovolume are not independent of each other (intercorrelated traits, see **Figure 3** and **Supplementary Table 3**), and therefore we tested several models with different combinations of these traits.

Morphotype- and Trait-Based Transfer Functions

For morphotypes, the best performing transfer functions are shown in **Table 2** (for the other models see **Supplementary Table 4**). The inverse weighted averaging model (68 samples, 143 morphotypes) showed the best performance according to the cross-validation methods (**Figure 4**). Depending on the cross-validation method, R^2 varied between 70 and 71% and RMSEP varied between 17.7 and 18.7 cm. After screening for outliers (**Figure 4**), the samples with a residual greater than 25 cm (absolute value) were removed (**Supplementary Table 5**). This improved the performance of the transfer function (R^2 = 77%, RMSEP = 15–16 cm, 63 samples, 139 morphotypes, WTD range 0–120 cm, SD = 31.4 cm). Tolerance and optimum values for the 87 morphotypes of testate amoebae were analysed and plotted in **Figure 5**. The functional traits were generally characterised by linear relationships to WTD and therefore were better modelled by multiple regression

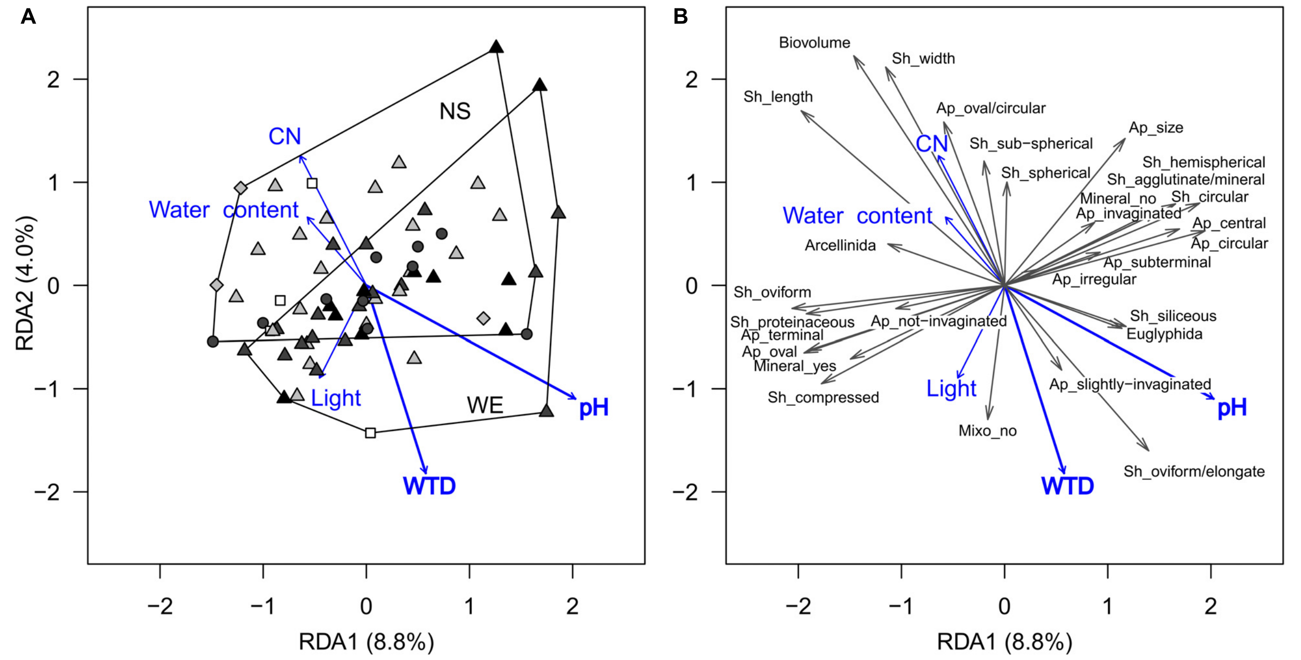

FIGURE 3 | Ordination diagrams based on the result of RDA showing the variation in testate amoeba functional traits composition (scaled data, only traits observed in more than 15 samples are shown) in surface samples from the Sungai Buluh peatland. **(A)** Sample scores: the polygons encircle samples from the same transect (NS or WE); symbol colour denotes the microrelief (black – hummock, dark grey – flat, light grey – hollow, white – pool), symbol shape denotes the substrate type (circle – herbal litter, square – pool sediments, diamond – rooted soil, triangles – tree litter). **(B)** Functional trait scores (multiplied by 2 to improve readability). The number in parentheses is the proportion of the total variance explained by the axis. Significant environmental variables (WTD and pH) are shown in bold. Trait abbreviations: Sh – shell, Ap – aperture, Mineral – mineral presence, Mixo – mixotrophy. WTD, for water table depth; CN, for C-to-N ratio; Light, for light intensity.

(MR) (**Supplementary Table 6**). Nonetheless, the performance of MR model for functional traits was weaker as compared to the WA inverse for morphotypes even after the removal of outliers with the residuals greater than 40 cm (absolute values; **Table 2**).

DISCUSSION

Testate Amoeba Communities in the Sungai Buluh Peatland

In the studied peatland we found 145 taxa from 25 genera in the 70 samples taken (**Figure 6**). This is similar to other ecosystems in Jambi Province, including rainforests and agricultural lands, where about 150 taxa were found in litter samples (Clough et al., 2016; Krashevska et al., 2016), and similar to moss samples in Java where about 126 taxa were found (Bartos, 1963). Many of the recorded morphospecies do have a cosmopolitan distribution. However, the variability in the morphological characteristics of the shell was unusually high in a number of these morphospecies. One of the most dominant and variable morphospecies was *H. subflava*, suggesting that it forms a species complex (**Figure 7**). Shell length of *H. subflava* ranged from 40 to 105 μm and shell width from 32 to 75 μm. We identified 17 morphotypes in this morphospecies and these morphotypes represented about 50% of the total number of individuals of testate amoebae in our samples. This great variability, to the best of our knowledge, has not yet been documented for any other *Hyalosphenia* in higher latitude ecosystems, but regularly reported for other tropical regions, including Indonesia, Peru, and Panama (Hoogenraad and de Groot, 1942; Biagioni et al., 2015; Krashevska et al., 2016; Swindles et al., 2016, 2018a). *H. subflava* has been generally considered as an indicator of dry conditions in high- and mid-latitude peatlands (Charman et al., 2000). However, our study shows that, in tropics, this abundant and morphologically variable species may form a species complex comprising of morphotypes which differ in ecological preferences (see "Relationships of Testate Amoeba Morphospecies and Traits With Environmental Variables"). The second dominant morphospecies was a cosmopolitan species cf. *C. eurystoma* v. *parvula* (**Figure 6C**), which also showed variability in the shell length and potentially represented a species complex. It is noteworthy that the morphological differences between *C. eurystoma* v. *parvula* and *Phryganella acropodia* are difficult to detect with light microscopy. Therefore, these morphospecies should be treated with caution, especially while comparing different studies, and interpreting their indicator value.

The most diverse genus was *Centropyxis* with about 28 morphotypes, however, it represented only 1.9% of the total abundance of testate amoebae. We did not find any species restricted to the Southern Hemisphere that is in contrast to the study of Hoogenraad and de Groot (1940), which reported *Apodera vas*, a typical Southern Hemisphere species,

TABLE 2 | Performance statistics for the morphotype- and trait-based transfer functions (weighted averaging (WA) and multiple regression (MR) models) for reconstruction of water table depth.

	Apparent			**Leave-one-out**			**Boot-strap**		
Model	**RMSE**	**R^2**	**Max Bias**	**RMSEP**	**R^2**	**Max Bias**	**RMSEP**	**R^2**	**Max Bias**
Morphotype-based, full training set (68 samples, 143 morphotypes)									
WA inverse	**14.6**	**0.80**	**29.9**	**17.7**	**0.71**	**36.1**	**18.7**	**0.70**	**38.3**
WA classical	16.4	0.80	20.5	18.2	0.71	27.9	18.8	0.71	31.6
WA-Tol inverse	16.3	0.75	40.8	19.2	0.66	32.0	23.7	0.68	34.7
WA-Tol classical	18.8	0.75	32.0	21.4	0.66	26.8	25.7	0.68	29.4
Morphotype-based, screened training set									
(samples with residuals >25 cm absolute values were removed; 63 samples, 139 morphotypes)									
WA inverse	**11.8**	**0.86**	**20.9**	**15.0**	**0.77**	**41.1**	**16.0**	**0.77**	**41.6**
WA classical	12.8	0.86	15.0	14.9	0.78	38.4	15.7	0.77	39.3
WA-Tol inverse	15.3	0.76	38.4	20.0	0.59	38.8	23.3	0.66	45.4
WA-Tol classical	17.5	0.76	31.1	20.9	0.59	33.4	24.5	0.66	42.9
Trait-based, full training set (68 samples, 5 functional traits)									
MR	**25.0**	**0.41**	**47.5**	**28.4**	**0.27**	**50.1**	**28.4**	**0.27**	**50.1**
Trait-based, screened training set (samples with residuals >40 cm absolute values were removed; 62 samples, 5 functional traits)									
MR	**18.0**	**0.67**	**27.5**	**21.2**	**0.56**	**33.8**	**23.1**	**0.56**	**34.4**

The best performing models are shown in bold.

in the Kerinci mountains in Sumatra. However, we also found some endemic species such as *C. minuta* with an elliptic aperture (Bartos, 1963), *Jungia sundanensis* cf. *ovoidalis, J. intermedia* as well as cf. *Trigonopyxis microstoma*, which was also characterised by a pronounced shell variability (**Figure 6**). Surprisingly, we found only two shells of *Archerella flavum* and *A. jollyi*, which are very common in high latitude peatlands, but very rare in peatlands in New Zealand and absent in other peatlands of the Southern Hemisphere (Charman, 1997; Charman et al., 2000; van Bellen et al., 2014). Further, twelve taxa could not be determined to the morphospecies level, and twelve taxa were ambiguous, therefore they were signed as similar to the ones known (cf.), see **Figure 6**.

Relationships of Testate Amoeba Morphospecies and Traits With Environmental Variables

From all measured environmental variables, only WTD and pH explained a significant fraction of the variation in morphotype composition. For the development of transfer functions, WTD with its wide range (from 0 to 120 cm) seems to be more appropriate than pH with its restricted variation (from 2.5 to 3.8). Further, our study once again showed that the substrate water content is inferior to the WTD in explaining community composition of testate amoebae (Charman and Blundell, 2007; van Bellen et al., 2014). This can be explained by a greater stability of WTD in comparison to surface wetness. Supporting the suggestion of Swindles et al. (2018a), our results underline the sensitivity of testate amoebae to hydrological fluctuation in tropical ecosystems.

The wet localities at our study site were dominated by *C.* cf. *aculeata oblonga* morph 3*, C. kahli*, *C.* cf. *aplanata microstoma* morph 1, cf. *T. microstoma* and *Spenoderia lenta* morph 1. Similarly, *C. aculeata* have been suggested to indicate wet conditions in other regions (Swindles et al., 2018a,b). *C. aculeata* WTD optima, ranging between 20 and 30 cm, were similar to those from Continental Canada (Charman and Warner, 1992), West Russia (Bobrov et al., 1999) and Panama (Swindles et al., 2018a). Thus, for wet indicators such as *C. aculeata*, which are well known from the Northern Hemisphere, we confirm their value as an indicator of wet conditions in tropics. In addition, the WTD optimum of *S. lenta* at our study site (27.4 cm) was similar to that reported from a bog in Continental Canada [22.4 cm; Charman and Warner (1992)] and Patagonian peatlands in the Southern Hemisphere [ca. 32.0 cm; van Bellen et al. (2014)]. In contrast to the well-known and globally distributed *C. aculeata*, little is known about the ecology of rare species such as cf. *T. microstoma* (**Figure 6P**). *T. microstoma* has only been recorded from southern Patagonia (Argentina and Chile), Ecuador, Vietnam and New Zealand. The WTD optimum for this species has only been reported from south Patagonia (15 cm), which is similar to 20 cm optimum of smaller morphotype of cf. *T. microstoma* morph 1 (Hoogenraad and de Groot, 1948; Bartos, 1963; Zapata et al., 2008; Bobrov et al., 2010; Krashevska et al., 2012; van Bellen et al., 2014). We suggest considering cf. *T. microstoma* as an indicator for of wet conditions. Notably, for our individuals of cf. *T. microstoma*, the shell-to-aperture ratio was lower (4.6) compared to the originally described ones (8.2) by Hoogenraad and de Groot (1948), therefore this morphospecies may well be classified as a new species.

Dry locations in the studied peatland were dominated by a number of morphospecies of *Trinema* and *Euglypha*, which

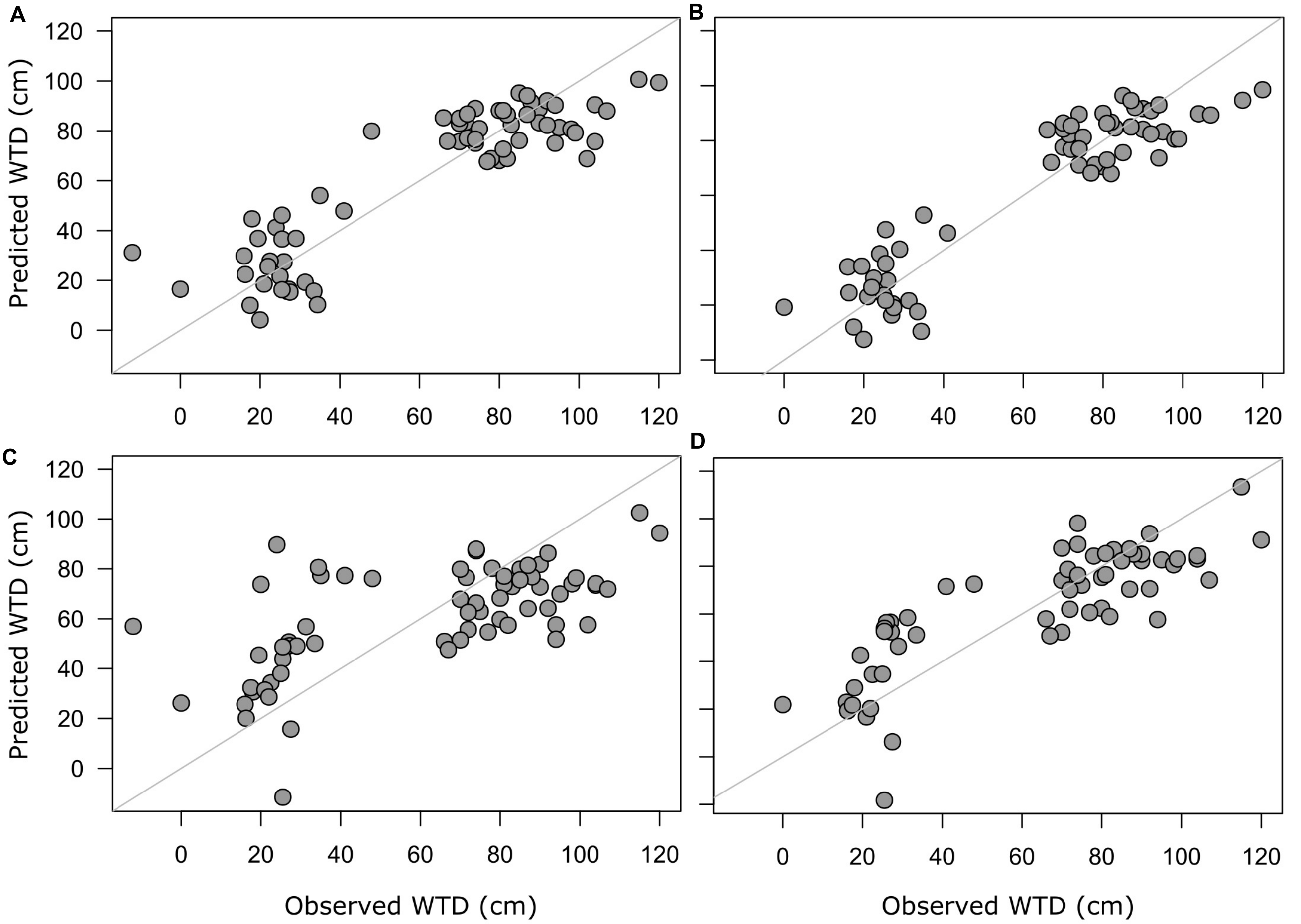

FIGURE 4 | Predicted WTD values plotted against observed WTD values. Predicted values are based on model estimates; full training set of morphotype-based WA model **(A)** and screened training set with the removed outliers for morphotype-based WA model **(B)**, the functional trait-based model **(C)** with selected functional traits and full training set and screened training set with the removed outliers for selected functional trait-based model **(D)**.

is in line with the results of the studies from North America and Europe (Charman et al., 2000). Notably, preferences for WTD depended on the size of the morphotypes, i.e., shells were larger in wetter locations. For example, *T. arcula* with large shells (130 μm) had a WTD optimum at 40.8 cm, whereas smaller morphotypes (95 μm) had their optimum at 63.9 cm. Similar patterns were observed by Schulz et al. (2018) in another tropical region, Ecuadorian Andes, showing that the shells of *T. arcula* were smaller in habitats characterised by low precipitation and low water availability. The WTD optima of the two smaller morphotypes of *T. arcula* (morph 1 and morph 2) were similar to the previously reported values (60 cm) in the Jura Mountains (Mitchell et al., 2001). Furthermore, the shell size of *Trigonopyxis* morphotypes with irregular aperture (similar to *T. arcula major*; **Figure 4**) decreases from 95 to 84 to 64 μm parallel to the increase in WTD optimum from 32.7 to 69.7 to 76.6 cm.

The WTD optima of the most abundant morphospecies *H. subflava* ranged from 27.5 to 74.1 cm. Small morphotypes, such as *H. subflava* morph 1 and morph 2, dominated in dry locations, whereas larger *H. subflava* morph 3, 5, 7, and 10 were abundant in wet locations. Previous studies suggested the WTD optimum of for *H. subflava* is 14.9 cm in peatlands of New Zealand (Charman, 1997) and 59.0 cm in bogs of the Jura Mountains in Switzerland (Mitchell et al., 2001). Thus, the indicator value of *H. subflava* needs to be reconsidered for tropical regions. Different optima of the morphotypes of *H. subflava* in the studied peatland indicate strong sensitivity of these taxa to WTD fluctuations. Therefore, we assume that the large variation in shell size of *H. subflava*, i.e., the co-existence of morphotypes differing in shell size in the studied peatland, is an indicator of an unstable hydrological regime. This is in line with the observation of Sullivan and Booth (2011) and Swindles et al. (2014) showing that some taxa, including *H. subflava*, are more abundant at more variable hydrological conditions.

For the second most abundant species complex, cf. *C. eurystoma* v. *parvula* morph 2 and morph 3, WTD optima varied from 53.2 to 62.2 cm and for cf. *C. eurystoma* v. *parvula* morph 1, the WTD optimum was at 75.9 cm. In comparison,

FIGURE 5 | Water table depth (WTD, cm) optima and tolerances for 87 taxa (present in >3 samples, for the exact number, see number in brackets) based on the inverse weighted averaging model after removal of samples with high (>25 cm, absolute values) residual values. For species abbreviations, see **Supplementary Table 2**.

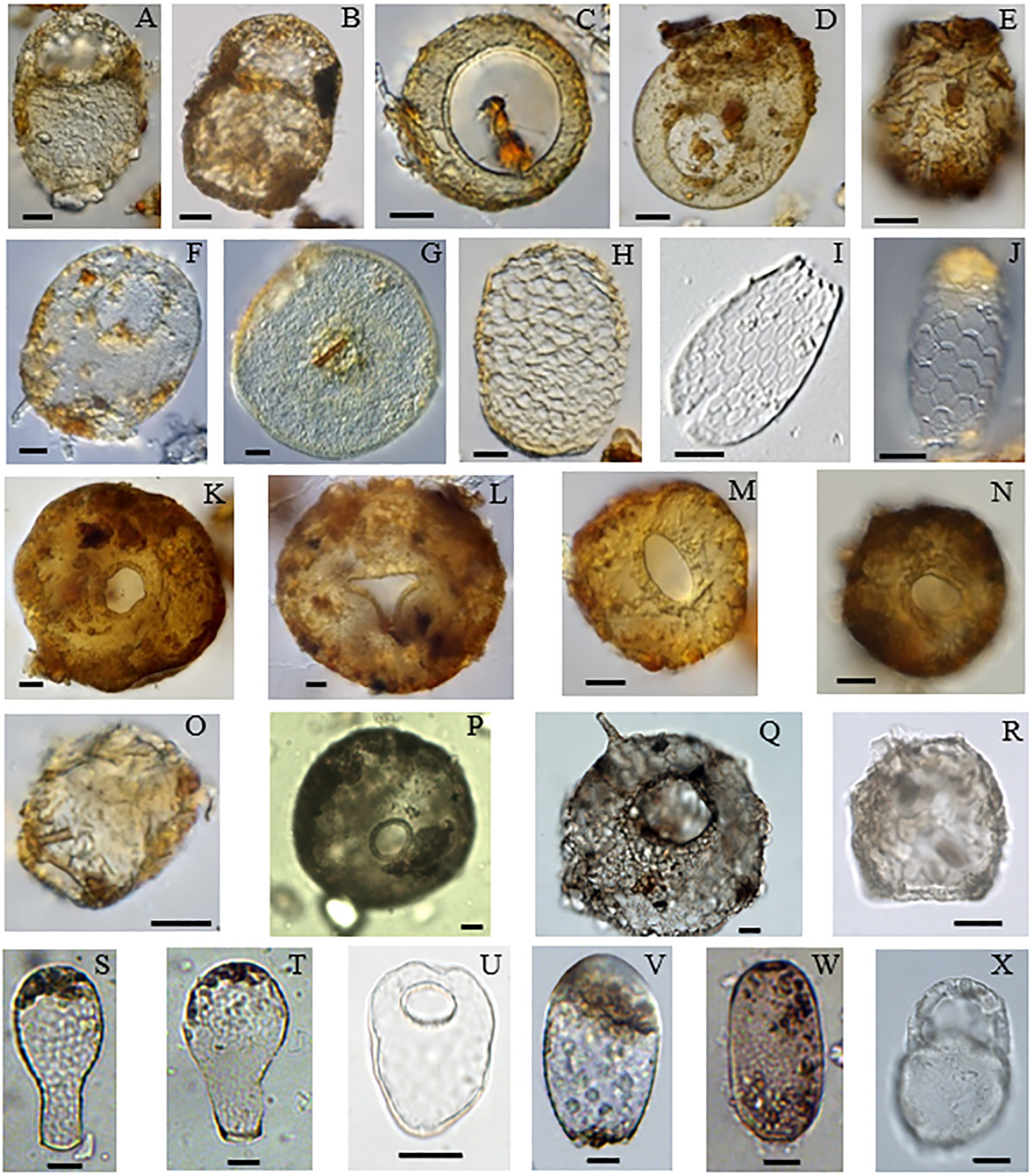

FIGURE 6 | Some taxa found in the samples. **(A)** *Centropyxis constricta*, **(B)** *Centropyxis aerophila* morph 1, **(C)** cf. *Cyclopyxis eurystoma* v. *parvula* morph 2, **(D)** *Centropyxis aculeata oblonga* morph 2, **(E)** *Difflugia penardi* morph 1, **(F)** *Centropyxis aculeata oblonga* morph 1, **(G)** *Arcella arenaria* v. *compressa*, **(H)** *Heleopera sylvatica*, **(I)** *Euglypha rotunda*, **(J)** *Tracheleuglypha dentata* morph 2, **(K)** *Trigonopyxis* sp1 morph 3, **(L)** *Trigonopyxis arcula* v. *major*, **(M)** *Cyclopyxis* sp1 morph 1, **(N)** *Cyclopyxis* sp1 morph 2, **(O)** cf. *Cyclopyxis eurystoma* f. *alta*, **(P)** cf. *Trigonopyxis microstoma* morph 1, **(Q)** *Centropyxis aculeata* morph 2, **(R)** *Difflugia angulostoma*, **(S)** *Padaungiella tubulata* morph 1, **(T)** *Padaungiella wailesi* morph 1, **(U)** *Trinema complanatum* morph 2, **(V)** *Jungia sundanensis* cf. *ovoidalis*, **(W)** *Archerella flavum*, and **(X)** *Centropyxis platistoma* morph 2. For taxa details, see **Supplementary Table 2**. Scale bares = 10 μm. Image A-O by F. Siemensma, P-X by A.S. Esaulov.

the optima of cf. *C. eurystoma* v. *parvula* in ombrotrophic and minerotrophic peatlands of the southern taiga of West Siberia were estimated as 27.6 cm and 6.9 cm, in the peats with WTD range of 0 to 46 cm (Kurina and Li, 2019).

In our samples we found representatives of the genera *Pyxidicula* and *Microchlamys* as well as species *Tracheleuglypha dentata*, *Centropyxis ecornis* and *Nebela collaris*, which are known to preferentially inhabit rich fens (rich in calcium, with pH range

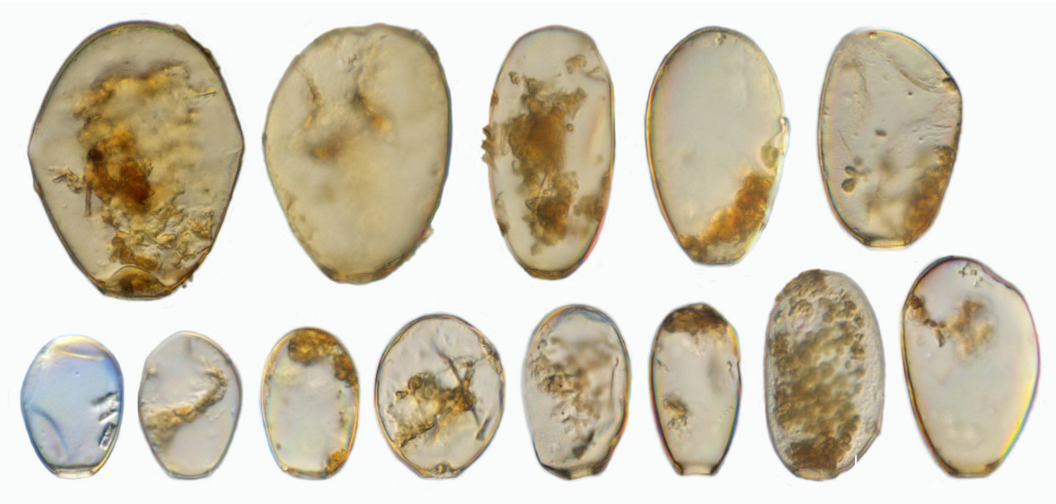

FIGURE 7 | Examples of different *Hyalopshenia subflava* morphotypes with a shell length of 40 to 105 μm from one location (NS13). Image by F. Siemensma.

from 6 to 8, but nutrient-poor), while in poor fens (nutrient poor, slightly acidic) and bogs they have not been recorded (see Marcisz et al., 2020, submitted). However, pH values in the studied peatland were very low (indicating ombrotrophic conditions), but C-to-N ratio of the substrate was low too, potentially indicating slightly minerotrophic conditions at least at some sampling points (Andersson et al., 2012). Such sites are typically characterised by the absence or low abundance of mixotrophic testate amoeba species (Jassey et al., 2014).

Again, we want to emphasise that some of the studied testate amoeba taxa have similar hydrological preferences across the globe, whereas others do not. Documentation of these differences, as well as the preferences of rare species with restricted geographical distribution, and the survey of species in underrepresented in literature tropical regions needs more attention. Further, species complexes, such as *Trigonopyxis* and *Hyalosphenia*, should be investigated in more detail including morphological and genetic traits potentially related to their hydrological preferences that may explain their wide range of hydrological tolerance.

Similar to morphotypes, only WTD and pH explained a significant fraction of the variation in the selected functional traits composition. Shell length, shell width and shell biovolume (which all were correlated) showed the strongest negative correlation with the WTD. In line with the results of our study, the predominance of smaller shells of testate amoebae at higher WTDs was shown in a field manipulation experiment (Koenig et al., 2018). Furthermore, testate amoebae with oviform/elongate shells dominated in dry biotopes, while sub-spherical testate amoebae (shell compression) and those with oval/circular aperture (aperture shape) were common in wet locations. This is similar to other studies, which showed that compressed shells and shells with small aperture preferentially occurred in dry habitats (Fournier et al., 2016; van Bellen et al., 2017; Koenig et al., 2018). By contrast, aperture position, presence of mineral particles on shell surface or mixotrophy did not correlate with WTD, with the latter being barely surprising as the abundance of mixotrophs was low (Lamentowicz et al., 2015; van Bellen et al., 2017).

Morphotype- and Trait-Based Transfer Functions

Both leave-one-out and bootstrap cross-validation provided relatively low model performance for the WTD transfer function based on morphotype data (RMSEP varied from 15 to 16 cm). By comparison, earlier studies on WTD transfer functions based on testate amoeba species showed RMSEP in the range of 2–8 cm (Payne and Mitchell, 2007; Booth et al., 2010; Swindles et al., 2018a). The wide range of WTD values in our study (0 to 120 cm) may explain this difference (van Bellen et al., 2014). The WTD gradient investigated in this study was 50% or even much wider than in most published training sets, e.g., over 60 cm in the study of Booth et al. (2010) and over 15 cm in the study of Payne and Mitchell (2007). Moreover, the RMSEP of our study is similar to that reported by van Bellen et al. (2014) (RMSEP 13 – 14 cm) in the ombrotrophic bogs in southern Patagonia which were characterised by a wide WTD range (0–104 cm). Notably, taking into account the WTD range, the relative errors in our study site (12.5–13.3%) showed values similar to the above-mentioned studies (11.5–14.5%), irrespective of the region. Thus, despite the high value of RMSEP in our study, the predictive power of WTD still remains at the same scale.

Weighted averaging resulted in the best model performance for the morphotype transfer function, whereas multiple regression gave the best model performance for functional traits. However, the trait-based transfer function provided lower model performance as compared to the morphotype-based one. The best RMSEP of 20.7 cm was obtained by including five traits in the model, i.e., aperture shape (oval/circular), aperture invagination (slightly invaginated), shell shape (oviform/elongate), shell compression (sub-spherical) and shell width. Among the traits related to the shell size, the shell width had a greater predictive power than others (**Supplementary Table 6**), even though shell width could probably be more casually related to the substrate wetness reflecting the width of the substrate pores. However, shell length may be a better indicator if used as intraspecific rather than interspecific trait, e.g., in *Trigonopyxis* and *Hyalosphenia*. Size stability in some species and variability in others may reflect the polyphyletic nature of testate amoebae, when shifts in shell size could have evolved differently in each lineage. Similarly, in oribatid mites it has been shown that phylogenetic signal of the body size and shape in some clades evolved independently several times but was conserved in others (Schaefer and Caruso, 2019).

Overall, RMSEP values of morphotype-based model (15–16 cm) and trait-based model (20.7 cm) are lower than the standard deviation of WTD in the studied peatland (33.4 cm), reflecting the predictive power of both transfer functions. The 4 cm difference in RMSEP between the morphotype- and the trait-based models is rather small considering the wide range of WTD (0–120 cm) in the studied peatland. The results of this study show that both morphotype-based and trait-based WTD transfer functions enable reliable reconstruction of palaeohydrological conditions in the study area, confirming the applicability of the trait-based approach. Thus, our weighted averaging transfer function will allow the first quantitative WTD reconstructions from a peat swamp in Central Sumatra. The trait-based reconstruction may be used to predict ecological preferences of unknown, rare or difficult for identification taxa. Moreover, considering that many morphospecies cannot be accurately determined taxonomically, the trait-based transfer function may provide additional realistic information avoiding inaccuracies due to identification errors and changing taxonomic affiliations.

CONCLUSION

The results of the present study contribute to the knowledge on testate amoebae in tropical ecosystems and indicate that for the development of transfer functions the ecological preferences of morphotypes within morphospecies also need to be considered and included in future studies. Moreover, there is an urgent need to fill the knowledge gap on geographical variations in the indicator value of cosmopolitan species to allow better palaeoecological reconstructions. Unfortunately, with few exceptions (Swindles et al., 2014, 2018a), transfer functions have only been established for European and North American peatlands until today. Overall, this study has presented the first testate amoebae transfer functions for reconstructing hydrological regime in the Southeast Asia and the third one based on functional traits. Our investigation also provided new insight into ecological preferences of a number of testate amoebae in tropical peatlands. Although hydrological preferences of some morphospecies were similar to those in high and middle latitudes, latitudinal differences in morphospecies and variations in environmental relationships of species require the development of region-specific transfer functions. Further, we intend to study subfossil testate amoebae from the Sungai Buluh peatlands and apply the developed transfer functions to reconstruct the palaeohydrological dynamics of peatlands in Sumatra with the scope to understand peat swamp ecosystem hydrological dynamics during the Holocene.

DATA AVAILABILITY STATEMENT

All datasets presented in this study are included in the article/**Supplementary Material.**

AUTHOR CONTRIBUTIONS

VK and SB conceived and designed the study. SB, VK, and AE performed the sampling and extractions. AE, VK, SB, and KH analysed the samples. VK and AT prepared and analysed the data and wrote the first draft of the manuscript. VK, YM, HB, and SB contributed reagents, material, and analysis tools. YM secured funding for microscopic and the data analyses and contributed to the data analysis methodology. AS, SS, SB, and HB provided field logistic support and acquired the funding. All authors contributed to writing and editing the manuscript and gave final approval for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00225/full#supplementary-material

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Abrupt Fen-Bog Transition Across Southern Patagonia: Timing, Causes, and Impacts on Carbon Sequestration

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Fens and bogs are distinct in terms of their biogeochemistry, water table behavior, and net peat-accumulation regimes. While most peatlands start developing as fens, a large fraction of them eventually shift to bogs in a step-like ecosystem shift. This transition has traditionally been assumed to be primarily controlled by the ecosystem itself (autogenic control). Here we use 90 peat profiles from southernmost South America (SSA) as a case study that illustrates a synchronous, regional-scale shift from fen to bog around 4200 years ago. In light of these results, we propose and discuss conceptual models that link environmental change (allogenic control) as a trigger to the fen-bog transition (FBT). In addition, our stratigraphic analyses show that *Sphagnum* deposits are associated with greater peat masses, larger soil-carbon stocks, and higher rates of peat-carbon accumulation than their non-*Sphagnum* counterparts, with *Sphagnum* bogs being characterized by soil-carbon densities over twice that of non-*Sphagnum* peatlands (medians = 141 vs. 56 kgC/m^2). Since fens and bogs also behave differently in terms of their carbon exchanges with the atmosphere, a better appraisal of the processes involved in the FBT could help elucidate the role of this critical ecosystem shift in the past and future global carbon cycle.

Keywords: carbon sink function, Holocene, Mires, tipping point, regime shift, complex adaptive system, Paleoecology

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INTRODUCTION

Peatlands cover about 3% of the global land area and account for roughly half of the wetlands worldwide (Rydin and Jeglum, 2013; Mitsch and Gosselink, 2015). Peatland soils are water-saturated; as a result, plant decomposition occurs slowly, which causes the net accumulation of partly decomposed plant matter over centennial to millennial timescales. Peatlands are predominantly found across the mid- and high-latitude regions (∼45–70°), though they can be regionally abundant in the subtropical belt (∼15–30°) (Xu et al., 2018).

Peatlands are classified into two main types, fens and bogs, on the basis of their hydrological and bioclimatic conditions, chemistry and nutrient status, and floristic composition (Charman, 2002; Vitt, 2006). While fens depend on mineral-rich water inputs from groundwater and overland flow, bogs are ombrotrophic, i.e., their water supply comes exclusively from rain and snow

(Damman, 1986; Vitt et al., 2003; Mitsch and Gosselink, 2015). Bogs tend to be acidic, while fens are typically characterized by neutral or alkaline pH conditions (Rydin and Jeglum, 2013). This trophic gradient, from rich fens to poor bogs, influences plant community dynamics, including biomass growth and floristic composition (e.g., Malmer, 1986; Glaser, 1992). Plant communities in fens tend to be dominated by sedges, grasses, rushes, and brown moss species. Bogs often harbor continuous *Sphagnum* moss carpets with some woody shrubs and sedge clusters. In continental regions, drier bogs can also be colonized by small trees. In general, fens are characterized by higher net primary productivity, greater plant diversity, and more rapid peat decomposition rates than bogs (Zoltai and Vitt, 1995; Granath et al., 2010).

Fens and bogs also differ in terms of their ecosystem functions. It has been repeatedly shown (e.g., Blodau, 2002; Turetsky et al., 2014; Abdalla et al., 2016) that fens emit substantially more methane (CH_4) than bogs [100:1 according to Laine and Vasander (1996)]. Fens are characterized by high water table levels, which limit CH_4 oxidation within the unsaturated ("acrotelm") upper peat layer (Moore and Roulet, 1993; Whiting and Chanton, 2001), thus facilitating CH_4 emission to the atmosphere. Fen vegetation cover, often dominated by sedges, acts as CH_4 conduits from deep peats to the atmosphere, further contributing to elevated CH_4 emissions from these peatlands (Bubier, 1995; Segers, 1998). Bogs, on the other hand, are typically characterized by lower water table levels and significantly less sedges than fens (Turetsky et al., 2014). Overall, it can be argued that fens and bogs are also distinct in terms of their biogeochemistry and water table (and moisture) behavior.

The estimated extent of fens and bogs at the global scale remains unclear. Best estimates put the global fen and bog areas around 1.1 and 2.1 Mkm^2, respectively (Loisel et al., 2017a). This knowledge is based on limited country inventories combined with broad geographic distribution patterns. For example, across the temperate, boreal, and arctic biomes, peatland distribution tends to follow climatic gradients of precipitation and temperature, from oceanic to continental, wet to dry, and mild to cold (Vitt, 2006; Parviainen and Luoto, 2007). For example, in Canada, most bogs are found in the boreal region (though there are permafrost bogs in the Arctic), while fens are typically clustered along the southern edge of the peatland distribution, in mountainous areas, and across the (sub-)Arctic lowlands (Tarnocai et al., 2002).

The classic peatland hydroseral succession that leads to the formation of an ombrotrophic peatland (bog) consists of a transition from an aquatic environment (pond, marsh) to a minerotrophic peatland (fen) and, subsequently, to a peat bog (Zobel, 1988). This general developmental scheme from fen to bog has been attributed to one key autogenic process, namely long-term peat accumulation that eventually reaches an elevation beyond the influence of the mineral groundwater (Kuhry et al., 1993). This autogenic succession paradigm is engrained in our understanding of the fen-bog transition (FBT); as such, process-based peatland models such as the Holocene Peat Model assume that the FBT occurs when 1–2 m of peat has accumulated (Frolking et al., 2001, 2010). In nature, an intermediate stage between the fen and bog states does not really exist (e.g., Walker, 1970; Kuhry et al., 1993; Bunting and Warner, 1998), suggesting that the transition from fen to bog might occur relatively quickly, likely over few decades to centuries; this approximation is based on the thinness (typically only a few cm) of fen-bog transitional layers (e.g., Hughes and Barber, 2003; Ronkainen et al., 2014). Also noteworthy is that alternative developmental pathways have been observed. For example, in permafrost regions, an ombrotrophic peat plateau can "revert" or "cycle" back to a fen following thermokarst collapse (Treat et al., 2015); such cyclical succession between fen and bog has been observed across the discontinuous permafrost zone (e.g., Zoltai, 1993; Camill and Clark, 2000). That said, for the purpose of this paper, the focus remains on the classic succession from fen to bog.

In contrast to the autogenic succession theory, a few studies have proposed that hydroclimatic conditions (allogenic factors) may exert a major influence on the passage from a wetter and richer fen to a poorer and drier bog (e.g., Hughes, 2000; Hughes and Dumayne-Peaty, 2002; Hughes and Barber, 2003). In these cases, the ombrotrophication process is triggered by small changes in allogenic controls such as increased effective moisture (the "wet route") or prolonged droughts (the "dry route"). An allogenic change can thus "speed up" autogenic succession (e.g., Väliranta et al., 2017); succession could also make the system more susceptible to allogenic factors. For example, in oceanic settings, it has been suggested that an increase in effective precipitation (precipitation minus evaporation) could flush mineral-rich water and enhance *Sphagnum* growth, which would lead to the formation of a perched water table and the initiation of ombrotrophy (Hughes and Barber, 2003). Under this "wet route" scenario, *Sphagnum* species isolate themselves from the groundwater by growing "upward." While the ombrotrophication process is ultimately driven by *Sphagnum* growth (autogenic control), it is triggered by increased moisture levels (allogenic control). Another example was observed in continental settings, where droughts could trigger ombrotrophication processes by enhancing peat decomposition, which leads to the creation of dense peat layers and to a relatively impermeable substrate, which ultimately isolates living biomass from minerotrophic groundwaters. Sedge remnants (e.g., *Eriophorum vaginatum*) have been identified along cores, at the FBT, across continental regions (Hughes, 2000; Hughes and Barber, 2004). This plant type, which creates dense tussocks, exhibits strong resistance to decay (Hughes and Barber, 2004) and has a great water-holding capacity (Hughes, 2000). Under this "dry route" scenario, sedge tussocks could provide *Sphagnum* species with micro-habitats above the groundwater influence, potentially allowing *Sphagnum* to rapidly grow upward, leading to a quick switch to ombrotrophy.

Both the autogenic and allogenic FBT pathways described above are in line with a growing body of evidence suggesting that peatland dynamics are non-linear. Rather than displaying gradual changes in structure and function that match the frequency of external forcing, peatlands can show long periods of little change that are punctuated with step-like transitions to alternative states in response to seemingly small internal or external forcing mechanisms (Levin, 1998; Belyea and Baird, 2006; Andersen et al., 2009; Belyea, 2009). Such abrupt transitions from one steady state to another are described as bifurcations (or tipping points), and they occur once the stability properties of an ecosystem are suddenly lost to the expense of a new (or alternative) stable state (Scheffer et al., 2001; Lenton, 2013; Milner et al., in press). The alternative ecosystem state may be maintained by self-regulating physical, biological, and/or chemical negative feedbacks, making it difficult or impossible to reverse (Scheffer and Carpenter, 2003). The difficulty to foresee these non-linear responses is concerning because changes in ecosystem structure may lead to critical shifts in ecosystem function (Lenton, 2013). As abrupt changes in peatland dynamics may directly impact peat-C sequestration rates, significant effects on the global C cycle and the climate system can result from such a non-linear behavior.

Rationale and Research Objectives

In an effort to broadly understand the response of peatlands to past, present, and future environmental change, a predictive modeling approach that links fen vs. bog traits with C exchange functions is needed. Although C dynamics are spatially variable within a single peatland due to small-scale heterogeneity associated with hummock-hollow microtopographic gradients (Waddington et al., 1996; Baird et al., 2009), there is merit in distinguishing between fens and bogs for the general purpose of coupling dynamic peatland models with Earth System Models (Frolking et al., 2009). Likewise, landscape-scale mapping of peatland types would lead to better estimates of peatland-atmosphere C exchanges (Baird et al., 2009). Also, knowing the timing of critical ecosystem shifts such as the FBT, both in the past and the future, could help model changes in peatland C dynamics and their role in the global C cycle. In particular, knowing how the extent of fens and bogs has changed throughout the Holocene would provide constraints on peatland-atmosphere greenhouse gas exchange (e.g., Frolking and Roulet, 2007; Yu et al., 2013). Indeed, it has been suggested that the progressive decrease in atmospheric CH_4 concentration that occurred from 8000 to 4000 years ago might relate to the FBT across the boreal biome (MacDonald et al., 2006; Loisel et al., 2015). Likewise, Yu (2011) suggested that peat-C accumulation could have contributed to the Holocene changes in atmospheric carbon dioxide (CO_2) concentration and $\delta^{13}CO_2$ observed along Antarctic ice cores. It is also possible that, in the near future, large swaths of wet arctic tundra switch to bogs, following landscape-scale permafrost thaw and drainage (Yu et al., 2009; Hugelius et al., in press). Despite its key role in the Holocene global C cycle, little is known about the timing and mechanisms of the FBT across the mid- and high-latitude biomes.

In this study, a data synthesis of FBT timing across 90 southern Patagonian peatlands is presented. We show a regional-scale switch from fens to bogs across the region around 4200 years ago, pointing toward a regional-scale control on the FBT. We present two non-exclusive hypotheses that could explain this regional shift in peatland type. A conceptual model of the FBT is then discussed. To our knowledge, no models provide a conceptual understanding of the FBT [but see Loisel et al. (2012), where a preliminary version of this model was presented]. In this model, allogenic factors push the fen beyond its resilience capacity, inducing a regime shift to ombrotrophy. Overall, this information may be of use to improve ecological models via the identification and quantification of ecosystem functional traits between bogs and fens; it also has applications for peatland management efforts such as rehabilitation, restoration, and conservation.

MATERIALS AND METHODS

Study Region

The study region is southernmost South America (SSA), which in this case refers to the landmass located between ∼ 50 and 56°S. This area includes Chile's Magallanes region and Argentina's portion of Tierra del Fuego (**Figure 1**). The regional climate is heavily influenced by the southern westerly winds, which carry moisture and heat from the Pacific Ocean onto the continent, in a west-to-east direction (Garreaud, 2009). By forcing the maritime air upward, the Andes Mountains create a strong precipitation gradient across the region (Paruelo et al., 1998). Three main peatland types are found across this gradient. First, cushion bogs are established under hyper-oceanic conditions. These ecosystems are dominated by *Astelia pumila* and *Donatia fascicularis*, two species that are tolerant of windy, stormy, and cold conditions (Auer, 1958; Moore, 1983; Pisano, 1983). Second, ombrotrophic peat bogs are located under humid to dry conditions (∼ 400–1000 mm/yr); these ecosystems are dominated by *Sphagnum magellanicum* carpets and scarce stunted trees (Pisano, 1983; Grootjans et al., 2010). Lastly, groundwater-fed herbaceous fens are found under dry to arid conditions (<400 mm/yr) and are usually dominated by grasses, sedges (*Carex spp.*), rushes (*Marsippospermum grandiflorum*), and a few shrubs. Peat bogs today thus occur within a narrow band of land located along the lee side of the Andes (**Figure 1**), where mean annual temperature is around 5°C (Loisel and Yu, 2013a), the temperature seasonality (difference between the warmest and coldest months) is around 10°C (Loisel and Yu, 2013a), and frost is rare (Schneider et al., 2003; Arroyo et al., 2005). These peat bogs are embedded within the deciduous and evergreen forests of southern Patagonia; the bogs are rain-fed and are typically found in valley bottoms atop lacustrine sediments or marine clays and silts (Bentley and McCulloch, 2005; Rabassa et al., 2006).

New Peat Core Data

This study includes a total of 90 peatlands (**Supplementary Figure S1**), of which 12 were sampled by our team over the course of two field seasons (**Supplementary Table S1**). In 2010 (PAT-10), four peat bogs were visited and a core was retrieved at each one of the following sites (unofficial names): Harberton (HB), Cerro Negro (CN), Upper Andorra Valley (UAV), and Escondido (ESC). Some results from these cores have been published elsewhere (Loisel and Yu, 2013a,b; Loisel, 2015). In 2018 (PAT-18), eight new sites were cored: Beef-Penguin (BP), Mercedes (MP), Jen (JB), Rasmussen (RAS), Ariel (AP), Cura (CP), Pat-Andy-Nat (PAN), and Flarks (FP). Some of the results from cores MP and BP are published (Bunsen and Loisel, 2020),

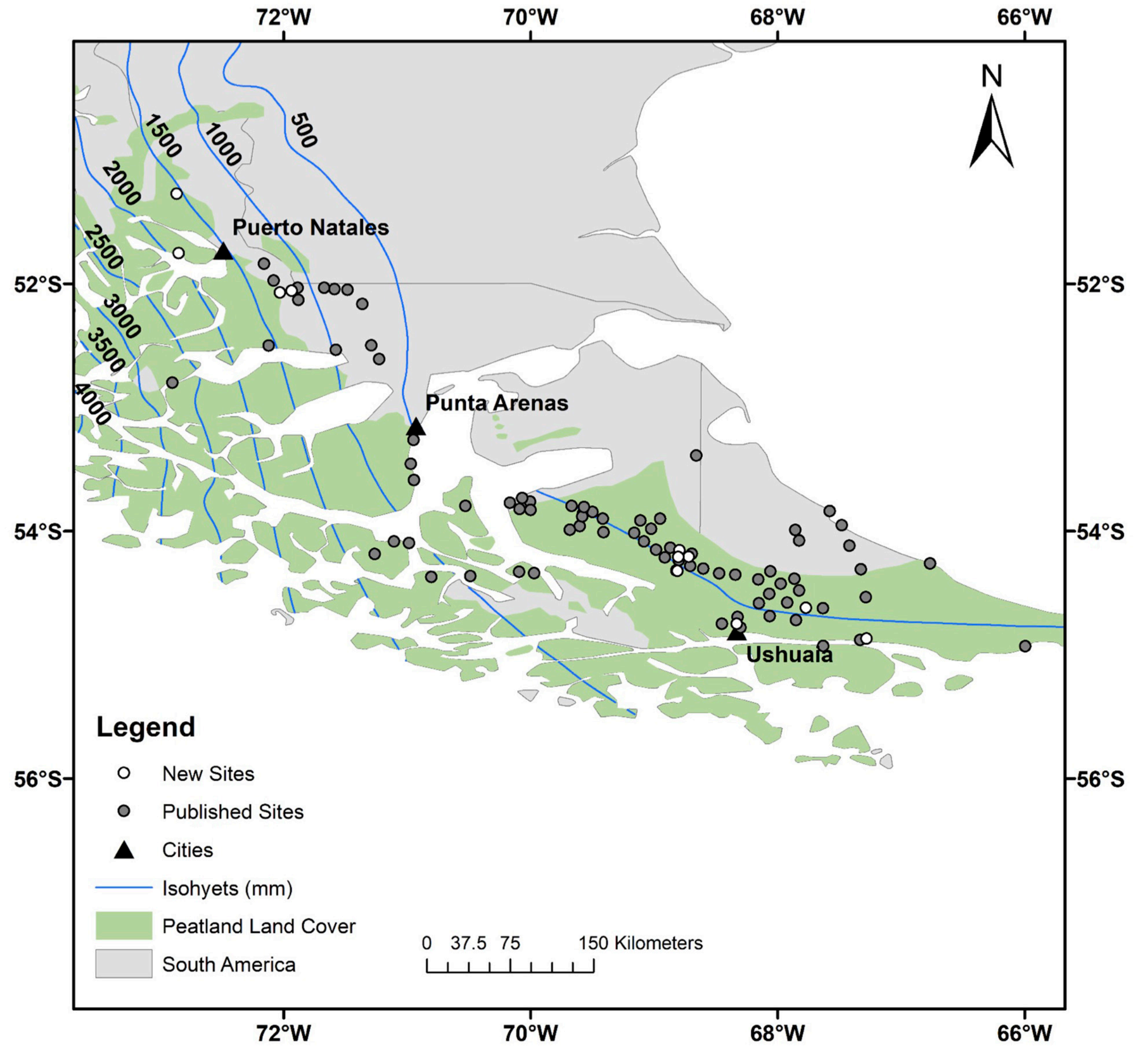

FIGURE 1 | Study region, southern South America. The open circles indicate our 12 new cores, the gray circles show the location of the 78 sites from the literature review, and the black triangles locate the main towns. The peatland area is from Yu et al. (2010) and the dataset used to generate the isohyets is from New et al. (2002). Detailed maps with site names can be found in the **Supplementary Information** (**Supplementary Figure S1**).

while those from the other cores remain unpublished at the moment (but see Bunsen, 2020). Note that Ariel Peatland has also been studied by Xia et al. (2018, 2020); likewise, Harberton Bog was the object of previous studies (e.g., Markgraf, 1991; Pendall et al., 2001).

Eleven of the twelve sites are *S. magellanicum* peat bogs that are characterized by microtopographic gradients, from dry hummocks to wet hollows. Shrubs *Empetrum rubrum* and *Nothofagus antarctica* were identified at all sites, with the exception of Jen Bog and Mercedes Peatland, where *N. antarctica* was replaced by cypress tree *Pilgerodendron uviferum*, a conifer only found in hyper-humid habitats of Patagonia (Moore, 1983). The sedge and grass communities typically included *Marsippospermum grandiflorum*, *Tetroncium magellanicum*, *Alopecurus magellanicus*, *Carex magellanica*, and *Carex curta*. The 12th site (Cura Peatland) is a spring-fed fen dominated by herbaceous plants and Bryales. Cores were extracted in 50-cm increments using a Russian auger. The stratigraphy of each section was described in the field in terms of main peat types (moss, herbaceous, ligneous, etc.) and soil properties (texture, particle size, color). The cores were then wrapped in plastic and foil, and stored in PVC pipe for shipping. Cores were kept at 4°C until lab analyses. Inception age varies between 15,550 and 1450 calibrated years Before Present (cal. yr BP). All 11 peat bog cores exhibit a clear, sharp switch from herbaceous-dominated fen peat to *Sphagnum*-dominated bog peat (**Figure 2**). The FBT has thus occurred at some point during the Holocene at these sites.

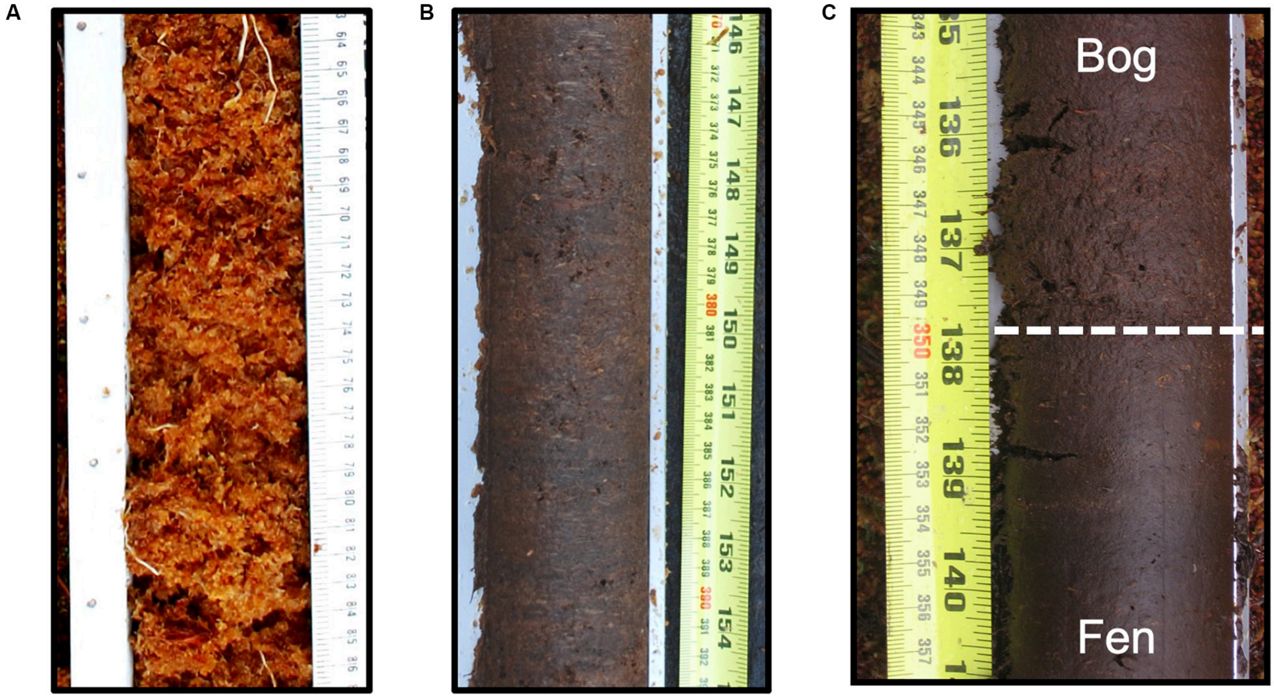

FIGURE 2 | Peat stratigraphic characteristics. **(A)** *Sphagnum* peat, poorly decomposed (core PAT10-HB). **(B)** Herbaceous peat, highly decomposed (core PAT10-CN). **(C)** Fen-bog transition along a peat core (site PAT10-CN).

