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Filling in the Gaps: Adopting Ultraconserved Elements Alongside COI to Strengthen Metabarcoding Studies

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Metabarcoding is rapidly gaining popularity as a means of conducting biodiversity studies. Using DNA barcodes to identify and catalog biodiversity has many advantages, and compares favorably with traditional methods based on morphological examination. Ease of use, taxonomic coverage, and increased efficiency are qualities that make metabarcoding a valuable ecological tool, particularly in light of the drastic anthropogenically induced ecosystem changes currently underway. However, limitations and challenges pertaining to existing barcodes create gaps from which inaccuracies can arise, contributing to skepticism regarding the value of metabarcoding based methods. Developing novel ways to address these limitations is crucial to improve metabarcoding methods and dispel doubt about their utility. Ultraconserved genomic elements (UCEs), genetic markers that have been used successfully in the field of phylogenomics, possess advantageous qualities that may be applied to fill in the gaps of existing metabarcoding methods. Here, I outline the strengths of UCEs and discuss their potential for complementing and strengthening existing metabarcoding methods based on the mitochondrial marker cytochrome oxidase I (COI).

Keywords: biomonitoring, DNA barcodes, marker multiplexing, metabarcoding, ultraconserved elements, biodiversity surveys

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INTRODUCTION

Researchers are increasingly using metabarcoding to address questions across a wide range of scientific fields. For example, metabarcoding has been used in recent studies to assess parasitism in an invasive species (Kitson et al., 2018), characterize hidden cryptic diversity in a reef ecosystem (Carvalho et al., 2019), and identify dietary choices of a prairie bird (Sullins et al., 2018), to name just a few. Over recent years, studies evaluating the performance of these methods have consistently demonstrated that metabarcoding can match, and in many cases exceed, the performance of traditional morphology-based methods (Ji et al., 2013; Deiner et al., 2017; Bush et al., 2019). Particular strengths of metabarcoding include taxonomic comprehensiveness and resolution, independence from taxonomic expertise, ability to overcome misidentifications, and efficiency in terms of time, manpower, and cost (Ji et al., 2013; Bush et al., 2019).

However, significant limitations and challenges to metabarcoding remain (Zinger et al., 2019). These include inherent issues like estimating abundance (Piñol et al., 2018), as well as logistical challenges such as selecting robust barcodes that work accurately across a wide taxonomic range

(Kress et al., 2015). Barcodes must meet certain criteria (Taberlet et al., 2007), and no universal genetic marker meeting these criteria has yet been identified (Valentini et al., 2009). Consequently, a range of markers has emerged, each utilized by researchers focusing on different taxonomic groups (Porter and Hajibabaei, 2018). For animals, the mitochondrial gene cytochrome c oxidase subunit 1 (COI) has emerged as the most commonly used marker for barcoding. This marker choice has many advantages, as reflected by the extent to which it is used and its thorough coverage in reference databases, a critical point for effective metabarcoding studies (Andújar et al., 2018).

Unfortunately, several issues associated with COI create potential sources for error, including incomplete lineage sorting, heteroplasmy, introgression, and the presence of pseudogenes (Rubinoff et al., 2006). More importantly, COI does not amplify equally well across all animal taxa, a major limitation for metabarcoding surveys aiming to achieve maximal taxonomic coverage (Kress et al., 2015). Overcoming these obstacles is therefore an important goal, both to increase metabarcoding accuracy and to dispel skepticism inhibiting a more widespread adoption of metabarcoding methods. Solutions that are relatively easy to incorporate into existing pipelines, such as marker multiplexing (Zhang et al., 2018), will be especially valuable. A phylogenomic approach of growing popularity utilizing ultraconserved elements (UCEs) may provide a complementary approach. Primarily used for reconstructing evolutionary relationships, several unique qualities of UCEs make them promising candidates for complementing and strengthening COI-based metabarcoding studies.

ULTRACONSERVED ELEMENTS

UCEs are conserved genomic regions found in large numbers throughout the genome. They consist of a highly-conserved core region flanked by more variable sequence (Faircloth et al., 2012), and have been identified in a wide range of eukaryotic groups, including plants, fungi, invertebrates, and vertebrates (Siepel et al., 2005; Reneker et al., 2012). UCEs are identified by aligning two or more genomes and scanning for regions of high fidelity, from which bait sets are then designed to extract DNA fragments containing UCE regions during targeted enrichment (Faircloth, 2017). Several advantages of UCEs have made them valuable tools in phylogenomics, where they have been used to high success in a number of animal groups (McCormack et al., 2013; Branstetter et al., 2017a; Alfaro et al., 2018). These include their high level of sequence conservation, robustness to duplication, strong phylogenetic signal, and the large number of alternate UCE loci present in the nuclear genome (Derti et al., 2006; Stephen et al., 2008; Faircloth et al., 2012). These same advantages of UCEs have application to metabarcoding, and may help fill in the gaps created by the limitations of COI.

