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Improving Metabarcoding Taxonomic Assignment:

A Case Study of Fishes in a Large Marine Ecosystem

12S Taxonomic Assignment Performance

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ABSTRACT

DNA metabarcoding is an important tool for molecular ecology. However, its effectiveness hinges on the quality of reference sequence databases and classification parameters employed. Here we evaluate the performance of MiFish *12S* taxonomic assignments using a case study of California Current Large Marine Ecosystem fishes to determine best practices for metabarcoding. Specifically, we use a taxonomy cross-validation by identity framework to compare classification performance between a global database comprised of all available sequences and a curated database that only includes sequences of fishes from the California Current Large Marine Ecosystem. We demonstrate that the curated, regional database provides higher assignment accuracy than the comprehensive global database. We also document a tradeoff between accuracy and misclassification across a range of taxonomic cutoff scores, highlighting the importance of parameter selection for taxonomic classification. Furthermore, we compared assignment accuracy with and without the inclusion of additionally generated reference sequences. To this end, we sequenced tissue from 597 species using the MiFish *12S* primers, adding 252 species to GenBank's existing 550 California Current Large Marine Ecosystem fish sequences. We then compared species and reads identified from seawater environmental DNA samples using global databases with and without our generated references, and the regional database. The addition of new references allowed for the identification of 16 additional native taxa representing 17.0% of total sequence reads from eDNA samples, including species with vast ecological and economic value. Together these results demonstrate the importance of comprehensive and curated reference databases for effective metabarcoding and the need for locus-specific validation efforts.

KEYWORDS: metabarcoding, MiFish primers, California Current Large Marine Ecosystem, eDNA, environmental DNA, reference database

INTRODUCTION

Metabarcoding is a process in which multiple species are identified from bulk DNA (e.g. homogenized gut contents, settlement tile scrapings, etc.) or environmental samples (Bohmann et al., 2014; Deiner et al., 2017; Taberlet, Coissac, Pompanon, et al., 2012). Metabarcoding is increasingly used to study marine ecosystems as the ability to sequence tens to hundreds of millions of reads in a single sequencing run allows the development of novel research questions, including species mapping, biomonitoring, gut content analyses, and population genomics, all of which aid understanding of the ecology of marine ecosystems (Baetscher et al., 2019; Closek et al., 2019; Goodwin et al., 2017; Guo, 2017; Kelly, Port, Yamahara, Martone, et al., 2014; Sanders et al., 2015; Thompson et al., 2017; Yamahara et al., 2019). In particular, metabarcoding of environmental DNA (eDNA), freely associated DNA obtained from environmental samples, is an increasingly attractive approach for marine ecosystem characterization because it can detect a broad range of diversity from a single liter of seawater, and has the potential to transform marine biomonitoring efforts (Kelly, Port, Yamahara, Martone, et al., 2014).

Metabarcoding typically employs PCR amplification and sequencing of a target gene (Goodwin et al., 2017) followed by comparison of these sequences to a database of known reference sequences to identify species present in the sample (Taberlet, Coissac, Hajibabaei, et al., 2012). Incomplete databases cannot identify all species present, leading to a lack of assignment despite the actual detection and capture of the sequences, potentially biasing the

interpretation of results (Boyer et al., 2016; Deiner et al., 2017; Machida et al., 2017). Thus, building complete and accurate reference databases is paramount to the success of molecular ecology monitoring efforts (Schenekar et al., 2020).

One approach for maximizing metabarcoding taxonomic assignment is to compare query sequences to a global database of archived sequences (Camacho et al., 2009; Edgar, 2018b). Global databases, such as GenBank, include nearly all publicly available sequences for specific barcode loci and are thus inherently comprehensive (Benson et al., 2018). However, the inclusion of reference barcodes from non-target or biologically irrelevant species may potentially bias taxonomic assignment algorithms (Curd et al., 2019). This issue is particularly problematic for lowest common ancestor taxonomic assignment methods that make inherent assumptions that each best sequence alignment is equally valid, irrespective of the geographic distributions and ecologies of these taxa (Curd et al., 2019; Gao et al., 2017), potentially leading to assignments of biologically implausible species. This problem can be compounded by the occurrence of mis-annotated sequences, a well-known problem in global reference databases (Heller et al., 2018; Leray et al., 2019; Nobre et al., 2016; Wakeling et al., 2019).

An alternative approach to using global databases for taxonomic classification is to employ a curated reference database that includes only appropriately annotated sequences for taxa that occur in a given region (Macheriotou et al., 2019; Poloczanska et al., 2013; Richardson et al., 2018). However, the inclusion or exclusion of barcodes from a reference database can affect metabarcoding taxonomic assignments (Macheriotou et al., 2019; Poloczanska et al., 2013; Richardson et al., 2018), yet few studies systematically addressed this problem (Bergsten et al., 2012; Stoeckle et al., 2020). As such, it is currently unclear whether global or regional reference

databases produce more accurate taxonomic assignments. Systematically quantifying error and bias associated with global and curated database is essential to identifying best practices for metabarcoding taxonomic assignment.

Critical to such assessments are methods that validate taxonomy prediction and evaluate the sensitivity to bioinformatic and database parameters. One key method for comparing the performance of taxonomic classification across different reference databases or classification parameters is the taxonomy cross-validation by identity (TAXXI) framework (Edgar, 2018a). The TAXXI framework is executed by using a reference database with known taxonomic identities that is split into test and training sets and then assigning taxonomy to the training set using the test set. The TAXXI framework can then be applied to allow taxonomic assignment performance to be compared across different metabarcodes, reference databases, and different assignment parameters.

Critically, TAXXI approaches allow for comparing the performance of bioinformatic pipelines within and across loci, including informing the proper selection of classifier parameters for a given metabarcoding locus (Boyer et al., 2016; Machida et al., 2017). Taxonomic assignments made by metabarcoding classifiers are particularly influenced by taxonomic cutoff scores (e.g., exact alignment match or 97% identity threshold) (Edgar, 2018c, 2018a). Using this cross-validation approach to evaluate the performance of taxonomic assignments for *16S* and fungal *ITS* metabarcoding loci across a range of classification parameters revealed that percent identities below 95% had poor classification performance (Edgar 2018a), and highlighted key tradeoffs between assignment confidence and taxonomic resolution (Edgar, 2018c, 2018a). Frequently, attempts to balance confidence–resolution tradeoffs leads to the selection of

conservative taxonomic cutoff scores to avoid over-classification errors (Alberdi et al., 2018; Camacho et al., 2009; Port et al., 2015; Siegwald et al., 2017; Wood & Salzberg, 2014). However, parameter selection is rarely systematically evaluated across different taxonomic groups or metabarcoding loci, inadvertently leading to poorer quality taxonomic assignments (Curd et al., 2019; Edgar, 2018a, 2018c). Importantly, the few studies that explored classification parameter performance across metabarcoding loci found that a "one size fits all" approach (e.g., 97% identity threshold) is inappropriate across different metabarcoding loci (Curd et al., 2019; Edgar, 2018c, 2018a). Thus, evaluating the performance of taxonomic assignments across a range of cutoff scores for a given metabarcoding target is important for maximizing the accuracy of metabarcoding efforts (Balakirev et al., 2017; Bokulich et al., 2018; Hassanin et al., 2010).

Using the TAXXI framework, Curd *et al.* (2019) compared the performance of reference databases for taxonomic assignment, demonstrating the utility of custom reference libraries. The *Creating Reference libraries Using eXisting tools* (*CRUX*) module of the *Anacapa Toolkit* constructs custom reference databases by querying public sequence archives based on primer sets defined by the user. Curd et al. (2019) showed that *CRUX*-generated custom reference databases were more comprehensive and provided improved taxonomic assignment compared to previously published *CO1* reference databases [Midori (Machida et al., 2017) and CO-Arbitrator (Heller et al., 2018)], yielding results nearly equal to heavily curated reference databases for *16S* [SILVA (Quast et al., 2012)] and *12S* [MitoFish (Sato et al., 2018)] metabarcodes. The TAXXI framework thus provides a critical set of tools to evaluate the performance of taxonomic assignment across classification parameters and reference databases for any metabarcoding locus of interest.

The MiFish Universal Teleost and MiFish Elasmobranch primer sets (Miya et al., 2015) target the same portions of the mitochondrial *12S* RNA gene, but differ by a few critical base pairs on the forward primer. These metabarcodes are vertebrate specific, provide species-level resolution for many fishes, and are well suited to short read-length next-generation DNA sequencing, such as Illumina platforms (Collins et al., 2019; Jo et al., 2017; Miya et al., 2015; Valsecchi et al., 2019). As such, they are becoming the standard barcode locus for marine vertebrate metabarcoding studies (Bista et al., 2017; Closek et al., 2019; Miya et al., 2015; Thomsen et al., 2016; Valsecchi et al., 2019; Yamamoto et al., 2017). However, *12S* fish reference databases are relatively incomplete compared to traditional barcoding loci, such as the 655 bp region of the mitochondrial Cytochrome Oxidase I (*COI*) gene (Ardura et al., 2013; Duke & Burton, 2020; Hastings & Burton, 2008; Ward et al., 2009). For example, there is an extensive *CO1* barcode database of fishes of the California Current Large Marine Ecosystem (Hastings & Burton, 2008) that, according to the MitoHelper query of the MitoFish database (accessed April 2021) includes 878 of 1,144 (76.7%) species (Iwasaki et al., 2013; Lim & Thompson, 2021)] facilitating numerous recent metabarcoding studies (Closek et al., 2019; Djurhuus et al., 2020; Pitz et al., 2020). However, there are relatively few reference *12S* sequences that overlap with the MiFish primer sets, limiting the utility of *12S* metabarcoding approaches in this region.

The California Current Large Marine Ecosystem is a highly productive coastal ecosystem that extends approximately 3,000 km across most of the Northeast Pacific from Baja California, Mexico to British Columbia, Canada (Checkley Jr & Barth, 2009; Coleman, 2008; Ekstrom, 2009; Koslow & Davison, 2016). This large marine ecosystem has enormous regional and global importance (Ekstrom, 2009; Sherman, 1991; Wells et al., 2020), driving an ocean economy

valued at over $56 billion USD, employing over 675,000 people (Block et al., 2011; Koslow & Davison, 2016; NMFS, 2017) and supporting food security of the region. The California Current Large Marine Ecosystem also plays a vital role in the cultures and traditional practices of coastal North American tribes and First Nations by supporting species such as Pacific salmon (*Oncorhynchus* spp.), orcas (*Orcinus orca*), eulachon (*Thaleichthys pacificus*), and abalone (*Haliotis* spp.) (Armstrong, 2017; Braje et al., 2017; Brooks et al., 2012; Lepofsky et al., 2017; Norgaard, 2019; Wadewitz, 2012).

Unfortunately, this ecosystem is increasingly facing numerous threats including overexploitation (Koslow & Davison, 2016), ocean acidification and hypoxia (Chan et al., 2008; Crozier et al., 2019; Hofmann et al., 2014; Samhouri et al., 2017), pollution (Good et al., 2020; Halpern et al., 2009), and climate change induced marine heat waves (Rogers-Bennett & Catton, 2019; Santora et al., 2020). Metabarcoding has the power to address many critical management questions in this region, ranging from shifting species distributions, effectiveness of marine protected areas, and seasonal patterns of larval fish recruitment, among others (Duke & Burton, 2020; Kelly, Port, Yamahara, Martone, et al., 2014; Port et al., 2015). However, the ability of metabarcoding efforts to address these important questions hinges on the availability of comprehensive reference databases and appropriate methods of bioinformatic analysis.

