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Against the Flow: The Colonisation of the Lesotho Highlands by Freshwater Limpets

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To date, limited research has taken place on the evolutionary history of biodiversity in the high-altitude zones of southern Africa, particularly the Lesotho Highlands. The few studies that do exist point to similar high-altitude zones being either prolific museums (i.e., refugia and cradles) for cold-evolved species or sinks for temperate species post the Last Glacial Maximum, yet the role this zone has played for freshwater biodiversity is unknown for almost all freshwater taxa. In this study, we address this lack of knowledge by looking at the phylogeography of the freshwater limpet genus, *Burnupia*, across its southern and eastern African range, but particularly focusing on the Lesotho Highlands. We used COI data to reconstruct the evolutionary history of the genus, quantify phylogenetic species diversity, test both isolation by distance (IBD) and by elevation (IBE) and model ancestral area estimation "in" and "out" of the Highlands to determine: 1) The diversity and endemicity of *Burnupia* spp. in the Highlands in comparison to the broader southern African region and 2) when did the colonisation of the Highlands happen. Our results showed that at least two of the nine southern African phylogenetic species delimited occur in the Highlands (which appears average for the geographical extent of this area in comparison to the broader southern African region) and that the genus has been present in the Lesotho Highlands for somewhere between 1.38–0.23 million years. However, we found the endemicity of at least one of the two Highland species, supported by weak but significant IBD and IBE in *Burnupia*. Therefore we favour the notion that the Highlands are likely an important haven for cold-evolved species. As our results also generated a lot of data useful for *Burnupia* systematics, we discuss some taxonomic implications of our findings.

Keywords: biogeography, *Burnupia*, freshwater ecology, high-altitude, molluscs, phylogeography, southern Africa

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INTRODUCTION

The Lesotho Highlands, primarily located in Lesotho and marginal parts of South Africa (and which includes a large portion of the World Heritage, transfrontier Maloti-Drakensberg Park), are some of the highest elevated land in Africa (maximum elevation = 3,482 m.a.s.l.) which maintain a particularly cooler climate against the backdrop of the broader, temperate southern African region (Norström et al., 2018; Hoogendoorn et al., 2020; **Figure 1A**). Although it is part of the Drakensberg mountain range and the Great Escarpment, which stretches from the Eastern Cape to

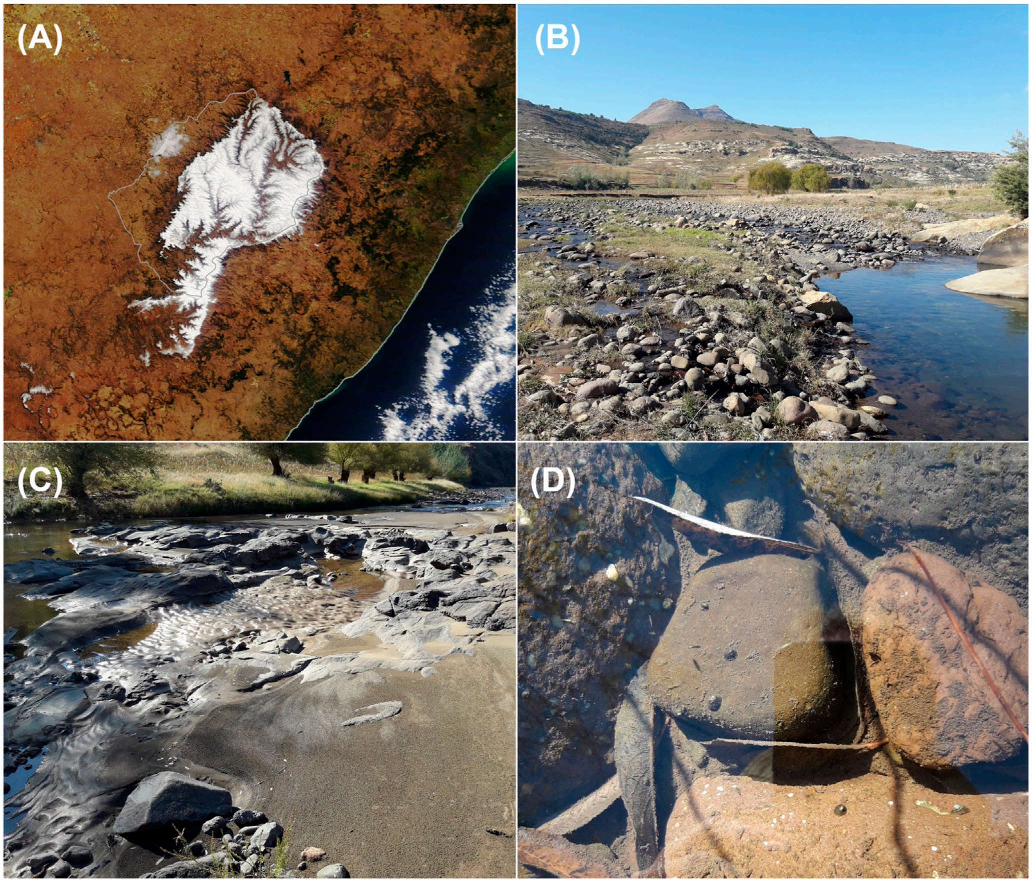

FIGURE 1 | (A) Lesotho Highlands covered in winter snow from space (NASA Earth Observatory images by Lauren Dauphin, using MODIS data from LANCE/ EOSDIS Rapid Response), **(B,C)** Typical freshwater streams in the Lesotho Highlands and **(D)** *Burnupia* attached to rocks in a Lesotho Highland stream.

