Molecular Investigations of the Diversity of Freshwater Fishes across Three Continents

by

Malorie M. Hayes

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Approved by

Jonathan W. Armbruster, Chair, Professor and Director Auburn University Museum of Natural History Department of Biological Sciences
Jason E. Bond, Professor and Schlinger Chair in Insect Systematics University of California, Davis
Scott R. Santos, Professor and Chair of the Department of Biological Sciences at Auburn University
John P. Friel, Director of the Alabama Museum of Natural History

Abstract

Fishes are the most speciose vertebrates, and incredible diversity can be found within different groups of fish. Due to their physiological limitations, fish are confined to waters, and in freshwater fish, this is restricted to lakes, rivers, and streams. With a constrained habitat like a freshwater system, it can be expected that freshwater fish will show varying levels of diversity depending on a suite of characteristics. Within this dissertation, I examine the diversity of three fish groups: the speciose *Enteromius* of West Africa, the population genetic diversity of *Pteronotropis euryzonus* in Alabama and Georgia, and the unexpectedly species rich *Trichomycterus* from the Guyana highlands. I use molecular methods and geometric morphometrics to determine the systematics of the species and uncover the hidden diversity within their respective groups. When it comes to diversity, the small barbs of Africa are vastly understudied and require a taxonomic revision. From that revision, a better understanding of the diversity leads to a new genus description. For the small-bodied minnow *Pteronotropis*, moving across a river was believed to be unlikely. Highland catfishes like *Trichomycterus* were believed to be limited not only in their dispersal ability, but also in their diversity. The diversity of freshwater fishes is reflected within the chapters of this dissertation.

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Chapter 1 – Introduction

Of the >40,000 described species of fish on earth, nearly 12,000 species are freshwater fish (Fricke, Eschmeyer, & van der Laan, 2018). Fish are the most diverse vertebrate group, and species are still being discovered to this day. Species numbers are growing as exploration of understudied regions develops and genetic analysis becomes standard. The diversity of freshwater fishes can be categorized in a variety of ways. Within this dissertation, I present four chapters exploring three different levels of freshwater fish biodiversity. First, I examine the fundamentals of taxonomy and systematic understanding of a complicated genus of fish, the former '*Barbus*' of Africa. From that study, the next chapter was developed in which I name and describe a new genus of west African barbs. The following paper changes from examining higher-order systematics of African taxa to focusing on population-level differences within a North American minnow species. The final chapter changes continents again to South America. I use genetic methods to place newly discovered species of South American *Trichomycterus* within a systematic context of the genus.

For some fish, such as the small African barbs, the extensive diversity has been overwhelming and convergent morphology has proven to be an impediment for future studies of the group. The genus *Enteromius* Cope 1867 was recently elevated to rename the small African '*Barbus*'; however, this designation has been controversial. Opponents to this change argue the designation was based on poorly supported phylogenies and state these changes would disrupt currently well-supported monophyletic groups. My work on this group, presented in this dissertation, establishes a definition of the group and clarifies

which species are members of *Enteromius.* This is done with respect to the closely related and currently recognized genera. While I do not sink any existing groups or raise new genera, this chapter sets up future studies to systematically approach the taxonomic problems associated with *Enteromius* and other small African barbs.

For other groups, such as North American minnows, taxonomy is relatively stable, and recent studies often focus on population-level diversity. This is true for species like *Pteronotropis euryzonus,* the Broadstripe Shiner. Unlike the *Enteromius* of Africa, *Pteronotropis* has a well-defined membership and a small range (limited to the southeastern United States). While their taxonomy is stable, little is known regarding the biology of the Broadstripe Shiner, particularly its dispersal capabilities. It is important to understand how well these little fish move because of their threatened status.

Pteronotropis euryzonus is a state-listed species in both Alabama and Georgia where it occurs. The species has a limited distribution in the Chattahoochee River system and populations are decreasing. Furthermore, populations are now physically separated due to the installation of a dam in the 1960s. Previous studies have speculated whether the dam has separated a connected population with clinal variation or if the dam is exacerbating an already established break between two diverging populations. I use population genetic methods to assess the historical and present genetic connectivity between populations of *P. euryzonus*, testing the hypothesis of clinal variation across its range. The results of this study can inform non-game fisheries managers in Alabama and Georgia regarding management plans for the shiner.

Anthropogenic threats are common among freshwater fishes. Like the dam potentially influencing the genetic connectivity of the Broadstripe Shiner, dams,

deforestation, and mining operations are a threat to the incredible freshwater diversity found in South America. In many regions, a lack of knowledge of biodiversity in streams mean species may be lost without knowing they existed. The Pakaraima Mountains of Guyana and Venezuela is an area under extreme threat by gold and diamond mining. These operations pour toxic waste into nearby rivers and streams, completely devastating the rivers.

In order to know what is at risk from these threats, the diversity of fishes in the rivers needs to be known. The Pakaraima mountains have been relatively poorly explored. In 2016, an expedition surveyed multiple streams in the highlands which drained to several different river systems. Until now, only two species of *Trichomycterus* – pencil catfish – were known from the region: *T. guianensis* and *T. conradi*. During the course of sampling, multiple morphologically different forms were collected. Using the morphology as a starting point, the putative species were examined using multiple genetic markers. I found that the morphological diversity was congruent with genetic diversity, and there are at least six more *Trichomycterus* species in the highlands rather than the two previously described species.

Throughout this dissertation, the importance of understanding biodiversity through different lenses is presented. This may be to create a solid base for other researchers, describe newly discovered diversity, inform local governments regarding threatened species, or update other biologists about a region likely rich with yet unknown species. Using molecular methods as well as geometric morphometrics, I assess the diversity of these groups, leading to a better overall understanding of freshwater fishes across three continents.

Chapter 2 – The Taxonomy and Relationships of the African Small Barbs (Cypriniformes: Cyprinidae)

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Abstract

Through a series of phylogenetic and taxonomic works, the unwieldy and non-monophyletic genus *Barbus* was reduced to just the typical *Barbus* of Europe and east Asia and most of the small, African, diploid barbs ('*Barbus*'). The genus *Enteromius* Cope 1867 was recently elevated to rename most of the small African '*Barbus*'; however, this designation has been controversial. Opponents of this change argue the designation was based on poorly supported phylogenies and state these changes would disrupt currently well-supported monophyletic groups; however, the genus is currently in use, and it is necessary to determine the species that should be recognized as *Enteromius*. Herein, we present a list of species placed in *Enteromius*, and provide a description of the genus. We list the species of the other small African barbs (*Barboides, Barbopsis, Caecobarbus, Clypeobarbus, Pseudobarbus*, and '*Pseudobarbus*'). We also contribute 36 cytochrome b sequences of *Enteromius* to further build an understanding of the phylogenetic relationships among members of this group, and to aid in further taxonomic decisions.

Introduction

Myers (1960: 213) stated that the genus *Barbus* was a "monstrous aggregation", and indeed *Barbus* was once a massive genus of cyprinids, with over 800 accepted species spread throughout Europe, Asia, and Africa. As of February 2017, a search for

Barbus in the catalog of fishes (Eschmeyer, Fricke, & van der Laan, 2017) returns 1047 names; a daunting problem for any taxonomist. Through a series of phylogenetic and taxonomic works, *Barbus* has since been reduced to include only the typical tetraploid *Barbus* of Europe and east Asia (*Barbus sensu stricto*) and most of the small, African, diploid barbs that are usually referred to as '*Barbus*' in single quotes to designate the taxonomic uncertainty (Berrebi, Kottelat, Skelton, & Ráb, 1996). These '*Barbus*' remain a large group with nearly 240 species considered valid (this study); thus, they likely represent the largest group of vertebrates without a valid taxonomic placement. This generic uncertainty is the antithesis to the goals of systematics and taxonomy, acting as a long-term barrier to effective scientific communication and understanding.

Yang et al., (2015) provided a phylogeny for the Cyprininae, and included several examples of '*Barbus*'. These results confirmed that the African diploid barbs were not closely related to *Barbus s.s.*, a result recovered by other researchers with smaller taxon sampling (Golubtsov & Krysanov, 2002; Rab, Machordom, Perdices, & Guegan, 1995; Rainboth, 1981; Tsigenopoulos, Ráb, Naran, & Berrebi, 2002). Yang et al., (2015) further resurrected the genus *Enteromius* Cope 1867 with type species *E. potomogalis* for all African diploid barbs that were not already placed into *Barboides* Brüning 1929, *Barbopsis* Di Caporiacco 1926, *Caecobarbus* Boulenger 1921, and *Clypeobarbus* Fowler 1936. South African tetraploid species not currently recognized as *Pseudobarbus* Smith 1841 were referred to as '*Pseudobarbus'*.

Schmidt and Bart (2015) reject the elevation of *Enteromius* and do not support the treatment of south African tetraploids sister to *Pseudobarbus* as '*Pseudobarbus*'. Schmidt and Bart argue that the phylogeny of the barbs is not well-supported, is based solely on

mitochondrial markers, and *Enteromius* is not monophyletic due to an unresolved polytomy; therefore, recognition of *Enteromius* would disrupt currently well-supported monophyletic groups such as *Barboides, Clypeobarbus,* and *Pseudobarbus*. Schmidt and Bart suggest that more taxa and more genes are required to establish monophyletic entities within the small African diploid barbs, and that species without the defining characteristics of the currently accepted, monophyletic, and well supported related taxa should remain as '*Barbus*' until more complete studies are accomplished.

More complete studies have been lacking since the use of the uncertain taxon '*Barbus*' was initiated (Berrebi et al., 1996). The paucity of phylogenetic studies on this group can be attributed to the unwieldy size of the taxon, uncertainty of sister-group relationships, and uncertainty of the species that belong to the group. The enormous amount of data collection needed to satisfactorily establish the genus as monophyletic is further hindered by limited access to tissue specimens.

While we agree with many of the arguments made by Schmidt and Bart (2015), we also recognize the importance of identifying the small African barbs as a taxonomically distinct unit as proposed by Yang et al., (2015). Unfortunately, this designation and the subsequent rebuttal has created taxonomic confusion regarding the barbs. Five publications have been released using the new classification (Armbruster, Stout, & Hayes, 2016; Decru, 2015; Decru et al., 2016; P. H. Skelton, 2016; Vreven, Musschoot, Snoeks, & Schliewen, 2016), whereas three publications use *'Barbus'* (Ren & Mayden, 2016; M. L.J. Stiassny & Sakharova, 2016; Melanie L.J. Stiassny, Liyandja, & Iyaba, 2016). Ren and Mayden (2016) provide no justification for the use of '*Barbus*' over *Enteromius,* and while some nuclear markers were included in their analysis, most

species were represented by a single gene, cytochrome *b*. Stiassny et al. (2016) and Stiassny and Sakharova (2016) echo Schmidt and Bart's reasoning, and use '*Barbus*' as the more conservative choice; however, Stiassny and Sakharova (2016) further state that the use of '*Barbus*' is likely to change based on the results of this manuscript. Despite the imperfection of *Enteromius*, it is a useful designation that is more taxonomically informative than '*Barbus*', as was also recently articulated by Skelton (2016). The use of *Enteromius* reflects the current available data regarding the systematic relationships, and places the taxon within the smiliogastrin lineage (Yang et al., 2015; Skelton, 2016). Further, by attributing the species to *Enteromius* vs. '*Barbus*' one can more easily search taxonomic databases such as "The Catalog of Fishes" (Eschmeyer et al., 2017) for species of interest.

Taxonomic lists of species of African barbs have been riddled with errors and misplaced species. With Vreven et al. (2016) clarifying the identity of the large African barbs (*Labeobarbus*), the generic identities of the small African barbs remain incomplete. Herein, we elaborate and clarify the membership of *Enteromius* and other small African barb genera, providing a list of member taxa, as well as a description of *Enteromius*. Additionally, we reanalyze the data used in Yang et al., (2015) and incorporate 36 additional cytochrome b sequences to further build an understanding of the phylogenetic relationships among members of this group.

Materials and Methods

Determining members of Enteromius

To determine the species of *Enteromius* (Table 2-1), The Catalog of Fishes (Eschmeyer et al., 2017) was searched for "*Barbus*". The resulting text file was moved into Microsoft Word, and had tabs entered using find and replace commands to minimally circumscribe all species and the category "Current Status". This file was entered into Microsoft Excel and sorted by the "Current Status" column to find all species currently placed in *Barbus*. All species from Europe and Asia were then eliminated. The list was then checked against a recent publication on the large African barbs of *Labeobarbus* (Vreven et al., 2016). Other genera were searched in Eschmeyer et al., as well, and species ascribed to '*Pseudobarbus*' were based on current phylogenetic information and the sawfin barb designation (P. Skelton, 2001). Synonymies listed in Eschmeyer et al., (2017) were accepted. Species are listed in Table 2-1. Original searches were conducted in July 2015, and species described since then were added (Stiassny & Sakharova, 2016; Stiassny et al., 2016). Photographs of syntypes of *Enteromius potamogalis* Cope were obtained from ANSP.