Peat core chronology was constrained using radiocarbon (^{14}C) dating (**Supplementary Table S2**). Hand-picked plant macrofossils were cleaned with deionized water, dried, and ^{14}C-dated using AMS at the University of California – Irvine's Keck AMS Carbon Cycle Lab (samples collected in 2010) and at Lawrence Livermore National Lab's CAMS Radiocarbon Lab (samples collected in 2018). When the samples were too decomposed for the identification and cleaning of plant macrofossils, root-free bulk peat samples (63–125 μm) were used instead. As the regional tephrochronology is relatively well known (e.g., Stern, 2008), a few volcanic tephras were identified along our peat profiles on the basis of visual examination (in the field) as well as changes in bulk density (in the lab). The tephra layers that were identified are: Aguilera I, Burney I and II, Hudson I, and Reclus I, as well as cryptotephras Hudson a and b (Mansilla et al., 2016; **Supplementary Table S3**). While they were not used to build the chronologies, the tephras were used to confirm our age-depth models and compare core stratigraphy between sites.

Age-depth models for the four PAT-10 cores (HB, CN, UAV, and ESC) have been published in Loisel and Yu (2013a) but have been updated for this study, in light of more recent calibration curves being available. Age-depth models for the eight PAT-18 cores (BP, MP, JB, AP, CP, FP PAN, and RAS) are available in Bunsen (2020). All raw and calibrated ^{14}C data, as well as our final age-depth models, are included in the Supplements (**Supplementary Table S2** and **Supplementary Figure S2**), along with tephra ages obtained from the literature (**Supplementary Table S3**) and bulk density profiles from all 12 cores to show the stratigraphic location of those tephras (**Supplementary Figure S3**). We used the SHCal13 (Hogg et al., 2013; Reimer et al., 2013) and SHZ 1-2 calibration curves (Hua and Barbetti, 2004), along with Bacon version 2.3.5 (Blaauw and Christen, 2011) to constrain peat chronologies.

Geochemical measurements were performed at high resolution along all 12 cores (**Supplementary Figure S3**). Along the PAT-10 cores, contiguous sub-samples (1 cm^3) were analyzed every cm; the sample interval was increased to 2-cm increments along the PAT-18 cores, though sub-samples (2 cm^3) were still measured contiguously. Loss-on-ignition (LOI) was performed to determine peat water content, organic matter content, dry bulk density, and organic matter density on each sub-sample (total = 3854 samples); standard procedures were followed (Dean, 1974). Changes in peat water content and organic matter density were used to infer stratigraphic changes in peat type along the cores for which plant macrofossil analysis has not yet been performed. This inference is based on a statistical analysis that was performed on 438 Patagonian peat samples for which plant fossil analysis as well as geochemical measurements were performed (see Loisel and Yu, 2013a). Results indicate that low water content and high density are associated with herbaceous peat, and high water content and low density are correlated with *Sphagnum* peat (Loisel and Yu, 2013a). Lastly, peat-carbon accumulation rates (PCAR) were obtained by multiplying the organic matter density of each depth increment (in g OM/cm^3) by the interpolated deposition rate of each sample (from the age-depth models, in cm/yr) and by an assumed 50% C content (Loisel and Yu, 2013a).

Data Synthesis

A compilation of 78 peat core stratigraphies from southernmost Patagonia was used to infer the timing of the FBT across the region. The quality of data varies across the different sources, from visual descriptions of peat stratigraphic changes (Auer, 1965) to semi-quantitative plant macrofossil-based reconstructions (De Vleeschouwer et al., 2014). Radiocarbon-based age-depth models were unavailable for Auer's (1965) cores; instead, peat stratigraphic changes are described in relation with three tephra layers of known ages (Stern, 2008): Reclus I (15,780–14,040 cal. yr BP; median age = 14,900 cal. yr BP), Hudson I (7960–7420 cal. yr BP; median age = 7700 cal. yr BP), and Burney II (5290–3230 cal. yr BP; median age = 4200 cal. yr BP). We used these three tephra layers to compare changes in peat type, total peat mass (gOM/cm^2), and PCAR (gC/m^2/yr) between all 78 cores from the literature as well as our 12 new cores. Lastly, peat depths were converted to organic matter density, peat mass, and PCAR using the following values: 0.05 g OM cm^3 for *Sphagnum* peat and 0.07 g OM cm^3 for herbaceous (i.e., *Carex* spp., *Marsippospermum* sp.) and other non-*Sphagnum* (i.e., Bryales) peat types (Loisel and Yu, 2013a).

RESULTS

Peat Stratigraphy and FBT Timing Across Southern Patagonia

New Cores

The stratigraphic records from our 12 new cores are presented in **Figure 3**. Each site started its development as a fen, and 11 out of 12 sites evolved into a bog during the mid- and late-Holocene time periods (**Figure 3**). Of these 11 sites, six switched to bogs right after the deposition of tephra layers (CN, MP, FP, PAN, RAS, and AP). While the FBT at site AP followed the Hudson I eruption (7960–7420 cal. yr BP), the transition occurred after the Burney II eruption (5290–3230 cal. yr BP) at the other five sites. Sites ESC and BP switched to bogs prior to those eruptions, around 4700 and 10,000 cal. yr BP, respectively. One site (HB) is too young for having been affected by any eruption and records a FBT around 1000 cal. yr BP. The last two sites (JB and UAV) transitioned into bogs around 1200 and 2300 cal. yr BP, respectively.

Data Synthesis

The synthesis included 78 peat profiles; the majority (63 out of 78 sites) started their development as herbaceous-dominated or Bryales-dominated fens (**Supplementary Figure S4**). Of the remainder, two sites started as *Donatia*-dominated cushion bogs, another one began its development as a forested peatland, and the remainder (11 out of 78 sites) started as *Sphagnum* bogs. As for today's vegetation communities, 47 of the 78 sites are now *Sphagnum*-dominated bogs, five are cushion bogs, and 26 are fens. At a few sites (T1, T2, and T3), *Sphagnum* deposits that predate the Reclus I eruption (14,900 cal. yr BP) have been identified (**Supplementary Figure S4**). Those are exceptional cases, with most *Sphagnum* deposits developing during the mid- and late-Holocene. Indeed, 35 out of the 47 *Sphagnum*-dominated sites transitioned from fen to bog right after the deposition of tephra layers. As is the case along our own cores, some peatlands switched to bogs following the Hudson I eruption (7960–7420 cal. yr BP; $n = 5$), though the majority transitioned after the Burney II eruption (5290–3230 cal. yr BP; $n = 30$). While sites T3, T5, and HAB switched to bogs prior to those eruptions, five other sites switched between those events (T14, T15, T56, T62, and P52); T29 and Onamonte transitioned after the Burney II eruption (**Supplementary Figure S4**).

Peat Accumulation Across the FBT in Southern Patagonia

New Cores

In general, our 12 study peatlands have been effective C sinks over the Holocene (**Figure 3** and **Supplementary Table S4**). Core analysis reveals a median thickness of 452 cm (min = 290, max = 770) and a median soil C density of 130 kgC/m^2 (min = 89, max = 220). The rate of peat accumulation has not been constant through time across our sites (refer to **Supplementary Figure S3** for details). For instance, mean PCAR for the time period between Reclus I (14,900 cal. yr BP) and Hudson I (7700 cal. yr BP) amounts to 7 gC/m^2/yr (**Supplementary Table S4**). This figure reaches 13 gC/m^2/yr during the time period between Hudson 1 and Burney II (4200 cal. yr BP) eruptions. It then rises up to 22 gC/m^2/yr for the last 4200 cal. yr BP (**Supplementary Table S4**). While these figures account differentially for long-term decomposition and peat compaction on the basis of their age (i.e., the young peat has not decomposed or compacted as much as the old one), they are still informative.

Data Synthesis

There are large differences in terms of peat depth and peat C density across the 78 sites described in the literature (**Supplementary Figure S4** and **Supplementary Table S4**). Median peat thickness amounts to 345 cm (min = 60, max = 1400), which translates to a median soil C density of 119 kgC/m^2 (min = 21, max = 375). Temporal changes in the rate of peat accumulation have also occurred across the 78 sites (refer to **Supplementary Figure S3** for detailed results). In general, and similar to the findings from our own cores, PCARs were lower than average between the Reclus I (14,900 cal. yr BP) and Hudson I (7700 cal. yr BP) eruptions, with a mean of 7 gC/m^2/yr (**Supplementary Table S4**). This figure increases to 11 gC/m^2/yr during the time period between Hudson 1 and Burney II (4200 cal. yr BP) eruptions, and remains about the same (12 gC/m^2/yr) over the past 4200 cal. yr BP (**Supplementary Table S4**).

DISCUSSION

Spatial and Temporal Patterns of the FBT Across Southern Patagonia

Altogether, 58 out of the 90 surveyed peatlands are bogs today (**Figure 3** and **Supplementary Figure S4**). Out of these 58 *Sphagnum*-dominated bogs, 35 transitioned from their fen state

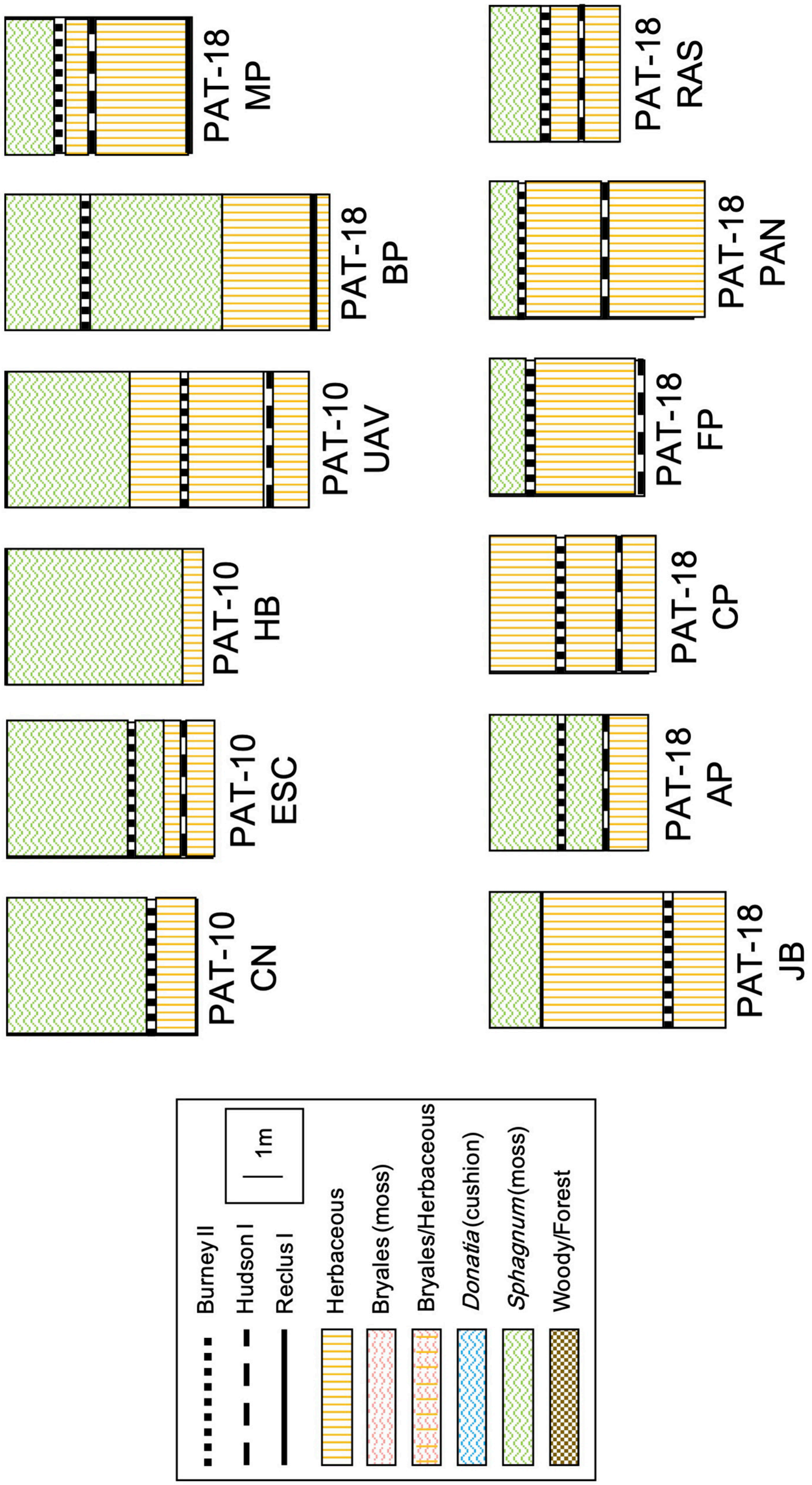

FIGURE 3 | Peat stratigraphic profiles for the 12 new sites included in this study. The sketches are true to scale. The remaining 78 peat profiles from the literature review can be found in the **Supplementary Information** (**Supplementary Figure S4**).

at 4200 cal. yr BP (Burney II eruption), six switched to bogs at 7700 cal. yr BP (Hudson I eruption), and 13 underwent the FBT at other times (**Figure 4**). As for the sites that did not respond to those eruptions and switched to bogs at later stages (e.g., JB, UAV, ONA, and RR), it is possible that the eruption (or its tephra) was not sufficient to destabilize the fen conditions. As for the sites that switched prior to those eruptions (e.g., ESC, BP, and HAB), we do not have sufficient evidence to suggest alternative triggering mechanisms at this time, though drying conditions during the mid-Holocene might have facilitated the colonization by *Sphagnum* (e.g., Hughes, 2000; Hughes and Barber, 2004). Of the remaining 32 sites, 27 have persisted as fens for their entire history while the other five are *Donatia*-dominated cushion bogs today (**Supplementary Figure S4**). The temporal pattern offered by the FBT is striking, with a majority of sites simultaneously switching to bogs across SSA (**Figure 4**).

Some peatland-rich areas of southernmost Patagonia are found outside the *Sphagnum* moss domain. As such, the five cushion bogs cataloged in this study (T40, T41, T42, T60, and SKY-2) are located off the western Chilean coast and along the Strait of Magellan, in areas of either very high precipitation, high wind, or storminess (**Supplementary Figure S4**; see **Supplementary Figure S1** for detailed location of these sites). Though tephra layers were identified along these core profiles,

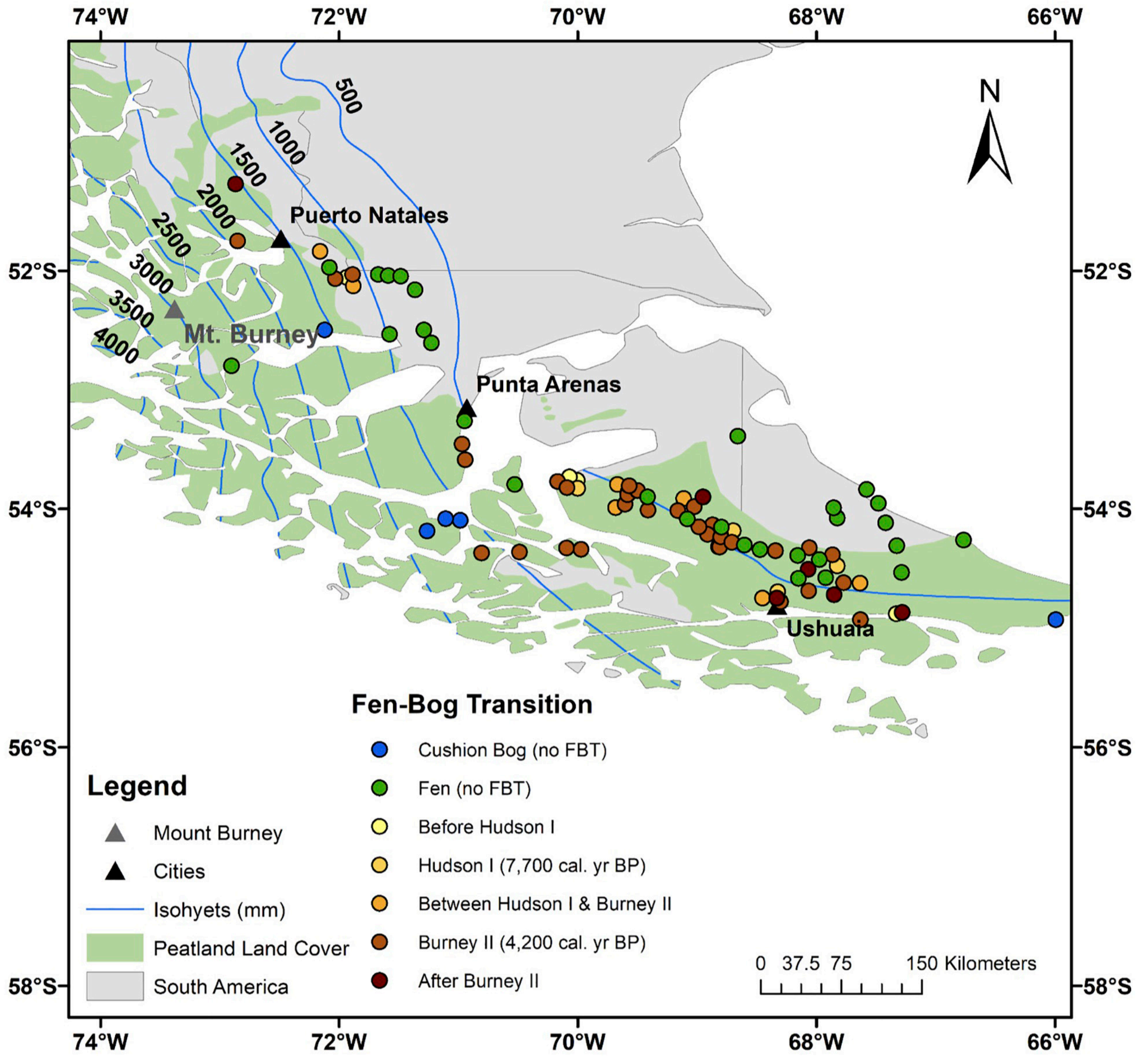

FIGURE 4 | Fen-bog transition timing across southern South America. Non-*Sphagnum* peatlands (fens and cushion bogs) are also shown. The location of Mount Burney is indicated by a gray triangle. Mount Hudson (45.9 S, 72.97 W) is located outside the area shown on the map. The peatland area is from Yu et al. (2010) and the dataset used to generate the isohyets is from New et al. (2002).

Sphagnum moss is not typically encountered in these two regions today, due to climatic limitations (Pisano, 1983). That said, the co-occurrence of *Donatia* and *Sphagnum* has been observed in a few peatlands located near the current limit of *Sphagnum* bogs (near sites PAT-18-MP, between sites PAT-10-HB and T60, and at site SKY). In this area, to our knowledge, at least one peat core harbors switches between cushion- and *Sphagnum*-dominated assemblages (Mathijssen et al., 2019). But for the sake of this study, which focuses on the FBT, we will not investigate this topic further.

Another outstanding spatial pattern from our analysis is the location of 27 "persistent fens." These peatlands have not undergone the FBT despite having been influenced by volcanic eruptions (**Figure 3** and **Supplementary Figure S4**). Of these persistent fens, 13 sites are clearly found in the steppe ecoregion (T36, T37, T51, T64, T65, T66, T67, T69, P57, P58, P60, P61, and P65), making it unlikely for *Sphagnum* to colonize these areas due to local hydroclimatic limitations (**Supplementary Figure S4**; see **Supplementary Figure S1** for detailed locations). That said, several sites that are either located near the modern-day boundary between the steppe and forest ecoregions (T7, T19, T30, T31, T32, T63, and P63) or within the *Sphagnum* bog domain (PAT-18-CP, T23, T26, T58, T61, P53, and P56) also persisted as fens. In those cases, a number of local conditions that would prohibit oligotrophy, including insufficient peat thickness, the presence of mineral-rich springs or surface runoff from surrounding hillslopes, and other conditions such as inadequate parent material or topography, could have prevented the switch to bog conditions. In particular, many of these peatlands are found in complex moraine basins that are probably strongly influenced by groundwater (Auer, 1965; Coronato et al., 2006; Rabassa et al., 2006).

Spatial and Temporal Patterns of Peat Accumulation Across the FBT

A comparative analysis of *Sphagnum* vs. non-*Sphagnum* peat mass revealed that *Sphagnum*-dominated peatlands are characterized by greater peat stores, and thus larger soil C stocks, than their non-*Sphagnum* counterparts (Tukey's HSD: $p < 0.001$, except for the period prior to 14,900 cal. yr BP, where the difference between the two peat types was not statistically significant). This finding is consistent through the different time periods (**Figure 5**). We observed relatively consistent peat mass values for both *Sphagnum* (median $\sim$ 12 g/cm^2) and non-*Sphagnum* (median $\sim$4 g/cm^2) peat types through time, though these figures should not be directly compared, as each time period has a different duration. To alleviate this issue, PCAR was calculated to standardize changes in peat mass over time.

There are two outstanding patterns that come out of the PCAR data (**Figure 6**). First, *Sphagnum* peat is consistently accumulating at greater rates than its non-*Sphagnum* counterpart (Tukey's HSD: $p < 0.001$, except for the period between 14,900 and 7700 cal. yr BP, where the difference between the two peat types was not statistically significant), with median apparent rates of C accumulation that progressively increase through time, from 10 gC/m^2/yr between Reclus I and Hudson I, to 16 gC/m^2/yr

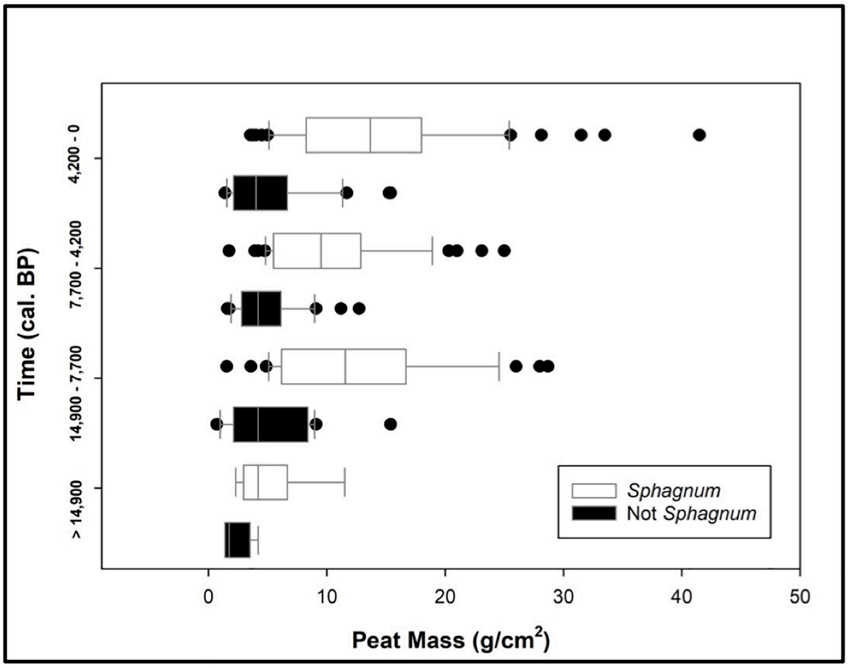

FIGURE 5 | A comparative analysis of *Sphagnum* vs. non-*Sphagnum* peat masses over time. The time periods are dictated by three tephra layers: Reclus I (14,900 cal. yr BP), Hudson I (7700 cal. yr BP), and Burney II (4200 cal. yr BP). *Sphagnum* and non-*Sphagnum* peat masses are statistically different for every time period (Tukey's HSD: $p < 0.0001$), except prior to 14,900 cal. yr BP.

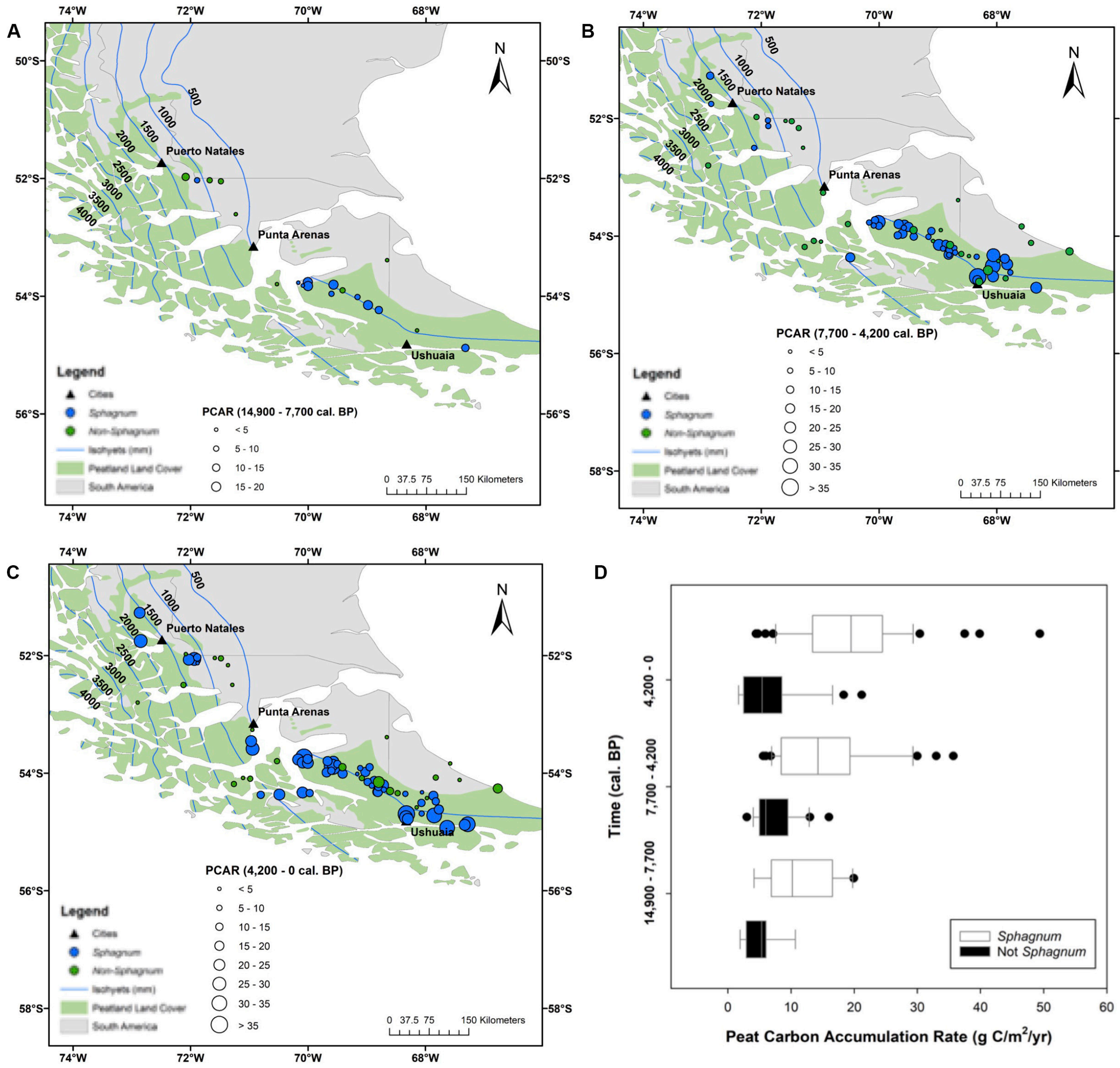

FIGURE 6 | A comparative analysis of *Sphagnum* vs. non-*Sphagnum* peat-carbon accumulation rates (PCAR) over time. **(A–C)** Spatial distribution of *Sphagnum* (blue circles) and non-*Sphagnum* (in green) peatlands and associated PCAR (circle size). **(D)** Temporal distribution of PCAR changes between *Sphagnum* (white boxes) and non-*Sphagnum* (black boxes) peat types. The time periods are dictated by three tephra layers: Reclus I (14,900 cal. yr BP), Hudson I (7700 cal. yr BP), and Burney II (4200 yr cal. BP). *Sphagnum* and non-*Sphagnum* peat masses are statistically different for every time period (Tukey's HSD: $p < 0.001$), except from 14,900 to 7700 cal. yr BP. The peatland area is from Yu et al. (2010) and the dataset used to generate the isohyets is from New et al. (2002).

between Hudson I and Burney II, and to 20 gC/m^2/yr from 4200 cal. yr BP to modern times (**Figure 6**). Second, non-*Sphagnum* PCARs remain uniform throughout all three time periods, with a median of 7 gC/m^2/yr (**Figure 6**). To normalize PCAR values for each peatland developmental history, time-weighted PCARs were calculated for the *Sphagnum* and non-*Sphagnum* peatland sites (**Figure 7**). This calculation multiplies the PCAR from each time period by its duration to ensure that each period is adequately represented in the "whole core PCAR." We find, once again, that *Sphagnum* sites have been characterized by an overall greater C-sink capacity than non-*Sphagnum* sites (Tukey's HSD: $p < 0.00001$), with median time-weighted PCAR values of 17 gC/m^2/yr (min = 4, max = 49) for *Sphagnum* and 8 gC/m^2/yr (min = 2, max = 33) for non-*Sphagnum*. Note that, amongst the non-*Sphagnum* sites, time-weighted PCAR ranged from 7 to 10 gC/m^2/yr for cushion bogs and from 2 to 33 gC/m^2/yr for persistent fens.

A comparison of the *Sphagnum* vs. non-*Sphagnum* portions of the cores reveals that, since the Reclus I eruption, approximately 42% of the total peat mass accumulated at these 90 sites is made of *Sphagnum* peat (**Figure 3**). This figure considers organic matter density differences between *Sphagnum* (0.05 gOM/cm^3) and non-*Sphagnum* peat (0.07 gOM/cm^3). For comparison, *Sphagnum* peat has dominated the regional peatland landscapes during

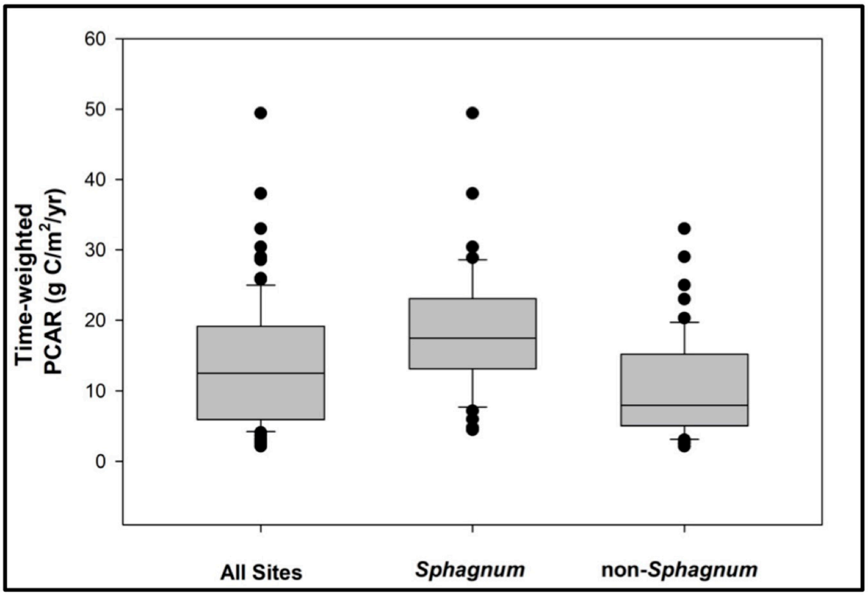

FIGURE 7 | A comparative analysis of time-weighted peat-carbon accumulation rates (PCAR) for *Sphagnum* vs. non-*Sphagnum* peat types. The *Sphagnum* sites have been characterized by an overall greater C-sink capacity than the non-*Sphagnum* sites (Tukey's HSD: $p < 0.00001$).

approximately 30% of the entire peat history, as reconstructed from the cores. In other words, though *Sphagnum* peat is less dense than its non-*Sphagnum* counterpart, it has built a peat deposit that is greater (42%) than the total amount of time (30%) these peatlands have been *Sphagnum*-dominated bogs. Likewise, core analysis reveals a median peat C mass (or median soil C density) of 119 kgC/m^2 (**Figure 8** and **Supplementary Table S4**), with modern-day *Sphagnum* bogs having significantly greater C storage (141 kgC/m^2) than their non-*Sphagnum* counterparts (56 kgC/m^2; Tukey's HSD: $p < 0.00001$). A regional map of peatland C density, which also reports whether each site is a *Sphagnum* or non-*Sphagnum* peatland, suggests that the greatest peat C stocks might be found along the eastern and southern portions of Isla Grande, in Tierra del Fuego (**Figure 8**).

Given the high proportion of sites that exhibit their FBT at 4200 cal. yr BP, we specifically analyzed peat mass and PCAR differences between sites that switched to bogs after the Burney II eruption vs. those that did not (**Figure 9**). We find that, on average, sites that switched to bogs accumulated significantly more peat (and stored more C) than sites that remained fens or cushion bogs (Tukey's HSD: $p < 0.00001$). These figures amount to a median peat mass of 15 g/cm^2, equivalent to 19 gC/m^2/yr for the 58 bogs, vs. a median peat mass of 4 g/cm^2 or 5 gC/m^2/yr for the other 32 sites (**Figure 9**).

Potential Mechanisms for the FBT

In light of tipping point theory, we argue that destabilizing forces are responsible for the FBT. These positive feedbacks consist of autogenic processes that can be triggered by a perturbation that helps "tip" a fen system into a bog state, by either dampening or amplifying a number of peatland processes. We propose two mechanisms that could explain the surge of *Sphagnum* during the late-Holocene: (1) volcanic impact, and (2) change in hydroclimate.

Volcanic Impact

Auer (1965) was the first to suggest that tephra layers might have aided the formation of *Sphagnum* bogs. He speculated that these tephra layers might have provided a dry and acidic substrate that also prevented evaporation across large swaths of land, thereby facilitating *Sphagnum* invasion. We postulate that the idea of a volcanic impact capable of inducing a regional-scale FBT is plausible. In fact, Kilian et al. (2006) suggested that the Burney II eruption led to long-lasting damage to forest, aquatic and peatland ecosystems in the vicinity of the stratovolcano. Our scenario implies a much broader, regional-scale impact associated with this eruption. We consider a scenario under which the combined effects of volcanic fallout (i.e., tephra) and sulfur dioxide (SO_2) plumes caused intense peatland acidification because of fens weak buffering capacity (Gorham et al., 1984), which led to nutrient seepage and an abrupt ecosystem shift to bog. While it is possible that the tephra itself provided a "dry" substrate for *Sphagnum*, as proposed by Auer (1965), we are less comfortable with this idea, given that our stratigraphic evidence suggests that the Burney II ash layer was often less than 1 cm in thickness. Conversely, the potential impact of an acidic SO_2 plume on the regional vegetation communities is worth considering, as those plumes could take place over the course of many years as the volcano is degassing, dissimilar to the 1-time tephra fallout event. This "volcanic impact" scenario suggests that a disturbance induced an abrupt acidification of the peatland surface, which facilitated *Sphagnum* establishment, which led to the FBT. In light of tipping point theory, this scenario implies that the new conditions brought about by the tephra and/or the

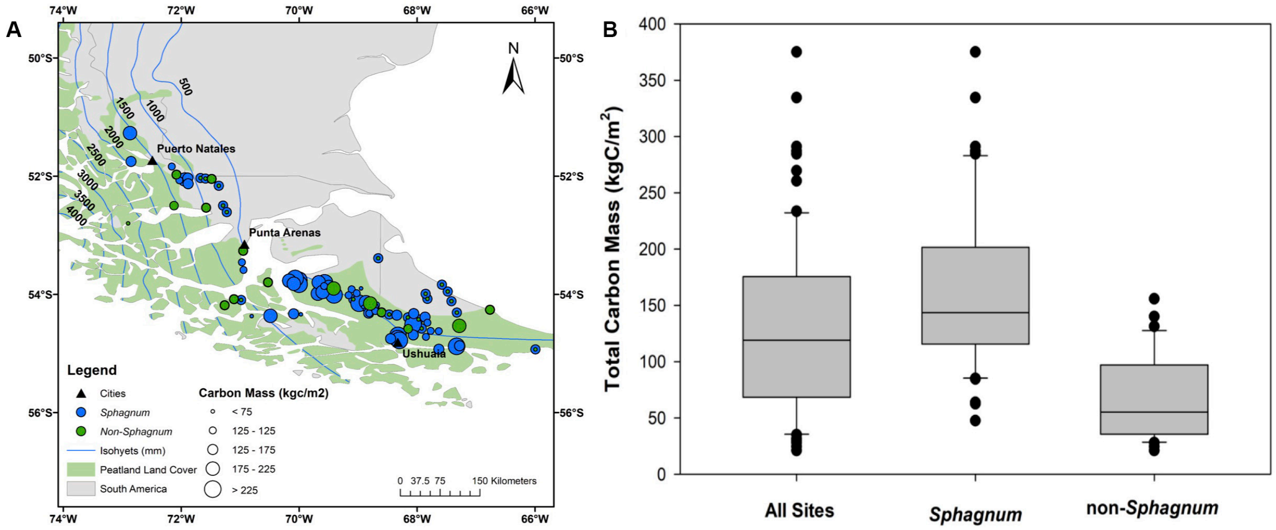

FIGURE 8 | A comparative analysis of *Sphagnum* vs. non-*Sphagnum* peat carbon mass per unit area (also referred to as soil carbon density in the text). **(A)** Spatial distribution of *Sphagnum* (blue circles) and non-*Sphagnum* (in green) peatlands and associated carbon mass (circle size). **(B)** Statistical representation of carbon mass for *Sphagnum* and non-*Sphagnum* peat types compared to all sites combined. The peatland area is from Yu et al. (2010) and the dataset used to generate the isohyets is from New et al. (2002).

SO_2 plume were able to promote the bog state at the expense of the fen state.

Change in Hydroclimate

While the broad-scale synchronicity of the FBT with a tephra layer makes the volcanic scenario a strong candidate for the observed pattern, the influence of climate on the FBT cannot be overlooked. The Burney II tephra was deposited toward the end of the co-called sub-Boreal time period (Auer, 1965), which was characterized by generally dry conditions. It is indeed possible that fens were drying up and that peat accumulation was slowing down due to enhanced decomposition. While the volcanic eruption might have facilitated the FBT, the underlying dry conditions could have been essential to the establishment of bog conditions. This dry climate scenario could also explain why a number of sites switched to bogs between Hudson I and Burney II, without "help" from a volcanic eruption. These ideas make intuitive sense and are corroborated by recent paleoclimate reconstructions. In particular, we know that many glaciers in the Patagonian Icefield, on South Georgia and James Ross Islands, and along the Antarctic Peninsula receded between ∼ 4500 and 2700 cal. yr BP (Mulvaney et al., 2012; Sterken et al., 2012; Anyia, 2013; Strelin et al., 2014; Kaplan et al., 2016; Oppedal et al., 2018). Peatlands expanded across sub-Antarctica during these times as well (e.g., Yu et al., 2016; Loisel et al., 2017b). Another aspect of the regional paleoclimate is a subsequent increase in effective moisture around 2700 cal. yr BP (e.g., Moreno et al., 2018; Oppedal et al., 2018). Glacial advances have been described for this time period; it is possible that *Sphagnum* moss benefited from wetter conditions, which could also explain the recent increases in peat mass and PCAR (**Figure 9**). The FBT across southern Patagonia is also contemporaneous with the "4.2 ka event," which has been observed in many parts of the world (e.g., Bond et al., 2001; Wanner et al., 2008) and associated with dry and cool climatic conditions. In light of tipping point theory, this "climate" scenario implies that the dry conditions brought about by the change in regional hydroclimate were able to promote the bog state at the expense of the fen state.

Wind

One last effect that should be discussed is the importance of wind in controlling peatland dynamics in southern Patagonia. Anyone who has seen bog surfaces from this region probably noticed the very tall and often desiccated *Sphagnum* hummocks that dot these peatlands. Often, the shape and orientation of these hummocks are determined by the main wind direction. While *Sphagnum* might be able to develop over fen deposits during dry periods, *Sphagnum* communities also have to persist amid strong directional winds. In many cases, *Sphagnum* is eventually destroyed by the influence of such strong winds and associated dry conditions, leading to retrogressive bog patches that give way to shrubby landscapes (Auer, 1965). Considering the negative impact of wind on bog development, the ∼ 2700 cal. yr BP increase in effective moisture becomes even more important in maintaining, and perhaps promoting, *Sphagnum* growth across the region.

A CONCEPTUAL MODEL FOR THE FBT

In light of complex adaptive system theory, a peatland could respond in a step-like way when a slowly varying internal driver (here: peat thickness) exceeds a threshold value, but where the threshold value depends upon the direction of the driver (increase or decrease). In other words, the transition from fen to bog could take place at a different threshold than the

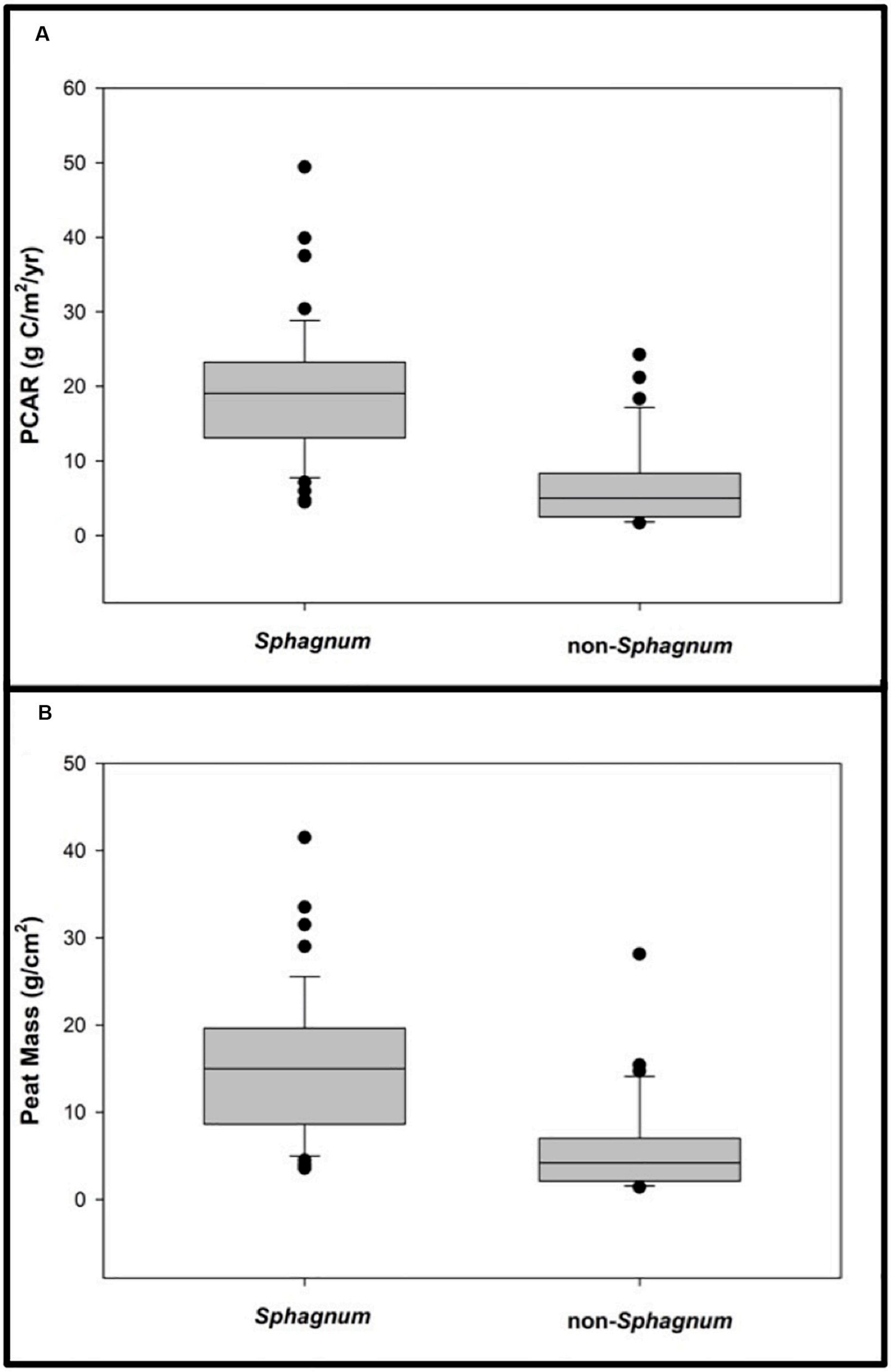

FIGURE 9 | A comparative analysis of *Sphagnum* vs. non-*Sphagnum* peat properties since the Mount Burney II eruption (4200 cal. yr BP). **(A)** *Sphagnum* vs. non-*Sphagnum* peat carbon accumulation rates since 4200 cal. yr BP. **(B)** *Sphagnum* vs. non-*Sphagnum* peat masses. Both analyses reveal that sites that switched to bogs accumulated significantly more peat (and stored more carbon) than sites that remained fens or cushion bogs (Tukey's HSD: $p < 0.00001$).

transition from bog to fen would, inducing a hysteresis loop that makes the fen and bog possible "alternative states" under a series of conditions (**Figure 10**). This model considers the resilience and ecological memory of the fen and bog stable states (Belyea, 2009). Allogenic factors can push a mature fen beyond its resilience capacity and induce a shift to bog conditions, and vice versa, but at different thresholds values (Hughes and Dumayne-Peaty, 2002). We postulate that changes in climate (moisture and temperature) and stochastic disturbance events (e.g., drought, flood, volcanic eruption, fire, pollution) become increasingly important over time in facilitating the FBT (i.e., the fen state becomes less stable over time). In other words, the stabilizing forces that were promoting the fen persistence eventually lose to a perturbation that promotes the bog state. The bog is then maintained by another set of stabilizing forces, and a return to the fen state would require different driver conditions than those that promoted the FBT.

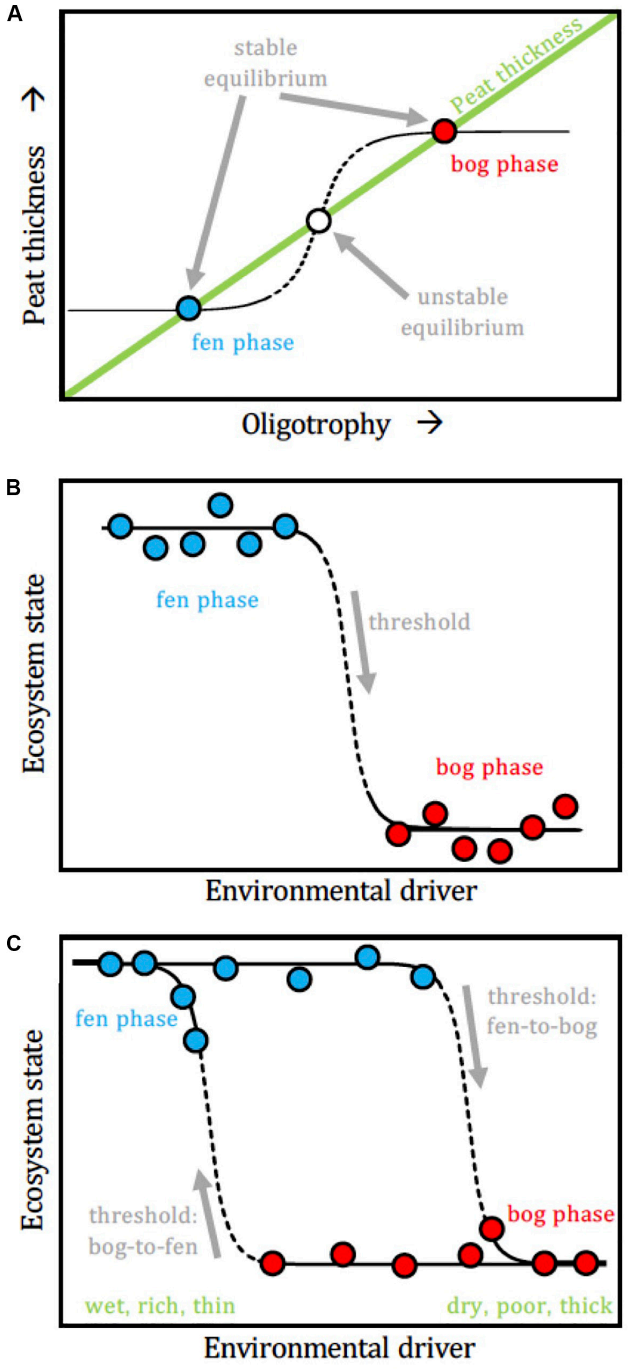

FIGURE 10 | Conceptual models for non-linear fen-bog transition scenarios. **(A)** Overall pattern, where the peatland state (fen vs. bog) responds non-linearly as oligotrophy increases along with peat thickness. There is no "intermediate" state between fen and bog but rather, once a threshold is passed, the system switches from one state to the other. **(B)** State-threshold model, where the regime shift in the ecosystem state takes place once a threshold in an internal driver (e.g., peat thickness) is exceeded. **(C)** Driver-state hysteresis, where a loop links the ecosystem state to the environmental driver, which results in jumps between two alternative stable states.

Bog Stabilizing Properties

The stabilizing properties of bogs occur over multiple spatial scales, from microforms (10^0–10^1 m) to mesoforms (10^1–10^2 m) and whole ecosystems (10^2–10^3 m). These stabilizing forces include a number of negative feedback mechanisms that relate to the presence of *Sphagnum*, changes in peat water storage, vegetation mosaics, and the rate of peat formation, which are briefly described below.

Presence of *Sphagnum*

The colonization by *Sphagnum* helps shape and maintain an acidic, nutrient-poor, and insulated habitat that further promotes *Sphagnum* persistence (Rydin et al., 2006); it can also absorb pollutants. This genus has a great water-holding capacity and is resistant to decay, making it an excellent peat builder that drives peat formation (van Breemen, 1995). The colonization by *Sphagnum* is likely associated to a local increase in soil wetness, which may be caused by decreased soil permeability (Rydin and Jeglum, 2013) or the formation of a groundwater mound (Ingram, 1982), both of which can be autogenically- or allogenically-induced.

Peat Water Storage

Following rainfall and snowmelt, excess water can be stored in the acrotelm (the unsaturated uppermost portion of the peat profile), where near-surface peat is pliable and pore spaces are large (Belyea, 2009; Rydin and Jeglum, 2013). By the same token, any superfluous water can be rapidly discharged, thus helping the bog maintain an adequate water supply without exceeding it, which could otherwise change the nature of soil water chemistry. In contrast, during a drought, water table levels drop, but near-surface transmissivity is also reduced, leading to water conservation. This process is accompanied by surface peat contraction, which also limits water loss (Glaser et al., 2004). In addition, mosses can lose their pigments, leading to high albedo and thus increased reflectance that results in slower water loss via evaporation (Gerdol et al., 1996).

Mosaics of Hummocks and Hollows

Vegetation mosaics can increase peat bog resilience by promoting water storage (drought persistence) and by easing drainage (flood persistence). Indeed, flooded hollows can expand to either store or discharge excess water during floods; conversely, hollows can shrink during droughts to limit water loss to evaporation (Quinton and Roulet, 1998; Belyea, 2009). These local water reallocation strategies seem to benefit the system as a whole when compared to a homogenous vegetation cover (Quinton and Roulet, 1998). However, it has been argued that vegetation mosaics can promote perturbations over long timescales. This is particularly the case in continental settings, as they could lead to the expansion of dry hummocks with vascular plants that pump water from the adjacent hollows (Eppinga et al., 2009). In this case, hummocks do not act as drought buffers; rather, they amplify the initial condition, which can lead to self-perpetuating dry conditions.

Rate of Peat Formation

In itself, peat formation plays a self-regulating role in maintaining bog conditions. Vertical peat accretion is a result of the net positive balance between plant production and peat decomposition. Under optimal growth conditions, which have been observed in "lawns" and low hummocks (Belyea and Clymo, 2001), plant production is maximized, leading to vertical peat accumulation that is accompanied by a rising groundwater mound (Belyea and Baird, 2006). If the rate of peat formation was to "outpace" the capacity of the peatland to keep itself wet enough, a hummock would form. Plant production would then be reduced and peat decomposition would increase due to aerobic conditions, thereby constraining vertical peat accretion and bringing the system back to the low hummock/lawn conditions of optimal growth. In this case, the non-linear relationship between the thickness of the acrotelm and the rate of peat formation brings stability to the system (e.g., Belyea and Clymo, 2001; Belyea, 2009). Conversely, if wet years were to occur, the lawns would become wetter, perhaps turning into hollows, which would decrease both the rate of peat formation and the acrotelm thickness, which could tip the system into a self-perpetuating wet state.

System Properties Leading to Fen Persistence

While it can be said that a number of negative feedbacks promote the persistence and homeostasis of the bog state (see above), the situation is different for the fen state. In the literature, it has been suggested that peatlands in general, and bogs in particular, are complex adaptive systems (Belyea and Baird, 2006). But not much has been said about the persistence capabilities of fens. Indeed, fens tend to be topographically constrained, that is, they develop in wet depressions and can "swamp" low slopes as they expand horizontally (e.g., Korhola et al., 2010; Loisel et al., 2013). It has also been suggested that they tend to be more sensitive to climate and hydrological changes then bogs (e.g., Hilbert et al., 2000), perhaps as a result of their incapacity to self-regulate the way bogs do. From this perspective, the stabilizing forces that promote a fen persistence are more difficult to identify. In an attempt to fill this knowledge gap, we propose that lateral expansion and intensified peat decay are potential properties that lead to persistence, albeit being "passive" negative feedbacks, as both these processes contribute to maintaining high surface wetness, particularly near the center of the fen.