Limpopo, the Lesotho Highlands are isolated by being ~1,500 m higher due to Jurassic vulcanism which led to the extra basaltic cover giving the highland its pronounced elevation (see Eriksson, 1981; Smith et al., 1993). The Highlands act as the catchment and water tower for several large drainages that filter off in different directions across the southern African landscape. As such, the region holds a diverse array of freshwater environments from rivers and streams to ponds and wetlands (Norström et al., 2018; **Figures 1B,C**), but species richness is virtually unknown for most aquatic groups. Similar cooler, high-altitude regions in temperate landscapes have been shown to support high species richness due to acting as a melting pot of museums (i.e., refugia) for freshwater fauna that evolved under the Pleistocene glacial periods (and require cooler conditions to survive—i.e.,"cold-evolved" species), as well as sinks for opportunistic, temperate species that are expanding their ranges post the Last Glacial Maximum (Füreder et al., 2006; Rahbek et al., 2019; Hågvar et al., 2020; Brighenti et al., 2021; Clewing et al., 2022). As such, similar has been predicted for the Lesotho Highlands aquatic environments (Skelton, 2000) and the region could therefore be important for the conservation of freshwater biota (certainly elevated diversity and/or high endemism has been shown in several terrestrial groups, including land molluscs; Hamer and Slotow, 2009; Kopij, 2015; Perera et al., 2021), but it is equally possible that the generally reduced dispersal capabilities of aquatic fauna in conjunction with glacial cycles, if these were dramatic enough, could have caused several turnover events—where the current species assemblages are all the result of very recent colonisations, thus being relatively species poor.

In the case of freshwater species conservation in the Lesotho Highlands, knowing if there is indeed high relative species richness and cold-evolved species is paramount given growing anthropogenic activity (e.g., waste pollution and agriculture; see Pullanikkatil and Urama, 2011; Chatanga and Seleteng-Kose, 2021; Turpie et al., 2021) and global warming—this is particularly relevant for southern Africa and cold-evolved species, where climatic change is likely to increase substantially over the next century (Dallas and Rivers-Moore, 2014; Serdeczny et al., 2016; Archer et al., 2018; Bentley et al., 2018; Weber et al., 2018). However, conservation relies on accurately being able to quantify relative biodiversity richness in the Highlands as compared with the surroundings and, in this case, decipher cold-evolved species from not (i.e., through determining the evolutionary and spatial origins of species). Molecular phylogenetic techniques can provide a suitable way of establishing the above when dated with molecular clocks and can circumvent some of the pitfalls quantifying species richness through traditional morphology-based methods

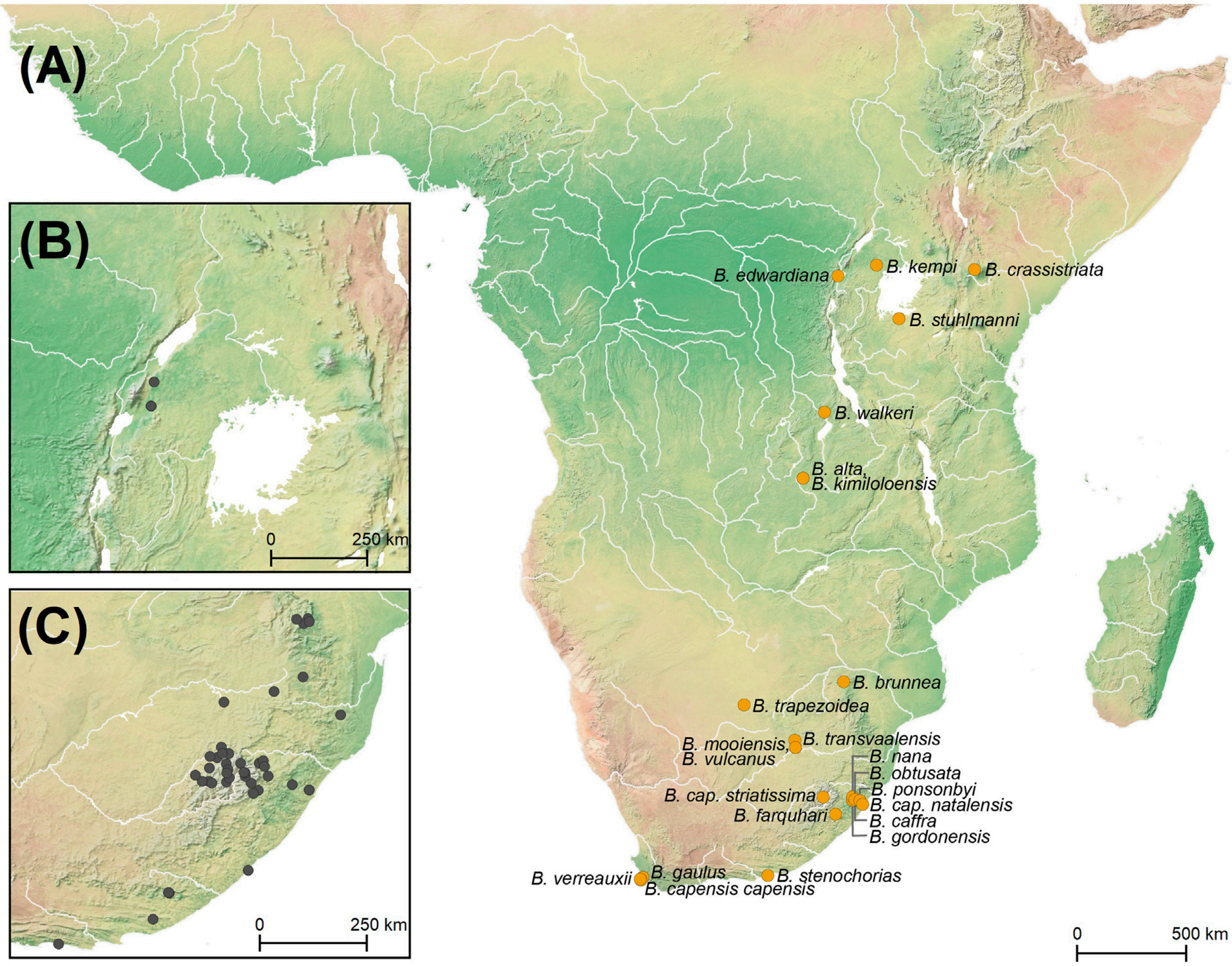

FIGURE 2 | (A) Map of Africa with the type localities of 22 *Burnupia* species and three subspecies of *Burnupia capensis* marked in gold dots, with sub maps showing **(B)** the sampling localities of *Burnupia* incorporated into the current study from eastern Africa and **(C)** southern Africa (inclusive of the Lesotho Highlands) in grey dots.

(e.g., which often have the inability to identify cryptic species). Sadly, there has been a neglect of modern molecular evolutionary aquatic research in the Lesotho Highlands and few studies exist that can even attempt to address these questions in freshwater biota (e.g., Daniels et al., 2003; Swartz, 2005; Swartz et al., 2009; Tolley et al., 2010; Phiri et al., 2016).

