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Development of a 16S Reference Library for eDNA Metabarcoding the Freshwater Fishes of Western Ecuador.

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Development of a 16S Reference Library for eDNA Metabarcoding the Freshwater Fishes of Western Ecuador

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ABSTRACT This project examines the use of the 16S locus to amplify neotropical freshwater fishes native to Western Ecuador in a newly created 16S reference library for DNA barcoding and eDNA metabarcoding applications. Among the orders Characiformes, Siluriformes, Cichliformes, Gobiiformes, Cryprinodontiformes, Gymnotiformes, and Perciformes, a compendium of 105 specimens were sequenced, with 43 representing new 16S sequences previously unavailable on Genbank.

INTRODUCTION

Life in the Anthropocene can be a balancing act between technological innovation and responsive species management during climatic consequences of those advancements. Significant losses in biodiversity, particularly endemic species isolated to one geographic location, is linked to halting vital ecosystem functions, and even ecosystem collapse (Dobson et al., 2006; Aguirre et al., 2021). Western Ecuador contains high levels of endemic species across taxonomic groups and includes two of the 24 global biodiversity hotspots (Myers et al., 2000). The high level of endemism present in

this area, coupled with its persistent habitat destruction, marks this region of concern for conservation priority. Driven primarily by the commercial use of aquatic resources, overexploitation of local river systems is dwindling freshwater fish numbers and posing a threat to the aquatic neotropical reservoirs that house 10% of all vertebrate species (Albert and Reis, 2011). If the rate of the current sixth mass-extinction continues unobstructed, one-third to two-thirds of life will likely be eradicated (Myers et al., 2000; Cowie et al., 2022). This cascade of extinction is damaging to all trophic

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levels. A loss in nearly half of endemic plant species could result in the loss of equal or more insect species, not including losses across other taxonomic groups (Myers et al., 2000). Freshwater biodiversity in Ecuador is of particular economic importance as the country is ranked globally at position 19 in total fisheries production among nations (World Bank Group Archives, 2023). Additionally, citizens often rely on locally sourced nutrition, such as organisms captured in nearby river systems (Aguirre et al., 2021). Loss of these species could cause irreparable trophic damage, and emphasizes a need to conserve these biodiverse habitats. A lack of data on the freshwater ecosystems of Western Ecuador highlights the urgent need to develop new scientific tools for identifying and tracking species in these diverse watersheds. Fortunately, advancements in molecular conservation methods are tipping the scales in favor of taxonomy, allowing for new, rapid methods of identifying species before they are lost to extinction.

DNA barcoding and metabarcoding have emerged as powerful techniques to fill in gaps in data on species distributions, habitat use, and taxonomic identifications (Hebert et al., 2003; Zaiko et al., 2022). In DNA barcoding, a DNA sequence is used as an identifying barcode for matching an obtained sequence with a reference sequence stored in a digital library. The main advantage to this is allowing for species identification through genetic means versus relying on traditional methods such as physical capture. DNA Metabarcoding, a newer technique in which many specimens are sequenced for the DNA barcoding gene while pooled in the same sample, particularly requires complete reference libraries of local systems to minimize misidentification across taxonomic groups (Zaiko et al., 2022). A popular application of metabarcoding is sequencing the barcoding gene from DNA extracted from environmental samples like filtered water (eDNA). Using DNA metabarcoding and techniques to analyze the genetic make-up of fish inhabiting these areas of high biodiversity in Western Ecuador, not only fills in data gaps, but it also provides insights into the evolution of the organisms in the region, and the ways in which

habitat destruction may influence species distribution throughout time. Unfortunately, applications of DNA barcoding are limited to available reference libraries for the species in question.