Lateral Expansion

Peat accumulation in a fen basin raises the water table locally. Due to an increase in the hydraulic head caused by greater vertical peat accumulation near the center, subsurface water has been shown to flow from the center of the fen toward the margins (Morris et al., 2011), allowing peat to spread laterally through "swamping" of adjacent mineral soils (Belyea, 2009; Korhola et al., 2010; Ruppel et al., 2013). The resulting effect is an increase in the total fen area, which further increases water availability and stability toward the center, thereby maintaining high surface wetness (Loisel et al., 2013). This prevents colonizing *Sphagnum* species from invading the fen (Granath et al., 2010). By the same token, when a fen reaches a stage where it can no longer spread laterally, its capacity to maintain wet conditions might be lost and perturbations could lead to a regime shift to dry/bog conditions.

Intensified Decay

Despite their high surface wetness, peat decay is intensive in most fens due to highly fluctuating water table levels and nutrient-rich water, which promote microbial diversity and activity, respectively (Vitt, 2006). Intensified aerobic decay has the effect of slowing down net vertical accumulation, which reduces hydraulic conductivity by creating dense peat deposits, further promoting a thin acrotelm and wet surface conditions. In addition, water table depth variability has been found to be most pronounced near the edges of fens (e.g., Loisel et al., 2013), which could lead to intensified peat decay (and thus higher bulk density) near the margins, which then reduces lateral water losses but maintains high surface wetness toward the center of the fen (Morris et al., 2011).

CONCLUSION

In light of complex adaptive system theory, we propose that allogenic controls can trigger the FBT in peatland systems. The case study presented here indicates that, out of 58 *Sphagnum*-dominated bogs from southern Patagonia, 35 transitioned from their fen state at 4200 cal. yr BP (Burney II eruption) while another six switched to bogs at 7700 cal. yr BP (Hudson I eruption); the remainder underwent the FBT at other times. Given this striking pattern, it is plausible that the volcanic eruptions facilitated the FBT. Pertaining to the Burney II eruption in particular, which is also synchronous with large-scale climate cooling and drying, it is possible that fens were drying up and that peat accumulation was slowing down due to enhanced decomposition. While the volcanic eruption might have facilitated the FBT, the underlying dry conditions could have been essential to the establishment of bog conditions. In any case, there is strong evidence that those external factors induced the FBT.

In terms of carbon storage, the stratigraphic analyses presented in this study show that, across southern Patagonia, *Sphagnum* deposits are associated with greater peat masses, larger soil carbon stocks, and higher peat-carbon accumulation rate values than their non-*Sphagnum* counterparts. The median peat mass for modern-day *Sphagnum* bogs is over twice that of non-*Sphagnum* peatlands (141 vs. 56 kgC/m^2). Focusing on the peat deposits that have accumulated since Burney II allows us to confirm these long-term trends. Indeed, we find that, on average, bogs stored carbon at a rate almost four times greater than the sites that remained fens or cushion bogs (median = 19 gC/m^2/yr for the 58 bogs vs. 5 gC/m^2/yr for the other 32 sites). Our dataset demonstrates that the carbon-sink capacity of those peatlands that switched to bogs is much greater than those that did not.

Overall, our paleoecological study demonstrates that fens and bogs across SSA are characterized by distinct soil carbon masses, densities, and accumulation rates. By governing the peatland long-term carbon sequestration, the bog vs. fen status of a peatland can be seen as an ecosystem trait that can be translated into a functional ecosystem property, that is, long-term carbon storage in peatlands; it can thus be used for integrating peatland types into process-based and physics-based ecological models. Additional studies that analyze the timing and driving mechanisms of the FBT, as well as its impacts on carbon sequestration, are needed to provide a broader perspective on this ecosystem transition and to better constrain such models. Additional functional properties such as carbon dioxide and methane gas exchange differences between fens and bogs could similarly be inferred and used to predict spatial and temporal variations in peatland carbon fluxes. This information may be of use for peatland management efforts, in particular, those interested in rehabilitation, restoration, and conservation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JL conceived the study and wrote most of the manuscript. MB contributed to data and analyses, helped writing the text, and made the majority of the figures and tables presented in the study. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.00273/full#supplementary-material

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REVIEW
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Testate Amoeba Functional Traits and Their Use in Paleoecology

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This review provides a synthesis of current knowledge on the morphological and functional traits of testate amoebae, a polyphyletic group of protists commonly used as proxies of past hydrological changes in paleoecological investigations from peatland, lake sediment and soil archives. A trait-based approach to understanding testate amoebae ecology and paleoecology has gained in popularity in recent years, with research showing that morphological characteristics provide complementary information to the commonly used environmental inferences based on testate amoeba (morpho-)species data. We provide a broad overview of testate amoeba morphological and functional traits and trait-environment relationships in the context of ecology, evolution, genetics, biogeography, and paleoecology. As examples we report upon previous ecological and paleoecological studies that used trait-based approaches, and describe key testate amoebae traits that can be used to improve the interpretation of environmental studies. We also highlight knowledge gaps and speculate on potential future directions for the application of trait-based approaches in testate amoeba research.

Keywords: protists, functional traits, morphological traits, ecology, peatlands, lakes, soils, trait-based approaches

INTRODUCTION

Paleoecological reconstructions based on subfossil species assemblage data are commonly used to reconstruct past environmental changes over long time-scales (from hundreds of years to millennia) (Willis and MacDonald, 2011; Roberts, 2013). Assuming that species ecological requirements are stable over time, the main premise in paleoecology is that changes in species assemblages over time similarly reflect past environmental changes within a study site or region (Birks et al., 2016). While this approach informs on changes in key abiotic drivers of ecosystem processes (e.g., peatland water table depth, air temperature, pH, nutrient loading, road salt and metal contamination in lakes), it does not provide direct evidence for functional changes. The functional trait approach aims to link community composition to ecological drivers and the functional consequences of these changes. This approach, which was first developed to better understand modern community structure has been increasingly used to infer long-term changes in ecosystem functioning (Walker and Cwynar, 2006; Luoto and Ojala, 2018; Peppe et al., 2018; van der Sande et al., 2019).

A functional trait, as defined by Violle et al. (2007), is any morphological, physiological or phenological feature measurable (or identifiable) at the individual level. These measurements need not refer to environmental variables or any other levels of organization (population, community, or ecosystem) (McGill et al., 2006; Violle et al., 2007; Nock et al., 2016; Céréghino et al., 2018). As such, functional traits can vary both within (intraspecific) and between (interspecific) species and can be further classified into two categories: (1) effect traits, which drive these changes in ecosystem functions; and (2) response traits, which drive the response of community structure to changes in environmental conditions (Lavorel and Garnier, 2002; Naeem and Wright, 2003; Suding et al., 2008). Response traits are adaptive to a specific environmental pressure, as they provide a higher fitness to the organisms that possess it.

Trait-based approaches are used to gain a mechanistic understanding of key processes in community ecology (Díaz and Cabido, 2001). They have predictive capabilities, and as such, have been adopted by a wide array of researchers through the last decade to, for example, explain the distribution of species along various abiotic gradients (e.g., Kaustuv et al., 2001; Berg et al., 2010; Diamond et al., 2011), the processes underlying the assembly of species into communities (e.g., Kraft et al., 2008; Jung et al., 2010; Kraft and Ackerly, 2010), the relationship between ecological community structure and ecosystem functions and services (e.g., de Bello et al., 2010; Lavorel and Grigulis, 2012; Robleño et al., 2018), and the impact of climate change on species assemblages (Bjorkman et al., 2018; Henn et al., 2018). The response/effect traits framework has been used in various fields including ecological restoration (Laughlin, 2014), environmental change prediction (Suding et al., 2008), and ecosystem services (Lavorel and Grigulis, 2012). In general, trait-based approaches are expected to lead to generalized predictions across organizational and spatial scales, independent of taxonomy (Shipley et al., 2016; Robroek et al., 2017). Across fossil records, species are gained or lost, and are sometimes not easy to identify due to non-optimal preservation of fossil material. However, certain traits remain and can be similarly influenced by environmental conditions regardless of species identity. Therefore, the trait approach is a powerful way of gaining insight on how past changes influenced communities functions and related ecological processes (Lamentowicz et al., 2019). In turn, its use may improve predictions on how future species assemblages and their function will change along with current climate change in response to emerging environmental stressors (e.g., climate change; Heilmeier, 2019).

The first trait-based paleoecological studies focused on plant macrofossils and pollen, and explored the response of life-history traits to environmental and climate changes (Lacourse, 2009; Butterfield et al., 2019; Birks, 2020). Trait-based approaches appeared to be particularly suited for application to testate amoebae, a polyphyletic group of protists. These unicellular amoeboid organisms are protected by a test (shell), whose shape, ornamentation and composition are characteristic for each species and used for morphology-based species identification. The tests can be preserved over millennia in peat (Charman, 2001) and lake sediments (Patterson and Kumar, 2002), and fossils strikingly similar to some modern taxa date to the Neoproteozoic (Porter and Knoll, 2000; Porter et al., 2003; Porter, 2011). Past environmental conditions can be inferred based on the observation of testate amoeba community makeup and previous knowledge on individual species ecological requirements. Testate amoebae are commonly used as proxies for peatland water table depth (DWT; Booth, 2002; Booth et al., 2004) and pH (Booth, 2001), the two major environmental gradients in peatlands (Hájek et al., 2006; Rydin and Jeglum, 2006), as well as lake trophic status (Patterson et al., 2012; Watchorn et al., 2013; Nasser et al., 2020b). Testate amoeba community composition data and associated measurements of environmental variables have been used to determine the environmental preferences of each species. Training sets have been developed across different geographical regions for both peatland and lake taxa in both the Northern and the Southern Hemisphere (Woodland et al., 1998; Wilmshurst et al., 2003; Lamentowicz and Mitchell, 2005; Booth, 2008; Patterson et al., 2012; Qin et al., 2012). These have been used to develop statistical models (transfer functions) for the reconstruction of long-term environmental changes based on species community data (Birks, 2012).

Testate amoeba tests can be used to measure several morphological traits, while knowledge of species' ecological preferences can provide additional relevant measures, e.g., trophic status of particular species or their hydrological preferences (Fournier et al., 2012; Céréghino et al., 2018). As such, testate amoeba functional traits in lake sediment and peat records constitute unique ecological archives of past changes in community structure that can be related to past environmental conditions and/or ecosystem functions. Several studies have successfully used trait-based approaches to investigate past trends in functional diversity (Marcisz et al., 2020), and to reconstruct past environmental conditions (Fournier et al., 2015; Lamentowicz et al., 2015; Marcisz et al., 2016; Gałka et al., 2017; van Bellen et al., 2017). This review summarizes the current knowledge of functional traits in testate amoebae and outlines some open questions and options for future applications in paleoecology.

Benefits of Testate Amoeba Functional Trait-Based Approaches in Paleoecology

In the specific context of paleoecology, the use of testate amoeba trait-based approaches has several notable advantages:

(1) **Improved mechanistic understanding and predictability of ecological processes**. Testate amoebae are important top predators in the microbial food web (Gilbert and Mitchell, 2006; Jassey et al., 2013b). They contribute to soil nutrient

cycling (Wilkinson and Mitchell, 2010), especially the cycles of C, N, and Si in soils (Schröter et al., 2003; Nguyen-Viet et al., 2004; Aoki et al., 2007; Jassey et al., 2015; Puppe, 2020). Their community structure is also strongly correlated to peatland water table depth, soil moisture regime (Woodland et al., 1998; Mitchell et al., 1999; Sullivan and Booth, 2011), shade/hydrology (Marcisz et al., 2014b; Lamentowicz et al., 2020), and chemistry (Tolonen et al., 1992; Hajkova et al., 2011; Singer et al., 2018); contaminant runoff into lakes (Neville et al., 2014; Roe and Patterson, 2014; Gavel et al., 2018); sea level change (Patterson et al., 1985; Charman et al., 1998, 2002); and has been shown to respond to experimental water table changes both in the field (Marcisz et al., 2014a) and in mesocosm studies (Koenig et al., 2017, 2018b). Testate amoeba community dynamics and how they relate to ecosystem functions is a dynamic field of research, although many knowledge gaps remain (but see Fournier et al., 2012; Jassey et al., 2013a, 2015; Lamentowicz et al., 2013a; Macumber et al., 2017). Investigation of past long-term changes in both testate amoeba assemblages and traits could be used to better understand community assembly rules, trait-filtering over time, and – most importantly – the possible existence of critical transitions of community functions, and thus of past ecosystem functioning. This would help to better understand current community dynamics and enable predictions of future environmental changes under ongoing global warming.

(2) **Independence from taxonomic bias and improved transferability of models across biogeographic regions and temporal and spatial scales**. Most soil life have not yet been described and this knowledge gap is larger for the smaller organisms (prokaryotes, protists and other microeukaryotes) (Decaëns, 2010). Testate amoebae have been thoroughly studied due to the well-defined morphological characteristics of the test that usually permits species-level identification for living and dead specimens. Their morphological identification can thus be considered simpler than for many other microbial groups. However, species boundaries, and the definition of species (Schlegel and Meisterfeld, 2003) requires additional research. Morphologic analysis and more recently molecular analysis has explored the phenotypic plasticity and cryptic diversity among several groups (Medioli et al., 1987; Wanner, 1999; Kosakyan et al., 2012; Singer et al., 2018; Macumber et al., 2020). This clearly illustrates the need for basic taxonomic research as a prerequisite for interpretations of the ecology and/or biogeography of taxa (Heger et al., 2009). The degree to which current taxonomic issues undermine the use of testate amoebae in palaeoecology is unclear, but *in silico* experiments in which the identity of morphologically similar species was switched, showed the potentially significant impact of inadequate baseline taxonomic research (Payne et al., 2011). One informal solution to overcome this problem would be to lump species into morphospecies and species complexes in ecological and paleoecological studies. This approach is principally used with small taxa, e.g., reporting *Corythion* and *Trinema* species as *Corythion-Trinema* type although they can be identified based on scale shape (oval vs. round) (Charman et al., 2000; Amesbury et al., 2016, 2018). However, merging taxa into larger complexes or morphospecies that possess different functional traits results in the loss of potentially useful ecological information, especially when focusing on species responses along narrow ecological gradients. Moreover, using a lower taxonomic resolution in paleoecological studies makes it impossible to fully exploit insights derived from observational or experimental studies that would have been possible if a higher taxonomic resolution was used. As described below in section "Lacustrine Environments" another approach widely utilized since the 1980s within the lacustrine testate amoebae research community is to designate informal infrasubspecific 'strains' associated with test morphologies, not necessarily linked to formal taxa, but which can be linked to specific environmental stressors. During multivariate statistical analysis, this ecophenotypic approach permits delineation of subtle environmental gradients that would not otherwise be possible (Patterson and Kumar, 2002). Alternatively, using testate amoebae functional traits in addition to, and not instead of, species data in paleoecology provides complementary information and reduces the risk associated with taxonomic confusion as the measurements of traits are less ambiguous than formal taxonomic criteria. The resulting transfer functions would potentially enable broader interpretations of community changes and functional processes over long time-scales.

(3) **A useful additional tool for multi-proxy paleoecological reconstructions**. Paleoecological investigations and interpretations often use several biological and geochemical proxies to obtain a wide range of information about past environmental changes. For instance, pollen, spores, plant macrofossils, and charcoal are commonly analyzed to reconstruct past vegetation changes, fires, and human impact over time (Schwörer et al., 2014; Feurdean et al., 2020), whereas geochemical data are used to assess changes in entire lake basins (Bonk et al., 2016; Lu et al., 2017; Jensen et al., 2020). Within projects involving a multi-proxy approach testate amoeba species assemblage data provide quantitative hydrological (e.g., Mitchell et al., 2001; Booth et al., 2012; Lamentowicz et al., 2015) and pH (Mitchell et al., 1999; Lamentowicz et al., 2008; Patterson et al., 2013); road salt contamination (e.g., Roe et al., 2010); nutrient loading (e.g., Patterson et al., 2012; Gavel et al., 2018), and mining induced metal contamination (e.g., Kihlman and Kauppila, 2009, 2010; Nasser et al., 2020b) reconstructions. Integrating trait-based approaches in multi-proxy investigations can provide information on ecological processes and may also help to elucidate functional roles based on correlations with other proxies. These then need to be tested in observational or experimental studies, illustrating a linkage between neo-ecology and paleoecology (Hunter, 1998; Rull, 2010;

Buma et al., 2019). Moreover, multi-proxy data can elucidate long-term trait-environment relationships, giving an insight into eco-evolutionary processes at time scales beyond the range of ecological studies.

A Functional Characterization of Testate Amoebae

Functional traits have been well studied in various taxonomic groups such as plants (Weiher et al., 1999; Diaz et al., 2004; Wright et al., 2004; Cornwell et al., 2008; Kleyer et al., 2008; Kattge et al., 2020), invertebrates (Andriuzzi and Wall, 2018; Céréghino et al., 2018), birds (Bregman et al., 2014), and mammals (Porto et al., 2013). In the case of testate amoebae, some functional traits relevant to understanding the response of these organisms to environmental changes and their functional role in the soil ecosystem have also been identified (Fournier et al., 2012, 2015; Jassey et al., 2013a, 2015, 2016; Lamentowicz et al., 2015; Marcisz et al., 2016; Koenig et al., 2017). Traits can be measured on tests preserved in lake sediments and peat and are thus relevant for paleoecological studies. However, much work still needs to be done to: (1) quantitatively link these traits to organisms' fitness under different conditions; (2) assess trade-offs among functional traits; (3) identify effect traits of specific ecosystem functions; (4) resolve the problem of collinearity among traits (cf. Lamentowicz et al., 2015); and (5) to relate traits to key factors such as climate.

Trait-based approaches have been increasingly applied in testate amoeba studies. It is therefore useful to provide a synthesis of the field of testate amoeba functional ecology and paleoecology and to outline some proposed future research avenues. Section "Current Knowledge and Characterization of Traits of Testate Amoebae" presents the current state of knowledge about testate amoeba traits linked to ecological, evolutionary, genetic and biogeographic contexts. Section "Description of Key Testate Amoeba Traits for Paleoecology" highlights the key traits and their significance to paleoecology, showcases the potential of using traits in long-term studies, emphasizes both the advantages, as well as the strengths and limitations of their application in paleoecology. Section "Conclusion, Knowledge Gaps and Perspectives for the Use of Testate Amoeba Traits in Paleoecology" summarizes knowledge gaps and future perspectives for the use of testate amoeba traits in paleoecology. A list of relevant testate amoeba functional traits and their characterization are presented in **Table 1** and **Figures 1–3**.

CURRENT KNOWLEDGE AND CHARACTERIZATION OF TRAITS OF TESTATE AMOEBAE

Testate Amoeba Traits and Ecological Roles Before "Functional Traits"

Early ecological studies on testate amoebae assessed the distribution of species in various ecosystems and later attributed the environmental gradients to which particular species assemblages were correlated (Penard, 1902; Harnisch, 1925; Sandon, 1927). The traits of testate amoebae in these studies were generally based on their taxonomic descriptions, in the context of well-defined, measurable taxonomy, usually based at the individual level and used comparatively across species (Nock et al., 2016). At the same time, the first attempts were made to develop ecological typologies of plant and animal species aimed at associating the performance of different species to ecologically relevant features of their morphology or physiology. The terms "guilds" (Schimper, 1903; Clements, 1905) and "life forms" (Raunkiaer, 1934) were introduced first by plant ecologists and similar attempts were subsequently made by zoologists e.g., Koepcke (1956), see Laureto et al. (2015). Unfortunately, the available information relating to the ecological preferences of testate amoebae was insufficient then to make any broad generalizations; therefore their functional role in ecosystem processes was rarely explicitly stated. For example, Deflandre (1937) reported that the largest species of all genera were purely aquatic. Other, early community ecology studies identified soil water content and pH as important factors explaining the distribution of testate amoebae in *Sphagnum* bogs (Bartoš, 1940, 1946; de Graaf, 1956, 1957; Bonnet, 1961a). Several studies observed that the variation in test size of certain species was related to the water content of the habitat (Jung, 1936; Heal, 1961, 1963). Bonnet (1961a) recognized that the tests of soil-dwelling testate amoebae were not only smaller in size than those from wetter biotopes, but that they were also different in shape. He observed that in wet *Sphagnum* mosses, tests were generally ovoid or pyriform, whereas in dry mosses and soils they are hemispherical or sub-hemispherical. Bonnet (1959) also noted that soil-dwelling species of *Centropyxis* Stein, 1859 lacked the characteristic hollow "horns" commonly found in aquatic species (e.g., *C. aculeata* Ehrenberg, 1832).

By the mid-1970s the terms "guild" (Root, 1967) and "functional groups" (Cummins, 1974) were formalized in ecological studies with the latter term having a more general connotation [see Blondel (2003) for a discussion of the key differences between the terms]. In line with this approach, Bonnet (1975) classified testate amoebae into sixteen morphological types according to the shape and symmetry of the tests and the structure of the aperture (or pseudostome). The morphological types were found to be closely related to the habitat types and could be used as indicators of ecological conditions and in understanding morphological evolutionary trends (Schönborn, 1989). For instance, species with a terminal and comparatively large aperture of acrostomic and trachelostomic morphological types were observed to be dominant in aquatic habitats and wet mosses. In dry habitats the proportion of species with a slit-like opening (compressed acrostomic type), with an aperture covered by an anterior visor or lip (plagiostomic or cryptostomic types) or by modified complex structures (propylostomic and diplostomic types) was found to be higher. The cotylostomic morphotype was determined to be characterized by an enlarged collar around the aperture which is used for fixation in unstable coastal marine interstitial biotopes (Schönborn, 1989). However, the application of this classification in subsequent functional ecology studies was limited.

TABLE 1 | List of relevant testate amoeba functional traits explored so far in ecological and palaeoecological studies.

Trait (unit; if relevant)	Description	Trait function, ecological meaning, link to ecosystem functions (if known)		Used in paleo?	References
Test (shell) size					
Biovolume (μm^3)	Volume of the test	Related to the metabolic rate and the capacity of the food web to process energy	Size features of testate amoebae are linked to dissolved organic carbon and nitrogen in peatlands, and thus related to C and nitrogen cycles. Small species better overcome difficult conditions and colonize new habitats faster than large ones as they are lighter	Yes	Lousier, 1974; Wanner and Xylander, 2005; Laggoun-Défarge et al., 2008; Fournier et al., 2012, 2015; Jassey et al., 2013a,b, 2016; Fiałkiewicz-Kozieł et al., 2015; Lamentowicz et al., 2015; Arrieira et al., 2016; Krashevska et al., 2016, 2020; Marcisz et al., 2016, Marcisz et al., 2020; Gałka et al., 2017; Koenig et al., 2017, 2018a,b; McKeown et al., 2019; Macumber et al., 2020
Length/size/shell size (μm)	Length of the test	Larger taxa have longer generation times than small taxa and are thus expected both to be more sensitive to perturbation and to recover more slowly from perturbations		Yes	
Height (μm)	Height of the test	–		No	
Width/breadth (μm)	Width of the test	–		No	
Diameter (μm)	Diameter of the test	–		No	
Body range (μm)	(body length species x – maximum body length within the community)/minimum body length within the community	-		Yes	
Centroid size	Geometric morphometrics: square root of the non-weighted sum of squared distances from the joint centroid to landmarks.	Large, pyriform taxa are associated with low nutrients, benthic habitats and mixotrophy.		Yes	Macumber et al., 2020, this issue
Test construction					
Test material origin/ composition/type	(1) tests made of secreted biosilica plates (idiosomes), (2) organic-coated idiosomes (tests with thick organic coating), (3) proteinaceous tests, (4) tests built from recycled organic or mineral particles (xenosomes, agglutinated)	May allow an environmental filter to operate, in relation to the availability of the different substrates (e.g., mineral particles of adequate size, fungal hyphae, silica) or the relative cost of building a self-secreted test by comparison with an agglutinated one; Availability of material and/or prey to construct the test. Source of material appear to be a major regulator of abundance and repartition of testate amoeba along *Sphagnum*		Yes	Lamentowicz et al., 2009a; Fournier et al., 2012; Gomaa et al., 2012; Fiałkiewicz-Kozieł et al., 2015; Krashevska et al., 2016, 2020; Schwind et al., 2016a,b; Koenig et al., 2017, 2018a,b; van Bellen et al., 2018; Marcisz et al., 2019, 2020

(Continued)

TABLE 1 | Continued

Trait (unit; if relevant)	Description	Trait function, ecological meaning, link to ecosystem functions (if known)	Used in paleo?	References
Test shape/morphology/outline	Test shapes: discoid, pyriform, globular, circular, oviform, flask-shaped	In lake Arcellinida, taxa with discoid and hemispherical tests are associated with planktic/epiphytic habitats.	No	Gomaa et al., 2012; Lansac-Tôha et al., 2014; Lahr et al., 2019; Krashevska et al., 2020; Macumber et al., 2020
	Geometric morphometrics: principal component scores and shape models.	In lake Arcellinida, taxa with large pyriform tests are associated with oxygenated benthic habitats, taxa with oviform tests with epiphytic habitats, and taxa with globular tests with planktic/epiphytic habitats.	Yes	Macumber et al., this issue
Presence of a neck	Yes or no	Important trait in Arcellinida	No	Gomaa et al., 2012; Lahr et al., 2019
Test compression	Compressed, sub-spheric, spheric, hemispherical	An adaptation to living in a thin water film and allowing the amoebae to remain active longer when the soil moisture content decreases; Survival potential in drier situations and thus potential contribution to the food web in dry conditions	Yes	Lousier, 1974; Fournier et al., 2012, 2015, 2016; Koenig et al., 2017, 2018a; Krashevska et al., 2020
Test aperture (pseudostome)				
Aperture position	Position of the aperture within the test: acrostomic, plagiostomic, axial	Protected/hidden apertures are interpreted as adapted to decreasing soil moisture content; Aperture position represents the ability to survive in thin water film and thus the ability to remain active and contribute to the food web in dry conditions	Yes	Bonnet, 1964; Lousier, 1974; Chardez and Lambert, 1981; Laggoun-Défarge et al., 2008; Fournier et al., 2012; Krashevska et al., 2012, 2020; Jassey et al., 2013a,b; Fiałkiewicz-Kozieł et al., 2015; Fournier et al., 2015; Lamentowicz et al., 2015; Marcisz et al., 2016; Koenig et al., 2017, 2018a,b; Marcisz et al., 2020
Aperture shape	Shape of the aperture can be circular, oval, slit-like, irregular	In lake Arcellinda, *Netzelia* sp. with teeth-like apertures have been observed to be planktic carnivores and have been found to be associated with the rise of other planktic carnivores such as *Daphnia* sp. highlighting success of strategy.	Yes	Han et al., 2011; Gomaa et al., 2012; Macumber et al., this issue; Lahr et al., 2019; Krashevska et al., 2020
Aperture size (μm)	Width of the shell aperture	Related to prey size and food web functioning	Yes	Jassey et al., 2013a,b, 2016; Meyer et al., 2013; Gomaa et al., 2014; Fournier et al., 2015; Marcisz et al., 2016; Koenig et al., 2017, 2018a,b; Krashevska et al., 2020; Marcisz et al., 2020

(Continued)

TABLE 1 | Continued

Trait (unit; if relevant)	Description	Trait function, ecological meaning, link to ecosystem functions (if known)	Used in paleo?	References
Phylogeny				
Pseudopod type	Lobose, filose	This trait was used as lobose/filose TA index where lobose TA represent K-strategic species whereas filose TA represent r-strategic species. More developed communities are dominated by K-strategic lobose TA.	No	Mattheeussen et al., 2005; Wanner and Xylander, 2005; Beyens et al., 2009; Fournier et al., 2012;
Phylogeny	Euglyphida vs. Arcellinida; “Pyriform” vs. "Lanceolate" vs. other	Different evolutionary stable strategy to cope with environmental settings	No	Fournier et al., 2012; Krashevska et al., 2020; Macumber et al., 2020
Trophy				
Trophic status (heterotrophy/ mixotrophy)	Presence or not of photosynthetic endosymbionts	Mixotrophy is strongly related to the presence of wet *Sphagnum* and carbon sequestration in peatlands. It can also be used as a proxy for species survival in extremely oligotrophic conditions in *Sphagnum* capitula. Among lake Arcellinida, mixotrophy, especially in large pyriform taxa may indicate oligotrophy, clear water and an oxygenated benthic environment.	Yes	Heal, 1964; Schönborn, 1965; Yeates and Foissner, 1995; Gilbert et al., 2000, 2003; Jassey et al., 2012, 2015; Krashevska et al., 2012, 2020; Marcisz et al., 2014b, 2015, 2016, 2019, 2020; Fournier et al., 2015; Payne et al., 2016; Gałka et al., 2017; Koenig et al., 2017, 2018a,b; Ratcliffe et al., 2017; Creevy et al., 2018; van Bellen et al., 2018; Herbert et al., 2019; Basińska et al., 2020; Lamentowicz et al., 2020; Macumber et al., 2020, this issue; Zhang et al., 2020a,b
Trophic level	Species with a low test-aperture size over body size ratio are considered as having low trophic position (bacterivores, algivores), whereas species with high ratio as having a high trophic position (predators of other protists and micro-metazoans)	Reflects the complexity of the food web. Dominance of high trophic level species points to a more complex microbial food web in the peatland.	No	Jassey et al., 2012, 2013a,b, 2014, 2016; Lamentowicz et al., 2013a; Fournier et al., 2016; Payne et al., 2016; Creevy et al., 2018
Ecological preferences				
Preferred DWT (cm)	Wet vs. intermediate vs. dry indicators		Yes	Marcisz et al., 2016; Krashevska et al., 2020
Preferred pH	Species pH preferences (higher or lower pH values)		Yes	Marcisz et al., 2016; Krashevska et al., 2020
Other				
Gas vacuoles	Presence or absence	Structures used by testate amoebae to move vertically in the water column. They are used to float the organism from the bottom to the surface of the water in an inverted position.	No	Ogden, 1991; Schwind et al., 2016b

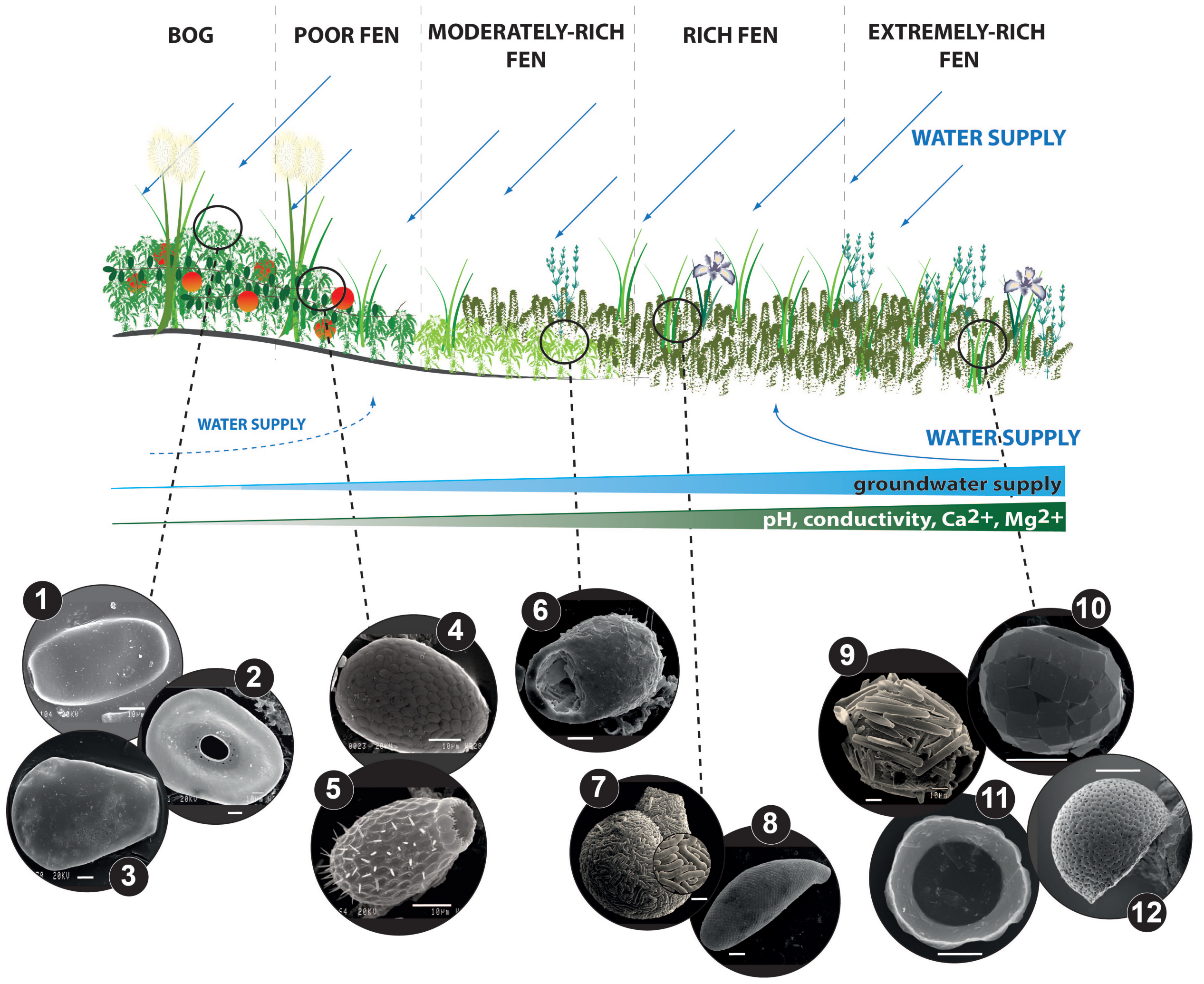

FIGURE 1 | An example of testate amoebae common in various types of peatlands along the bog-rich fen gradient: **(1)** *Archerella flavum* (Archer 1877) Loeblich et Tappan 1961, **(2)** *Arcella catinus* Penard 1890, **(3)** *Hyalosphenia papilio* Leidy 1874, **(4)** *Assulina muscorum* Greeff 1888, **(5)** *Euglypha strigosa* Ehrenberg 1848, **(6)** *Centropyxis aerophila* Deflandre 1929, **(7)** *Lesquereusia spiralis* Ehrenberg 1840, **(8)** *Cyphoderia* cf. *ampulla* Ehrenberg 1840, **(9)** *Difflugia bacillariarum* Perty 1849, **(10)** *Paraquadrula irregularis* Wallich 1863, **(11)** *Microchlamys patella* (Claparède et Lachmann 1859) Cockerell 1911, **(12)** *Pyxidicula patens* Claparède et Lachman, 1858. The distribution of species along the poor-rich gradient defines the bog-fen habitat that is mainly determined by hydrological dynamics, especially higher supplies of groundwater and nutrient input in the rich end of the gradient. Other variables influencing testate amoebae also vary between the peatland type, e.g., the poor end of the gradient is characterized by a low pH whereas the rich end of the gradient possesses a higher pH and high ground water Ca and Mg concentrations. SEM photos credit: **(1–11)**: Łukasz Lamentowicz and Mariusz Lamentowicz, **(12)**: Eckhard Völcker. White scale on each of the photos equals 10 μm.

Knowledge of the feeding preferences and the functional role of testate amoebae in relation to nutrient cycling and primary production is scarce [see Wilkinson and Mitchell (2010) for an overview]. Six distinct feeding groups were identified that encompassed freshwater protozoa: photosynthetic autotrophs, bacterivores/detritivores, saprotrophs, algivores, non-selective-omnivores and predators (Pratt and Cairns, 1985). These groups were also successfully applied to soil communities with the addition of the mycophagous group (Coûteaux and Darbyshire, 1998). However, these generalizations have not been formulated for testate amoebae due to a lack of important basic knowledge for most taxa. Most testate amoebae are predators with their primary diet being comprised of a wide range of food sources including bacteria (Coûteaux and Pussard, 1983), other protists (Deflandre, 1936b; Gilbert et al., 2000), microalgae (Bles, 1929), fungi (Coûteaux and Dévaux, 1983) and small metazoa (i.e., rotifers, Mast and Root, 1916). Although prey size is often limited by the size of the aperture (Ogden and Hedley, 1980), testate amoebae are also able to feed on food sources much larger than themselves (e.g., filamentous algae, Stump, 1935). They also feed on prey such as significantly larger nematodes (Yeates and Foissner, 1995), which they have been observed to hunt in packs (Gilbert et al., 2000; Geisen et al., 2015). Schönborn et al. (1987) suggested that some species (i.e., *Schoenbornia humicola* Schönborn 1964) might also be saprophagic with different feeding phases. Bonnet (1958) and Heal (1964) found that light

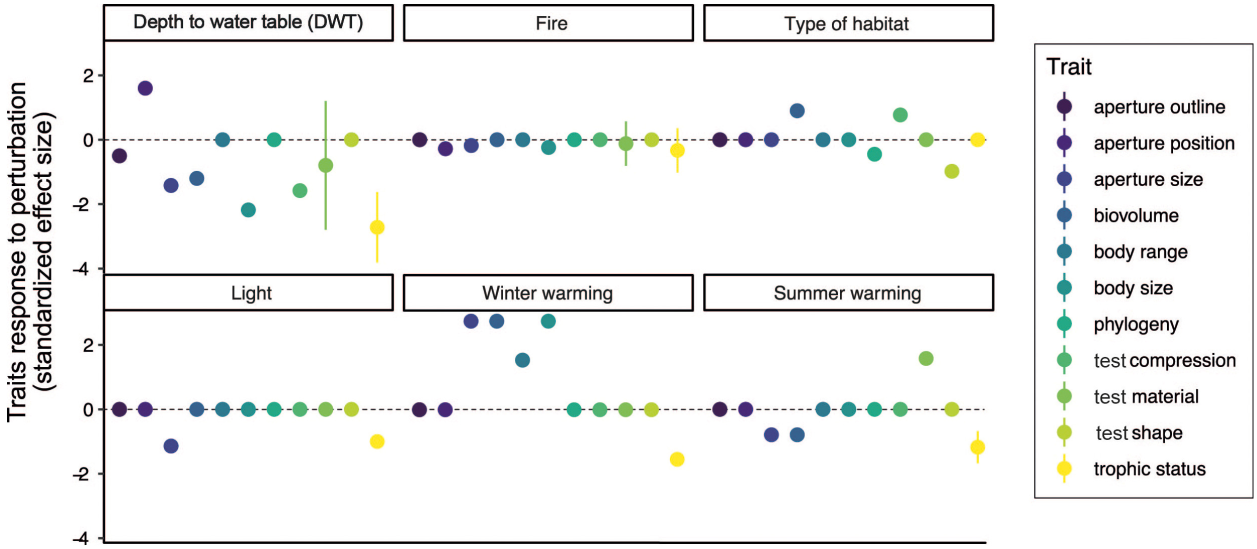

FIGURE 2 | Testate amoeba trait responses to environmental factors. Standardized effect size was calculated based on published testate amoeba trait studies that investigated the effect of various perturbations on testate amoeba trait composition.

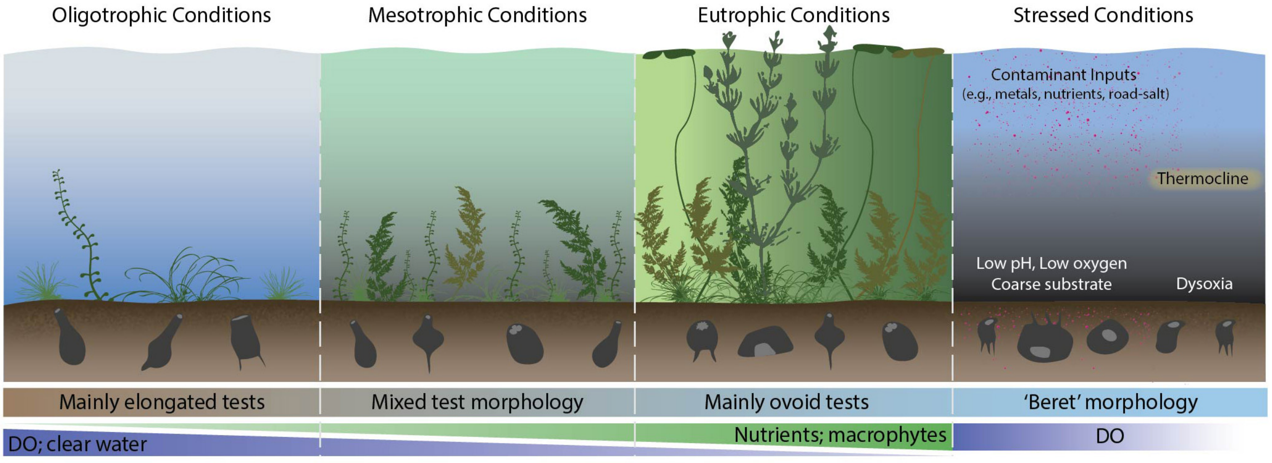

FIGURE 3 | An example of Arcellinida morphological variability in various types of lakes. DO, dissolved oxygen.

could determine the distribution of mixotrophic (zoochlorellae containing) species and Schönborn (1965) concluded that their occurrence was linked to the low nutrient content in *Sphagnum*-dominated bogs. However, a quantitative determination of the contribution of mixotrophic taxa to primary production in bogs was only recently demonstrated (Jassey et al., 2015).

Bonnet (1976b) suggested that filose testate amoebae (e.g., *Euglypha*, *Corythion*, *Trinema*) were assumed to be *r*-strategists (i.e., small test size, short generation times, etc.), whereas lobose testate amoebae (e.g., *Arcella, Nebela, Centropyxis, Difflugia*) are in contrast *K*-strategists (larger test size, longer generation times, etc.) and introduced the L/F index to estimate the developmental stage of testate amoebae communities (or ecosystems). The L/F index, which represents the relative proportion of lobose vs. filose testate amoebae, has been applied in ecological and paleoecological studies to estimate ecosystem resilience and recovery after perturbation (see details in section "Soil and Other Wetland Habitats"). Unfortunately, only a few species have been cultured in laboratory settings (Wanner, 1999), and further research is thus required to validate the assumption concerning the life strategies of testate amoebae (Creevy, 2013). That said, unpublished research based on cultures of >100 taxa indicates that rather than there being a "filose-lobose" difference in growth rates, the rate of growth seems to depend primarily on biovolume, with smaller species reproducing faster than larger ones. Unfortunately, most of this research data has not been published (Ralf Meisterfeld, personal communication). The most consistent attempt to classify testate amoebae following the concept of "vital attributes" (Noble and Slatyer, 1980) was undertaken by Jax (1992) in order to describe the succession and long-term dynamics within freshwater testate amoebae communities. In that work, testate amoebae were grouped according to their dispersion ability, the preference of particular successional phases and overall long-term ecosystem dominance. However, with only a few exceptions (e.g., Chardez, 1965; Raikov et al., 1989), there is a basic lack of information on the ability of most testate amoebae to, for example, form cysts, which is an important functional trait related to ecosystems resilience and recovery rate. Nevertheless, the available studies provide a considerable amount of data that can be used to infer the functional role of individual testate amoeba taxa, which can be considered within the frameworks of functional ecology to form the basis for future studies.

Evolution vs. Testate Amoeba Morphological Traits

Within the constraint of being unilocular, testate amoebae are characterized by a myriad of test morphologies that differ in general shape, size and composition, which are therefore useful for species identification. Additionally, Arcellinida testate amoebae have a very long fossil record that makes the group an important component of evolutionary studies on early eukaryotes (Schönborn et al., 1999; Porter and Knoll, 2000; Porter et al., 2003; Schmidt et al., 2006; Schmidt et al., 2010; Porter, 2011). Attempts to resolve the systematics and evolutionary relationships within the group began in the early 20th century (Deflandre, 1936a; Jung, 1942), and initially pseudopod morphology and test composition were used to classify testate amoebae into "filose" (euglyphids and amphitremids) and "lobose" (arcellinids), as well as the major suborders within these groups (Bovee, 1985; Meisterfeld, 2002).

Application of molecular methods changed the understanding of the deep evolutionary relationships within the testate amoebae resulting in several major taxonomic revisions. Studies based on single-gene phylogenetic reconstructions (i.e., SSU rRNA, mt COI) demonstrated that several genera (i.e., *Nebela*, *Quadrulella*, *Difflugia)* are paraphyletic, and that test morphology appeared to be one of the most consistent traits constraining deep phylogeny (Lara et al., 2008; Lahr et al., 2011; Gomaa et al., 2012). More recently, combined approaches using hundreds of genes as well as detailed morphological analyses have been used to more robustly reconstruct the major lineages within the Arcellinida (Lahr et al., 2019). In addition, reconstruction of ancient Tonian (Neoproterozoic, 730 million years ago) test morphologies of vase-shaped microfossils revealed that they display a remarkable similarity to modern arcellinidans (Lahr et al., 2019). These results, building on previous research, indicate that testate amoebae had already diversified before the beginning of the Sturtian glaciation, 717 million years ago (Porter and Knoll, 2000; Porter et al., 2003; Porter, 2011). The morphological diversity in place by the Neoproterozoic and apparent evolutionary conservatism since suggests that distinct functional types of testate amoebae related to ecological niches, feeding behaviors and distinct evolutionary strategies developed early in their evolutionary history (Lahr et al., 2019). The recognition of arcellinidan cryptotaxa provides additional evidence of the important role of functional traits in constraining test morphology.

It can therefore be concluded that, at least in Arcellinida, test morphology has played a substantial role in shaping the evolution of testate amoebae. While the overall test outline is the most important trait defining deep phylogenetic groups, in some groups such as the Organoconcha, comprising genera *Pyxidicula* and *Microchlamys*, the characteristic very wide aperture may be of evolutionary significance. Furthermore, at the genus and species level other morphological traits can be equally important. For example, in the genus *Quadrulella,* the size and placement of siliceous test plates are species-specific (Kosakyan et al., 2016).

Testate Amoeba Molecular Variability vs. Trait Variability

Testate amoeba species identification based on test morphology has always been a challenge. Phenotypic plasticity is common among testate amoebae, making it difficult to determine valid morphological identification criteria (Medioli et al., 1987). Culturing studies by Jennings (1916) early on demonstrated the wide variation that could be observed within clonal lineages of *Difflugia*. Factors such as silicon availability (Wanner et al., 2016), food or temperature were shown experimentally to influence aperture shape and test size, both in the field (Wanner and Meisterfeld, 1994) and *in vitro* (Wanner, 1994). Given the importance of morphology-based taxonomic identification for applications such as paleoecology and biomonitoring (Mitchell et al., 2014), and the comparability of observations and experiments carried out within various research laboratories, taxonomic revisions, or the development of an alternative objective classification scheme is necessary. The existing testate amoeba classification schemes have been challenged by the results of molecular studies (see section "Evolution vs. Testate Amoeba Morphological Traits"). DNA barcoding of individual specimens based on variable mitochondrial markers has led to both the recognition of distinct new species within existing species complexes and the revision of phylogenetic relationships among taxa, which has further led to the description, or redefinition, of both genera and families (Singer et al., 2015; Kosakyan et al., 2016; Duckert et al., 2018). Of ecological relevance, some closely related and morphologically similar (pseudo-cryptic) species (e.g., *Nebela guttata* Kosakyan et Lara 2012 and *Nebela gimlii* Singer et Lara 2015) were shown to occupy different ecological niches (Singer et al., 2018). *Hyalosphenia papilio* Leidy, 1874, a common species in northern *Sphagnum* peatlands was found to be a species complex comprising at least 13 distinct genetic lineages with geographically limited distributions (Heger et al., 2013; Singer et al., 2019).

The unresolved state of testate amoeba taxonomy makes the use of morphology-based community descriptions particularly difficult, especially because closely-resembling species can occupy divergent niches and lumping them blurs taxon related ecological indication signals. In order to overcome these biases and to produce objective and comparable data, metabarcoding approaches are currently developed. Whole environmental DNA is extracted on concentrated testate amoeba cells, and a group-specific PCR protocol is applied to obtain DNA sequences of the studied organisms (Kosakyan et al., 2015). These promising initiatives are however, at the moment, of limited applicability given the sparse molecular information available for most groups of testate amoebae; in the future, however, they could also be applied to fossil DNA. Likewise, in the future, tracking functional genes expressed by testate amoebae during identified and environmentally relevant processes within metatranscriptomes or ancient DNA metagenomes will probably increase the relevance of these protists as bioindicators (Kosakyan and Lara, 2019).

Trait analysis overcomes the biases linked to species identification, and importantly, can integrate any information derived from ecophenotypic plasticity. Likewise, a correspondence has been observed between measurable traits (such as test size and the number of pores) and climatic/environmental factors (Mulot et al., 2017). In addition, large test size has been reported as a trait related to humidity when considering the whole community in peatland settings (Koenig et al., 2018b), and climate variability in lacustrine Arctic studies (Dallimore et al., 2000). Paradoxically the largest species with the otherwise primarily wetland genus *Quadrulella* has been discovered in biocrusts from a Mexican tropical desert (albeit receiving substantial water through condensation; Pérez-Juárez et al., 2017).

These data are relevant for characterizing environmental change over time, but would be omitted in species only based studies, regardless of the level of taxonomic resolution analyzed. Studying the correspondence between observed traits, phylogenetic position and environmental factors is still a wide-open field of research. *Sphagnum*-associated species characterizing Palearctic peatlands are relatively well studied. However, many species may still be left to be described when taxonomists eventually take a closer look at individual species complexes (Kosakyan et al., 2016). Other biogeographic regions (e.g., the Neotropics), and other ecosystem types (e.g., rich fens) are still relatively less studied.

Testate Amoeba Trait-Environment Relations in Different Habitats

Sphagnum-Dominated Peatlands

Studies on testate amoeba-environment relationships in *Sphagnum*-dominated peatlands have been mainly carried out on raised ombrotrophic bogs (but see Opravilova and Hajek, 2006; Hajkova et al., 2011). Peatlands of this type are highly acidic (pH 3.5-5.5), have no contact with groundwater, and are supplied exclusively by rainwater (Rydin and Jeglum, 2006). Therefore, they only receive allochthonous material from precipitation (rain and dry deposition) but not from e.g., ground water from catchment, streams or rivers. Those ombrogenic conditions result in rather straightforward species-environment relationships that are relatively easy to explore in comparison with other peatland types (e.g., fens, spring-fed fens or landslide mountain fens; **Figures 1** and **2**). Early studies mostly focused on the diversity and community composition of testate amoebae along ecological gradients (e.g., Heinis, 1910; Harnisch, 1927; Grospietsch, 1953; Warner, 1987), later leading to more quantitative and experimental studies (e.g., Tolonen et al., 1992; Warner and Chmielewski, 1992; Tolonen et al., 1994; Gilbert et al., 1998b; Mitchell et al., 1999). Most work on the ecology of peatland testate amoebae has focused on community patterns along the hydrological gradient (typically water table depth) and trophic gradients (typically pH). However, other factors such as peatland type, landscape openness, light intensity/shading, nutrient concentration and temperature changes have also been studied (**Figure 2**). Most research aimed at understanding testate amoebae morphological traits and their relationship to environmental gradients and changes, and the functional implications of these changes has in fact focused on peatlands (**Table 1**):

(1) **Trophic level and testate amoeba functional traits.** Testate amoeba community composition plays an important role in regulating the peatland microbial food web structure (Gilbert et al., 1998a, 2003; Jassey et al., 2012, 2013a,b; Lamentowicz et al., 2013a; Meyer et al., 2013), with mounting evidences that shifts in microbial food web structure may be linked to shifts in the functional trait composition of testate amoebae (Jassey et al., 2013b; Lamentowicz et al., 2013a; Hamard et al., 2019). For instance, it has been recently shown that the loss of mixotrophic testate amoebae (**Table 1**) destabilizes peatland microbial food webs by changing trophic interactions and decreasing connectivity within the microbial network (Hamard et al., 2019). Other research has further demonstrated that shifts in environmental conditions select for testate amoeba taxa with either a high or low trophic level based on their body size features (Lamentowicz et al., 2013a; Jassey et al., 2014; Fournier et al., 2016). Functional trait shifts toward either high and low trophic level testate amoebae species has often been the consequence of abrupt environmental changes connected with natural or anthropogenic influence (Jassey et al., 2013a, 2016; Lamentowicz et al., 2013a; Meyer et al., 2013; Krashevska et al., 2016; **Figure 2**). Other studies along open-to-forested bog transects have further shown that tree encroachment reduces the trophic level of testate amoeba communities leading to an increased abundance of smaller species (Payne et al., 2016; Creevy et al., 2018). Loss of testate amoeba functional diversity and a decrease in abundance of larger species have also been linked to extreme weather conditions; harder soil frosts (Jassey et al., 2016) as well as warming (Jassey et al., 2013a), suggesting that smaller species are more resistant to extreme climatic condition. In New Zealand peatlands, smaller species were found to be more closely correlated to water table depth and conductivity while larger species were correlated to pH, suggesting that these two components of the community may provide contrasting ecological signals (McKeown et al., 2019). Among direct anthropogenic impacts, atmospheric pollution has been shown to

negatively influence high trophic level testate amoeba species (Meyer et al., 2012, 2013), again emphasizing the generally higher sensitivity of larger testate amoeba taxa to environmental disturbances (**Table 1**).

(2) **Individual trait responses.** There has been an increase in research through the last decade on assessing the response of individual testate amoeba functional traits to environmental changes in peatlands. To identify general patterns in trait responses to environmental changes, we summarized the responses of individual testate amoeba traits to disturbances (DWT change, warming, light intensity and habitat change) by calculating standardized effect sizes. To do so, coefficients of correlations and/or F-values were extracted from tables, figures, the main text and/or supplementary materials available in the literature. Then, *F*-values and/or coefficient of correlations were converted into an effect size using the *compute.es* package in *R* (R Development Core Team, 2020); the results are presented in **Figure 2**. Overall, this analysis revealed that testate amoeba traits strongly respond to DWT and warming, while fire, light intensity and habitat shift had only weak and/or specific effects on individual testate amoeba traits. We also observed a strong, and primarily negative, response for most testate amoeba traits to water level changes (**Figure 2**). Most studies (Fournier et al., 2012; Jassey et al., 2013a; Marcisz et al., 2014b; Koenig et al., 2017, 2018a,b) have reported a decrease in test size (length, width, range) with decreasing water level, while mixotrophic species tended to disappear (**Figure 2**). Documented effects on test material and type have been more variable, with sometimes conflicting results (Fournier et al., 2016), suggesting that some postulated trait-hydrology relationships may be context-dependent, and might be more closely related to the initial water levels and peatland trophic status. Often confounded with hydrology, the most recent analyses reported that light intensity and habitat shifts had similar effects, and that both were more strongly correlated than hydrology to testate amoeba traits such as trophic status and test characteristics (Marcisz et al., 2014b; Payne et al., 2016; Ratcliffe et al., 2017; Creevy et al., 2018; Herbert et al., 2019; Lamentowicz et al., 2020; **Figure 2**). Overall, such findings show that not only testate amoeba community structure (Charman and Warner, 1992; Mitchell et al., 1999; Lamentowicz and Mitchell, 2005), but also the testate amoeba functional traits present can be directly correlated to peatland hydrology (Lamentowicz et al., 2020). These results further support recent attempts of using testate amoeba functional traits in transfer function models to quantitatively reconstruct past water table levels (van Bellen et al., 2017). Besides hydrology, studies focusing on the impact of climate change effects (e.g., precipitation, fire) have shown that testate amoeba body size, aperture size and trophic status can commonly be correlated to environmental changes (**Figure 2**), with related potentially important impacts on food webs and biogeochemical cycles (Jassey et al., 2015; Basińska et al., 2020). It has also been observed that the trophic status (i.e., mixotrophy) of testate amoebae is a sensitive trait to most environmental disturbances. The importance of mixotrophs for peatland carbon cycling (Jassey et al., 2015) and microbial food web structure (Hamard et al., 2019) confirms that investigations of testate amoeba functional traits are as important to consider as species taxonomy and morphology. Identifying dominant testate amoeba traits that respond to environmental changes over long timescales (hundreds of years to millennia) can potentially serve as a sensitive paleoenvironmental indicator (e.g., documentation of shift of *Sphagnum* peatlands from a carbon sink to a carbon source; Zhang et al., 2020a,b).