To improve the utility of *12S* metabarcoding of marine fishes for the California Current Large Marine Ecosystem and to address larger questions regarding the impact of bioinformatic processes on taxonomic classification, we 1) generated and contributed 741 additional MiFish *12S* sequences representing 597 fish species to global sequence databases; 2) used these additional sequences to create a reference database curated specifically for the California Current

Large Marine Ecosystem; 3) compared the performance of taxonomic assignments made by this regional curated reference database to those made by global marine vertebrate reference databases; and 4) assessed the effect of classifier parameters on phylum through species level assignments of MiFish *12S* sequences to identify optimal locus-specific bioinformatic parameters.

METHODS

Reference Barcode Generation from Fish Tissue Samples

To generate a more complete *12S* barcode reference database for California Current Large Marine Ecosystem fishes, we assembled a list of the 1,144 marine teleost and elasmobranch species that occur in this system (Allen & Horn, 2006; Froese & Pauly, 2010; Hastings & Burton, 2008; Love, & Passarelli, 2020) (Table S1). From this list, we acquired 741 ethanol-preserved voucher specimens representing 597 species (Table S1, Table S2) from the Scripps Institution of Oceanography Marine Vertebrate Collection at the University of California San Diego. DNA was extracted from each tissue sample using a Chelex 100 extraction method (Walsh, Metzger, & Higuchi, 1991), as described in the Supplemental Methods. We amplified all teleost DNA extracts (n=701) using the MiFish Universal Teleost Primers (Miya et al., 2015), and all elasmobranchs (n=55) using the MiFish Elasmobranch primers (Miya et al., 2015) following the thermocycler profile of Curd et al., (2019) (Table S3). We Sanger sequenced purified amplicons (see Supplemental Methods for details), and aligned and trimmed forward and reverse sequences in Sequencher version 5.4.6 (Nishimura, 2000). We used *R* package *taxize* (version 0.9.99) (Chamberlain & Szöcs, 2013) to synonymize taxonomic names of all vouchered

specimens and GenBank. We then checked the accuracy of generated reference barcodes by building a UPGMA phylogenetic tree of all reference sequences and California Current Large Marine Ecosystem fishes using *phangorn* (2.5.5). In addition, we queried each sequence using *blastn* (Camacho et al., 2009) and removed any sequence that did not cluster or align to known taxonomic lineages (data available at https://doi.org/10.5068/D1H963). The resulting *12S* reference barcodes were deposited into GenBank (SAMN19289093–SAMN19289810; Table S2).

Reference Database Creation

To test variation in taxonomic assignment among reference databases, we generated three distinct reference sequence databases: "CRUX-GenBank", "global", and "regional" (Table 1 and Table 2). CRUX-GenBank is a custom *12S* reference database generated using Creating Reference libraries Using eXisting tools (*CRUX*) module of the Anacapa Toolkit to query GenBank for reference barcodes conducted with standard search parameters (Benson et al., 2018; Curd et al., 2019) and MiFish Universal *12S* sequences (Table S1) as the user-defined primers. Briefly, we created this reference database by running *in silico* PCR (Ficetola et al., 2010) on the European Molecular Biology Laboratory (EMBL) standard nucleotide database (Stoesser et al., 2002) to generate a seed library of *12S* references. Next, we used *blastn* (Camacho et al., 2009) to capture reference barcodes without included primer sequences and to query the seed database against the NCBI non-redundant nucleotide database (Gold, 2020; Pruitt et al., 2005; sequences downloaded in October 2019). The resulting *blastn* hits were de-replicated by retaining only the longest version of each sequence and taxonomy for each accession was retrieved using

Entrez-qiime (Baker, 2016). The resulting set of reference sequences in the CRUX-GenBank database included any GenBank reference barcodes that *in silico* amplified to the MiFish *12S* primers at the time of this analysis.

We created the global database to evaluate whether increasing database completeness improves taxonomic assignment. To create the global database, we supplemented the CRUX-GenBank database with 741 additional California Current Large Marine Ecosystem fish *12S* barcodes generated for this study (Table S2). Thus, the global database includes all fish *12S* reference sequences available at the time of download. From this global database, we created the regional database, including only *12S* sequences of fishes known to occur in the California Current Large Marine Ecosystem. We created this database to specifically test whether databases curated to specific ecosystems enhance taxonomic assignment performance relative to more comprehensive databases ("global"). Because of the high degree of similarity between the MiFish Universal and Elasmobranch loci and the flexibility built into *CRUX,* a single *CRUX* generated *12S* reference database performs well for both markers (Curd et al., 2019), so we did not create separate teleost and elasmobranch databases. Additionally, because the MiFish primer set amplifies nearly all vertebrate taxa (Miya et al., 2015; Valsecchi et al., 2019), the global database include teleosts, elasmobranchs, mammals, reptiles, amphibians, birds, etc. All databases are available at https://doi.org/10.5068/D1H963.

Taxonomy cross-validation by identity comparisons

We implemented the taxonomy cross-validation by identity (TAXXI) framework developed by (Edgar, 2018a) to 1) compare taxonomic assignment performance metrics for global versus

regional reference databases, 2) determine the resolution of taxonomic assignments for all available MiFish barcodes in the global database, and 3) understand the performance of the MiFish barcode across taxonomic classifier cutoff scores. Although we use three databases (global, CRUX-GenBank and regional) on our test dataset below, we did not include the CRUX-GenBank database in taxonomic cross validation comparisons because the global database contains all these sequences.

The TAXXI analyses were implemented using scripts from Curd et al. (2019) which adapted TAXXI to the *Anacapa Toolkit* (https://drive5.com/taxxi/doc/index.html and https://github.com/limey-bean/Anacapa). We conducted taxonomic assignments using the *Anacapa Toolkit classifier* which implements the Bayesian Lowest Common Ancestor (BLCA) classifier (Gao et al., 2017) modified to incorporate sequences from *Bowtie2* (Langmead & Salzberg, 2012). In brief, amplicon sequence variants (ASVs; exact unique sequences dereplicated from generated metabarcoding data) are first aligned to reference barcodes using *Bowtie2* retaining the top 100 alignments. Then the BLCA classifier conducts multiple sequence alignment for each query ASV to inform a weighted Bayesian posterior probability of taxonomic assignment. Taxonomy is then ultimately assigned based on the lowest common ancestor of the total weighted reference database matches; reliability is evaluated through bootstrap confidence scores which are analogous to percent identity metrics provided by other metabarcoding classifiers (Gao et al., 2017; See Curd et al. 2019 for full description).

We evaluated taxonomic assignment performance by comparing the following metrics: 1) true positive rate – the number of correct taxonomic assignments divided by the total opportunities for correct classification, 2) over-classification rate - the number of assignments

incorrectly made to additional lower taxonomic ranks divided by the total opportunities to make an over-classification error, 3) under-classification rate - the number of assignments incorrectly made to fewer taxonomic ranks divided by the total opportunities to make an under-classification error, 4) misclassification rate - the number of assignments incorrectly predicted divided by the opportunities for correct classification, and 5) accuracy - the number of correct assignments divided by the taxonomic assignment opportunities for which correctness can be determined (R. C. Edgar, 2018a). The 6) sensitivity was calculated as the true positive rate / (true positive rate + under-classification rate) as under-classification is analogous to a false negative rate. The 7) specificity was calculated as 1- (misclassification rate + over-classification rate) as the combination of the misclassification rate and over-classification rate is analogous to the false positive rate.

Taxonomic Resolution of the MiFish *12S* primer

To provide insights into which fishes can be resolved to species level using the MiFish *12S* primer set, we conducted TAXXI comparisons using the global database as both the test and training database to assign taxonomy to itself. We then calculated the seven taxonomic assignment metrics described above. Additionally, we identified families and genera of fishes for which the MiFish *12S* locus performed poorly, defined as frequently failing to assign species level identification. Although all vertebrate sequences in the global database were used in the taxonomic cross validation, only results for fishes are discussed here.

Regional vs. global reference databases

To compare the relative ability of regional versus global reference databases to accurately assign taxonomy, we conducted two additional TAXXI comparisons using the reference databases created for this study. First, we used the global reference database as a training database to assign taxonomy to the regional reference database that only contained sequences for fishes known from the California Current Large Marine Ecosystem. Second, we used the regional reference database as both the test and training database to assign taxonomy against itself. The taxonomic assignments made by the global and regional reference databases were compared across the taxonomic assignment metrics described above.

Effect of Bootstrap Confidence Scores on Taxonomic Assignment

To understand the performance of the MiFish barcode across a range of taxonomic classifier cutoff scores, we repeated each of the three TAXXI analyses described above (global-regional, regional-regional, global-global) using bootstrap confidence cutoff scores of 40, 50, 60, 70, 80, 90, 95, and 100. We then evaluated the effect of bootstrap confidence cutoff scores across the various taxonomic assignment metrics, as described above.

eDNA Metabarcoding Case Study

Seawater Sample Collection, DNA Extraction, and Library Generation

To specifically test the impact of *12S* database design on taxonomic assignment in real world applications, we compared the performance of the three databases in assigning taxonomy to existing eDNA sequence data as a test case. Briefly, we used MiFish *12S* metabarcoding

sequence data generated from three seawater samples collected from 10 m depth from three sites off eastern Santa Cruz Island, CA in 2017 that were part of a larger ecological study of biodiversity patterns within rocky reef ecosystems. These sequences were generated using standard eDNA collection, processing, and sequencing methods, as outlined in Gold et al., (2021).

We processed this eDNA metabarcoding data three separate times using the *Anacapa Toolkit* (Curd et al., 2019), assigning taxonomy using the CRUX-GenBank, global, and regional reference databases (Table 2). We used the default *Anacapa Toolkit* parameters and a bootstrap confidence cutoff score of 60. We then examined the total number of ASVs and taxonomic ranks identified by each of the three reference databases. We also investigated differences in taxonomic assignment between single direction ASVs (comprised of forward- and reverse-only sequence reads) and merged ASVs (merged paired-end sequence reads) to understand the importance of full length vs. partial length sequences for taxonomic assignment (See Supplemental Results and Discussion).

RESULTS

Generation of Novel Barcodes and 3 References Databases

We generated 741new *12S* MiFish barcode sequences for 597 California Current Large Marine Ecosystem fishes (Table S1 and Table S2), 545 teleosts (bony fishes), 49 elasmobranchs (cartilaginous fishes), and 3 cyclostomatan (jawless fishes) (Table S2). This dataset includes 252 that had no previous *12S* reference barcodes (Table S1).

CRUX created a custom *12S* database comprised of 14,066 taxa and 44,140 sequences with existing entries in GenBank. Adding the 741 novel sequences, above, resulted in a global database comprised of 14,321 species and 44,882 sequences. Restricting these sequences to only fishes from the California Current Large Marine Ecosystem resulted in a curated regional database that includes 706 out of 1,144 (61.7%) reference *12S* barcodes from fishes known from this region. Excluding 382 species missing from the database that are rare in California (n=357) or not coastal (n=25), resulted in a total coverage of 92.7% of the 763 common coastal fishes in this region.

