Putting COI Metabarcoding in Context: The Utility of Exact Sequence Variants (ESVs) in Biodiversity Analysis

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DNA barcoding and metabarcoding are techniques that focus on signature genomic regions that in theory provide species level resolution, but in practice this is not always possible. We place animal-focused COI metabarcoding in context with respect to the use of marker gene sequencing in microbial and fungal ecology. We focus on three specific aspects of metabarcodes: (1) the process of metabarcode sequence clustering, (2) how metabarcode cluster types affect the results of biodiversity analyses, and (3) the current state of reference sequence databases used for metabarcode identification. Using examples from the arthropod COI metabarcode literature, we show that exact sequence variants (ESVs) detect more unique taxa than operational taxonomic units (OTUs) but with similar patterns in taxonomic resolution. We also show that the difference between ordinations based on ESVs or OTUs recover similar groupings. We compile a list of reference sequence databases useful for multi-marker metabarcoding and present a list of reference sequence databases specifically formatted for use with a naive Bayesian classifier for rigorous metabarcode taxonomic assignments. Sophisticated tools and reference databases are available for analyzing COI sequences, and these compare favorably with those available for other metabarcode markers such as the ribosomal RNA genes used to target microbes and fungi.

Keywords: exact sequence variant, amplicon sequence variant, operational taxonomic unit, DNA barcode, mini-barcode, metabarcode, taxonomic assignment

BACKGROUND

The objective of DNA barcoding is to permit specimen identification to the species rank. Part of the DNA barcoding process involves building a high-quality reference database containing geographic, morphological, and taxonomic information that is submitted along with a high-quality reference sequence providing species-level resolution (Hebert et al., 2003). DNA barcodes can then be used to help identify unknown specimens when compared to a reference sequence database. Cytochrome c oxidase subunit I (COI) mitochondrial DNA (mtDNA) barcodes for animal species are about 650 bp, the length supported by Sanger sequencing, but modern barcoding has been able to scale up by using newer sequencing technology (Box 1). In practice, however, only a proportion of DNA barcode records themselves represent fully-identified specimens at the species rank.
BOX 1 | Scaling up DNA barcoding.

Though DNA barcodes can be generated for a few samples at a time to help fill out a reference dataset for a particular study, the process can also be scaled up tremendously where researchers have access to automation, liquid handling machines, and high throughput sequencing technology (Hebert et al., 2003, 2018; Hajibabaei et al., 2005). Initially, DNA barcodes were generated in batches using Sanger sequencing. Later, protocols were adapted for high throughput sequencing using an Illumina MiSeq platform where multiple overlapping mini-barcode regions were targeted and then assembled into full length barcodes (Shokralla et al., 2016b). More recently, scalability has been increased and overall cost per sequence decreased by using asymmetric unique molecular identifier (UMI) tagging to track individual samples with single molecule real time (SMRT) technology on the PacBio SEQUEL system (Hebert et al., 2018). This new system ramps up the throughput from 96-sample batches using Sanger sequencing up to 10,000 samples per SEQUEL run. For example, the International Barcodes of Life (iBOL) consortium has released more than 2.6 million DNA barcode sequences from 500,000 species as a part of the BARCODE 500K project (available from https://www.boldsystems.org). Most recently, the current BIOSCAN project is expected to generate DNA barcode sequences for more than 2 million species (Hobbern and Hebert, 2019; Hobbern, 2020).

(Porter and Hajibabaei, 2018b). Some issues that hamper rapid taxonomic identification include dwindling taxonomic expertise (Ebach et al., 2011); hyperdiversity in certain taxa such as insects, microbes, and fungi (Lozupone and Knight, 2007; Blackwell, 2011; Basset et al., 2012; Tedersoo et al., 2014); and lack of morphological characters at certain life stages such as immature insect larva or asexual fungal cultures. Even specimens with degraded DNA, however, such as food products or archival specimens, have been successfully sequenced using mini-barcodes (Box 2). The commonality of these challenges across multiple fields of study, from microbes to animals, has driven the development of DNA-based methods to detect and identify organisms.

The fields of microbial ecology and animal biodiversity each came up with their own solution to a shared problem: How do you consistently label sequences from specimens that cannot be identified to the species rank? In mycology, internal transcribed spacer region of ribosomal DNA (ITS rDNA) sequences are clustered into species hypotheses (SHs) that are given a numeric identifier and can be used as a common label for sequences that cannot be identified to the species rank (Koljalg et al., 2013). In the field of COI barcoding, the barcode index number (BIN) serves a similar purpose (Ratnasingham and Hebert, 2013). Specialized databases such as BOLD for COI mtDNA and UNITE for ITS rDNA barcodes house reference sequences and their corresponding BINS or SHs that attempt to approximate species units (Ratnasingham and Hebert, 2007, 2013; Koljalg et al., 2013). In the future, it is possible that BINS could be adapted to include high quality metabarcode (environmental) sequences lacking a physical specimen in the way that fungal species hypotheses (SHs) currently do (Koljalg et al., 2019; Nilsson et al., 2019).