Fens

In contrast to ombrotrophic bogs, fens are characterized by the influence of ground or surface water, and hence are typically more minerotrophic. The dominant environmental gradients in fens are water pH and Ca content (Bridgham et al., 1996; Hájek et al., 2006, 2014; Hájková et al., 2012). Fens cover a much wider range of habitats and abiotic conditions than ombrotrophic bogs, ranging from *Sphagnum*-dominated poor-fens to alkaline rich fens dominated by brown mosses (Gąbka and Lamentowicz, 2008) (**Figure 1**). This wider range of possible hydrological and hydrochemical conditions makes it more challenging to determine the testate amoeba species-environment relationships in fens than in ombrotrophic bogs. While testate amoeba community structure typically differs markedly between bogs and fens (Heal, 1961), some testate amoeba functional traits may be characteristic of both environments. For example, the abundance of mixotrophs and heterotrophs in peatlands differs along the poor-rich and wetness gradient, with higher abundances of mixotrophs in bogs and poor fens. Indeed, *H. papilio* and *Archerella flavum* (Archer 1877) tolerate slightly minerotrophic conditions such as existing in *Sphagnum*-dominated poor-fens where they can be very abundant, but they do not occur in calcium-rich fens dominated by brown mosses (Jassey et al., 2014).

Specific taxa restricted to extremely rich fens, such as *Pyxidicula* and *Microchlamys*, possess disk-like tests with a wide aperture (**Figure 1**). In *Microchlamys patella* (Claparède & Lachmann 1859) for example, the cell is enclosed within a membranous sac, which is fixed to the test at several points, but is lost in empty tests (Siemensma, 2019). These species are associated with high groundwater Ca and Mg concentrations, and they often occur at the base of peat deposits, indicating the past presence of calcium-rich or extremely calcium-rich fen stages in the early development of some peatlands (Lamentowicz et al., 2013b; Gałka et al., 2017). The enriched part of the gradient (Lamentowicz et al., 2011) is typically characterized by *Paraquadrula irregularis* Wallich 1863, a small species that builds its test from secreted quadratic or rectangular calcite plates, as well as *Quadrulella symmetrica* (Wallich 1863) which produces siliceous square plates, regularly arranged in rows (Siemensma, 2019).

Jassey et al. (2014) showed that small testate amoebae were more abundant in rich and extremely rich fens than in poor and moderately rich fens. This study also showed that species with a central aperture such as *Arcella discoides* Ehrenberg 1843 and *M. patella*, as well as idiosomic species such as *P. irregularis* and *Tracheleuglypha dentata* (Vejdovsky 1882), dominate communities extending toward the richer part of the gradient. In a study of subalpine peatlands covering the full bog to rich fen gradient, the species richness of testate amoebae was highest in calcium-rich and extremely calcium-rich fens when compared to calcium-poor fens and bogs (Lamentowicz et al., 2010). New Zealand peatlands in contrast, are characterized by higher relative abundances of small taxa in areas at the acidic end of the pH gradient (pH 4.0–4.5) as well as in wetter habitats (DWT −10 to 20 cm), as compared to higher pH (5.0–5.5) and drier habitats (DWT 25–50 cm; McKeown et al., 2019). These results clearly indicate the need for more detailed studies on determining the nature of testate amoeba-environment relationships in fens.

Tropical Peatlands

Tropical peatlands, which are mainly located in lowlands (Page and Baird, 2016), differ from temperate, boreal and montane peatlands in their hydrology, vegetation, nutrient distribution, oxygenation, as well as by these environments being characterized by generally higher temperatures (Lawson et al., 2014; University of Leeds Peat Club et al., 2017). Unfortunately, in comparison to high- and mid-latitude peatlands, there have been relatively few studies carried out on assessing the diversity and ecology of testate amoebae from tropical peatlands. Since the taxonomic composition in tropical peatlands may vary due to biogeographic limitations, there is a need to carry out additional studies on the communities composition, species richness, and functional traits for each region separately, in order to detect similarities or differences in the ecological constraints characterizing similar species, as well as for geographically restricted ones.

For example, in a study assessing the ecological influence of changes in hydrology in tropical montane rainforests along an elevation gradient, testate amoeba species richness did not respond to a 64% reduction of precipitation, but at the same times population density decreased by 91% (Krashevska et al., 2012). In contrast, in the subarctic it was observed that simulated extreme rainfall was correlated with a reduction in the mean abundance and species richness of testate amoebae (Tsyganov et al., 2013). Thus, although all testate amoebae need water to be active (i.e., to move, feed and reproduce), different taxa in different regions and ecosystems may have different tolerances related to soil water availability. It is therefore required that the environmental conditions influencing the morphological or functional traits characterizing the most representative taxa in the community should be emphasized.

Such traits as testate amoeba test size have been shown to be related to hydrology and temperature in temperate zones (Lousier, 1974; Bobrov et al., 1999; Mitchell et al., 2008a). This correlation is also true for the taxa frequently found in tropical systems (e.g., *Trigonopyxis arcula s.l.* Penard 1912, *Hyalosphenia subflava s.l.* Cash and Hopkinson 1909). For instance, in both a tropical montane rainforest of Ecuador, and in lowland tropical rainforests and peatlands of Indonesia, over 40 different morphotypes of *T. arcula* were observed, with test size varying from 60 to 210 μm (Krashevska et al., 2007, 2010, 2016; Schulz et al., 2018). It was shown for *Trigonopyxis* that test size, increased on average by 20 μm, with decreasing temperature (from 19 to 9°C mean annual temperature) and increasing precipitation (from 2200 to 4500 mm year $^{-1}$; Schulz et al., 2018). Similarly, the test size of *H. subflava* in peatlands of Indonesia, Peru and Panama ranged from 35 to 140 μm, and different test sizes correlated differently with water table depth in both modern and paleo samples of peatlands (Swindles et al., 2014, 2016, 2018; Biagioni et al., 2015; Krashevska et al., 2020). Therefore, *H. subflava* test size changes from small to large can be considered as an indicator trait of frequent hydrological changes from drier to wetter conditions in tropical peatlands, whereas in high- and mid-latitude peatlands this species is considered as an unambiguous indicator of dry conditions (Charman et al., 2000).

Moreover, test composition has been shown to be a useful trait reflecting anthropogenic environmental changes in tropical lowlands. For example, the decrease in the abundance of testate amoebae with siliceous tests in oil palm plantations compared to that in rainforest environments has been hypothesized to indicate the influence of anthropogenic desilication on community structure. Further, species with siliceous tests showed a negative correlation with soil temperature and a positive correlation with water content and the amount of litter (Krashevska et al., 2016).

Similar to traits connected with the test, the trophic status (mixotrophy vs. heterotrophy) can be a potentially good indicator trait of habitat openness (Payne et al., 2016; Herbert et al., 2019), yet a less frequent studied one, especially in the tropics (Krashevska et al., 2012). A mixotroph, such as *H. papilio*, widespread in the Northern Hemisphere peatlands, is absent or very rarely found in tropical regions, underscoring the need for experimental studies to identify representative mixotrophs in the tropics. However, mixotrophic species within the genera *Placocista* and *Archerella,* as well as some species of the genus *Heleopera,* and heterotrophic species of *Assulina* and *Euglypha*, often found in the tropics, have been observed to respond to change in light intensity, making them potentially suitable indicators of plant habitat openness in tropical regions (Schönborn, 1965; Searles et al., 2001; Robson et al., 2005; Krashevska et al., 2012; Payne et al., 2016).

Lacustrine Environments

The principal testate amoebae studied in lacustrine environments are Arcellinida. Arcellinidans play a key role in aquatic ecosystems where they exert considerable predatory pressure on bacteria and smaller eukaryotic microbes and represent an important intermediary food web component (Beyens and Meisterfeld, 2001; Patterson and Kumar, 2002; Anderson, 2012). Tests typically range in size from 5 to 500 μm, and are comprised primarily of high-preservation potential agglutinated material (e.g., mineral grains and diatom frustules with autogenous cement), although species characterized by idiosomic tests are also observed (Medioli and Scott, 1988).

Arcellinidans are found in lacustrine environments from the equator to poles, although they are most abundant in temperate environments where they are often present in great abundances (500–3000 specimens per ml; Patterson and Kumar, 2002; Patterson et al., 2015).

Lakes are comprised of lentic ecosystems ranging in size from ponds to thousands of square kilometers that can be subdivided into a myriad of habitats influenced by numerous climatic and physio-chemical constraints (e.g., open water, shallow littoral habitats with macrophytes, varying trophic status, thermoclines, pH). Arcellinida are mostly restricted to benthic habitats, but species with at least a partial planktic habit have been documented (Schönborn, 1962; Medioli et al., 1987; Yu et al., 2014). A large body of research has been produced that has documented the complex community responses of Arcellinida in these subenvironments, and due to the large number of environmental variables typical of lacustrine systems considerable intra-lake and inter-lake assemblage variability has been observed (Patterson, 1996; Roe et al., 2010; Nasser et al., 2016, 2020a; Steele et al., 2018; Cockburn et al., 2020). There has been considerable taxonomic confusion within the Arcellinida due to a historical proliferation of new species as a result of the considerable morphological variability observed within some taxa, and the common practice of describing new species within a regional context with little consideration of research elsewhere, (e.g., Medioli et al., 1990; Wanner and Meisterfeld, 1994; Bobrov et al., 1999; Wanner, 1999; Charman et al., 2000). For Arcellinida found in lacustrine settings, Medioli and Scott (1983) proposed a possible solution by suggesting that species be considered as widely variable groups that collectively, for any given wild population, accommodate 75% or more of the morphological variability within the entire population. Through the late 1980s and 1990s this approach was refined with the establishment of an informal infrasubspecific nomenclature, which in the lacustrine research community became known as 'strains' (e.g., Medioli et al., 1987; Asioli et al., 1996). The 'strain' approach came out of the recognition that the morphology of certain specimen, not necessarily linked to specific taxa, could be associated with specific environmental stressors, which during multivariate statistical analysis permitted the recognition of more subtle environmental subdivision than would be otherwise possible (Patterson, 1996; Patterson and Kumar, 2002).

The increase in the number of functional taxonomic units afforded by deployment of the 'strain' concept has permitted lacustrine researchers to more effectively explore the spatio-temporal effects of various sedimentological, geochemical, and organic variables, as well as the influence of lacustrine parameters on community structure and dynamics (Collins et al., 1990; Patterson, 1996; Reinhardt et al., 1998; Kumar and Patterson, 2000; Escobar et al., 2008; Neville et al., 2010; Roe et al., 2010; Nasser et al., 2016; Gavel et al., 2018; Steele et al., 2018; Cockburn et al., 2020). For instance, many studies have confirmed the sensitivity of arcellinidan communities to variability in pH (e.g., Asioli et al., 1996; Kumar and Patterson, 2000; Escobar et al., 2008; Neville et al., 2010; Patterson et al., 2013). Changes in the trophic state of lakes have also been shown to profoundly impact arcellinidan distribution (e.g., Reinhardt et al., 2005; Roe et al., 2010; Patterson et al., 2012; Gavel et al., 2018; Prentice et al., 2018). Several researchers have also linked community dynamics to changes in lake salinity (e.g., Roe et al., 2010; Roe and Patterson, 2014; Cockburn et al., 2020). The recognition that arcellinidan species and 'strains' are sensitive to industrial contamination has attracted particularly close attention. For example, studies from Italy (Asioli et al., 1996) and Canada (Patterson, 1996; Reinhardt et al., 1998) were amongst the first to demonstrate the sensitivity of Arcellinida populations to the impact of mine-induced metal contamination, albeit in a semi-quantitative manner. The advent of more sophisticated multivariate statistical techniques resulted in the quantification of the spatio-temporal relationship between Arcellinida assemblages and mine-derived contaminants (Kauppila et al., 2006; Kihlman and Kauppila, 2009, 2010, 2012; Patterson et al., 2013; Nasser et al., 2016; Gavel et al., 2018; Cockburn et al., 2020; Nasser et al., 2020a,b). The great diversity and variety in arcellinidan lacustrine research, coupled with the use of multivariate statistics, has not only permitted identification of the environmental controls on arcellinidan assemblage distribution, but also established better links between functional 'strain' traits and specific environments.

In spite of the significant advances in understanding species/'strain'-environment relationships, very few studies have focused on the characterization of specific traits in lake environments (e.g., Macumber et al., 2014, 2020; Steele et al., 2020). The lack of studies may be the result a number of factors, including:

(1) **Challenges associated with the counting medium**. In contrast to the analysis of peatland testate amoeba, which are counted under cover-slips using light microscopy, the size range of lake Arcellinida examined are typically in the 37–200 μm range and enumerated *in vitro* in Petri dishes using relatively low power stereomicroscopy, which hinders the measurement of some traits (e.g., process length and aperture size; Patterson and Kumar, 2002; Wall et al., 2010a,b). There are also complications relating to the 3D morphology of specimens examined in Petri dishes vs. the compressed 2D morphology of specimens observed under coverslips.

(2) **Taxonomic challenges**. As outlined above, phenotypic plasticity, especially within the difflugiids (Medioli and Scott, 1983; Wanner and Meisterfeld, 1994; Wanner, 1999), has led to challenges in determining the relationship between morphological characteristics and environmental drivers, or other factors (e.g., stochastic processes; cf. Medioli et al., 1987; Prentice et al., 2018; Macumber et al., 2020).

(3) **Taphonomic challenges**. Although the relative abundance of live vs. dead specimens are documented in applied lacustrine research, the emphasis is on providing an overview of average annual conditions so total specimen counts, which include both live and dead specimens, are analyzed, with little consideration of seasonal changes in standing crop and associated taphonomic processes (e.g., Nasser et al., 2020a,b). The

> attraction of this approach is that average environmental conditions are measured, which would otherwise require multiple expensive geochemical analyses to replicate. Two studies, one carried out as part of a seasonal assessment of the effectiveness of an oil sands reclamation project in northern Alberta, Canada (Neville et al., 2010), and a second that looked at the impact of seasonality on a lake on the Ganga-Yamura Plains of North India (Farooqui et al., 2012), both demonstrated that there are significant seasonal changes in arcellinidan standing crop that can be directly related to taphonomic processes. These studies demonstrated the complexity of the response of Arcellinida to a range of environmental drivers, but also present the promise that the group can be used as a high temporal resolution biomonitoring tool.

Notwithstanding these issues, there has been a recognition of the significance of the role of test morphology between specific environments and across gradients. For example, Velho et al. (2003) and Lansac-Tôha et al. (2014) classified taxa according to dominant test morphology (e.g., hemispherial, flattened, spherical and elongated forms) in floodplain lakes in southern Brazil and linked this to habitat types and hydrological flow regimes. Macumber et al. (this issue) applied geometric morphometrics (Zelditch et al., 2012) to examine morphological responses across a temporal gradient of nutrient enrichment in a shallow Scottish lake (dominant test morphology, aperture size; biovolume) and compared this to species-based approaches. A further earlier study focused on an autecological approach to quantify taxonomic divisions between two strains of *Difflugia protaeiformis* LeClerc in Lamarck 1816, in relation to a wide array of environmental proxy data from urban lakes (Macumber et al., 2014). The more time-consuming characterization of traits in lake Arcellinida, in particular quantification of subtle changes in morphology (e.g., tapering of the neck, nature of complex basal processes, apertural characteristics), which are not readily captured by simple quantitative 2-dimensional metrics, has limited the application of traits-based analysis in lake Arcellinida. However, the introduction of new technologies may lead to a significant reduction in analytical time. A novel experiment carried out by Steele et al. (2020), demonstrated that a Flowcam flow cytometer used in conjunction with machine learning software could not only significantly decrease specimen enumeration time, but using a library of >40 morphometric properties, was able to group tests into morpho-groups that could be used to effectively identify distinct limnological habitats.

Soil and Other Wetland Habitats

Studies on the functional ecology of testate amoebae were first developed for soils with the test typology developed by Louis Bonnet (Bonnet, 1964, 1975). Bonnet developed a classification of testate amoebae communities based on a phytosociological hierarchical classification scheme and established correlations between soil and humus developmental stages and testate amoeba communities (Bonnet, 1961b). Based on this, he identified indicators for different soil types and also compiled extensive lists of ecological preferences for soil testate amoebae (Bonnet, 1976a, 1984, 1985, 1986, 1988, 1989, 1991a,b, 1992). The ecology of testate amoebae was also studied in agro-ecosystems and the impact of farming practices and pesticides on community structure was assessed (Foissner, 1987, 1997, 1999; Heger et al., 2012). These studies generally showed a negative impact of intensive farming on the diversity and abundance of testate amoebae. However, only a small number of studies have investigated functional traits of testate amoebae in soils, and these included terrestrial soils (Wanner and Xylander, 2005), various aquatic substrate types in the arctic tundra (Mattheeussen et al., 2005; Beyens et al., 2009), and floodplain soils (Fournier et al., 2012). All these studies included the phylogenetic grouping proposed by Bonnet (1976b) (i.e., the lobose vs. filose testate amoebae L/F index, see section "Testate Amoebae Traits and Ecological Roles Before "Functional Traits"").

Studies carried out in both high Arctic and sandy inland dune settings revealed similar recruitment patterns despite contrasting environmental conditions/filters (Mattheeussen et al., 2005; Wanner and Xylander, 2005; Beyens et al., 2009). In these habitats filose species typically fulfilled the role of rapid generalist colonizers, whereas lobose amoebae characterized more mature stable habitats. The lobose species that became part of these ecosystems during later successional stages did not outcompete and replace the earlier colonizers, but instead enlarged the existing microbial loop (Wanner and Xylander, 2005). The ready addition of lobose testate amoebae to these environments is in part related to these taxa being more resistant to warmer soil conditions, and associated desiccation that is better than filose species (Beyens et al., 2009). These findings confirmed previous assumptions that a high L/F index is characteristic of more stable and/or more developed habitats (Bonnet, 1976b).

The first ecological study of testate amoebae ever carried out in floodplain soils used not only functional traits, but also functional indices to characterize the testate amoebae communities, including: functional richness, divergence, dispersion, and evenness (Fournier et al., 2012). The results of this research revealed that although these environmental filters were significant in shaping the patterns of testate amoeba community structure, the main variable influencing amoeba diversity was soil moisture. Among functional traits analyzed, test-related traits, particularly compression and biovolume, were the most responsive to environmental changes.

The following studies that focused on the neotropical floodplains confirmed the significant role of environmental filters in the shaping of testate amoeba communities (Arrieira et al., 2015). Especially moisture was a variable to which testate amoebae species richness and abundance were significantly correlated (Arrieira et al., 2017). Among functional traits analyzed, morphological variability and test-related traits such as test composition, test height and breadth, and presence of gas vacuoles that support movement in the water column (Ogden, 1991), were the most responsive to environmental changes (Arrieira et al., 2016; Schwind et al., 2016a,b).

Marginal Marine Environments

Salt marsh testate amoebae are present both in brackish and intertidal zones next to diatoms and foraminifera (Riveiros et al., 2007; Barnett et al., 2017b), and have been shown to be important sea-level indicators (Patterson et al., 1985; Charman et al., 1998; Roe et al., 2002; Barnett et al., 2016, 2017a,b). Testate amoebae are strongly correlated with elevation and flooding duration and frequency in salt marsh environments (Charman et al., 1998; Roe et al., 2002). When it comes to distribution of salt marsh testate amoebae, especially important factors are salinity, particle size and organic matter content that vary locally (Charman et al., 2002; Ooms et al., 2011). Idiosomic tests typically dominate in high saltmarsh habitats above the mean high water mark and in supra-tidal settings, while in mid marsh settings low diversity agglutinating assemblages are observed (Gehrels et al., 2001).

Another very diverse group of testate amoebae are the interstitial testate amoebae typical of sandy saline freshwater and marine habitats. Because of their relatively late discovery and scarce subsequent investigation, little is known about their adaptive strategies for inhabiting marine and psammal supralittoral marine environments (Golemansky, 1969, 1978, 1998; Chardez, 1971, 1980; Golemansky and Ogden, 1980). The most characteristic morphological feature characterizing many interstitial testate amoeba taxa is the presence of a collar, which can be either small (e.g., *Centropyxiella lucida* Golemansky 1971) or very large, having a similar diameter as the maximum breadth of the test [e.g., *Messemvriella filosa* Golemansky, 1973, *Pseudocorythion acutum* Wailes 1927, *Ogdeniella elegans* (Golemansky 1970), *O. maxima* (Golemansky 1970), *Chardezia caudata* Golemansky 1970, *Corythionella* sp., *Micramphora pontica* Valkanov 1970] (Golemansky and Todorov, 2004, 2006, 2007). The molecular variability within this group of species is unknown (Todorov et al., 2009; Heger et al., 2011). As a result, the inferred general taxonomic relationships between these species are primarily based on species morphology (Golemansky, 2008).

DESCRIPTION OF KEY TESTATE AMOEBA TRAITS FOR PALEOECOLOGY

Although testate amoeba traits have only recently been used in paleoecology, several functional traits have been found to be particularly useful for paleoecological reconstructions, while others have yet to be tested in long-term studies (**Table 1**).

Key Testate Amoeba Traits for Paleoecological Studies of Peatlands

Mixotrophy

In both observational and experimental studies mixotrophic testate amoebae (e.g., *H. papilio, A. flavum, Amphitrema wrightianum* Archer, 1869*, Heleopera sphagni* Leidy, 1874*, Placocista spinosa* Penard, 1899) have been found to be useful for analysis of both wet and open, highly acidic *Sphagnum*-dominated peatlands (i.e., high light intensity, landscape openness and high water table; Schönborn, 1965; Lamentowicz and Mitchell, 2005; Mazei and Tsyganov, 2006). In these studies the appearance of mixotrophic taxa in a peat sequence has been interpreted to be indicative of either development of an opening of the landscape due to forest decline or deforestation, especially in small sites surrounded by forest cover. When peatlands develop over a siliceous impermeable bedrock following deforestation there is often a rise in the peatland water table due to increased surface runoff (Warner et al., 1989; Sillasoo et al., 2007). Such changes generally lead to higher abundances of mixotrophs due to both the higher water table changes, and an increase in solar radiation resulting from the disappearance of the forest canopy. The opposite case (i.e., the disappearance of mixotrophs in peat profiles) may indicate a lowering of the water table and/or tree encroachment and afforestation of the peatland. Such information is highly relevant for the interpretation of long-term changes in testate amoeba communities and has implications for land-use management (Davis and Wilkinson, 2004). In this way, testate amoebae provide not only water table depth changes information, but can also enhance our understanding of other paleoecological processes that took place in the studied site in the past.

Several previous paleoecological studies have examined long-term changes in the abundance of mixotrophic testate amoeba taxa in peat profiles, with the interpretation of the micropaleontological data being supported by multi-proxy biotic and abiotic evidence. This body of research includes research from Polish *Sphagnum*-dominated peatlands where it was determined that increased human peatland disturbance (e.g., deforestation, prescribed burns, peat extraction, establishment of drainage ditches) led to water table lowering; the effect on testate amoeba communities resulted in a rapid decline of mixotrophs (Lamentowicz et al., 2009b; Marcisz et al., 2015, 2016, 2019; Kołaczek et al., 2018). When no evidence of direct human peatland disturbance was observed, stratigraphic sections from an open bog were characterized by continuous *Sphagnum* growth and an associated stable hydrology, as indicated by the regular presence of mixotrophs within observed testate amoeba communities (Marcisz et al., 2020). As mixotrophs are the first functional group to respond to direct disturbances connected with either water table lowering or increased shading, it can be assumed that a loss of this trait from species in the testate amoeba community is an indicator of both water table lowering and peatland openness. A recent study by Zhang et al. (2020b) investigated peat cores from boreal bogs in Finland and compared paleoecological data with instrumental meteorological measurements. The authors confirmed that drying and associated lower carbon accumulation rates in the peatland were related to significant loss of mixotrophic testate amoebae (Zhang et al., 2020b). In another study, van Bellen et al. (2018) showed that the establishment of permafrost conditions in an ombrotrophic peatland in Canada coincided with the disappearance of mixotrophic testate amoebae. These researchers interpreted the of loss mixotrophs to be due to a significant reduction in peat accumulation, in conjunction with an input of dust on the surface of the peatland, which would impact food sources, giving heterotrophic testate amoebae a competitive advantage (van Bellen et al., 2018). These findings indicate that mixotrophs can easily outcompete heterotrophs under acidic peatland conditions by relying on photosynthetic symbionts for supplemental nutrition when prey abundance is scarce, whereas under nutrient-rich conditions heterotrophs have the competitive edge. The results of these studies indicate that tallying changes in the abundance of mixotrophic testate amoebae alone, or by comparing the ratio between mixotrophs and heterotrophs over time, provides a simple index of environmental changes integrating hydrology, landscape openness and carbon accumulation potential. These results provide further confirmation of just how interconnected functional traits and environmental drivers are, highlighting their potential in paleoecological reconstructions.

Position of Test Aperture

Louis Bonnet first demonstrated the clear relationship between apertural position and soil moisture conditions (see section "Soil and Other Wetland Habitats"). Apertural position is believed to have evolved as an adaptation of testate amoebae to variable soil moisture conditions (Lahr et al., 2019), and has been strongly correlated to both modern-day and reconstructed water table depths (Lamentowicz et al., 2015; Marcisz et al., 2016). Hydrological instability and the drying of peatlands was shown to lead to an increase in the abundance of testate amoebae with small plagiostomic apertures, which during dry seasons may prevent desiccation, and a decline in taxa with axial or acrostomic apertures (Lamentowicz et al., 2015; Marcisz et al., 2016). Lamentowicz et al. (2007, 2009a) showed that increased soil erosion after deforestation caused a *Sphagnum* bog to shift to a fen; a process which led to an increase in the relative abundance of the plagiostomic species *Centropyxis aerophila* Deflandre 1929 highlighting its possible adaptation to disturbed sandy habitats (**Figure 1**). Furthermore, a study covering the whole bog-to-fen gradient shows that hidden apertures are not only linked to lower water tables, but are also associated to higher mineral matter input (Lamentowicz et al., 2009a). These promising results illustrate the need for more studies covering the entire rich fen to bog gradient (see section "Fens").

Test and Aperture Size

Several observations and experiments have shown that the size of testate amoeba tests may be controlled by hydrological conditions, which suggests that species with small tests are probably better adapted to survive under dry conditions (see McKeown et al., 2019). Therefore, in peat cores, an observed shift in the dominant test size of testate amoebae may indicate a change in hydrology (or other relevant environmental factor), as confirmed by two paleoecological studies. An analysis of four peat records from North America and Europe revealed the co-existence of small and large testate amoebae during high water tables phases, but the abundance of larger species decreased after the water table declined (Fournier et al., 2015). A similar pattern was observed in two Central European peatlands, where the communities were dominated by small testate amoebae following direct human-induced impacts (Marcisz et al., 2016). Smaller testate amoeba species are characterized by smaller apertures, which may reduce desiccation, but because the tests are smaller in general makes them more prone to drying out. In terms of a peatland trophic status, a shift to smaller species expressed by a decrease in aperture size, body length and biovolume has also been linked to decreasing pH in *Sphagnum*-dominated peatland (Gałka et al., 2017). These observations indicate that interpreting the morphological adaptation of species to different microhabitats present on the surface of bogs can be complicated (i.e., hummock and hollows structure).

Test Type/Construction

Testate amoebae build their tests from various available material. The most commonly observed test types are those constructing: idiosomic tests produced using biosilica or calcite plates; organic-coated idiosomic tests; proteinaceous tests; and xenosomic (agglutinated) tests comprised of recycled organic or mineral material (Mitchell et al., 2008b). Several paleoecological studies have investigated whether changes in erosion processes or an increase in dust deposition translates into changes in the testate amoeba community structure. Increased input of mineral matter should stimulate the growth of species building idiosomic and xenosomic tests. Therefore, an increased abundance of these species in the stratigraphic record could indicate significant environmental change. Lamentowicz et al. (2009a) showed how vast deforestations led to increased catchment erosion, which increased the input of nutrients into the peatland, and led to a rapid increase in pH values from acidic to alkaline. This ecological shift caused a rapid decrease of testate amoebae possessing proteinaceous and organic-coated tests, and was directly linked to the subsequent appearance and dominance of *C. aerophila* and *Phryganella acropodia* (Hertwig et Lesser 1874), two species characterized by a xenosomic test (Lamentowicz et al., 2009a). A similar shift was described in another study based on a raised bog, where deforestation and severe disturbance by fire caused a temporal decrease in *Sphagnum* and an encroachment of vascular vegetation (Marcisz et al., 2019). In response to this environmental shift, testate amoebae with proteinaceous tests decreased in abundance in favor of those constructing tests utilizing xenosomes and organic-coated idiosomes (Marcisz et al., 2019).

Studies on dust deposition have significantly contributed to our understanding of testate amoeba trait variability over time. High levels of atmospheric dust deposition derived from southern Poland anthropogenic sources during the 1980s significantly impacted *Sphagnum* bog microbial communities (Fiałkiewicz-Kozieł et al., 2015). Testate amoebae with proteinaceous tests were observed to decrease and communities became dominated by xenosomic *C. aerophila* and *P. acropodia*. In addition, Al, Ti, Cr, Ni, and Cu concentrations were found to be highly correlated with aperture position/body size ratio and aperture position (Fiałkiewicz-Kozieł et al., 2015). In contrast, van Bellen et al. (2018) did not find any relationship between dust flux and the relative abundance of agglutinating testate amoebae. They hypothesized that the mineral particles typically being transported to mires were too large for testate amoeba to incorporate into their tests (van Bellen et al., 2018). In contrast, another study found that testate amoebae incorporated volcanic dust into their tests based solely on particle size with little or no selection by mineral type (Delaine et al., 2016). These results suggest that changes in test type and construction can therefore be used as an additional anthropogenic indicator, providing environmental information related to deforestation, human-induced changes in trophic status, dust deposition from anthropogenic industrial sources, as well as influences related to natural events (e.g., volcanic eruptions). As generation times of testate amoebae are short, their response to external factors influencing grain size input is much faster than that of vegetation (be it local bryophytes and vascular plants, or the surrounding upland vegetation), adding to their value as indicators of both anthropogenic and natural grain-size related inputs.

Key Testate Amoeba Traits for Paleoecological Studies of Lakes

Test Type/Construction

Lake testate amoeba communities are overwhelmingly dominated by agglutinated species, although proteinaceous forms (notably *Arcella* species) are commonly observed. *Arcella vulgaris* Ehrenberg, 1830, for example, is a key component of ’stressed’ assemblages associated with low oxygen and/or low pH conditions, and sediments strongly impacted by contaminants (e.g., heavy metals, winter de-icing salts; Dalby et al., 2000; Neville et al., 2011; Roe and Patterson, 2014; **Figure 3**). Idiosomic species, in contrast, typically form a minor assemblage constituent and are sometimes absent from paleolimnological records. This may partly reflect preparation techniques that result in the removal of smaller tests (notably the use of coarse sieve meshes to remove mineral content, but see Wall et al., 2010a,b), or preservation issues (Roe and Patterson, 2006). Some lake taxa exhibit a composite wall structure that may reflect changes in the availability of mineral material for test construction. *Cucurbitella tricuspis* (Carter, 1856), for example, displays complex changes in test wall composition related to life-cycle stage, switching from a dominance of xenosomic to idiosomic test construction between benthic and pelagic phases (Schönborn, 1962; Medioli et al., 1987; Meisterfeld, 1991). This transition is thought to be controlled by the ingestion of lipids from filamentous green algae, which aids buoyancy (Schönborn, 1962; Medioli et al., 1987). Given the significance of this species as an eutrophic indicator taxon, understanding the temporal controls on test composition (along with any associated changes in morphology) will continue to be important in paleolimnological studies (Prentice, 2019).

Test Morphology

Lake Arcellinida display enormous variability in test morphology, and there has been growing interest in assessing whether this is an adaptive response to aquatic conditions. Velho et al. (2003), for example, noted that in the floodplain environments of the Upper Paraná River, Brazil, species with a flattened (compressed) or an elongated test were most abundant in lotic (moving water) habitats associated with higher values of dissolved oxygen and pH, while spherical and hemispherical taxa were most abundant in lentic (still water) environments with greater concentrations of chlorophyll *a*. In this study and others, compressed morphologies, along with the presence of gas vacuoles (as observed, for example, in *Arcella* species) were hypothesized to minimize resistance to water, allowing floating and movement in the water column (Ogden, 1991; Velho et al., 2003; Arrieira et al., 2015, 2016).

Many shape-related observations have been made in macrophyte-enriched lakes. Lansac-Tôha et al. (2014) observed higher frequencies of spherical and elongated taxa in floodplain lakes rich in aquatic macrophytes, and a greater abundance of elongate species in sedimentary floodplain habitats. Macumber et al. (this issue) similarly noted that spherical and ovoid shape groups increased in sediments impacted by cultural eutrophication in a well-studied core from a large, shallow Scottish lake, while elongated taxa were dominant in sediments with oligo- to mesotrophic plant macrofossil assemblages (Salgado et al., 2010). Intra-species variations in morphology were also observed as the nutrient status of the lake changed. The functional diversity of floodplain testate amoeba communities in the Upper Paraná River, Brazil was also reported to be controlled by nutrient dynamics (Arrieira et al., 2015). These studies hold considerable promise, although it is likely that in macrophyte-dominated lake settings, testate amoeba communities are influenced by multiple drivers, including light and oxygen availability, substrate characteristics and the character of microbial food webs, as well as nutrient fluxes (Wiik et al., 2015; Prentice et al., 2018; Roe et al., unpublished data). Future work to elucidate traits in paleolimnological records must consider these complex, inter-related controls.

In addition to overall test morphology (i.e., test outline), the presence of basal processes (spines), or aperture-related features (e.g., serrated collars or teeth) have long been thought to play a functional role in lake Arcellinida (e.g., Penard, 1902). Spines may act to stabilize the organism during feeding or aid in the entrapment of prey (Han et al., 2008) and may provide protection against predation (Gomaa et al., 2015), while teeth may be used as a cutting tool to perforate algal membranes (Medioli et al., 1987).

Test and Aperture Size

Amongst the lake Arcellinida studies that have considered the significance of test size (or biovolume), a study of the assemblages characterizing thermokarst lakes on Richard’s Island in the Canadian Arctic is noteworthy (Dallimore et al., 2000). They reported tests which were larger than those typically observed in other studies, including ‘giant’ specimens which were 2–3 times greater in size, and suggested that this might reflect low ambient temperatures and a short growing season. They hypothesized that the organisms might feed and grow for a period of time, reaching a large size, then wait for a few days to weeks to reproduce under optimal conditions, giving rise to exceptionally large test dimensions (Dallimore et al., 2000). Macumber et al. (this issue) also reported a decrease in centroid size (biovolume) and changes in aperture size in response to nutrient enrichment and lake level lowering in the core study in Scotland. As the aperture provides the organism’s main interaction with the environment, variations in its size are likely to be adaptive (Schönborn, 1962; Medioli et al., 1987; Collins et al., 1990; Bobrov and Mazei, 2004), although further systematic analysis is required to assess this, and the relationship with other traits.

Mixotrophy

The significance of mixotrophy in lake testate amoeba communities is poorly understood. This partly reflects a lack of understanding of the distribution of algal symbionts in limnological taxa. Feeding strategies/mixotrophy could nevertheless be important predictors in the structure of lake communities. Penard (1902) noted that mixotrophic testate amoebae can tolerate dysoxic conditions and lack of nutrition longer than non-mixotrophic specimens and hypothesized that it was due to the presence of zoochlorellae. Interestingly, Macumber et al. (2020) have recently demonstrated that mixotrophy and morphology are related within selected Difflugiidae clades, and this may also hold phylogenetic significance.

Trait-Based Transfer Function Model

In the past transfer function models have been typically based on testate amoeba species assemblage data, which are now routinely used to reconstruct past depth-to-water table (DWT) in long-term studies worldwide (Charman, 1997; Mitchell et al., 1999; Booth, 2002; Payne and Mitchell, 2007; Lamentowicz et al., 2008; Amesbury et al., 2016). A novel trait-based transfer function was recently developed by van Bellen et al. (2017) using a previously published training set (van Bellen et al., 2014), and including new measurements of several functional traits: test biovolume and compression, aperture size and position, mixotrophy, phylogenetic group, and mineral matter presence. This approach made it possible to include traits of all observed taxa, including rare ones, which is unusual in most species-based transfer function models, where rare taxa are excluded (van Bellen et al., 2017). Both the species-based and trait-based DWT reconstructions provided similar reconstructions, confirming the relevance of the trait-based approach in paleoecological reconstructions. In particular, trait-based transfer functions can be useful in regions where classical transfer function models are absent, or when an existing training set does not include a large number of taxa.

CONCLUSION, KNOWLEDGE GAPS AND PERSPECTIVES FOR THE USE OF TESTATE AMOEBA TRAITS IN PALEOECOLOGY

The development of testate amoeba functional traits as an area of active investigation has opened novel research avenues by providing a powerful tool for future ecological and paleoecological research. Their application can bridge long-term (paleoecological) and short-term (observational and experimental) studies addressing various aspects of not only testate amoebae ecology, but also microbial functionality and related ecosystem processes from all ecosystems where testate amoebae occur. Trait analysis can deliver important additional information that can be used to enhance that gleaned from species and community data, informing sounder paleoecological inferences.

Based on the current level of knowledge, testate amoeba morphological and functional traits can specifically provide important information on regime shifts and tipping points in ecosystem functioning caused by both natural and anthropogenic processes (Domínguez-García et al., 2019; Lamentowicz et al., 2019). Examples of systems where ecosystem functions and traits provide important monitoring data include:

- detection of forest decline, anthropogenic deforestation and landscape opening (mixotrophy);
- support for inferences of hydrological changes (i.e., dry shifts detection; mixotrophy, test size, aperture size and position);
- detection of increased nutrient deposition within a sedimentary basin (test type/construction);
- identification of autogenous environmental changes such as fen-to-bog transition (test and aperture size);
- identification of natural and anthropogenic dust deposition (test type/construction);
- assessment of carbon accumulation potential (mixotrophy);
- recognition of changes within microbial food web structures (proportion between mixotrophs and heterotrophs, L/F index).

Testate amoeba trait patterns observed in contrasting habitats show that functional traits can be a useful measure when comparing ecological shifts observed in different habitats and ecosystems where species composition may vary, but similar traits may be present.

Promising as this line of research has proven thus far, a trait-based approach applied to testate amoebae still requires substantial development in the research subdisciplines of paleoecology, as well as in observational and experimental ecology. The most significant knowledge gaps relate to the functional significance of specific traits related to testate amoeba communities, relevant biotic and abiotic characteristics in the environment, and ecosystem functioning. Many of these relationships can be inferred statistically (e.g., the correlation between peatland carbon accumulation rates and the relative abundance of mixotrophs; Zhang et al., 2020b).

Another important research question relates to the definition of response vs. effect traits. Distinguishing the two is not always obvious and in some cases, a trait can belong to both categories. An example of the latter is mixotrophy, which for example in peatlands, both responds to moisture and openness, and contributes to carbon sequestration.

In this review an outline of the current theoretical and empirical state of research of a functional traits approach applied to testate amoebae has been presented. Although this field of research is still in its infancy it presents great promise as an important tool in future ecological and paleoecological studies. We look forward to seeing its development in the near future!

AUTHOR CONTRIBUTIONS

KM led the study. All the authors contributed to the review with discussions, ideas, photos, diagrams, comments, and text.

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Freshwater Testate Amoebae (Arcellinida) Response to Eutrophication as Revealed by Test Size and Shape Indices

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We review the potential for applying traits-based approaches to freshwater testate amoeba, a diverse protist group that are abundant in lakes and are valuable ecological indicators. We investigated the efficacy of geometric morphometric analysis to define Arcellinida test size and shape indices that could summarize freshwater testate amoeba community dynamics along a temporal gradient of eutrophication in Loch Leven, Scotland (United Kingdom). A cluster analysis of test size and shape indices yielded three clusters, each dominated by a single shape: elongate, spherical and ovoid. When plotted stratigraphically, we observed increases in spherical tests, decreases in elongate tests and shrinking of test size coeval with eutrophication in Loch Leven. Decreases in the elongate cluster may reflect benthic conditions with reduced oxygen levels, while increases in the spherical cluster are likely associated with an expanding macrophyte community that promoted pelagic and epibiotic life habits. Shrinking of test size may be a stress response to eutrophication and/or warming temperatures. Tracking community dynamics using test size and shape indices was found to be as effective as using species-based approaches to summarize key palaeolimnological changes, with the added benefits of being free from taxonomic bias and error. The approach thus shows significant potential for future studies of aquatic community change in nutrient impacted lakes.

Keywords: Arcellinida, ecology, lake, morphometrics, paleolimnology, Loch Leven, land-use change

INTRODUCTION

The ever-increasing impact of humans on their environment via urbanization, industrialization and climate change has led to an 80% decline in freshwater biodiversity in the last 50 years (Grooten and Almond, 2018). This emphasizes the need to model the trajectory of impacted natural freshwater systems. Lakes being complex natural systems are best understood by characterizing multiple proxies representing various levels of biological organization and assessing how their functional interactions vary through time and space (Blois et al., 2013).

Testate amoeba are a diverse protist group that enclose their cell body within a test (i.e., a hard shell) that can be used to identify species and preserves well in sediments. They are valuable ecological indicators due to their (1) abundance in freshwater, moist soils and wetlands; (2) sensitivity to environmental conditions; (3) high preservation potential; and (4) rapid generation times (Patterson and Kumar, 2002; Mitchell et al., 2008). In lakes, lobose testate amoeba dominate (i.e., Arcellinida) and have been used as indicators for: lake acidity (Kumar and Patterson, 2000; Patterson et al., 2013); land-use change (Patterson et al., 2002); industrial impacts (Nasser et al., 2016); water quality (Roe et al., 2010); ecosystem health and seasonal environmental change (Neville et al., 2011); nutrient loading (Patterson et al., 2012; Prentice et al., 2018); and climate change (McCarthy et al., 1995; Boudreau et al., 2005), amongst other variables.

Arcellinida studies typically present community dynamics by tracking the abundances of multiple species. The expertise required to identify Arcellinida species and associated taxonomic error and bias limits the comparability of the results (Lamentowicz et al., 2015). Summaries of the ecological function of the group (e.g., life habits, metabolism), as tracked by functional traits, has the potential to offer a complementary approach and potentially provide more robust predictions that could translate across time and space (Blois et al., 2013; Lamentowicz et al., 2015; Marcisz et al., 2020). The success of using functional traits, rather than species abundances, to track community dynamics is demonstrated by studies of peatland testate amoeba where researchers have modeled the response to drier climates (Fournier et al., 2015), autogenous plant succession from lake to bog (Lamentowicz et al., 2015) and peatland disturbance (Jassey et al., 2016; Marcisz et al., 2016). van Bellen et al. (2017) also demonstrated that water table depth reconstructions (i.e., transfer functions) based on functional traits were comparable to species-based reconstructions. Unlike their peatland counterparts, the functional ecology of lake Arcellinida is poorly understood.

Based on four morphological traits (pseudopod characteristics, presence of gas vacuoles, test compression and test composition) Arrieira et al. (2015) observed that functional diversity of Arcellinida communities from the upper Paraná River floodplain, Brazil were controlled by nutrient dynamics. Lansac-Tôha et al. (2014) characterized habitats of Guaraná Lake, Brazil (i.e., epipelic, epibiotic, and planktic) based on the relative abundance of three Arcellinida test morphologies: elongate, spherical and hemispherical. These modern ecological studies highlight the promise of using Arcellinida functional traits to summarize their ecological function in lakes. One property that both studies share with species-based approaches is they all transform a continuous variable (i.e., test morphology) in to a discrete one (i.e., groupings and labels). Morphological variability was shown to be effectively continuous, with many transitional forms blurring the boundaries between morphotypes (Bobrov and Mazei, 2004). It is still uncertain whether this inherent morphological plasticity (i.e., phenotypic plasticity) is completely random (Wanner, 1999), an adaptive response to variable environmental conditions (Bobrov and Mazei, 2004) or perhaps species specific. Thus, there is value in capturing morphological variation as a continuous variable to preserve potential environmental signals present in the many transitional forms.

Geometric morphometric analysis has been applied successfully to characterize continuous shape variation in a number of biological and paleontological studies (Zelditch et al., 2012; Adams et al., 2013), including for other shelled microfossil groups (e.g., diatoms; Beszteri, 2005). Macumber et al. (2020) used geometric morphometric analysis to demonstrate that two novel clades of Arcellinida could be differentiated based on test size. Shapes are defined by the geometric configuration of landmarks (fixed anatomically definable locations found on all specimens) and by boundary curves drawn between the landmarks (Zelditch et al., 2012). Geometric morphometric analysis of similarly shaped specimens (i.e., Arcellinida tests) models a morphological space where each test (i.e., shape) occupies a unique place, providing continuous measures of morphological variability (Zelditch et al., 2012). A generalized Procrustes analysis removes variation due to differences in size and orientation, capturing size variation as an independent variable. Multivariate statistical methods can test for shape differences among groups, covariation between shape and other continuous variables and visualize patterns of shape variation (Adams et al., 2013).

As the functional ecology of lake Arcellinida is poorly understood there is value in using continuous measures of morphological variation (i.e., geometric morphometric analysis) to highlight important Arcellinida test traits that are associated with environmental change. Highlighted traits could form the basis of future functional trait studies of lake Arcellinida. We investigated a previously described Arcellinida assemblage from a lake sediment core collected from Loch Leven, Scotland, a large shallow lake which has a well documented history of nutrient enrichment and associated biological change (Salgado et al., 2010; Prentice et al., 2018). Our aims were to (1) characterize test morphological variability in response to environmental change, specifically lake eutrophication induced shifts in the macrophyte community; (2) test the efficacy of geometric morphometric analysis in summarizing community dynamics as compared to conventional paleoecological (i.e., species- and assemblage based) approaches; (3) identify inter- and intraspecific variability in relation to the observed changes in test size and morphology; and (4) make basic inferences regarding the role of shape and size in Arcellinida ecology.

MATERIALS AND METHODS

Study Site

Loch Leven situated in central Scotland (56°11′55′′ N, 3°22′46′′ W), is a large (13.3 km^2), shallow (mean = 3.9 m, max = 25.4 m), lowland (107 m.a.s.l) freshwater lake (**Figure 1**). It is moderately eutrophic, with mean annual average total phosphorus and chlorophyll *a* concentrations (2008–2010) of 33 and 21 μg L^{-1}, respectively (Dudley et al., 2012). There are four inflows sourcing

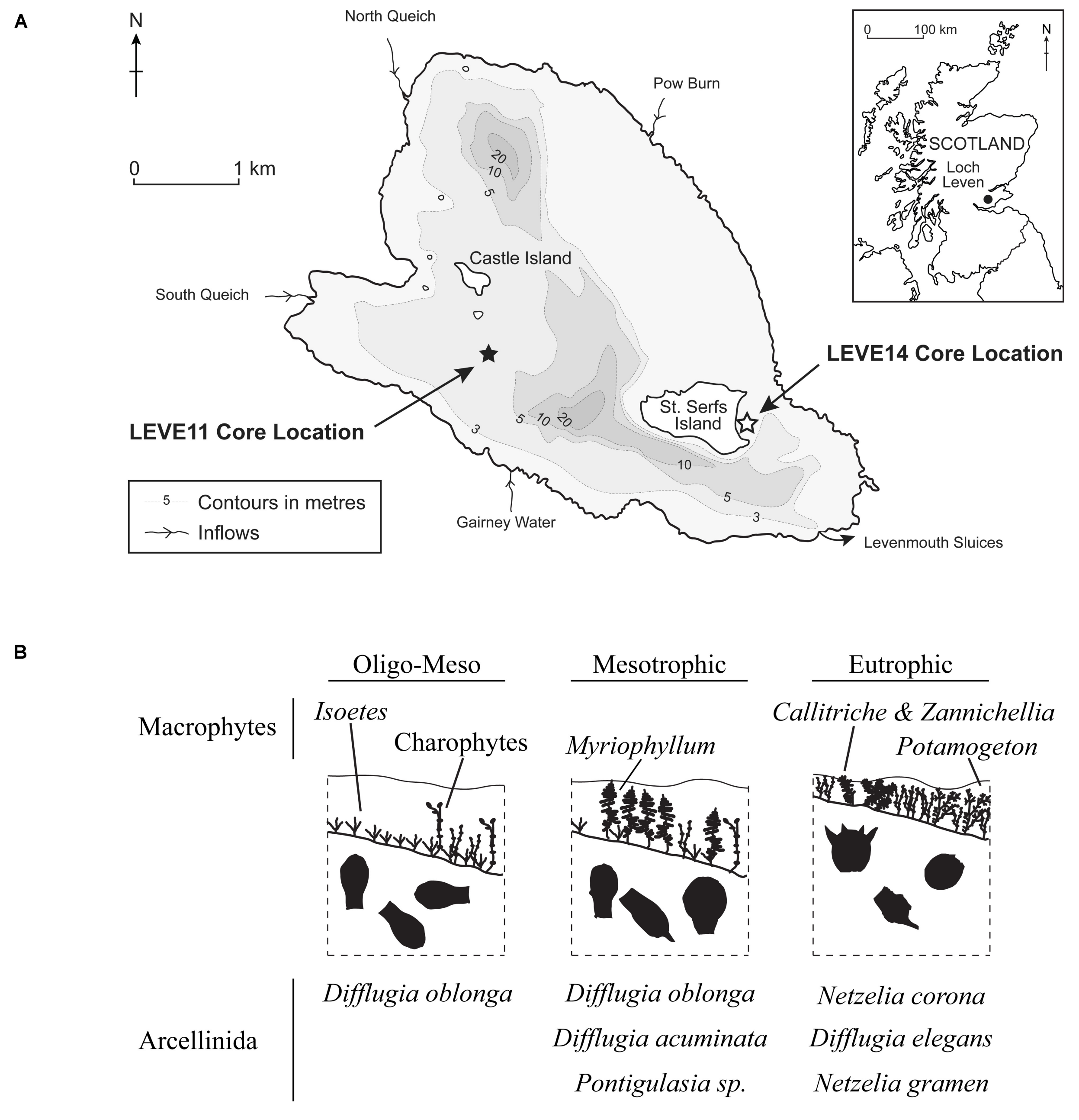

FIGURE 1 | (A) Map showing the location and bathymetry of Loch Leven and the locations of sediment cores from previous studies of plant macrofossils (LEVE14) (Salgado et al., 2010), testate amoebae (LEVE14) (Prentice et al., 2018) and diatoms (LEVE11) (Bennion et al., 2012). Core LEVE14 is used in this study. **(B)** Three phases of progressive nutrient enrichment as inferred from plant macrofossils (Salgado et al., 2010) and testate amoeba (Prentice et al., 2018).

water from farms, villages and the nearby town of Kinross (population ∼5000).

Core Collection

The sediment core LEVE14, previously studied by Salgado et al. (2010) and Prentice et al. (2018), was collected in shallow-water (depth = 2.2 m) using an adapted Livingstone (7.4 cm diameter) piston corer in May 2006 near the eastern shore of St. Serf's Island in the southeast corner of Loch Leven (**Figure 1**). The 141-cm core was extruded (i.e., subsampled) in the field at 1-cm intervals.

Chronology

The core was dated radiometrically (^{210}Pb, ^{226}Ra, and ^{137}Cs isotope analysis) using thirteen sediment samples from the top 60 cm (Salgado et al., 2010) using standard methods (Appleby and Oldfield, 1978). The ^{210}Pb chronology suggests a constant sedimentation rate from the late 1920s through the late 1990s

(0.29 cm year^{-1}), although this increased to 1.1 cm year^{-1} from the early 2000s (Salgado et al., 2010). Extrapolation of this sedimentation rate would give a basal date of AD 1492 (Salgado et al., 2010).

To extend the age depth model, Prentice et al. (2018) obtained three samples for AMS ^{14}C radiocarbon dating: bulk sediment from 68 to 69 cm (1882 ± 37 year BP), a charcoal sample from 96 to 97 cm (827 ± 34 year BP) and bulk sediment from 134 to 135 cm (2420 ± 34 year BP). The sedimentation rate based on the charcoal date (0.11 cm year^{-1}) estimates a basal date of AD 828. Unfortunately, the AMS ^{14}C dates do not correspond well with the extrapolated ^{210}Pb chronology (see Prentice et al., 2018 for further discussion). Interpretation of the ages is further compounded by a lithostratigraphic change between 120 and 90 cm. Thus, estimating ages for depths greater than 90 cm in the core is contentious, but the basal part of the record is likely older than inferred by the ^{210}Pb age-depth model.

Lithology

Fifty one samples were analyzed for loss-on-ignition (LOI) by Salgado et al. (2010) and an additional eighteen samples by Prentice et al. (2018), providing the relative abundance of organic carbon, inorganic carbon and unignitable minerogenic matter (Dean, 1974). Prentice et al. (2018) completed particle size analysis (% sand, silt, clay) of forty-two samples determined by laser diffraction using a Malvern Mastersizer-2000.

Study Design

Geometric morphometric analysis of Arcellinida tests requires more analytical time than conventional Arcellinida enumeration, thus our study was limited to a subset of the 42 Arcellinida samples counted from the LEVE14 core by Prentice et al. (2018). In order to examine morphological variability at key points of change in the sediment history of Loch Leven we targeted core depths immediately above and below CONISS zone boundaries (Grimm, 1987) defined by Prentice et al. (2018). We iteratively compared the CONISS zones based on sample subsets to the zones identified by Prentice et al. (2018) and continued to increase the number of samples in our subset until both agreed. This occurred with a subset of 14 samples (see **Supplementary Table S1** and **Supplementary Figure S1**).

Arcellinida Identification

We enumerated Arcellinida tests from uncounted subsamples from core LEVE14 interval splits [see Prentice et al. (2018) for details of subsample preparation]. Arcellinida tests were quantified in aqueous solution using a Nikon light dissection binocular microscope at x50-93 magnification with reference to various identification keys and published images (**Supplementary Table S2**). Our taxonomic approach differs from Prentice et al. (2018) who employed a strain-based taxonomic approach (Patterson and Kumar, 2002). All specimens in our study were matched with a previously described species; this resulted in some inconsistencies and highlights some of the difficulties with species-based approaches (**Supplementary Table S2**). We identified individual specimens until our total counts were enough to pass the inflection points of the accumulation curves (see **Supplementary Figure S1**).