Taxonomy Cross-validation by Identity Comparisons

Regional Versus Global Reference Database Comparisons

The TAXXI quality metrics indicate that the regional reference database yielded more reliable taxonomy at genus and species ranks relative to the global reference databases across all bootstrap confidence scores; regional database species level accuracy ranged from 64.2-94.2% compared to 51.3-90.8% for the global database (Table 2 & Table S4; Figures S1 and S2). This difference was driven by higher misclassification and under-classification rates for the global reference databases. In particular, database misclassification rates were higher for the global compared to the regional reference database across all bootstrap confidence cutoff scores less than 60 (global reference database misclassification 1.8-4.5%, regional database misclassification rate 1.3-3.1%) (Table S4). Likewise, global reference database under-classification rates were higher than regional reference database under-classification rates across

all bootstrap confidence cutoff scores (global reference database under-classification 4.8-48.7%, regional database under-classification rate 2.8-35.8%).

Taxonomic Resolution of the MiFish *12S* primer

Cross validation of the 44,896 sequences within the global database demonstrated that the MiFish primer set delivered 88.0% sensitivity [true positive rate / (true positive rate + under-classification rate)] and 98.2% specificity [1- (misclassification rate + over-classification rate)] at a bootstrap cutoff score of 60 (Table 2, Table S5), providing species level taxonomic assignments to 6,762 fish species, genus level resolution to 923 fish species, family level assignments to 180 fish species, and class level assignments to 2 fish species while overclassifying 214 fish species (Table S5). While poor taxonomic resolution with the MiFish primer sets (e.g. assigned taxonomic rank above species) spanned a large number of genera and families, the genus *Sebastes* and families Cichlidae, Cyprinidae, and Pleuronectidae were particularly problematic (Figures 4 and 5). Of these, *Sebastes* and Pleuronectidae are highly prevalent within the California Current Large Marine Ecosystem. A full breakdown of taxonomic assignment resolution is provided in the Supplemental Results.

Effect of Bootstrap Confidence Scores on Taxonomic Assignment

Across all TAXXI comparisons, accuracy and true positive rates increased with decreasing bootstrap confidence cutoff scores (Figure 1, Figures S1 and S2, Table S4). Likewise, the proportion of species level assignments also increased with decreasing bootstrap confidence score (Figure 2, Figures S3 and S4). We also found that misclassification rates increased with

decreasing bootstrap confidence cutoff score, but at much lower rate (Figure 3, Figures S5 and S6). These results indicate a clear tradeoff between under-classification and misclassification across bootstrap confidence cutoff scores.

eDNA Metabarcoding Example

Unassigned MiFish *12S* ASVs

The *Anacapa Toolkit* failed to assign taxonomy to 49.6% (169/341) of ASVs representing 24.5% (81,002/330,877) of all reads using all three reference databases investigated in this study (Table S6). Of the 169 unassigned ASVs, 16 were forward-only reads, and 153 were merged reads. To explore the origins of these unassigned reads, we used BLAST to query all GenBank sequences, revealing that 94.7% (160/169) of these ASVs aligned to marine prokaryotic and eukaryotic *16S* sequences (Max Alignment Scores 87.9-475). Of these aligned ASVs, 85% (136/160) matched to uncultured sequences generated from marine metagenomic studies. 80.0% (128/160) of successfully aligned ASVs matched to bacterial barcodes including those from *Psychromonas* sp., *Photococcus caeruleum*, *Loktanella* sp., *Leucothrix* sp., and *Gimesia* sp., and cyanobacteria. A smaller fraction of assigned ASVs (18.8%; 30/160) best aligned to eukaryotic sequences including those from diatoms (e.g. *Nitzschia alba* and *Eucampia antarctica*) and other marine microalgae (e.g. Picobiliphytes, *Heterosigma akashiwo*, *Mesopedinella arctica*, and *Phacus warszewiczii*). Given that these 169 unassigned sequences were non-vertebrate, we excluded these ASVs from all subsequent comparisons. All remaining 172 ASVs were assigned to a class of vertebrates by at least one of the three reference databases used. Of these vertebrate ASVs, 58 were merged, 107 were forward-only, and 7 were reverse only reads.

372

373 **Comparisons of CRUX-GenBank, Global, and Regional Reference Database Taxonomic**
374 **Assignments**

375 The inclusion of additional reference barcodes increased the total number of ASVs and reads
376 assigned to marine fishes resident in the California Current Large Marine Ecosystem (Tables 2 &
377 Table S7). Importantly, the inclusion of novel voucher sequences within the global database
378 resulted in species-level identification for 11 additional California Current Large Marine
379 Ecosystem fishes including Kelp Bass (*Paralabrax clathratus*), California Moray (*Gymnothorax*
380 *mordax*), Opaleye (*Girella nigricans*), Giant Kelpfish (*Heterostichus rostratus*), Ocean
381 Whitefish (*Caulolatilus princeps*), and California Halibut (*Paralichthys californicus*) (Table S8).
382 Use of the regional database largely increased accuracy of taxonomic assignments. The
383 regional database assigned an ASV to the Black Croaker (*Cheilotrema saturnum*) that was only
384 assigned to the family Sciaenidae by the global database. Additionally, the regional database
385 assigned one ASV as Bat Ray (*Myliobatis californica*) and another as Jack Mackerel (*Trachurus*
386 *symmetricus*), species native to the California Current Large Marine Ecosystem, that the global
387 database assigned to the non-native species, Common Eagle Ray (*Myliobatis aquila*) and Rough
388 Scad (*Trachurus lathami*), respectively. However, the regional reference database failed to
389 resolve the taxonomy of one ASV that the global database assigned to the family of Delphinidae.

390

391 # DISCUSSION

392 Taxonomic assignment in metabarcoding studies typically employ large public sequence
393 databases such as GenBank or Barcode of Life (Leray & Knowlton, 2015; Schenekar et al.,

2020; Stat et al., 2017), or databases that are curated to specific barcoding markers or taxonomic groups without consideration of species distributions (e.g. Curd et al. 2019). However, systematic comparison of these approaches to a curated, region-specific reference database shows that the region-specific database outperforms the global databases in metabarcoding taxonomic assignment (Table 1). Accuracy of eDNA metabarcoding only improved by including GenBank sequences from fishes native to the California Current Large Marine Ecosystem and supplementing these sequences with additional reference barcodes. Furthermore, examination of taxonomic assignment over a range of bootstrap cutoff scores revealed key tradeoffs, with lower bootstrap confidence cutoffs yielding more accurate species assignment, but at the cost of higher misclassification rates. Combined, these results highlight the importance of reference database and bootstrap cutoff selection in obtaining the best results from metabarcoding studies.

In a test dataset for fish eDNA extracted from seawater collected from three sites on Santa Cruz Island, the regional database performed the best. The regional database identified 16 additional ASVs to species not identified by the CRUX-GenBank database, and an additional 3 fishes that were misidentified by the global database (Table 2). Higher accuracy with increased database completeness echoes previous research on the importance of complete reference databases in metabarcoding (Leray et al., 2012; Machida et al., 2017), and greatly improves the utility of eDNA for monitoring the California Current Large Marine Ecosystem.

Although the *12S* barcodes and reference databases tested here performed well with regard to annotating fish species (e.g., 91.3% sensitivity and 98.3% specificity across MiFish reference barcodes), almost half of the ASVs and a quarter of all reads generated in our eDNA test datasets were not assigned to any fish reference barcode (Table 2). While other

metabarcoding studies report similar levels of unassigned taxa (Leray & Knowlton, 2017) and others have encountered this issue (Goodwin, personal communication), this issue isn't widely reported in the literature, particularly considering the popularity of the MiFish primers. Further investigation showed that the vast majority of unassigned ASVs were uncultured bacteria *16S* loci (Table S6) derived from marine shotgun sequencing metagenomic studies (Bork et al., 2015). This result highlights that the MiFish Teleost *12S* primer set, while extremely useful for targeting vertebrate *12S* loci, can also amplify non-target *16S* genes, raising the possibility that non-target amplification may at best result in lower returns of target sequences, and at worst artificially increase estimates of fish diversity.

Importance of Regional reference databases

Given that increased reference database completeness increases the ability to assign ASV's to species (Table 2), it is logical to assume that databases with more taxonomic coverage are universally better (Curd et al., 2019). However, our results suggest an unexpected trade-off between greater diversity of barcodes and ecologically informed taxonomic assignment. For example, using only the regional database specific to California Current Large Marine Ecosystem marine fishes, we identified important native taxa like Black Croaker (*Cheilotrema saturnum*) and Bat Ray (*Myliobatis californica*) in eDNA isolated from seawater samples. However, while the global database contained the largest total number of barcodes, including all taxa in the regional database, Black Croaker was not identified and Bat Ray was inconsistently identified across multiple ASVs. The global database failed to identify Black Croaker due to the high similarity of *12S* barcode sequences within the Family Sciaenidae, specifically within the

clade that includes *Cheilotrema,* a genus native to California, as well as *Equetus* and *Pareques,* non-native coral reef-associated genera (Table S8). Similarity of barcode sequences also explains the loss of taxonomic resolution in *Myliobatis*.

By excluding highly similar non-native *12S* barcodes, the database curated for the region of interest provided more accurate species-level assignments and far fewer under-classifications and misclassifications, demonstrating that a database comprised of only local taxa is preferred to maximize identification of local species. Yet, this improvement was not universal. For example, the regional database failed to classify one ASV belonging to the family Delphinidae that was identified by both the CRUX-GenBank and global databases. This result stems from the regional database being specific to California Current Large Marine Ecosystem fishes, and could thus not identify a marine mammal. This shortcoming easily could be overcome, however, by appending the regional database with barcodes for other marine-associated vertebrate taxa of regional management interests (Valsecchi et al., 2019). An alternative and taxon agnostic approach currently employed by the co-authors is to conduct taxonomic assignments twice. First, taxonomic assignments are conducted using a regional reference database to get the best taxonomic assignment for focal taxa of interest, and second using a global reference database to identify as many remaining unidentified ASVs as possible (Gold et al., 2021). We did not directly report the results of the two-step taxonomic assignment method here as the only difference between this approach and the taxonomic assignments made using the regional database alone is the additional assignment of the single Delphinidae ASV.

These results highlight the tradeoff between identifying local species from clades with little genetic variation and providing taxonomic coverage across a broad range of species. As

such, researchers need to identify their research priorities when deciding on which reference databases to use, with a particular focus on defining the scope of the target taxa. Future work could alleviate this tradeoff by building bioinformatic pipelines that prioritize assignments to a reference set of resident species, perhaps by including information on species ranges and sample locations in the assignment algorithm. However, an advantage of the two-step approach outlined above is that it allows for eDNA studies to address specific ecological questions without having a specific target list in mind. This approach is particularly important for eDNA studies which directly test for the presence of invasive species or range shifts associated with climate change (Bohmann et al., 2014; Klymus et al., 2017).

Importance of Taxonomic Cutoff Scores

Taxonomic cutoff scores, or percent identity, strongly influenced taxonomic assignments (Edgar, 2018c). Patterns for the MiFish *12S* locus showed a similar pattern with higher true positive and misclassification rates and lower under-classification rates at lower bootstrap confidence cutoff scores (Edgar, 2018a). These results highlight a key tradeoff between under-classification and misclassification for metabarcoding taxonomic assignment, and demonstrate that the decision of which taxonomic cutoff score can strongly influence results (Edgar, 2018a). Lower bootstrap confidence cutoffs ensure a higher overall accuracy in species-level identification but come at the cost of higher misclassification rates to an incorrect species-level assignment.