To transition from sampling individuals (DNA barcoding) to whole communities (DNA metabarcoding) requires the use of “culture-free” and “capture-free” approaches based on targeting environmental DNA (Box 3). DNA metabarcoding is a technique similar to the culture independent marker gene sequencing routinely used in the microbial and fungal ecology literature. The term DNA metabarcoding, however, also implies species-level taxonomic assignment (Taberlet et al., 2012b). Species level resolution of metabarcodes, however, may not be possible if there are gaps in the reference sequence database, the chosen marker lacks species-level resolution (Hajibabaei et al., 2011; Hajibabaei, 2012), or if the metabarcode sequences are too short to provide enough variable characters for a confident assignment (Porter and Hajibabaei, 2018a). In the microbial literature, it is accepted that 16S rRNA gene sequences may only provide genus level taxonomic assignments (Wang et al., 2007). Popular bioinformatic pipelines used in the microbial ecology and microbiome literature, such as QIIME, produce rank-flexible taxonomic assignments (Caporaso et al., 2010). In the DNA barcoding and metabarcoding literature, this type of rank flexible taxonomic assignment was specifically termed “metasystematics” (Hajibabaei, 2012).

From microbes to macrofauna, DNA metabarcoding can be conducted without having to isolate or identify individuals using morphological characters and leverages the sequence and taxonomic information contained in reference databases built from DNA barcodes (Hajibabaei et al., 2011; Taberlet et al., 2012b; Yu et al., 2012). Often, metabarcodes range from about 200–400 bp to correspond to the length supported by current high throughput sequencing platforms such as the Illumina MiSeq (Hajibabaei et al., 2011; Taberlet et al., 2012b). For some applications, such as with ancient DNA, even shorter regions may be targeted (D’Costa et al., 2011). In this paper, we focus on how metabarcodes are generated, analyzed, and identified. We ask three questions: (1) Why do we cluster metabarcode reads? (2) Does metabarcode cluster type affect the results of biodiversity analyses? (3) What resources are available for metabarcode identification?

WHY DO WE CLUSTER METABARCODE READS?

If the DNA metabarcode sequences themselves provide the finest level of resolution, why do many metabarcode bioinformatic pipelines include a clustering step (Box 4)? First, clustering metabarcode sequences allows users to reduce the size of the data files and facilitate downstream processing. Second, sequence clustering may absorb artificial sequences caused by PCR or sequencing error. This clustering step was needed because the early methods of denoising were computationally intensive and difficult to implement on large datasets (Reeder and Knight, 2009). Current denoising methods are incorporated into several existing programs and pipelines such as DADA2, USEARCH, VSEARCH, and Deblur (Callahan et al., 2016; Edgar, 2016; Rognes et al., 2016; Amir et al., 2017; Nearing et al., 2018). Reads may be clustered to approximate species units. In the field of microbial ecology, it was shown that if a phylogenetic species definition requires at least 70% or greater DNA similarity, this
corresponds to ~97% sequence similarity in the 16S rRNA gene region (Stackebrandt and Goebel, 1994). A recent study, however, suggests that 99–100% thresholds may be more appropriate (Edgar, 2018b). In current fungal ecology, 97–99% cutoffs for the ITS rDNA are sometimes used to approximate species (Koljalg et al., 2013). In COI metabarcoding studies, a variety of sequence similarity cutoffs have been used ranging from 95–100% to maximize genetic diversity recovered while controlling for the effect of sequence errors, resulting in species-like groupings (Elbrecht et al., 2017; Braukmann et al., 2019; Tapolczai et al., 2019). In many cases, a 97% sequence similarity cutoff is used because existing bioinformatic pipelines were originally developed to process microbial rRNA gene sequences, and this threshold is often a default value. In all cases, use of a single sequence similarity threshold, such as 97% OTUs, may not reproduce species units across all taxa defined by traditional species concepts or across the variety of markers used for metabarcoding today.