Geometric Morphometric Analysis

We used a modified geometric morphometric protocol based on MacLeod (2008) and Zelditch et al. (2012) to capture shape and size indices for the Arcellinida tests. Geometric morphometric analysis creates a shape space in which the morphology of each specimen in a dataset is represented as a unique point and every other point in the shape space represents a hypothetical transitional morphology. Trends in shape variation are reduced to principal components [via principal component analysis (PCA)], the scores from which can be illustrated as hypothetical shape models. It should be noted that because landmarks need to be consistently placed on each specimen (see **Figure 2**) only similarly shaped specimens could be included in the dataset. We therefore focused on Arcellinida species whose test have terminal apertures, and tests that could not be oriented in a consistent manner (e.g., Centropyxidae taxa which are characterized by angled apertures and a random distributions of spines) were excluded from the study.

Arcellinida tests (n = 1505) were photographed in aqueous solution using a digital camera mounted to a Nikon binocular dissecting light microscope at x93 magnification. Images were digitized using the tps series of software (Rohlf, 2015). We chose three landmark locations on the outer wall of each specimen that could be consistently identified: (1) at the furthest point of the fundus away from the aperture, (2) the left edge of the aperture and (3) the right edge of the aperture (**Figure 2**). Semilandmarks can be anchored and equally spaced between landmarks and are ideal for tracking curves. Two sets of thirteen equally spaced semilandmarks were anchored between landmarks 1 and 2 and landmarks 1 and 3 (**Figure 2**). The combination of landmarks and semilandmarks were used to quantify variability related to test vertical elongation, horizontal widening and tapering.

Statistical Analysis

To capture size indices for the Arcellinida tests we carried out a Procrustes superimposition on the landmark and semilandmark coordinates (**Figure 2**) using sliders to align all specimens and isolate size variation as an independent variable (Zelditch et al., 2012; Rohlf, 2015). Size variation was quantified as the square root of the non-weighted sum of squared distances from the joint centroid (MacLeod, 2008).

To capture shape indices for the Arcellinida tests we modeled the Arcellinida test shape space through a PCA of the Procrustes superimposed landmarks using the "geomorph" R-package (Adams and Otarola-Castillo, 2013). We excluded all principal components that described less than 5% of the variance as this was less than the error of geometric morphometric analysis (see below).

We used a Procrustes ANOVA to characterize error and assess the repeatability of our imaging and landmark placement (Fruciano, 2016). We used Arcellinida tests isolated from a subsample at 80 cm core depth as our test group following the protocol illustrated in **Supplementary Figure S2**. To quantify

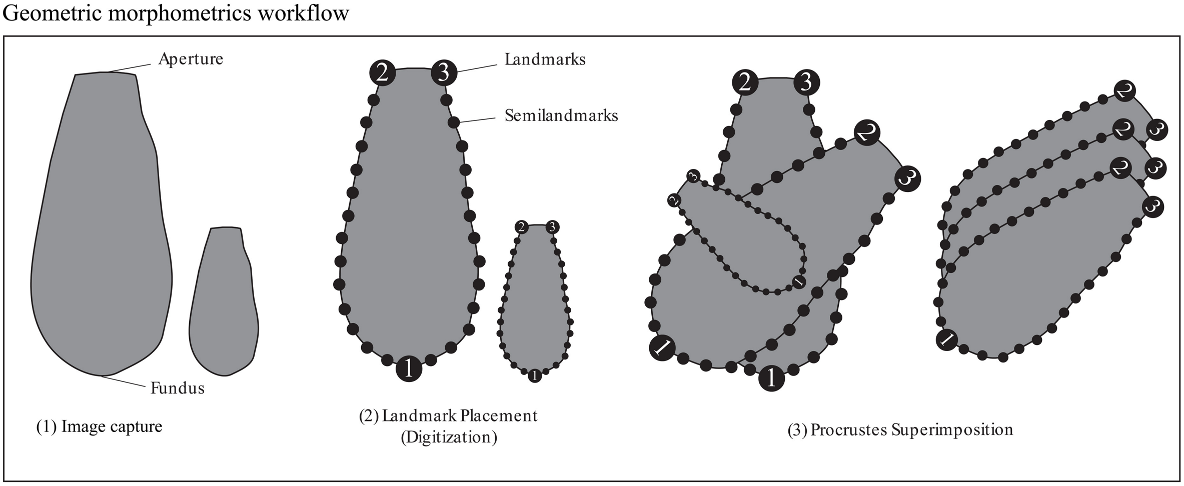

FIGURE 2 | Figure illustrating placement of landmarks and semilandmarks for the geometric morphometric analyses Procrustes-PCA and Aperture-PCA. The line drawings are not to scale.

the repeatability of our imaging method we compared the mean square error between image replicates (**Supplementary Figure S2** and **Supplementary Table S3**). To quantify the repeatability of our landmark placement we compared the mean square error between landmark placement replicates (**Supplementary Figure S2** and **Supplementary Table S3**). Both the imaging and landmark placement had repeatability measures greater than 95%.

To summarize the principal components of the Procrustes PCA into groupings based on shape indices, we visualized significant breaks in Procrustes principal component 1 and 2 scores using a Wards cluster analysis implemented with the "ward.D" method in the hclust function in the stats package (R Core Team, 2016). Before cluster analysis we created an Euclidean distance matrix using the vegdist function in the vegan package in R (Oksanen et al., 2017). Following Borcard et al. (2011), we constrained the number of clusters using the Rousseeuw quality index and the Mantel statistic. To compare within and between species trait variation (i.e., centroid size, PC1 and PC2 scores) we grouped specimens by species and displayed variance in their trait values as boxplots.

We defined temporal communities represented by the CONISS zones as defined by Prentice et al. (2018): Zone 1 (141–94 cm), Zone 2 (94–41 cm), Zone 3 (41–3 cm), and Zone 4 (3–0 cm). Using these temporal communities, we can investigate how shape (PC1 and PC2 scores) and size (centroid size) indices vary in response to eutrophication in a freshwater lake (Salgado et al., 2010; Prentice et al., 2018). To understand the contribution of intraspecific trait variance to the total within-community trait variance (sum of interspecific and intraspecific trait variance), we calculated the relative contribution of intraspecific trait variance to within-community trait variance as described by Siefert et al. (2015). To compare Arcellinida test shape (i.e., median PC1 scores) and size (i.e., median centroid size) to Arcellinida species dynamics (i.e., over time) we used a series of biplots, each representing specimen values from a temporal community representing different environmental conditions.

RESULTS

Temporal Communities and Environmental Conditions

The palaeolimnological studies of Salgado et al. (2010) and Prentice et al. (2018) which utilized plant macrofossils and Arcellinida, respectively, identified three main long-term phases of ecological change in the Loch Leven (LEVE14) core in response to gradual eutrophication: (1) an early phase of oligo-mesotrophic conditions, (2) an intermediate phase of mesotrophic conditions which began after circa 1200 AD, and (3) an upper eutrophic phase. Prentice et al. (2018) observed a fourth phase in the testate amoeba assemblage captured in the uppermost two samples (0–3 cm). They observed a significant drop in species diversity with a coeval increase in test concentrations (tests/cc). We adopt the four phases (or zones) defined by Prentice et al. (2018) through CONISS analysis of the plant macrofossils and Arcellinida results. The four phases demarcate temporal communities each associated with different environmental conditions and hereafter referred to as Zones 1 to 4.

The sediments of Zone 1 (141–94 cm) are characterized by increasing sand content and decreasing organic matter (LOI = 1–11%) toward Zone 2. The oligo-mesotrophic conditions are characterized by tests of *Difflugia oblonga* Ehrenberg 1838 (Prentice et al., 2018) and isoetid (e.g., *Isoetes lacustris*) macrophytes (Salgado et al., 2010). Zone 2 (94–41 cm) is characterized by an increase in organic matter and decreased sand content in comparison to Zone 1. The mesotrophic conditions are characterized by a greater diversity in the Arcellinida assemblage with a decreasing trend in *D. oblonga* (Prentice et al., 2018) and a reduction in isoetids

(Salgado et al., 2010). The sediments of Zone 3 (41–3 cm) become less sandy and continue to increase in organics toward Zone 4. The eutrophic conditions are characterized by tests of *Cucurbitella tricuspis* (Carter 1856) and *Mediolus corona* (Wallich 1864) Patterson et al., 2014 (i.e., *Netzelia* sp. see **Supplementary Table S2**), the disappearance of isoetid plants, and the proliferation of elodeid (e.g., *Zannichellia palustris*, *Potamogeton pusillus*) macrophytes. There is no change in the lithology for Zone 4 (3–0 cm) but there is a 15-fold increase in Arcellinida test concentrations compared to Zone 3 and Arcellinida diversity decreased substantially.

Geometric Morphometric Analysis

The Procrustes PCA yielded two axes of shape variation (**Supplementary Table S4**): principal component one (PC1) explained 72.3% and PC2 explained 13.5% of the test shape variation in the Loch Leven Arcellinida microfossil assemblages. We kept the first two principal components (PC1 and PC2) as all other principal components explained less than 5% of the shape variation, less than methodological error (**Supplementary Table S3, S4**).

Shape change in the Arcellinida tests along PC1 (**Figure 3**) represents the gradual transition from elongated specimens (negative values) to spherical specimens (positive values) as illustrated by the end-members found to the left (negative end member) and right (positive end member) of the panels in **Figure 3**. Important modes of change include compression of the vertical axis, widening of the horizontal axis, and the loss of a distinctive neck (**Figure 3**). Based on PC1 scores, the basal process-type taxa could be divided into an elongate-type (i.e., *Difflugia protaeiformis* Lamarck 1816 and *Difflugia acuminata* Ehrenberg, 1838) and the ovoid-type group (i.e., *Difflugia*

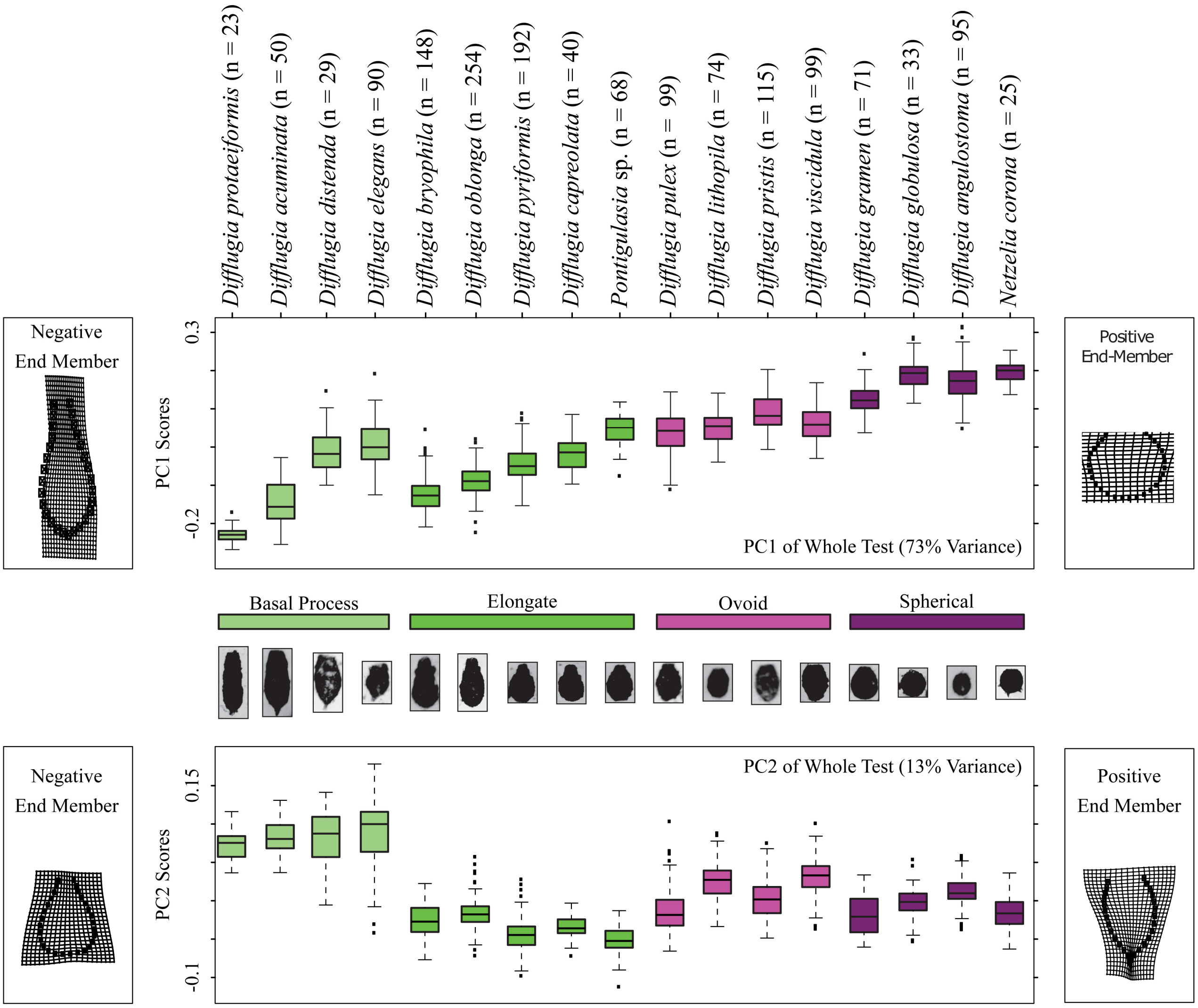

FIGURE 3 | Boxplots showing the distributions of Procrustes Principal Component Analysis axis 1 and 2 scores, top and bottom panels, respectively, for each taxon and color coded by shape group. The models on the left and right sides of the diagrams illustrate shape end-members on deformation grids representing the deviation in shape from the mean shape. The number of specimens analyzed for each taxon is shown.

distenda Ogden 1983 and *Difflugia elegans* Penard 1890). Shape change along PC2 varies between tests with wide bases and narrow apertures to tests with pointed bases and wide apertures, and effectively separates the basal process-type taxa from all other types.

Not all taxa can be distinguished based on shape alone. For example, *D. distenda* and *D. elegans* or *Difflugia lithophila* Penard 1902 and *Difflugia viscidula* Penard 1902 can only be differentiated based on centroid size (**Figure 4**). Several taxa, especially the elongate type but also *D. acuminata* and *Difflugia globulosa* Dujardin 1837, have wide test centroid size ranges. The elongate-type taxa [except *Difflugia bryophila* (Penard 1902) Jung 1942] also have the largest test centroid sizes, as do *D. protaeiformis*, *D. viscidula*, and *Netzelia corona* (Wallich 1864) Gomaa et al., 2017. Ovoid-type taxa have narrow ranges and apart from *D. viscidula* have low mean test centroid sizes (**Figure 4**).

Intraspecific Trait Variation (ITV)

For each Arcellinida test trait (centroid size, PC1, PC2 scores), we calculated the contribution of intraspecific trait variation (ITV) to the total within community trait variation (i.e., the sum of intraspecific and interspecific trait variation; **Figure 5**).

For the PC1 scores, ITV contributes relatively little variation to the total within community trait variation (median = 11.54%, mad = 0.01%), and this is true across all temporal communities. For the PC2 scores, ITV contribution is twice as large (median = 22.67%, mad = 0.01%) as compared to the PC1 scores, with the ITV in Zone 4 being significantly higher (mean = 38.36%) then all other communities that are clustered around the median.

When compared to the shape indices (i.e., PC1 and PC2), ITV was found to explain a greater amount of variation (median = 32.80%, mad = 10.92%) to total within community centroid size variation. In addition, the communities (i.e., Zone 1–4) form two groupings on either size of the median: Zones 1 and 4 (range = 20.52–27.29%) and Zones 2 and 3 (range = 38.32–42.01%).

Traits and Species Dynamics

Figure 6 displays the temporal communities (CONISS zones) as a series of biplots of Arcellinida species with median PC1

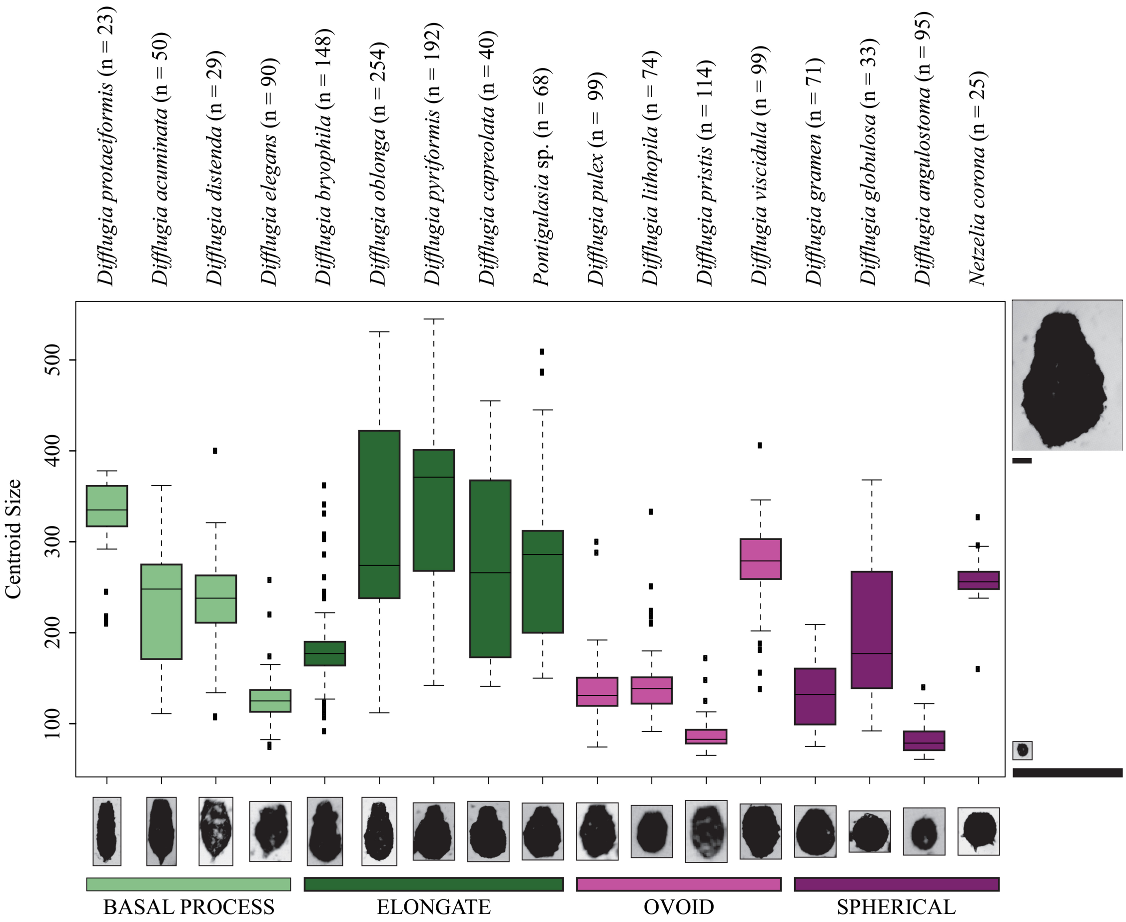

FIGURE 4 | Boxplots showing the centroid size ranges for Arcellinida taxa. Color represents shape groups. The number of specimens analyzed for each taxon is given beside the taxon name. The images to the left and right of the figure are to scale relative to one another demonstrating the difference between the maximum and minimum centroid size. The taxon images shown along the *x*-axis are not to scale.

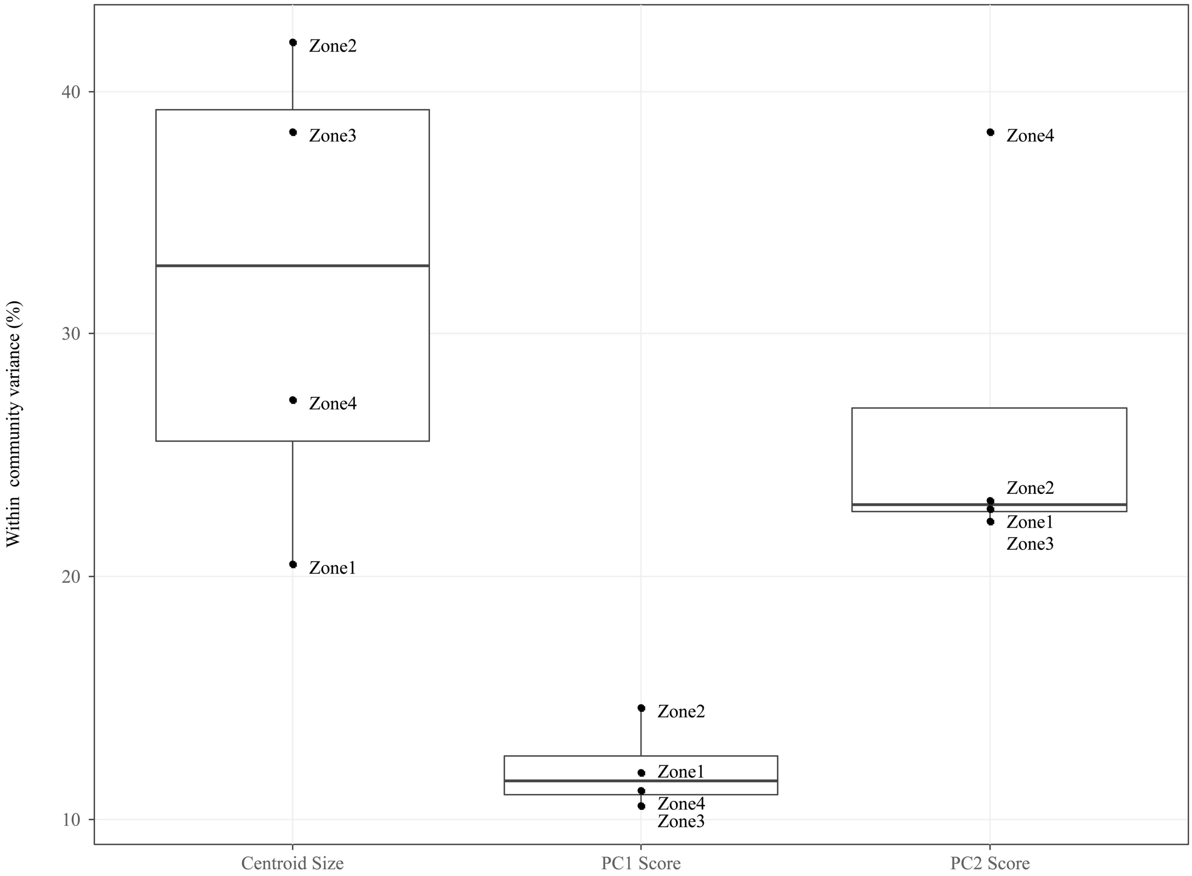

FIGURE 5 | Boxplots comparing the mean of within community (i.e., CONISS zones) intraspecific variance grouped by traits: Centroid size, PC1 Scores and PC2 scores. For each trait we measured the intraspecific variance for each species within a temporal community and took the mean value for all species.

score along the x-axis and median centroid size along the *y*-axis. Interestingly, between Zone 1 to Zone 4 the average community test shape changes from a dominance of elongate-ovoid to ovoid-spherical shape types, while the average test size decreases. The greatest range in both centroid size and test shape is found in Zone 3, followed by Zone 2. Zone 1 is restricted, as compared to Zone 2 and 3, in its shape range, while Zone 4, as compared to all other zones, is restricted in both shape and size.

There are several taxa that display relatively large changes in median centroid size (e.g., *Difflugia capreolata* Penard 1902, *D. distenda*, *D. globulosa* and *D. oblonga*) as compared to the other taxa. The communities differ in their size structure, with Zone 1 comprising two groupings, large (>300) and small tests (<200); in Zone 2 there is the development of a medium test size (200–300), increased representation of the small test size and decrease in the large test size; Zone 3 continues the trend seen in Zone 2, with a further decrease in large tests and an increase in the number of medium sized taxa; finally in Zone 4 both large and medium tests are not found and only small tests remain.

Cluster Analysis

Cluster analysis on the Procrustes PC1 and PC2 scores divided the dataset into three test shape clusters (**Figure 7**). Each cluster is dominated (and so named) by a shape-group (i.e., Elongate, Ovoid and Spherical). The cluster analysis further divides the basal process shape group between the Elongate (30%) and Ovoid (69%) clusters. This distinction of taxa based on a simplified morphological descriptor reliably represented the cluster assignment (e.g., elongate-type taxa were assigned to the Elongate cluster); however, several exceptions exist: *D. acuminata*, *D. capreolata*, *Netzelia gramen* (Penard 1902) Gomaa et al. (2017), *D. globulosa* and *Difflugia angulostoma* Gauthier-Lièvre and Thomas (1958) (**Figure 7**). Cluster analysis divides these taxa into several clusters.

Stratigraphic Variability

In **Figure 8** we display the stratigraphic variation of our test size and shape data alongside the species-based results of the Salgado et al. (2010) plant macrofossil and the Prentice et al. (2018) Arcellinida studies. Significantly, our size and shape indices display stratigraphic trends that correspond with the CONISS zone boundaries defined by the testate amoeba and plant macrofossil datasets, especially the initiations of Zones 3 and 4 (**Figure 8**). We observed four phases of test shape cluster change: (1) a stable phase comprising only the elongate and ovoid tests

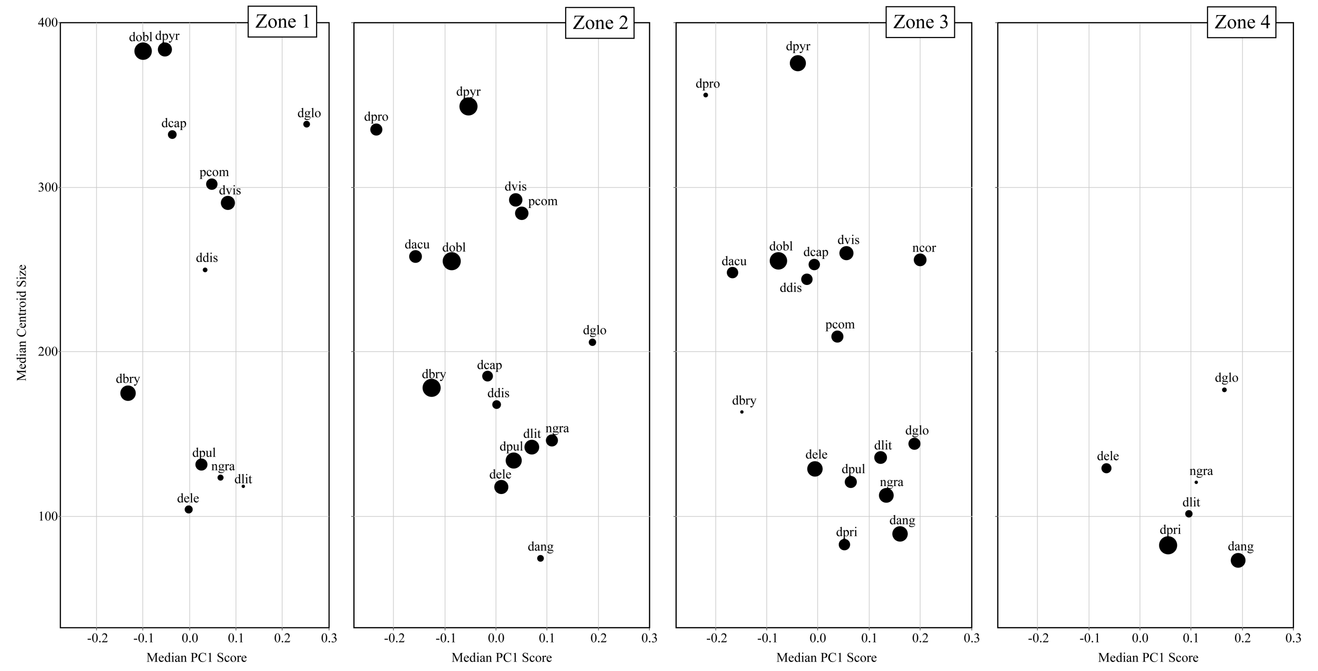

FIGURE 6 | A series of biplots comparing median centroid size to median PC1 scores for each taxa. Each biplot is a different temporal community (i.e., CONISS zones). The size of the dots is relative to species abundance with larger dots reflecting greater relative abundances.

through Zones 1 and 2; (2) the appearance of spherical tests at 40 cm (Zone 3); (3) the initiation of a decline in elongate tests at 36 cm; and (4) the disappearance of elongate tests above 6 cm (Zone 4). The median centroid size follows a similar stratigraphic pattern to the elongate tests, with a noticeable decrease in median centroid size above 6 cm.

DISCUSSION

The four phases of ecological change recorded in Loch Leven core LEVE14 in response to gradual eutrophication, as identified by the palaeolimnological studies of Salgado et al. (2010) and Prentice et al. (2018), provide an excellent framework to see whether geometric morphometric analysis applied to freshwater Arcellinida tests can identify functional traits which provide complementary summaries of ecological function without taxonomic expertise. In addition, assess the impact of intraspecific trait variability.

Pre-enrichment Phase (pre-1830)

Zone 1 (141–90 cm)

Salgado et al. (2010) inferred that the Zone 1 plant macrofossil assemblage of the LEVE14 sediment core was typical of a nutrient poor oligotrophic-mesotrophic lake, with very few dense stands of macrophytes and relatively deep and open water conditions (**Figure 1B**). The Arcellinida assemblage is comprised of mainly of elongate tests (60%; **Figures 7**, **8**). Elongate difflugids, are inferred to reflect a benthic life habit (Patterson and Kumar, 2002; Lansac-Tôha et al., 2014; Prentice et al., 2018) and in fact, their abundances have been observed to decrease as benthic conditions become stressed (e.g., in reduced oxygen environments, Roe et al., 2010).

Taxa in the ovoid cluster make up 30% of the Zone 1 community (**Figure 6**). Lansac-Tôha et al. (2014), in a study of living testate amoeba from different habitats of a shallow lake in Brazil, observed assemblages composed of "elongate" tests in benthic habitats, also in association with macrophyte habitats (i.e., epibiotic). Several of the taxa classified as "elongate" by Lansac-Tôha et al. (2014) are classified by our geometric morphometric analysis as ovoid (e.g., *D. elegans*; **Figure 7**). Thus, we infer that taxa of the ovoid cluster might represent the success of an epibiotic life habit (i.e., stands of macrophytes) in addition to an oxygenated benthic environment.

Zone 1 has the greatest median centroid size (**Figure 8**), with more species characterized by large tests (centroid size >200) than any other temporal community (**Figure 6**). This is typified by *Difflugia pyriformis* Perty 1849 a large elongate difflugid known to be mixotrophic (Leidy, 1879). Macumber et al. (2020) using high-resolution phylogenetics (NAD9-NAD7 genes) and geometric morphometrics demonstrated two novel clades of elongate type *Difflugia* species that could be distinguished based on test centroid size: a pyriform clade and a lanceolate clade. The "pyriform" clade was characterized by larger centroid sizes and members known to be mixotrophic, while the "lanceolate" clade had smaller test centroid sizes and only heterotrophic taxa. The larger average centroid size and the higher abundance of elongate type taxa recorded in Zone 1 could therefore reflect relatively higher abundances of mixotrophic as compared to heterotrophic taxa. The oligotrophic-mesotrophic conditions inferred for the

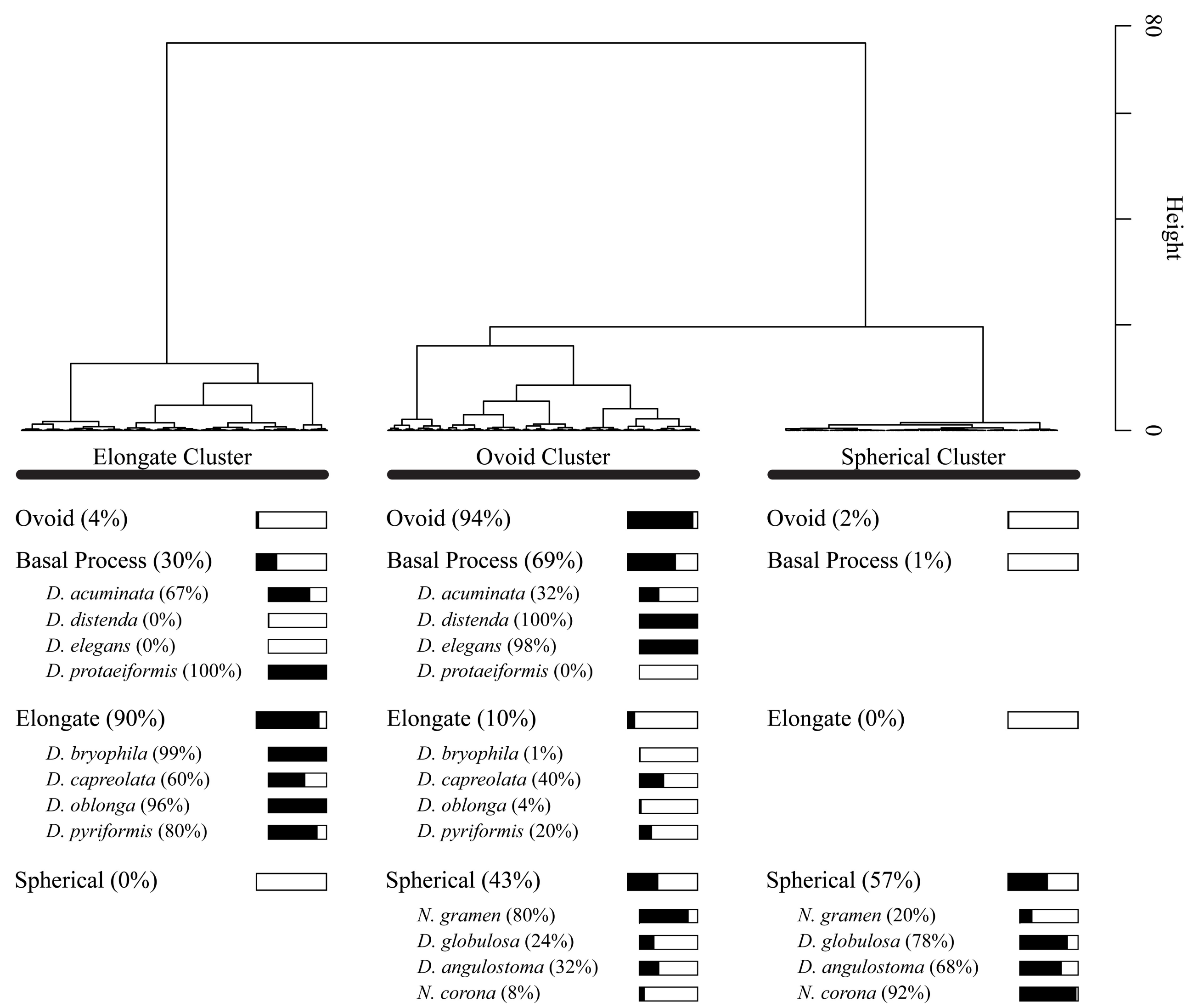

FIGURE 7 | Dendrogram showing Wards cluster analysis of Principal Component Analysis PC1 and PC2 scores for all 1503 specimens. For each shape group, we show what percentage of the specimens are present within the cluster groups. For example, 57% of specimens in the spherical shape group are found in the Spherical Cluster.

loch at this time would have promoted a benthic mixotrophic life habit and thus may well explain the presence of large elongate difflugids.

Zone 2 (90–41 cm)

From Zone 1 to Zone 2, macrofossils of the submerged taller growing macrophytes *Myriophyllum* and *Chara* sp., expanded coinciding with a reduction in isoetid plants. These changes were associated with increasing eutrophication of the lake, which was driven by expanding agricultural production in the catchment (Salgado et al., 2010). Denser stands of macrophytes would have provided increased habitat availability for epibiotic taxa such as members of the ovoid cluster. Accordingly, we observe an increase in the ovoid cluster (33 to 44%) as represented by increases in *D. elegans*, *Difflugia pulex* Penard 1902, and *D. lithophila*, with the appearance of *D. distenda* (**Figures 6**, **8**). This increase in ovoid taxa could also explain the coeval decrease in median centroid size (**Figure 8**), which is driven by a decrease in median size of several species, notably *D. oblonga*, *D. protaeiformis*, *D. pyriformis*, and *D. globulosa* (**Figure 6**). Such shifts in median centroid size for several species would also explain why Zone 2 has the greatest amount of intraspecific centroid size variation (**Figure 5**).

Water Level Lowering and Nutrient Enrichment Phase (1830–1985)

The 1830s lowering of Loch Leven by 1.5 m was coincident with increased catchment agriculture and was inferred by a sedimentological color change at 41 cm in the LEVE14 core (Salgado et al., 2010; **Figure 8**). Nutrient enrichment led

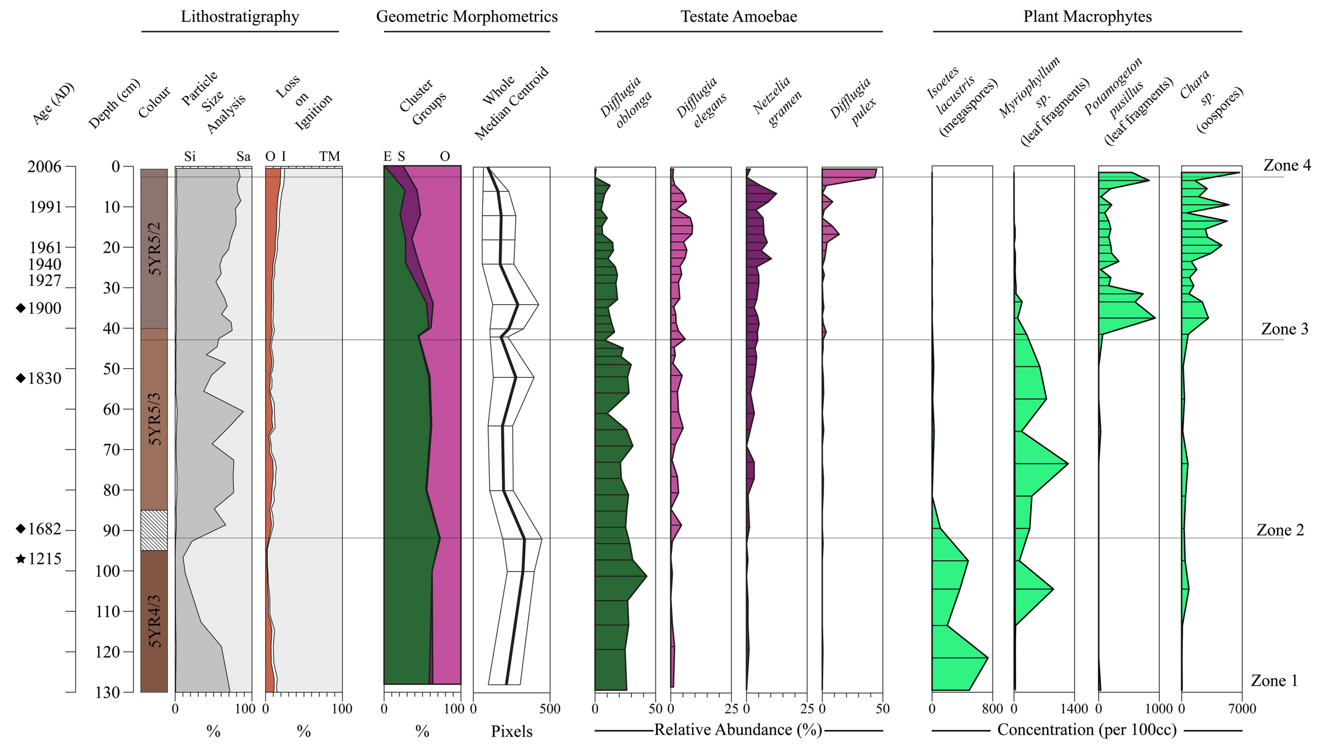

FIGURE 8 | Stratigraphic summary diagram for Loch Leven. Core chronology is based on modeled ^{210}Pb dates (Salgado et al., 2010), extended ^{210}Pb model dates (diamonds) and a single radiocarbon date (1215) marked by a star at 96 cm (Prentice et al., 2018). Lithostratigraphic variables are shown as stacked profiles: loss-on-ignition results are percent organic carbon (OC), percent inorganic carbon (IC) and percent minerogenic matter (TM) (Salgado et al., 2010); particle size data as percent silt (Si) and sand (Sa) (Prentice et al., 2018). The percentage of specimens belonging to each shape group identified by cluster analysis of the Whole-PC1 and -PC2 scores are shown as a stacked profile: (O) Ovoid; (S) Spherical; (E) Elongate. Median centroid size of the Procrustes-PCA landmark dataset is displayed as a line graph. Select Arcellinida (Prentice et al., 2018) and plant macrofossil taxa (Salgado et al., 2010) are illustrated. CONISS zones are from the Arcellinida dataset presented by Prentice et al. (2018).

to frequent phytoplankton blooms, reducing light penetration and favoring macrophyte canopy forming species that grew close to the water surface (e.g., *Potamogeton* and *Chara* sp.; **Figure 1B**; Salgado et al., 2010). The expansion of dense elodeid stands (e.g., *Myriophyllum* and *Chara* sp.) could have resulted in benthic conditions low in oxygen (Lindholm et al., 2008; Tarkowska-Kukuryk and Kornijów, 2008) as supported by decreases in benthic diatoms (Bennion et al., 2012). Oxygen levels could have been further impacted by stands of *Chara* sp. reducing water flow (Vermaat et al., 2000). Reduced bottom water oxygen levels would have stressed benthic taxa. Indeed, we observe coeval decreases in the relative abundance of the elongate cluster, inferred to reflect benthic life habits (**Figure 8**).

At 40 cm there is also an increase in taxa from the spherical cluster: *N. corona* and *D. globulosa* and other taxa transitioning to more spherical shapes: *D. angulostoma*, *D. lithophila*, and *N. gramen* (**Figures 6**, **8**). These shifts in test shape are characterized by a relative low amount of intraspecific variability as compared to centroid size (**Figure 5**). The taxon *N. gramen* (i.e., *C. tricuspis*; **Supplementary Table S2**), assigned to the ovoid and spherical clusters, can switch from benthic to planktic life habits during the summer through the addition of fat droplets and gas bubbles into the test to aid buoyancy (Schönborn, 1962; Arndt, 1993). In their study of Guaraná Lake in Brazil, Lansac-Tôha et al. (2014) found that planktic testate amoeba assemblages were composed of hemispherical and spherical tests. Wiik et al. (2015) also observed coincident increases in *C. tricuspis* (i.e., *N. gramen*), planktic diatoms and *Daphnia* sp., in response to increasing eutrophication of a small marl lake, Cunswick Tarn (United Kingdom). Interestingly, Han et al. (2008, 2011) described the feeding agility and wide prey range of several planktic Arcellinida taxa including *N. tuberspinifera*, which is morphologically and phylogenetically related to *N. corona* (Gomaa et al., 2017) which is assigned to the spherical cluster (**Figure 7**). Taking these points together, it is likely that the observed increase in the spherical cluster in our study reflects the expansion of planktic and epibiotic life strategies, both for Arcellinida and their prey, driven by the expansion of canopy forming macrophyte forming denser stands in the shallower waters.

The existence of an ecological separation between taxa in the spherical (i.e., planktic or epibiotic) and elongate cluster (i.e., benthic) is mirrored by differences in their phylogenetic placement. Molecular barcoding results demonstrate that spherical cluster taxa (e.g., *Netzelia* sp.) previously classified as part of the genus *Difflugia* in fact belong to the family Netzellidae and were assigned to the genus *Netzelia* (Kosakyan et al., 2016; Blandenier et al., 2017; Gomaa et al., 2017). The genetic divergence between these two groups could reflect a speciation event related to divergent life habits (i.e., planktic/epibiotic vs. benthic; Weisse, 2008).

The period from 1969 to 1987 represents one of the most productive periods in the history of Loch Leven with cyanobacteria blooms regularly occurring and planktic diatoms dominating the diatom assemblages (Bennion et al., 2012). This period coincided with a peak in tests from the spherical cluster at 12 cm (*c.* 1986 AD). This was broadly coeval with a reported *Daphnia* sp. bloom in the 1970s (May et al., 2012) suggesting that a zooplankton life strategy was very successful at this time and perhaps why there is an increase in small spherical tests. An increase in the availability of zooplanktonic prey would support planktic carnivory, potentially explaining the increase in *N. corona* (Han et al., 2008, 2011).

Remediation Phase (1985 to Present)

Remediation measures to reduce nutrient loading of Loch Leven have been in place since the mid-1980s (Salgado et al., 2010). Bennion et al. (2012) observed little evidence in the Loch Leven diatom assemblage for remediation success though and hypothesized that it was masked by the increasing influence of climate change. The Arcellinida data support this observation as test morphological trends continue along trajectories established before remediation efforts (**Figure 8**). The disappearance of the elongate cluster from 2000 AD is inferred to represent the development of an inhospitable benthic zone, while planktic and epibiotic life habits continued to be successful (**Figures 6**, **8**). Prentice et al. (2018) hypothesized that continued *Chara* sp. expansion from 2000 to 2006 AD (Salgado et al., 2010) could have further reduced bottom water oxygen levels.

At *c.* 2006 AD, very small tests, having a median centroid size 58% less than in Zone 3, dominate the sample at unprecedented concentrations (**Figures 6**, **8**). Prentice et al. (2018) inferred stressed environmental conditions prevailed in the loch at this time based on a decrease in Arcellinida species diversity. Assemblage dwarfing was observed in response to a stressed environment (e.g., fire and peat farming) by Marcisz et al. (2016) in their study of *Sphagnum* peatlands in Poland, as well as in other protist groups after abrupt perturbations (e.g., foraminifera; Hsiang et al., 2016).

Leading up to the interval representing *c.* 2006 AD centroid size is relatively stable, suggesting that an additional stressor in addition to nutrient enrichment may have impacted the Arcellinida of this uppermost part of the record, for example, warming water temperatures. Spring temperatures are certainly known to have risen in the loch between 1968 and 2007 (Carvalho et al., 2012). A relationship between Arcellinida test size and temperature has been observed experimentally (Wanner, 1999) and in field studies (Collins et al., 1990; Dallimore et al., 2000). A temperature increase in 10°C doubles cellular respiration while oxygen diffusion increases proportionally (Wanner, 1999). Thus, testate lobose amoeba may respond to temperature rises by increasing their surface area to volume (e.g., smaller cell sizes) and increasing the size of their apertures.

Efficacy of Geometric Morphometrics to Summarize Arcellinida Community Dynamics as Compared to Species-Based Approaches

This study has shown that geometric morphometric analysis of Arcellinida tests was able to capture the key phases of environmental variability recorded in the Loch Leven LEVE14 sediment core using only three variables (i.e., PC1, PC2, and centroid size). This is far fewer variables than a conventional species-based approach and requires less taxonomic expertise, making it more accessible to non-specialists. Our identification of three shape types (ovoid, elongate, and spherical) in freshwater Arcellinida tests adds support to ecological (Lansac-Tôha et al., 2014) and molecular (Gomaa et al., 2017) studies which have shown that there is a link between test morphology and life habit. In the current study, spherical tests have been shown to be associated with the expansion of both planktic and macrophytic habitats, while elongate tests are related to benthic habitat conditions. We recommend future experimental studies to test the relationship between Arcellinida and dissolved oxygen levels, to further observe macrophyte and Arcellinida associations and to undertake lake surveys along nutrient gradients to characterize the test morphological response to nutrient-enrichment.

Size variability, as an independent measure, was found to be valuable for inferring a response to eutrophication and/or warming temperatures and illustrating that shape groups (e.g., the elongate cluster) and species vary greatly in their range of sizes (**Figures 4–6**). This high degree of infraspecific variation could reflect an environmental response or could be related to the lumping of distinct taxa during the enumeration process. More research is needed to understand whether size is a specific trait and/or an intraspecific response to environmental variability. Other potential evidence of infraspecific variation was observed by certain taxa belonging to more than one shape cluster. The ability to capture this variation is an added benefit of geometric morphometrics, and future studies could assess the importance of this variation as a response to environmental variability.

Future Development

Geometric morphometric analysis requires Arcellinida tests to be photographed and digitized (i.e., landmark placement) in a consistent manner. This increases analytical time, excludes those taxa that cannot be oriented in a consistent manner (e.g., centropyxids) and, depending on the camera resolution, means that fine features may not be captured. Photography and digitization could potentially be automated reducing analytical time (Hsiang et al., 2016; Steele et al., 2020). Using 3D imaging techniques (e.g., nano-ct) could facilitate the inclusion of all test shapes. The sediment record of LEVE14 core, illustrates an environmental history of plant macrofossil community turnover associated with enrichment, resulting in significant habitat changes. Whether morphological changes in freshwater

Arcellinida tests can track other types of environmental changes should be the focus of future research.

CONCLUSION

This study is the first paleoecological study to characterize Arcellinida test morphological variability using geometric morphometric analysis, providing continuous and independent measures of shape and size variation. Four morphological parameters (centroid size, spherical, ovoid and elongate clusters) accorded well with species-based reconstructions of limnological change in Loch Leven in terms of timing and nature. Fewer parameters that do not require taxonomic expertise and associated with ecological functioning provided intuitive results that could be made accessible to non-specialists facilitating their incorporation into larger multi-proxy studies. Our results support and build upon the hypothesis that freshwater Arcellinida test morphology reflects life habit, with spherical tests representing planktic habitat settings, ovoid tests signaling epibiotic living modes and elongate tests associated with benthic habitats. Our research illustrates the promise of freshwater Arcellinida test morphological variability in tracking environmental change.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

AM and HR conceived and designed the analyses. SP and CS provided valuable insight about the previous Loch Leven study and access to the subsamples. HR provided research space and resources. AM performed data collection, analyses, and wrote the manuscript. All authors revised and edited the manuscript, read, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.568904/full#supplementary-material

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Chironomidae Morphological Types and Functional Feeding Groups as a Habitat Complexity Vestige

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Chironomid (Diptera: Chironomidae) larvae play an important role in a wide range of aquatic ecosystems. The study focuses on Chironomidae trophic guilds and morphological types as indicator traits in reconstructions of habitat changes in shallow water bodies. Mentum and ventromental plates are important mouthparts whose shape depends on food type and feeding behavior. Chironomidae larvae strongly vary in the mode of life and feeding habits, representing almost every feeding group. Here we classified the mentum types into 16 groups and tested if they indicated similar past habitat changes as the Chironomidae functional feeding groups (FFGs), and tribes/subfamilies. Paleoecological data of biotic and abiotic proxies were derived from short sequences from a Late Glacial oxbow and a nearby medieval moat located in Central Poland. The study revealed that the habitat substratum structure, vegetation and physicochemical conditions are associated both with the feeding types and morphological traits. This provides a valuable tool for future reconstructions of habitat changes.

Keywords: Diptera, functional feeding groups, trophic guilds, paleoecology, paleolimnology, habitat reconstruction, environmental changes, mouthpart morphology

INTRODUCTION

Chironomidae are one of the most abundant and ubiquitous groups of aquatic insects. Their sensitivity to changing limnological conditions and species-specific environmental preferences make them good ecological indicators (Armitage et al., 1995; Porinchu and MacDonald, 2003). A variety of environmental variables affects Chironomidae larvae both in a direct and indirect way. Among local-scale abiotic factors, some of the most important for chironomids are pH, lake depth and water nutrient status (Walker, 2001; Brooks et al., 2007; Płóciennik et al., 2020). As there are some truly rheophile taxa, chironomid subfossils can indicate a connection of peripheral river valley water bodies to the main stream during overbank episodes (Gandouin et al., 2006;

Howard et al., 2010; Kittel et al., 2016). They also indicate a stagnant water level in a quantitative way (Luoto, 2009a). While a relation of littoral versus profundal taxa reflects lake-level changes, semi-terrestrial taxa indicate paludification and terrestrialisation processes (Frouz, 1999; Brooks et al., 2007; Lamentowicz et al., 2009). When pH drops below 6.0, chironomid assemblages are mostly replaced by Chaoboridae or Ceratopogonidae (Henrikson et al., 1982; Walker et al., 1985). Even though most chironomid larvae prefer circum-neutral pH, certain taxa are characteristic to acidic or slightly alkaline conditions (Walker, 2001; Brooks et al., 2007). Generally, water pH exceeding 7.5 eliminates many taxa, reducing species richness. It is associated with increased eutrophication and oxygen deficiencies (Brooks et al., 2007). The extraordinarily wide spectrum of preferred water trophic states makes chironomids good indicators of lake nutrient status (Saether, 1979; Walker, 2001; Porinchu and MacDonald, 2003).

Chironomidae are applied in inference models developed as a tool for many quantitative climatic and environmental reconstructions (e.g., Brooks et al., 2001; Luoto, 2009b; Heiri et al., 2011; Kotrys et al., 2020). However, the use of chironomid autecology for reconstruction of aquatic habitats is a time-consuming method and requires high competition in subfossil identification. The acquisition of necessary experience for chironomid identification takes years of work and there are still too few specialists to meet the needs of European paleoecological projects. But, while standard analysis is impossible, the functional trait approach may be applied. This method allows for tracking past shifts in the functional groups of organisms, and thus studying the long-term ecosystem response to past environmental changes (Gregory-Eaves and Beisner, 2011). So far, functional traits of several groups of organisms have been studied according to their application in paleoecology (e.g., Vogt et al., 2010; Fournier et al., 2015; Nevalainen and Luoto, 2017). The functional traits carried by chironomid larvae have so far been studied mainly by Serra et al. (2016, 2017). Here we want to verify the applicability of this method in Chironomidae subfossil analysis.

Functional traits are the features of organisms indicating environmental changes (response traits) or driving such changes in the ecosystems (effect traits) (Nock et al., 2016). They are widely used in community ecology, studying the ecological niche (Kearney et al., 2010), improving ecological process analyses and quantifying the influence of assemblage shifts (Díaz and Cabido, 2001; Nock et al., 2016). Community-trait approach, which takes into considering community weighted means of traits (CWMs), is a noteworthy method (Garnier et al., 2007; Kleyer et al., 2012). Several indices were constructed for functional diversity calculation (reviewed in Pla et al., 2012), providing a broader view for ecosystem complexity than species richness (e.g., Stuart-Smith et al., 2013). The Chironomidae taxonomic and functional diversity across various environmental gradients have been already studied in several regions of the world (e.g., Milošević et al., 2018; Jiang et al., 2019; Motta and Massaferro, 2019; Ni et al., 2020), while Nevalainen et al. (2015) focused on the results provided by the subfossil chironomid assemblages. Kivilä et al. (2019, 2020) used chironomid functional feeding groups (FFGs) to track not only recent habitat changes, but also climate warming in northern Finland. However, food preferences of some taxa are still not well-specified. To avoid loss of data, morphological traits such as the mouthpart structure may serve as a good surrogate. Several studies have proven that morphological features of some organisms, such as testate amoebae (e.g., Fournier et al., 2015; Lamentowicz et al., 2015), cladocerans (Nevalainen and Luoto, 2017) and plants (e.g., Woodward, 1987; Reitalu et al., 2015; Carvalho et al., 2019) can be used to infer past environmental changes, both on a micro- and macroscale. The study by Barboni et al. (2004) indicates that plant response to climate changes (reflected in pollen analysis) is more diagnostic in combinations of traits rather than in individual traits. This assumption is likely applicable to every biological proxy, including chironomids. Consequently, future studies on shifts in habitat structure should apply appropriate methods that take into consideration the complexity of aquatic ecosystems.