Our results suggest that a TAXXI bootstrap confidence cutoff score of 60 provides a balance between maximizing species-level assignment accuracy (89.7%, global reference database) while minimizing misclassification rates (1.7%, global reference database), matching

the general findings of Curd et al. (2019). However, in instances in which metabarcoding results may influence management or health decisions with substantial legal or economic ramifications (i.e., detection of an endangered or invasive species or discriminating a putative disease causing microbe) a misclassification error may be valued as a far less desirable outcome than an under-classification error (Bohmann et al., 2014; Lodge et al., 2012; Wakeling et al., 2019). In such cases, results indicate that there isn't one single bootstrap confidence cutoff score that completely ameliorates these tradeoffs (Figure 1).

Given that previous work demonstrates that results may not be consistent across loci (Curd et al., 2019), we can only generalize our results to the MiFish *12S* primer set. Determining confidence–resolution tradeoffs in other widely used primer sets will be fundamental for effectively interpreting metabarcoding results from those loci. Combining the capabilities of *CRUX* with the TAXXI framework provides a critical set of tools to both generate and evaluate the performance of a range of metabarcoding loci and reference databases (Table 1; Curd et al., 2019; Edgar, 2018a), facilitating such studies. Given the growing number of metabarcoding applications across a broad range of ecosystems and taxa (Curd et al., 2019; Deiner et al., 2017; Edgar, 2018a), assessing the performance of barcoding markers in the taxonomic group of interest is critical.

Importance of Complete Reference Databases

Previous eDNA metabarcoding efforts in the California Current Large Marine Ecosystem report poor species-level identification and frequent taxonomic assignment to non-native sister taxa (Closek et al., 2019; Kelly, Port, Yamahara, & Crowder, 2014; Port et al., 2015). For example,

an eDNA metabarcoding study in Southern California (Curd et al., 2019) assigned multiple *12S* ASVs to *Girella simplicidens*, the Gulf Opaleye, a fish that does not occur in California Current Large Marine Ecosystem coastal waters (Froese & Pauly, 2010; Love & Passarelli, 2020). This incorrect assignment occurred due to the lack of *12S* reference sequences for the local native Opaleye, *G. nigricans*. By maximizing the number of local reference barcodes, regional databases allow the reads to be correctly assigned to ecologically and geographically relevant species.

In our eDNA samples, the regional database improved species-level assignments, identifying an additional 17.0% of the total vertebrate sequence reads. Much of this improvement was due to the inclusion of reference barcodes for Kelp Bass (*Paralabrax clathratus*), one of the most abundant marine species in Southern California kelp forest ecosystems and an important sport fishery target (Pondella II et al., 2015). By including a reference barcode for this species, the regional database assigned 20 previously unidentified ASVs to *P. clathratus,* which accounted for 16.4% of our total sequence reads. Thus, even the inclusion of reference barcodes for a few key native taxa can dramatically improve metabarcoding efforts.

Taxonomic Assignment Limitations of MiFish primers

Of the 8,084 fishes represented in the global database, the MiFish primers were unable to provide species level taxonomic assignments to 1,322 species (See Table S5 for complete list of putative *in silico* taxonomic assignments). Thus, although the MiFish primer set has broad utility for fish metabarcoding, this portion of *12S* cannot resolve many fishes to species (Miya et al., 2015). These results highlight the tradeoff between breadth and specificity of any metabarcoding

primer set, a result consistent with previous investigations of the MiFish primer set and universal barcodes in general (Deiner et al., 2017; Miya et al., 2015). Critically, these results provide much needed insights into taxonomic blind spots of the MiFish primers, informing primer selection for future fish metabarcoding applications both in the California Current Large Marine Ecosystem and globally (Figures 4 and 5).

Another key limitation to metabarcoding taxonomic assignment is the prevalence of sequence misannotations in public sequence repositories. Misannotations arise predominantly from subtle incidental issues, such as mislabeling of sequences, and thus are particularly difficult to address bioinformatically (Heller et al., 2018; Nobre et al., 2016; Wakeling et al., 2019). To date, the onus of identifying and preventing misannotations are on the user and research community and there remain few systematic methods for identifying and removing misannotated sequences although Kozlov et al., 2016 is a notable exception (we also note there is a process to flag and report such sequences are available through GenBank). One potential solution to the issue of misannotated sequences is the development and maintenance of global curated datasets (e.g., MitoFish, Silva, and UNITE) (Nilsson et al., 2018; Quast et al., 2012; Sato et al., 2018). However, while these approaches may work well for a handful of key loci and taxonomic targets, these approaches are not scalable with the rapid development of additional metabarcoding loci and targets of interest (Curd et al., 2019). Thus, further efforts to systemically prevent and address mis-annotations in public sequence repositories clearly are warranted.

Limitations of Barcoding Efforts

The regional database did not include barcodes for all California Current Large Marine Ecosystem fishes (Table S1) due to a combination of limited resources, difficulties amplifying vouchered tissue samples, the onset of the COVID-19 pandemic (Omary et al., 2020), and a lack of some vouchered reference material within the Marine Vertebrates Collection of the Scripps Institution of Oceanography. In total, our regional database did not include 438 of 1,144 (38.3%) California Current Large Marine Ecosystem fishes. However, the vast majority of these (n=357) are rare in the state of California (the focus of the collection and study), others (n=25) are common but not coastal species. Discounting these, our barcoding efforts provide coverage for 92.7% of the 763 marine fishes common in this ecosystem, making it an important tool for metabarcoding studies, despite a small number (n=53) of common coastal species missing from the database (Table S9).

The one major shortcoming of our barcoding efforts is that 7.3% (n=32) of the missing taxa are rockfishes in the genus *Sebastes*. Rockfishes are ecologically important (Hyde & Vetter, 2007), form the basis of many commercial and recreational fisheries (Lea et al., 1999; Williams et al., 2010), and declines in rockfish stocks led to the establishment of the largest marine protected areas in southern California, the Cowcod Conservation Areas (Thompson et al., 2017). Unfortunately, this shortcoming cannot be easily overcome through additional *12S* barcoding because rockfish are a recent and diverse radiation comprised of 110 species (Ingram & Kai, 2014) and *12S* fails to resolve most *Sebastes* to species-level (Hyde & Vetter, 2007; Yamamoto et al., 2017). Thus, effective metabarcoding of *Sebastes* will require designing novel *Sebastes*-specific metabarcoding primers that target a more rapidly evolving region of the mitochondrial

568 genome (e.g. *CytB*) (Min et al., 2020; Thompson et al., 2017). Importantly, this *Sebastes*
569 example highlights the importance of comprehensively evaluating the taxonomic performance of
570 a particular locus (here MiFish *12S*) for a given taxonomic group and the difficulty of using
571 metabarcoding methods for delineating species within an adaptive radiation.

572 Despite these limitations, however, the current regional California Current Large Marine
573 Ecosystem *12S*-specific reference database includes all but one non-*Sebastes* nearshore species
574 monitored by the Channel Islands National Kelp Forest Monitoring Program (n=80, Sprague et
575 al., 2013), as well as by PISCO, the Partnership for Interdisciplinary Studies of Coastal Oceans
576 (n=76; the only missing species is White Sea Bass *Atractoscion nobilis*; Caselle, Rassweiler,
577 Hamilton, & Warner, 2015; Pondella II et al., 2015). Further, there is now a *12S* reference
578 sequence for 98 of the 100 most abundant ichthyoplankton species collected by the California
579 Cooperative Oceanic Fisheries Investigation (CalCOFI) from the California Current Large
580 Marine Ecosystem between 1951-2019 (only missing Showy Bristlemouth *Cyclothone signata*
581 and White Barracudina, *Arctozenus risso*) (Moser, 1993). Moreover, in real world application,
582 this reference barcode database assigned taxonomy to over 90% of vertebrate ASVs detecting a
583 broad range of ecologically and commercially important nearshore rocky reef species (Pondella
584 II et al., 2019). As such, our barcoding efforts represents an important genetic resource for
585 coastal California marine metabarcoding monitoring efforts.
586

587 ## Off Target Limitations of MiFish primers

588 High numbers of unidentified ASVs are a common feature of barcoding and metabarcoding
589 studies (e.g. Leray & Knowlton, 2017). These unidentified ASVs are typically attributed to

incomplete reference databases (Curd et al., 2019; Ransome et al., 2017; Schenekar et al., 2020) and/or novel biodiversity (Barber & Boyce, 2006; Boussarie et al., 2018). However, given that the regional database includes 92.7% of fishes common in this coastal ecosystem, it was extremely surprising that half of all ASVs and a quarter of all sequences generated in our eDNA test datasets could not be assigned.

In fact, the vast majority of these sequences and ASVs did not belong to vertebrates, but instead uncultured marine bacteria, specifically matching to *16S*, rather than the target *12S* locus. Since mitochondria represent the capture of microbial endosymbionts by ancient eukaryotes (Roger et al., 2017) and that this capture occurred in the sea, it perhaps is not surprising that primers designed to target vertebrate *12S* might also capture marine prokaryotes. Similarly, the homology between vertebrate *12S* and prokaryotic and bacterial *16S* genes is well known (Crews & Attardi, 1980) suggesting capturing microbial *16S* with vertebrate *12S* primers is not surprising. However, this particular feature of the MiFish primer set previously has not been widely reported in the scientific literature (Minamoto et al., 2020), potentially impacting the interpretation of unidentified ASVs in other fish metabarcoding studies.

These findings highlight the importance of accurate universal metabarcoding primer design, especially in outlining both target and non-target sequences. In the design of the MiFish Teleost *12S* primers, uncultured marine microbe *16S* sequences were not considered as potential alternative targets for the primer set, resulting in the selection of a metabarcoding locus with a high degree of non-target amplification (Miya et al., 2015). This finding is important for the marine vertebrate eDNA community, which has recently converged on the MiFish *12S* primers as the vertebrate barcode of choice (Closek et al., 2019; Miya et al., 2020; O'Donnell et al.,

612 2017; Valsecchi et al., 2019; Yamahara et al., 2019). At best, this non-target amplification of
613 microbial DNA will lead to wasted sequencing effort, as every microbial sequence generated
614 reduces the number of vertebrate sequences captured. Such a situation would be particularly
615 problematic for relatively rare targets. At worst, it could result in incorrect interpretation of
616 unidentified ASVs and lead to incorrect biomonitoring assessments (Cordier et al., 2018). This
617 problem is of particular concern in biodiversity hotspots such as the Coral Triangle where
618 reference databases are incomplete, as well as in environments with high abundance of bacteria
619 relative to vertebrate biomass such as in some pelagic midwater and deep-sea habitats where
620 recent eDNA sample collection efforts have struggled to detect vertebrate sequences (K. Pitz
621 personal communication).

622 Previous applications of MiFish *12S* primer sets did not identify high rates of non-
623 homologous sequences (Collins et al., 2019; Miya et al., 2015). Interestingly, these studies used
624 higher annealing temperatures (60-65˚C) and fewer PCR cycles than those used in this study
625 which may potentially explain why we observed high rates of *16S* amplification. We note that we
626 used the touchdown PCR method in order to successfully amplify eDNA from sea water
627 samples. A white paper from *The eDNA Society* in Japan using the original 65˚C annealing
628 methods highlighted that the application of a size-selection step during library preparation (either
629 via gel extraction or dual size-selection bead clean up) can be used remove off-target sequences
630 and help ameliorate this issue (Minamoto et al., 2020; Miya & Sado, 2019). We also confirm
631 here that non-target ASVs are substantially longer in length than vertebrate *12S* fragments.
632 Incorporating these practices to reduce microbial cross-amplification will improve the
633 application of MiFish *12S* metabarcoding efforts. Ultimately, understanding the full scope of

taxa that can be amplified by a given metabarcoding primer is critical for the successful application and interpretation of results and concerted efforts to validate markers are clearly warranted.