The reasons for clustering metabarcodes may vary, but the result are two types of metabarcode clusters, operational taxonomic units (OTUs) or exact sequence variants (ESVs). OTUs, or molecular OTUs (mOTUs), represent a cloud of similar sequences whose composition may vary depending on the order of the sequences being clustered, making them difficult to reproduce and compare across studies (He et al., 2015). Any single OTU is usually represented by a single sequence, such as the centroid, and the remaining sequences in the OTU are disregarded in further analyses obscuring the underlying nucleotide variation within any single OTU. On the other hand, exact sequences variants (ESVs), also known as amplicon sequence variants (ASVs) (Callahan et al., 2017), zero-radius OTUs (Edgar, 2016), or simply error-corrected OTUs defined by 100% sequence identity, each represent sequence variation down to single-nucleotide resolution. To ensure high quality ESVs, steps need to be taken to remove artifactual sequences such as putative chimeras, sequences with predicted errors, and contaminants (Callahan et al., 2016; Edgar, 2016). We make the case here that ESVs are appropriate for analyzing metabarcodes from any taxon, from microbes to arthropods, using any marker from rRNA genes to COI. The advantages of using ESVs includes improved taxonomic resolution down to single nucleotides as well as improved reproducibility and comparability across studies that use the same marker (Callahan et al., 2017). In theory, ESVs are comparable to haplotypes used commonly in population genetics and phylogeography (Callahan et al., 2017) and are already starting to be treated as such in the COI metabarcoding
BOX 4 | A general bioinformatic pipeline for metabarcode clusters based on operational taxonomic units (OTUs) or exact sequence variants (ESVs). DNA metabarcodes are often generated using paired-end Illumina sequencing. Forward and reverse reads are paired, then the ends of the sequence matching the primers are removed. In some pipelines, primers are trimmed first, then forward and reverse reads are paired. Each of these steps may require the user to set a minimum Phred quality score cutoff as well as a cutoff for the number of mismatches tolerated.

At this point, sequence files belonging to each sample are often pooled together for a “global” analysis. Dereplication involves obtaining just the unique sequences from the set. The number of reads matching each unique sequence is retained as this information is needed for both the clustering and denoising methods described below. The output is usually sorted by decreasing read abundance, but other sort orders are possible. Because many clustering methods are “greedy” to improve computation time, changing the input order of the sequences can change the composition of the resulting OTUs.

The operational taxonomic unit (OTU) clustering part of the pipeline is shown in orange. In USEARCH or VSEARCH, each OTU is represented by a single centroid sequence in any future analyses (Edgar, 2013; Rognes et al., 2016). To create an OTU, the OTU centroid sequence in the database. This step may require numerous parameters to be chosen such as the identity threshold, for example, 0.97, to retain sequences with at least 97% sequence similarity to an OTU centroid sequence.

The exact sequence variant (ESV) denoising pipeline is shown in orange. In USEARCH or VSEARCH, the UNOISE3 algorithm performs denoising (Edgar, 2016) by clustering identical sequences together, similar to using an identity threshold of 1.0 to cluster sequences that have 100% sequence similarity. During this process, sequences with predicted sequence errors, putative PhiX carry-over from Illumina sequencing, putative chimeric sequences, and rare sequences are removed. Each denoised ESV is represented by a single sequence in any future analysis. To create an ESV, each sample table containing read numbers, primer-trimmed paired sequences can be aligned to each unique ESV sequence in the database. This step may require numerous parameters to be chosen such as the identity threshold of 1.0 to retain sequences with at least 100% sequence similarity to a denoised ESV sequence.

Several metabarcode denoising programs have been compared and the USEARCH UNOISE3 algorithm was shown to be the fastest and DADA2 was found to generate the greatest number of ESVs (Callahan et al., 2016; Nearing et al., 2018). USEARCH is proprietary software with a free 32-bit version available and DADA2 is open source software. VSEARCH is another useful open source software program that allows you to use as much memory as your system supports to facilitate large analyses, and it can also run the UNOISE3 algorithm.

Metabarcodification can be performed in a number of different ways using similarity-, phylogeny-, or composition-based methods (Porter and Hajibabaei, 2018c). The most popular method for high-throughput identification of large batches of COI metabarcodes is to perform BLAST comparisons against the GenBank nucleotide or other custom databases. We have developed the COI classifier v4 that uses a method initially developed to taxonomically assign rRNA gene sequences. This naive Bayesian classifier was trained on a curated set of COI sequences from BOLD and GenBank to make rapid, accurate taxonomic assignments (Altschul et al., 1997; Wang et al., 2007; Porter and Hajibabaei, 2018a). Recently, a python package called BOLDgigger has been developed to help automate batch query submissions to the BOLD identification engine and can be used to identify COI, ITS, rbcL, and matK sequences (Buchner and Leese, 2020). For each of these methods, there are trade-offs in terms of ease of use, speed, and rigor. Users should carefully consider the output: Similarity-based methods provide a measure of how similar a query sequence is to a target sequence whereas taxonomic assignment methods provide a statistical measure of confidence for a taxonomic placement at each rank. Each of these approaches relies on comparing unknown metabarcodes sequences against a reference sequence database of known sequences. The quality, coverage, and availability of these reference sequences can be quite varied for COI and other popular metabarcodes markers and is discussed below (also see Table 1).