Taxon Sampling, DNA Extraction, Amplification and Sequencing

Samples of *Enteromius* and other freshwater fish species were collected from Democratic Republic of Congo, Cameroon, and Guinea. Specimens were captured using 10-foot seines and a Smith-Root electrofisher. Specimens were anesthetized with MS-222, then fin clips and muscle tissue were collected in 95% ethanol for DNA preservation. Specimens were then fixed in 10% formalin and deposited at the following museums: Auburn University Natural History Museum, (AUM) in Auburn, Alabama, Tulane Museum (TU) in New Orleans, Louisiana, and Cornell University (CU) in Ithaca,

New York. Additional tissues of *Enteromius* species preserved in 95% ethanol were obtained from other institutions from separate expeditions to various countries in Africa. GenBank Accession numbers of sequences generated in this and other studies are listed in Table 2-2.

Whole genomic DNA was extracted from fin clippings using an E.Z.N.A. Tissue DNA Kit (Omega BioTek Norcross, GA, USA). This study used the mitochondrial gene cytochrome b (cytb) to generate data for *Enteromius*. The gene was isolated by polymerase chain reaction (PCR) using the previously developed primers H16461 and L15267 (Briolay, Galtier, Brito, & Bouvet, 1998). The temperature profile is as follows: initial denaturation of 94°C for 2 min, followed by 30 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min, with a final extension of 72° C for 3 mins. PCR products were size-verified on a 0.8% agarose gel through electrophoresis. PCR purification and sample preparation for Sanger sequencing were performed at Beckman Coulter (Danvers, MA). Chromatographs from forward and reverse reads were assembled together, and assembled contiguous sequences were aligned and edited by eye using the program Geneious v 6.0.6 (Kearse et al., 2012). Due to variations in the length of cytb products deposited on GenBank, the sequences were trimmed to 1118bp length. Sequences shorter than this length had missed base pairs coded as N. Tissues of the Congo Cave Barb (*Caecobarbus*) and the Somali Cave Barb (*Barbopsis*) are not currently available; therefore, these monotypic genera are excluded from the analysis.

Data Analysis

Phylogenetic analysis was performed on the cytb dataset, using *Puntius sophore* as an outgroup. Best-fit models of evolution were tested using PartitionFinder (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017). Bayesian Inference (BI) was run in MrBayes 3.2.2 on CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010; Ronquist et al., 2012). The BI had two runs with four chains each for 10,000,000 generations and was sampled every 1,000 generations. A 25% burnin was calculated using the sump option and a 50% consensus tree was created.

Results

Enteromius Cope 1867

Figure 2.

Enteromius Cope 1867: 405. Type species: *Enteromius potomogalis* Cope 1867.

Hemigrammopuntius Pellegrin 1923: 128. Type species: *Barbus salessei* Pellegrin 1908.

Nicholsopuntius Pellegrin 1933: 107. Type species: *Barbus candens* Nichols and Griscom 1917.

Agrammobarbus Pellegrin 1935: 382. Type species: *Barbus apleurogramma* Boulenger 1911.

Beirabarbus Herre 1936: 99. Type species: *Beirabarbus palustris* Herre 1936.

Vanderbiltella Fowler 1936: 273. Type species: *Barbus lepidura* Fowler 1936.

Mannichthys Schultz 1942: 320. Type species: *Mannichthys lucileae* Schultz 1942.

Hemigrammocapoeta Estève 1952: 177 (as a subgenus of *Barbus*). Type species: *Barbus (Hemigrammocapoeta) mirei* Estève 1952. Objectively invalid, preoccupied by *Hemigrammocapoeta* Pellegrin 1927; replaced by *Estevea* Whitley 1952.

Estevea Whitley 1953: 133. Type species: *Barbus (Hemigrammocapoeta) mirei* Estève 1952. Replacement name for *Hemigrammocapoeta* Estève 1952.

Afropuntio Karaman 1971: 190. Type species: *Barbus pobeguini* Pellegrin 1911.

Parapuntius Karaman 1971: 190. Type species: *Barbus anema* Boulenger 1903.

Species Included: Table 2-1.

Diagnosis: A genus of small-bodied diploid minnows endemic to the African continent possessing scales with radial striae and seven to eight dorsal-fin rays. *Enteromius* can be separated from other small African diploid barbs as follows: from *Barboides* by having the dorsal-fin placement beginning at or slightly posterior of anal-fin origin (vs dorsal fin beginning before the anal-fin origin), with seven or more branched dorsal fin rays (vs six branched rays), and a pair of nostrils on either side of the snout (vs the single, figure *8*-shaped nostril on either side of snout); from *Barbopsis* by having the eye large and free from the orbital rim (vs. very reduced eyes without a free orbital rim), from *Caecobarbus* by having eyes and pigment (vs. eyes and pigment absent), and from *Clypeobarbus* by lacking a midlateral row of greatly enlarged, shield-like scales edged with dark pigment and deepest and widest below dorsal fin. In addition, *Enteromius* can be distinguished from all other African Smiliogastrini, by being diploid (vs. tetraploid or hexaploid), and

can further be distinguished from *Labeobarbus, Luciobarbus, Sanagia,* and *Varicorhinus* by having radial striae on the scales (vs. parallel striae) and by usually having 7–8 dorsal-fin rays (vs. 10–11), and from *Pseudobarbus* by possessing from five to nine well developed supraneural bones (vs. supraneural bones absent or vestigial), and from '*Pseudobarbus*' by typically lacking serrations on the primary dorsal-fin ray (vs. dorsal spines always being serrated).

Phylogenetic Results

An additional 36 samples of *Enteromius* cytochrome b were included in this analysis along with 87 sequences available on GenBank including sequences of *Barboides, Enteromius, Pseudobarbus, 'Pseudobarbus',* and *Systomus* (Durand, Tsigenopoulos, Ünlü, & Berrebi, 2002; Machordom & Doadrio, 2001; Rüber, Kottelat, Tan, Ng, & Britz, 2007; Swartz, n.d.; Tsigenopoulos et al., 2002). The Bayesian Inference (BI) recovers a deep polytomy between clades of *Barboides, Enteromius, Pseudobarbus,* '*Pseudobarbus*', and *Systomus* (Figure 1). The deeper relationships between these taxa are unresolved, meaning sister-group relationships are not understood based on this analysis; however, examination of the resulting well-supported clades (posterior probability >95%) furthers the case of the practical utility of a taxon such as *Enteromius.*

The *Enteromius* I clade is composed of exclusively *Enteromius* species (Figure 1), and is recovered with 100% posterior probability. The membership of this clade is equivalent to one of the *Enteromius* clades designated by Yang et al., (2015). A second well-supported clade recovered in this analysis, *Enteromius* II, corresponds to the second

Enteromius clade found in Yang et al., (2015). Similar to their results, we recover two species of *Clypeobarbus* Fowler 1936, a genus recently elevated from '*Barbus*' (Conway & Stiassny, 2008) nested in this clade. *Clypeobarbus* is sister to a clade consisting of specimens identified as *E. fasciolatus, E. punctitaeniatus,* and *E. trispilos*. These taxa should be examined for characteristics that may unite or differentiate them from *Clypeobarbus*. Unfortunately, the locality information for the specimen of *E. fasciolatus* is missing as it is an aquarium specimen (although the species is rather easy to identify), and no voucher specimen is listed for the specimen of *E. trispilos,* an uncertain species identification, given that other *E. trispilos* taxa (with vouchers) are in the other *Enteromius* clade. The genus *Clypeobarbus* is distinguishable from other *Enteromius* based on their enlarged midlateral row of scales edged with dark pigment and large occipital fontanel (Conway & Stiassny, 2008). The other *Enteromius* in this clade do not possess the characters to consider them *Clypeobarbus*. Rather than reverting all members of this clade to *Enteromius,* we retain the *Clypeobarbus* designation for those species already described and recognize the uncertain taxa as *Enteromius* until more information becomes available.

A small clade composed exclusively of *Enteromius jae* is recovered as distinct from the other *Enteromius* clades. Putative members of this clade include *E. condei, E. nounensis, and E. parajae* (Van den Bergh & Teugels, 1998). These taxa are genetically and morphologically distinct from other genera in this analysis, and a generic-level diagnosis for this clade is underway by the authors.

Sister to *Barboides* is *Enteromius hulstaerti*. This specimen is separated from *Barboides* by long branch-lengths, but its placement as sister to *Barboides* has high

posterior probability. Fortunately, this specimen is vouchered at the Natural History Museum and Institute, Chiba (CBM), and may be examined for accurate identification and synapomorphic traits. The original description of *E. hulstaerti* from the Momboyo River in DR Congo describes three spots down the body, the presence of seven branched dorsal-fin rays, and a maximum total length of 30mm (Poll, 1945). The holotype drawing of the specimen shows the dorsal-fin origin beginning more anterior than the anal-fin origin. The first and smallest spot corresponds to location of the humeral organ of *Barboides* and the placement of the dorsal-fin origin relative to the anal fin agrees with the description of *Barboides* (Conway & Moritz, 2006). Despite these shared characters, the species has 7 (vs 6) branched dorsal rays, and the maximum length exceeds that of *Barboides*. *Enteromius hulstaerti* is putatively sister to *E. candens* and *E. papilio*, all sharing a small adult size and reduced or lost lateral line tubules (Banister & Bailey, 1979). We recognize these taxa in *Enteromius*, but they likely represent a separate genus. *Nicholsopuntius* was described for *B. candens* by Pellegrin (1933), and it is likely that this genus will be elevated.

The final well-supported clade in this analysis is a tetraploid clade. It includes the described *Pseudobarbus* as well as the other tetraploid barbs, which have previously retained the generic name '*Barbus'*. Yang et al., (2015), suggested moving all southern African tetraploids not assigned as *Pseudobarbus* to '*Pseudobarbus*'. Our results are similar to those of Yang et al., (2015) with the exception of high posterior probabilities and the placement of '*Pseudobarbus*' *trevelyani* as basal to the sawfin barb clade (Skelton, 2001). These taxa have been recognized to have serrated dorsal fins, which distinguishes them from *Pseudobarbus*. Therefore, we recognize the redfin minnows are

Pseudobarbus Smith 1841 and the sawfin barbs are temporarily placed in '*Pseudobarbus*' as proposed by Yang et al., (2015) and accepted by Skelton (2016). Serrate dorsal fins are also present in some species of *Enteromius*; however, '*Pseudobarbus*' differs from them by being tetraploid (vs. diploid).

Discussion

On the identity of Enteromius potamogalis

Enteromius potamogalis was described as a new genus and species for a series of fishes collected "in streams and rivulets fifty to sixty miles north of the equator, and the same distance from the ocean" (Cope, 1869:406). This likely places the species in the Rio Muni on the border of Gabon and Equatorial Guinea (Roberts, 2010). Problematically, (de Weirdt, Getahun, Tshibwabwa, & Teugels, 2007) do not discuss *E. potamogalis* nor indicate any similar species in the Muni in their treatment of the barbs of the Lower Guinea ichthyoprovince. The species of *Enteromius* listed from the Muni and neighboring basins are *E. camptacanthus*, *E. holotaenia*, *E. rubrostigma,* and *E. trispilomimus,* which are each distinct from *E. potamogalis*. *Enteromius potamogalis* has a lateral stripe, differentiating it from *E. trispilomimus* and *E. camptacanthus*, and has a flexible second dorsal-fin ray, which distinguishes it from *E. holotaenia*. The species is similar to *E. rubrostigma*; however, the syntype specimens key to *E. chlorotaenia* based on a higher lateral scale count (de Weirdt et al., 2007). *Enteromius chlorotaeina* is from the Volta River in northern Cameroon, and lacks a black spot in the dorsal fin (which is present in some of the types of *E. potamogalis*, though most of the color is faded in the specimens).

Boulenger (1911) placed *Enteromius potamogalis* into the synonymy of *E. ablabes*, but this is incorrect as *E. ablabes* has 3.5 scales between the lateral line and dorsal-fin origin (vs. 4.5) and pit lines on the head (vs. no pit lines; de Weirdt et al., 2007). Roberts (2010) examined the syntypes of *E. potamogalis* (ANSP 7607), and stated that half were distinct from *E. ablabes*, but he did not elaborate on differences among the specimens. We do not see any compelling differences amongst the photos of the syntype series except for one individual that may have lower lateral line scale count and other differences. The specimens are in poor shape, and will need to be examined in greater detail before a lectotype can be designated; however, it is clear that none of the specimens are *E. ablabes*.

Our phylogeny did not include genetic samples of the most similar species (*E. chlorotaenia*); however, *E. chlorotaenia* is replaced by *E. bigornei* in the west (Stiassny et al., 2007), and the genetic specimen of *E. bigornei* is a member of Clade I. This clade also contains most of the larger, more robust species of *Enteromius* that are similar to the types of *E. potamogalis*. Unfortunately, no samples of *Enteromius,* whole or tissue, appear to be available from the Rio Muni. It will be necessary to obtain some topotypic material to precisely determine the placement of *E. potamogalis,* but the evidence available suggests strongly that it is a member of the clade *Enteromius* I.