The mitochondrial cytochrome oxidase subunit 1 (COI) gene has long been the traditionally used locus for applications of DNA barcoding. This is in part because of the region's highly conserved nature among interspecific individuals (Hebert et al., 2003), but also because of an abundance of information present across taxonomic units. Large reference libraries for this gene have been created and posted on publicly available DNA databases to facilitate species identification. However, the COI locus historically has high rates of non-specific amplification of taxa, particularly favoring microbial communities (Collins et al., 2012). This can result in biases when using the locus as a sole DNA region for species identification. With the development of metabarcoding techniques, particularly in environmental DNA applications, researchers have begun to explore the use of other markers that can be amplified more reliably for a wider range of taxa. The mitochondrial 16S region of organisms allows for less nonspecific amplification among taxa, as well as higher variability between individuals of a population (Vences et al., 2005).

This study examines the potential use of the mitochondrial 16S gene in DNA metabarcoding applications for freshwater fishes of Western Ecuador. Through the creation of a 16S reference library, further studies can be conducted using metabarcoding techniques with environmental DNA (eDNA), an actively- evolving collection-to-analysis technique. The key to eDNA taking the lead in future studies of DNA analysis is the ability to generate genetic sequences from environmental factors, such as soil or water, instead of requiring collection from the organism itself. Environmental DNA assays foreshadow a paradigm shift for molecular ecology in areas where collections or sightings of organisms, particularly endemic species, are rare, or where barriers lead to data inaccessibility (Sales et al., 2021; Srivathsan et al., 2021). Further uses in eDNA backed by

metabarcoding would be particularly useful in impoverished areas facing the brunt of anthropogenic climate consequences where research is minimal and data gaps are vast among taxonomic species.

METHODS

Specimen Collection

Various lab archive specimens for this project were collected from 2008-2018 within Western Ecuador as part of field collections for prior neotropical-focused projects. The most recent samples were collected in 2022 across an eight-day period from various river-access points and local fishing markets in the Guayas River Basin (GRB) depicted in Figure 1. For the collections made in 2022, specimens of commercially important species were collected from fish markets in the region, while fresh specimens were collected using electrofishing methods on August 19, 2022, in the Chague Grande (2.17724 °S, 79.13347 °W) and Limon (2.12050

°S, 79.11552 °W) rivers, tributaries in the Guayas River basin. Specimens were kept cold on ice until arrival at the lab located at *la Escuela Superior Politécnica del Litoral* in Guayaquil, Ecuador. From there, fin clips were collected following aseptic techniques and stored in 98% ethanol. Specimens were also photographed fresh for verification of field identification. They were then fixed in 10% formaldehyde and deposited in the *Instituto Nacional de Biodiversidad's* fish collection in Quito, Ecuador. Whereas these specimens were solely collected in the GRB, the lab archive of specimens consisted of organisms collected throughout Western Ecuador.

DNA Extraction

Fin clips from specimens collected in the summer of 2022 field collection in Guayaquil, Ecuador were stored in 95% ethanol under cool, dark conditions. Upon return to the United States, the samples were stored in a -20°C freezer. Fin tissue was lysed and DNA extracted according to Qiagen DNeasy Blood and Tissue Kit Protocol, producing a working stock of eluted DNA between 100 to 200 µL per sample (QIAGEN, 2023). Archival specimens used in this project