In terms of reproducibility and comparability, it is relatively straightforward to align new reads using a 100% sequence similarity threshold to an ESV reference database. It is more complicated to align new reads to an OTU reference database because an arbitrary similarity threshold needs to be chosen or to regenerate OTUs from scratch since greedy algorithms are affected by sequence input order and may not be chosen or to regenerate OTUs from scratch since greedy algorithms are affected by sequence input order and may not generate OTUs with the same composition as before (He et al., 2015). For studies that require species estimates, fungal ITS or animal COI ESVs can be aligned to ITS SHs or COI BINS using a meaningful threshold for sequence similarity, say 97% sequence similarity. In the fungal literature, ESVs and OTUs were both shown to recover similar ecological patterns (Glassman and Martiny, 2018). In this paper, we show how the analysis of COI metabarcodes clusters based on ESVs and OTUs affects biodiversity analyses (see next section).
resolution that can be made with confidence (Table 1; Porter and Hajibabaei, 2018a). Filtering for bootstrap support values that exceed cutoff values can also help reduce the rate of false positive taxonomic assignments (Porter and Hajibabaei, 2018a). This may be an important consideration in cases where the cost of making a false-positive assignment is high, such as where falsely detecting an invasive species could be a cause for alarm. Methods that use a naive Bayesian classifier such as the RDP classifier, phylogenetic-based taxonomic assignment such as SAP, Bayesian multinomial regression such as PROTAX, or non-Bayesian k-mer based methods such as SINTAX each produce measures of confidence for taxonomic assignments for each rank (Wang et al., 2007; Munch et al., 2008; Huson et al., 2016; Somervuo et al., 2016). Some methods even take into consideration species that exist but may not have a reference sequence, new species, and mislabeled sequences (Somervuo et al., 2016, 2017).

HOW DOES CLUSTER METHOD CHOICE AFFECT DIVERSITY ANALYSES?

For biodiversity analyses, the choice between using ESVs or OTUs can affect recovered alpha diversity/richness (Hajibabaei et al., 2019). We reanalyzed the data from a study that used COI metabarcoding to assess invertebrates directly from forest soils and directly compared the data reanalyzed two ways:

| ESV                   | Rank             | Taxon               | COI Classifier v4 bootstrap support* |
|-----------------------|------------------|---------------------|--------------------------------------|
| F230R_Otu231          | Root             | Cellular organisms | 1.0                                  |
|                       | Superkingdom     | Eukaryota           | 1.0                                  |
|                       | Kingdom          | Metazoa             | 1.0                                  |
|                       | Phylum           | Arthropoda          | 1.0                                  |
|                       | Class            | Arachnida           | 1.0                                  |
|                       | Order            | Araneae             | 1.0                                  |
|                       | Family           | Amaurobiidae        | 1.0                                  |
|                       | Genus            | Amaurobius          | 1.0                                  |
|                       | Species          | Amaurobius borealis| 1.0**                                |
| F230R_Otu1794         | Root             | Cellular organisms | 1.0                                  |
|                       | Superkingdom     | Eukaryota           | 1.0                                  |
|                       | Kingdom          | Metazoa             | 1.0                                  |
|                       | Phylum           | Arthropoda          | 1.0                                  |
|                       | Class            | Insecta             | 1.0                                  |
|                       | Order            | Diptera             | 0.94**                               |
|                       | Family           | Hybotidae           | 0.16                                 |
|                       | Genus            | Crossopalpus        | 0.13                                 |
|                       | Species          | Crossopalpus nightellus | 0.13                           |

*Bootstrap support ranges from 0 to 1. These values can be filtered using appropriate cutoff values that vary according to taxonomic rank and query sequence length to ensure 95 or 99% accuracy. Assumes that the query sequence is in the reference sequence database. **Indicates the finest resolution for the taxonomic assignment to ensure 99% correct assignments for a COI metabarc ode ~200 bp in length.
Observed community composition can vary based on choice of COI amplicon. Data is from a study that assessed arthropod diversity using COI metabarcoding of 6 amplicons from freshwater kick net samples (Hajibabaei et al., 2019). Number of unique taxa detected from each primer set is indicated below the COI amplicon name on the x-axis. Results are based on ESVs whose taxonomic assignments have been summarized to the family rank where possible on the y-axis and ordered by decreasing read number (see legend). A UPGMA dendrogram is shown above the heatmap, indicating which amplicons recover communities that are most similar to each other. Fields in "gray" indicate that zero reads were detected.
FIGURE 4 | Sample sites and soil strata are similarly distinguished using either ESV or OTU clusters. Data is from a study that assessed arthropod diversity using COI metabarcoding from forest soil (Porter et al., 2019). Parts (A,B) show that non-metric multidimensional scaling plots based on binary Bray Curtis (Sorensen) dissimilarities. A Procrustes analysis (least squares orthogonal mapping) was used in part to assess differences in the ordinations based on the analysis of ESVs and OTUs. The vector residuals plotted in (C) show the differences between the original ESV ordination and the OTU ordination. Smaller residuals indicate smaller differences between the ordinations.

at the species rank and 99% accuracy at all other ranks. As expected, we detected greater number of unique ESVs (3,357) than OTUs (2,078) (Figure 1). We also, however, found a similar distribution in taxonomic assignment resolution with almost half the clusters being identified to species, and just over half resolved to more inclusive ranks from genus to order. In the original study, analyzing the data with ESVs or OTUs did not make a difference to our final conclusions, so the final data was presented using ESVs.