Trait-based studies help researchers understand the mechanisms of ecological processes and, therefore, their potential in paleoenvironmental reconstructions cannot be ignored (Marcisz et al., 2020). The studied organisms were affected over time by a range of direct and indirect variables. One of such indirect factors was human activity, which impacted aquatic habitats in many ways. Therefore, the study includes both truly natural and artificial water bodies. The results of the previous study concerning the moat system of the motte-and-bailey stronghold in Rozprza (Kittel et al., 2018a) were utilized. In its case, human impact turned out to be one of the main factors. The inhabitants caused the increase of water trophy, but also changed water dynamics by creating artificial channels (Kittel et al., 2018a). In order to check the applicability of the proposed method in a natural ecosystem, the results of paleoecological studies of the nearby paleo-oxbow were also included.

Morphological characters and well-established ecological preferences are key traits in paleolimnology (Gregory-Eaves and Beisner, 2011). The study focuses on the morphological traits (mouthparts type) which are thought to be (at least partially) dependent on feeding preferences, and thus also associated with the habitat character. The habitat structure is expressed not only by physicochemical features but also by biological variables, such as macrophytes and diatoms, included in our study. Langdon et al. (2010) state that biotic variables are even more important than the abiotic ones in determining zoobenthos communities in temperate shallow lakes, and chironomids are very good indicators of past changes in the primary production, as well as macrophyte abundance and structure. Our primary goal is to recognize if the traits carried by the Chironomidae at different organization levels indicate similar habitat changes in the context of paleoenvironmental reconstructions.

Chironomidae vary not only in their environmental preferences but also in their diet type. Although the larval feeding behavior depends on many factors, such as larval size, food quality, and availability (Berg, 1995), each species seems to have specific preferences, often associated with its habitat type. Representatives of almost every feeding group can be found among chironomids. There are sediment collectors such as *Chironomus plumosus*-type, phytophagous shredders such as *Glyptotendipes caulicola*-type, and scrapers (many Orthocladiinae). Such a great variety is associated with their diverse modes of life – some of them live in tubes built out of sediment (e.g., *Chironomus* and Tanytarsini), while others are free-living and predatory, such as Tanypodinae. Chironomids include detritus feeders, parasites, commensals, and plant miners (Berg, 1995; Walker, 2001; Beiger, 2004; Vallenduuk and Moller Pillot, 2007; Moller Pillot, 2009, 2013; Schiffels, 2014).

Larvae morphology, especially their mouthparts, had to be adapted to their diet and feeding behavior. The relationship between mandibles and chironomid autecology was studied using the geometric morphometrics approach (Đurđević et al., 2017). Mandibles and labrum are important features, useful in diagnostics, but they are often missing from the subfossil material. Other mouthparts, likely related to the diet, are mentum and ventromental plates. This is the reason for choosing them as the key characteristics for morphological types in our study. The shape of the mentum may be similar even between taxonomically distant taxa. For example, the concave mentum of predatory *Cryptochironomus* resembles the dorsomentum of many Tanypodinae (e.g., *Procladius*), while striated plates are often characteristic of filtrating Chironominae (Olafsson, 1992; Moller Pillot, 2009).

Considering this, we can assume that some generalization in the morpho-functional traits of Chironomidae can be done. However, we need to be careful with some morphologically close species which vary in their feeding behavior (Monakov, 1972). In paleoecological studies, chironomids are mostly identified to the morphotype level, which usually includes closely related species with similar ecology. On the other hand, it is sometimes impossible to distinguish subfossil head capsules of ecologically different taxa, such as *Micropsectra insignilobus* and *M. atrofasciata, Chironomus tenuistylus* and *Ch. plumosus,* or *Stempelinella* and *Zavrelia* (Brooks et al., 2007). Nevertheless, chironomid mouthparts are closely related to their feeding type, which often reflects the habitat structure. It raises the question if the knowledge of certain species' autecology is always required for reconstructions of environmental conditions? Perhaps some general assessments can be done, even by non-specialists, on the basis of mentum shape. In this case, such preliminary chironomid analyses based on the proposed mentum classification could bring valuable insight into past ecosystems, even when undertaken during a macrofossil analysis.

Mentum and ventromental plates are also the main differing features between subfamilies and tribes. Although there are eleven Chironomidae subfamilies, three of them are the most species-rich: Chironominae, Orthocladiinae, and Tanypodinae (Brooks et al., 2007; Ferrington, 2008). In this study, we focus on these subfamilies, as their representatives were found in examined sediments. Chironominae are usually characterized by distinctive, striated, large ventromental plates and a slightly convex mentum. The mentum of Orthocladiinae is often strongly convex, their head capsules are usually smaller, and ventromental plates are mostly inconspicuous. Tanypodinae have a toothed ligula, a less developed mentum, and no ventromental plates. Some chironomid subfamilies are divided into tribes. In the studied sequences, taxa belonging to Macropelopiini, Natarsiini, Pentaneurini, Procladiini, Tanypodini (Tanypodinae), as well as to Chironomini and Tanytarsini (Chironominae), and to Orthocladiinae were recorded. Among Tanypodinae, only Macropelopiini, Procladiini and Tanypodini have dorsomental teeth. Natarsiini and Pentaneurini are characterized by a bright, elongated head and a membranous toothless mentum that is not well-preserved in subfossil material. Chironomini have relatively large, fan-shaped ventromental plates, while the plates of Tanytarsini are usually bar-shaped and straight (Brooks et al., 2007; Andersen et al., 2013). The Orthocladiinae are rarely divided into tribes (Spies, 2005), so we left this group undivided at the subfamily level.

Finally, the goal of this study is to check whether (and how) some chironomid groups (mentum types, trophic guilds, or tribes/subfamilies) are correlated with other indicators within studied proxies (macrophytes and other plants, diatoms, sediment type or geochemical components). The applicability of morphological functional traits in paleoecological research was verified. Presumably, they could serve as surrogate indicators where it could be applicable.

STUDY AREA

The study area (51°18′07″ N; 19°40′04″ E; 182–183 m a.s.l.) is situated in Central Poland, approx. 60 km south of Łódź. It is located in the valley floor of the Luciąża River, a 3rd-order river in the Vistula River basin.

The region is a part of the Odranian (Saalian) formerly glaciated area – the last ice sheet was present here in the Wartanian Cold Stage of the Odranian Glaciation (Marks, 2011). Intense transformation of river valleys took place during the Weichselian glacial period under periglacial conditions. While the valley floor is strongly expanding in the Rozprza area, residual terraces occur within the Holocene floodplain (Goździk, 1982; Kittel et al., 2018b).

In the opinion of Wachecka-Kotkowska (2004a,b), the morphology of the Luciąża valley floor was obliterated by the deposition of modern overbank alluvium. However, this landscape reconstruction has not been confirmed by a detailed examination of surficial geology in the Rozprza area (Kittel et al., 2018a,b). In the recent research, numerous subfossil paleomeanders of different sizes have been discovered in the valley floor (**Figure 1**). A large paleochannel (W4) was recorded as a strong curvilinear magnetic anomaly with a width of ca. 10 m, and a radius of ca. 15 m. It is filled with organic mud and coarse-detritus gyttja reaching 1.3 m thickness and underlain by channel alluvia of sands and gravels with organic admixtures and laminations of organic mud. Radiocarbon data evidences the channel's cut-off in the Late Allerød. The paleochannel fill is overlain by the overbank alluvia of sandy organic mud (Kittel et al., 2018b).

The Rozprza motte-and-bailey moat system was established in the 1330s AD and is now filled with organic (gyttja and peat) and partially inorganic deposits containing rich remains of wood. The medieval age of the features has been confirmed by dendrochronological and radiocarbon AMS date sets. The fill of

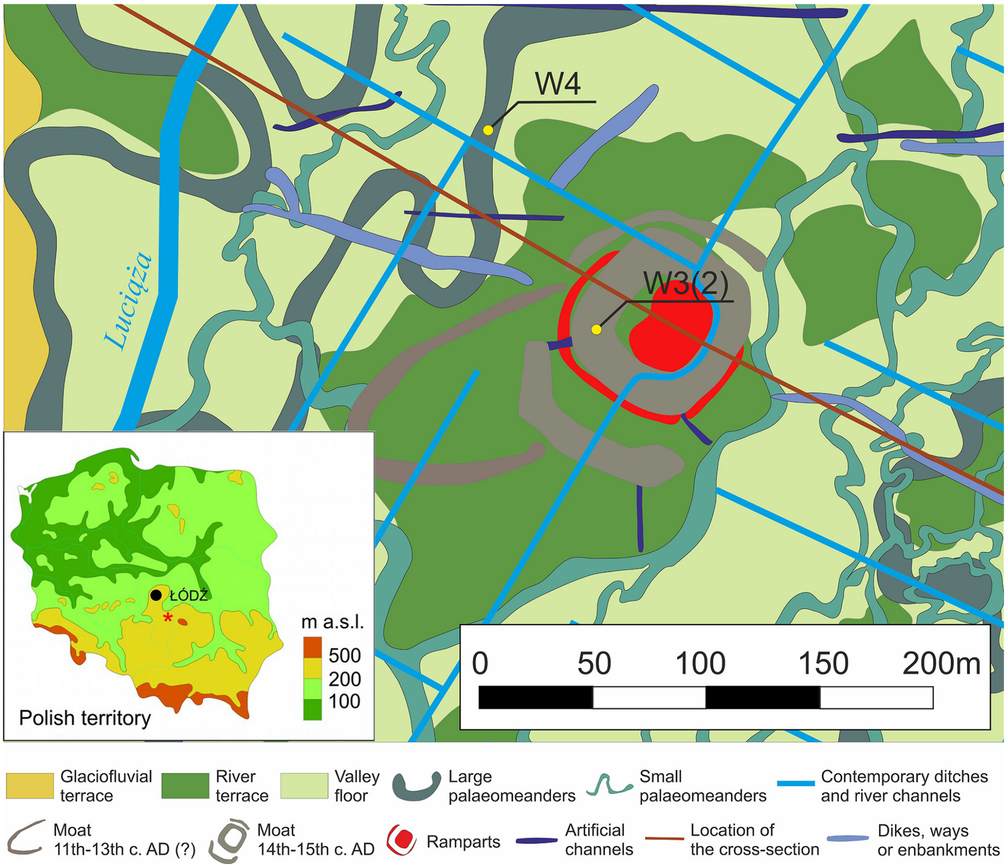


FIGURE 1 | The geomorphological map of the vicinity of Rozprza archaeological site with the location of W4 (paleo-oxbow) and W3(2) (moat) profiles (marked as yellow dots) (after Kittel et al., 2015, 2018b, modified). In the map of Poland, Rozprza is marked with an asterisk.

the main moat was a subject of a detailed paleoenvironmental study (Kittel et al., 2018a). The accumulation of overbank silty sandy organic mud took place within the moat ditch system as late as in the 18th or 19th c. CE (Kittel et al., 2018a,b).

The potential natural vegetation of the Luciąża River valley would have been mostly lime-oak-hornbeam forests representing the *Tilio-Carpinetum* association and, to a lesser extent, the *Potentillo albae-Quercetum* typicum. Immediate surroundings of the water courses would have been overgrown by lowland ash-alder and alder forests of *Fraxino-Alnetum* and *Carici elongatae-Alnetum* associations (Matuszkiewicz, 2008).

The sediment cores were taken from the same area, but they are dated to different time periods. The W3(2) sequence originates from the Late Medieval moat, while the W4 core covers the Late Weichselian paleochannel history (**Figure 1**).

MATERIALS AND METHODS

Fieldwork and Geochronology

The fieldwork in Rozprza was conducted in 2015–2016, as part of a multidisciplinary archaeological investigation of the medieval ringfort remnants. In order to choose proper sampling sites in the field, not only was topography and geomorphology of studied area thoroughly mapped but also the combination of other non-destructive methods (including aerial photography, geophysical, and geochemical survey) was used (Sikora et al., 2015, 2019; Kittel et al., 2018b).

The sediments for paleoecological analyses were collected from the trench walls as monoliths using metal boxes (50 × 10 × 10 cm each). With this method, the undisturbed structure of the sediments was preserved. Five selected samples of bulk organic deposits collected from the W3(2) profile and two bulk samples from the W4 profile were dated with the radiocarbon (^{14}C) method, using the liquid scintillation technique (LST). Two samples of selected terrestrial plant macrofossils from the W3(2) core and three samples from the W4 core were dated using accelerator mass spectrometry technique (AMS). Moreover, a dendrochronological analysis was performed to determine the age of the moat fill (Kittel et al., 2018a).

Geochemical and Sedimentological Analyses

For geochemical analyses, samples from both cores were dried at 105°C and homogenized. The organic matter content was obtained using the loss on ignition (Heiri et al., 2001).

Carbonate content was determined using the volumetric method with Scheibler's apparatus, and pH by a mean potentiometric method in distilled water. The ash samples were dissolved (with HCl, HNO_3, and H_2O_2) in Teflon bombs using a microwave mineralizer. For the solution analyzed concentrations of such elements as: Na, K, Mg, Ca, Fe, Mn, Cu, Zn, and Pb, the atomic absorption spectrometry (AAS) was used. Grain size composition of the ash samples, remaining after the solution was made in accordance with Clift et al. (2019), was determined using a Mastersizer 3000 laser particle-size analyzer (Malvern). The relationship between the mean grain size and the sorting index (the so-called coordinate system) follows Mycielska-Dowgiałło and Ludwikowska-Kêdzia (2011).

Biotic Proxies

Chironomidae sample processing followed the methods outlined in Brooks et al. (2007). The sediment volume ranged between 1 and 10.5 cm^3 per sample. The sediments were passed through a 63 μm mesh sieve. If necessary, kerosene flotation was used according to the method of Rolland and Larocque (2007). Processed sediment was put into a Bogorov counting tray and scanned under a stereo-binocular microscope. Where applicable, at least 50 (preferably 100) chironomid head capsules from each sample were picked and mounted in Euparal® on microscope slides. Identification of chironomid head capsules followed keys by Schmid (1993), Brooks et al. (2007), and Andersen et al. (2013). Chironomidae taxa were classified in three ways: taxonomically (subfamilies and tribes), morphologically and by feeding group. Sixteen morphological types were distinguished based on the larvae mouthpart structure, namely: the mentum ratio (width/height), the median and lateral mentum teeth structure, as well as the size and shape of ventromental plates (**Figure 2**). Most types were distinguished within Chironomini and Orthocladiinae. Tanypodinae with toothed dorsomentum were grouped together with *Cryptochironomus*, while for Pentaneurini and *Natarsia* (with toothless mentum) a separate group was created. Tanytarsini were classified into two types, depending on the shape and length of ventromental plates (**Table 1**). The recorded taxa were also divided into 12 functional feeding groups (FFGs): collector-gatherers (C), collector-filterers (C/F), collectors/grazers (C/G), shredders/filterers (S/F), shredders/collectors (S/C), grazers/scrapers (G), grazers/collectors (G/C), filterers/collectors (F/C), filterers/grazers (F/G), miners (M), predators (P), and predators/collectors (P/C). The feeding preferences of identified taxa are based mainly on Franquet (1999), Vallenduuk and Moller Pillot (2007), Moller Pillot (2009, 2013), and Serra et al. (2016). The stratigraphic diagrams were created with C2 software (Juggins, 2007).

Samples for a plant macrofossil analysis were wet-sieved in a 200 μm mesh, then boiled with KOH to reduce the amount of sediment and remove humic matter. The material was examined under a microscope. Conservation of plant remains was done with a standard mixture of alcohol, water and glycerine, with addition of thymol. Fragments of plants were then dehydrated in 50% ethyl alcohol. Macrofossils were identified using plant keys, atlases (e.g., Greguss, 1945; Kats et al., 1965; Grosser, 1977; Schweingruber, 1978; Berggren, 1981; Cappers et al., 2006; Velichkevich and Zastawniak, 2006, 2008; Schweingruber et al., 2011), scientific descriptions and publications, a reference collection of modern seeds, fruits, wood and charcoal, and a collection of fossil floras of the W. Szafer Institute of Botany, Polish Academy of Sciences, in Kraków.

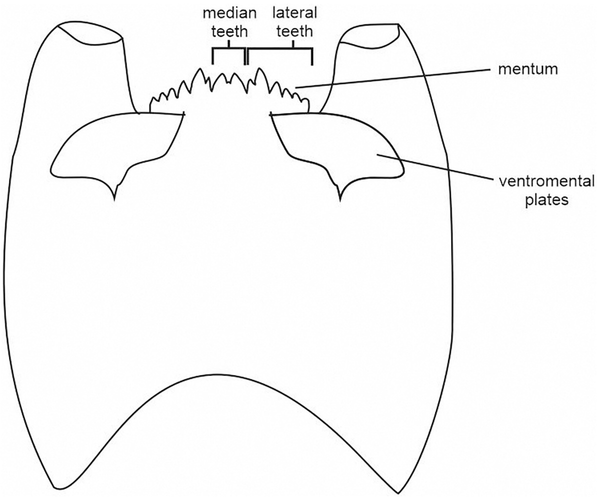


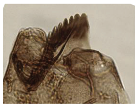
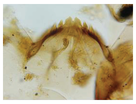
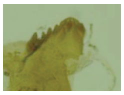
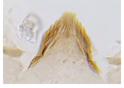
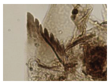
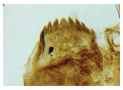
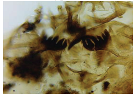
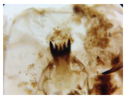
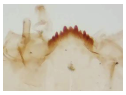
FIGURE 2 | Chironomidae head capsule with mouthpart structure.

A diatom analysis was conducted according to the method by Battarbee (1986). Samples of 1 cm^3 each from the cores were processed. Sediments were treated in 10% HCl to remove calcium carbonate and washed several times in distilled water. Afterward, the samples were boiled in 30% H_2O_2 to digest the organic matter. Finally, the samples were washed several times in distilled water. Microspheres were added to each sample in order to determine the frequency of the diatoms in each sample (Battarbee and Kneen, 1982).

Statistical Analyses

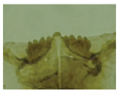
All statistical analyses were made in R software (R Core Team, 2020). Firstly, environmental data were standardized using the robustHD package (Alfons, 2019). The corrplot package (Wei and Simko, 2017) was used to compute Pearson's correlation matrix in order to check which environmental variables were correlated (correlation >0.70) with each other (**Supplementary Figures 1**, **2**). The most autocorrelated predictors were removed from the analysis. Finally, eight variables: pH, $CaCO_3$, organic matter (OM), K, sand, wood, Carex sp., and U. dioica were included in further analysis (**Supplementary Table 1**). Next, a dataset for each group of Chironomidae was square-root transformed, in order to reduce the influence of outliers. The "decorana" function from the vegan package (Oksanen et al., 2019) was used to fit the best ordination analysis to the datasets of each group. A Redundancy Analysis (RDA) was performed for each Chironomidae typology using the vegan package (Oksanen et al., 2019). To determine collinearity between environmental factors shaping chironomid composition, variance inflation factors (VIF) analysis was used

TABLE 1 | Chironomidae morphological types.

Morphological type	Description	Morphospecies from this study	Example
1	- One broad or several smaller median teeth - Narrow plates with setae - Mentum ratio > 3	*Acricotopus, Diplocladius, Psectrocladius barbatipes*-type, *P. flavus*-type	 *Diplocladius*
2	- Two median teeth - Broadening/narrow ventromental plates - Mentum ratio 2.2–2.9	*Chaetocladius piger*-type, *Metriocnemus fuscipes*-type, *Heterotrissocladius marcidus*-type, *H. grimshawi*-type, *Hydrobaenus conformis*-type, *Nanocladius rectinervis*-type, *Psectrocladius sordidellus*-type, *P. limbatellus*-type	 *Chaetocladius piger*-type
3	- Large, fan-shaped plates - One/trifid median tooth - Mentum ratio 2.9–3.9	*Glyptotendipes barbipes*-type, *G. pallens*-type, *Chironomus plumosus*-type, *Ch. anthracinus*-type	 *Chironomus anthracinus*-type
4	- Fan-shaped plates - Cluster of outermost lateral teeth	*Cladopelma viridulum*-type, *Cladopelma goetghebueri*-type	 *Cladopelma viridulum*-type
5	- Tanytarsini - Long ventromental plates, close together	*Micropsectra contracta*-type, *M. insignilobus*-type, *M. pallidula*-type, *Tanytarsus chinyensis*-type, *T. lactescens*-type, *T. lugens*-type, *T. mendax*-type, *T. nemorosus*-type, *T. pallidicornis*-type, *Paratanytarsus austriacus*-type, *P. penicillatus*-type, *P.* type A, *Rheotanytarsus*	 *Micropsectra contracta*-type
6	- Broadening/narrow ventromental plates - Mentum ratio < 2.2	*Corynoneura arctica*-type, *C. coronata*-type, *C. edwardsi*-type, *Rheocricotopus effusus*-type, *Eukiefferiella coerulescens*-type	 *Corynoneura arctica*-type
7	- One broad median tooth - Narrow ventromental plates - Mentum ratio ca. 2	*Cricotopus bicinctus*-type, *C. cylindraceus*-type	 *Cricotopus cylindraceus*-type
8	- One median tooth - narrow ventromental plates - Mentum ratio 2–2.9	*Cricotopus intersectus*-type, *C. laricomalis*-type, *Orthocladius trigonolabis*-type, *O.* type S, *O.* type I, *Metriocnemus terrester*-type	 *Metriocnemus terrester*-type
9	- Concave mentum	*Cryptochironomus, Procladius, Tanypus, Psectrotanypus, Derotanypus*	 *Cryptochironomus*
10	- Fan-shaped plates - Mentum ratio 2–2.5	*Dicrotendipes nervosus*-type, *D. notatus*-type, *Glyptotendipes caulicola*-type, *G. severini*-type	 *Glyptotendipes severini*-type
11	- No teeth on mentum	*Natarsia, Ablabesmyia, Clinotanypus nervosus, Guttipelopia, Larsia, Krenopelopia, Monopelopia, Paramerina, Thienemannimyia group, Zavrelimyia*	 *Ablabesmyia*
12	- Fan-shaped plates - 3–4 median teeth higher than lateral ones - Mentum ratio 2.9–4.1	*Endochironomus tendens*-type, *E. impar*-type, *E. albipennis*-type, *Phaenopsectra* type A, *P. flavipes*-type, *Stictochironomus roesenschoeldi*-type, *Sergentia coracina*-type, *Polypedilum sordens*-type	 *Endochironomus tendens*-type

(Continued)

TABLE 1 | Continued

Morphological type	Description	Morphospecies from this study	Example
13	- Fan-shaped plates - 3–4 median teeth brighter than lateral ones - Mentum ratio 3.2–3.7	*Microtendipes pedellus*-type, *Paratendipes albipennis*-type, *P. nudisquama*-type	 *Microtendipes pedellus*-type
14	- Large fan-shaped plates - 2 median teeth - First lateral tooth lower	*Polypedilum nubeculosum*-type, *P. nubifer*-type, *Lauterborniella, Zavreliella*	 *Polypedilum nubeculosum*-type
15	- Ventromental plates narrow or foot-shaped - One broad median tooth or two median teeth - 4–5 lateral teeth - Mentum ratio ca. 3	*Metriocnemus eurynotus*-type, *Parametriocnemus-Paraphaenocladius, Smittia foliacea*-type, *Pseudosmittia, Pseudorthocladius, Limnophyes, Paralimnophyes*	 *Metriocnemus eurynotus*-type
16	- Tanytarsini - Broad, well separated ventromental plates	*Constempellina-Thienemanniola, Stempelinella-Zavrelia*	 *Constempellina -Thienemanniola*

(Fox and Weisberg, 2011). In addition, ANOVA was conducted to determine statistically significant variables (Oksanen et al., 2019). These variables were used in subsequent RDA analysis. Adjusted R^2 indicated how much variability was explained by the RDA analysis. Eigenvalues of RDA axes (RDA1 and RDA2) were computed to compare the proportions of variance explained between each analyzed group of chironomids. Eight environmental factors (pH, $CaCO_3$, organic matter, K, sand, wood, Carex sp., and U. dioica) were used in generalized linear models (GLMs) as fixed effects to assess their influence on the richness of chironomid tribes/subfamilies, mentum types and feeding types. Models were selected using the "dredge" function from the MuMIn package (Bartoń, 2018). Next, the most parsimonious models (with $\Delta AIC < 2$) were averaged into one model to determine statistically significant environmental variables. In addition, the hier.part package (Walsh and Mac Nally, 2013) was used to calculate the independent effects of each variable on the richness as well as its significance. Charts were created using the ggplot2 package (Wickham, 2016).

RESULTS

Chironomidae Stratigraphy

From a short, Late Weichselian paleochannel sequence (W4), 1138 chironomid head capsules were collected and identified to 56 morphospecies. The chironomid fauna in the paleochannel underwent significant fluctuations. After the favorable conditions for chironomid assemblages in the Allerød and Younger Dryas, their number suddenly dropped in the Holocene. Because of a very low chironomid concentration in that period (<50 head capsules), further analyses were based only on the Late Weichselian part of the sequence (ca. 13,000 – 12,200 cal. BP). During this whole period, collectors, represented mainly by Tanytarsini with mentum type 5, clearly dominated. Their relative proportion ranged between 15 and 60%, with the peak in the Late Allerød (194–198 cm b.g.l.). This was the time when the share of subdominant grazers (G, G/C) dropped. Interestingly, in the Younger Dryas (180–190 cm b.g.l.), the share of collectors (here mentum type 5, predominantly *Micropsectra*) slightly decreased, which coincides with the increase in the proportion of larvae with mentum type 1 (mainly *Acricotopus*). Besides that, the share of semiterrestrial taxa (grazers/collectors, mostly *Limnophyes-Paralimnophyes* and *Parametriocmenus-Paraphaenocladius*) was significant (**Figure 3**).

In the Late Holocene moat sequence [W3(2)], as many as 2488 head capsules of 83 Chironomidae morphospecies were recorded. The sequence reveals clear shifts in chironomid composition. The first phase is characterized by high complexity of chironomid assemblages. Worth noting is a particularly high share of predatory taxa (mainly Tanypodinae), shredders/collectors (mainly *Psectrocladius*) and grazers (mainly *Corynoneura*), but also many other groups, including grazers/collectors represented here by *Cricotopus*. When the abundance of chironomid larvae decreased in the 2nd half of the 16th century AD, semiterrestrial grazers/collectors started to dominate, accompanied by shredders/filterers (*Polypedilum nubifer*-type and *P. nubeculosum*-type). In the last phase, very few Chironomidae were found: firstly grazers/collectors, later mainly collectors (mentum type 10), collector/filterers and filterer/grazers (both representing mentum type 3) (**Figure 3**). For detailed reconstruction of the moat development, see Kittel et al. (2018a).

Tribes/Subfamilies

The ANOVA analysis for tribes/subfamilies showed that pH, K and organic matter played a significant role in shaping Chironomidae assemblages within analyzed cores (**Table 2**). The RDA plot (**Figure 4A**) revealed that W4 samples were associated

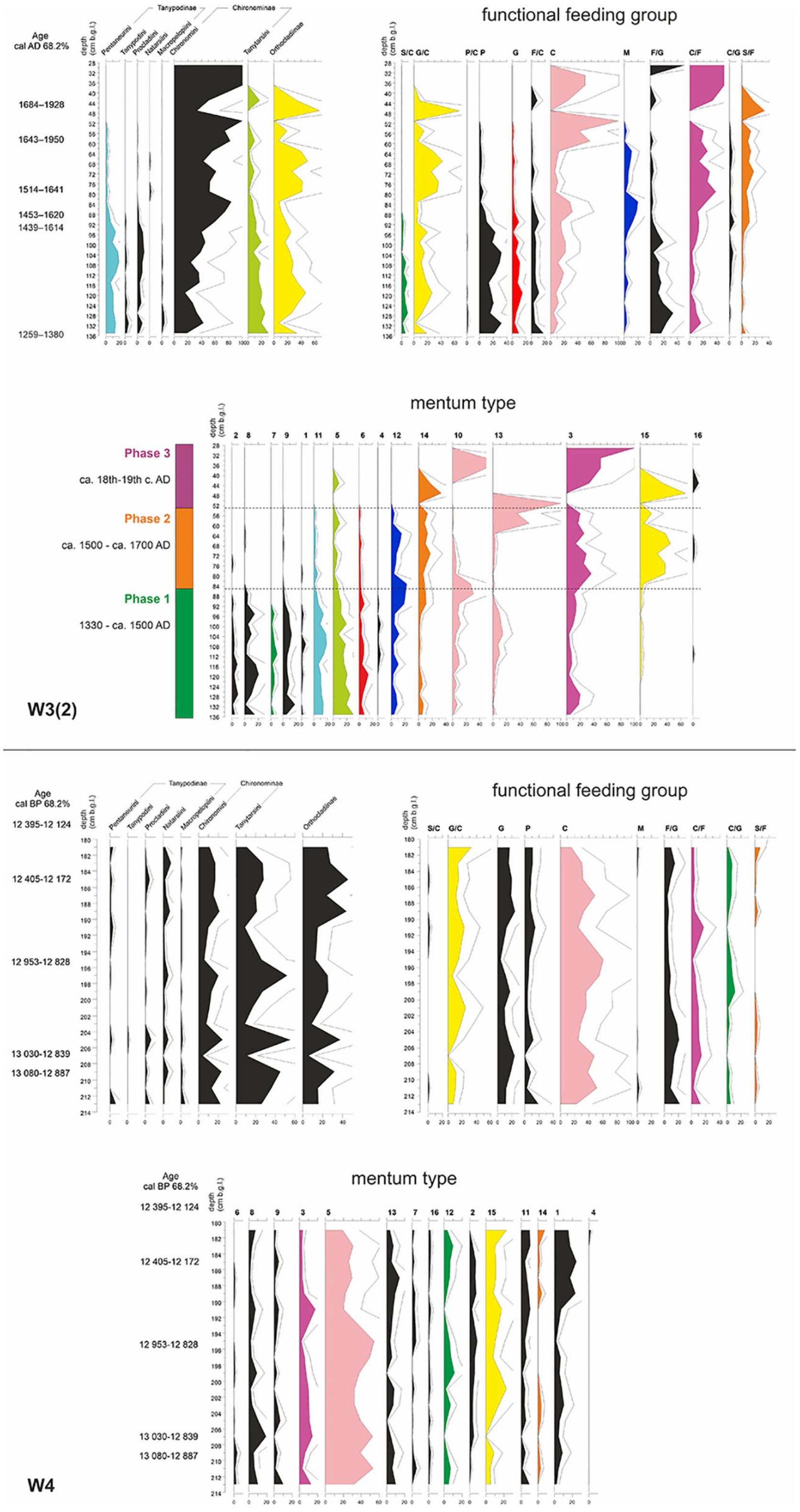


FIGURE 3 | Chironomidae stratigraphic diagrams of the W3(2) (top) and W4 (bottom) cores, according to three typologies: subfamily/tribe, functional feeding groups and mentum types. Similar trends in some groups are outlined by the same graph color. The bar alongside W3(2) diagram represents three phases of the moat development based on Chironomidae assemblages (Kittel et al., 2018a). The abbreviations used for functional feeding groups were as follows: C, collector-gatherers; C/F, collector-filterers; C/G, collectors/grazers; S/F, shredders/filterers; S/C, shredders/collectors; G, grazers/scrapers; G/C, grazers/collectors; F/C, filterers/collectors; F/G, filterers/grazers; M, miners; P, predators; and P/C, predators/collectors. The symbols used for morphological types are explained in **Table 1**.

TABLE 2 | Variance influence factors (VIFs) and significance of eight environmental factors selected from correlation matrices.

	Variable	VIF	Significance
Tribes/subfamilies	**pH**	**2.323**	**0.001**
	$CaCO_3$	1.267	0.092
	OM	**4.247**	**0.043**
	K	**2.925**	**0.002**
	Sand	2.635	0.418
	U. dioica	2.211	0.563
	Carex sp.	1.969	0.579
	Wood	2.098	0.064
Mentum type	**pH**	**2.324**	**0.001**
	$CaCO_3$	**1.267**	**0.033**
	OM	**4.247**	**0.003**
	K	**2.925**	**0.001**
	Sand	2.635	0.277
	U. dioica	2.211	0.662
	Carex sp.	1.969	0.326
	Wood	**2.098**	**0.028**
Feeding type	**pH**	**2.324**	**0.001**
	$CaCO_3$	1.267	0.275
	OM	**4.247**	**0.001**
	K	**2.925**	**0.001**
	Sand	2.635	0.864
	U. dioica	2.211	0.120
	Carex sp.	1.969	0.283
	Wood	**2.098**	**0.001**

Bolded variables were used in redundancy analysis.

with relatively low content of organic matter and K. Those two factors were correlated with the RDA2 axis. The samples from W3(2) core representing phases of moat development were clearly separated in the plot. The first phase was influenced by high pH and K values, as well as by abundance of Procladiini and Pentaneurini. Samples from the second phase were characterized by a dominance of Chironomini and high content of organic matter. The third phase, in turn, was characterized by low pH. The RDA explained 38% of variance from the analysis. While the RDA2 axis explained 11% of variance, the RDA1 axis described 31% of total variance. GLMs for Chironomidae tribes/subfamilies were initially calculated for eight predictors. Seven of them (pH, OM, $CaCO_3$, K, sand, wood, and *Urtica dioica*) were included in the averaged model. Among them, only pH was statistically significant and positively correlated with richness (**Figure 5A**). On the other hand, hierarchical partitioning (HP) revealed that both pH (independent effects: 70%) and *Urtica dioica* (independent effects: 14%) were statistically significant and had positive impact on tribes/subfamilies richness (**Figure 6A**).

Mentum Types

Five environmental factors: pH, organic matter, $CaCO_3$, K, and wood macrofossils were statistically significant in the ANOVA analysis for mentum types (**Table 2**). The RDA analysis showed that samples from different cores and phases were clearly distinguished. Chironomids with mentum types 1 and 5 were the most abundant in the W4 core, which may be associated with low content of wood, organic matter and potassium (factors correlated with the RDA2 axis). On the other hand, high pH (correlated with the RDA1 axis) and K values impacted the higher abundance of chironomids with 7, 8, 9, and 11 mentum types in the first phase of W3(2) core. The second phase was shaped by high content of wood, organic matter and low content of $CaCO_3$. Low pH values characterized phase 3 of the W3(2) core. The first two RDA axes explained 21% (RDA1) and 14% (RDA2) of variance, but with Adjusted R^2, the whole RDA analysis described 34% of variance. Six out of eight predictors: pH, $CaCO_3$, organic matter, K, sand, and wood macrofossils were included in the GLMs for richness of Chironomidae mentum types. However, only pH, organic matter and potassium were statistically significant and positively correlated with richness (**Figure 5B**). Hierarchical partitioning results for mentum types were similar to those for tribes/subfamilies. They revealed that both pH and *Urtica dioica* were significant and had positive correlation with independent effects reaching 64% and 16%, respectively (**Figure 6B**).

Feeding Groups

The ANOVA analysis revealed that four factors: pH, OM, K, and wood were significant for the composition of chironomid feeding groups (**Table 2**). RDA analysis supported the results for other chironomid typologies, where samples representing each core and phase created clear aggregations (**Figure 4C**). W4 samples were characterized by a low content of organic matter, wood and K (factors correlated with the RDA2 axis), as well as high abundance of collectors (C). Samples from the W3(2) core were distributed along the RDA1 axis. The first-phase samples were under the influence of pH (correlated with the RDA1 axis), with high abundance of S/C, P, and F/C feeding types. High content of organic matter and wood affected the aquatic organisms in the second phase, which might be reflected in the abundance of shredders/filterers (S/F). Phase 3 assemblages were shaped by low pH. The coefficient of determination (Adj R^2) for this dataset is 0.36. The RDA axis 1 described 26% of variance, while the second axis explained only 8%. Generalized Linear Models for feeding type richness included seven predictors: pH, organic matter, K, sand, *Urtica dioica*, *Carex* sp., and wood. However, only pH, organic matter and wood were statistically significant and showed positive correlation with richness (**Figure 5C**). Moreover, hierarchical partitioning indicated pH, OM, *Urtica dioica*, and wood (independent effect: 59, 11, 18, and 12%, respectively) as significant for shaping chironomid feeding types. All these factors were positively correlated with richness of feeding groups (**Figure 6C**).

The VIF values of all environmental variables in each RDA analysis were <10.0 (**Table 2**). Generally, pH seems to be the main factor shaping Chironomidae composition in each typology. Besides that, redundancy analyses revealed the importance of K and organic matter (**Figure 4**). In W3(2) samples, pH is strongly correlated (0.88) with diatom concentration (**Supplementary Figure 1**), while they are absent from the W4 sequence.

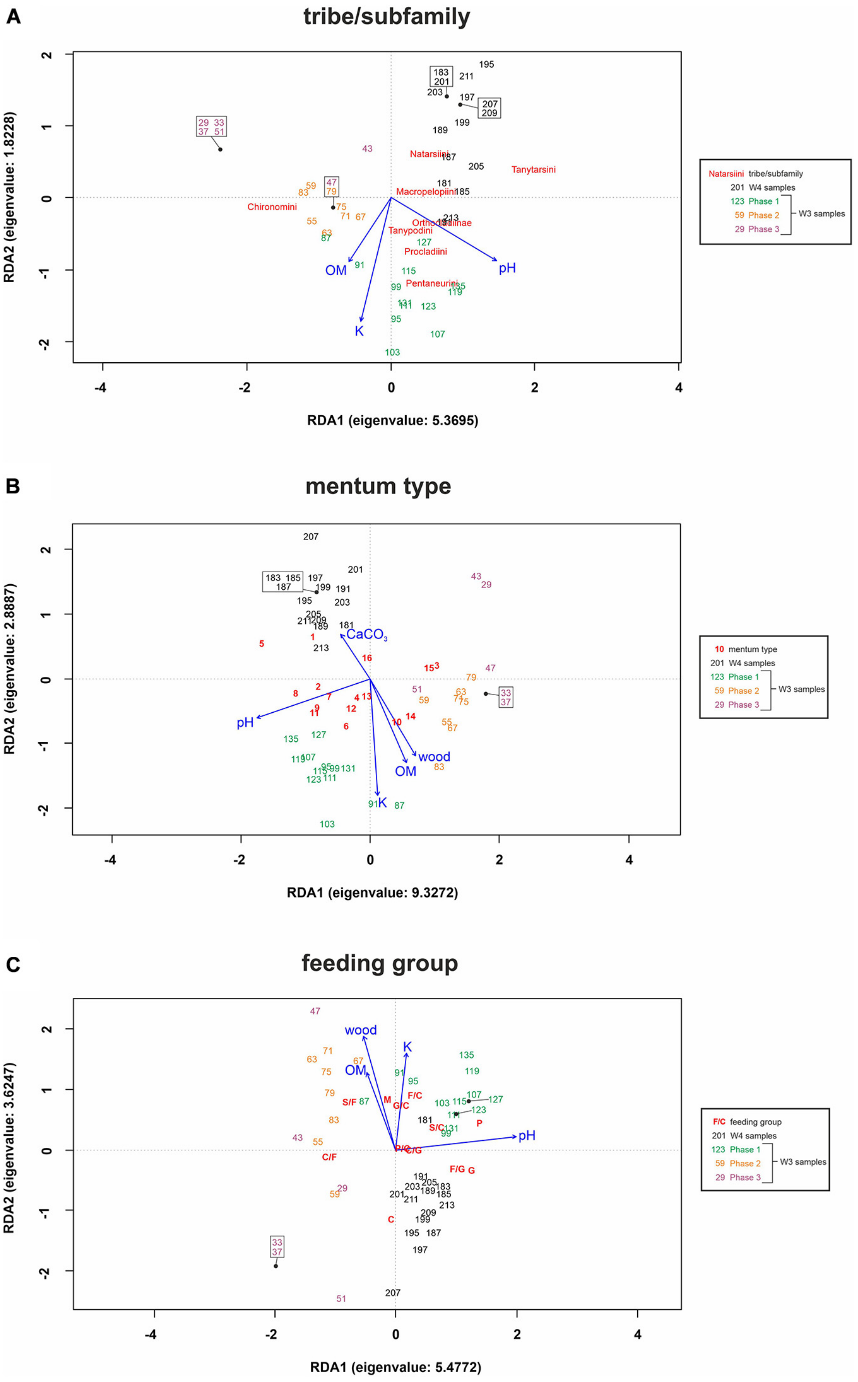


FIGURE 4 | Redundancy Analysis for tribes/subfamilies **(A)**, mentum types **(B)** and feeding groups **(C)** showing composition differences according to selected environmental factors (blue) in samples within two cores [W3(2) and W4]. The abbreviations used for functional feeding groups were as follows: C – collector-gatherers; C/F – collector-filterers; C/G – collectors/grazers; S/F – shredders/filterers; S/C – shredders/collectors; G – grazers/scrapers; G/C – grazers/collectors; F/C – filterers/collectors; F/G – filterers/grazers; M – miners; P – predators; and P/C – predators/collectors. The symbols used for morphological types are explained in **Table 1**. For full names of variables, see **Supplementary Table 1**.

This is the reason why, although they are an important food source for some chironomid larvae, they were not included in the analysis. Another important factor is *Urtica dioica,* which reveals higher significance treated individually in the hierarchical partitioning than in the averaged GLMs.

All combinations of the most parsimonious models used to compute average models for each analysis are listed in

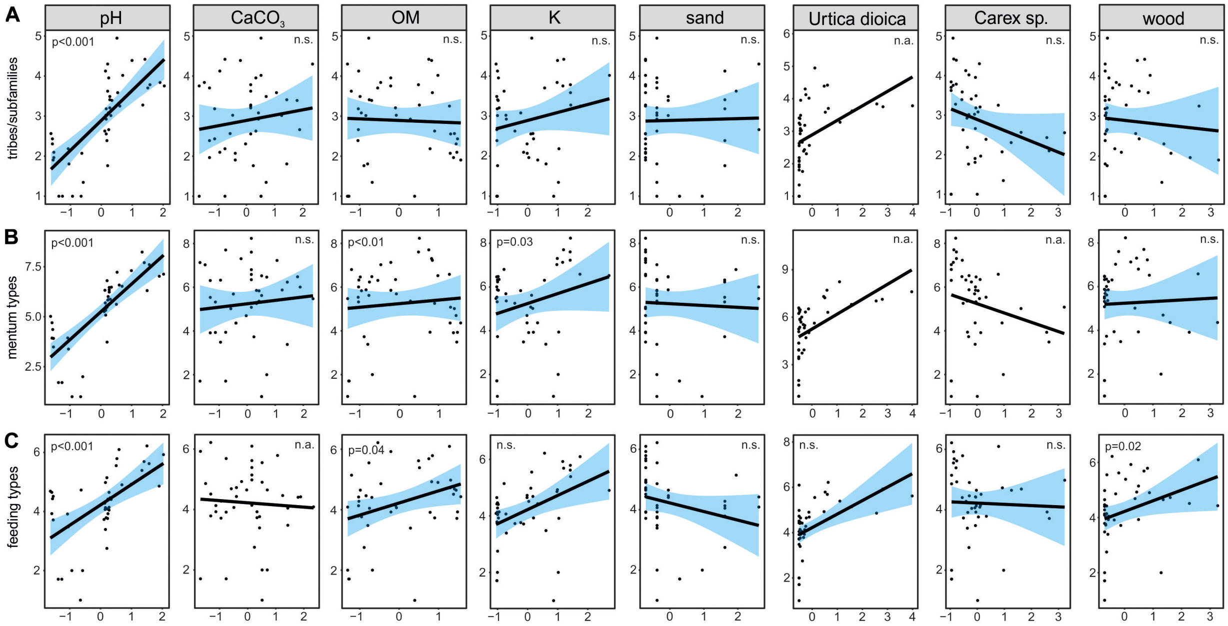


FIGURE 5 | Visualization of generalized linear models testing for effects of selected environmental factors on richness of chironomid tribes/subfamilies **(A)**, mentum types **(B)** and feeding type **(C)**, where "n.a." means that the factor was not included in the models, and "n.s." that its explanatory power was not significant.

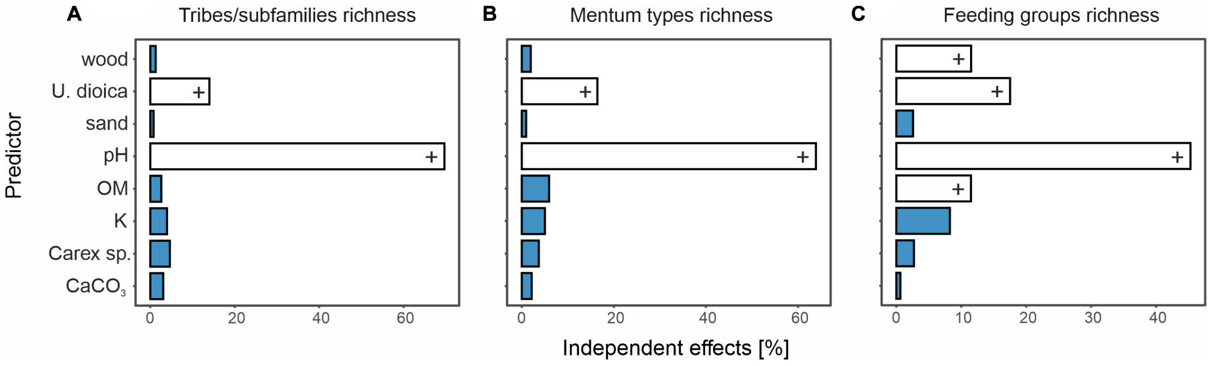


FIGURE 6 | Relative contribution of each predictor to shared variability of full models testing for effects of environmental factors and richness of tribes/subfamilies **(A)**, mentum types **(B)**, and feeding types **(C)**. A positive impact of environmental variables was marked with a plus (+) and negative impact with a minus (–). Predictors with statistical significance of response variables are given in white. For full predictor names, see **Supplementary Table 1**.

Supplementary Table 2. For final values of each environmental factor in averaged models, see **Supplementary Table 3**.

DISCUSSION

Chironomidae Habitat Preferences in the Paleo-Oxbow and the Moat

Both generalized linear models (**Figure 5**) and hierarchical partitioning (**Figure 6**) clearly show that the crucial factors shaping Chironomidae functional composition are pH and OM. However, each site is different, depending on the levels of these factors. In the paleochannel (W4), pH was stable and circum-neutral, as indicated by the geochemical analysis. That provided favorable conditions for a complex, functionally diverse ecosystem. In the moat system (W3), pH conditions underwent significant change, from slightly alkaline in the first phase of its development to slightly acidic in the following phases. Those changes are associated with primary human-induced eutrophication and water acidification after ringfort abandonment (Kittel et al., 2018a; **Figure 4**). The K, OM, and wood positively shaped Chironomidae assemblages in the first phase of moat development, during human settlement in the ringfort (Kittel et al., 2018a). Taxa typical of permanent stagnant, partly overgrown water bodies (e.g., *Paratanytarsus penicillatus*-type, *Glyptotendipes pallens*-type and *Cricotupus intersectus*-type) were dominant. Mining chironomids were represented mainly by *Endochironomus tendens*-type and *Glyptotendipes pallens*-type.

In the group of eutrophic species that are mining several macrophyte species (as *Endochironomus impar*-type in Berg, 1995; Beiger, 2004; Moller Pillot, 2009), many just live in coarse organic detritus, without direct association with living plant tissue (Bijlmakers, 1983). Tóth et al. (2012) state that the above-mentioned chironomids are in fact mostly opportunistic in selecting macrophyte species, while the structure of vegetation is more important. They could also settle on decaying wood particles, bark and small branches. Among terrestrial plants, *Urtica dioica* is strongly correlated with chironomid community richness according to its independent effect (**Figure 6**). This is an indicator of wet, nitrogen-rich soil (Hill et al., 1999). *Urtica* is often found in swampy habitats, but also in great abundance in habitats where high trophic state is the result of human impact (Šrùtek and Teckelmann, 1998). Both *Urtica* abundance and Chironomidae species richness result from high trophic conditions in the moat (high OM and pH) (**Figure 6**). Potassium (K) that shaped communities with high significance, indirectly marks denudation processes associated with human influence on the moat, in its first stage of development (W3). The ringfort surroundings were strongly exposed to trampling, lithogenic elements were also moved from local gardens and fields (Kittel et al., 2018a). Denudation processes influenced moat bank habitats affecting chironomid communities even on a high taxonomic level. These processes are associated with the inwash of organic and inorganic suspension, including fine particulate organic matter. Thus, the filtrators and collector-gatherers were abundant. The moat history ends with a sudden decrease of Chironomidae abundance and its transformation into the semiterrestrial peaty habitat (low pH but high OM, K, and wood accumulation) (**Figure 4**). It is associated with the complete change of the conditions in the moat, which were no longer favorable for most chironomid taxa. The scarcity of water, acidification, and macrophyte composition transformation meant that only taxa adapted to such conditions could survive. Within the chironomid fauna of that time, there were single individuals of *Limnophyes-Paralimnophyes*, *Pseudosmittia,* and *Pseudorthocladius* (mentum type 15), along with some Chironominae (mostly collectors) typical of muddy bottoms of the retain ditch of the moat (Kittel et al., 2018a).

Whereas the samples from W3(2) are more scattered and arranged according to depth, the W4 samples are aggregated closely together in the ordination analysis (**Figure 4**). This results from the habitat conditions in the paleochannel (W4) during Late Weichselian, which were more uniform and stable for a long time than in the moat habitat. They reveal significantly lower importance of lithogenic elements (like K) than in W3(2), indicating that Medieval human settlement in the valley caused higher denudation than during Allerød-Younger Dryas transition. Lower trophic state in this case does not follow acidification like in W3(2) but comes from generally low OM content in the Lateglacial landscape (Birks and Birks, 2004). Initial plant communities (Feurdean et al., 2014) did not generate much wood substrate for the mining chironomid communities. The morphological traits varied across the $CaCO_3$ gradient from W3(2) to W4 mostly due to its high content in W4, as opposed to low content in W3(2). The influence of this variable on chironomids is associated with the periods of increased leaching from the shallow groundwater. During the Late Glacial oxbows in the regional river valleys were supplied by carbonate groundwater (Płóciennik et al., 2015; Pawłowski et al., 2016a). The water supply (precipitation, floods, and groundwater) is important for the biota composition in river valley wetlands. When groundwater seeps to the oxbows there appear taxa typical to cold, alkaline springs and brooks (Płóciennik et al., 2015; Pawłowski et al., 2016b).

Despite covering a similar timespan (ca. 600 years), out of both studied cores, the W3(2) sequence was far more ecologically diverse. Without doubt, human impact was crucial here. It changed both physical and chemical water composition, and thus also flora and fauna within the moat system and in its immediate vicinity (Kittel et al., 2018a). Ringfort inhabitants supplied the moat system with a significant amount of wood (mostly *Quercus* sp.), which served as a hard substrate for periphyton, including diatoms. This way, even if it was not a direct food source for the xylophiles, it created a suitable microhabitat for a range of trophic guilds, mostly scrapers. Such coarse organic matter is important not only for phytophilous grazers and filter feeders (such as *Glyptotendipes pallens*-type and *Dicrotendipes nervosus*-type) but also for collector-gatherers and shredders, represented here by larvae with mentum types 12 and 14 (*Polypedilum, Phaenopsectra,* and *Endochironomus*). Lower habitat variability and higher environmental stability (manifested here, e.g., by the narrow pH gradient) in the paleo-oxbow implied lower diversity of chironomid groups. In the W4 sequence, only 15 (out of 16) mentum types and 10 (out of 12) functional feeding groups were recorded (**Figure 3**).

Comparison of Typologies

It is not surprising that the results of ordination analyses based on three Chironomidae typologies are to some extent convergent. They reveal similar trends as they are based on the same data, though classified differently. However, mentum types and FFGs allow for a more detailed ecological interpretation than tribe classification. As feeding ecology strongly influenced mouthparts evolution, FFGs may aggregate taxa phylogenetically distant but having similar mouthparts (Armitage et al., 1995). Among the examples are Procladiini and *Cryptochironomus* with a similar, concave mentum, belonging to two different subfamilies, semiterrestrial Orthocladiinae representing different genera, and phytophile taxa from mentum types 10 and 12. The mentum shape fits feeding ecology across the taxonomic classification, as can be seen in the stratigraphic diagrams presented in **Figure 3**. The FFGs and mentum type stratigraphy are consistent, because habitat transformations cause changes in the available food – from suitable for P and G throughout G/C, C/F, and M typical of eutrophic, overgrown shallow water, to C and C/F living in wet moss and soil, and thus having a different food base. In fact, chironomid communities often consist of taxa with a similar type of mentum according to habitat conditions (pers. obs.).

Functional Groups as Habitat Indicators

Chironomidae functional traits have so far been studied mainly by Serra et al. (2016, 2017), who developed a trait database for

European genera, analyzed it for subfamilies and compared it with the North American database (USEPA, 2012). However, such a taxonomic level seems to be too general for several traits. Creating such a database for larvae morphotypes, commonly used in paleolimnology, is worth considering.

Birks (2020) indicates that the functional trait approach in plant paleoecology has several limitations. While pollen-based ecological information is generalized, as it often can be identified only to the family or genus level, plant macrofossil analyses are problematic because of mixed data types and the possibility of 'false absences' (Birks, 2014, 2020). Here, chironomid subfossils could play a significant complementing role. The functional groups approach seems to work well in microhabitat reconstructions. Chironomidae FFGs and mentum types correspond to several environmental factors, such as the substrate and vegetation type, as well as physicochemical conditions. Although dividing taxa into groups is itself a generalization and may lead to loss of some data, it is also an easier and more systematized method to obtain comparable results. As functional guilds work well in climate reconstructions (see Kivilä et al., 2019), why not use them in tracking changes in the habitat structure? The chironomids serve well at the morphotype level as indicators of habitat complexity in terms of plant richness and density (Langdon et al., 2010). In fact, their association with vegetation is generally established within FFGs and, most likely, also morphological groups.