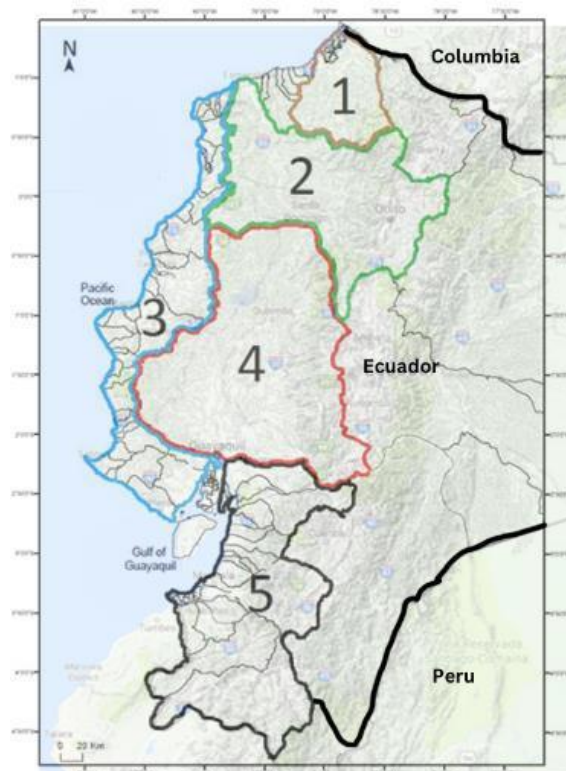


Figure 1. Map of the major drainage basins in Western Ecuador. From north to south, the drainage basins are as follows: (1) Santiago-Cayapas drainage, (2) Esmeraldas drainage, (3) the Northern Coastal System, (4) Guayas drainage, and the (5) Southern Coastal System (Adapted from Aguirre et al., 2017).

were the result of past fin clippings that were also stored in 95% ethanol.

Primer Selection

The primer pair 16SAR (5'-CGCCTGTTTAAACAAAACAT-3') and 16SBR(5'-CCGTTTGAAGTTCAGATCACGT-3') was used to amplify the mitochondrial 16S gene, targeting a region of 500-700 bp fragments (Palumbi, 1996). Compared to other primers created for 16S amplification for metabarcoding, the combination of 16SAR and 16SBR allows for a larger read length in sequence recovery. This is demonstrated in Figure 2, highlighting the importance of the 16SAR and 16SBR as end caps compared to other regions targeted by other primers.

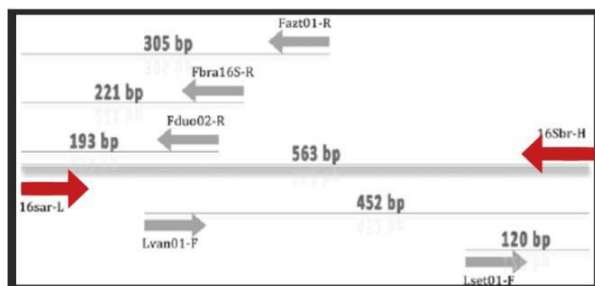


Figure 2. Each primer is labeled in the diagram as well as associated with an arrow indicating the direction of base pair reconstruction after binding. Primers used in this project are indicated with red arrows (Adapted from Alvarado et al., 2010).

Polymerase Chain Reaction

Each PCR reaction contained 19.5 μL of distilled water, 10 μL of BSA, 5 μL buffer, 1.25 μL dNTP, 0.5 μL magnesium, 0.5 μL of Taq polymerase, 1.75 μL of 16SAR primer, 1.75 μL of 16SBR primer, and 8-10 μL of specimen DNA. PCR conditions consisted of 35 total cycles [1 cycle at 95°C for 1 minute and 45 seconds, 52°C for 45 seconds, 72°C for 45 seconds; 4 cycles at 94°C for 45 seconds, 52°C for 45 seconds, and 72°C for 45 seconds; 30 cycles at 92°C for 30 seconds, 52°C for 45 seconds, 72°C for 45 seconds] with a final extension period of 7 minutes at 72°C. Subsequently, 10 μL of each PCR product was run on a 1.5% agarose gel (400 mA, 90 Voltz, 30 minutes) to verify amplification occurred and the targeted fragment length was achieved. The remaining 40 μL of each viable PCR reaction were sent to the Arizona Genetics Core (AzGC) located at the University of Arizona for sequencing.

Sanger Sequencing, Chromatogram Analysis

At the AzGC, samples were standardized in DNA concentration by addition of buffer volume, cleaned for sequencing, and run on a capillary Sanger sequencer. Resulting sequence chromatograms were manually edited in Geneious Prime v. 2023.0.1 and aligned and edited in the program Bioedit v. 7.2.6 at 640 bp, 643 bp, and 660 bp for the orders Characiformes, Siluriformes, and the Miscellaneous order category (containing Cichliformes, Gobiiformes, Cyprinodontiformes, Gymnotiformes, and Perciformes), respectively.