In the absence of suitable literature and having molecular evolutionary data for a multitude of species, reconstructing the evolutionary history of a model group may help provide some indications on the relative levels, origins and types of diversity in the Lesotho Highlands. A model taxon needs to be well distributed across southern Africa, have several species, lack a free-swimming larval stage to avoid dispersal biases associated with currents or flow and be a running-water species to avoid dispersal and temporal biases that may be caused by species that occur in transient or short-lived aquatic environments. In this regard, the freshwater limpet genus *Burnupia* meets all the criteria (**Figure 1D**): They are found in high-oxygenated, moving waters from the Western Cape in South Africa right up, through Lesotho, into Botswana, Zimbabwe, Mozambique, Eswatini and even further into the burbling littoral zones of the great lakes of eastern Africa (Brown, 1994; de Kock and Wolmarans, 2009, 2016, 2017; Albrecht and Clewing, 2019). Moreover, there are 22 morphospecies described from across Africa (with 15 occurring specifically within the southern African region; Connolly, 1939; Brown, 1994; **Figure 2A**) which lay a stationary egg mass from which fully formed juvenile limpets emerge (Davies-Coleman, 2001).

In this study, we sampled the genus *Burnupia* across southern and eastern Africa to gain evolutionary insights into the relative freshwater biodiversity of the Lesotho Highlands using molecular phylo- and population-genetic approaches. Specifically, we aimed to; 1) determine if molecular species diversity is rich in the Highlands in comparison to the broader region, 2) assess the evolutionary history of the genus to determine their origin in/colonisation history of the Highlands and the degree of endemism occurring there, as well as, 3) establish if cold-evolved species exist for the genus in the Highlands. To address these aims, we first reconstruct the evolutionary history of the genus using multiple phylogenetic approaches and place a temporal perspective on divergence events using an established

TABLE 1 | Preparation numbers, GPS coordinates (Longitude and Latitude), altitude and GenBank accession numbers of all *Burnupia* specimens used in the current study and the *Protancylus* outgroup (Prep. number: 20024).

Prep. number	GPS Lat.	GPS Long.	Altitude (m a.s.l.)	GenBank accession number	Prep. number	GPS Lat.	GPS Long.	Altitude (m a.s.l.)	GenBank accession number
27789	–26.61000	30.67777	1,260	ON256428	**27816**	–28.73638	28.61833	2,615	ON256537
27790	–27.66222	31.72083	273	ON256429	27817	–28.81527	28.09556	1,578	ON256538
27791	–29.75667	29.43444	1,488	ON256515	27818	–24.99333	30.81444	1,229	ON256539
27792	–29.84527	29.29944	1,632	ON256516	27819	–25.13083	30.69472	1,125	ON256540
27795	–29.00250	29.46138	1,178	ON256517	27820	–25.07388	30.85083	890	ON256541
27796	–28.93611	29.57638	1,064	ON256518	27821	–25.00861	30.49972	1,337	ON256542
27797	–29.09583	29.60861	1,554	ON256519	**27822**	–28.55694	28.41250	1701	ON256543
27798	–29.36361	29.70972	1,655	ON256520	27823	–27.30833	28.48361	1,501	ON256544
27799	–29.55269	29.23520	2,789	ON256521	27826	–31.97861	29.15583	27	ON256545
27800	–29.00805	28.94527	2,342	ON256522	20936	–34.02175	23.89737	34	ON256546
27801	–29.52750	28.55333	2,301	ON256523	20937	–32.60727	26.96279	1,080	ON256547
27802	–29.50805	28.06333	2,207	ON256524	20938	–32.59489	26.94708	1,149	ON256548
27803	–29.50472	27.88056	1,837	ON256525	20939	–32.59489	26.94708	1,149	ON256549
27804	–29.33611	27.69166	1,549	ON256526	20943	–27.01371	29.87412	1,624	ON256550
27805	–29.55298	28.14870	1823	ON256527	**20944**	–28.73313	28.43822	1,673	ON256551
27806	–29.40777	28.63222	1,863	ON256528	20946	–29.74703	30.84878	435	ON256552
27807	–29.36805	29.07444	2,200	ON256529	20947	–29.74703	30.84878	435	ON256553
27809	–29.27111	29.06639	2,073	ON256530	24770	0.64320	30.39399	1,431	ON256557
27810	–29.25472	29.03972	2,059	ON256531	24771	0.06782	30.32354	1,039	ON256554
27811	–29.02027	28.54861	2,102	ON256532	24772	0.06782	30.32354	1,039	ON256555
27812	–29.18722	28.59416	2,141	ON256533	24752	0.06782	30.32354	1,039	ON256556
27813	–29.27611	28.56361	2,061	ON256534	AY282584	–29.60000	30.38000	635	AY282584
27814	–28.84916	28.29166	1,639	ON256535	AY282585	–33.33330	26.51660	639	AY282585
27815	–29.13694	28.07944	1,628	ON256536	20024	–1.78883	120.63465	536	MT925561

Preparation numbers in bold denote those from the Lesotho Highlands.

molecular clock rate. Secondly, we delimit phylospecies to determine the diversity in the Highlands and surroundings. Thirdly, we determine the temporal and spatial origin and/or colonisation history of Highland species by modelling ancestral area estimations and, finally, assess for cold-evolved species therein. As these outcomes and methodological approaches generate a lot of data useful for the *Burnupia* systematics, and several authors have encouraged a molecular review of the genus (Brown, 1994; Albrecht et al., 2004; de Kock and Wolmarans, 2016, 2017; Albrecht and Clewing, 2019), we also discuss the taxonomic implications of our work.

It is hoped that the outcomes of this research will provide us with a preliminary indication of when and where the Highland's freshwater diversity originated and if it is an important area for conservation consideration in respect to relative diversity and/or cold-evolved species. Moreover, we hope the results will encourage and provide guidance and direction for freshwater biogeographic research in the Highlands going forward.