Towards improved metabarcoding efforts

The curated California Current Large Marine Ecosystem *12S*-specific reference database was designed to improve effectiveness of metabarcoding of California Current Large Marine Ecosystem fishes. To further improve and expand the taxonomic coverage of the database, we generated a website that identifies species needing *12S* reference barcodes and provides the research community targets for additional barcoding efforts (Zack Gold, 2020). The ability to update and expand the regional reference database will be especially important as climate change leads to range expansions of sub-tropical species that may become resident within the California Current Large Marine Ecosystem (Gentemann et al., 2017; Harvell et al., 2019; Sanford et al., 2019; Walker et al., 2020). The importance of expanding the database is highlighted by our detection of Finescale Triggerfish, *Balistes polylepis,* in the eDNA samples, a species that has only recently become more common off Santa Cruz Island and La Jolla since the 2014-2016 marine heatwave (B. Frable & S. McMillan, personal communication).

Additionally, while the MiFish Teleost and Elasmobranch *12S* loci are important targets for current marine metabarcoding studies, future efforts and different applications of marine metabarcoding will likely rely on additional barcoding targets. Here we used the same primer set to both generate reference barcodes as well as conduct metabarcoding. Although, this choice limits the applicability and usefulness of our reference barcode generation beyond

metabarcoding efforts, it allowed us to more easily and rapidly sequence and generate barcodes for our intended purpose. Furthermore, recent efforts have found success multiplexing *CO1* and *16S* loci simultaneously provides more species-level identifications than either marker alone, demonstrating complimentary genetic loci can improve metabarcoding assignments (Duke & Burton, 2020). Thus future efforts to develop rapid and affordable multilocus barcoding or whole mitogenomic tools will provide greater resources for marine metabarcoding and population genomic efforts (Coissac et al., 2016). As these new barcode loci are developed (e.g., *Sebastes*-specific barcodes), the California Current Large Marine Ecosystem specific reference database can be expanded to include these loci. Additionally, resources like the SIO Marine Vertebrate Collection will continue to provide important voucher specimens for advancing marine molecular ecology resources as they accession new material.

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DATA ACCESSIBILITY

Reference databases and metabarcoding data are publicly available and stored on a Dryad repository (https://doi.org/10.5068/D1H963). All reference barcode sequences have been uploaded to GenBank (BioProject PRJNA731549). Additional supporting information is available at https://github.com/zjgold/FishCARD.

AUTHOR CONTRIBUTIONS

- Conceptualization ZG, ESC, DK, BF, RSB, KDG, ART, PHB, HJW
- Performed Research ZG, ESC, DK, BF, ART
- Funding Acquisition ZG, PHB, ART, KDG, DK, RSB
- Data Curation ZG, ESC, DK, BF, HJW
- Formal Analysis ZG, EEC
- Writing – Original Draft Preparation ZG, EEC, ESC, DK, BF, RSB, KDG, ART, PHB, HJW

CONFLICS OF INTEREST

The authors have no conflicts of interest to report.

TABLES

Table 1. Summary of Cross Validation Results. Comparison of performance metrics for taxonomic assignments using the global database as a reference to annotate sequences in the global database (global-global)[(test database-training database)], the regional database (global-regional), and using the regional database as a reference to annotate sequences in itself (regional-regional). Reporting metrics calculated using a taxonomic cutoff score of 60.

Metric	Global-Global	Global-Regional	Regional-Regional
Under-classification Rate	8.6%	11.8%	7.8%
Misclassification Rate	1.7%	1.8%	1.3%
Over-classification Rate	0.0%	0.0%	0.0%
Accuracy	89.7%	86.5%	90.9%
True Positive Rate	89.7%	86.5%	90.9%
Sensitivity	91.3%	88.0%	92.1%
Specificity	98.3%	98.2%	98.7%

Table 2. Summary of Seawater eDNA Metabarcoding Taxonomic Assignments for Tested Reference Databases.

Metric		Reference Database		
		CRUX-GenBank	**Global**	**Regional**
Database	Reference Barcode Origin	GenBank	GenBank + Generated	GenBank + Generated
	Species Included	**All**	**All**	**California Fishes**
Reads	Total Reads	330,877		
	Assigned to NA	81,014	81,002	81,006
	Assigned to Class Level	54,090	-	-
	Assigned to Order Level	727	-	-
	Assigned to Family Level	1,286	1,409	131
	Assigned to Genus Level	952	1,068	1,063
	Assigned to Species Level	192,808	247,398	248,677
ASVs	Total ASVs	341		
	Assigned to NA	172	169	170
	Assigned to Class Level	12	-	-
	Assigned to Order Level	3	-	-
	Assigned to Family Level	5	13	11
	Assigned to Genus Level	4	6	4
	Assigned to Species Level	145	153	156
Taxonomy	Unique Families Identified	31	28	27
	Unique Genera Identified	39	38	39
	Unique Species Identified	38	38	37
	CA Native Species	25	36	37
	Avg. ASVs Per Species	3.8	4.1	4.2

FIGURES

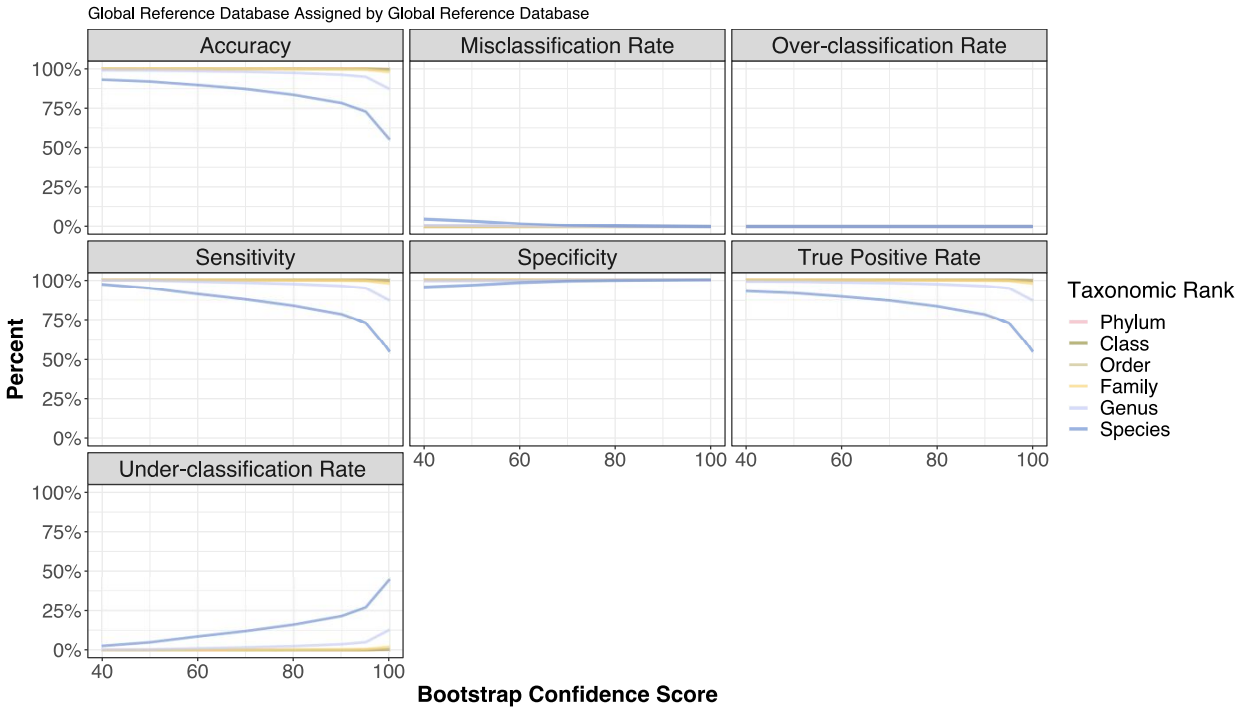
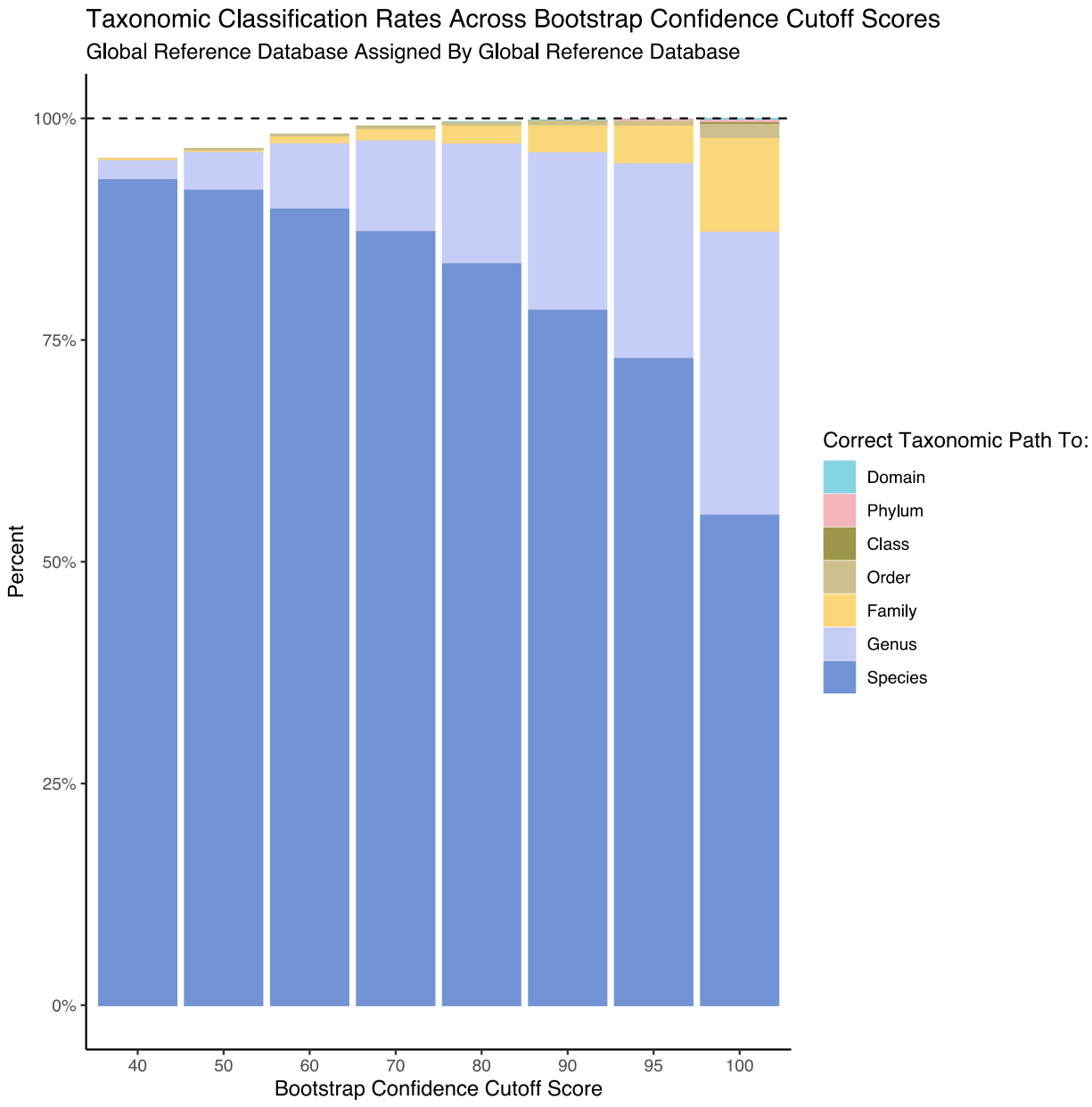


Figure 1. Effect of TAXXI Bootstrap Confidence Cutoff Scores on Taxonomic Assignment Metrics. Taxonomy cross-validation by identity (TAXXI) results for taxonomic assignments generated by using the global database as a reference to annotate the sequences in that same database. Accuracy, true positive rate, sensitivity, and misclassification increased with relaxed bootstrap confidence cutoff scores. Under-classification and specificity decreased with relaxed bootstrap confidence cutoff scores. Results for each taxonomic rank are colored.