In this study, we classified each recorded Chironomidae morphospecies to a morphological group. The groups were distinguished on the basis of mentum and ventromental plates, as these elements are usually preserved well in the sediment. Whereas this classification may be enhanced and complemented in the future, it may serve as a good surrogate for functional feeding groups in habitat reconstructions. However, we need to be aware that, while the mouthpart type is strongly associated with feeding behavior, it is not the only factor shaping it. Mouthpart morphology is associated with many aspects of species biology and ecology, so it is not as strong an indicator of habitat changes as feeding groups. Moreover, as food preferences may change throughout life of chironomid larvae, only the 3rd and 4th instars should be included in such analyses. In fact, head capsules of the 1st and 2nd instars of chironomid larvae hardly ever preserve well in the sediment. In the future, the feeding groups and morphological types should also be tested within the contemporary assemblages ranging through measured ecological gradients. It would give a picture of more direct linkages between chironomid larvae and their habitat.

CONCLUSION

The results indicate that Chironomidae FFGs and morphological types reveal similar reactions to the biotic and abiotic environmental factors. Thus, they could serve as surrogate indicators where applicable. A well-designed mouthpart typology would be an easy and systematized method to obtain basic results comparable with functional feeding groups. Chironomidae morphological types can be easily recognized, e.g., during plant or beetle macrofossil analysis, and pre-analyzed without knowing the exact ecology of each taxon. If such preliminary examination give promising results, further detailed analysis should be undertaken by a specialist. Such an approach would enable the implementation of Chironomidae analyses in a wider range of paleolimnological and archeological research. The trait-based approach in subfossil Chironomidae studies is worth developing and can prove useful in the future application in paleolimnology. The potential use of morphological types should be checked during further studies based on modern Chironomidae assemblages ranging through known ecological gradients.

To conclude, such simplified para-taxonomic analyses based on generalized morphological types never fully replace detailed species-specific autecological approach. However, in cases where a comprehensive chironomid study is not possible, the proposed method can serve as a useful tool to obtain some ecological information.

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DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

OA-O and MP conceived the study. RS-R, MR, and DO contributed paleobotanical and geochemical data. PK analyzed the geomorphology and paleogeography of the area and conducted the fieldwork at the study site. MK provided radiocarbon and dendrological dates. RS provided statistical analyses. OA-O, RS, and MP interpreted the data and wrote the manuscript with contributions from all authors. RS, OA-O, AM, and PK created the figures. JS, AM, and MS edited the final manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.583831/full#supplementary-material

Supplementary Figure 1 | Correlation matrices with Pearson's r correlation coefficients calculated for the W3(2) core: **(A)** all predictors and **(B)** predictors left for further analyses. For full predictor names, see **Supplementary Table 1**.

Supplementary Figure 2 | Correlation matrices with Pearson's r correlation coefficients calculated for the W4 core: **(A)** all predictors and **(B)** predictors left for further analyses. For full predictor names, see **Supplementary Table 1**.

Supplementary Table 1 | Variables for cores analyzed in the study. The data was standardized during analyses. Bolded variables were included in further analysis.

Supplementary Table 2 | Most supported (ΔAIC $<$ 2) models testing for impact of environmental factors on richness of chironomid groups in each typology.

Supplementary Table 3 | Impact of environmental factors used in averaged models on richness of analyzed chironomid groups (generalized linear regression).

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Functional Paleoecology and the Pollen-Plant Functional Trait Linkage

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Paleoecological investigations using the pollen-plant functional trait linkage are increasing in value as new insights to past ecological function and dynamics are revealed. These retrospective approaches link pollen sequences to plant functional trait measurements to reveal long-term changes in ecosystem properties that are difficult to resolve using traditional paleoecological methods. Despite these methodological improvements and the newfound perspectives, there has yet to be thorough testing of whether transforming pollen to ecological function tracks functional trait distributions in geographic space. We assess this in North America by linking surface pollen samples to measurements of three functional traits that represent major axes of plant ecological strategy. Pollen-derived estimates of function were first used to investigate occupied trait space at different scales. These estimates were used to reconstruct the latitudinal functional diversity gradient of North America, and results were compared to the continent's functional diversity gradient estimated from tree assemblages and gradients based on pollen richness and evenness. Results indicated that the patterns in pollen-based function sufficiently track ecological function in trait and geographic space and the macroecological biodiversity pattern was reconstructed, although there were minor differences between the slopes of the functional diversity and each of the pollen index gradients. Taken together, the outcomes of our investigation indicate reliability in extending the pollen-plant functional trait linkage into deeper time, at least for examining North American functional dynamics.

Keywords: ecological function, functional diversity, latitudinal functional diversity gradient, paleoecology, plant functional traits, pollen-derived function, pollen-plant functional trait linkage, trait space

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INTRODUCTION

Sediment-based sub-fossil pollen is frequently used as a proxy for local and regional changes in vegetation composition over time (Faegri and Iversen, 1989). The application of pollen in paleoecological analysis has been to categorize "who was there" through compositional changes and presence and absence in time (Dodd and Stanton, 1990), with infrequent interpretations of changes in ecosystem function. Yet, these data contain untapped potential. A new tool emerging in paleoecology focuses on measuring ecosystem function by applying ecological theory of modern plant floristics to paleoecological records. Ecosystem function (ecosystem property), or the biological, geochemical, and physical processes that take place within an ecosystem, are those that reflect the capacity of ecosystems to fulfill human needs (ecosystem service) (de Groot et al., 2002). Analysis of long-term functional responses to environmental change, by linking plant functional traits to fossil pollen assemblages, sheds new light on past ecosystem dynamics and provides complementary information to taxonomic-based analyses. Novel interpretations of past functional dynamics that are not typically obtainable from conventional paleoecological methods (Wingard et al., 2017) are increasing in value as a consequence of making the pollen-plant functional trait linkage.

Plant functional traits are defined as any morpho-, physio-, and phenological plant characteristics affecting overall plant fitness through their influence on survival, growth, and reproduction (Violle et al., 2007). These plant characteristics are biological adaptations to the environment and represent fundamental tradeoffs between individual species, as they direct the acquisition, processing, and investment of resources in plants (Chapin et al., 2000). Variations in functional traits impact ecosystem properties (e.g., biologic diversity, cycling of nutrients, soil characteristics) (Hooper et al., 2005) as species respond to and are filtered by climate, disturbance, and biotic interactions (Lavorel and Garnier, 2002). Plant functional trait data, in the form of species-level trait measurements, are increasingly accessible from large databases, such as the Botanical Information and Ecology Network (BIEN Database[1]; Enquist et al., 2016) and the TRY Plant Trait Database[2] (Kattge et al., 2020). Conceptual developments in functional trait theory have led to the identification of a wide variety of plant traits linked to various aspects of ecosystem function and processes (Pérez-Harguindeguy et al., 2013).

Early plant functional trait theory identified three traits as the major axes of plant strategies governing ecosystem function (i.e., plant height, seed mass, leaf area) (Westoby, 1998). These indicators of plant fitness were recently found to occupy primary axes of global plant form and function (Díaz et al., 2016) and were used to identify the latitudinal functional diversity gradient (Lamanna et al., 2014). Tall plant heights are advantageous for dispersing seed and intercepting light (Gaudet and Keddy, 1988; Westoby, 1998; Westoby et al., 2002; Muller-Landau et al., 2008; Moles et al., 2009), although construction and maintenance costs are incurred along with higher risks of breaking (Niklas, 1993; Poorter et al., 2008). High leaf area is advantageous for increasing photosynthetic capacity through capturing light at the expense of increased construction costs and produces large boundary layers responsible for reducing transpiration rates and thus heat exchange and carbon dioxide diffusion to the surrounding air (Díaz et al., 2016). Low seed mass is advantageous for dispersal and colonization ability. Seeds with low weights are generally produced in large numbers that can be dispersed farther, thus allowing for establishing a persistent seed bank, and conversely, high seed mass is advantageous for surviving early recruitment stages and may offer a passive dispersal advantage depending on if and whether fruit is formed (Leishman and Westoby, 1994; Westoby et al., 2002; Muller-Landau, 2010; Thomson et al., 2011; Ben-Hur et al., 2012).

The central tenet of functional analysis is that ecosystem properties can be evaluated through the procedure of classifying plant species by their functional characteristics, information typically unavailable when examining shifts in taxonomic abundances alone (Diaz and Cabido, 2001). Classifying species by their functional characteristics has primarily been an exercise of ecological studies based on direct observation and botanical surveys (e.g., Mccormack et al., 2012; Enright et al., 2014) although the application of plant functional trait theory to remotely sensed imagery is growing (Jetz et al., 2016). In paleoecological contexts, plant functional traits have been previously linked to pollen records to model macro-scale changes in past terrestrial ecosystems making use of broad taxonomic classifications identifying plant functional types (Prentice et al., 1996; Williams et al., 2001, 2004; Marchant et al., 2010; Ni et al., 2010). Functional paleoecology, although in its infancy, links plant functional traits to pollen taxa and transforms pollen percentages (i.e., relative abundances) to represent ecological function variations through space and time. Doing so helps to bridge the gap in paleoecological and neontological analysis (Fritz et al., 2013) and can provide new perspectives of past ecosystem change resulting from biotic and abiotic interactions at various spatial and temporal scales.

Although currently sparse, the field of functional paleoecology is growing. Recent applications have shown the effectiveness of making the pollen-plant functional trait linkage, primarily assessing functional responses to climate variations. Evaluating modern geographic trait distributions related to climate in the European Mediterranean region, Barboni et al. (2004) showed plant functional traits (e.g., life form: tree, shrub; leaf traits: type, size, waxiness; plant traits: leaf phenology: thorniness, phenology) were related to either plant water availability or mean temperature of the coldest month, or a combination of the two. Knight et al. (2020) showed pollen assemblage responses to changing climate were highly variable through the Quaternary in eastern North America and high seed mass within assemblages generally resulted in more matches with climate. Methods for estimating long-term functional trait compositions in fen peatland communities were developed based on the best-fit linear mixed-effects modeling of leaf traits linked to modern pollen samples that acted as a filter for extending back in time (Carvalho et al., 2019). Neogene and Quaternary tree species in eastern North America were found to occupy similar albeit more densely packed trait space compared to Europe despite greater species richness (Ordonez and Svenning, 2018). Within the same time periods, Ordonez and Svenning (2020) developed a conceptual framework for understanding the legacies of environmental changes on ecosystem processes; species diversity variations via response traits were shown to have the potential to impact effect traits that govern ecosystem processes. A regional study on the relationships between pollen-derived function, climate, and human influences in Estonia and Latvia showed cold late-glacial climates were associated with low plant heights as well as small seeds and low leaf area (Reitalu et al., 2015). The transition to a progressively warmer Holocene climate

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[1] http://bien.nceas.ucsb.edu/bien/

[2] https://www.try-db.org

led to taller vegetation with larger seeds and greater leaf area and consequently, functional diversity increased through time.

Functional relationships to disturbances have also been investigated. An analysis of functional trait variations in the Pacific Northwest, United States showed how increased Holocene fire activity led to changes in community-level fire adaptation and structure (Brussel et al., 2018). Site-level functional diversity and overlap estimates provided evidence of an ecosystem shift from seral to stable aspen (Morris et al., 2019). In the Amazon, the effects of precipitation variation and human-caused environmental changes on community traits were estimated and indicated plant functional traits responded more clearly to drivers than pollen taxa, and different anthropogenic disturbances, e.g., erosion and fire, increased the dominance of distinct functional traits (van der Sande et al., 2019).

The utility of making pollen-plant functional trait linkages requires evaluating how well transforming pollen data to ecological function captures the spatial distribution of modern functional traits. The overarching question we work to address is, does the pollen-plant functional trait linkage represent ecological function in North America? We test the linkage by integrating surface pollen samples from the Neotoma Paleoecology Database (Williams et al., 2018) and plant functional traits from the BIEN Database (Enquist et al., 2016). Functional trait data representing primary axes of plant strategy are used to assess spatial relationships between the pollen-plant functional trait linkage (i.e., estimates of site-level function via individual traits) and the functional trait data used to inform these pollen-derived values. We then use these functional estimates from North American sites to quantify functional diversity, the biodiversity metric that governs ecosystem function, and therefore the ability of ecosystems to provide services for humans (Diaz et al., 2007). To present a macroecological example of the utility of making the pollen-plant functional linkage, we compared pollen-based functional diversity estimates to those from modern tree assemblages in the form of latitudinal functional diversity gradient, a pattern found to be embedded within the latitudinal biodiversity gradient for the western hemisphere (i.e., North and South America) (Lamanna et al., 2014). We hypothesize that pollen-derived functional diversity estimates will corroborate the recently observed latitudinal gradient in functional diversity of North America, as there is a non-linear relationship between plants and pollen. The pollen-based functional diversity gradient is next compared to gradients in the richness and evenness of pollen taxa through latitude, as these correspond to measures of the number and distribution of species abundances, respectively. We then briefly discuss potential avenues for investigating biogeographic questions that are difficult to resolve by employing traditional paleoecological methods.

MATERIALS AND METHODS

Pollen Data and Plant Functional Trait Measurements

The Neotoma Paleoecology Database (accessed November 2019) was queried for North American surface pollen samples using the R package “neotoma” version 1.7.2 (Goring et al., 2015; Williams et al., 2018) returning pollen sequences for 3033 sites. Each pollen count dataset with standard taxonomies was converted to pollen percentages for tree and herb taxa. Sample density was low in Mexico, west of the United States’ Rocky Mountains, the west coast of Canada and southern Alaska. A request for trait measurements of leaf area, plant height, and seed mass from the BIEN Database (R package "bien" version 4.1.1, Enquist et al., 2016; Maitner et al., 2017) returned over 10 million standardized trait observations. The list of species with trait measurements was restricted to only those that are native to continental North America (Mexico, United States, and Canada) in a multi-step process. A list of standardized plant species native to the United States and Canada was obtained from the PLANTS Database (USDA and NRCS, 2019) and species lists of native and introduced plants of Mexico were obtained (Villaseñor and Espinosa-Garcia, 2004; Villaseñor, 2016), which were then combined. Plants of the World Online[3] was consulted to reduce the list of species with trait measurements to only those plant species that are native to the entire continent. If a species was listed as introduced to Mexico, but native to the United States or Canada, the species was retained. Conversely, if a species was listed as introduced to one of the countries but not designated as a native species in one of the others, it was removed from the list. The final data set for North America included 9,487,396 plant height measurements from 2,146 species, 13,103 leaf area measurements from 1,016 species, and 16,621 seed mass measurements from 3,580 species.

Pollen Taxa X Species Functional Trait Matrix

Following the identification of functional trait measurements for plant species native to North America, the species within the list were then linked to their respective pollen taxonomies (i.e., species-, genus-, and family-level pollen types) by constructing a species with trait measurements X pollen taxon matrix. The “taxize” R package version 0.7.5 (Chamberlain et al., 2016) allows for retrieving both higher and lower standardized taxonomies by accessing different databases that house these datasets, and so the National Center for Biotechnology Information (NCBI) and Integrated Taxonomic Information System (ITIS) databases were employed to link individual species to their respective taxonomies. The surface pollen dataset contains pollen grains varying in taxonomic resolution. Some pollen types are identified to the species level and others to the pollen-type level, such that they could be linked to multiple species (e.g., *Acer spicatum, Acer* undifferentiated). We opted to retain the original pollen taxonomy as identified by pollen analysts that were submitted to the Neotoma Paleoecology Database (Williams et al., 2018), opposed to harmonizing pollen taxa to standardize pollen types (see, e.g., Giesecke et al., 2019). This makes the assumption pollen analysts use an informed approach to identify pollen types to the species level (e.g., local species presence surrounding a depositional environment; a regional absence of any other species; geographic context). Doing so maximizes the amount

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[3] http://plantsoftheworldonline.org

of data that was used to inform trait value estimates while also estimating the most precise trait values.

Trait measurements were log-transformed and scaled to normalize and standardize distributions. We then assigned values for the three traits to each pollen taxon. As the majority of pollen taxa are associated with multiple observations in the BIEN Database, due to repeated measurements or from linking lower taxonomic resolution pollen taxa to species names, we represent the possible range of functional trait values for each taxon as the mean and standard deviation of all associated values. Remarkable efforts have been put forward to catalog plant functional traits and changes in these through time (Meeus, 2018; Meineke et al., 2018; Kissling et al., 2019). However, and because there has yet to be a comprehensive list of measurements for species-level functional traits across different geographic localities compiled, we assume these values are stationary across space. The result was a list of pollen taxa and the mean and standard deviation of their trait values (**Supplementary Table 1**).

Functional Diversity Estimates

Functional diversity estimates were calculated using multidimensional hypervolumes that were based on the set of trait values for each pollen taxon at each surface pollen site using box kernel density estimations in the R package "hypervolume" version 2.0.8 (Blonder et al., 2018). These estimates quantify the range of occupied trait space of a set of functional traits at each pollen sample, consistent with a measure of functional richness (Villéger et al., 2008) and correspond to a realized niche (Hutchinson, 1957). To quantify accurate representations of functional diversity on the landscape, we opted to use only surface sample sites that had all traits quantified for no less than five pollen taxa (i.e., each pollen taxon had a trait value estimated in earlier steps), and from these, only those taxa with pollen percentages greater than 0.5%. Retaining only those pollen taxa that had estimates for each trait and sites that had no less than five pollen taxon level functional estimates strives to represent accurate site-scale functional diversity estimates. The threshold is used to restrict pollen species that may not be present on the landscape from diversity estimates. To represent the uncertainty in the trait assignments, we used a Monte Carlo approach, where at each site, we resample a trait value for each taxon from a normal distribution defined by the mean and standard deviation described above, and then calculate the hypervolume of each surface sample. This is then repeated 500 times with the result as pollen-derived, occupied trait space at each site measured in standard deviations (Blonder et al., 2018).

The set of functional diversity estimates were then gridded at a 0.5° resolution by taking the median values of the full set of Monte Carlo estimates for all sites within each grid cell, resulting in 828 gridded functional diversity estimates. Doing so minimizes variability that may have arisen from inconsistent pollen sampling spatially and taxonomically. These gridded functional diversity estimates were then used to reconstruct the latitudinal functional diversity gradient (Lamanna et al., 2014) by estimating a linear model against latitude (**Supplementary Table 2**).

Comparisons: Pollen-Derived Function and BIEN Data; Latitudinal Functional Diversity Gradient and Pollen Indices

To identify the capacity of the pollen-plant functional trait linkage to accurately represent ecological function, we compared the occupied trait space (i.e., range of functional estimates) of those sites that had hypervolumes quantified, the subset of plant species that are native to North America from the BIEN Database (Enquist et al., 2016) that were used for informing functional diversity estimations, and also just the BIEN Database's North American tree species. We also made comparisons between the North American latitudinal functional diversity gradients estimated from the full set of terrestrial pollen taxa and tree assemblages (Lamanna et al., 2014), and pollen-based richness and evenness gradients. Constituents of the functional diversity gradient of the western hemisphere, the estimates from tree assemblages were quantified based on the presence of tree species with diameters at breast height > 10 cm in 0.1 ha plots (see Lamanna et al., 2014 for their detailed methods). We limited their dataset to only functional diversity estimates of North America. Evenness was calculated as the slope of the rank-order versus log-abundance curve for each surface pollen sample, and richness was calculated using rarefaction to 500 pollen grain counts (Giesecke et al., 2014). Each surface sample's richness and evenness estimates were gridded as previously described and all pollen-based estimates were mapped against the continent's Level 1 ecoregions (Omernik, 1987). Generalized least squares regression was used to estimate all latitudinal gradients, including trends in the traits themselves. An exponential spatial covariance function was incorporated into each model to account for spatial dependency (**Supplementary Table 2**). **Table 1** reports the model coefficients and the results of Moran's I tests for residual spatial autocorrelation. Pollen-based functional diversity, richness, and evenness were correlated using Pearson's product-moment correlation with the R package "Hmisc" (Harrell, 2004).

RESULTS AND DISCUSSION

Comparison of Pollen-Based Trait Space and Ecological Datasets

As a first test of the pollen-plant functional trait linkage, we compare the trait space of North America derived from pollen samples to the full list of BIEN trait measurements for native species and a subset of the measurements for native trees (Enquist et al., 2016). Compared to the full list of native BIEN species functional trait measurements, all three aspects of pollen-based function occupy the upper ranges of the BIEN data. The pollen-plant functional trait linkage collapses the spread of interquartile ranges of all three traits with few outliers (**Figure 1**). As related to a subset of BIEN Database tree species, pollen-derived values for leaf area were lower and seed mass values were matched more similarly. We thus posit linking surface pollen samples to plant functional traits reconstructs ecological function, such that the method may be carried forward for further analysis and into recent geologic time. Further, these results allow for an ability

TABLE 1 | Outcomes of generalized least squares models of functional traits, functional diversity, and pollen evenness and richness against latitude in North America.

	Leaf area	Plant height	Seed mass	Functional diversity	Evenness	Richness
Intercept	−0.024	−0.526**	1.541***	137.563***	9.189***	30.913***
Latitude	0.003	0.005	−0.029***	−1.251***	−0.065	−0.285***
Moran's I	0.001	−0.055	−0.012	−0.04	−0.008	−0.028

$^{**}p < 0.01$; $^{***}p < 0.001$.

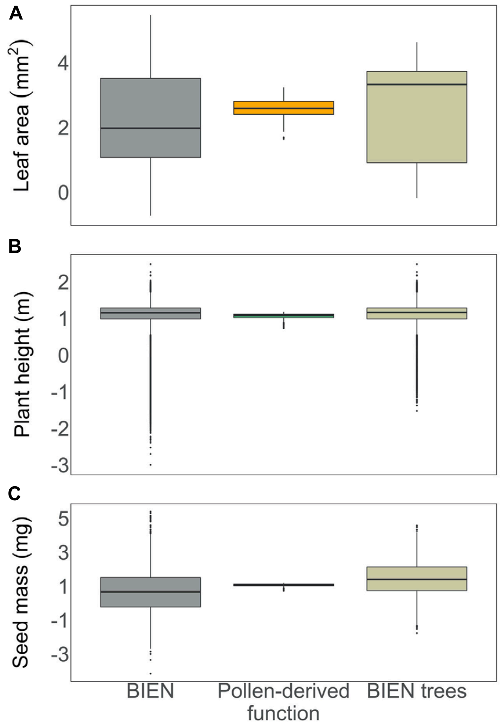

FIGURE 1 | Comparisons between the trait spaces of leaf area **(A)**, plant height **(B)**, and seed mass **(C)** from the list of trait measurements for species native to North America from the BIEN Database (Enquist et al., 2016), surface pollen samples (Williams et al., 2018), and native tree species (Enquist et al., 2016). Ranges of functional estimates from the pollen-plant functional trait linkage were generated using median trait values in each 0.5° × 0.5° grid cell. All traits were log-transformed. See **Supplementary Table 1** for pollen-based trait values.

to examine the dynamics of ecological function in addition to taxonomic composition.

Spatial Patterns of the Pollen-Plant Functional Trait Linkage

The distribution of pollen-derived ecological function in geographic space is more complicated, but a useful comparison can be made at the scale of ecoregions (Omernik, 1987). Leaf area values were highest in the Eastern Temperate Forest and the eastern margins of the Great Plains (**Figure 2A**). Leaves with the smallest area were found primarily in the southern ranges of Northwestern Forested Mountains and the Marine West Coast Forest, ecoregions dominated by coniferous trees, and North American Deserts, where small leaves are advantageous for limiting water loss during transpiration. Pollen-derived leaf area was intermediate in high latitude ecoregions, where temperate coniferous trees dominate and smaller leaf area would be expected.

The pollen-plant functional trait linkage indicated most tree vegetation occupies the mid- to upper- ranges of pollen-derived plant height trait space (**Figure 2B**). The shortest plant heights were found generally at the southern extent of the Eastern Temperate Forest and northern Alaska. The general consistency of plant height through geographic space in North America suggests competition for light is generally low. A different climatic forcing, potentially water availability through precipitation in the wettest month, may govern the spatial distribution of plant heights in North America (Moles et al., 2009).

The continent's spatial distribution of seed mass estimates from pollen showed a largely coherent latitudinal trend (Moles and Westoby, 2003) (**Figure 2C** and **Supplementary Figure 1**), as this was the sole pollen-based functional trait to decline significantly with movement toward the pole (**Table 1**). Heavy seeds were found at southern latitudes, and with movement to the north, seeds became lighter across all ecoregions. Plants bearing seeds with light weights are expected to invest resources for producing large numbers of seeds that can disperse farther distances and also develop persistent seed banks. Low seed mass values at high latitudes may suggest environmental controls (e.g., the timing of spring snowmelt, poor soil conditions, and frequent fires) influence plants to establish and grow rapidly once an opportunity arises. Greater seed weights in southern latitudes indicate longer-term investments to capitalize on canopy openings when opportunities arise.

Post-glacial Dispersal as an Interpretation for the Spatial Patterns of Seed Mass

Many examples have shown individual tree species migrated from southern latitudes at different rates in post-glacial North America and Europe (Davis, 1976; Cwynar and MacDonald, 1987; Webb, 1987; Birks, 1989). It is generally accepted that periodic, long-distance dispersal characterizes colonization and range expansion events from the late-glacial to present (Cain et al., 1998; Clark, 1998). Considering this, the nearly systematic decline in seed mass through latitude (**Figure 2C** and **Supplementary Figure 1**) may be a result of functional selection that occurred from the late-glacial period to modern day, opposed to species selection. Cwynar and MacDonald (1987) showed directional selection for lighter seeds of *Pinus contorta* spp. *latifolia* as the species

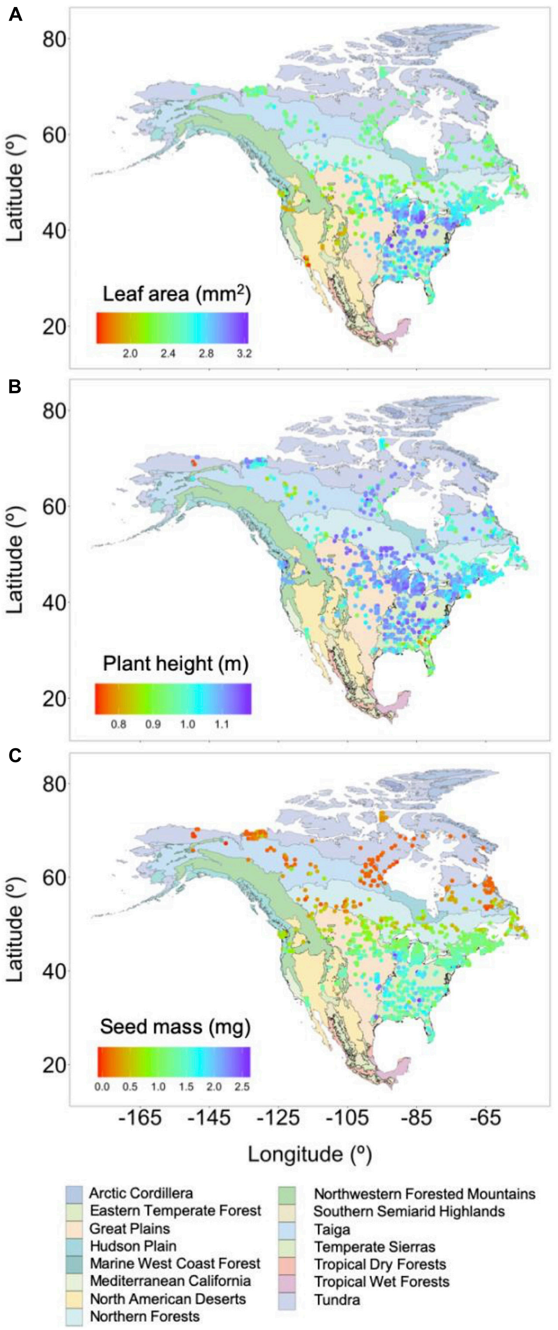

FIGURE 2 | Gridded and log-transformed leaf area **(A)**, plant height **(B)**, and seed mass **(C)** estimates from surface pollen samples against a backdrop of the continent's ecoregions (Omernik, 1987) with mean grid cell values colored on a log scale. Generalized least squares regression found the seed mass trend was significantly related to latitude.

migrated in post-glacial western Canada; as new populations established northward, allelic diversity decreased and seeds became more readily dispersible. With the modern distribution of seed mass in North America, and the results of Cwynar and MacDonald (1987), we provisionally suggest the consistent trend in seed mass may be a factor of directional selection, such that species with low seed weights at high latitudes may have been under the strongest selection for light seeds in post-glacial North America. To make this conclusion, though, would require continued phylogenetic investigations of post-glacial migrations (Abbott et al., 2000; Griffin and Barrett, 2004) yet the potential for range expansion and colonization via birds assisting in seed dispersal in post-glacial North America may be a constraint (Porter, 1984). Another potential cause for the seed mass gradient, which may be either independent of or entwined within the notion of functional selection, is the prospect of a macroscale driver such as temperature, precipitation, or insolation (Lacourse, 2009; Knight et al., 2020).

Macroecological Application Using the Pollen-Plant Functional Trait Linkage

We used estimates of pollen-based function to quantify functional diversity for reconstructing the latitudinal functional diversity gradient of North America. This macroecological pattern was recently found to coincide with the latitudinal biodiversity gradient and showed that functional diversity in the western hemisphere trees declined with increasing latitude, supporting environmental filtering theory (Lamanna et al., 2014). We hypothesized the functional diversity gradient estimated from surface pollen samples would exhibit a similar slope compared to that estimated from modern tree assemblages (Lamanna et al., 2014). Estimated from surface pollen samples, the latitudinal functional diversity gradient exhibited a stronger slope compared to the gradient estimated from tree assemblages (**Figure 3A** and **Table 1**, slope from surface pollen samples = −1.251, slope from tree assemblages = −0.2; Lamanna et al., 2014).

The discrepancies in slopes may result from several differences between the studies and datasets used. The reference study we made comparisons to Lamanna et al. (2014) quantified functional diversity based on trees sampled in 0.1 ha plots numbering 47 in North America from the latitudinal range of 15°–53° N with patchy longitudinal coverage. Our estimations were based on a wider set of terrestrial taxa (trees and herbs) that convey vegetation composition effectively sampled in larger areas (Jacobson and Bradshaw, 1981) from 3033 surface pollen samples that after gridding resulted in 828 functional diversity estimates ranging a broader latitudinal transect (25.25°–73.75° N). Individually or combinedly, these differences in estimating the functional diversity gradient may have resulted in wider occupied trait space in grid cells and thus a steeper functional diversity gradient slope. Including both tree and herbaceous pollen types may reflect site openness. Pollen-based trait values for functional diversity estimates and relationships between can be found in **Supplementary Table 1** and **Supplementary Figure 2**. We recommend the criteria employed herein for future investigations of functional diversity dynamics in paleocontexts.

Gradients in Functional Diversity and Pollen Species Richness, Evenness

Following comparisons of the latitudinal functional diversity gradients estimated from surface pollen samples and tree

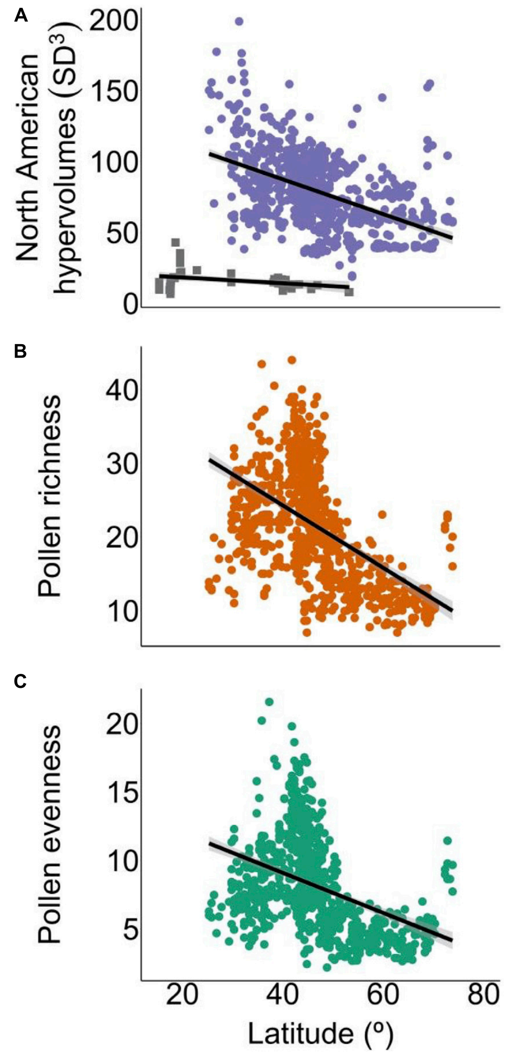

FIGURE 3 | The North American functional diversity gradient estimated from surface pollen samples (purple points, estimated in this study based on data from the Neotoma Paleoecology Database, Williams et al., 2018) and tree assemblages (gray points, Lamanna et al., 2014) **(A)**, with pollen indices of richness **(B)** and evenness **(C)**. Slopes of each gradient were identified using linear modeling (black lines). Regressions indicated the functional diversity **(A)** and pollen richness **(B)** gradients were significantly related to latitude.

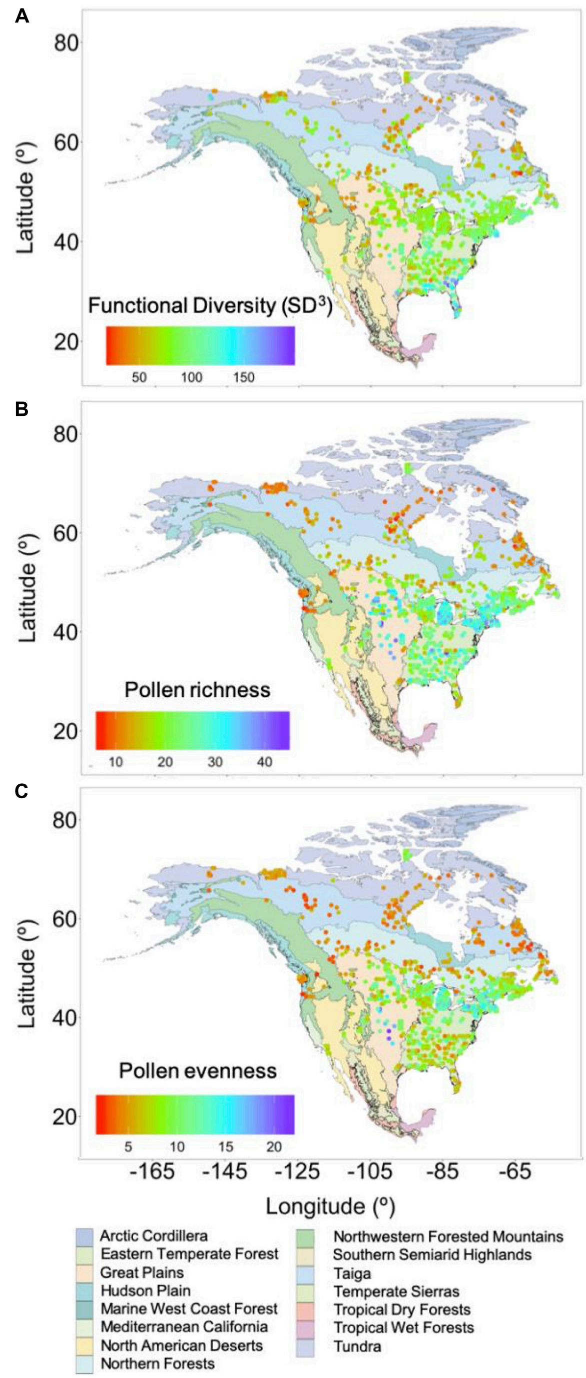

FIGURE 4 | Estimates of functional diversity **(A)**, pollen richness **(B)**, and pollen evenness **(C)** estimated from surface pollen samples presented as the mean values of all estimates within a grid cell plotted in relation to different ecoregions of North America (Omernik, 1987).

assemblages (Lamanna et al., 2014), we evaluated relationships between pollen-based gradients in functional diversity, richness and evenness (**Figures 3**, **4**). Pollen richness approximates the number of pollen taxa that would be expected using a standardized pollen count. Pollen evenness describes the potential dominance of pollen taxa within an ecosystem, with low evenness suggesting ecosystem function being driven by lower numbers of pollen taxa. Slopes of both pollen indices declined through latitude like that of the functional diversity gradient, suggesting ecosystem function is dominated by high species numbers at low latitudes and low species numbers at high latitudes (**Figures 3**, **4**). Surprisingly, while pollen richness had a significant relationship with latitude, pollen evenness did not, once spatial autocorrelation was accounted for **Table 1**. As the calculation of evenness incorporates pollen abundances, this may bias the result due to the non-linear representation of plant abundances.

Amongst the three pollen-based estimates, Pearson's product-moment correlation coefficients indicated significant but weak correlations between functional diversity, and pollen richness and evenness estimates (richness: $r = 0.36$, $P < 0.001$; evenness: $r = 0.31$, $P < 0.001$). These results are contrary to what might be presumed regarding species richness and correspondingly, the dominance of individual pollen taxa within ecosystems as they relate to the range of function within ecosystems along latitudinal gradients (e.g., Lamanna et al., 2014). Instead, weak correlations suggest there may be different drivers of each gradient, for instance, environmental constraints (Klopfer and MacArthur, 1961; Janzen, 1967; Kerkhoff et al., 2014) or competition (Dobzhansky, 1950; Tilman et al., 1997).

When looking to functional diversity estimates and their values in each ecoregion, significant differences in ecoregion's hypervolumes generally occurred between ecoregions with large geographic distance between them (**Supplementary Table 3**). Median functional diversity varied by ecoregion with the highest and lowest average hypervolumes found in Tropical Wet Forests (median hypervolume = 147.0) and Arctic Cordillera (median hypervolume = 45.4; the ecoregion with the most outliers was Eastern Temperate Forests that also had the highest number of surface samples (**Supplementary Figure 3**).

Future Directions Using the Pollen-Plant Functional Trait Linkage

The analysis of pollen-based functional trait space in principle would entail the use of georeferenced functional trait measurements, although there is a dearth of these datasets. As such, we estimated pollen-based functional traits and diversity based on all species that belong to a pollen taxon notwithstanding variation in functional trait variation through geographic space, which may influence these estimates (e.g., low variation in pollen-derived plant height). Future research may look to incorporate plant species distributions in large-scale (i.e., continental) analyses of ecosystem function, to constrain the trait measurements associated with any pollen assemblage.

Given suitable functional datasets, the pollen-plant functional trait linkage can be applied to capture enhanced explanations of ecological, evolutionary, and biodiversity dynamics, and place ecological theory based on modern plant floristics into the dynamic perspective of long-term analysis that was previously difficult to obtain. Many of these aspects are aligned with priority research questions for the paleoecological community (Seddon et al., 2014) and include complex community-level processes such as competition and succession, and also ecosystem processes like decomposition and biogeochemical cycling. Past ecosystem services can also be explored, such as the capacity to provide climate regulation, pollination, or soil stability. Gaining these new perspectives of long-term, ecosystem function changes provides a new route by which paleoecological analysis can contribute to sustainable conservations and management (Willis et al., 2007). The prospective assessment of functional responses to natural ranges of climate variability, disturbances, and anthropogenic pressure at varying spatiotemporal scales, especially in comparison to modern landscape changes, offers a new avenue of using the past to understand how the future of terrestrial ecosystems may unravel.

CONCLUSION

The focus of our investigation was to determine if pollen-derived aspects of ecological function track functional trait distributions in North America. Results showed that pollen-based function sufficiently tracks ecological function at the continental-scale on account of site-level estimates. At large-regional scales, the analysis shows strong linkages with pollen-inferred ecological function across a latitudinal gradient of functional diversity, which also followed the latitudinal trends of pollen richness and evenness. When considered with the robust interpretations of functional paleoecological analyses to date, our results indicate the transformation of pollen abundances to ecological function can be extended to deeper time, at least for North American functional dynamics. Making pollen-plant functional trait linkages allows for new interpretations of spatiotemporal biogeographic dynamics beyond the observational record. Functional paleoecology provides new perspectives that have great potential for placing the field into important discussions on how current and future ecosystems should be conserved and managed.

DATA AVAILABILITY STATEMENT

The surface pollen trait values (**Supplementary Table 1**) generated for this study are available at: https://doi.org/10.1594/PANGAEA.922040.

AUTHOR CONTRIBUTIONS

TB and SB designed and performed the research. TB led the analysis and writing. Both authors contributed to writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2020.564609/full#supplementary-material

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Searching for Function: Reconstructing Adaptive Niche Changes Using Geochemical and Morphological Data in Planktonic Foraminifera

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Dead species remain dead. The diversity record of life is littered with examples of declines and radiations, yet no species has ever re-evolved following its true extinction. In contrast, functional traits can transcend diversity declines, often develop iteratively and are taxon-free allowing application across taxa, environments and time. Planktonic foraminifera have an unrivaled, near continuous fossil record for the past 200 million years making them a perfect test organism to understand trait changes through time, but the functional role of morphology in determining habitat occupation has been questioned. Here, we use single specimen stable isotopes to reconstruct the water depth habitat of individual planktonic foraminifera in the genus *Subbotina* alongside morphological measurements of the tests to understand trait changes through the Middle Eocene Climatic Optimum [MECO: ∼40 Myr ago (mega annum, Ma)]. The MECO is a geologically transient global warming interval that marks the beginning of widespread biotic reorganizations in marine organisms spanning a size spectrum from diatoms to whales. In contrast to other planktonic foraminiferal genera, the subbotinids flourished through this interval despite multiple climatic perturbations superimposed on a changing background climate. Through coupled trait and geochemical analysis, we show that *Subbotina* survival through this climatically dynamic interval was aided by trait plasticity and a wider ecological niche than previously thought for a subthermocline dwelling genus supporting a generalist life strategy. We also show how individually resolved oxygen isotopes can track shifts in depth occupancy through climatic upheaval. During and following the MECO, temperature changes were substantial in the thermocline and subthermocline in comparison to the muted responses of the surface ocean. In our post-MECO samples, we observe restoration of planktonic foraminifera depth stratification. Despite these changing temperatures and occupied depths, we do not detect a contemporaneous morphological response implying that readily available traits such as test size and shape do not have a clear functional role in this generalist genus. Modern imaging measurement technologies offer a promising route to gather more informative morphological traits for functional analysis, rather than the traditional candidates that are most easily measured.

Keywords: functional trait, ecological niches, paleoclimate, paleoecology, planktonic foraminifera

INTRODUCTION

Existence in an ecosystem implies importance. Abundance estimates and extinction rates are key measures used to monitor efforts to conserve species but counting alone cannot accurately demonstrate the health of an ecosystem and the species within it (Akçakaya et al., 2020). To be present in an ecosystem an organism must have a role, and thus a function (Jax, 2005), which is fluid through time, space and biota (Akçakaya et al., 2020). The key is therefore not to merely note an organism's presence but to understand the traits that allows it to function within an ecosystem such as pollinator body and face hairiness which aids pollination (Stavert et al., 2016) or wing length which influences foraging distance (Brousseau et al., 2018). Functional loss through species extinction can be undetectable, if another species has the same or a similar function, or non-linear and saturating (Cardinale et al., 2012), with the smallest of functions having large impacts on ecosystem health (Akçakaya et al., 2020). But function is not yet used effectively in conservation. For example, functional rarity is highest for species not identified at risk on the IUCN red list in global coral reefs (Grenié et al., 2018) and simulations based on Californian bee populations show the most functionally efficient and important bees (from an analysis of 12 species/genera) are most at risk of extinction (Larsen et al., 2005). Therefore, identifying and understanding function is of paramount importance.

Simply described, a function typically describes some form of ecological process (Farnsworth et al., 2017) yet applying this definition in ecology has led to much ambiguity (Jax, 2005; Farnsworth et al., 2017). In modern ecosystems, assigning function to a trait can be done through observation and experiments with direct reference to human requirements and usefulness (Mace et al., 2014). *In situ* observations are one dimensional, however, and if the past is the key to predicting the future (Tierney et al., 2020), then we need to test modes of inference of how organisms lived thousands to millions of years ago and we need to be able to integrate modern and fossil functional indices. In the fossil record, direct observations of physiology and ecological function are typically not possible, so we are often left instead to infer an organism's functional role in its community from the preserved morphological traits. Such traits may therefore be a viable currency to "bind the past and present together" (Eronen et al., 2010). Here, we relate morphological traits in fossilized planktonic foraminifera to inferred depth habitats, derived from geochemical measurements, through climatic change during the middle Eocene (∼48 to 38 Ma).

We define a trait as any morphological, physiological, phenological or behavioral feature measurable at the individual level (Violle et al., 2007). Traits are the avenue through which an organism interacts with its environment (biotic and abiotic) (Lacourse, 2009; Oksanen et al., 2019) and determine whether an organism survives or meets its demise in a changing environment (McGuire and Lauer, 2020). Thus, traits capture the most important aspects of the environment, and consequently it is the trait and not taxonomic identity that is crucial (Lacourse, 2009). For a trait to be classed as functional, it must, directly or indirectly, impact individual performance and fitness of species (McGill et al., 2006).

Taxon-free traits are a specific class of traits that transcend taxonomic classification and provide a commonality to allow comparisons across communities in different climatic and geographical settings (McGill et al., 2006). Often these traits are phenotypic such as dental morphology (e.g., Renaud et al., 2005; Žliobaitë et al., 2016; Oksanen et al., 2019; McGuire and Lauer, 2020), overall body geometry (Bregman et al., 2014; Pimiento et al., 2017; Macumber et al., 2020; Antczak-Orlewska et al., 2021; Di Martino and Liow, 2021) and, in our study system planktonic foraminifera, test morphology (Baumfalk et al., 1987; Huber et al., 1997; Renaud and Schmidt, 2003; Schmidt et al., 2004, 2006; Payne et al., 2012; Rego et al., 2012; Weinkauf et al., 2014, 2019; Weiner et al., 2015; Brombacher et al., 2017; Kucera et al., 2017). Traits can also include ecological traits like habitat (Bregman et al., 2014; Pimiento et al., 2017) and feeding behavior (Bregman et al., 2014). Despite morphological traits being collected in abundance (Parr et al., 2012), trait based research on fauna is reduced compared to flora (Lavorel and Garnier, 2002; Lacourse, 2009; Steinthorsdottir et al., 2016; Fried et al., 2019; Birks, 2020), which has resulted in a plethora of plant-based data bases [e.g., TRY (Kattge et al., 2020); LEDA (Kleyer et al., 2008) and BIEN (Enquist et al., 2016)]. One reason for this discrepancy, is that faunal morphological traits were collected before the birth of trait-based ecology and are therefore not located in easy-to-access databases and require advancements and applications of text mining tools (Parr et al., 2012). Another fundamental issue is that plants make up 81% of global biomass compared to the 0.73% made up by protists (including planktonic foraminifera) and 0.37% made up by animals (Bar-On et al., 2018), meaning flora is more accessible for research. One promising approach then is to apply trait-based methods in the geological record to understand faunal responses to environmental change.

We have the clearest indication of the impact of extinction on species richness/taxon counts in the deep time fossil record, but assignment of a functional trait in extinct species implies some knowledge of the environment (Violle et al., 2007), as well as an observation of how the trait reacts to climatic fluctuations (Eronen et al., 2010). This correlative relationship allows us to infer the (biotic and abiotic) environment from the traits observed, or vice versa if we wish (Eronen et al., 2010). If function only exists within the context of the broader community, then this inference is particularly challenging and prone to inconsistent extrapolation. Ideally, we should measure traits with an inferred ecological function alongside an environmental indicator that indicates habitat, and then seek to match the two signals.

Our goal in this study is to understand trait changes within the context of a broader community undergoing large-scale abiotically induced change. We present new morphological and geochemical data on planktonic foraminifera. Planktonic foraminifera are extant, holoplanktonic, single celled organisms that build calcite shells (tests) that, upon death, rain down in abundance to the seafloor contributing significantly to deep-sea biogenic carbonate (Vincent and Berger, 1981). Foraminifera tests provide a near continuous, spatially and temporally high-resolution fossil record dating back ∼200 Myrs to the Jurassic (Fraass et al., 2015). Additionally, planktonic foraminifera are currently represented by ∼50 extant species (Kucera, 2007) meaning specimens can be cultured in the lab (e.g., Bé et al., 1981, 1982; Bijma et al., 1990; Spero and Lea, 1993; Henehan et al., 2017; Burke et al., 2018; Fehrenbacher et al., 2018; Holland et al., 2020), yielding an ever-growing knowledge of micro and macroscale influences on foraminifera life-history. In addition, our understanding of what are functional traits in foraminifera is increasing. Some traits have obvious function such as spines for feeding (Hemleben et al., 1991) or the presence of symbionts (Bé et al., 1977, 1981, 1982; Takagi et al., 2019). Recent studies, building on previous hypotheses, have shown that pores on foraminifera tests may be functionally linked to gas exchange (Bé, 1968; Baumfalk et al., 1987; Burke et al., 2018) but are only measured reliably from the inside of the test requiring scanning electron microscope (SEM) images or micro-CT scanning (Constandache et al., 2013; Burke et al., 2018). In contrast, traits such as test shape and size must have a function, but what those functions are remain highly debated (Renaud and Schmidt, 2003; Caromel et al., 2014; Burke and Hull, 2017).

A high-resolution fossil record, advances in morphological measurements and increasing knowledge of function mean planktonic foraminifera are an ideal candidate to investigate trait-based responses to environmental change in the geological record. Furthermore, geochemical methods are advancing at an astounding rate making ecological inferences such as life history, metabolic rate, gene flow and geolocations accessible in deep time (Trueman et al., 2016). In planktonic foraminifera these advances have allowed geochemical measurements to be taken at the level of the individual, enhancing our knowledge of paleoclimate (Thirumalai et al., 2013; Schmitt et al., 2019; Glaubke et al., 2021) and the impact of individual planktonic foraminifera ecology on geochemical signatures (Eggins et al., 2003, 2004; Friedrich et al., 2012; Fehrenbacher et al., 2018; Groeneveld et al., 2019; Weinkauf et al., 2020). We leverage these advances in analytical techniques alongside the exemplary evolutionary record of planktonic foraminifera to investigate trait, organismal and community responses to climatic change on geological timescales. Here, we focus on planktonic foraminifera trait changes across a transient warming event known as the Middle Eocene Climatic Optimum (MECO).

The Eocene was a time of global climatic and biotic restructuring. Following the "Hothouse" interval from the Paleocene-Eocene Thermal Maximum [PETM; 56 Ma (Westerhold et al., 2020)] through the Early Eocene Climatic Optimum [EECO; 48 Ma (Westerhold et al., 2018, 2020)], global climate gradually cooled (Zachos et al., 2001, 2008; Cramer et al., 2009). This long-term cooling trend culminated at the Eocene-Oligocene Climatic Transition [EOT; 34 Ma (Westerhold et al., 2020; Hutchinson et al., 2021)] with the onset of large-scale glaciation on Antarctica (Zachos et al., 1996; Coxall et al., 2005). The early to middle Eocene is punctuated by multiple short-lived (∼40–200 kyrs) transient global warming events or "hyperthermals" (Westerhold et al., 2020). During the middle Eocene there was a ∼270–500-kyr transient warming event known as the Middle Eocene Climatic Optimum that interrupted the long-term cooling trend (Bohaty and Zachos, 2003; Bohaty et al., 2009; Rivero-Cuesta et al., 2019; Edgar et al., 2020). The MECO was recognized by a progressive shift to lower deep-sea $\delta^{18}O$ records between ∼40.6 and 40 Ma followed by an abrupt return to higher $\delta^{18}O$ values (Bohaty et al., 2009). Interpretations of this shift suggest a gradual ∼3–6°C increase in surface and deep waters (Bohaty and Zachos, 2003; Bohaty et al., 2009; Bijl et al., 2010; Cramwinckel et al., 2019; Henehan et al., 2020) followed by a rapid 200-kyr cooling to pre-excursion temperatures (Bohaty et al., 2009). This upheaval is the pre-cursor to the restructuring of planktonic foraminifera communities in the proceeding 6 Myrs, which included the progressive loss of characteristic Eocene surface dwellers that were host to algal photosymbionts (Wade, 2004; Ezard et al., 2011; Fraass et al., 2015), a reduction in morphological (Schmidt, 2004) and assemblage complexity (Schmidt, 2004; Wade and Pearson, 2008). We use the MECO as an exemplar to test for functional shifts amongst the contemporaneous planktonic foraminifera community.

MATERIALS AND METHODS

Material and Sample Preparation

Samples were taken from two scientific drillholes on the North Atlantic Southeast Newfoundland Ridge in the northwest Atlantic Ocean, ∼700 km east-southeast of Newfoundland, which were cored during International Ocean Discovery Program (IODP) Expedition 342 at Sites U1408 (41°26.3′N, 49°47.1′W) and U1410 (41°19.6993′N, 49°10.1847′W) (Norris et al., 2014). The material collected from these sites is dominated by clay-rich drift sediments that were deposited at seafloor depths well above the average late Paleogene carbonate compensation depth (CCD) (Norris et al., 2014; Boyle et al., 2017). The clay-rich lithology resulted in good to excellent preservation of carbonate microfossils including foraminifera (Norris et al., 2014; Boyle et al., 2017), with most specimens appearing "glassy" under light microscope observations indicating little or no diagenetic alteration of the calcite (Sexton et al., 2006a). Sample ages were calculated based on an age-depth model constructed using available biostratigraphic and magnetostratigraphic data for Sites U1408 and U1410 (Norris et al., 2014; Yamamoto et al., 2018; Cappelli et al., 2019). Age calibrations from the 2012 geologic timescale were used for middle Eocene geomagnetic polarity reversals [GTS2012; (Gradstein et al., 2012)]. Six samples that span the MECO (∼38.50–43.50 Ma) were selected with a ∼0.5–1-Myr sample spacing (**Supplementary Table 1**).

Each sample was dried at 40°C for 4 days and then soaked in sodium hexametaphosphate for a minimum of 4 days on a shaker table to disaggregate the sediment. Samples were then washed over a 38 μm sieve using deionized water until the water ran clear and then dried overnight in a 40°C oven overnight before being transferred to vials. Each sample was subsequently dry sieved to allow picking of individual specimens under the light microscope. For multi-specimen analysis (Section "Stable isotope analysis") foraminifera were picked from a narrow sieve size fraction of 250–315 μm to avoid the effects of foraminifera size on geochemical analysis. For the individual foraminifera analysis (Section "Individual foraminifera analysis") we picked from the > 180 μm size fraction to capture the widest range of morphological variation from the adult population.