METHODS

Sampling Design, Laboratory Protocols and Sequence Alignment

Forty-five *Burnupia* specimens were hand-collected from hard substrate in shallow water streams and river pools and dams (e.g., **Figures 1D**, **2B,C**). *Protancylus pileolus* was used as an outgroup to root downstream phylogenetic analyses (**Table 1**). Specimens were preserved in 80% ethanol, before being utilised for DNA extraction. Emphasis was placed on collecting *Burnupia* from as many locations across the Lesotho Highlands and surrounding regions as possible (**Figures 2B,C**; **Table 1**). Genomic DNA was extracted from the foot tissue of each specimen using a DNeasy Blood and Tissue kit (QIAGEN) or *via* the CTAB extraction method (Winnepenninckx, 1993). Amplification occurred for a 655 bp region of the mitochondrial DNA cytochrome *c* oxidase subunit 1 (COI) gene through PCR with the primers LCO1490 (Folmer et al., 1994) and COR722b (Wilke and Davis, 2000) and under the following cyclic conditions: 95°C—5 min; 30 cycles of 95°C—10 s, 40°C—30 s, 70°C—1 min; final elongation at 72°C—10 min. The success of PCR amplifications was confirmed by 1% agarose gel electrophoresis, before purification of gene fragments and bidirectional Sanger sequencing were carried out by LGC Ltd. (Berlin, Germany). Sequence ends were trimmed in Geneious 10.1.2 (Biomatters Ltd., 2017). To expand our dataset, all additional sequences from *Burnupia* specimens with corresponding COI data published on GenBank (www.ncbi.nlm.nih.gov/genbank) where incorporated (i.e., two sequences; **Table 1**). Alignments of the 48 gene fragments were performed using Geneious 10.1.2 and the Geneious alignment algorithm.

Phylogenetic Analyses and Molecular Dating

To reconstruct the evolutionary history of *Burnupia*, phylogenetic relationships were established following the methods of Sands et al. (2019a) with only minor differences. In summary, PAUP 4.0b (Swofford, 2002) and MrBayes 3.2.6 (Ronquist et al., 2012) were used to generate unweighted maximum parsimony (MP) and model-informed Bayesian inference (BI) based phylogenies respectively conducted through the CIPRES Science Gateway (Miller et al., 2010). Firstly, the MP analysis was constructed using the heuristic search function, with TBR branch swapping and 100 random taxon additions. Only the best tree was saved during each replicate and the robustness of nodes were assessed by 10,000 bootstrap (BS) replicates (where values ≥70% were considered supported; Felsenstein, 1985). Secondly, the BI analysis was performed to determine the posterior probabilities (PP) of associations. The HKY+I+Γ was selected as the best-fit model of sequence evolution (determined using jModelTest 2.1.10; Darriba et al., 2012) using the Akaike Information Criterion (AIC; Akaike, 1973) and two parallel Markov Chain Monte Carlo (MCMC) simulations used five chains for 100,000,000 generations, saving one tree in every 2,000 generations. The BI trees were summarised after the first 20% of trees were discarded as burn-in, as assessed by parameter convergence in Tracer 1.6 (Rambaut et al., 2014). Nodes with PP ≥ 0.95 where considered supported. Finally, as the BI and MP topologies were highly comparable and no conflict was found between supported nodes of these methods, BS values ≥70% from the MP phylogeny (phylogeny not shown) where transferred onto the BI phylogeny.

To place a temporal perspective on divergence events, to observe when the Lesotho Highlands were colonised and if these overlap with major climatic or geological changes, a dated phylogeny was constructed with the BEAST package (Bouckaert et al., 2014). Four independent runs of 100,000,000 MCMC generations, saving one tree in every 10,000 generations, were constructed in BEAUti 2.6.5 and implemented in BEAST 2.6.3 through the CIPRES Science Gateway (Miller et al., 2010). For each run, a lognormal relaxed clock and the birth–death tree prior were selected. bModelTest 1.1.2 (Bouckaert and Drummond, 2017), as implemented in BEAST, was used to determine the best-fit model for the COI dataset which was subsequently determined to be a variant of the HKY (with an additional group for the rates rct and rgt; 121,323). As fossil dating is challenging for *Burnupia*, the phylogeny was calibrated using published molecular clock rate for the COI gene in Wilke et al. (2009). The clock rate was set to the 95% confidence intervals found therein and linearly distributed [COI = 1.695% per million years; standard deviation (SD) = 1.33%–2.06%]. Thereafter, LogCombiner 2.5.2 (Bouckaert et al., 2014) was used to combine trees and log files of each run with 75% burn-in removed. Validation of the convergence and mixing of the combined log file was assessed in Tracer 1.7.1 (Rambaut et al., 2014) to ensure that all effective sample size (ESS) values were ≥200 and TreeAnnotator 2.5.2 (Bouckaert et al., 2014) was used to summarise trees, with no further burn-in removed.

Species Delimitation

Three species delimitation methods were followed to get an idea on the species richness and population structure in *Burnupia* in the Lesotho Highlands and surroundings: These included the General Mixed Yule-coalescent (GMYC), bayesian Poisson Tree Processes (bPTP) and Discriminant Analysis of Principal Components (DAPC). In all species delimitation instances, the outgroup (*P. pileolus*) was removed from sequence alignment files or input topologies (using TreeGraph 2; Stöver and Müller, 2010) to limit impacting *Burnupia* spp. delimitation results. The GMYC species delimitation method made use of the amended time-calibrated, ultrametric tree (generated in BEAST) and run using single and multiple thresholds, while the bPTP species delimitation method made use of the amended Bayesian topology (generated through MrBayes). All settings were otherwise kept as default for these approaches and the analyses were run through the online server at https://species.h-its.org/ptp/. The DAPC approach was performed using the adegenet 1.4-1 (Jombart and Bateman, 2008) package as run in the R statistical environment 4.0.2 (R Core Team, 2020) using a fasta alignment of all *Burnupia* haplotypes. Herein, Bayesian Information Criterion (BIC; Schwarz, 1978) was used to determine the optimal number of genetic clusters (k) before the DAPC analysis defined the specimens in each cluster.

Ancestral Distribution Analyses

To gain better perspectives of the evolutionary origins or colonisations of the established *Burnupia* phylospecies in the Lesotho Highlands ancestral area estimation was assessed across the dated phylogeny. BioGeoBEARS (Matzke, 2013) was implemented in RASP 4.2 (Yu et al., 2015) and used to estimate ancestral areas under six different biogeographical models for the entire dated phylogeny. This included the DEC, DIVA-like and BayArea-like models, including the +J parameter for each. Here, a simplified analysis with default settings in which only two areas were predefined (namely "within the Lesotho Highlands" and "outside the Lesotho Highlands") was run and the best-fit model was determined by using the AIC—as implemented in BioGeoBEARS.