Bioinformatics

Maximum likelihood phylogenies were constructed in MEGA X (Kumar et al., 2018), with 500 bootstrap replicates to check for inconsistencies in species identification and examine the extent to which the 16S gene could distinguish different species. The phylogenetic trees were inferred using the Minimum Evolution method (Rzhetsky and Nei, 1992). Given the taxonomic diversity, phylogenetic analyses were conducted separately for the order Characiformes, the order Siluriformes, and a Miscellaneous orders group. The 16S gene lacks the power to properly resolve relationships among such distantly related species, and the evolutionary distances among species from such distinct groups would also make sequence alignment difficult given the lack of codon structure and presence of indels. The optimal trees had varying branch lengths, the sums being 0.695 for the order of Characiformes, 0.459 for Siluriformes, and 0.767 for the miscellaneous orders. The percentage of replication with a bootstrap value of 500 is shown next to the branches where each branch length is also the same unit as the evolutionary distances inferred on the phylogenetic trees (Felsenstein, 1985). Distances between branches are in units of base substitutions per site computed using the Tamura-Nei method (Tamura and Nei, 1993). The final dataset total positions ranged from 638 to 656 based on variation in the number of insertions and deletions.

RESULTS

A total of 115 individuals were sequenced at the 16S locus, with five of the sequences excluded from consensus analysis because of failed sequencing reactions. With the remaining 110 viable sequences, five were then excluded from bootstrap analysis because of a suspected sample contamination (e.g., sequenced as human or sequenced as a species from an unrelated taxonomic group). In total, 105 sequences were used for the analysis of the 16S locus indicating a 91.3% successful sequencing from the 16SAR and 16SBR primer pairing. Sequences successful to the amplification of the 16S gene are depicted in phylogenetic trees shown in Figures 3, 4, and 5 grouped in relation to taxonomic order, as well as

Table 1 portraying the relation of sequence identification comparison to the online DNA database GenBank.

A total of 21 species were sequenced and organized in Figure 3 depicting similarities for a 640 base pair region of the mitochondrial 16s locus in the order of Characiformes. Thirteen species were identified from the sequencing of the 16S locus for the order Siluriformes, and an additional 11 species were sequenced for the other orders. The total number of individuals per species identified by the study, as well as their comparison to files on GenBank, is listed in Table 1. A total of 61 specimens sequenced were from the order Characiformes, 25 specimens were from the order Siluriformes, and 19 specimens sequenced were in other orders.

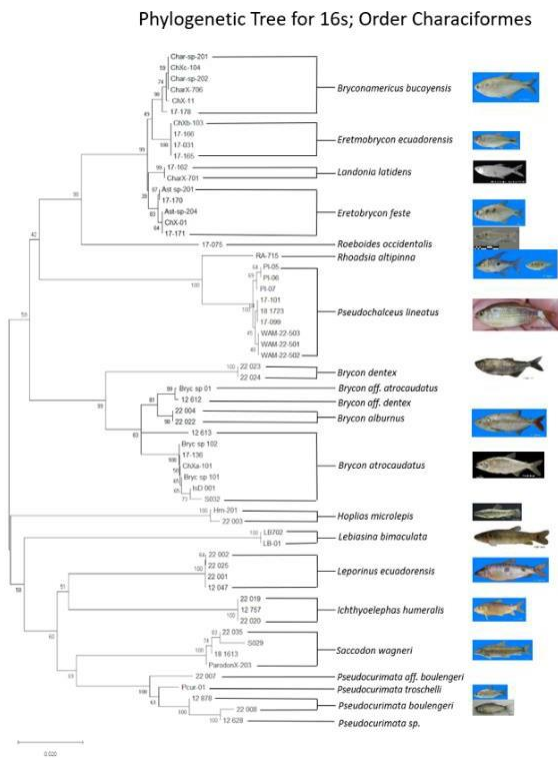


Figure 3. Phylogenetic tree of the 16S locus for species in the order Characiformes. The photos were taken from Aguirre et al. (2017).