UCEs AND BARCODE CRITERIA

Not all genetic markers can be used as barcodes, and not all barcodes work equally well across or within taxonomic groups (Kress and Erickson, 2008). Selecting appropriate barcoding regions is critically important for biodiversity surveys, with major implications for the organismal groups that can be studied (Deagle et al., 2014), and candidate genetic markers should meet certain criteria (Taberlet et al., 2007; Valentini et al., 2009). Furthermore, the best choice of barcode will depend on the individual priorities and goals of a given study. Metabarcoding studies prioritize accurate, high throughput species recovery from samples of unknown taxonomic composition, typically containing degraded DNA, and therefore should use genetic markers with strengths in these areas (Taberlet et al., 2012). Although COI-based metabarcoding has been shown to work well-compared to traditional morphological approaches, its weaknesses limit the ability of metabarcoding studies to accurately recover the full range of animal species present in an environment. Incorporating other markers with complementary strengths will increase the accuracy and reliability of existing metabarcoding methods. Below, I summarize the relative strengths and weakness of UCEs and COI in the context of metabarcoding based on previously suggested criteria (Taberlet et al., 2007; Valentini et al., 2009), and discuss how UCEs may be used to complement and strengthen metabarcoding methods.

Species Discrimination

DNA barcodes should discriminate species effectively, having high intraspecific fidelity while being variable between species. COI has been used to successfully identify and differentiate species in many groups (Hebert et al., 2003), particularly when used as part of an integrative approach to taxonomy (Will et al., 2005; Janzen et al., 2009). However, issues like incomplete lineage sorting, heteroplasmy, introgression, and the existence of pseudogenes, may result in incongruence between the number and identity of COI sequences and species or populations represented in a sample, resulting in false estimates and misidentifications (Moritz and Cicero, 2004; Will et al., 2005; Rubinoff et al., 2006). Additionally, single-locus methods are vulnerable to overlapping character variation (Will et al., 2005). These issues limit the ability of COI to accurately and reliably differentiate species, particularly uncharacterized taxa. This is especially problematic for metabarcoding studies where using additional verification methods is generally not desirable. Conversely, UCEs are robust to such issues. UCE loci have been found to be depleted from duplicated gene regions, are present in high numbers throughout the genome, and the bait design workflow removes loci deemed likely to be paralogs (Derti et al., 2006; Stephen et al., 2008; Faircloth, 2017). Though UCEs may be occasionally duplicated in some taxa or missing in others, the large number of UCEs available can provide consensus estimates, and problematic UCE loci can be pruned from bait sets as these become more refined through the increasing availability of sequenced genomes.

Universal Standardization

A truly universal barcode will have functionality across the Tree of Life, working equally well across and within all groups. Because no genetic marker fitting this criterion has yet been identified, utilizing barcodes that work well across different

taxonomic ranges is the best possible alternative. As such, different *de facto* universal barcodes have emerged for different taxonomic groups (Taberlet et al., 2007). COI has become the *de facto* universal barcode for the metazoa. However, it is not equally effective across or within all metazoan lineages (Deagle et al., 2014). Some groups will require different barcodes, resulting in fragmentation of metabarcoding methodologies and sequence databases. Although no single UCE locus is likely to be universal, comprehensive UCE bait sets can be designed with wide taxonomic coverage, as has been demonstrated for diverse groups such as amniotes (Faircloth et al., 2012), fish (Faircloth et al., 2013; Alfaro et al., 2018), and several hyper-diverse invertebrate groups (Starrett et al., 2016; Baca et al., 2017; Branstetter et al., 2017b; Quattrini et al., 2018). While the number of orthologous UCE loci drops as a function of phylogenetic distance between taxa, hundreds to thousands of UCEs are still available covering groups separated by hundreds of millions of years of evolution (Faircloth et al., 2012). Eventually, UCE bait sets with universal coverage of metazoan groups may be designed to consistently recover the full range of species represented in environmental and bulk samples.