Taxonomic challenges of Enteromius

Much work is needed to resolve the taxonomy of *Enteromius.* Resolution of the relationships between *Enteromius* Clade I and *Enteromius* Clade II is needed, but will come only with increased taxon sampling and additional loci. *Hemigrammopuntius* is an

available name for Clade II; however, it is unclear if this clade is diagnosable and what species would belong in it.

Major barriers to inferring relationships across Cyprinidae are also barriers to clarifying the relationships among members of *Enteromius*: incomplete taxon sampling due to inaccessibility of samples, multiple genome duplication events, few loci analyzed, and homoplasious morphological characteristics (Chen & Mayden, 2009; Tsigenopoulos et al., 2002; Yang et al., 2015). Although these small minnows have a pan-African distribution, accessibility into countries of interest and to field sites is limited, often due to political instability (Day et al., 2013; Williams & Kniveton, 2011). This low accessibility, as well as few professional curators and taxonomists across the continent, makes voucher specimens, and especially tissue samples, rare (P. H. Skelton & Swartz, 2011).

In addition to the rarity of specimens, an uninformative and confusing taxonomy has halted forward progress for the African cyprinids. The polyphyletic arrangement of *Barbus s.l.* has been recognized since Myers (1960:213) decried this "monstrous aggregation", and molecular work since the 1990s has continued to verify the fact that the small African barbs are not closely related to *Barbus*, despite bearing the name (Berrebi et al., 1996; Pethiyagoda, Meegaskumbura, & Maduwage, 2012; Tsigenopoulos et al., 2002; Wang, Li, & He, 2007). Although the monophyly of *Enteromius* group remains unclear, morphological characteristics and ploidy level unite these taxa, and distinguish them from other historical members of the genus.

There are three certainties about the small African diploid '*Barbus*': they are not *Barbus*, they are not closely related to *Barbus*, and they will never be placed back in

Barbus. Rather than reverting into an uninformative genus as proposed by Schmidt and Bart (2015), we use *Enteromius* to reflect those diploid taxa which bear radial striae on their scales and possess seven to eight branched dorsal rays until a more complete examination of the group is possible. Although *Enteromius* is non-monophyletic, recognizing *Enteromius* as a valid genus is the more taxonomically sound choice than to continue to use a genus name known to be incorrect. We provide a list of the taxa which should be considered *Enteromius,* and do not advocate at this time disturbing the taxonomic rankings of genera whose distinctiveness is sufficiently described based on morphological and molecular data (*Barboides*, *Barbopsis*, *Caecobarbus*, *Clypeobarbus*, and *Pseudobarbus*). Combined with Vreven et al. (2016), there is finally a list of species in each of the African barb genera, and ichthyologists can now better handle future taxonomic revisions.

The future of Enteromius

Puntius sensu lato (the small, diploid Asian barbs) represents a model for examining the small, diploid African barbs. The elevation of *Puntius* generated increased taxonomic interest (description of new species and genera) since it was separated from *Barbus* (Pethiyagoda et al., 2012; Kottelat, 2013). Like *Enteromius*, when *Puntius* was separated from *Barbus*, it was non-monophyletic. Based on our current results, it is clear that *Enteromius* will likely be broken into more genera as additional molecular and morphological studies resolve the relationships among *Enteromius* and related taxa, and it is the goal of this study to provide the framework for these future taxonomic decisions. However, it is not possible to break the two larger *Enteromius* clades into genera based

on current data. It is clear that there are many misidentifications of the taxa available on GenBank, and it will require much greater effort to correctly identify the species. It is likely that *Nicholsopuntius* for *E. candens, E. hulstaerti,* and *E. papilio* will be elevated and a new genus for *E. condei, E. jae, E. nounensis,* and *E. parajae* will be described; however, this is beyond the scope of this current study.

We recognize that our phylogeny is based on a single gene, and understand that with additional taxa and loci these relationships may change; however, the species we recognize in *Enteromius* will never again be placed in *Barbus*, and we believe continuing to recognize the species in a distantly related genus would only hamper further taxonomic work.

Figures

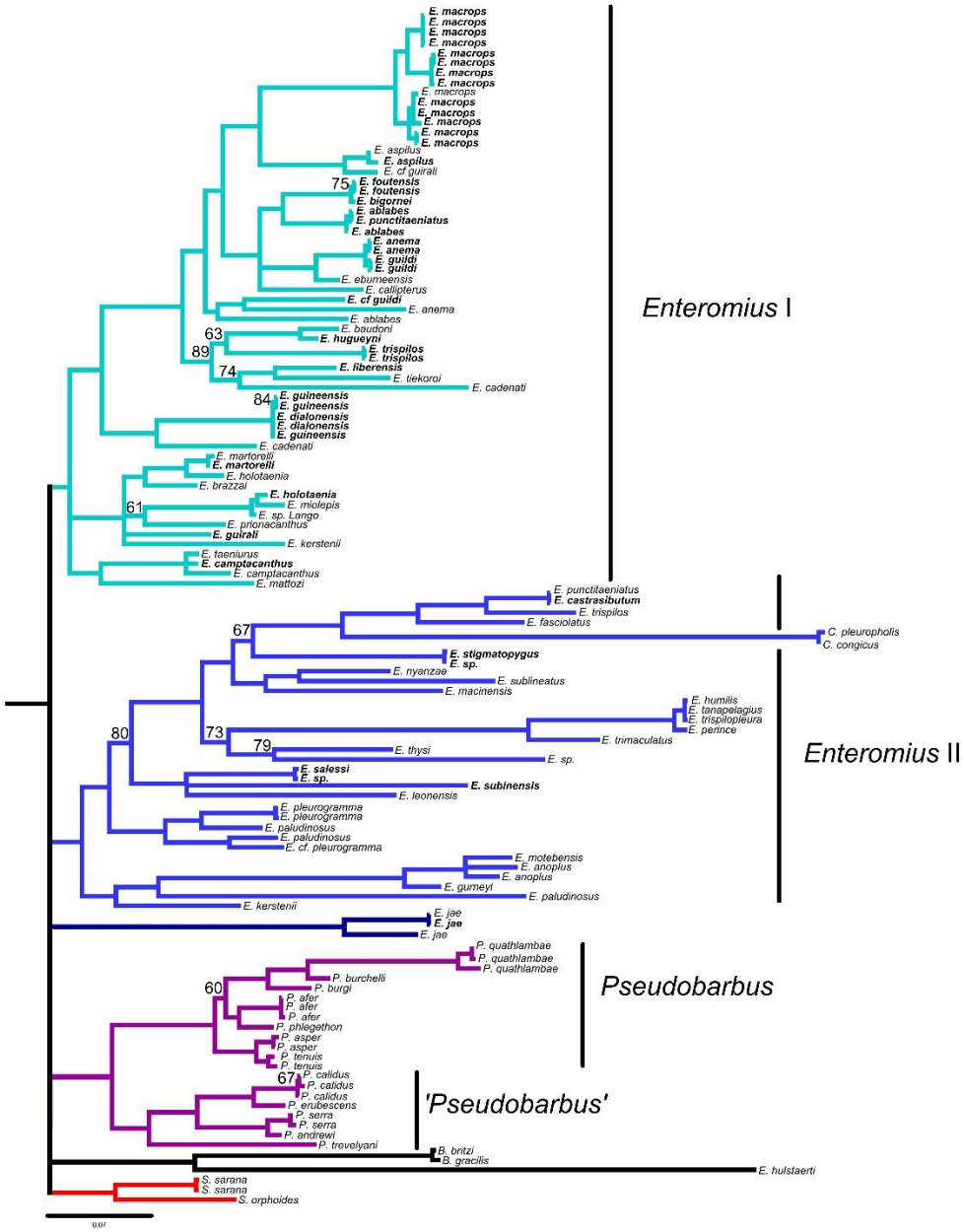

Figure 2-1. Fifty-percent majority rule consensus tree from Bayesian Inference of cytochrome b sequences. Each branch represents a posterior probability >90% unless otherwise noted as values at nodes. Any node with support <60% was collapsed into a polytomy. Taxa in bold represent sequences unique to this study.

Figure 2-2. Lateral view of *Enteromius potamogalis* from Academy of Natural Sciences, Philadelphia, ANSP 7607-12.

Tables

Table 2-1. Valid species of African barbs of the genera *Barboides*, *Barbopsis*, *Caecobarbus*, *Clypeobarbus*, *Enteromius*, *Pseudobarbus*, and '*Pseudobarbus'* based on Eschmeyer et al., (2017).

Current name	Original Name	Author	Synonyms
Barboides britzi	*Barboides britzi*	Conway and Moritz 2006	
Barboides gracilis	*Barboides gracilis*	Brüning 1929	*Raddabarbus camerunensis* Thys van den Audenaerde 1971; *Barbus lorenzi* Loiselle and Welcomme 1971;
Barbopsis devecchii	*Barbopsis devecchii*	Di Caporiacco 1926	*Eilichthys microphthalmus* Pellegrin 1929; *Barbopsis stefaninii* Gianferrari 1930
Caecobarbus geertsii	*Caecobarbus geertsii*	Boulenger 1921	
Clypeobarbus bellcrossi	*Barbus bellcrossi*	R. A. Jubb 1965	
Clypeobarbus bomokandi	*Barbus bomokandi*	G. S. Myers 1924	*Barbus rubripinnis* Nichols and Griscom 1917
Clypeobarbus breviclipeus	*Clypeobarbus breviclipeus*	Stiassny & Sakharov 2016	
Clypeobarbus congicus	*Barbus congicus*	Boulenger 1899	
Clypeobarbus hypsolepis	*Barbus hypsolepis*	Daget 1959	
Clypeobarbus pleuropholis	*Barbus pleuropholis*	Boulenger 1899	*Barbus gribinguensis* Pellegrin 1919; *Barbus kemoensis* Fowler 1936; *Barbus uellensis* Boulenger 1919
Clypeobarbus pseudognathodon	*Barbus pseudognathodon*	Boulenger 1915	
Clypeobarbus schoutedeni	*Barbus schoutedeni*	Poll & J. G. Lambert 1961	

Current name	Original Name	Author	Synonyms
Enteromius ablabes	*Puntius (Barbodes) ablabes*	Bleeker 1863	*Barbus spurrelli* Boulenger 1913
Enteromius aboinensis	*Barbus aboinensis*	Boulenger 1911	
Enteromius afrohamiltoni	*Barbus afrohamiltoni*	Crass 1960	*Barbus hamiltoni* Gilchrist and Thompson 1913
Enteromius afrovernayi	*Barbus afrovernayi*	Nichols & Boulton 1927	
Enteromius aliciae	*Barbus aliciae*	Bigorne & Lévêque 1993	
Enteromius aloyi	*Barbus aloyi*	Roman 1971	
Enteromius altidorsalis	*Barbus altidorsalis*	Boulenger 1908	
Enteromius alvarezi	*Barbus alvarezi*	Roman 1971	
Enteromius amanpoae	*Barbus amanpoae*	Lambert 1961	
Enteromius amatolicus	*Barbus amatolicus*	Skelton 1990	
Enteromius anema	*Barbus anema*	Boulenger 1903	
Enteromius annectens	*Barbus annectens*	Gilchrist & Thompson 1917	
Enteromius anniae	*Barbus anniae*	Lévêque 1983	
Enteromius anoplus	*Barbus anoplus*	Weber 1897	*Barbus anoplus oraniensis* Barnard 1943; *Barbus anoplus* typica Barnard 1943; *Barbus cernuus* Barnard 1836; *Barbus karkensis* Gilchrist & Thompson 1913
Enteromius apleurogramma	*Barbus apleurogramma*	Boulenger 1911	*Barbus* amboseli Banister 1980; *Barbus* (*Agrammobarbus*) *babaulti* Pellegrin 1935 *Barbus lapsus* Banister 1973; *Barbus scheemani* Klausewitz 1957; *Barbus*

Current name	Original Name	Author	Synonyms
			zanzibaricus paucior Hilgendorf 1905
Enteromius arcislongae	*Barbus trispilopleura arcislongae*	Keilhack 1908	
Enteromius argenteus	*Barbus argenteus*	Günther 1868	*Barbus crocodilensis* Fowler 1934
Enteromius aspilus	*Barbus aspilus*	Boulenger 1907	
Enteromius atakorensis	*Barbus atakorensis*	Daget 1957	
Enteromius atkinsoni	*Barbus atkinsoni*	Bailey 1969	
Enteromius atromaculatus	*Barbus atromaculatus*	Nichols & Griscom 1917	*Barbus lepidura* Fowler 1936
Enteromius bagbwensis	*Barbus bagbwensis*	Norman 1932	
Enteromius barnardi	*Barbus barnardi*	Jubb 1965	
Enteromius barotseensis	*Barbus radiatus barotseensis*	Pellegrin 1920	
Enteromius baudoni	*Barbus baudoni*	Boulenger 1918	*Barbus baudoni ubangensis* Pellegrin 1920; *Barbus svenssoni* Johnels 1954; *Barbus voltae* Hopson 1965
Enteromius bawkuensis	*Barbus bawkuensis*	Hopson 1965	
Enteromius bifrenatus	*Barbus bifrenatus*	Fowler 1935	
Enteromius bigornei	*Barbus bigornei*	Lévêque Teugels & Thys van den Audenaerde 1988	
Enteromius boboi	*Barbus boboi*	Schultz 1942	
Enteromius bourdariei	*Barbus bourdariei*	Pellegrin 1928	
Enteromius brachygramma	*Barbus brachygramma*	Boulenger 1915	
Enteromius brazzai	*Barbus brazzai*	Pellegrin 1901	*Barbus tshopoensis* De Vos 1991
Enteromius breviceps	*Barbus breviceps*	Trewavas 1936	
Enteromius brevidorsalis	*Barbus brevidorsalis*	Boulenger 1915	*Barbus puellus* Nichols & Boulton 1927