We also assessed whether community composition patterns were affected by the use of ESVs or OTUs (Figure 2). The top 10 most abundant taxa are found in similar proportions whether the data are analyzed according to ESVs or OTUs. Again, in the original study, the analysis of ESVs or OTUs showed similar patterns and the final results were shown using ESVs. The taxonomic resolution of these results are typical of most studies, where many sequence clusters cannot yet be assigned to the species rank with confidence, and
indicate where to target additional barcoding efforts. This is especially important for geographic locations that are poorly sampled, where diversity is high, and where the reference database is incomplete.

Both richness and community composition can be assessed based on metabarcoding data generated using a single primer set, but how would these results be affected if the primers were found to be biased in some way? Some of the early microbiome literature used only a single primer set to produce single amplicon datasets, and this has facilitated large scale studies and brought a measure of standardization to the field (Gilbert et al., 2014; Thompson et al., 2017). There are many examples, however, showing the effect of primer bias for a variety of commonly used metabarcoding primers (Hong et al., 2009; Bellemain et al., 2010; Clarke et al., 2014; Gibson et al., 2014; Elbrecht et al., 2019; Hajibabaei et al., 2019). There is also difficulty in designing “universal” COI primers to capture broad swaths of phylogenetic diversity and a switch to a multi-marker approach has been proposed for assessing animal diversity (Deagle et al., 2014). In the microbiome literature, there has been a shift to the use of PCR-free metagenomic methods to both avoid PCR-bias as well as to aid in quantitative assessments (Nayfach and Pollard, 2016). PCR-free methods have also been proposed to study terrestrial arthropod biodiversity, but these approaches are not often used due to cost and technical challenges for application in large scale studies (Zhou et al., 2013; Shokralla et al., 2016). For now, the most cost-effective approach to capture a wide array of phylogenetic diversity using COI metabarcoding is to use multiple primers sets.

To look at the effect of primer bias, we reanalyzed the data from a study that used 6 different COI metabarcode amplicons to sample arthropods from freshwater kick net samples (Hajibabaei et al., 2019). This study includes two COI amplicons that we have routinely used in our own work to survey freshwater macroinvertebrates, BR5 (B/ArR5) and F230R (LCO1490/230_R) (Folmer et al., 1994; Hajibabaei et al., 2012; Gibson et al., 2014, 2015); a primer set designed for marine taxa but has been shown to perform well for detecting arthropods in other environments, ml-jg (mlCOIintF/jgHCO2198) (Geller et al., 2013; Leray et al., 2013); as well as a few other recently published primer sets that look promising for macroinvertebrate
biomonitoring, BF1R2 (BF2/BR2), BF2R2 (BF2/BR2) (Elbrecht and Leese, 2017), and fwh1 (fwhF1/fwhR1) (Vamos et al., 2017). Taxonomic assignments were carried out as described above using the naive Bayesian classifier and summarized to the family rank where possible. Read number was normalized across each amplicon using rarefaction to account for differences in library size. We compared the results for each COI amplicon and found similarities among taxa represented by the greatest number of reads and many differences among taxa represented by fewer reads (Figure 3). Binary data was also used to create a Jaccard dissimilarity matrix to generate the UPGMA dendrogram clustering the COI amplicons. Community dissimilarities across amplicons ranged from 32 to 56%, with the community detected by ml-jg and BF1R2 being the most similar. The number of unique taxa detected by each amplicon ranged from 44 to 65, with the highest number of unique taxa detected by BF1R2.