Isolation by Elevation and Distance

To assess the level of genetic diversity that can be attributed to Isolation by Distance (IBD) and Elevation (IBE), and thus help identify if the genus may contain cold-evolved species (i.e., those restricted by elevation and/or geographic distance at high altitudes), analyses of IBD and IBE were calculated within the genus as a whole following the methods of Sands et al. (2019b). In summary IBE and IBD was calculated using genetic, elevation and geographic distance matrices between samples (as compiled in GenAlEx 6.5; Peakall and Smouse, 2006) and following the distance-based redundancy analysis method (db-RDA; Legendre and Anderson, 1999; Legendre and Fortin, 2010) with the package vegan 2.5-4 (Oksanen et al., 2019) in the R statistical environment 4.0.2 (R Core Team, 2020).

RESULTS

Phylogenetics and Species Diversity

Between the BI and MP topologies generated in MrBayes and Paup respectively there are 19 supported nodes among the

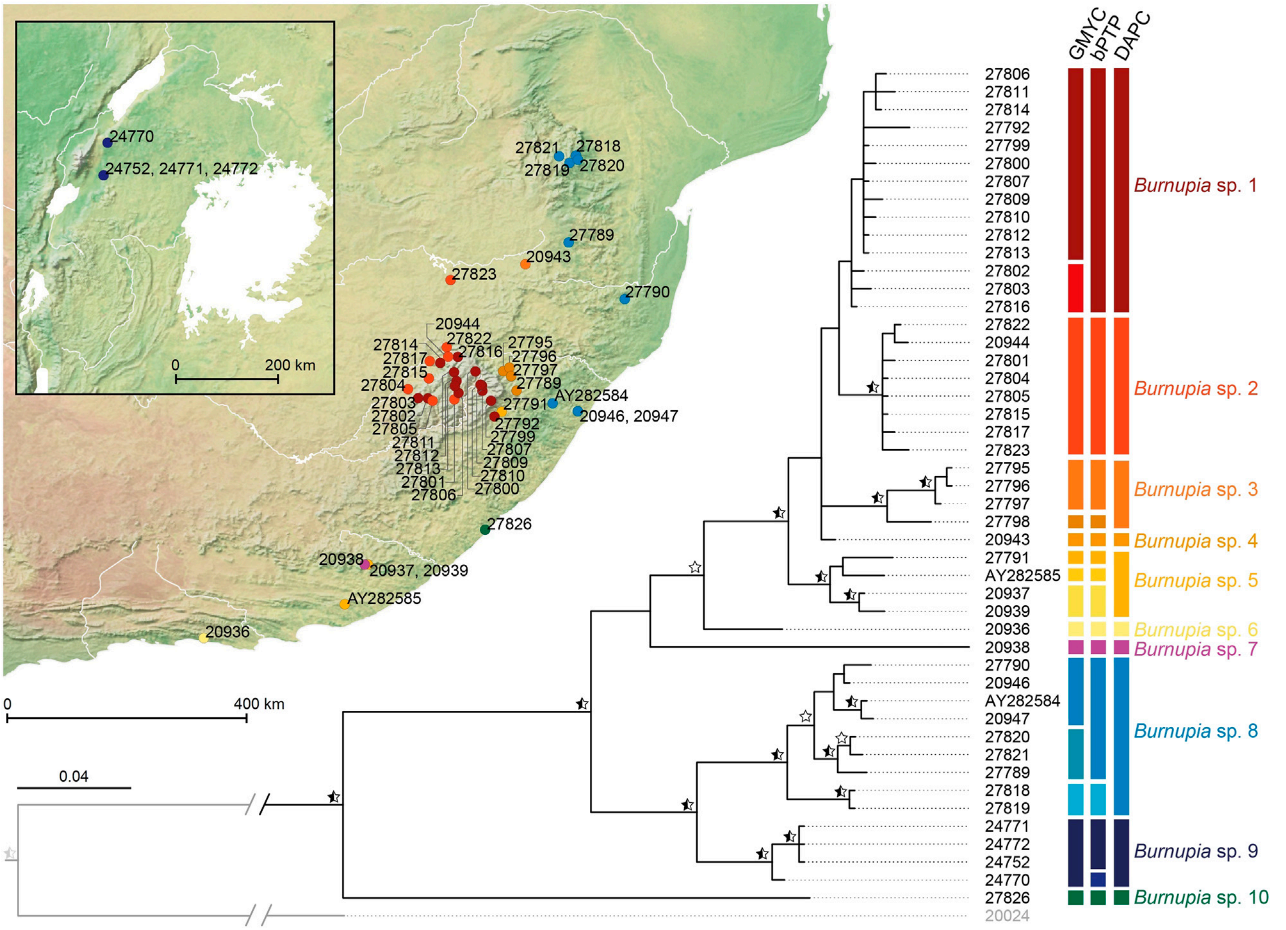

FIGURE 3 | Bayesian phylogeny of *Burnupia* generated in MrBayes. Supported nodes (PP ≥ 0.95) are marked with black stars. Nodes additionally supported by parsimony analyses (Paup; BS ≥ 70) are marked with white stars or half black/white stars where PP support already exists. Results of the species delimitation methods (i.e., GMYC, bPTP and DAPC) are given alongside the tips in the form of coloured bars where different colours within each method denote different species and shared colours across methods indicate shared species. A distribution map with dots coloured and following the DAPC derived species are also presented. Further details on the samples and localities can be found in **Table 1** following the specimen alphanumeric labels.

relationships of the 47 *Burnupia* specimens (PP ≥ 0.95 and/or BS ≥ 70; **Figure 3**). Species delimitation results range from between 10–16 *Burnupia* spp. among the dataset (GMYC = 16, bPTP = 15, DAPC = 10; **Figure 3**). The DAPC approach is the most conservative and, although not always reflective of phylogenetic support, best corresponds to the supported clade structures of the phylogenies. The other methods (i.e., GMYC and bPTP) tend to define species across multiple unsupported nodes creating increased complications with phylogenetic support. The ten species classified by the DAPC approach and regions where specimens of each were found are as follows: *Burnupia* sp. 1 (Lesotho Highlands), *Burnupia* sp. 2 (northern and western Lesotho Highlands and the South African Highveld), *Burnupia* sp. 3 (South African Highveld) and *Burnupia* sp. 4 (lower slopes of the eastern Drakensberg, KwaZulu-Natal), *Burnupia* sp. 5 (lower slopes of the south-eastern Drakensberg, KwaZulu-Natal down into the Eastern Cape), *Burnupia* sp. 6 (Tsitsikamma), *Burnupia* sp. 7 (Katberg Mountains), *Burnupia* sp. 8 (South African Lowveld and KwaZulu-Natal), *Burnupia* sp. 9 (Great Lake drainages of western Uganda) and *Burnupia* sp. 10 (Wild Coast) (**Figure 3**).