Phylogenetic Signal

Barcodes must contain a sufficient phylogenetic signal to assign taxonomy to recovered sequences. Ultimately this requires the availability of comprehensive open-access databases of taxonomically verified sequences for comparison. Such databases already exist for COI, and this is arguably the greatest strength of this marker as a barcode choice (Andújar et al., 2018). Because UCEs are flanked by regions of increasing variability, they are useful for resolving both deep (Crawford et al., 2012) and shallow relationships (Smith et al., 2014). This suggests that UCEs would be effective for both discriminating species and assigning taxonomy in metabarcoding studies. However, because taxonomically comprehensive databases of UCE sequences from a wide range of species do not exist at present, assigning taxonomy at lower levels (e.g., family and below) to UCEs recovered during metabarcoding represents a challenge. Combining both marker types would allow users to generate consensus diversity estimates and pinpoint possible sources of error, while leveraging the taxonomic coverage of COI reference databases.

Robustness and Recoverability

For barcodes to be effective they must be reliably amplified, containing both highly conserved and variable regions (Taberlet et al., 2007). Sequence conservation is especially important for metabarcoding, which uses DNA extracted from environmental and bulk samples containing a wide taxonomic range of species of an unknown composition. However, the conserved region of COI, necessary for effective primer binding, is not sufficiently conserved to work equally well across or within all animal groups (Deagle et al., 2014). Because of this, the amplification step may introduce biases in both copy number and taxonomic representation. The core regions of UCEs are, as the name implies, highly conserved, and are reliably recovered using targeted enrichment (Gnirke et al., 2009; Faircloth et al., 2012). The targeted enrichment approach does not require amplification with universal primers, reducing the opportunity for bias. Moreover, UCEs are identified by comparing the genomes of highly divergent taxa, but importantly the bait sets designed to target them have been demonstrated to work well on a multitude of intermediate taxa, an underpinning of the approach (Faircloth et al., 2012).

Environmental and Degraded DNA

Environmental DNA is a major component of metabarcoding studies (Deiner et al., 2017), which obtain DNA from diverse sources like seawater (Boussarie et al., 2018) and fecal samples (Sullins et al., 2018), and which may be from either modern or ancient environments (Thomsen and Willerslev, 2015). Environmental DNA is generally degraded, and the proportion of amplifiable fragments drops off with increasing amplicon size (Deagle et al., 2006). Longer barcodes will be difficult to amplify, and it has been recommended that markers used for amplifying degraded DNA be no longer than 150 bp (Valentini et al., 2009). COI is several times this length, creating a need for developing shorter barcodes for use with degraded DNA (Hajibabaei et al., 2006). By contrast, UCE loci have a wide range of lengths (Bejerano et al., 2004), bait sets targeting shorter UCE loci can be specified, and at 120 bp, the baits used to enrich UCE loci fit within the commended maximum length. Furthermore, UCEs have been demonstrated to work successfully with old and degraded DNA such as that obtained from museum specimens stored in suboptimal conditions (Blaimer et al., 2016; McCormack et al., 2016).

DISCUSSION

COI does not make a perfect metabarcode, and its widespread use reflects the lack of apposite substitutes rather than its suitability as a marker. As noted by Deagle et al. (2014), even the best metabarcoding studies using COI have pointed out its limitations, underscoring the importance of developing alternative markers. At the heart of this lies the fact that COI cannot be reliably and consistently amplified from all animal groups or from environmental samples containing degraded DNA, both of which are crucial points for metabarcoding. Utilizing multiple barcoding regions and markers better suited for use with degraded DNA will likely become a matter of routine, as multiplexing markers can improve the accuracy and reliability of species recovery (De Barba et al., 2014; Zhang et al., 2018). Taberlet et al. (2012) also discuss potential methods for overcoming amplification bias. Direct sequencing methods, similar to genome skimming (Dodsworth, 2015) or metagenomics (Quince et al., 2017), are one possible solution. Direct sequencing methods produce large amounts of data without the bias introduced during amplification. However, most of the data is likely to be taxonomically unassignable or prokaryotic in origin, and direct sequencing has been shown to significantly underperform compared to metabarcoding in regard to evaluating eukaryotic diversity (Stat et al., 2017). Another solution identified by Taberlet et al. (2012) involves sequence capture, using hundreds of baits to target different taxonomic groups. Targeted enrichment using UCEs fits this

proposed solution neatly, with the significant advantage of established bait sets and open-access workflows, minimizing the cost and effort required to adapt these methods to metabarcoding studies.