Current name	Original Name	Author	Synonyms
Enteromius brevilateralis	*Barbus brevilateralis*	Poll 1967	
Enteromius brevipinnis	*Barbus brevipinnis*	Jubb 1966	
Enteromius brichardi	*Barbus brichardi*	Poll & Lambert 1959	
Enteromius cadenati	*Barbus cadenati*	Daget 1962	
Enteromius callipterus	*Barbus callipterus*	Boulenger 1907	
Enteromius camptacanthus	*Puntius (Barbodes) camptacanthus*	(Bleeker 1863)	*Puntius tholonianus* Thominot 1886
Enteromius candens	*Barbus candens*	Nichols & Griscom 1917	
Enteromius carcharhinoides	*Barbus carcharhinoides*	Stiassny 1991	
Enteromius carens	*Barbus carens*	Boulenger 1912	
Enteromius castrasibutum	*Barbus castrasibutum*	Fowler 1936	
Enteromius catenarius	*Barbus catenarius*	Poll & Lambert 1959	
Enteromius caudosignatus	*Barbus caudosignatus*	Poll 1967	
Enteromius cercops	*Barbus cercops*	Whitehead 1960	
Enteromius chicapaensis	*Barbus chicapaensis*	Poll 1967	
Enteromius chiumbeensis	*Barbus chiumbeensis*	Pellegrin 1936	
Enteromius chlorotaenia	*Barbus chlorotaenia*	Boulenger 1911	
Enteromius choloensis	*Barbus choloensis*	Norman 1925	
Enteromius citrinus	*Barbus citrinus*	Boulenger 1920	
Enteromius clauseni	*Barbus clauseni*	Thys van den Audenaerde 1976	
Enteromius collarti	*Barbus collarti*	Poll 1945	
Enteromius condei	*Barbus condei*	Mahnert & Géry 1982	
Enteromius deguidei	*Barbus deguidei*	Matthes 1964	
Enteromius deserti	*Barbus deserti*	Pellegrin 1909	
Enteromius dialonensis	*Barbus dialonensis*	Daget 1962	
Enteromius diamouanganai	*Barbus diamouanganai*	Teugels & Mamonekene 1992	
Enteromius ditinensis	*Barbus ditinensis*	Daget 1962	
Enteromius dorsolineatus	*Barbus dorsolineatus*	Trewavas 1936	
Enteromius eburneensis	*Barbus eburneensis*	Poll 1941	*Barbus flomoi* Schultz 1942

Current name	Original Name	Author	Synonyms
Enteromius erythrozonus	*Barbus erythrozonus*	Poll & Lambert 1959	
Enteromius eutaenia	*Barbus eutaenia*	Boulenger 1904	
Enteromius evansi	*Barbus evansi*	Fowler 1930	
Enteromius fasciolatus	*Barbus fasciolatus*	Günther 1868	*Barbus barilioides* Boulenger 1914
Enteromius foutensis	*Barbus foutensis*	Lévêque Teugels & Thys van den Audenaerde 1988	
Enteromius fritschii	*Barbus atlanticus*	(Boulenger 1902)	
Enteromius greenwoodi	*Barbus greenwoodi*	Poll 1967	
Enteromius guildi	*Barbus (Enteromius) guildi*	Loiselle 1973	
Enteromius guineensis	*Barbus guineensis*	Pellegrin 1913	
Enteromius guirali	*Barbus guirali*	Thominot 1886	*Barbus camptacanthus melanepiptera* Pellegrin 1924
Enteromius cf. guirali			
Enteromius gurneyi	*Barbus gurneyi*	Günther 1868	
Enteromius haasianus	*Barbus haasianus*	David 1936	*Barbus woehlerti* Trewavas 1938
Enteromius holotaenia	*Barbus holotaenia*	Boulenger 1904	*Barbus holotaenia ovomaculata* Pellegrin 1930
Enteromius hospes	*Barbus hospes*	Barnard 1938	
Enteromius huguenyi	*Barbus huguenyi*	Bigorne & Lévêque 1993	
Enteromius huloti	*Barbus huloti*	Banister 1976	
Enteromius hulstaerti	*Barbus hulstaerti*	Poll 1945	
Enteromius humeralis	*Barbus humeralis*	Boulenger 1902	*Barbus dolichosoma* Nichols & Griscom 1917
Enteromius humilis	*Barbus humilis*	Boulenger 1902	
Enteromius inaequalis	*Barbus inaequalis*	Lévêque Teugels & Thys van den Audenaerde 1988	

Current name	Original Name	Author	Synonyms
Enteromius innocens	*Barbus innocens*	Pfeffer 1896	
Enteromius jacksoni	*Barbus jacksoni*	Günther 1889	*Barbus jacksonii mitior* Hilgendorf 1905; *Barbus nummifer* Boulenger 1904; *Barbus pappenheimi* Boulenger 1905
Enteromius jae	*Barbus jae*	Boulenger 1903	
Enteromius janssensi	*Barbus janssensi*	Poll 1976	
Enteromius kamolondoensis	*Barbus kamolondoensis*	Poll 1938	
Enteromius kerstenii	*Barbus kerstenii*	Peters 1868	*Barbus akeleyi* Hubbs 1918; *Barbus lumiensis* Boulenger 1903; *Barbus minchini* Boulenger 1906; *Barbus nigrolinea* Pfeffer 1889; *Barbus salmo* Pfeffer 1896
Enteromius kessleri	*Barbus kessleri*	(Steindachner 1866)	*Barbus caudimacula* Günther 1868
Enteromius kissiensis	*Barbus kissiensis*	Daget 1954	
Enteromius kuiluensis	*Barbus kuiluensis*	Pellegrin 1930	
Enteromius lamani	*Barbus lamani*	Lönnberg & Rendahl 1920	
Enteromius laticeps	*Barbus laticeps*	Pfeffer 1889	
Enteromius lauzannei	*Barbus lauzannei*	Lévêque & Paugy 1982	
Enteromius leonensis	*Barbus leonensis*	Boulenger 1915	
Enteromius liberiensis	*Barbus camptacanthus liberiensis*	Steindachner 1894	
Enteromius lineomaculatus	*Barbus lineamaculatus*	Boulenger 1903	
Enteromius litamba	*Barbus litamba*	Keilhack 1908	
Enteromius lornae	*Barbus lornae*	Ricardo-Bertram 1943	
Enteromius loveridgii	*Barbus loveridgii*	Boulenger 1916	

Current name	Original Name	Author	Synonyms
Enteromius lufukiensis	*Barbus lufukiensis*	Boulenger 1917	
Enteromius luikae	*Barbus luikae*	Ricardo 1939	
Enteromius lujae	*Barbus lujae*	Boulenger 1913	
Enteromius lukindae	*Barbus lukindae*	Boulenger 1915	
Enteromius lukusiensis	*Barbus lukusiensis*	David & Poll 1937	
Enteromius luluae	*Barbus luluae*	Fowler 1930	
Enteromius machadoi	*Barbus machadoi*	Poll 1967	
Enteromius macinensis	*Barbus macinensis*	Daget 1954	
Enteromius macrops	*Barbus macrops*	Boulenger 1911	*Barbus francisci* Boulenger 1916; *Barbus gambiensis* Svensson 1933; *Barbus weidholzii* Holly 1928
Enteromius macrotaenia	*Barbus macrotaenia*	Worthington 1933	
Enteromius magdalenae	*Barbus magdalenae*	Boulenger 1906	
Enteromius manicensis	*Barbus manicensis*	Pellegrin 1919	*Barbus hondeensis* Määr 1962
Enteromius marmoratus	*Barbus marmoratus*	David & Poll 1937	
Enteromius martorelli	*Barbus martorelli*	Roman 1971	
Enteromius matthesi	*Barbus matthesi*	Poll & Gosse 1963	
Enteromius mattozi	*Barbus mattozi*	Guimarães 1884	*Barbus rapax* Steindachner 1894; *Barbus sauvagei* Pellegrin 1912; *Barbus serrula* Gilchrist & Thompson 1913
Enteromius mawambi	*Barbus mawambi*	Pappenheim 1914	
Enteromius mediosquamatus	*Barbus mediosquamatus*	Poll 1967	
Enteromius melanotaenia	*Barbus melanotaenia*	Stiassny 1991	
Enteromius mimus	*Barbus mimus*	Boulenger 1912	
Enteromius miolepis	*Barbus miolepis*	Boulenger 1902	*Barbus camptacanthus cotesi* Pellegrin 1907; *Barbus decioi* Fowler 1958;

Current name	Original Name	Author	Synonyms
			Barbus holotaenia macracantha Pellegrin 1930; *Barbus nicholsi* Vinciguerra 1928; *Barbus squamosissimus* Steindachner 1912; *Barbus treadwelli* Pellegrin 1933
Enteromius mocoensis	*Barbus mocoensis*	Trewavas 1936	
Enteromius mohasicus	*Barbus mohasicus*	Pappenheim 1914	*Barbus mohasicus paucisquamata* Pellegrin 1933
Enteromius motebensis	*Barbus motebensis*	Steindachner 1894	*Barbus motebensis kamaiae* David & Poll 1937
Enteromius multilineatus	*Barbus multilineatus*	Worthington 1933	*Puntius carpenteri* Fowler 1949
Enteromius musumbi	*Barbus musumbi*	Boulenger 1910	
Enteromius neefi	*Barbus neefi*	Greenwood 1962	
Enteromius neglectus	*Barbus neglectus*	Boulenger 1903	
Enteromius neumayeri	*Barbus neumayeri*	Fischer 1884	*Barbus carpio* Pfeffer 1896; *Barbus luazomela* Lönnberg 1911; *Barbus luhondo* Pappenheim 1914; *Barbus nairobiensis* Boulenger 1911; *Barbus percivali kitalensis* Pellegrin 1935; *Barbus* percivali Boulenger 1903; *Barbus portali* Boulenger 1906; *Barbus serrifer* Boulenger 1900

Current name	Original Name	Author	Synonyms
Enteromius nigeriensis	*Barbus nigeriensis*	Boulenger 1903	
Enteromius nigrifilis	*Barbus nigrifilis*	Nichols 1928	
Enteromius nigroluteus	*Barbus nigroluteus*	Pellegrin 1930	
Enteromius niokoloensis	*Barbus niokoloensis*	Daget 1959	
Enteromius nounensis	*Barbus nounensis*	Van den Bergh & Teugels 1998	
Enteromius nyanzae	*Barbus nyanzae*	Whitehead 1960	
Enteromius okae	*Puntius okae*	Fowler 1949	
Enteromius oligogrammus	*Barbus oligogrammus*	David 1937	
Enteromius olivaceus	*Barbus olivaceus*	Seegers 1996	
Enteromius owenae	*Barbus owenae*	Ricardo-Bertram 1943	
Enteromius pallidus	*Barbus (Pseudobarbus) pallidus*	Smith 1841	*Barbus hemipleurogramma* Boulenger 1911
Enteromius paludinosus	*Barbus paludinosus*	Peters 1852	*Barbus akakianus* Boulenger 1911; *Barbus amphigramma* Boulenger 1903; *Barbus gibbosus* Peters 1852; *Barbus helleri* Hubbs 1918; *Barbus ivongoensis* Fowler 1934; *Barbus longicauda* Boulenger 1905; *Barbus macropristis* Boulenger 1904; *Barbus macropristis meruensis* Lönnberg 1907; *Barbus meruensis* Lönnberg 1907; *Barbus thikensis* Boulenger 1905; *Barbus tsotsorogensis* Fowler 1935; *Barbus*