1103

1104 **Figure 2. Taxonomic Classification Rates Across Bootstrap Confidence Cutoff Scores.**

1105 Results from taxonomy cross-validation by identity (TAXXI) using the global database as the reference to assign

1106 taxonomy to all sequences in that database. Correct species level matches increase with more relaxed bootstrap

1107 confidence cutoff scores. Correct taxonomic level matches are colored by the lowest common ancestor match.

1108 Dotted line indicates 100% and all mismatches were excluded.

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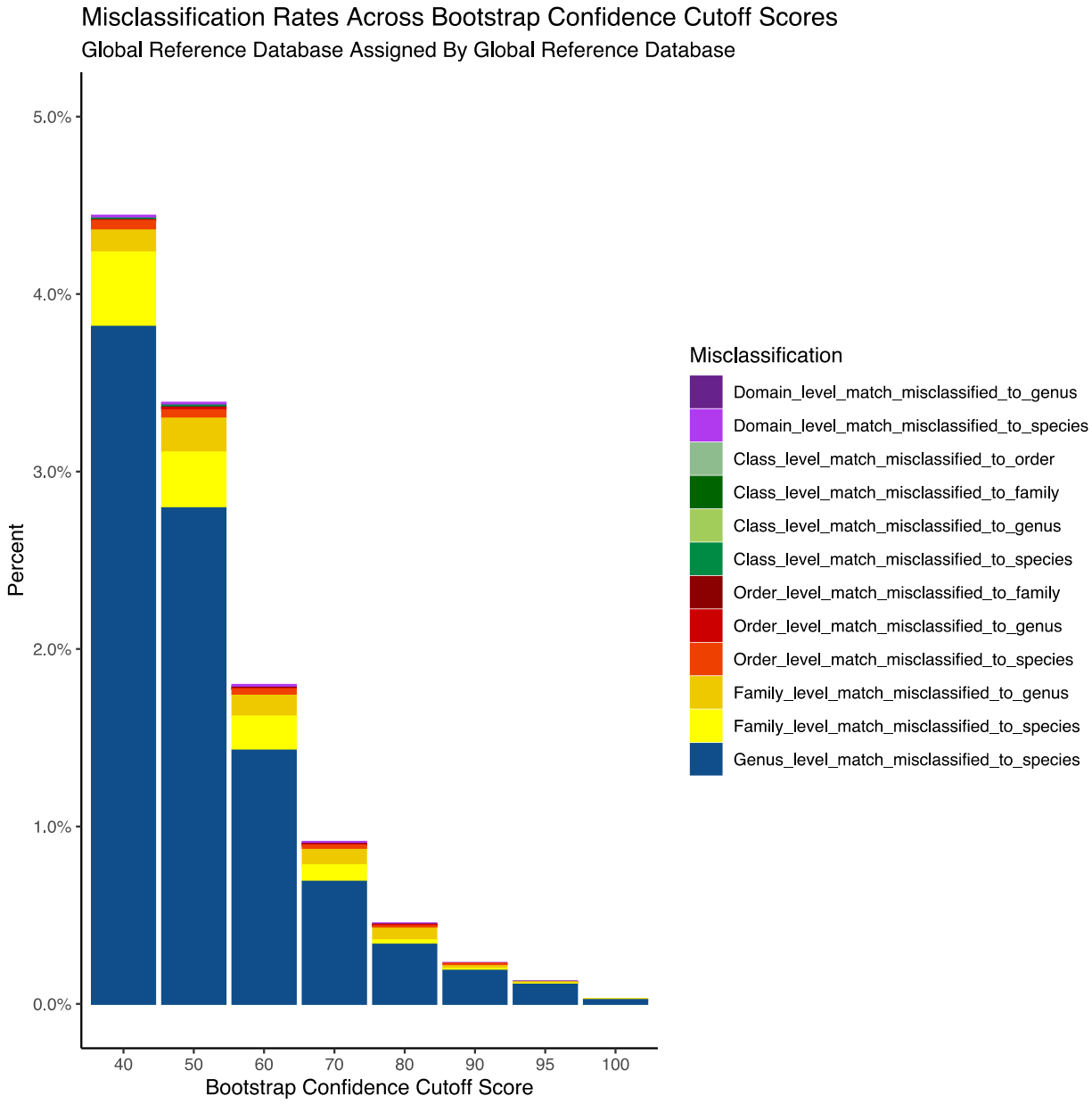


Figure 3. Misclassification Rates Across Bootstrap Confidence Cutoff Scores. Results from taxonomy cross-validation by identity (TAXXI) using the global reference database to assign taxonomy to all sequences in that database. Misclassification increased with relaxed bootstrap confidence cutoff scores. Misclassification types are colored.

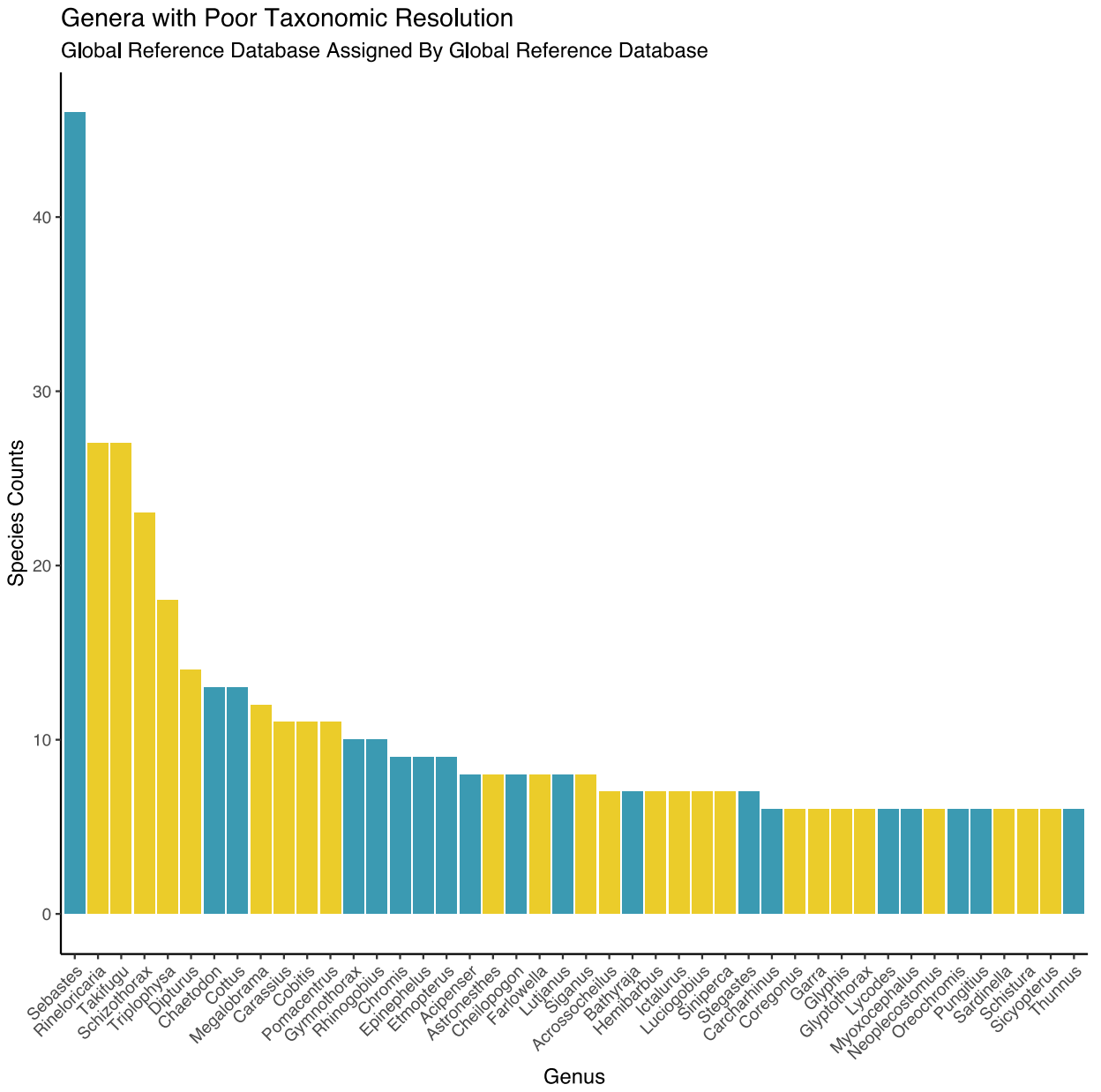


Figure 4. Genera with Poor Taxonomic Resolution. Genera poorly resolved to the species level by the MiFish *12S* barcode based on results from taxonomy cross-validation by identity (TAXXI) using the global

reference database to assign taxonomy to all sequences in that database using a bootstrap confidence cutoff of 60. Genera in blue occur in the California Current Large Marine Ecosystem.