It is important to note that the limitations of COI described here apply to metabarcoding studies that utilize DNA extracted directly from bulk community and environmental samples containing an unknown species composition (*sensu* Stat et al., 2017; Ritter et al., 2019). In regard to standard barcoding studies, which benefit from a narrower approach focusing on single specimens and usually complemented by alternative methods like morphological examination, COI has been used to a high degree of success (Janzen et al., 2009). Even with its limitations, COI has been used successfully in a variety of metabarcoding applications and retains several advantageous qualities. Chief among these is the public availability of millions of taxonomically verified COI sequences from hundreds of thousands of species (Ratnasingham and Hebert, 2007, 2013), which alone is sufficient to justify the continued usefulness of COI in metabarcoding studies (Andújar et al., 2018). The way forward lies not in replacing COI as a metabarcode, but rather in developing suites of markers to use in parallel, which can then complement one another's strengths and shortcomings.

UCEs may offer a way to strengthen results obtained using COI by redressing its limitations, such as the amplification step, as well as by providing replication. Furthermore, their utility may stretch to fungi and plants (Siepel et al., 2005; Reneker et al., 2012), groups beyond the reach of COI (Kress et al., 2015). Already available are bait sets covering a number of broad taxonomic groups, and open-source workflows for identifying UCEs in other taxa (Faircloth, 2017). Used together, UCEs may be able to provide a way to generate comprehensive bait sets that can reliably recover species from across the tree of life, for parallel use with standard barcodes like COI that can leverage the verified taxonomic coverage available in standard barcode databases. Obtaining both UCE and COI data simultaneously is efficient and cost-effective, as mitochondrial DNA is captured concomitantly during targeted enrichment of UCEs as "bycatch" (Raposo do Amaral et al., 2015), as demonstrated in several phylogenomic studies utilizing UCEs (Pierce et al., 2017; Zarza et al., 2018; Branstetter and Longino, 2019). The relative ease with which these markers have been used together in phylogenomics suggests a similar feasibility for metabarcoding. Despite the many possible advantages, thorough testing will be required to determine the feasibility and advantage of utilizing UCEs in a metabarcoding context.

Several distinct challenges would need to be overcome to obtain the full added benefit of implementing UCEs in metabarcoding. Chief among these is the lack of a comprehensive UCE reference database, posing a challenge to taxonomic assignment. Creating such a database will be time and resource intensive, and in the meantime species identification of UCE loci will be limited based on available GenBank data. This underscores the importance of building on the COI framework and utilizing its extensive database, and using UCEs to provide replication, fill in gaps where COI does not work well, and identify potential sources of error. Given the multi-locus nature of UCEs, combining UCE data from single organisms in a mixed sample would represent another major challenge. Without linking data from intra-individual UCE loci, each locus would act independently as a barcode sequence, limiting the added value of UCEs to providing support and validation for conventional metabarcoding methods. Though still valuable, linking the combined multi-locus data would be able to provide much stronger phylogenetic signal, potentially allowing elucidation of evolutionary relationships or population level analysis from mixed samples, greatly enhancing the added benefit of UCEs to metabarcoding analyses. The linkage problem is further compounded for degraded DNA, given that shorter DNA fragments are likely to contain less phylogenetic signal, further limiting the usefulness of unlinked loci. Potential solutions to the linkage problem include progressive match-based and/or distance-based binning, and machine or deep learning algorithms based on input data from taxon-specific studies. Whether these or other possible solutions will work is unclear, and solving this analytical problem will be an important but challenging goal.

CONCLUDING REMARKS

As we move deeper into the Anthropocene, global biodiversity faces an unparalleled and worsening crisis. Scientists tasked with cataloging global diversity face two monumental challenges: the profound biological and ecological diversity of life, and the precipitous rate at which humanity is destroying and altering the environment. In the midst of anthropogenically induced mass extinction (Ceballos et al., 2015), environmental upheaval (Newbold et al., 2015; Seebens et al., 2018), and climate change (Bellard et al., 2012), the number of species on Earth remains unknown (Caley et al., 2014), the majority of species remain undiscovered (Mora et al., 2011), and their evolutionary histories and ecological interactions uncharacterized. In the face of these challenges, scientists must adopt and improve the most effective methods available to discover, catalog, and monitor biodiversity.

Of available methods for surveying biodiversity, metabarcoding provides the greatest balance of taxonomic coverage and resolution, sampling depth, accuracy, efficiency, and ease of use (Ji et al., 2013; Bush et al., 2019). However, there remain significant hurdles to overcome to improve its accuracy and reliability. Fundamental to this is the need to identify alternative barcodes that can fill in the gaps of standard barcodes (Deagle et al., 2014). Ultraconserved elements are one possible solution. Their many strengths, particularly in areas where standard barcodes have demonstrated weaknesses, make them promising candidates that merit consideration.

AUTHOR CONTRIBUTIONS

MP developed and wrote the manuscript.

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