Current name	Original Name	Author	Synonyms
			vinciguerraii Pfeffer 1896; *Barbus welwitschii* Günther 1868
Enteromius papilio	*Barbus papilio*	Banister & Bailey 1979	
Enteromius parablabes	*Barbus parablabes*	Daget 1957	
Enteromius parajae	*Barbus parajae*	Van den Bergh & Teugels 1998	
Enteromius perince	*Barbus perince*	Rüppell 1835	*Barbus donaldsonsmithi* Fowler 1958; *Barbus lawrae* Hopson 1965; *Barbus lepidus* Pfaff 1933
Enteromius petchkovskyi	*Barbus petchkovskyi*	Poll 1967	
Enteromius pleurogramma			
Enteromius cf. pleurogramma			
Enteromius pobeguini	*Barbus pobeguini*	Pellegrin 1911	*Barbus pleurogramma* Boulenger 1902; *Barbus* (*Hemicapoeta*) *mirei* Estève 1952; *Barbus pobeguini mauritanica* Pellegrin 1937
Enteromius poechii	*Barbus poechii*	Steindachner 1911	*Barbus bernardcarpi* Jubb 1958; *Barbus poechii* Lohberger 1930
Enteromius potomogalis	*Enteromius potamogalis*	Cope 1867	
Enteromius prionacanthus	*Barbus prionacanthus*	Mahnert & Géry 1982	
Enteromius profundus	*Barbus radiatus profundus*	Greenwood 1970	
Enteromius pseudotoppini	*Barbus pseudotoppini*	Seegers 1996	
Enteromius pumilus	*Barbus pumilus*	Boulenger 1901	
Enteromius punctitaeniatus	*Barbus punctitaeniatus*	Daget 1954	
Enteromius pygmaeus	*Barbus pygmaeus*	Poll & Gosse 1963	

Current name	Original Name	Author	Synonyms
Enteromius quadrilineatus	*Barbus quadrilineatus*	David 1937	
Enteromius quadripunctatus	*Barbus quadripunctatus*	Pfeffer 1896	
Enteromius radiatus	*Barbus radiatus*	Peters 1853	*Barbus aurantiacus* Boulenger 1910; *Barbus bangwelensis* Boulenger 1905; *Barbus doggetti* Boulenger 1904; *Barbus myersi* Poll 1939; *BeiraBarbus okavangoensis* Barnard 1941; *Barbus rogersi* Boulenger 1911; *Barbus rubellus* Crass 1960; *Barbus radiatus* Günther 1868
Enteromius raimbaulti	*Barbus raimbaulti*	Daget 1962	
Enteromius rohani	*Barbus rohani*	Pellegrin 1921	
Enteromius roussellei	*Barbus roussellei*	Ladiges & Voelker 1961	
Enteromius rouxi	*Barbus rouxi*	Daget 1961	
Enteromius rubrostigma	*Barbus miolepis rubrostigma*	Poll & Lambert 1964	
Enteromius salessei	*Barbus salessei*	Pellegrin 1908	*Barbus apogonostomatus* Pellegrin 1913
Enteromius sensitivus	*Barbus sensitivus*	Roberts 2010	
Enteromius serengetiensis	*Barbus serengetiensis*	Farm 2000	
Enteromius sexradiatus	*Barbus sexradiatus*	Boulenger 1911	
Enteromius seymouri	*Barbus seymouri*	Tweddle & Skelton 2008	
Enteromius stanleyi	*Barbus stanleyi*	Poll & Gosse 1974	*Barbus gobioides* Poll & Gosse 1963
Enteromius stauchi	*Barbus stauchi*	Daget 1967	
Enteromius stigmasemion	*Barbus stigmasemion*	Fowler 1936	

Current name	Original Name	Author	Synonyms
Enteromius stigmatopygus	*Barbus stigmatopygus*	Boulenger 1903	*Barbus alberti* Poll 1939; *Barbus* (*Nicholspuntius*) *gourmansis* Pellegrin 1934; *Barbus karoualensis* Blache & Miton 1960; *Barbus miolepis* Boulenger 1903; *Barbus werneri* Boulenger 1905
Enteromius subinensis	*Barbus subinensis*	Hopson 1965	
Enteromius sublineatus	*Barbus sublineatus*	Daget 1954	
Enteromius sylvaticus	*Barbus (Enteromius) sylvaticus*	Loiselle & Welcomme 1971	
Enteromius syntrechalepis	*Puntius syntrechalepis*	Fowler 1949	
Enteromius taeniopleura	*Barbus taeniopleura*	Boulenger 1917	
Enteromius taeniurus	*Barbus taeniurus*	Boulenger 1903	
Enteromius tanapelagius	*Barbus tanapelagius*	Graaf Dejen Sibbing & Osse 2000	
Enteromius tangandensis	*Barbus tangandensis*	Jubb 1954	
Enteromius tegulifer	*Barbus tegulifer*	Fowler 1936	
Enteromius tetraspilus	*Barbus tetraspilus*	Pfeffer 1896	
Enteromius tetrastigma	*Barbus tetrastigma*	Boulenger 1913	
Enteromius teugelsi	*Barbus teugelsi*	Vreven and Snoeks 2011	
Enteromius thamalakanensis	*Barbus thamalakanensis*	Fowler 1935	*Barbus fitzsimonsi* Fowler 1935
Enteromius thysi	*Barbus thysi*	Trewavas 1974	
Enteromius tiekoroi	*Barbus tiekoroi*	Lévêque Teugels & Thys van den Audenaerde 1987	*Barbus trispilos quinquepunctata* Pellegrin 1911
Enteromius tomiensis	*Barbus tomiensis*	Fowler 1936	
Enteromius tongaensis	*Barbus tongaensis*	Rendahl 1935	

Current name	Original Name	Author	Synonyms
Enteromius toppini	*Barbus toppini*	Boulenger 1916	*Barbus umbeluziensis* Groenewald 1958
Enteromius trachypterus	*Barbus trachypterus*	Boulenger 1915	
Enteromius traorei	*Barbus traorei*	Lévêque Teugels & Thys van den Audenaerde 1987	
Enteromius treurensis	*Barbus treurensis*	Groenewald 1958	
Enteromius trimaculatus	*Barbus breijeri*	Weber 1897	
Enteromius trimaculatus	*Barbus (Dangila) trimaculatus*	Peters 1852	*Barbus breijeri* Weber 1897; *Barbus decipiens* Boulenger 1907; *Barbus katangae* Boulenger 1900; *Barbus kurumanni* Castelnau 1861
Enteromius trinotatus	*Barbus trinotatus*	Fowler 1936	
Enteromius trispiloides	*Barbus trispiloides*	Lévêque Teugels & Thys van den Audenaerde 1987	
Enteromius trispilomimus	*Barbus trispilomimus*	Boulenger 1907	
Enteromius trispilopleura	*Barbus trispilopleura*	Boulenger 1902	
Enteromius trispilos	*Barbus trispilos*	(Bleeker 1863)	
Enteromius turkanae	*Barbus turkanae*	Hopson & Hopson 1982	
Enteromius unitaeniatus	*Barbus unitaeniatus*	Günther 1866	*Barbus* inermoides Nichols & Boulton 1927; *Barbus labialis* Gilchrist & Thompson 1913; *Barbus macrurus* Gilchrist & Thompson 1913; *Barbus tristigmaturus* Fowler 1934
Enteromius urostigma	*Barbus urostigma*	Boulenger 1917	
Enteromius usambarae	*Barbus usambarae*	Lönnberg 1907	

Current name	Original Name	Author	Synonyms
Enteromius validus	*Barbus validus*	Stiassny, Liyandja, & Monsembula Iyaba 2016	
Enteromius vanderysti	*Barbus vanderysti*	Poll 1945	
Enteromius venustus	*Barbus venustus*	Bailey 1980	
Enteromius viktorianus	*Barbus viktorianus*	Lohberger 1929	
Enteromius viviparus	*Barbus viviparus*	Weber 1897	
Enteromius walkeri	*Barbus walkeri*	Boulenger 1904	
Enteromius wellmani	*Barbus wellmani*	Boulenger 1911	
Enteromius yeiensis	*Barbus yeiensis*	Johnsen 1926	
Enteromius yongei	*Barbus yongei*	Whitehead 1960	
Enteromius zalbiensis	*Barbus zalbiensis*	Blache & Miton 1960	
Enteromius zanzibaricus	*Barbus zanzibaricus*	Peters 1868	*Barbus altus* Pfeffer 1896; *Barbus argyrotaenia* Boulenger 1912; *Barbus kiperegensis* Steindachner 1914; *Barbus pfefferi* Boulenger 1905
Pseudobarbus afer	*Barbus (Capoeta) afer*	Peters 1864	Smith 1936
Pseudobarbus asper	*Barbus asper*	Boulenger 1911	
Pseudobarbus burchelli	*Barbus (Pseudobarbus) burchelli*	Smith 1841	*Barbus gobionides* Valenciennes 1842; *Barbus* multimaculatus Steindachner 1870; Gnathendalia vulnerata Castelnau 1861
Pseudobarbus burgi	*Barbus burgi*	Boulenger 1911	
Pseudobarbus phlegethon	*Barbus phlegethon*	Barnard 1938	
Pseudobarbus quathlambae	*Labeo quathlambae*	Barnard 1938	
Pseudobarbus skeltoni	*Pseudobarbus skeltoni*	Chakona & Swartz 2013	
Pseudobarbus tenuis	*Barbus tenuis*	Barnard 1938	

Current name	Original Name	Author	Synonyms
Pseudobarbus verloreni	*Pseudobarbus verloreni*	Chakona, Swartz, & Skelton 2014	
'Pseudobarbus' andrewi	*Barbus andrewi*	Barnard 1937	
'Pseudobarbus' capensis	*Barbus (Cheilobarbus) capensis*	Smith 1841	
'Pseudobarbus' erubescens	*Barbus erubescens*	Skelton 1974	
'Pseudobarbus' serra	*Barbus serra*	Peters 1864	
'Pseudobarbus' trevelyani	*Barbus trevelyani*	Günther 1877	*Barbus brookingi* Gilchrist & Thompson 1913

Table 2-2. Information of tissue specimens used in this study. Specimens which have no physical voucher associated have a photographic voucher.

Species	Tissue ID	Voucher Catalog #	Accession #	GenBank Accession Number	Drainage	Locality	Latitude	Longitude
Enteromius macrops	AUF 5350	59487	8621	MF135212	Forécariah River - Bofon River	Serguey Creek, near Bassia	9.53212	-13.022
Enteromius macrops	AUF 5351	59487	8621	MF135200	Forécariah River - Bofon River	Serguey Creek, near Bassia	9.53212	-13.022
Enteromius macrops	AUF 5354	59487	8621	MF135201	Forécariah River - Bofon River	Serguey Creek, near Bassia	9.53212	-13.022
Enteromius dialonensis	AUF 5359	59504	8623	MF135221	Gambie River	Diwet River, at Diwet, ~10 km past Sannou	11.4584	-12.064
Enteromius dialonensis	AUF 5361	59517	8624	MF135222	Rio Corubal	Dimmah River, in frontier between Sannou and Diogoma	11.4516	-12.167
Enteromius cadenati	AUF 5364	59519	8624	MF135224	Rio Corubal	Dimmah River, in frontier between Sannou and Diogoma	11.4516	-12.167
Enteromius guineensis	AUF 5366	59518	8624	MF135215	Rio Corubal	Dimmah River, in frontier between Sannou and Diogoma	11.4516	-12.167
Enteromius salessi	AUF 5367	59520	8624	MF135198	Rio Corubal	Dimmah River, in frontier between Sannou and Diogoma	11.4516	-12.167
Enteromius macrops	AUF 5371	59497	8625	MF135202	Rio Corubal	Koumba River, at crossing on Labe to Mali road	11.6634	-12.283
Enteromius macrops	AUF 5372	59497	8625	MF135203	Rio Corubal	Koumba River, at crossing on Labe to Mali road	11.6634	-12.283
Enteromius sublinensis	AUF 5378	None*	None	MF135195	Rio Corubal	Koumba River, at crossing on Labe to Mali road	11.6634	-12.283
Enteromius guineensis	AUF 5379	59521	8626	MF135216	Kakrima River - Konkouré River	Sala River, at N5, WNW of Labe	11.3765	-12.378
Enteromius sp	AUF 5392	None*	None	MF135197	Kakrima River - Konkouré River	Sala River, at road crossing near Diari	11.3039	-12.446
Enteromius ablabes	AUF 5431	59647	8634	MF135227	Bafing River - Senegal River	Bafing River, at Sokotoro, E of Timbo	10.6623	-11.752