| Database | Content/Markers (Taxa) | Number of reference sequences | Website | References |
|----------|------------------------|-------------------------------|---------|------------|
| International Nucleotide Sequence Database Collaboration (INSDC) | Repository for raw sequence data, alignments/annotations, sample/experimental metadata available through the NCBI, ENA, DDBJ * | 216,531,829 in GenBank [April 2020] | http://www.insdc.org/ | Cochrane et al., 2016 |
| Barcode of Life Data System v4 (BOLD) | COI (mostly), rbcL, matK, ITS (eukaryotes) | Available for searching: 7,389,964 COI (public and private BOLD + INSD); 2,027,132 COI (public BOLD + INSD) Available for download: 2,869,168 in data release packages | https://www.boldsystems.org/ | Ratnasingham and Hebert, 2007 |
| SILVA release 138 | 16S + 18S SSU, 23S + 28S LSU (bacteria, archaea, eukaryotes) | 510,984–9,469,656 SSU; TBD LSU** | https://www.arb-silva.de/ | Pruesse et al., 2007; Yilmaz et al., 2014 |
| Greengenes 13.5 | 16S (bacteria, archaea) | 1,262,986 | https://greengenes.secondgenome.com/ | Desantis et al., 2006; McDonald et al., 2012 |
| Genome Taxonomy Database (GTDB release 89) | 120 proteins and 16S SSU (bacteria, archaea) | 145,904 genomes; 284,051 SSU | https://gtdb.ecogenomic.org/ | Parks et al., 2020 |
| Ribosomal Database Project (RDP) release 11 | 16S SSU (bacteria + archaea), 28S LSU (Fungi)*** | 3,196,041 (bacteria) + 160,767 (archaea) SSU; 125,525 (fungi) LSU | https://rdp.cme.msu.edu/ | Cole et al., 2014 |
| The Ai-Species Living Tree Project (LTP) 132 (SSU) + 123 (LSU) | 16S + 23S type strains (bacteria, archaea) | 13,903 SSU; 1,614 LSU | https://www.arb-silva.de/projects/living-tree | Yilmaz et al., 2014 |
| The Protist Ribosomal Reference Database (PR2) v4.12.0 | 16S, 18S (protists plus metazoa, land plants, macrosporic fungi, and eukaryotic organelle outgroups) | 6,010 16S; 177,934 18S | https://pr2-database.org/ | Guillou et al., 2012 |
| ITS2 database V | ITS2 (eukaryotes) | 711,172 | http://its2.bioapps.biozentrum.uni-wuerzburg.de/ | Ankenbrand et al., 2015 |
| UNITE v8.2 | ITS fungi/eukaryotes (UNITE + iNSD) | 714,329 fungi; 1,796,591 eukaryotes | https://unite.ut.ee/ | Kõljalg et al., 2019 |
| PLANiTS | ITS (plants) | 104,584 ITS1; 101,584 ITS2; 104,342 ITS | https://github.com/apallavicini/PLANiTS | Banchi et al., 2020 |
| R-Syst:Diatom v7 | 18S, 28S, ITS, rbcL, COI (diatoms) | 2,647 18S; 315 28S; 293 COI; 83 ITS2; 3,504 rbcL | https://www6.inrae.fr/r-syst_eng/Databases/R-Syst-diatom | Rimet et al., 2019 |
| MitoFish | Mitochondrial genomes (fish) | 2,853 genomes | http://mitofish.aori.u-tokyo.ac.jp/ | Sato et al., 2018 |
| rbcL Bell | rbcL (plants) | 87,352 | https://figshare.com/collections/rbcL_reference_library/3466311 | Bell et al., 2017 |

*National Centre for Biotechnology Information (NCBI), European Nucleotide Archive (ENA), DNA Data Bank of Japan (DDBJ). **To be determined (TBD), LSU has not been released yet. ***A fungal ITS classifier is also provided.
The total number of unique taxa detected by all 6 COI amplicons was 109. It is clear from our example that taxa represented by the greatest number of reads tend to be similar across amplicons, but combining the results from multiple amplicons improves the overall recovery of the greatest diversity of taxa. In the original study, we showed that using at least two COI amplicons from this set of six could detect most species, genera, and families. Previous work has used in silico PCR using ITS primers to detect fungi (Bellemain et al., 2010) and mock community studies in bacterial (Brooks et al., 2015) and terrestrial arthropod communities (Elbrecht et al., 2019) to demonstrate the effect of PCR bias. Here we show the effect of primer bias on a real community with realistic complexity and template background.

We have shown that alpha diversity, richness, is sensitive both to choice of metabarcode cluster type and primer choice, but what does this mean for beta diversity? For arthropods sampled using COI metabarcoding from freshwater or soil samples, beta diversity assessments have been shown to be robust to both variations in primer choice and sampling method (Hajibabaei et al., 2019; Porter et al., 2019). Does this hold true for differences in clustering strategy and resolution of the matrix? In our research we have found that beta diversity estimates are robust to the use of either ESVs or OTUs (Figure 4). The difference between ordinations based on ESVs and OTUs is minimal, and the site and soil layer groupings are visually distinct using either sequence cluster type. In the original study, clustering patterns observed from NMDS plots and permutational analysis of variance (PERMANOVA) results were not affected by the analysis of ESVs or OTUs. As a result, we prefer the use of ESVs over OTUs to improve reproducibility, facilitate comparisons across studies, and permit within-species analyses.