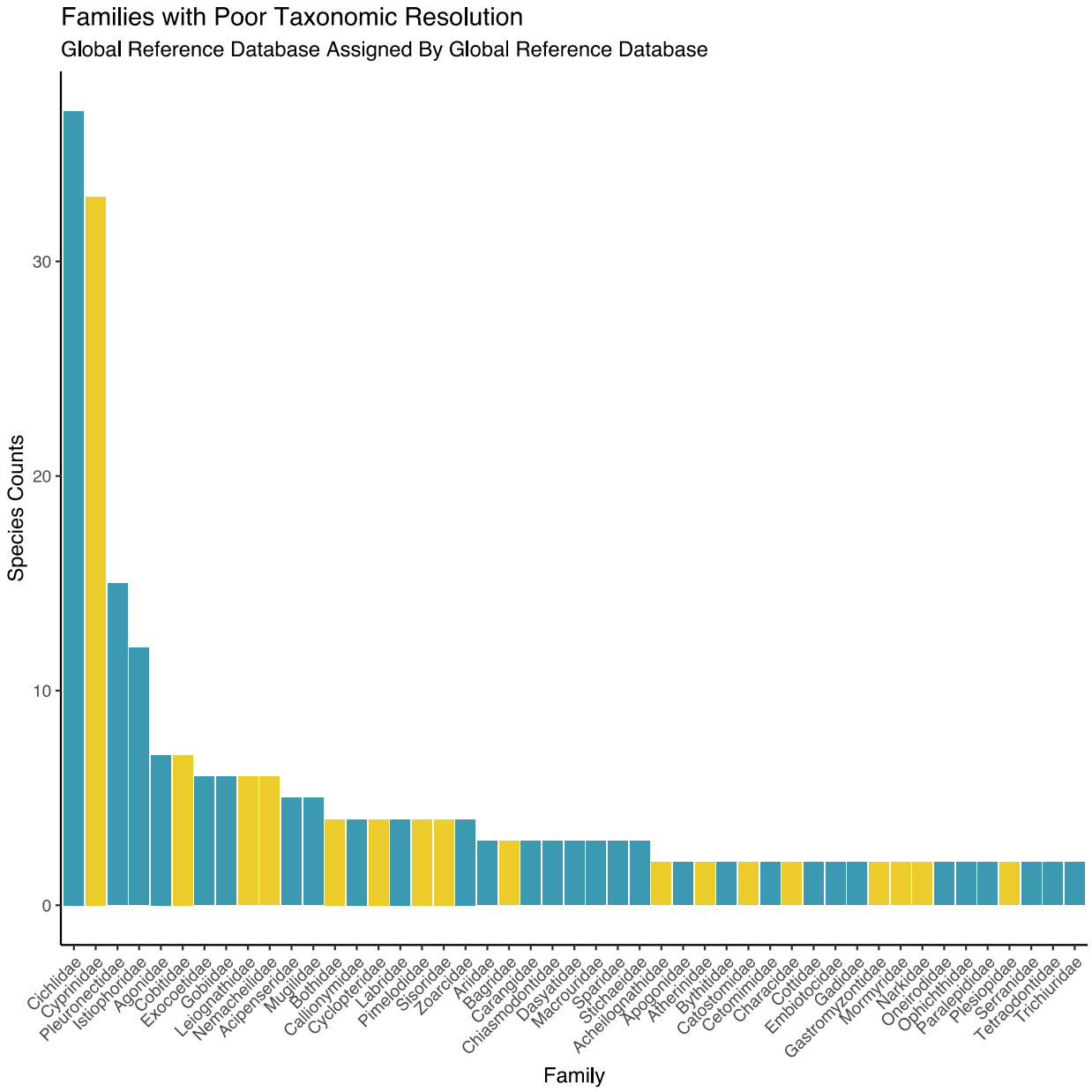
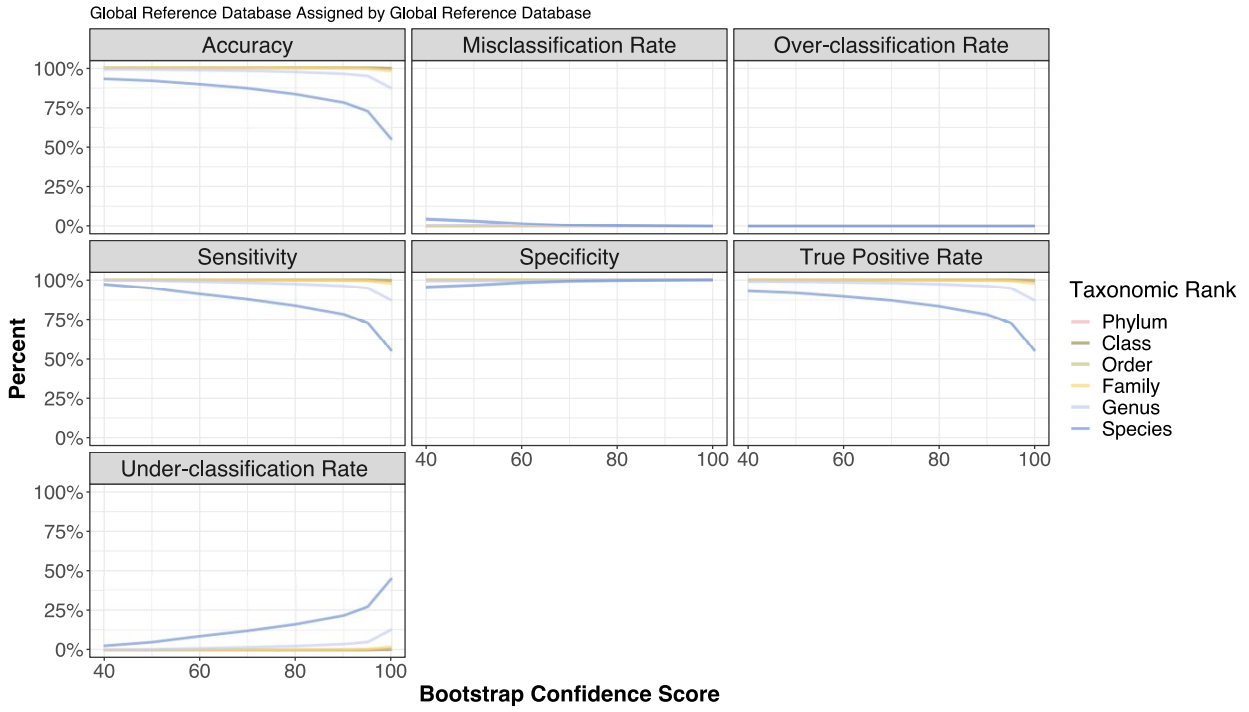


Figure 5. Families with Poor Taxonomic Resolution. Families poorly resolved to the species level by the MiFish *12S* barcode based on results from taxonomy cross-validation by identity (TAXXI) using the global

reference database to assign taxonomy to all sequences in that database using a bootstrap confidence cutoff (BCC) of 60. Families in blue that occur in the California Current Large Marine Ecosystem.



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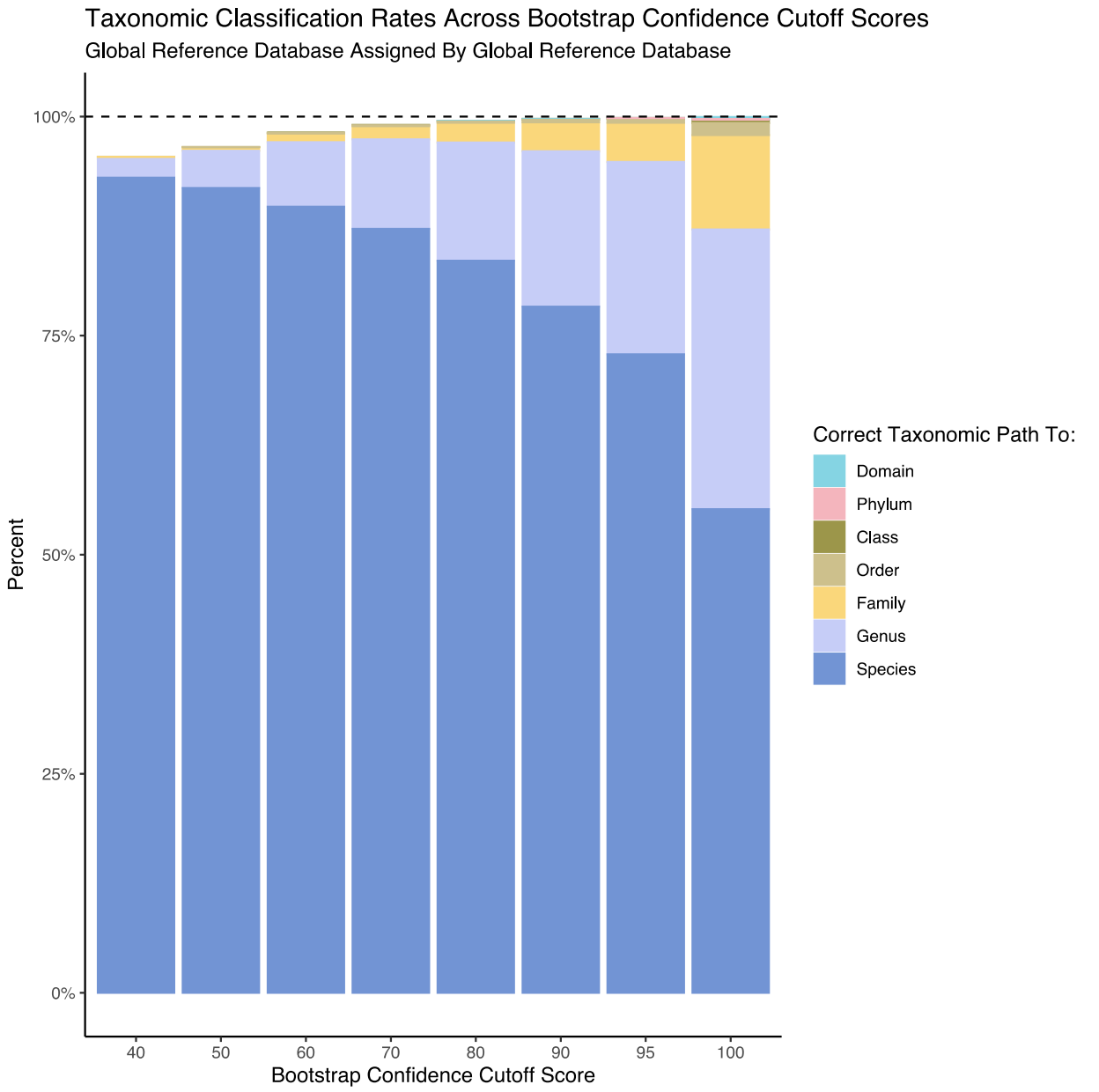
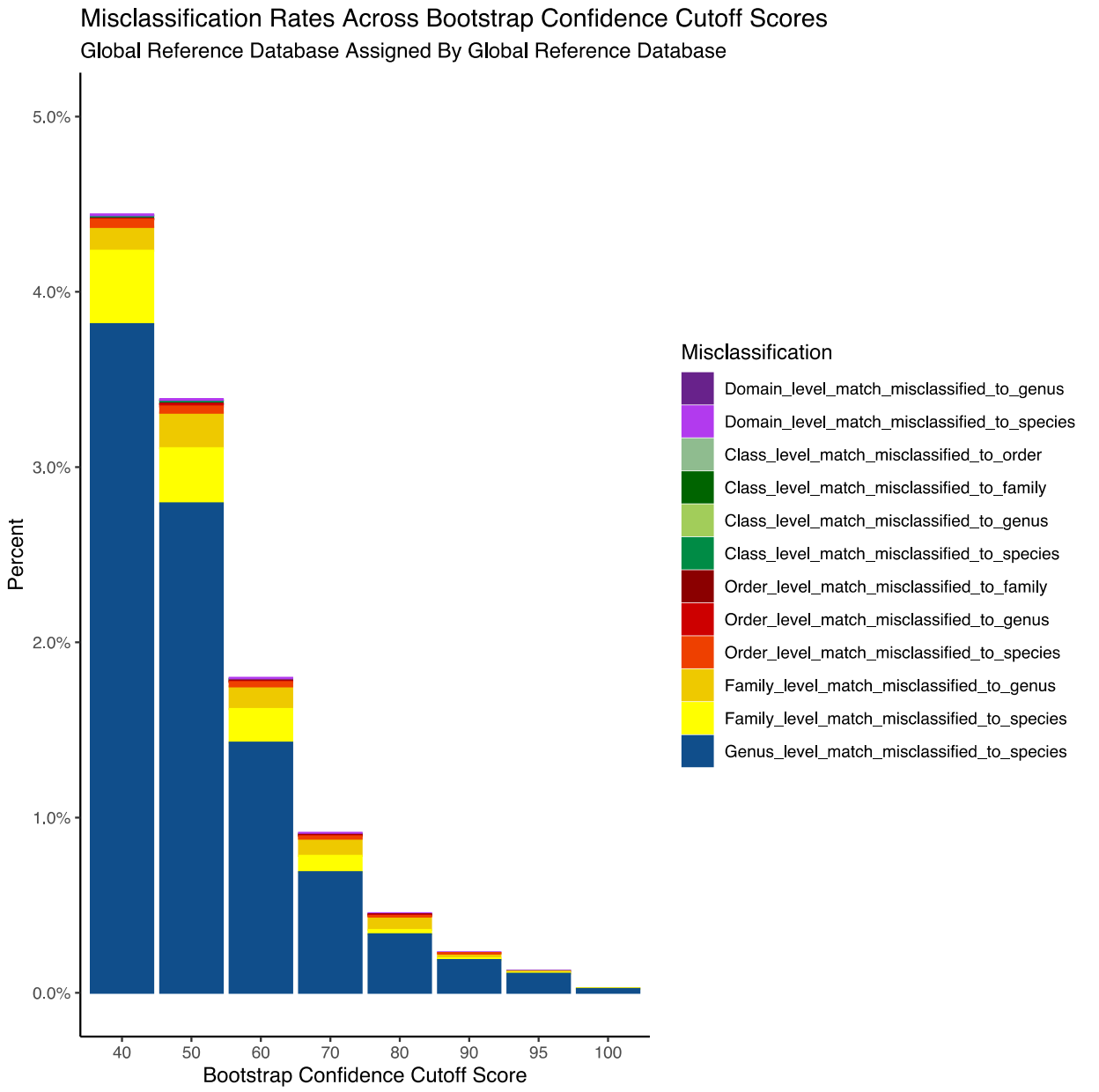


Figure 2. Taxonomic Classification Rates Across Bootstrap Confidence Cutoff Scores.