Dated Phylogeny and Ancestral Distributions

The genus *Burnupia* plausibly arouse somewhere between 25.95–5.64 Ma (data not displayed in **Figure 4**). While this study lacks several species of *Burnupia* (e.g., those from South America—which possibly represent a separate genus), the species occupying southern Africa likely began interspecific diversification from a common ancestor around 9.26 Ma [95% highest posterior density (HPD): 13.45–5.64 Ma; **Figure 4A**]. Following the DAPC species delamination results, by 0.60 Ma (95% HPD: 1.00–0.29 Ma; for *Burnupia* sp. 1 and *Burnupia* sp. 2) all species have diverged and the remaining genetic diversity is held intraspecifically (**Figure 4A**).

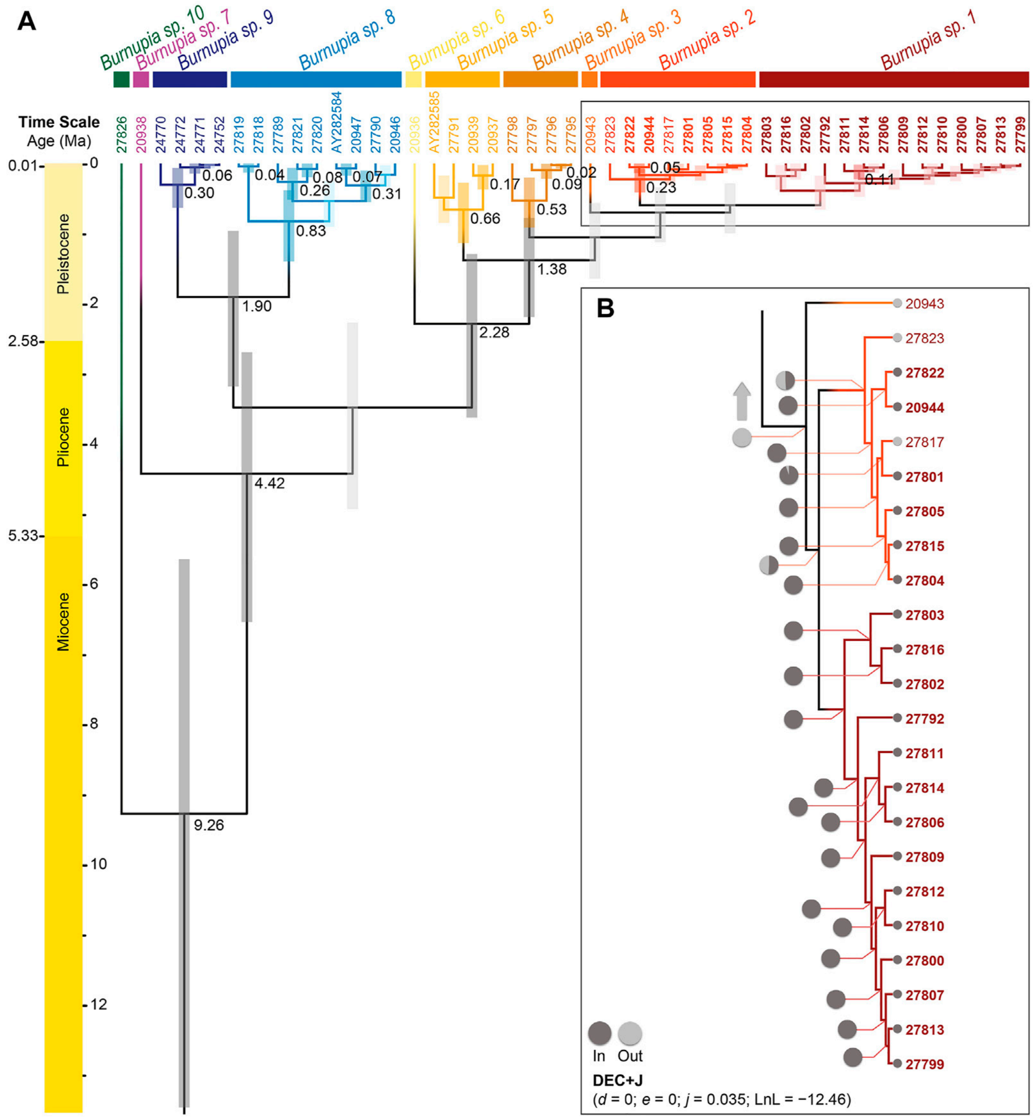

FIGURE 4 | (A) The dated phylogeny of *Burnupia* spp. sampled across southern and eastern Africa constructed in BEAST based on COI sequence data. Supported divergence events (PP ≥ 0.95) are indicated by written dates at the nodes (in millions of years ago; Ma) and brighter shaded 95% credibility interval (i.e., HPD) bars. Species or clades are partitioned and coloured according to the DAPC species delimitation results (see **Figure 2**). Tip labels correspond to the preparation numbers of specimens noted in **Table 1** and, therein, those in bold correspond to specimens occurring in the Lesotho Highlands. **(B)** The portion of the DEC+J results of the ancestral area estimation for *Burnupia* spp. on the dated phylogeny that correspond to Lesotho Highland occurrences. Ancestral area estimations were obtained from BioGeoBEARS run through RASP. Nodal pie charts represent the relative likelihood of the lineage occurring within the Lesotho Highlands (dark grey) and outside of the Lesotho Highlands (light grey). Smaller pies at the tips represent the current occurrence of each specimen. All other nodes across the phylogeny (data not shown; indicated by the light grey arrow) are equivalent to ancestral areas outside of the Lesotho Highlands.