**How can we leverage taxonomic coverage across reference databases?**

The composition, quality, and completeness of reference sequences databases determines our ability to identify unknown specimens using DNA barcodes and metabarcodes. BOLD has become the canonical COI reference sequence database, with official DNA barcode sequences available for download through data releases available from https://www.boldsystems.org/index.php/datarelease. The BOLD system also contains sequences mined from GenBank as well as private data that is available for comparison when using the BOLD identification engine (Ratnasingham and Hebert, 2007). Recently an R package was released that facilitates mining BOLD data; however, it can still be challenging to retrieve large amounts of data at one time, for example, the entire reference database of all arthropoda (Chamberlain, 2019). The NCBI nucleotide database, GenBank, has accumulated over 2.5 million COI sequences.

### Table 3: Curated reference sequence databases specifically formatted to work with the RDP naive Bayesian classifier.

| Marker | Name version (year) | Target taxa | Number of reference sequences | Availability | References* |
|--------|---------------------|-------------|--------------------------------|--------------|-------------|
| SSU (16S) | 16S trainsetNo16 (2016) | Prokaryotes | 13,212 | https://sourceforge.net/projects/rdp-classifier/ | Wang et al., 2007 |
| SSU (18S) | 18S classifier v4** (2020) | Eukaryotes | 42,301 | https://github.com/terrimporter/18SClassifier | Pruesse et al., 2007 |
| SSU | Diatom Classifier v1.0 (2020) | Diatoms | 2,962 | https://github.com/terrimporter/SSUdaiatomClassifier | Rimet et al., 2019 |
| LSU | Fungi LSU trainsetNo11 (2014) | Fungi | 11,442 | https://sourceforge.net/projects/rdp-classifier/ | Liu et al., 2012 |
| ITS | Fungalits UNITE 07042014 (2014) | Fungi | 145,019 | https://sourceforge.net/projects/rdp-classifier/ | Abarenkov et al., 2010 |
| ITS | Fungalits Warcup v2 (2016) | Fungi | 17,878 | https://sourceforge.net/projects/rdp-classifier/ | Deshpande et al., 2016 |
| rbcL | rbcL Classifier v1 (2020) | Eukaryotes | 164,454 | https://github.com/terrimporter/rbclClassifier | Benson et al., 2012 |
| rbcL | rbcL Diatom Classifier v1.0 (2020) | Diatoms | 3,504 | https://github.com/terrimporter/rbclDiatomClassifier | Rimet et al., 2019; Maitland et al., 2020 |
| COI | CO1 Classifier v4 (2019) | Eukaryotes | 1,221,528 | https://github.com/terrimporter/CO1Classifier | Porter and Hajibabaei, 2018a |
| 12S | 12S fish Classifier v1.0 (2020) | Fish | 2,853 | https://github.com/terrimporter/12SfishClassifier | Iwasaki et al., 2013 |

*References for the database where sequences were obtained and/or for the trained naive Bayesian classifier if available. **Based on SILVA 138 SSURef N99.
since the advent of the DNA barcoding initiative in 2003 (Hebert et al., 2003; Benson et al., 2012; Porter and Hajibabaei, 2018b). Since BOLD has a policy of depositing DNA barcodes in GenBank, much of the public BOLD data is also available through GenBank. Neither BOLD nor GenBank, however, is entirely complete, and each database provides complementary taxonomic coverage as has been shown for Canadian freshwater invertebrates (Curry et al., 2018). Combining these databases would improve both sequence and taxonomic coverage. Making the merged reference data available in plain text formats would make it relatively straightforward to reformat so they can be used as the basis for alternative taxonomic assignment tools such as those that provide rank-flexible statistical measures of confidence. For example, the BOLD_NCBI_Merger script provides a means to combine data from BOLD and the NCBI nucleotide database for use with MEGAN (Huson et al., 2016; Macher et al., 2017). Our own approach has been to update the underlying reference sequence database used by the COI classifier v4 to combine data from BOLD and GenBank, and it is available from https://github.com/terrimporter/CO1Classifier (Wang et al., 2007; Porter and Hajibabaei, 2018a). We demonstrate the improved taxonomic composition when COI reference sequences from the BOLD data releases are combined with COI sequences mined from GenBank (Figure 5). The combined reference set is available as a FASTA file as are the trained files needed to use these reference sets with the naive Bayesian classifier.