Species	Tissue ID	Voucher Catalog #	Accession #	GenBank Accession Number	Drainage	Locality	Latitude	Longitude
Enteromius ablabes	AUF 5441	59647	8634	MF135228	Bafing River - Senegal River	Bafing River, at Sokotoro, E of Timbo	10.6623	-11.752
Enteromius cf guildi	AUF 5443	None*	None	MF135223	Bafing River - Senegal River	Bafing River, at Sokotoro, E of Timbo	10.6623	-11.752
Enteromius macrops	AUF 5454	59541	8635	MF135204	Niger River	Tinkisso River, below Tinkisso dam	10.7279	-11.169
Enteromius macrops	AUF 5458	59541	8635	MF135205	Niger River	Tinkisso River, below Tinkisso dam	10.7279	-11.169
Enteromius macrops	AUF 5476	59615	8638	MF135206	Kolenté River	Kolenté River, at Kolenté, on Kindia to Mamou road	10.0953	-12.627
Enteromius macrops	AUF 5477	59615	8638	MF135207	Kolenté River	Kolenté River, at Kolenté, on Kindia to Mamou road	10.0953	-12.627
Enteromius macrops	AUF 5478	59615	8638	MF135208	Kolenté River	Kolenté River, at Kolenté, on Kindia to Mamou road	10.0953	-12.627
Enteromius macrops	AUF 5479	59615	8638	MF135209	Kolenté River	Kolenté River, at Kolenté, on Kindia to Mamou road	10.0953	-12.627
Enteromius macrops	AUF 5481	59666	8640	MF135210	Badi River - Konkouré River	Safa-Khoure River, at Camara-Bouyhe	9.90155	-13.022
Enteromius liberensis	AUF 5483	59671	8640	MF135213	Badi River - Konkouré River	Safa-Khoure River, at Camara-Bouyhe	9.90155	-13.022
Enteromius anema	AUF 5493	59674	8646	MF135225	Cavally River	Mia River, at Bourata village	7.65004	-8.3007
Enteromius anema	AUF 5494	59674	8646	MF135226	Cavally River	Mia River, at Bourata village	7.65004	-8.3007
Enteromius trispilos	AUF 5496	59673	8646	MF135193	Cavally River	Mia River, at Bourata village	7.65004	-8.3007
Enteromius trispilos	AUF 5498	59673	8646	MF135194	Cavally River	Mia River, at Bourata village	7.65004	-8.3007
Enteromius guildi	AUF 5504	59624	8647	MF135217	Lavally River	Zie River, W of Zera village	7.71696	-8.3622
Enteromius guildi	AUF 5505	59624	8647	MF135218	Lavally River	Zie River, W of Zera village	7.71696	-8.3622

Species	Tissue ID	Voucher Catalog #	Accession #	GenBank Accession Number	Drainage	Locality	Latitude	Longitude
Enteromius macrops	AUF 5525	59716	8650	MF135211	Sassandra River - Bafing River	Doulou River, at Kokota village	7.91706	-8.4993
Enteromius hugenyi	AUF 5589	59780	8658	MF135214	Makona River - Moa River	Masseni River, about 3 miles N of Konesseridou	8.7204	-9.5244
Enteromius stigmatopygus	AUF 5608	59758	8660	MF135196	Niger River	Mafou River, on N2 ~80 km S of Faranah	9.53072	-10.402
Enteromius punctitaeniatus	AUF 5610	59756	8660	MF135199	Niger River	Mafou River, on N2 ~80 km S of Faranah	9.53072	-10.402
Enteromius foutensis	AUF 5656	59589	8666	MF135219	Little Scarcies River	Penselli River, near Ourékaba, on N2	10.1557	-11.675
Enteromius foutensis	AUF 5657	59589	8666	MF135220	Little Scarcies River	Penselli River, near Ourékaba, on N2	10.1557	-11.675
Enteromius sp	AMNH 9021	254097	-	MF135192	-	Region de Conakry	9.0925	-9.0069

Chapter 3 – A new genus of minnow in West Africa (Cypriniformes, Cyprinidae, Smiliogastrini)

Abstract

The *Enteromius jae* species group (*E. jae*, *E. condei*, *E. nounensis*, and *E. parajae*) is a monophyletic group of African small barbs diagnosable by several characteristics including: a broad premaxilla, presence of a pseudotympanum, dark pigment on the skin above the pseudotympanum, an incomplete lateral line, barbels absent, a rectangular neurocranial fontanelle, and sensory pit lines on the head. As a monophyletic and diagnosable group, we recognize the *E. jae* species group as a new genus, *Baka*. *Baka* is an endemic of the Lower Guinea ichthyoprovinces with collections reported from Cameroon, Equatorial Guinea, Gabon, and the Republic of the Congo. It is also present in the Dja River (Congo River tributary) of the Democratic Republic of the Congo and Cameroon. *Baka* can be separated from the cave barbs (*Caecobarbus*) by having fully developed eyes and pigment; from *Barboides* by lacking a humeral organ; from *Clypeobarbus* by lacking a cleithral mark; and from *Enteromius* by having a short, chunky premaxilla. A description of the pseudotympanic structure, a 3D rendering of key skeletal elements, and a molecular phylogeny are also presented to demonstrate the distinctiveness of *Baka*.

Introduction

The taxonomy of African Cyprinidae is undergoing significant and necessary change. This is especially true regarding small barbs. The African small barbs were historically members of the genus *Barbus* Daudin, 1805; however, *Barbus* has undergone significant taxonomic revision that resulted in descriptions of many new genera across

Africa, Asia, and Europe that are recognized in several different subfamilies (Tan and Armbruster 2018). In Africa, the South African tetraploid barbs were elevated to *Pseudobarbus* Smith, 1841 (Paul H Skelton, 1988). This eventually led to clarification and recognition of the following new genera: *Cheilobarbus* Smith, 1841, *Amatolacypris*, *Namaquacypris*, and *Sedercypris* Skelton, Swartz, & Vreven, 2018. Some diploid barbs have recognized as different genera such as the large scale barbs *Clypeobarbus* Fowler, 1936 (Conway & Stiassny, 2008); two genera of cave barbs *Caecobarbus* Boulenger, 1921 and *Barbopsis* Di Caporiacco, 1926; and the paedomorphic *Barboides* Brüning, 1929 (Conway & Moritz, 2006). This left most of African small barbs as '*Barbus*' contained in single quotes to demonstrate taxonomic uncertainty (Berrebi, Kottelat, Skelton, & Ráb, 1996).

Based on molecular data, Yang et al. (2015) recommended that '*Barbus*' be moved to *Enteromius*, and this was accepted by Skelton (2016) and expounded upon in Hayes and Armbruster (2017). This move has not been without controversy (Schmidt & Bart, 2015), especially because the deep phylogenetic relationships among *Enteromius* remain unclear. Despite uncertainty of deeper relationships within the group, *Enteromius* is no longer recognized within the same subfamily as the genus *Barbus sensu lato* (Smiliogastrinae vs. Barbinae; Tan & Armbruster, 2018); thus, the member species could not remain in *Barbus*.

Boulenger (1911) differentiated *Barbus sensu lato* into two groups: the large barbs with parallel striae, which includes the true *Barbus* and taxa later recognized as *Labeobarbus* (Vreven, Musschoot, Snoeks, & Schliewen, 2016); and the small barbs with radial striae, which encompasses *Amatolacypris*, *Caecobarbus*, *Cheilobarbus*,

Clypeobarbus, *Barboides*, *Enteromius Sedercypris*, and *Namaquacypris*. Later, a third group was added to represent a single species, *Barbus jae* (Mahnert & Géry, 1977). This third category was created based on the unique morphology of the premaxilla and maxilla (Mahnert & Géry, 1977), but the taxon has remained grouped with the other small African barbs throughout subsequent taxonomic changes. Additional species have been described and attributed to this third category with the understanding that the group was artificially created to include species with an incomplete lateral line and lacking barbels (Van den Bergh & Teugels, 1998).

Barbus jae Boulenger, 1903 was described from the Dja River in southern Cameroon and, at the time, was compared to only *B. brazzai* Pellegrin, 1901 due to its lack of barbels (Boulenger, 1903). Later, *B. condei* was described from the Nounah River, Ivindo, Gabon (Mahnert & Géry, 1982). Two species similar to *B. jae* were described by Van den Bergh and Teugels (1998) from the Sanaga River basin of Cameroon: *B. nounensis* and *B. parajae.* These taxa are similar in morphology, but their phylogenetic relationships were unexamined, and the provided dichotomous key was made only for species from the Sanaga River. The paper also included a clarification of *B. condei*. With molecular data, *Barbus jae* (as *Enteromius*) was placed basal to a clade including *E. hulstaerti* and *Barboides gracilis* + *Barboides britzi* (Yang et al., 2015); however, the bootstrap support for this relationship was weak (<50%). Hayes and Armbruster (2017) included denser taxon sampling but used only a single mitochondrial marker and recovered a clade composed exclusively of *B. jae* distinct from other *Enteromius* clades (>90% posterior probability).

Here, additional specimens from new collection localities are analyzed using molecular and morphological methods. The placement of *Enteromius jae* (Boulenger 1903) in a phylogenetic framework suggests the species is its own distinct evolutionary lineage, and the clade is raised to represent a new genus. This new genus includes *Enteromius condei* (Mahnert and Géry 1982), *E. jae* Boulenger 1903, *E. nounensis* (Van den Bergh and Teugels 1998), and *E. parajae* (Van den Bergh and Teugels 1998) as member taxa.

Methods

Specimen Acquisition

Specimens for molecular and morphological analysis include individuals from a December 2011 collecting trip to the Dja River in Cameroon (the type locality of *Enteromius jae*) and a 2014 expedition to the Ogooué (or Ogowe) River in Gabon and are cataloged at the Auburn University Museum of Natural History (AUM) and Oregon State University, Department of Fisheries and Wildlife, Corvallis, Oregon (OS) respectively.

High-resolution photographs of specimens of *E. condei* and *E. jae* were provided by the Natural History Museum of Geneva (MHNG) and the British Museum of Natural History (BMNH). Accession numbers are as follows: *E. jae:* AUM 58053; AUM 58144; AUM 58060; BMNH 1903.7.28.237-239 (Syntypes); MHNG 1539.51-100; MHNG 1540.45-50; OS 19399; OS 19400; OS 19401; OS 19402; OS 19403; OS 19404; OS 19407; OS 19409; OS 19411. *E. condei:* MHNG 1544.29-48; MHNG1544.049 (Holotype).

Morphological Analysis

Geometric Morphometrics

Specimens were photographed in lateral view with a Nikon D90 digital SLR camera attached to a copy stand. The photograph files were then landmarked and digitized using TpsUtil 1.46 (F. J. Rohlf, 2004) and TpsDig 2.16 (F. James Rohlf, 2017) according to the standardized 18 homologous landmarks for cyprinids (Jonathan W. Armbruster, 2012). As some specimens were clearly bent from preservation, the Unbend function was implemented in TPSUtil. Three existing landmarks were used to unbend the specimens: snout tip, posterior-most edge of opercle, and end of vertebral column. The resultant .tps file was analyzed in MorphoJ (Klingenberg, 2011). Landmarks were aligned with a Generalized Procrustes Analysis (GPA), the data checked for outliers, a covariate matrix constructed, and a Principal Components Analyses (PCA) performed.

Specimen Dissection

Two individuals from the Auburn Museum of Natural History were chosen for dissection. The right lateral side was dissected to explore the subcutaneous layer at the level of the pseudotympanum. An additional 8 specimens were chosen for clearing and staining following techniques modified from Taylor and van Dyke (1985). Specimens were photographed using Leica dissection microscope attached to a camera system. Photographs were edited to z-stack at multiple depths-of-field using the program Zerene Stacker.

Molecular Methods

DNA was extracted from fin clips preserved in ethanol using Omega BioTek E.Z.N.A. Tissue DNA kits (omegabiotek.com) using methods provided by the

manufacturer with the tissue lysis time extended to overnight. A 1041 bp fragment of the cytochrome b mitochondrial gene was amplified for each specimen using primers and conditions developed by Briolay et al (1998). Sequence reads were assembled and using Geneious v.6.1.6 (http://www.geneious.com, Kearse et al., 2012). Cytochrome b sequences from additional taxa in *Barboides, Clypeobarbus, Enteromius, Pseudobarbus,* and *Systomus* were imported from GenBank. Best-fit models of evolution were tested using PartitionFinder2 (Lanfear, Frandsen, Wright, Senfeld, & Calcott, 2017). Bayesian Inference (BI) was run in MrBayes 3.2.2 on CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010; Ronquist et al., 2012) using *Puntius sophore* as an outgroup taxon. Each BI had two runs with four chains each for 10,000,000 generations and was sampled every 1,000 generations. A 25% burnin was calculated using the sump option and a 50% consensus tree was created.

Results

BAKA, Hayes and Armbruster, *NEW GENUS*

Figure 1

Type species

Barbus jae Boulenger 1903

Syntypes: BMNH 1903.7.28.287-289

Inclusive Taxa

Barbus condei Mahnert and Géry 1982

Barbus jae Boulenger 1903

Barbus nounensis Van den Bergh and Teugels 1998

Barbus parajae Van den Bergh and Teugels 1998

Diagnosis

Baka differs from all other African small barbs by the presence of a short, chunky premaxilla with pedicel as high as the rostral process (vs. thin, elongated, and a short rostral process; Figure 2); presence of a pseudotympanum with a patch of pigment on the skin above (unique among African small barbs, Figure 2; *Barboides* also has a pseudotympanum, but the pseudotmpanum lacks pigment and has a visible humeral organ); a reduced or absent lateral line (shared with *Barboides*, as well as some species of *Enteromius*); absence of barbels (shared with *Barboides* and some members of *Enteromius*); a large, rectangular cranial fontanelle (different in shape from *Barboides* and *Clypeobarbus*); and pit lines of the laterosensory system present on the head (shared with *Clypeobarbus* and some *Enteromius*). *Baka* can be further separated from *Barboides* by having 0–4 pores of the lateral line system generally present (vs. pores always absent), the presence of sensory pit-lines throughout the jaw (vs. absent), a larger maximum size (53.5 mm SL vs. 22.5 mm SL), and eight branched dorsal-fin rays (vs. six). The genus can also be differentiated from *Clypeobarbus* by lacking a cleithral mark (vs. a dark line present along the posterior edge of the pectoral girdle), by having scales above the lateral line just slightly taller than long (vs. about twice as tall as long), and by having a small, circular spot at the end of the caudal peduncle (vs a dark line running much of the depth of the caudal peduncle).