Results from taxonomy cross-validation by identity (TAXXI) using the global database as the reference to assign taxonomy to all sequences in that database. Correct species level matches increase with more relaxed bootstrap confidence cutoff scores. Correct taxonomic level matches are colored by the lowest common ancestor match. Dotted line indicates 100% and all mismatches were excluded.



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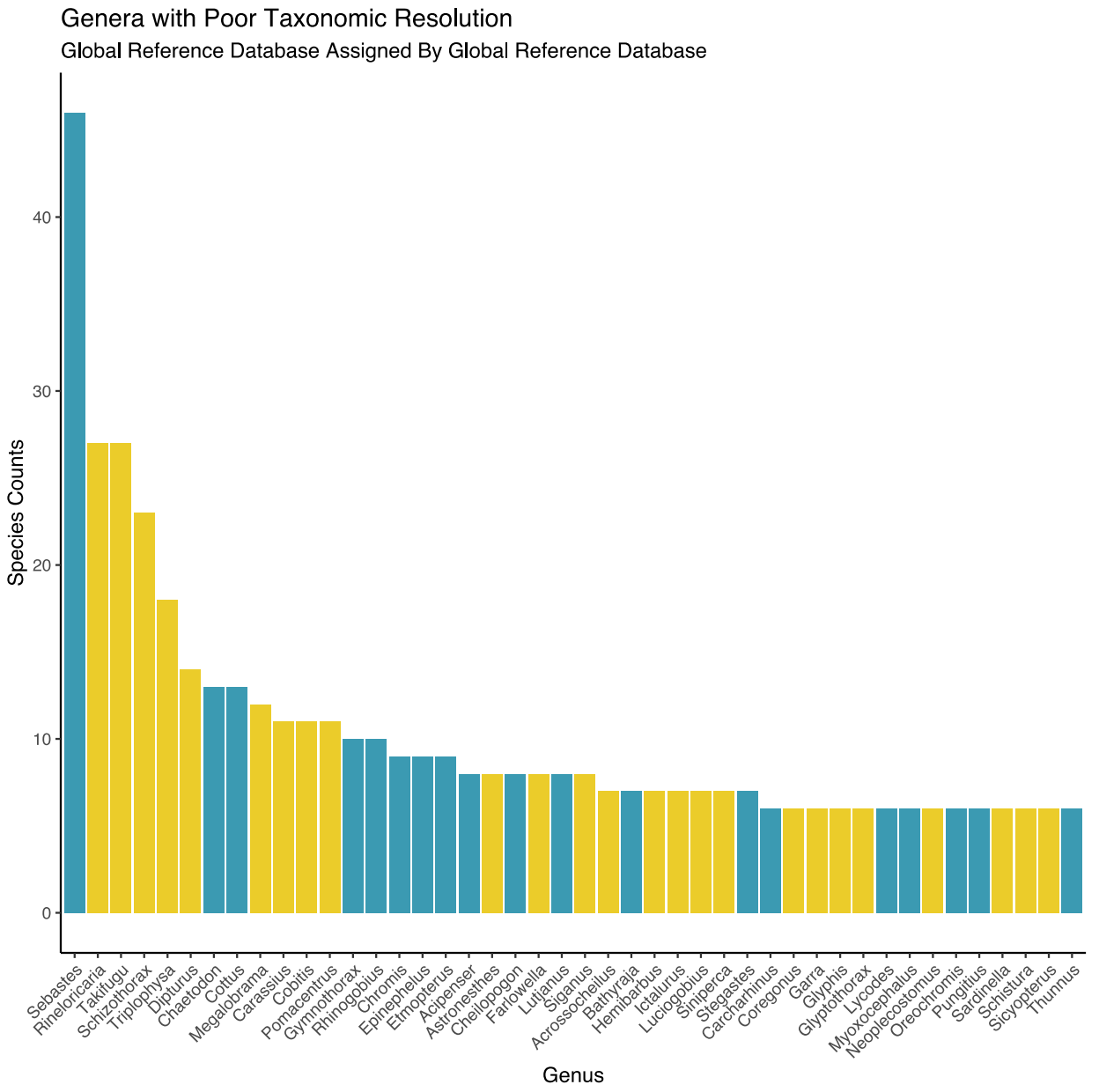

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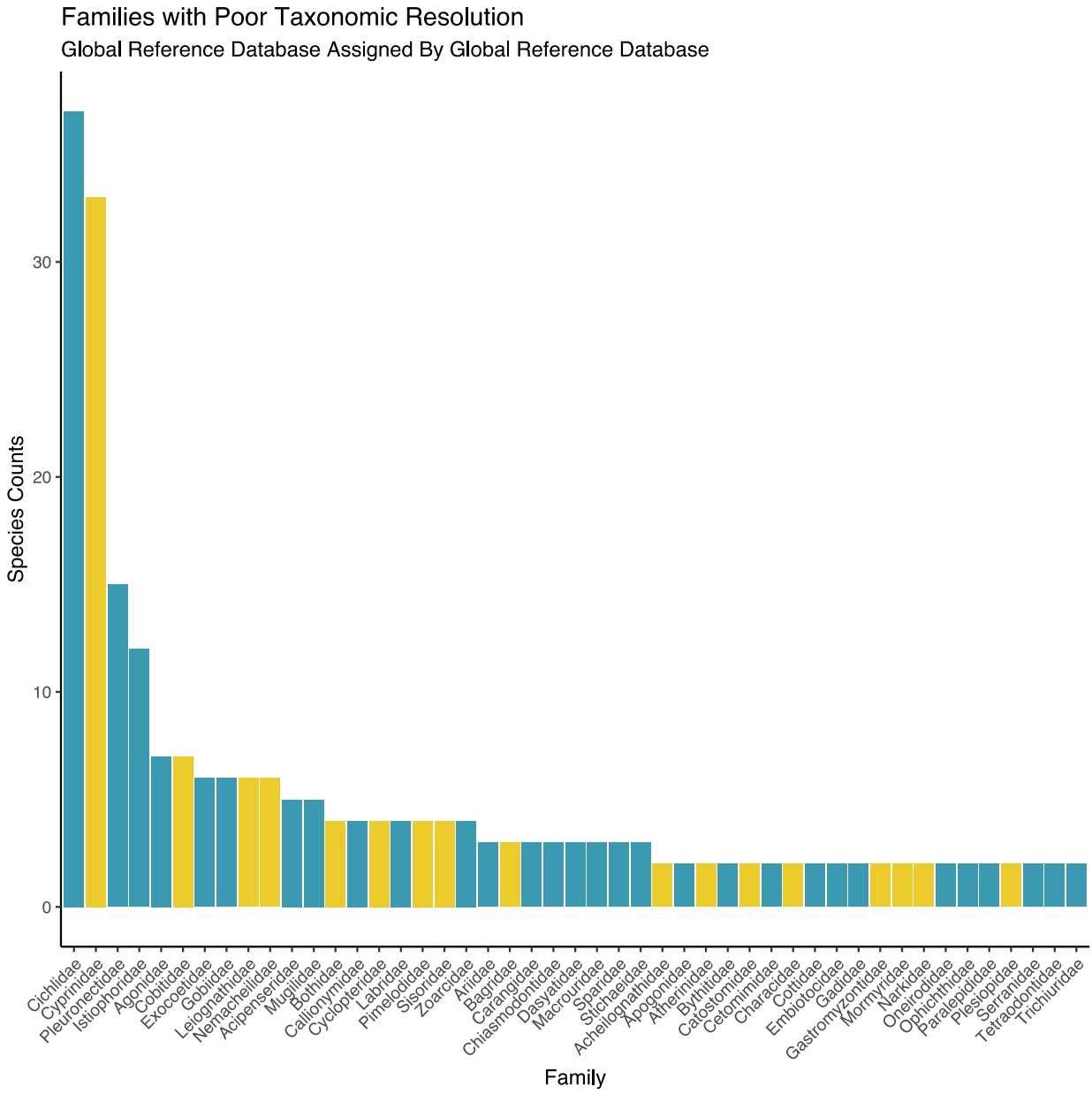

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Table 1. Summary of Cross Validation Results. Comparison of performance metrics for taxonomic assignments using the global database as a reference to annotate sequences in the global database (global-global)[(test database-training database)], the regional database (global-regional), and using the regional database as a reference to annotate sequences in itself (regional-regional). Reporting metrics calculated using a taxonomic cutoff score of 60.

Metric	Global-Global	Global-Regional	Regional-Regional
Under-classification Rate	8.6%	11.8%	7.8%
Misclassification Rate	1.7%	1.8%	1.3%
Over-classification Rate	0.0%	0.0%	0.0%
Accuracy	89.7%	86.5%	90.9%
True Positive Rate	89.7%	86.5%	90.9%
Sensitivity	91.3%	88.0%	92.1%
Specificity	98.3%	98.2%	98.7%

Table 2. Summary of Seawater eDNA Metabarcoding Taxonomic Assignments for Tested Reference Databases.

Metric		Reference Database		
		CRUX-GenBank	**Global**	**Regional**
Database	Reference Barcode Origin	GenBank	GenBank + Generated	GenBank + Generated
	Species Included	**All**	**All**	**California Fishes**
Reads	Total Reads	330,877		
	Assigned to NA	81,014	81,002	81,006
	Assigned to Class Level	54,090	-	-
	Assigned to Order Level	727	-	-
	Assigned to Family Level	1,286	1,409	131
	Assigned to Genus Level	952	1,068	1,063
	Assigned to Species Level	192,808	247,398	248,677
ASVs	Total ASVs	341		
	Assigned to NA	172	169	170
	Assigned to Class Level	12	-	-
	Assigned to Order Level	3	-	-
	Assigned to Family Level	5	13	11
	Assigned to Genus Level	4	6	4
	Assigned to Species Level	145	153	156
Taxonomy	Unique Families Identified	31	28	27
	Unique Genera Identified	39	38	39
	Unique Species Identified	38	38	37
	CA Native Species	25	36	37
	Avg. ASVs Per Species	3.8	4.1	4.2