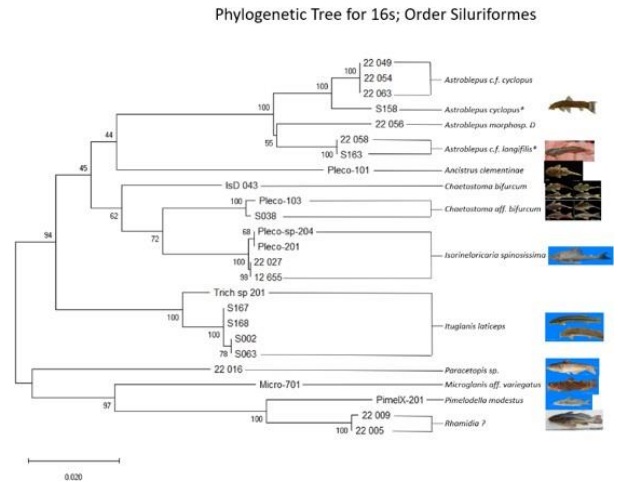


Figure 4. Phylogenetic tree of the 16S locus for species in the order Siluriformes. The photos were taken from Aguirre et al. (2017) except for species marked with an asterisk that were obtained through the online site FishBase (Froese and Pauly, 2022).

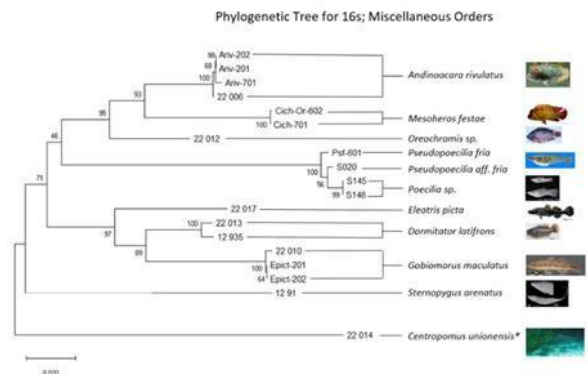


Figure 5. Phylogenetic tree of the 16S locus for species belonging to miscellaneous orders. The photos were taken from Aguirre et al. (2017) except for species marked with an asterisk that were obtained through the online site FishBase (Froese and Pauly, 2022).

As depicted in Table 1, specimens were organized taxonomically. Those whose sequence identification comparison matched 99% of a sequence previously published on Genbank were considered a match for species already on file. Matches between 98 and 99% sequence identity with Genbank subjects were considered possible new contributions. Anything below a 98% identity to the closest match on GenBank were classified as new contributions to GenBank, amounting to a total of 43 new contributions out of the 105 specimens sequenced from this study (40.9%).

DISCUSSION

Western Ecuador is a hotspot for biodiversity (Myers et al., 2000) and a crucial reservoir of vertebrate diversity. Since the region is threatened by many factors including the imminent effects of climate change, such as coastal flooding and saltwater encroachment into freshwater systems (Renwick, 2018; Rasmussen, 2021), responsive species management is crucial. Metabarcoding applications hold the potential to allow for rapid biodiversity assessments in areas where recurrent analysis is necessary and minimization of habitat destruction is vital to prevent ecosystem collapse. Application of metabarcoding is minimally intrusive through eDNA sampling; however, the use of primers capable of broad- range taxonomic analysis is necessary to accurately target a multitude of species during DNA amplification.

This study provides a new 16S genetic reference library with a 91.3% success rate of the primer combination used across orders of neotropical freshwater fish native to Western Ecuador. This success rate is all the more remarkable given the taxonomic breadth of the fishes studied. Species from seven taxonomically diverse orders were successfully sequenced. The primer efficacy demonstrated here further adds to the evidence that 16S is an optimal locus to target for barcoding and metabarcoding applications. The historically used COI primers in metabarcoding surveys can result in mis-amplification of species due to biases in loss of organismal sequencing not accounted for in the mass microbial data (Collins et al., 2012). When used in combination with other loci such as COI or 12S, 16S extends coverage overlap across taxonomic groups during surveys of biodiversity and abundance.