Ancestral distribution estimations suggest that the common ancestors of all *Burnupia* species included in this study is not likely to have occurred in the Lesotho Highlands (**Figure 4B**). The analysis demonstrates only *Burnupia* sp. 1 and *Burnupia* sp. 2 have had historical presences in the Lesotho Highlands. The results point to the common ancestor of these two species likely colonising the Lesotho Highlands between 1.12–0.29 Ma, however significant PP and BS support for nodes surrounding this clade could not be found across our phylogenetic topologies (**Figure 4A**). Nevertheless, given species of *Burnupia* from the Lesotho Highlands seem to be closely related and *Burnupia* sp. 1, *Burnupia* sp. 2 and *Burnupia* sp. 3 diverged from *Burnupia* sp. 4 around 1.38 Ma (95% HPD: 2.19–0.78 Ma; PP ≥ 0.95 and BS ≥ 70; **Figures 3**, **4**), this would indicate colonisation only occurred after this point (**Figure 4**).

Isolation by Distance and Elevation

Within the genus *Burnupia* as a whole, both IBD and IBE were significant yet both very weak in accounting for genetic diversity between species within the genus. We found

$R^2 = 0.097$ ($p < 0.001$) for IBD and $R^2 = 0.102$ ($p < 0.001$) for IBE, respectively (also see **Supplementary Material**).

DISCUSSION

Diversity and Biogeography in the Highlands

Although not particularly species rich in *Burnupia*, our results point to the Lesotho Highlands as being an important area for freshwater diversity.

Our results support between 10–16 phylogenetic species established through the molecular delimitation methods across, primarily southern Africa but also Uganda, albeit limited sampling the latter (**Figures 2B**, **3**). Following the most conservative delimitation method, the DAPC approach, that best corresponds to our phylogenetic support and clade structure in our trees, two species can be found in the Lesotho Highlands, six can be found in the lower laying areas of KwaZulu-Natal and the Eastern Cape, three in the inland areas of South Africa (i.e., in the Highveld and Lowveld) and a single species was identified in the Western Region of Uganda (**Figure 3**). Diversity in the Lesotho Highlands is therefore to be seen as relatively average in comparison to at least the broader southern African region. However, given the temporal persistence of *Burnupia* in the Highlands and the endemicity of at least one of these species our results do suggest the region is important for cold-evolved freshwater biodiversity. Similar patterns have been observed for other aquatic organisms in the region (Daniels et al., 2003; Tolley et al., 2010). Our study is based on mitochondrial DNA variation. Studies in the European freshwater limpet genus *Ancylus* has demonstrated that diversity of young species can be underestimated—genome-wide approaches can help detecting hidden diversity in such cases (Weiss et al., 2018).

It is interesting that *Burnupia* partly overlaps with two other cold-evolved endemic species in the Highlands (Prinsloo and van Eeden, 1973). *Bulinus* sp. occurs up to 3,100 m.a.s.l. in Lesotho (Tumwebaze et al., 2022) and *Galba mweruensis* reaches similar altitudes (Mahulu et al., 2019). These two species are primarily found in stagnant and even temporary waters, whereas *Burnupia* species predominate on hard substrates as found in the permanent streams in the Highlands. A high oxygen demand and less drought tolerance has been shown for *Burnupia* (but see de Kock and Wolmarans, 2017) and some species have been used in lowland regions for ecotoxicological monitoring (e.g., Gerhardt and Palmer, 1998; Davies-Coleman and Palmer, 2004; Vellemu et al., 2018). Temperature sensitivity of *Burnupia* could potentially be established as a proxy for ongoing climatic changes, also in other Afromontane regions. In eastern Africa *Burnupia* also coincides with *Bulinus* and *Galba*, for example in the Ethiopian Highlands and the Aberdares Mountains, the Mau region and the Mount Kenya Massif (Brown, 1994; Mahulu et al., 2019; Tumwebaze et al., 2022). Comparative phylogeographical studies should be conducted to establish general patterns for freshwater benthic organisms in the Afromontane archipelago of sky-islands. Such studies could also help understanding better the means of colonisation across various taxa (Daniels et al., 2003; Tolley et al., 2010; Taylor et al., 2020). For *Burnupia*, the intrinsic characteristics of this limpet group, restrictive active dispersal and the narrow ecological tolerances question the actual dispersal and thus colonisation mechanism. Given that populations occur along basically whole drainage systems from lowland to sometimes up to the spring regions, jump dispersal is less likely. On the other hand, the nature of the habitat, high currents and the mobility of the (gravel) substrate restricts the ability of active dispersal upstream tremendously in aquatic mollusc (Kappes and Haase, 2012), more even for limpets (Albrecht et al., 2006). Birds as dispersal vector are probably not important in this case of upstream movement. Fish are often invoked as biological passive vector (Kappes and Haase, 2012). In fact, there are candidate species of fish including *Labeobarbus* yellow fishes (see Plug et al., 2010; Schrijvershof, 2015). Their role in the dispersal context of *Burnupia* should be studied in a comparative population-genetics framework.

The common ancestor of *Burnupia* sp. 1 and *Burnupia* sp. 2 was likely the first to colonise the region (**Figure 4B**). Our temporal reconstruction of the evolution of *Burnupia* suggests this may have occurred around 1.12–0.29 Ma, however phylogenetic support for the divergence events between *Burnupia* sp. 1, *Burnupia* sp. 2 and *Burnupia* sp. 3 are not well supported. Nevertheless, given the closest supported nodes, colonisation certainly happened post 1.38 Ma (95% HPD: 2.19–0.78 Ma; **Figure 4**) and prior to 0.23 Ma (95% HPD: 0.41–0.08 Ma; **Figure 4**). This Pleistocene period means *Burnupia*'s persistence in the Highlands likely overlapped with possibly several glacial maxima (Elderfield et al., 2012). Glacial cycles probably had two effects: firstly, glacial maxima caused the freezing of suitable freshwater environments in the Highlands (Mills et al., 2009, 2012; Hall and Meiklejohn, 2011)—this likely drove the several allopatric speciation events that can reasonably explain the speciation of *Burnupia* sp. 1 and *Burnupia* sp. 2 and the population structures therein. Secondly, glacial cycles probably drove several retreats and recolonisations (i.e., sinking) in *Burnupia* sp. 2 (**Figure 4**) and likely forced *Burnupia* sp. 1 to become cold-evolved given the endemicity of this species in the Highlands (**Figures 3**, **4**), which likely accounts for at least some signals of IBD and IBE within *Burnupia*. Given our results, the *Burnupia* might serve as an ideal model group for testing the existence and extent of refugia and generally the role of climatic changes in rapidly changing Afromontane environments and colonisation patterns and processes, including co-evolution with dispersal vectors. As such, *Burnupia* could well become an Austral pendant to freshwater limpets in the northern hemisphere such as *Ancylus* (Albrecht et al., 2006) or *Acroloxus* (Stelbrink et al., 2016). An important asset in such studies would be an enhanced and better resolved taxonomic framework for all African *Burnupia* spp.