We have mainly focused on using a single marker, such as COI for animal metabarcoding, but the field has progressed such that investigators are now using multi-marker approaches (Drummond et al., 2015) to conduct food web studies or comprehensive biodiversity monitoring across phylogenetically diverse taxa. As such, we should be aware of tools available for analyzing other widely used metabarcoding markers (Table 2). The largest source for reference sequence information is through the International Nucleotide Sequence Database Collaboration (INSDC) comprised of the NCBI (GenBank, Short Read Archive), EMBL-EBI, and DDBJ. In North America, most users are familiar with GenBank, a repository for marker gene sequences (also see European Nucleotide Archive and DDBJ), and the Short Read Archive (SRA) where raw metabarcode reads are stored. For COI barcodes, public data in BOLD is automatically transferred to GenBank, and additional barcode sequences are retrieved from GenBank to complement the BOLD database. Multi-marker or genome projects focused on particular taxonomic groups are also valuable sources of reference sequence information. For example, DNA barcodes found to be most useful for diatom identification includes 18S, 28S rDNA, internal transcribed spacer 2 (ITS2), rbcL cpDNA, and COI mtDNA and are available through the Diat barcode library (Chaumeil et al., 2018; Rimet et al., 2019). Additionally, though COI DNA barcodes are readily available for fish identification (Becker et al., 2011; Weigand et al., 2019), 12S mtDNA has a history of use for vertebrate detection (Kitano et al., 2007; Sato et al., 2018). Throughout the course of our own work, we have mined existing databases and created our own curated reference sets reformatted to work with a naive Bayesian classifier to make rank-flexible taxonomic assignments with a statistical measure of confidence (Table 3). Each of these curated datasets are also available as FASTA files. These resources show how the field of eukaryote metabarcoding is diversifying to use multiple markers and support a variety of taxonomic assignment methods.

Choosing a database for any given DNA barcode or marker often comes down to one’s preferred species concept, database coverage, as well as the availability and ease-of-use of related tools. The NCBI database is the primary source of raw sequence data for most of the databases listed in Table 2. What makes each of the rRNA gene databases unique, however, is that they filter the data using their own quality control standards, and they follow their own taxonomic roadmap (Balvoˇci¯utë and Huson, 2017). For example, a phylogenetic species concept is often preferred in microbial ecology where taxa are challenging to study and describe using traditional methods and undescribed environmental diversity is exceedingly high. In this case, both Greengenes and SILVA assume that trees based on available SSU sequences reflect evolutionary relatedness, and any taxonomic inconsistencies are resolved to make classification consistent with phylogeny. The RDP, however, follows Bergey’s classification system (Cole et al., 2014). When the goal is to identify unknown environmental sequences from metabarcode sequences, the so-called “dark taxa,” the microbial and fungal communities have come up with their own methods. For prokaryotes, the GTDB includes metagenome assembled genomes (MAGs) represented in their database (Chaumeil et al., 2019). The RDP, SILVA, and Greengenes databases each contain many environmental sequences for comparison, but the taxonomic assignment can be based on different criteria using an algorithm (RDP) or phylogenetic placement and manual curation (SILVA, Greengenes). For fungi, the UNITE database has made a concerted effort to incorporate fungal dark taxa in their SHs and have introduced Taxon Hypotheses (THs) to allow for the communication of SHs using different classification schemes at the same time (Nilsson et al., 2019). If a fungal or animal study requires species estimates, then using a database that attempts to approximate species using fungal SHs or animal COI BINs may be preferred. For studies where few taxa can be confidently identified, using a large database that includes environmental sequences will provide the most coverage, and using a method that provides a statistical measure of confidence can allow the user to adjust for the recovery of false negatives or false positives according to the study aims (Edgar, 2018a).

CONCLUDING REMARKS

Over the last 15 years the use of standardized DNA-based biodiversity markers such as DNA barcodes has become a routine practice in various scientific and socioeconomic endeavors. A much wider spatiotemporal biodiversity perspective is now achievable through bulk analysis of metabarcodes. Our ability to fully identify metabarcodes from particularly diverse taxonomic groups or samples may be currently limited, but...
with continued DNA barcoding efforts these databases are expected to become more representative over time. Insufficiently identified sequence clusters, those not confidently identified to the species rank, can still be used for biodiversity analyses including richness assessment, community composition, and beta diversity assessments. For improved reproducibility, comparison across studies, and nucleotide-level resolution, we encourage the use of ESV level analyses. For studies that require species estimates, we suggest aligning ESVs to fungal ITS SHS or animal COI BINs which both attempt to approximate species units. If representative BIN sequences were made available in an easily parsed format, this would allow taxonomic assignments to be made using tools outside the BOLD system built-in barcode identification engine and would allow inclusion in metabarcoding bioinformatic pipelines that are already widely used for analyzing large metabarcoding datasets. COI metabarcoding offers a sophisticated toolset and reference databases suitable for large scale studies; as such, it is now firmly established as a marker for animals in molecular ecological and biodiversity studies.

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DATA AVAILABILITY STATEMENT

Code used to generate figures is available on GitHub from https://github.com/terrimporter/PorterHajibabaei2020_ESVs_vs_OTUs.

AUTHOR CONTRIBUTIONS

MH conceived of the idea. TP and MH wrote and edited the manuscript. TP generated the figures. Both authors contributed to the article and approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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