The genetic information acquired at 16S will serve as a component to a broader genetic reference library for freshwater fishes in Western Ecuador, with an emphasis on eDNA metabarcoding applications. Progress in sequencing technology, such as with the Oxford Nanopore MinION, allows for rapid genomic assessment of aquatic and terrestrial species (Srivathsan et. al, 2021). Alternatively to traditional sampling methods, sequestering environmental DNA sequences minimizes habitat upheaval and damage to organisms. When considered with the portable nature of the

Genus	Species	N	Match	Blast Ident Spp.	ID%	Covr%	Accession
Order Characiformes							
Family Anostomidae							
<i>Leporinus</i>	<i>ecuadorensis</i>	4	Yes	<i>Leporinus ecuadorensis</i>	100%	96%	MF285871.1
Family Bryconidae							
<i>Brycon</i>	<i>alburnus</i>	2	Maybe	<i>Brycon</i> sp.	98.61%	93.00%	OP422488.1
<i>Brycon</i>	<i>aff. atrocaudatus A</i>	1	Maybe	<i>Chilobrycon deuterodon</i>	98.65%	96%	KF780004.1
<i>Brycon</i>	<i>atrocaudatus</i>	7	No				
<i>Brycon</i>	<i>dentex</i>	2	Yes	<i>Brycon charginensis</i>	99.66%	95%	KF779972.1
<i>Brycon</i>	<i>aff. dentex A</i>	1	Maybe	<i>Brycon</i> sp.	98.10%	93%	OP422488.1
Family Characidae							
<i>Bryconamericus</i>	<i>aff. bucyensis</i>	6	Yes	<i>Bryconamericus dahl</i>	99.10%	90%	KF209727.1
<i>Eretmobrycon</i>	<i>aff. ecuadorensis</i>	4	Yes	<i>Bryconamericus dahl</i>	99.82%	90%	KF209727.1
<i>Eretmobrycon</i>	<i>festae</i>	5	Yes	<i>Eretmobrycon breviostris</i>	100%	90%	KF209716.1
<i>Landonia</i>	<i>latidens</i>	2	No				
<i>Pseudochalceus</i>	<i>lineatus</i>	9	No				
<i>Rhoadsia</i>	<i>altipinna</i>	1	No				
<i>Roeboides</i>	<i>occidentalis</i>	1	Yes	<i>Roeboides guatemalensis</i>	99.49%	96%	HQ171262.1
Family Curimatidae							
<i>Pseudocurimata</i>	<i>troscelli</i>	1	No				
<i>Pseudocurimata</i>	<i>boulengeri</i>	2	No				
<i>Pseudocurimata</i>	<i>aff. boulengeri B</i>	1	No				
<i>Pseudocurimata</i>	<i>sp.</i>	1	No				
Family Erythrinidae							
<i>Hoplias</i>	<i>microlepis</i>	2	Yes	<i>Hoplias microlepis</i>	98.14%	95%	HQ171264.1
Family Lebiasinidae							
<i>Lebiasina</i>	<i>bimaculata</i>	2	No				
Family Parodontidae							
<i>Sacodon</i>	<i>wagneri</i>	4	No				
Family Prochilodontidae							
<i>Ichthyolephas</i>	<i>humeralis</i>	3	No				
Order Gymnotiformes							
Family Sternopygidae							
<i>Sternopygus</i>	<i>arenatus</i>	1	Maybe	<i>Sternopygus dariensis</i>	98.39%	100%	MK530707.1
Order Cyprinodontiformes							
Family Poeciliidae							
<i>Poecilia</i>	<i>sp.</i>	2	No				
<i>Pseudopoecilia</i>	<i>fra</i>	1	No				
<i>Pseudopoecilia</i>	<i>aff. fra</i>	1	No				
Order Cichliformes							
Family Cichlidae							
<i>Andinoacara</i>	<i>rivulatus A</i>	3	Yes	<i>Andinoacara rivulatus</i>	100%	96%	EF432885.1
<i>Andinoacara</i>	<i>rivulatus B</i>	1	Yes	<i>Andinoacara rivulatus</i>	99.81%	86%	MH444784.1
<i>Mesoheros</i>	<i>festae</i>	2	Yes	<i>Mesoheros festae</i>	100%	92%	DQ119187.1
<i>Oreochromis</i>	<i>sp.</i>	1	Yes	<i>Oreochromis niloticus</i>	100%	90%	M11567058.1
Order Gobiiformes							