Taxonomic Implications

Freshwater scientists operating in Africa have long been advocating for a molecular based review of the genus *Burnupia* given the distribution of type localities among described species and shallow morphological differences among certain groups (Brown, 1994; Albrecht and Clewing, 2019), yet our molecular results only partly elucidate the complex picture. Brown (1994) presumed a very high level of synonymies among the available names but still listed no less than 21 species of *Burnupia* in his seminal book.

Our sampling regime largely overlapped the type localities of 14 of the 22 *Burnupia* species described from Africa as well as two subspecies of *B. capensis* (**Figure 2**). It was therefore interesting to find between 10–16 phylogenetic species established through the molecular delimitation methods (**Figure 2**). While the DAPC approach showed the best correspondence between the phylogenetically supported clade structure and the expected morphological diversity, the distribution of phylogenetic species did not entirely always correspond well with the distribution of described species and their type localities (**Figures 2**, **3**). For example, at least six species have been described or noted in the KwaZulu-Natal Midlands (**Figure 2A**), yet through our sampling we could only find a single species in this area (i.e., *Burnupia* sp. 8; **Figure 3**). This species corresponds to *B. caffra* (Albrecht et al., 2004). While our sampling regime did not specifically target type localities, it is not unreasonable to suggest careful review and possible synonymisation of *B. caffra*, *B. capensis natalensis*, *B. gordonensis*, *B. nana*, *B. obtusala* and *B. ponsonbyi* may need to be looked at, especially given the broad distribution range of *Burnupia* sp. 8 (i.e., *B. caffra*) and that some of these described species share type localities in very close proximity to one another—sometimes even in the same drainage systems (Brown, 1994). Additionally, only a single species has been described (i.e., having a type locality) from the Eastern Cape region of South Africa (i.e., *B. stenochorias*; **Figure 2A**), yet we found three seemingly endemic lineages in this area (i.e., *Burnupia* sp. 6, *Burnupia* sp. 7 and *Burnupia* sp. 10; **Figure 3**). These could possibly represent the range extremities of Western Cape species whose type localities and systems were sampled but specimens failed to be amplified for COI (i.e., *B. capensis capensis*, *B. gaulus* and *B. verreauxii*), however, *B. c. capensis* and *B. gaulus* have been synonymised (see Brown, 1994) and it seems more realistic that they are as yet undescribed lineages given the lack of drainage collection between the southern Western Cape type localities and the large environmental and climatic shifts up the east coast of southern Africa (Werger, 1978; Abell et al., 2008; Linder et al., 2012). Placing names to phylogenetic species is therefore still a major challenge.

These naming uncertainties also extend to the *Burnupia* diversity in the Lesotho Highlands. For example, the distribution of *Burnupia* sp. 1 largely conforms with that attributed to *B. capensis striatissima*, while *Burnupia* sp. 2 conforms with that noted for *B. trapezoidea* (Brown, 1994; de Kock and Wolmarans, 2009, 2017). However, it seems unlikely that *B. capensis striatissima* is a subspecies of *B. capensis* given the endemicity in the Highlands and very close relationship with *Burnupia* sp. 2—noted as *B. trapezoidea* (see de Kock and Wolmarans, 2017; **Figures 3**, **4**). The name *B. trapezoidea* (described from modern day Botswana) for *Burnupia* sp. 2 itself also requires careful review given three species (i.e., *B. mooiensis*, *B. transvaalensis* and *B. vulcanus*) have been described across the Highveld, particularly in the Vaal River catchments (Brown, 1994; **Figures 2A**, **3**).

The taxonomic complications and conflicts in *Burnupia* are not unexpected. Other freshwater limpets have a similar history of splitting and lumping, which often masks real biodiversity (e.g., in *Ancylus*; Albrecht et al., 2006). Increasingly in aquatic malacology, when traditional shell-based taxonomic approaches are reviewed with molecular data and modern morphological methods, differences in shell shapes and patterns are noted to sometimes be poor tools for distinguishing species and likely to be influenced by environmental changes in some groups (Schultheiss et al., 2009; Sands et al., 2020; van Bocxlaer et al., 2021). Such changes have even been documented in a variety of molluscs in laboratory settings by small alterations in their environments (Neumann, 1959; Kistner and Dybdahl, 2013). If the same is corroborated for *Burnupia,* research involving type material is vitally needed to properly update the systematics of the genus and attribute the correct names to the phylogenetic species discovered.

CONCLUSION AND FUTURE OUTLOOKS

Our study revealed that the Lesotho Highlands are an important region for freshwater diversity and likely holds cold-evolved species. These Highlands were colonised roughly during Calabrian–Chibanian stages of the Pleistocene by *Burnupia* species and may be a useful model to study the evolution of the Highlands and likewise its impact on freshwater organisms given indications of population structure. It requires NGS approaches to be used which are more sensitive to reconstructing population-level history. The study has improved our knowledge of *Burnupia* systematics, but it still remains mostly unresolved. Further research is desperately needed using type material and establishing if morphological features such as shell shape and patterning is affected by environmental conditions.

DATA AVAILABILITY STATEMENT

The DNA sequence data presented in this study can be found in the NCBI's GenBank repository online (www.ncbi.nlm.nih.gov/genbank). The accession numbers can be found in **Table 1**.

ETHICS STATEMENT

Ethical review and approval was not required for the study. All material was sampled through bilateral agreements among collaborating institutes or with local permits where required.

AUTHOR CONTRIBUTIONS

The team of authors has a strong research focus on freshwater biogeography, phylogenetics and geology—especially in Africa. All authors contributed to the general framework of the article: CA, VSG, and FR performed fieldwork and collected samples; CA generated the data; AFS performed the analyses; AFS and CA led the writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2022.914272/full#supplementary-material

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