Description

Member of Cyprinidae: Smiliogastrinae *sensu* Tan and Armbruster (2018). Small barbs with maximum reported standard lengths ranging from 28 mm SL (*B. condei*) to 53.5 mm SL (*B. nounensis*).

Mouth subterminal, small, maxilla reaching just behind anterior margin of eye. *Barbus* type III maxilla and premaxilla (Mahnert & Géry, 1977): premaxilla deep-bodied with high pedicel and maxilla with well-defined posterior process (Figure 2A,B).

Pseudotympanum present; humeral organ absent (Figure 3). Pseudotympanum formed by gaps in hypaxial musculature at ribs 5, 6, and 7. Myomeres taper dorsally. Space between musculature filled with small fat globules. Pseudotympanum and fat globules visible without dissection. Pigmentation on skin above pseudotympanum.

Lateral line absent or incomplete, 0–4 pored scales. Barbels absent. Rectangular, narrow fontanelle bordered anteriorly by frontals, laterally by parietals, and posteriorly by supraoccipital (Figure 4). Head with exposed neuromasts of sensory system forming pit lines. Infraorbital bones with laterosensory canals; number of infraorbitals variable but reduced compared to *Enteromius* and *Clypeobarbus*. Eye large, depth greater than half head depth at middle of eye. Snout short, less than half eye length.

Rectangular to kite-shaped, deepest part of body at origin of dorsal fin and the origin of the pelvic fins and slope of dorsal profile linear between tip of snout and dorsal-fin origin and there to insertion of dorsal fin; angle of body decreases from insertion of dorsal fin to caudal fin until dorsal, procurrent, caudal-fin rays where body depth increases. Ventral surface from flat to convex from snout tip to anal-fin insertion with deepest point just anterior to dorsal fin; caudal peduncle convex from anal-fin insertion to caudal fin.

Coloration

Longitudinal bands or spots typically characteristic; however, coloration highly variable among and within member species. Number and intensity of bands vary between

and among populations. Rarely, specimens show only pigmentation above the pseudotympanum and a caudal spot. *Baka nounensis* lacks longitudinal bands instead possessing a faint dark lateral band running from above the pored scales to caudal fin; however, the presence of a pigmented pseudotympanum remains.

Distribution

Baka is an endemic of the Lower Guinea ichthyoprovinces with collections reported from Cameroon, Equatorial Guinea, Gabon, and the Republic of the Congo. It is also present in the Dja River (Congo River tributary, Democratic Republic of the Congo and Cameroon) and Kouilou and Loeme basins in Republic of the Congo of the Democratic Republic of the Congo.

Etymology

Named in honor of the Baka people of west Africa who, like these fish, are relatively small.

Key to the genera of small African barbs in West Africa

1.
   a. Eyes present, pigmentation on body ..... to 2
   b. Eyes absent or reduced, little to no pigmentation on the body ....*Caecobarbus*

2.
   a. Lacking a pseudotympanum; barbels present or absent; lateral line usually complete, running the full length of the body; if lateral line is incomplete, presence of a broad lateral band or distinct circular spots along the flank; dorsal fin sometimes serrated ..... to 3
   b. Possessing a pseudotympanum; barbels absent; lateral line absent or reduced; dorsal fin never serrated; circumorbital bones reduced in number; less than 50mm SL..... to 4

3.
   a. Presence of a cleithral streak (present, but muted in *C. schoutedeni*); pored lateral line scales pigmented along the edges and enlarged relative to the rows above and below with depth greatest below dorsal fin; unbranched dorsal fin ray flexible without serrations; infraorbital bones reduced in size *Clypeobarbu*

b. Coloration variable, but never possessing a distinct cleithral streak; pored lateral line scales similarly sized to surrounding scales; unbranched dorsal fin rays sometimes modified into a spine, and sometimes serrated; infraorbital series usually complete ..... *Enteromius*

4.

a. Lateral line absent; no pigmentation on pseudotympanum; fat globules beneath the pseudotympanum only in females; presence of humeral organ; maximum size <22.5mm SL; six branched dorsal fin rays; no exposed neuromasts on the cheek .....*Barboides*
b. Lateral line reduced (occasionally absent in *Baka jae*); dark pigmentation on skin covering the pseudotympanum; small fat globules present in pseudotympanic space; eight branched dorsal fin rays; exposed neuromasts on cheek… .....*Baka*

Geometric Morphometrics

Specimens of *Baka jae* from Cameroon and Gabon and *Baka condei* from Gabon were included in this analysis. The specimens of *B. condei* and the syntypes of *B. jae* were considerably bent; however, the unbend analysis corrected for most of this (see wireframes in Figure 5). PC1 explains 27% of the variance in the analysis. PC2, which explains 16% of the variation, is mostly attributed to remaining bend in the individuals, while PC3 explains 12% of the variation and does not seem influenced by specimen bending (see wireframe insert in Figure 5). Thus, the PCA presented includes PC1 and PC3 as the axes of variation among sampled populations.

Baka condei is somewhat distinct from the other *B. jae* specimens included in the analysis; however, the syntypes of *B. jae* are nested within the morphospace of *B. condei* (Figure 5). There is a large amount of spread in the *B. condei* individuals, likely because there are fewer of them compared to the other collections. Interestingly, the holotype of *B. condei* is close in morphospace to the syntype of *B. jae*.

The 95% confidence ellipses surrounding the Gabonese *B. jae* overlaps with both *B. condei* and with population of *B. jae* from Cameroon. Cameroonian and Gabonese *B.*

jae from AUM and MHNG occupy similar morphospace. Collections of Gabonese *B. jae* from OS span the range of morphospace in PC1 and connect the morphospaces of *B. condei* and *B. jae.* A single *B. jae* specimen from Cameroon is found outside of the confidence ellipses and on the most extreme PC1 axis. This individual is similar to the Type I *B. jae* of Van den Bergh and Teugels (1998), while all other appear to be Type II.

Molecular Results

Sequence data were obtained from six individuals morphologically identified as *Baka jae* or *B. cf. jae* from Gabon. These sequences were combined with materials available on GenBank including one *Baka jae* from Cameroon and another of unknown origin. Sequences were trimmed with respect to the shortest sequence, yielding a total fragment length of 1129bp. The sequence data were partitioned by codon with first and second codon positions modeled under GTR+I+Γ and the third codon position under HKY+I+Γ. The deep relationships among clades of African minnows are poorly resolved (Hayes and Armbruster, 2017); however, the monophyly of the *Baka* clade is recovered with 100% posterior probability (Figure 6).

Discussion

Baka is a diminutive African minnow (maximum 53.5 mm SL) that possesses a chunky premaxilla, pseudotympanum covered with pigmented skin, and an incomplete or absent lateral line (0-4 pored scales). Other characteristics not unique to the genus, but that are characteristic include: barbels absent; a large, rectangular fontanelle between the parietal, frontal, and supraoccipital bones; scales with radial striae; cephalic sensory pores on the cheek and lower jaw. The genus can be distinguished from *Enteromius* by its pseudotympanum, an area without muscle and with thin, transparent skin over the swim

bladder, and a large, rectangular fontanelle between the frontal, parietal, and supraoccipital bones. Two other west African genera possess similar features to *Baka: Barboides* Brüning 1929 and *Clypeobarbus* Fowler 1936.

Unlike members of *Enteromius*, *Clypeobarbus*, or *Caecobarbus*, *Baka* possess a pseudotympanum. The presence of a pseudotympanum is not unique to *Baka, Barboides* also has one, but the overall morphology is different between the two. In *Barboides*, the pseudotympanum is a single, wide opening in the musculature (Conway, Kubicek, & Britz, 2017). Hypaxial muscles attach to the middle of ribs 5 and 6, and a window is formed between the hypaxial and epaxial musculature. In *Baka*, the pseudotympanum is formed from multiple openings between hypaxial musculature (Figure 3) The hypaxial muscles reach to the dorsal portion of ribs 5, 6, and 7, but taper dorsally, creating voids. These spaces are filled with fat globules in all examined *Baka* and are visible without dissection. In *Barboides*, only females have fat globules (Conway & Moritz, 2006). Further, in *Barboides*, the pseudotympanum houses a humeral organ formed by a core of connective tissue surrounded by bulbous muscle (Conway et al., 2017). No such structure exists in the pseudotympanum of *Baka*. The evolution of the pseudotympanum, along with the potentially convergent miniaturization between the two taxa, is a question for future studies regarding the function of the structure. Whether body size and presence of a pseudotympanum are correlated remains to be determined.

Baka is most similar to *Barboides* Bruning 1929 with its lack of barbels, reduced circumorbital bones, scales with radial striae, pseudotympanum (but see above), and occipital fontanelle. *Baka* differs from *Barboides* in certain key characteristics: no humeral organ (vs. humeral organ present); maximum size of 41.5mm SL (vs 22.5mm);

dorsal fin with eight branched rays (vs. dorsal fin with six branched rays); head covered in cephalic sensory pores (vs absent); and incomplete lateral line with 0-4 pored scales (vs. absent lateral line).

The presence of an occipital fontanelle was previously recognized as a synapomorphy for *Clypeobarbus* (Conway & Stiassny, 2008; Stiassny & Sakharova, 2016); however, both *Barboides* and *Baka* have a similar cranial opening. The fontanelle of *Baka*, *Barboides,* and *Clypeobarbus* is formed anteriorly by the frontals, laterally by the parietals, and posteriorly by the supraoccipital. While these three genera have similar cranial foramina, the shape is markedly different. In *Barboides,* the fontanelle is teardrop shaped with the narrowest portion at the frontals; at its widest, the fontanelle is as wide as the frontals and approximately half the width of the cranium at that point. In *Clypeobarbus* the fontanelle is almost an hourglass shape and is narrow. The fontanelle of *Baka* is rectangular and narrow, with the opening as long as the frontals, but not as wide as them at any point.

Baka can be further distinguished from *Clypeobarbus* Fowler 1936 by possessing pigmentation on the pseudotympanum (vs. a pigmented cleithral streak); lacking barbels; having same-sized scales with diffuse pigmentation on the midline of the body (vs. enlarged midlateral scales edged with pigment); pigmentation on the caudal region no more than a caudal spot (vs. caudal bar); loss of some infraorbital bones (vs reduced in size); and by having a smaller maximum body size (41.5mm vs 66.2mm SL).

The most similar species of *Enteromius* to *Baka* in size and color is *E. fasciolatus*, which shares and orange-red body coloration and dark bars. However, the dark bars in *E. fasciolatus* are thinner than those of *Baka*, and the species lacks a pseudotympanum.

Thus far, *E. fasciolatus* has not been examined genetically. Armbruster et al. (2016) found significant convergence in shape and color in distantly related barbs, and it remains unknown if the similarity between *Baka* and *E. fasciolatus* is indicative of convergence or evolutionary descent.

Other miniature species of *Enteromius* include *E. candens*, *E. hulstaerti*, and *E. papilio*. These species also have reduced lateral lines (Banister & Bailey, 1979; Nichols & Griscom, 1917; Poll, 1945), and can be recognized from *Baka* by the lack of a pseudotympanum and differences in coloration (three large, round spots on the sides in *E. candens* and *E. hulstaerti* and a midlateral stripe in *E. papilio* vs. bars generally present or a combination of bars and spots in *Baka* or a single, thin stripe in *B. nounensis*). *Enteromius candens*, *E. hulstaerti*, and *E. papilio* also have a unique shape among African barbs, with short, oval bodies similar to pupfishes (Cyprinodontiformes) vs. the diamond shape of *Baka*. *Enteromius hulstaerti* was found to be sister to *Barboides* in the phylogenetic analysis suggesting that this group of barbs may also hold separate generic status, and a name is potentially available (*Nicholsopuntius* Pellegrin 1933).

The new genus, *Baka*, can be found throughout the Lower Guinea ichthyological province, from the Sanaga River in Cameroon southeastward to the Congo River in Democratic Republic of Congo. Despite belonging to different river basins, our genetic analysis shows no distinction between the *Baka jae* collected in Gabon and Cameroon. Stream capture between the Dja, Nyong, and Ogooué Rivers may explain this genetic similarity, but this hypothesis must be further explored. Unfortunately, genetic materials for this genus are desperately lacking. This is especially critical for validating *B. condei*,

as the species is described from few specimens and many in poor condition (Mahnert and Géry, 1977; Van den Bergh and Teugels 1998) as well as *B. parajae* and *B. nounensis*.