Family Eleotridae							
<i>Dormitator</i>	<i>latifrons</i>	2	Yes	<i>Dormitator latifrons</i>	99.64%	89%	KJ095211.1
<i>Eleotris</i>	<i>picta</i>	1	Yes	<i>Eleotris picta</i>	99.64%	100%	MF927492.1
<i>Gobionorus</i>	<i>maculatus</i>	3	Yes	<i>Gobionorus maculatus</i>	99.47%	92%	KF415378.1
Order Perciformes							
Family Centropomidae							
<i>Centropomus</i>	<i>sp.</i>	1	Yes	<i>Centropomus unionensis</i>	99.21%	99%	U85009.3
Order Siluriformes							
Family Astroblepidae							
<i>Astroblepus</i>	<i>c./ cyclopus</i>	3	Yes	<i>Astroblepus aff. trifasciatus</i>	98.99%	80%	MN541468.1
<i>Astroblepus</i>	<i>c./ longifilis</i>	2	Maybe	<i>Astroblepus sp. grisealvii</i>	98.59%	80%	MN541519.1
<i>Astroblepus</i>	<i>cyclopus</i>	1	Maybe	<i>Astroblepus</i> sp.	98.79%	81%	MN541441.1
<i>Astroblepus</i>	<i>morphosp. D</i>	1	No				
Family Cetopsidae							
<i>Paracetopsis</i>	<i>sp.</i>	1	No				
Family Heptapteridae							
<i>Rhamdia?</i>	<i>sp.</i>	2	No				
Family Loricariidae							
<i>Ancistrus</i>	<i>clementinae</i>	1	Yes	<i>Ancistrus clementinae</i>	99.10%	90%	KP959769.1
<i>Chaetostoma</i>	<i>bifurcum</i>	1	Yes	<i>Chaetostoma bifurcum</i>	100%	90%	OL303482.1
<i>Chaetostoma</i>	<i>aff. bifurcum</i>	2	Yes	<i>Cordylancistrus santarosensis</i>	100%	90%	KP95982.1
<i>Isoirineloricaria</i>	<i>spinosissima</i>	4	Yes	<i>Isoirineloricaria spinosissima</i>	99.82%	90%	KP959806.1
Family Pimelodidae							
<i>Microglanis</i>	<i>variegatus</i>	1	No				
<i>Pimelodella</i>	<i>modestus</i>	1	No				
Family Trichomycteridae							
<i>Iuglanis</i>	<i>aff. laticeps A</i>	1	Maybe	<i>Trichomycterus fasslii</i>	98.61%	93%	MW415500.1
<i>Iuglanis</i>	<i>aff. laticeps B</i>	2	Maybe	<i>Trichomycterus corduvensis</i>	98.10%	86%	FJ744633.1
<i>Iuglanis</i>	<i>aff. laticeps C</i>	2	Maybe	<i>Trichomycterus chiltoni</i>	98.25%	93%	MW415499.1

Table 1. Complete list of organisms sequenced and GenBank sequence accession codes for similar species already in existence on NCBI. This table represents the following taxonomic orders: Characiformes, Gymnotiformes, Cyprinodontiformes, Gobiiformes, Perciformes, and Siluriformes.

Oxford MinION and similar technologies, time of sample collection and rapid processing rates across large amounts of genetic information, the creation of a database for taxonomically broad primers may aid in efforts to fill in the gaps in knowledge of the ecology, distribution, and conservation status of freshwater fishes. In a country such as Ecuador where sequences are mostly sent off to other countries for analysis,

this library equips local researchers with the cloud information needed to take taxonomic assessment of native systems into their own hands. This not only reduces the need for international shipping charges but reduces the cost of sequencing per sample (Chang et al., 2020). Empowerment of local researchers would result in more frequent analysis of these genetically diverse areas, providing better data on ecosystem health and resilience.

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