Stable Isotope Analysis

Planktonic foraminifera occupy different depth niches within the water column creating an ecologically stratified community. As a result of isotopic fractionation in the water column, the stable isotopic signature of planktonic foraminifera tests reflects the depth habitat in which they live. The fractionation of oxygen into foraminifera calcite is temperature dependent, resulting in increasing foraminifera test δ^{18}O values with increasing depth paralleling the trend of decreasing temperature with depth in the oceans (Fairbanks et al., 1980, 1982). The opposite depth dependent trend is seen in carbon isotopes with foraminifera calcite δ^{13}C decreasing with depth. This relationship is due to the preferential uptake of ^{12}C during photosynthesis at shallow water depths and the export of particulate organic carbon from the upper water column. Remineralization of particulate organic carbon at depth then releases isotopically light carbon back into the dissolved organic carbon (DIC) pool. Thus, surface ocean symbiont-hosting foraminifera have relatively low δ^{18}O and high δ^{13}C values relative to non-symbiont subthermocline dwellers with thermocline dwelling sitting somewhere in-between (**Figure 1**). These relationships do assume isotopic equilibrium between the foraminifera test and seawater. However, this equilibrium is offset by a number of physical parameters such as salinity, carbonate ion concentration (Urey, 1947; Urey et al., 1951; Epstein et al., 1953; Epstein and Lowenstam, 1954; Spero et al., 1997; Pearson, 2012) as well as foraminifera biology and ecology often referred to as "vital effects" (Erez, 1978; Spero and Williams, 1989; Spero et al., 1991; Bemis et al., 1998; Friedrich et al., 2012; Birch et al., 2013). These factors, especially foraminifera biology (Edgar et al., 2017), tend to impact test δ^{13}C more than δ^{18}O and can be minimized, for example, by picking foraminifera within a narrow size fraction. Nevertheless, the broad patterns of depth ranking using δ^{18}O and δ^{13}C (**Figure 1**) remains true and can be used to reconstruct foraminifera depth habitats through geological time (Pearson, 1998; Spero, 1998; Pearson et al., 2001; Sexton et al., 2006a,b; Coxall et al., 2007; Birch et al., 2013). To investigate water column structure across our study interval, planktonic foraminifera from genera representing three depth ecologies were picked: *Globigerinatheka* (mixed layer), *Subbotina* (thermocline) and *Catapsydrax* (sub-thermocline) (**Figure 1**).

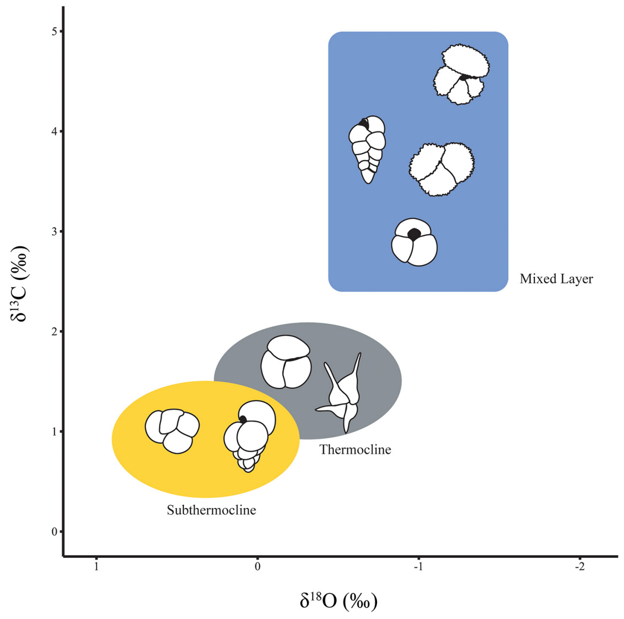


FIGURE 1 | Schematic figure showing the three main eco groups as defined in Aze et al. (2011) based on stable isotope measurements. This figure is illustrative and does not represent absolute values for each ecogroup. Top to bottom the mixed layer is represented by schematic outlines of *Acaranina* spp., *Chiloguembelina* spp., *Morozovelloides* spp. *and Globigerinatheka* spp. Left to right the thermocline is represented by *Subbotina* spp. and *Hantkenina* spp. and the subthermocline represented by *Catapsydrax* spp. and *Jenkinsina* spp. Note the reversed scale on the *x*-axis. Modified from Pearson (1998).

We use the symbiont bearing, deep mixed layer dwelling *Globigerinatheka* (Sexton et al., 2006b; Edgar et al., 2013) to represent the mixed layer (**Figure 1**) rather than other commonly used shallower mixed layer inhabitants *Acarinina* or *Morozovelloides*. *Morozovelloides* which were not abundant enough in our samples for statistically robust isotope or morphological analyses, whilst *Acarinina* were abundant but showed signs of reworking (stained and heavily fragmented) and recrystallization. We made every effort to pick individuals with no visible signs of either gametogenic calcite or recrystallized wall textures and were mindful of the caveats during interpretation. To represent the thermocline and subthermocline we used asymbiotic *Subbotina* and *Catapsydrax,* respectively (**Figure 1**).

Approximately 20 individuals from each of *Globigerinatheka* spp., *Subbotina* spp. *(S. utilisindex, S. eoceana, S. projecta, and S. linaperta)* and *Catapsydrax unicavus* were picked from each of the 6 samples following the taxonomy of Pearson et al. (2006). The 18 subsamples, each of 20 individuals, were then crushed, homogenized and weighed into vials. Each subsample weighed between 50 and 60 μg (**Supplementary Table 2**) and was then cleaned by ultrasonification in ethanol for 3–5 s, rinsed in deionized water and then placed in a 40°C oven for 1–2 h to dry. Coupled $\delta^{18}O$ and $\delta^{13}C$ measurements were analyzed in the Stable Isotope Mass Spectrometry Laboratory at the National Oceanographic Centre, University of Southampton, using a Thermo Fisher Scientific Kiel IV carbonate device coupled to a MAT253 stable isotope ratio mass spectrometer. All samples were measured against the reference standards NBS19 and NBS18, as well as an in-house quality control standard (GS1) and then standardized using a two-point calibration between NBS19 and NBS18 to Vienna Pee Dee Belemnite (VPDB). Long-term analytical precision based on repeat analysis of GS1 is estimated as $\pm$ 0.09‰ for $\delta^{18}O$ and $\delta^{13}C$.

Individual Foraminifera Analysis

To investigate the link between functional traits and environmental change in deep time we use individual foraminifera analysis (IFA) on the extinct, thermocline dwelling, asymbiotic planktonic foraminifera genus *Subbotina* (Edgar et al., 2013). *Subbotina* was present globally in Earth's oceans from the early Paleocene through to the end of the Oligocene (~65–23 Ma) (Aze et al., 2011; Wade et al., 2011). During the middle Eocene, Subbotinids increased in abundance at multiple sites across the globe flourishing over a period detrimental to many other groups (Macleod et al., 1990; Luciani et al., 2010). A possible reason for this survival is a hypothesized adaptable depth ecology, which is suggested in various intervals of the Eocene based on stable isotope measurements (Macleod et al., 1990; Bralower et al., 1995; Wade, 2004; Dutton et al., 2005; Wade and Pearson, 2008; Stap et al., 2010; Arimoto et al., 2020). Their ecology, diversity fluctuations and depth habitat make *Subbotina* a versatile group to investigate the link between functional traits and the environment.

Individual Morphological Analysis

To collect morphological traits, 50 individuals of *Subbotina* were picked from each of the six sample residues (discarding specimens showing gametogenic overgrowth and evidence of reworking) resulting in 300 individuals in total. To obtain 50 *Subbotina* individuals per sample, each sample was split using a microsplitter until approximately 300 foraminifera remained, shown to represent the diversity of a sample (Al-Sabouni et al., 2007). *Subbotina* were then picked from this "split." To avoid biasing because of an uneven distribution of individuals on the picking tray, individuals were picked from square cells on the picking tray chosen by a random number generator until 50 well-preserved *Subbotina* were picked per sample. *Subbotina* individuals were mounted on glass slides with the aperture facing upward (umbilical view) using double sided sticky tape in groups of up to 20 for morphological analysis (Brombacher et al., 2017). Images of each block of 20 individuals were taken using a Leica M205C stereo microscope with IC90HD camera illuminated from above and then processed using Image Pro 9.1 Software. Automatic measurements of test area (size) and aspect ratio (shape) (**Supplementary Figure 1**) were taken using the automated image macro in Image Pro (**Supplementary Table 3**), which have high reproducibility (Brombacher et al., 2017). To maintain a similar scale across all analyses, size was log transformed and mean-centered around 0.

Single-Specimen Stable Isotope Analysis

In addition to the 18 multi-specimen foraminifera samples analyzed, oxygen and carbon stable isotope ratios were determined for 120 *Subbotina* individuals. During the morphological analysis, described above, each individual from each sample was assigned a number from one to fifty. For each sample 20 individuals were chosen through computer generated random numbers with no replacement. Samples were cleaned in the same manner as the multi-specimen analysis described in section "Stable isotope analysis". Weights of individuals ranged from 13 to 21 μg with an average weight of 14 μg (**Supplementary Table 4**). To account for the range of weights, vials were loaded into the Kiel in batches, with each batch consisting of vials of individuals with similar weights so that the settings across the whole batch were optimized for a narrower size fraction. Eight samples out of the analyzed 120 failed to record a measurement due to insufficient weight.

Statistical Methods

Ordinary least squares linear models constructed in the R environment (version 4.0.3; R Core Team, 2020) were applied to investigate the drivers of planktonic foraminifera $\delta^{18}O$ and $\delta^{13}C$ values. Separate models were built with $\delta^{18}O$ and $\delta^{13}C$ as the dependent variable and a combination of environmental ($\delta^{18}O$ and Age), ecological ($\delta^{13}C$) and morphological (test area and test aspect ratio) traits as the independent variables. Numerous models were constructed with varying degrees of interaction. Models were compared using analysis of variance and the best model chosen based on likelihood ratio tests and Akaike Information Criterion (AIC). Clustering analysis was conducted with a Gaussian finite mixture model using the mclust package (Scrucca et al., 2016) with the best model and thus number of clusters chosen using the Bayesian Information Criterion (BIC). The influence of sample age on morphological and stable isotope variables was tested using one-way analysis of variance (ANOVA).

RESULTS

Paleoceanographic Changes Based on Multi-Specimen Analyses

To analyze our trait data in the context of local MECO paleoceanographic changes, we generated stable isotope records from three genera at each of the sites each with different depth habitats (**Figure 2** and **Supplementary Table 2**). *Globigerinatheka* show the lowest $\delta^{18}O$ across the interval compared to *Catapsydrax* and *Subbotina* (**Figure 2A**). Between 43.50 and 41.31 Myrs *Globigerinatheka* $\delta^{18}O$ increased by 0.25‰, over the same interval *Subbotina* $\delta^{18}O$ increased by 0.72‰ whilst *Catapsydrax* increased by 1.03‰ (**Figure 2A**). Then at 40.14 Ma all genera show a decrease in $\delta^{18}O$ with *Globigerinatheka* decreasing by 0.21‰, *Subbotina* by 0.64‰ and *Catapsydrax* by 1.50‰ (**Figure 2A**). Following the MECO to the end of our record at 38.50 Ma both *Globigerinatheka* and *Catapsydrax* show gradual increases in δ^{18}O (0.41 and 1.40‰, respectively; **Figure 2A**). In contrast *Subbotina* shows an increase in δ^{18}O of 0.89‰ at 39.56 Ma followed by a decrease of 0.17‰ at 38.50 Ma (**Figure 2A**).

The vertical thermal structure of the water column can be assessed by calculating the difference in δ^{18}O between surface water dwellers (*Globigerinatheka*) and deeper dwellers (*Subbotina* (thermocline) and *Catapsydrax* (subthermocline): $\Delta\delta^{18}O_{surface-deep} = \delta^{18}O_{Catapsydrax-Subbotina} - \delta^{18}O_{Globigerinatheka}$ (**Figure 3A** and **Supplementary Table 5**). A multiple linear regression using the δ^{18}O difference between *Globigerinatheka* and the two deeper dwelling genera (*Subbotina* and *Catapsydrax*) as the response variable showed a significant impact on the habitat differences in oxygen isotope space ($p < 0.001$) and in the MECO ($p < 0.01$) interval (**Supplementary Table 6**). There was a predicted 0.71 ± 0.11‰ overall decrease in the δ^{18}O gradient between *Globigerinatheka* and the deeper dwelling genera (*Subbotina* and *Catapsydrax*) across the study interval compared to the gradient change observed in δ^{13}C (**Figure 3** and **Supplementary Table 6**). Additionally, there was a predicted 0.56 ± 0.15‰ decrease in overall isotopic gradient (both δ^{18}O and δ^{13}C) during the MECO interval compared to the other time slices, which was primarily due to a reduction δ^{18}O gradient between all genera (**Figure 3** and **Supplementary Table 6**). An adjusted R^2 of 0.69 shows that 69% of variation in isotopic differences in **Figure 3** can be explained by a model that includes isotope grouping (carbon and oxygen) and interval grouping (MECO or not). The $\Delta\delta^{18}O_{surface-Catapsydrax}$ is between 0.52 and 1.30‰ for most of the interval except at 40.14 Ma where $\Delta\delta^{18}$O is 0.01 (**Figure 3A**). At this time, both *Globigerinatheka* and *Catapsydrax* have δ^{18}O values of −1.31 and −1.30‰, respectively. *Subbotina*, with an inferred intermediate depth habitat, records a δ^{18}O value of −1.12‰ (**Figure 2A**).

Globigerinatheka consistently show the highest δ^{13}C values of the measured genera throughout the study interval, and their values stay within a narrow δ^{13}C range throughout (between 2.15 and 2.92‰; **Figure 2B**) with the highest δ^{13}C value recorded immediately after the MECO. *Subbotina* and *Catapsydrax* consistently yield δ^{13}C values > 1.00‰ lower than *Globigerinatheka* and show their highest δ^{13}C values in the MECO and immediately after. *Subbotina* sits slightly above *Catapsydrax* in δ^{13}C space as expected based on their recorded depth habitat but following the MECO this relationship is reversed (**Figure 2B**).

Individual Geochemical Analysis of *Subbotina*

Individuals of *Subbotina* show variation around "batch" *Subbotina* measurements in both δ^{18}O and δ^{13}C space (**Figure 2**). However, the batch *Subbotina* measurements plot within 1 standard deviation of the median of the individual foraminifera analyses (IFA; black circle, **Figure 2**) in each time slice. This is expected and indicates that IFA analysis draws out intraindividual variation within a genus. The range in IFA δ^{18}O values at each time slice, apart from at 38.50 Ma, is consistently >1.00‰, with the widest ranges observed prior to the MECO where one to three analyses sit outside of two absolute deviations of the median (**Figure 2A**). These "extreme" values sit apart from the rest of the measurements in each sample. For example, at 41.31 Ma one positive δ^{18}O value leads to the largest range across the interval of 2.44‰ (**Figure 2A**). Only at 40.14 and 38.50 Ma do all measurements sit within 2.5 absolute deviations of the median. Although these points are more "extreme" there is no reason that they should be excluded. In all samples some individual *Subbotina* measurements plot within the same space or above that of the *Globigerinatheka* batch measurements (**Figure 2A**). In contrast to δ^{18}O, individual measurements of *Subbotina* in δ^{13}C space are overall more spaced out with limited or no clusters. The range of δ^{13}C values in each time slice is > 1‰ throughout with the largest range seen between 40.14–41.31 Ma around the MECO. The exception is at 39.56 Ma where it is 0.70‰ (**Figure 2B**). Unlike δ^{18}O, *Subbotina* individuals are consistently below and separated from batch *Globigerinatheka* δ^{13}C values albeit with a reduction in this separation at the MECO (**Figure 2B**).

Cross plots of the individual *Subbotina* δ^{13}C and δ^{18}O measurements shows an apparent cluster of points in the middle of the plot (0.50–1.25‰ in δ^{13}C and −0.20–1.20‰ in δ^{18}O space) with higher δ^{13}C values primarily from the MECO plotting to the side (**Figure 4**). To determine whether the MECO data genuinely represent a separate cluster of points, a clustering analysis was conducted using a Gaussian finite mixture model. The analysis revealed a spherical, varying volume model with two clusters fitted the data best with a BIC of -255 (**Supplementary Figure 2**). This model split the 112 data points into two relatively even clusters (Cluster 1: $n = 53$, Cluster 2: $n = 59$, **Supplementary Table 7**). The cluster classification does not cleanly follow the boundaries of our pre-defined time slices (**Figure 4**), with all but three data points from the MECO time slice and one from 43.50 Ma within a separate cluster (Cluster 1) along with some individuals from each other time slice. The MECO and oldest time slice cluster together as these two samples are climatically similar, as indicated by δ^{18}O values, thus the cluster captures the transient warming interval and the earliest stage of the global cooling trend, respectively.

Although clustering analysis identifies two clusters from the data, there is uncertainty particularly where the clusters are in close contact when δ^{18}O is around −1.00‰ (**Figure 4**). To investigate this signal further, one-way ANOVAs were fitted on carbon and oxygen separately with age as the groups. There was a statistically detectable difference between time slices in both δ^{18}O [$F(5,106) = 11.84$, $p < 0.001$] (**Supplementary Table 10**) and δ^{13}C [$F(5,106) = 9.84$, $p < 0.001$] (**Supplementary Table 12**). A *post hoc* TUKEY HSD test showed this difference to only be significant in δ^{18}O between the MECO sample (40.14 Ma) and all other samples except for 43.50 Ma at the beginning of the record ($p < 0.001$) supporting the clustering analysis (**Supplementary Table 11**). In δ^{13}C, the same *post hoc* test showed no detectable difference between the start (43.50 Ma) and end (38.50 Ma) of the record, but a supported a difference between the MECO and all pre-MECO samples ($p < 0.05$) (**Supplementary Table 13**). This,

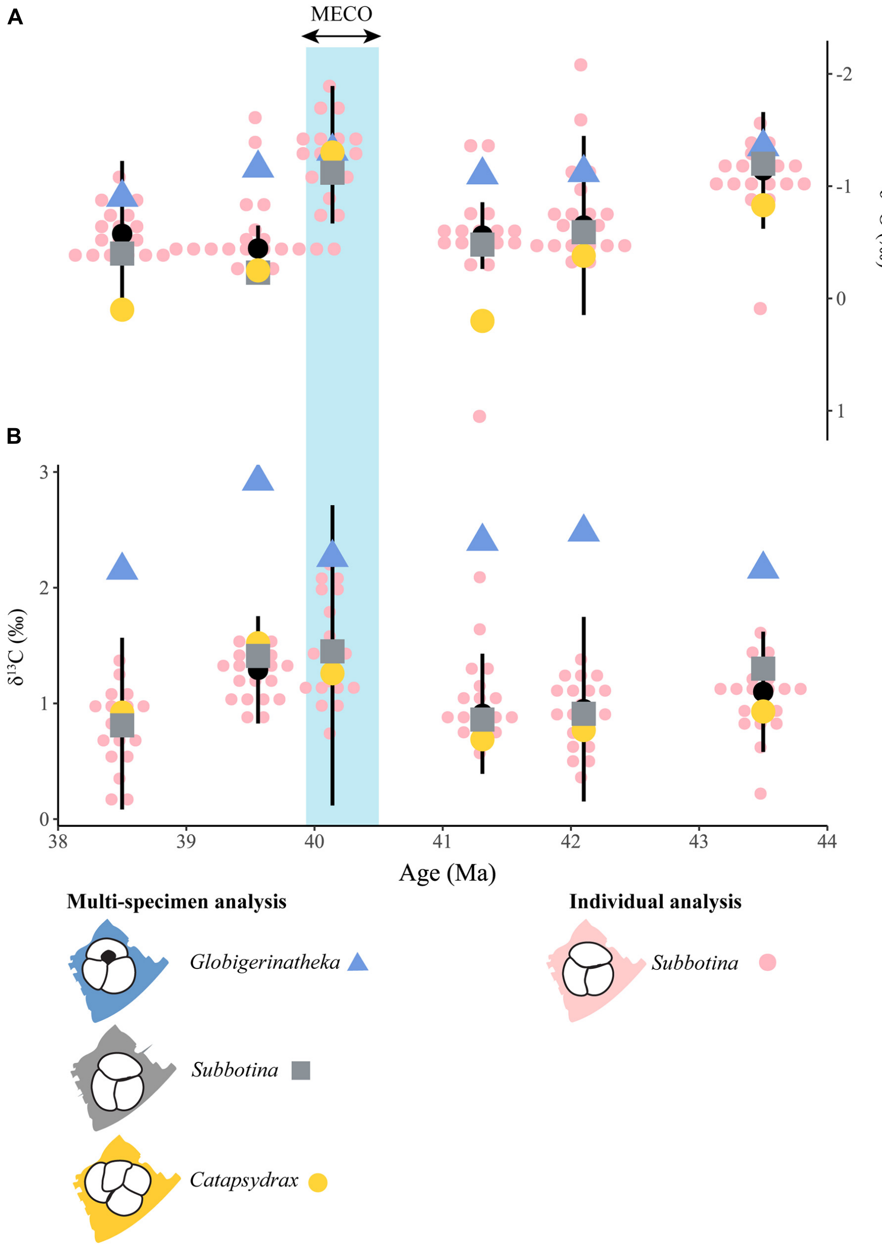


FIGURE 2 | Oxygen **(A)** and carbon **(B)** stable isotope results from individual foraminifera analysis (IFA) and batch multi-specimen analysis. Small pink circles show IFA of *Subbotina*, where circles are stacked horizontally multiple individuals have the same measurement. The genera used in batch analysis are represented by consistent coloring and shapes across each time slice: *Globigerinatheka* = blue triangles, *Subbotina* = gray squares and *Catapsydrax* = yellow circles. The black circle and vertical lines represent summary statistics from IFA with the circle representing the median and vertical line indicating 2.5 absolute standard deviations of the median (MAD). Note the reversed y- axis on panel A. The vertical blue box indicates the position of the MECO.

along with the clustering analysis, strengthens the inference that *Subbotina* isotopic variation is more nuanced in $\delta^{13}C$ than $\delta^{18}O$, likely through the increased role of biological "vital effects".

Understanding Drivers of Individual Foraminifera Analysis

Morphological traits of test shape (test aspect ratio) and size (mean-centered test area on log scale) were also made on 300 *Subbotina* individuals including the 120 individuals that were used for geochemical analysis. Considering the full morphological dataset, both size and shape show large ranges in all samples with no clear trend with sample age (**Figure 5**). A one-way ANOVA showed detectable differences between time slices in both mean shape [$F(5,294) = 3.171$, $p < 0.01$] (**Supplementary Table 14**) and size [$F(5,294) = 3.185$, $p < 0.01$] (**Supplementary Table 15**). Used in isolation these traits give us little information, so we integrated the 120 individuals that had both morphological (size) and stable isotope measurements

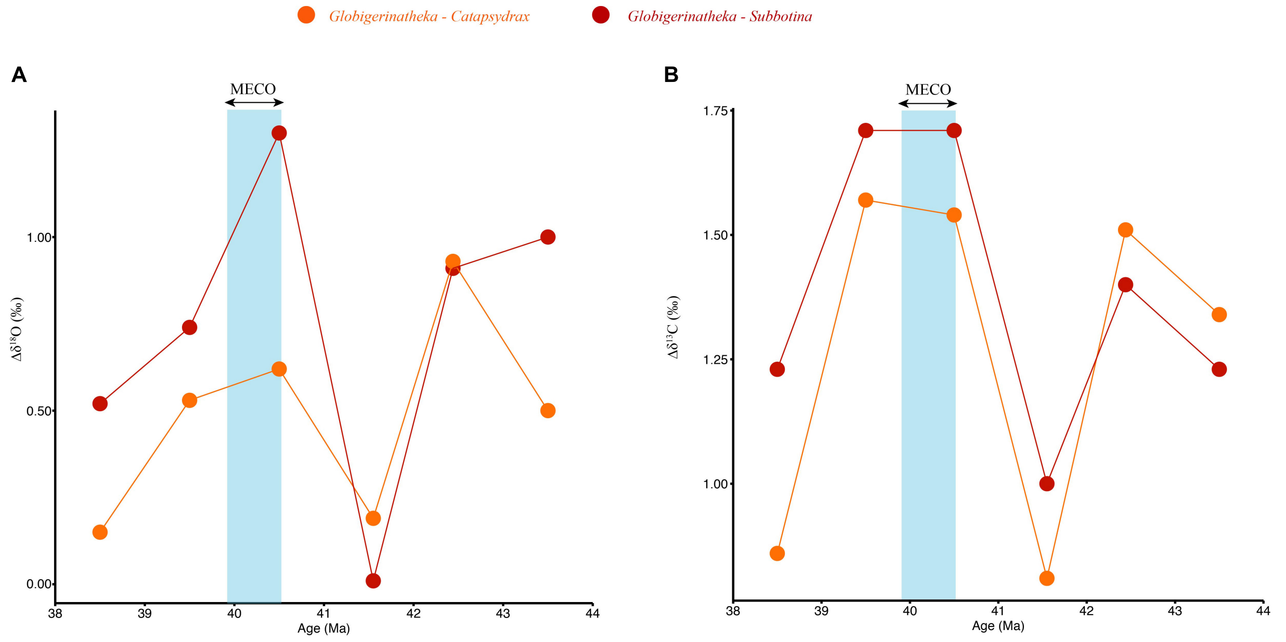


FIGURE 3 | Stable oxygen **(A)** and carbon **(B)** isotope gradients between *Globigerinatheka* (surface) and *Subbotina* (thermocline) and *Catapsydrax* (subthermocline) across the MECO interval. As the line approaches zero the isotopic gradient between the different genera is reduced, with 0 indicating no difference in isotopic signature between the different depth habitats. The vertical blue box indicates the MECO.

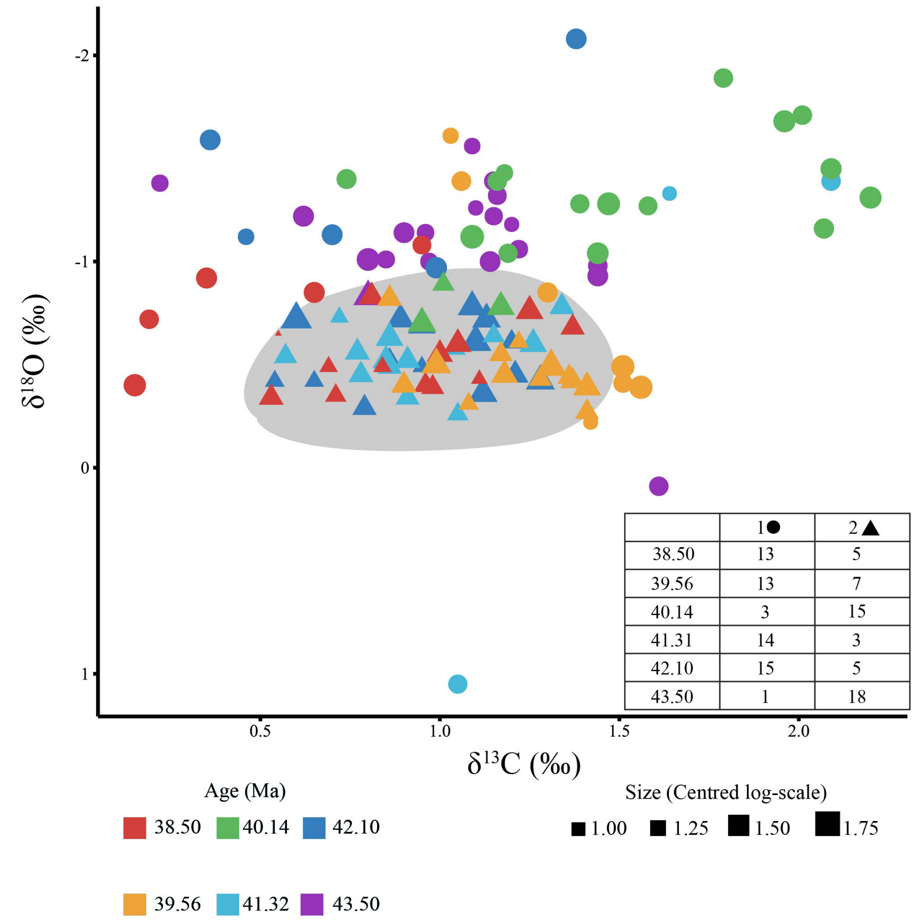
(**Supplementary Table 4**) into multiple linear regression models because size has an impact on stable isotope and trace element expression in planktonic foraminifera (Elderfield et al., 2002; Friedrich et al., 2012).

The best supported $\delta^{13}C$ model included an interaction between $\delta^{18}O$ and size with an adjusted R^2 of 0.28 (**Supplementary Table 16**). Of the predictors included, only age at 40.14 and 39.56 Ma differed detectably from the baseline $\delta^{13}C$ at 38.50 Ma ($p < 0.001$) (**Supplementary Table 17**). At 39.56 and 40.14 Ma (MECO) $\delta^{13}C$ is predicted to increase by 0.46 ± 0.11‰ and 0.60 ± 0.13‰, respectively, compared to $\delta^{13}C$ at 38.50 Ma (**Supplementary Table 17**). Our models predict a 0.10‰ decrease in Subbotina $\delta^{13}C$ values per log size unit increase, although this relationship is not significant (**Supplementary Table 17**). Removal of sample age and oxygen isotopes leaves size as the only explanatory variable results in a positive relationship between $\delta^{13}C$ and size [0.29 ± 0.15‰ increase per log(size) increase], but not one that is detectably different from 0 (**Supplementary Table 18**). For $\delta^{18}O$ a similar model with an interaction between $\delta^{13}C$ and size was the most supported to explain individual *Subbotina* $\delta^{18}O$ (**Supplementary Table 19**). Of the predictors $\delta^{13}C$, size and sample age, sample age was the main driver of individual Subbotina $\delta^{18}O$ ($p < 0.001$). At 40.13 Ma (MECO) and 43.50 Ma, $\delta^{18}O$ is predicted to decrease by 0.61 ± 0.15‰ and 0.44 ± 0.13‰, respectively, compared to $\delta^{18}O$ at 38.50 Ma (**Supplementary Table 20**). Replacing foraminifer size with weight yielded qualitatively similar results with best fitting model structure remaining the same (**Supplementary Tables 21–24**). The positive $\delta^{13}C$-weight relationship ($p < 0.05$) was qualitatively consistent with the inferred $\delta^{13}C$-size relationship, but weight explained more variation (lower residual sum of squares, **Supplementary Tables 17, 22**) than size.

Since we measured batch isotopes on foraminifera with three typically distinct depth ecologies (surface, thermocline and subthermocline), we can use the batch $\delta^{18}O$ values to understand the impact of water-depth temperature changes on *Subbotina* $\delta^{18}O$, a proposed thermocline dweller. We used the same model as above but with the age predictor replaced by surface, thermocline and subthermocline $\delta^{18}O$ measurements as a proxy for water depth temperature. It is the deeper ocean temperatures (thermocline and subthermocline), rather than surface ocean, that drive the individual foraminifera measurement of *Subbotina* $\delta^{18}O$ ($p < 0.05$) (**Supplementary Table 25**). A subthermocline and thermocline $\delta^{18}O$ increase of 1.00‰ is predicted to increase *Subbotina* $\delta^{18}O$ by 0.34 ± 0.16‰ and 0.44 ± 0.19‰, respectively, translating to a deepening depth habitat for *Subbotina* as the deeper ocean cools at a faster rate than the surface waters (**Supplementary Table 25**).

DISCUSSION

Unpicking ecological signals from paleoceanographic signals is difficult, but worthwhile because of the ability to study transient climatic events in deep time. The most common way to infer functionality in deep time is to look for a correlation between a morphological trait and ecological differences (Eronen et al., 2010). We show complicated morphological and geochemical responses to the Middle Eocene Climatic Optimum (MECO, **Figures 2–5**). The reduction of surface-deep $\delta^{18}O$ and $\delta^{13}C$



	1●	2▲
38.50	13	5
39.56	13	7
40.14	3	15
41.31	14	3
42.10	15	5
43.50	1	18

FIGURE 4 | Cross plot of carbon versus oxygen isotopes for individual *Subbotina* analysis. Individual point size represents the size (area) of each individual on a log scale. The symbol (triangle vs. circle) indicates the cluster that each individual was assigned to following cluster analysis. Circles represent cluster 1 and consist primarily of individuals from 40.14 and 43.50 Ma as highlighted in the table inset into the figure, while triangles represent cluster 2. The number of individuals in each time slice assigned to each cluster is shown in the inset table. The gray area visually represents cluster 2 as identified by the model.

gradients in our study at the MECO (**Figure 5**) could be explained by: (1) hydrological changes causing the changes in "batch" isotopes observed but depth habitat of foraminifera remaining constant through the interval (**Figure 6**) discussed in Section "Paleoenvironmental Changes"; or (2) ecological changes, with deeper dwelling foraminifera (*Catapsydrax* and *Subbotina*) migrating upward in the water column to occupy a similar thermal habitat to that of *Globigerinatheka* (**Figure 6**) discussed in Section "Measuring functionality in foraminifera traits."

The carbon isotope signature ($\delta^{13}C$) of planktonic foraminifera is controlled by biology (Edgar et al., 2017) and there are long established relationships between test size and $\delta^{13}C$ signal in planktonic foraminifera (Berger et al., 1978; Oppo and Fairbanks, 1989; Spero et al., 1991; Elderfield et al., 2002; Friedrich et al., 2012). The $\delta^{13}C$-test size relationship is commonly controlled in stable isotope analysis by using narrow size fractions intended to remove the relationship between $\delta^{13}C$ and size. Unfortunately, this pre-emptive censoring also removes the correlation needed to infer functionality. In this study, we use a wide size fraction (>180 μm) and detect no relationship between *Subbotina* $\delta^{13}C$ and size (~47,219–173,204 μm^2) in either multivariate or univariate models when the presence of explanatory variables that track climatic fluctuations are removed ($\delta^{18}O$ and sample age). Although only a single case study, the lack of size-$\delta^{13}C$ relationship here has implications for studying functional traits in deep time where vast climatic changes can occur instantaneously in geological terms. Our discussion focusses on the challenges of interpreting these biologically driven isotopic signatures through periods of substantive environmental change.

Paleoenvironmental Changes

Applying trait based studies on thousand-to-million year time scales requires a thorough understanding of climatic and environmental variables. To provide an environmental dimension to our study, we will focus on paleoenvironmental changes (**Figure 6**) across the 6 Myr focal interval using the inferred depth ecology of individual planktonic foraminifera alongside the measured $\delta^{13}C$ and $\delta^{18}O$ of the genera level

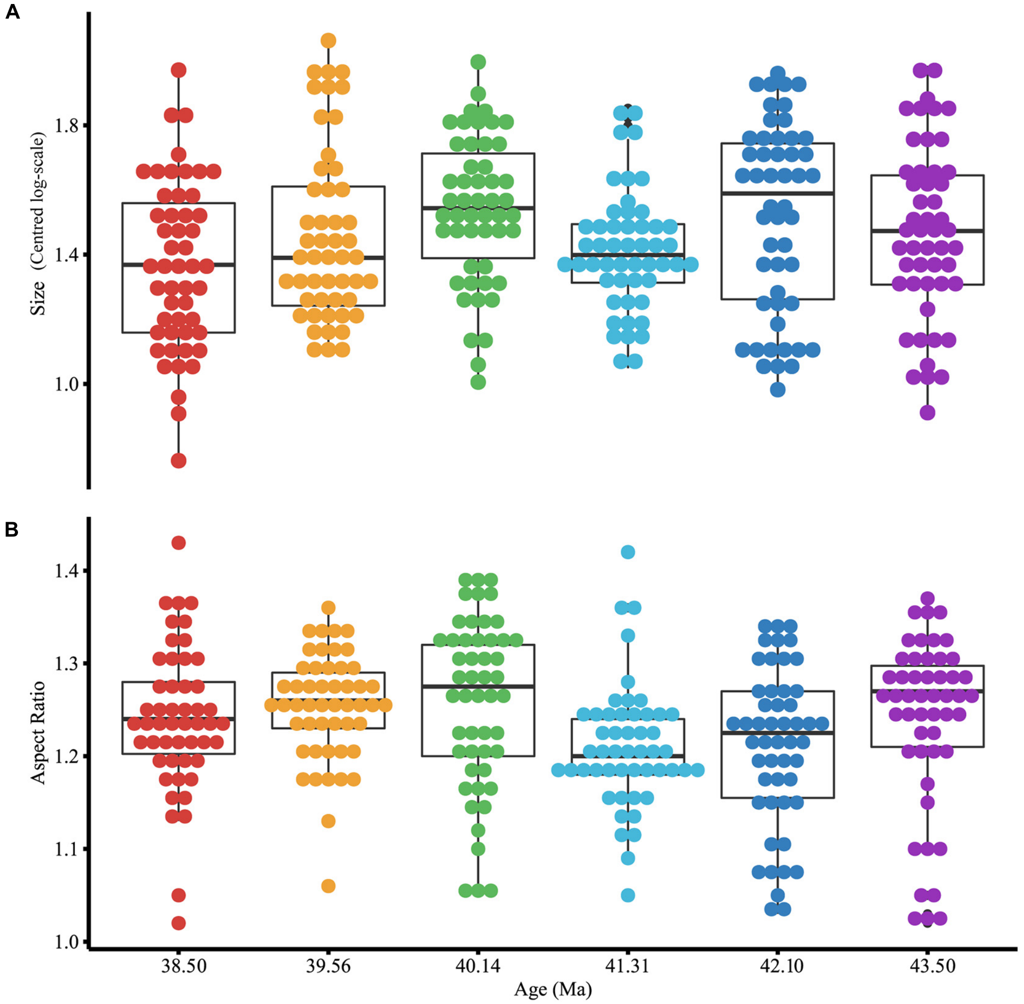


FIGURE 5 | Morphological [test size **(A)** and test aspect ratio **(B)**] variation of all *Subbotina* measured in this study (n = 300). Size is on a log scale so has no unit of measure. The box represents the interquartile range whilst the whiskers show 1.5*interquartile range. The black line represents the median. Individual filled symbols represent all individual specimens sampled.

isotopes. Through this comparison, we can infer potential water column thermal and trophic state changes across the MECO, which in concert with more temporally resolved studies can provide important climatic and environmental context for our trait-based analysis.

Water Column Thermal Structure Changes Across the Middle Eocene ($\delta^{18}O$)

The Eocene saw a gradual change from hothouse to icehouse climate punctuated by short-lived global warming events, the final globally recognized of which is the MECO (Cappelli et al., 2019). Prior to the MECO, a positive shift in benthic foraminifera $\delta^{18}O$ values at ~42 Ma in the Atlantic Ocean and other ocean basins (Coxall et al., 2000; Edgar et al., 2007; Cramwinckel et al., 2018; Westerhold et al., 2020) as well as nannofossil assemblage composition changes toward cool water taxa in the Southern Ocean at ~41.6 Ma (Villa et al., 2008, 2013) indicate that global temperatures were lower than in the early Eocene and that the transition from a hothouse to icehouse was already underway. Water column cooling resulted in increasing planktonic foraminifera depth habitat stratification (**Figures 2**, **3**).

In our record from the Newfoundland margin, *Catapsydrax* and *Subbotina* show progressive $\delta^{18}O$ increases of 0.45 and 0.61‰, respectively, suggesting up to 2°C cooling of deep water temperatures in comparison to the surface ocean by 41.44 Ma (**Figure 2A**). Thermal decoupling of the water column due to global cooling would result in the development of a strong, shallow thermocline and more thermally stratified water column prior to the MECO (43.50 to 41.44 Ma; **Figure 2A**), as suggested in previous research at this locality (Arimoto et al., 2020). In contrast, sea surface temperature change in the subtropical North Atlantic Ocean appear to be relatively minimal with *Globigerinatheka* $\Delta\delta^{18}O$ of ~0.23‰ across the same interval (**Figure 2A**) supported by minimal surface ocean changes in the South East Atlantic (Galazzo et al., 2014).

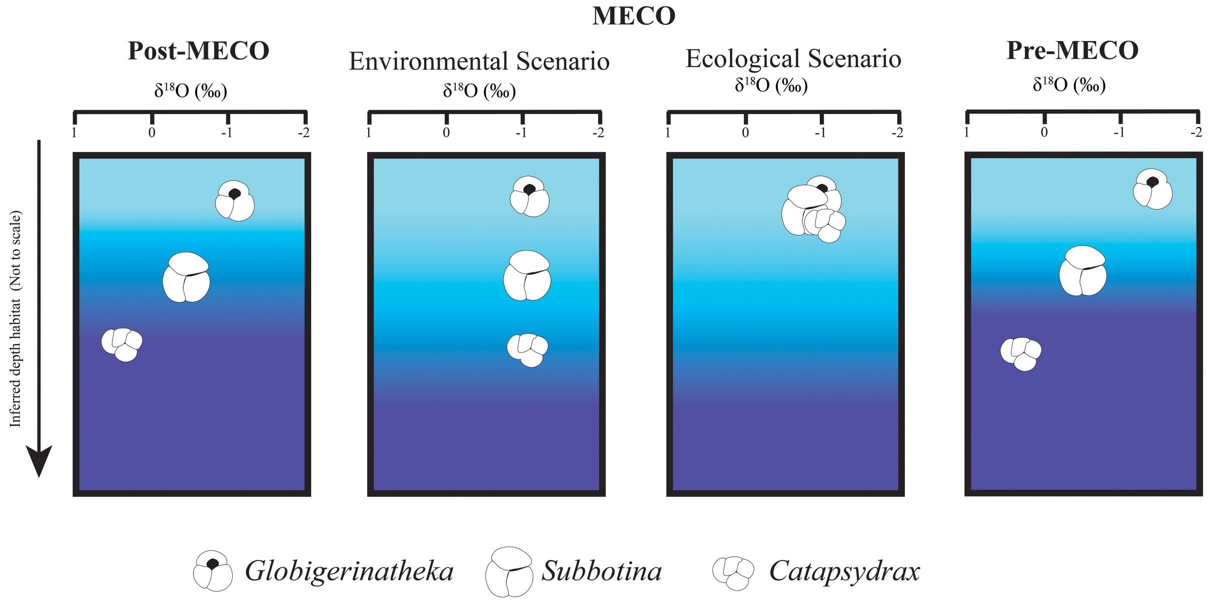


FIGURE 6 | Schematic illustration of environmental versus ecological scenarios to explain the $\delta^{18}O$ isotopic gradient changes between genera. Color gradient in all panels represents temperature change from warm (light blue) to cool (darker blue) temperatures.

Our record indicates that decoupled water column responses to environmental changes continued during the MECO. We see a reduction in $\delta^{18}O$ gradients between surface and deep water dwellers ($\Delta\delta^{18}O_{Globigerinatheka-Catapsydrax}$: 0.01‰; **Figure 3**) driven by large decreases in *Subbotina* and *Catapsydrax* $\delta^{18}O$ values ($\Delta\delta^{18}O$: 0.64 and 1.50‰, respectively) and only small decreases in *Globigerinatheka* $\delta^{18}O$ ($\Delta\delta^{18}O$: 0.21‰ compared to pre-MECO levels (**Figure 2A**). This suggests that warming was concentrated at thermocline and subthermocline depths (increasing by ~7°C) compared to the surface ocean (~1°C increase). This muted surface ocean response is not supported by high resolution stable isotope records at this locality that suggest a 4°C increase in surface ocean temperature (Arimoto et al., 2020) or most other global studies using various geochemical proxies which suggest between 3–6°C warming of sea surface temperatures across the MECO (Bohaty and Zachos, 2003; Bohaty et al., 2009; Bijl et al., 2010; Cramwinckel et al., 2018, 2019). Additional sites in the north Atlantic alongside temperature estimates from a greater range of proxies such as Mg/Ca and clumped isotopes are needed to understand the mismatch between this dataset and that of Arimoto et al. (2020) and any site-specific changes in the thermal structure of the water column.

Peak warming in the MECO was followed by globally rapid cooling (Villa et al., 2008; Bohaty et al., 2009; Arimoto et al., 2020) accompanied by increases in cool water taxa (Luciani et al., 2010; Villa et al., 2013), reduced abundances and eventual extinctions of oligotrophic, shallow mixed layer planktonic foraminifera *Acarinina* and *Morozovelloides* (Wade, 2004) and calcareous nannofossils discoasters (Villa et al., 2008). In our record, global cooling is represented by an increase in $\Delta\delta^{18}O_{Globigerinatheka-Catapsydrax}$ of ~1.00‰ (**Figure 3**) re-establishing the pre-MECO planktonic foraminifera depth stratification in $\delta^{18}O$ seen at 43.50 Ma by 38.50 Ma, indicating abrupt cooling of the thermocline following the MECO. Surprisingly, despite such rapid cooling, *Catapsydrax* takes ~2 Myrs to reappear below *Subbotina* in the water column (**Figure 2A**), possibly not returning to its position below *Subbotina* in $\delta^{18}O$ space until thermocline conditions became more stable at 38.50 Ma (**Figure 2A**). *Globigerinatheka* shows a slight increase of 0.15‰ in $\delta^{18}O$ values post-MECO, returning to pre-MECO values (**Figure 2A**) indicating only minor cooling of the surface ocean.

Trophic State Changes Across the Middle Eocene Climatic Optimum ($\delta^{13}C$)

Inferred trophic state changes are highly site specific (Witkowski et al., 2012, 2014; Moebius et al., 2015; Cramwinckel et al., 2019). Our study shows that, in tandem with the increased $\delta^{18}O$ depth stratification between 43.50 and 41.31 Ma [described in section "Water column thermal structure changes across the middle Eocene (δ18O)"], the $\delta^{13}C$ stratification also increased over the same pre-MECO period by 0.48‰ ($\Delta\delta^{13}C_{Globigerinatheka-Catapsydrax}$). This increasing separation between genera is interrupted by a transient reduction in $\Delta\delta^{13}C_{Globigerinatheka-Catapsydrax}$ to 1.00‰ and $\Delta\delta^{13}C_{Globigerinatheka-Subbotina}$ to 0.81‰ at 40.15 Ma. These $\Delta\delta^{13}C$ values during the peak of the MECO represent the lowest $\delta^{13}C$ gradients between the surface and deep-water dwellers recorded in our samples (**Figure 3B**). The decreasing gradient is a result of increasing *Subbotina* and *Catapsydrax* $\delta^{13}C$ values, coupled with a slight decline in *Globigerinatheka* of ~0.14‰ (**Figure 2B**) suggesting a possible decrease in water column primary productivity at Sites U1408 and U1410.

The interpreted reduction in primary productivity from our record during the peak of the MECO event at 40.15 Ma is supported by another study at the same locality (Arimoto et al., 2020) that observed weakened depth stratification between planktic foraminifera and interpreted a large reduction in planktonic foraminifera accumulation rates as an indicator of primary productivity reduction (Arimoto et al., 2020). In addition, decreased productivity during the MECO has been observed in the Southeast Atlantic (Galazzo et al., 2014). Open ocean mid latitude south Atlantic (Galazzo et al., 2014) and north Atlantic (Arimoto et al., 2020) locations are therefore recording a different trophic signal than observed at other sites across the MECO where primary productivity increases have been proposed based on benthic foraminifera accumulation rates and assemblage structure (Boscolo Galazzo et al., 2013; Moebius et al., 2015), increased deposition of organic rich layers in the Tethys ocean (Luciani et al., 2010; Spofforth et al., 2010), increased diatom flux in the Southern Ocean (Witkowski et al., 2012) and North Atlantic Ocean (Witkowski et al., 2014), as well as shifts in planktonic foraminifera communities toward more eutrophic, opportunists (Luciani et al., 2010). At continental margin sites, these changes are attributed to increased weathering and terrestrial input as a result of global warming across the MECO (Moebius et al., 2015). The open ocean setting of our study sites (Sites U1408 and U1410) mean a similar terrestrial input of nutrients is not plausible, which potentially explains the decreased δ^{13}C gradient and inferred productivity decrease we observe. Further studies combining micropalaeontological methods and geochemistry are needed in the North Atlantic to understand whether our results represent a local or regional signal. The described thermal and trophic state fluctuations above show the importance of including environmental change in functional trait-based studies. Whilst our study period and data reflect the globally observed rapid changes of the MECO, we also capture global background cooling (**Figure 2**). While background climatic changes are not often the focus of paleoecological studies, our low-resolution record shows how major changes to water column structure occur before and after large climatic fluctuations. These changes will have an impact on ecosystems and, as we have shown, on traits in planktonic foraminifera.

Functional Traits in Foraminifera in Deep Time

What Does “Functional” Mean for Foraminifera?

This study demonstrates that measurable morphological and ecological traits can be used to infer responses to abiotic forcing in paleoceanographically dynamic environments. However, inferring the functionality of measurable morphological traits is much harder for extinct than for extant species. Further, assigning functions to traits in deep time is often based on observations of extant taxa and the assumption that the observed functional relationship has not changed. Several studies indicate that this approach may be too simplistic (Wade et al., 2008; Eronen et al., 2010; Edgar et al., 2013). Presence of algal photosymbionts has been shown to be functional (Bé et al., 1977, 1981, 1982) in some modern planktonic foraminifera species (obligate symbiosis) (Takagi et al., 2019), with the only way of determining obligate symbiosis through direct observation. Other functional traits such as spines are not readily preserved *in situ* and require SEM images to identify. However, more complex morphological traits that are more tightly related to biogeochemical function (such as pore density) can now be easily measured through technological advances (Bé, 1968; Constandache et al., 2013; Burke et al., 2018). While gross morphology has been hypothesized to control buoyancy (Caromel et al., 2014), mathematical models suggest any potential relationship is weak at best (Caromel et al., 2017) and such features are variable even within a constant laboratory environment (Davis et al., 2020). One clear conclusion from these studies and our results is that simple measures of gross test morphology are not primary controls on organismal function, and that interdisciplinary developments offer promising avenues to extract more biogeochemically relevant signals.

Measuring Functionality in Foraminifera Traits

Implying functionality of foraminifera traits is further complicated by foraminifera trait diversity, analytical protocols and trait plasticity. The genus based approach used in this study likely expands the range of morphological or geochemical values compared to species level analyses. Genera have long been argued to represent biological reality (Mayr, 1942) and analysis at the generic level has advantages and disadvantages (Hendricks et al., 2014). Despite their highly resolved, species-level record, a genera based approach is appropriate for planktonic foraminifera as phenotypic and ecological traits are shared across species and genera resulting in morphogroup and ecogroup classifications (Aze et al., 2011).

Despite increasing morphological and geochemical niche breadth by measuring genera rather than species, and measuring almost 8 times as many individuals for morphological as geochemical analysis, we do not detect a morphological response in terms of either test size or shape to either the long-term Eocene cooling trend or the transient MECO. In addition, our analysis found no detectable relationship between size and δ^{13}C in *Subbotina* as expected for an asymbiotic foraminifera. Note though that we obtained statistical significance between specimen weight and δ^{13}C (**Supplementary Table 22**), re-emphasizing the importance of measuring the most relevant trait rather than the easiest to measure. This implies that either size is not a functional trait (assuming a δ^{13}C-size correlation infers functionality) or plasticity of *Subbotina* traits is sufficient to mask any functional relationship.

In contrast, to the lack of δ^{13}C-size relationship in adult *Subbotina*, species hosting dinoflagellate algal photosymbionts do have a positive test size-δ^{13}C relationship implying functionality (Berger et al., 1978; Oppo and Fairbanks, 1989; Spero et al., 1991; Elderfield et al., 2002; Friedrich et al., 2012; Edgar et al., 2017). The δ^{13}C-size relationship in symbiont bearers is a results of algal preferential uptake of isotopically light carbon. If a correlation implies functionality, size is functional in at least symbiont bearing planktonic foraminifera. To understand this relationship further we propose the need for investigations at the individual level outside of analytical size constraints in symbiotic genera and additional research on whether this relationship extends to asymbiotic genera at the individual level.

Individual analysis will also further our ability to constrain the degree of plasticity in planktonic foraminifera traits and therefore better infer their functionality. The discussions in section "Trophic state changes across the Middle Eocene Climatic Optimum (δ^{13}C)" focus on linking δ^{13}C changes in planktonic foraminifera to trophic changes in the water column. This one-dimensional view of δ^{13}C assumes that the depth ecology of planktonic foraminifera does not change and therefore paleohydrological changes drive stable isotope variations. Yet, studies based on extant foraminifera indicate that depth habitat can vary as a result of season, biogeography and environment (e.g., Jonkers and Kučera, 2017; Kretschmer et al., 2018; Taylor et al., 2018; Chernihovsky et al., 2020) as well as changes in life strategy (Darling et al., 2009). Additionally, studies of foraminifera species and across evolutionary lineages have shown changing depth habitats through evolutionary history (Norris et al., 1993; Coxall et al., 2000; Stewart et al., 2012). The increase in δ^{13}C values in *Subbotina* and *Catapsydrax* ($\Delta\delta^{13}$C: 0.57–0.59‰, respectively) across the MECO compared to pre-MECO (**Figure 2B**), reducing the overall surface-deep δ^{13}C depth gradient, suggests these genera could have migrated up in the water column during the MECO supporting an ecological scenario (**Figure 6**).

An adaptable depth ecology and generalist life strategy has been suggested to explain batch *Subbotina* variation in δ^{18}O and δ^{13}C at several points through the Eocene (Macleod et al., 1990; Bralower et al., 1995; Wade, 2004; Dutton et al., 2005; Wade and Pearson, 2008; Stap et al., 2010; Arimoto et al., 2020). In other studies these changes are most often associated with cooling of surface waters and/or increases in productivity (Macleod et al., 1990). However, with no symbionts and a preference for cooler thermocline waters it is hard to envisage this genus moving to a warmer (= shallower) part of the water column.

Utilizing single specimen analysis, we can explore this conundrum further. Depth habitat hypotheses of *Subbotina* through the Eocene have previously been based on multi-specimen (batch) isotope analyses hiding any interindividual variation (Macleod et al., 1990; Bralower et al., 1995; Wade, 2004; Dutton et al., 2005; Wade and Pearson, 2008; Stap et al., 2010; Arimoto et al., 2020). Assuming that our new individual isotopic measurements reflect a genuine isotopic signature of *Subbotina*, the wide range of individual isotopic values (**Figures 2**, **4**) with some individuals plotting in the same space as both *Globigerinatheka* (mixed layer) and *Catapsydrax* (subthermocline) suggests that *Subbotina* had a large ecological niche extending from the surface ocean to the subthermocline during the middle Eocene (**Figure 2**). It is this wide ecological niche that may have aided species' resilience through the MECO, multiple early Paleogene hyperthermal events and long-term Eocene cooling. More stable isotope studies of *Subbotina* at the individual level are needed to understand how the width of the *Subbotina* ecological niche changed through time and space during the Eocene and through to their demise in the Oligocene.

In this study, we have demonstrated that planktonic foraminifera are an ideal study organism for trait-based studies and can be integrated with paleoceanographic changes to investigate functional trait changes through climatic perturbations. Using an integrated approach with individual based analyses, we have demonstrated profound changes to ecosystems undergoing a transient global warming event. We did, however, detect no evidence that the measured morphological traits, and their relationship to stable isotopes, imply altered functionality across our time period. Further research is needed to reconcile the true meaning of which traits are functional for planktonic foraminifera in deep time, and how we can detect functional relationships statistically in "go-to" morphological traits such as size. We suggest that to truly investigate functionality in planktonic foraminifera we need to be measuring outside of size fractions, using the whole spectrum of genera diversity that planktonic foraminifera offer and leveraging developments in imaging techniques.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

LK, TE, and SN conceived the study. LK analyzed the data, took the lead on writing the manuscript, and collected the data with help from SB and KE. All authors commented on subsequent drafts and approved submission.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2021.679722/full#supplementary-material

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