Membership of Baka

A total of four species are recognized in *Baka: Baka condei, B. jae, B. nounensis,* and *B. parajae*; however genetic material exists only for *Barbus jae*. Given the overlap of the sampled populations and species in morphospace, it is likely that that some of these species are synonyms. Van den Bergh and Teugels (1998) recognized the incredible variation of *Baka* throughout its distribution and noted that morphological variation is not consistent with geographical proximity. This may be due to a plastic response to the environment rather than speciation. In characterizing the variation in shape of *B. jae*, Van den Bergh and Teugels (1998) discuss Type I and Type II body shapes. As seen in the PCA, *B. jae* has a range of body shapes from narrow to kite-shaped, and a specimen identified as likely Type I falls on that extreme of PCA1 (Figure 5).

Our genetic data show Gabonese and Cameroonian specimens are genetically similar, and it is very possible that morphologically distinct species are genetically identical. Member taxa of *Baka* are rare in academic collections, and genetic specimens more so due to the inaccessibility of many regions (Hayes & Armbruster, 2017). Collection of these species – as voucher specimens as well as tissue collections – is needed to understand the variation across the genus. With further excursions into the Lower Guinean ichthyoprovince and surrounding areas, genetic material should become more readily available, leading to a better understanding of the evolutionary relationships among these barbs.

Conclusion

The small, African, diploid barbs are undergoing renewed taxonomic interest (Hayes & Armbruster, 2017; Schmidt, Bart, & Nyingi, 2017; Paul H Skelton et al., 2018; Yang et al., 2015). Although the deeper relationships of these fishes are poorly resolved, identification of monophyletic subgroupings can aid in understanding the generic-level relationships of *Enteromius*-like taxa. The genus *Baka* described herein is recovered as a distinct molecular lineage with a unique set of morphological characteristics uniting member taxa. This is a small step in tackling the taxonomy and systematics of *Enteromius*-like barbs, but a necessary forward progression for the group.

Figures

Figure 1. *Baka jae* syntype BMNH 1903.7.28.237-239 from the Ja River at Bitye, Cameron, Africa. Photograph provided by the Trustees of the Natural History Museum, London

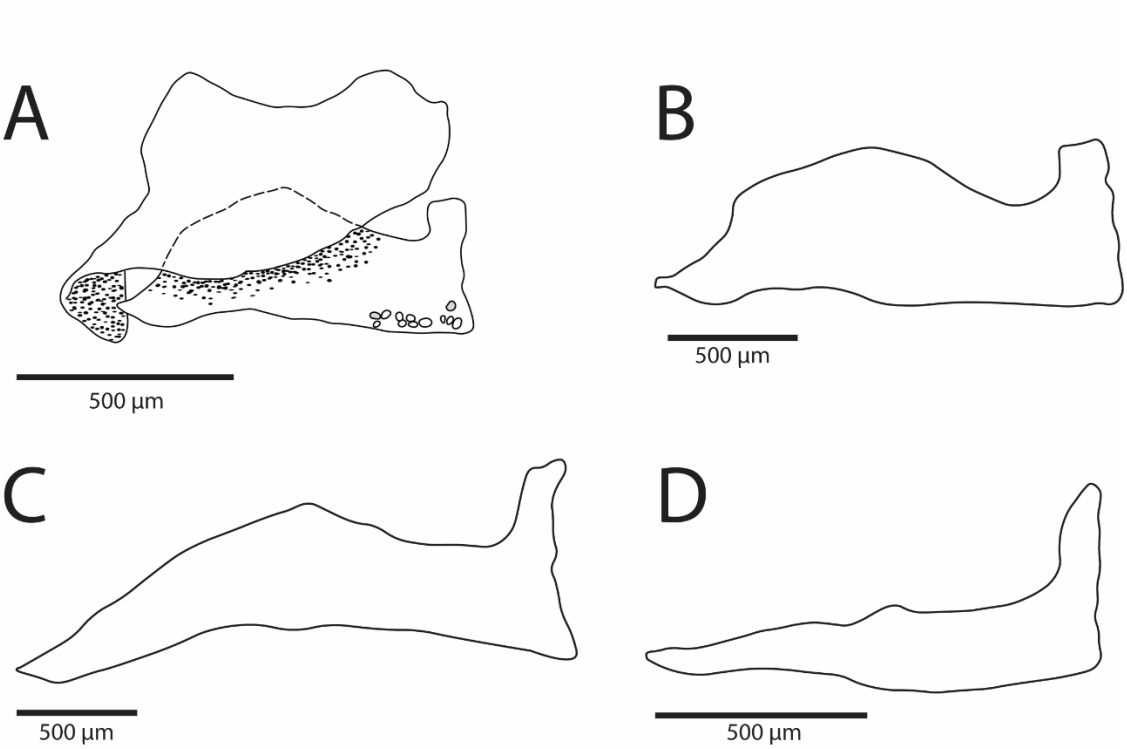

Figure 2. A) Line drawing of premaxilla as it articulates with the maxilla of *Baka jae* AUM 58060. B) *Baka jae* right premaxilla in lateral view AUM 58060. *Baka jae* has a short, chunky premaxilla with pedicel as high as the rostral process vs. thin, elongated, and a short rostral process typical of other barbs. C) *Barboides gracilis* premaxilla (redrawn from Conway et al. 2017) D) *Clypeobarbus pleuropholis* premaxilla AUM 51242.

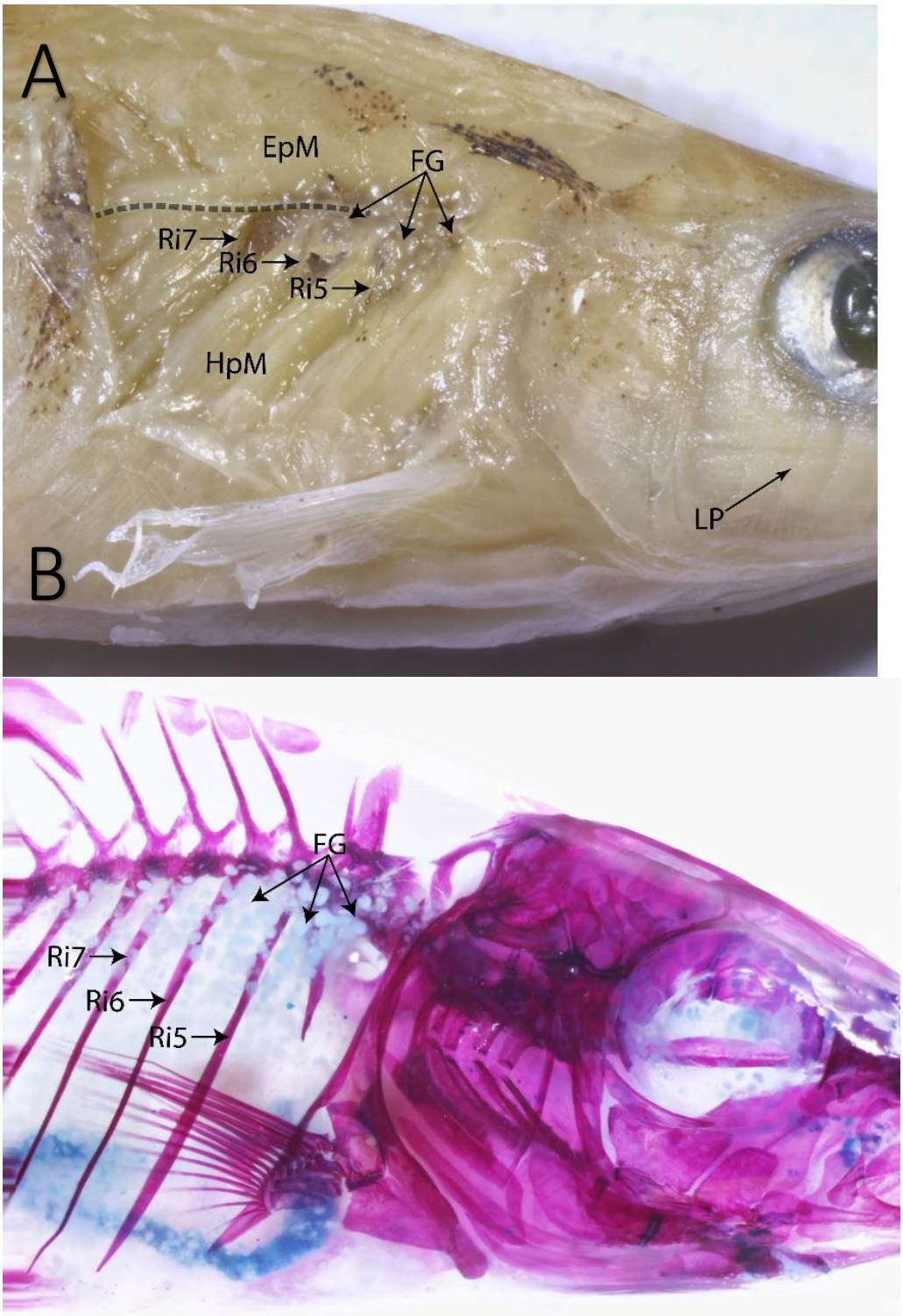

Figure 3. A) Pseudotympanum of *Baka jae* specimen AUM 58060. The top layer of skin has been cut and reflected to expose the variation in the musculature. Dashed line indicates the lateral line sensory canal. B) Cleared and stained specimen of *Baka jae* AUM 58060. No humeral organ is visible in either the dissected or cleared and stained views. Abbreviations: EpM, expaxial musculature; FG, fat globules; HpM, hypaxial musculature; LP, lateral sensory pores; Ri5-7, ribs 5-7.

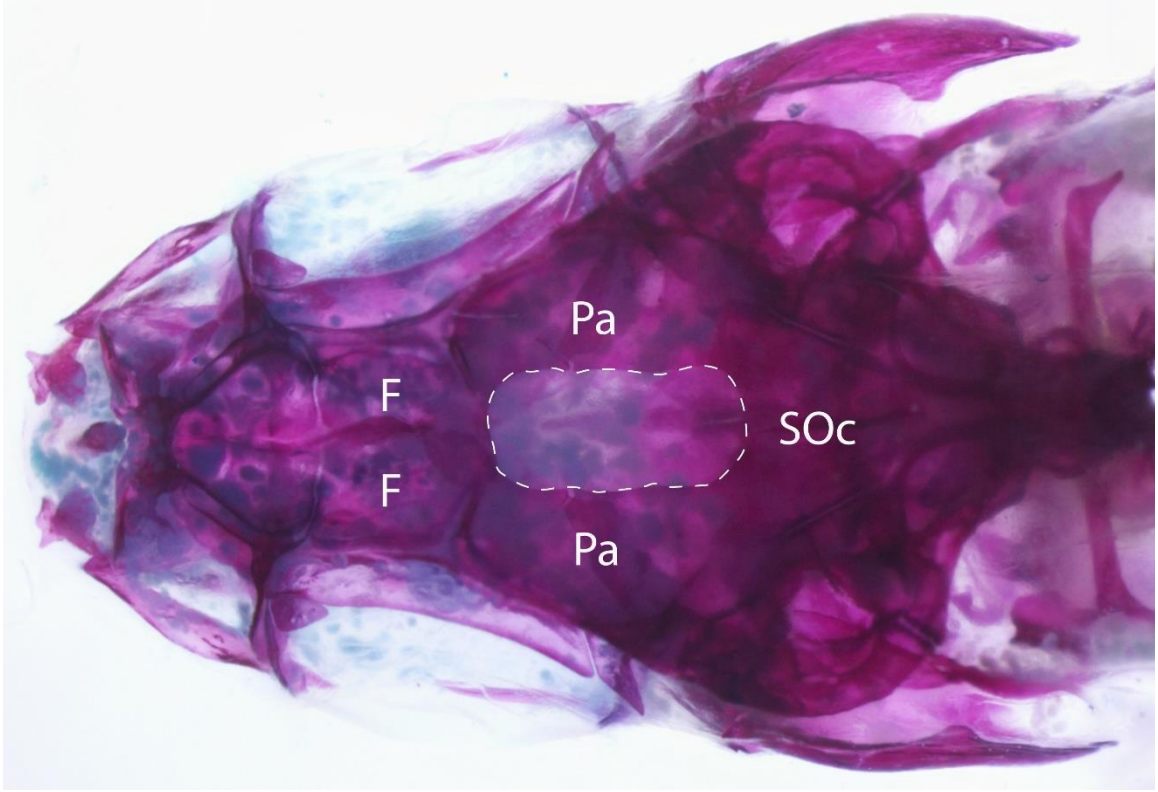

Figure 4. Cranial fontanelle of *Baka jae* AUM 58060 from the dorsal view. Specimen has been cleared and stained. Fontanelle is indicated by dashed lines. Abbreviations: F, frontal; Pa, parietal; SOc, supraoccipital.

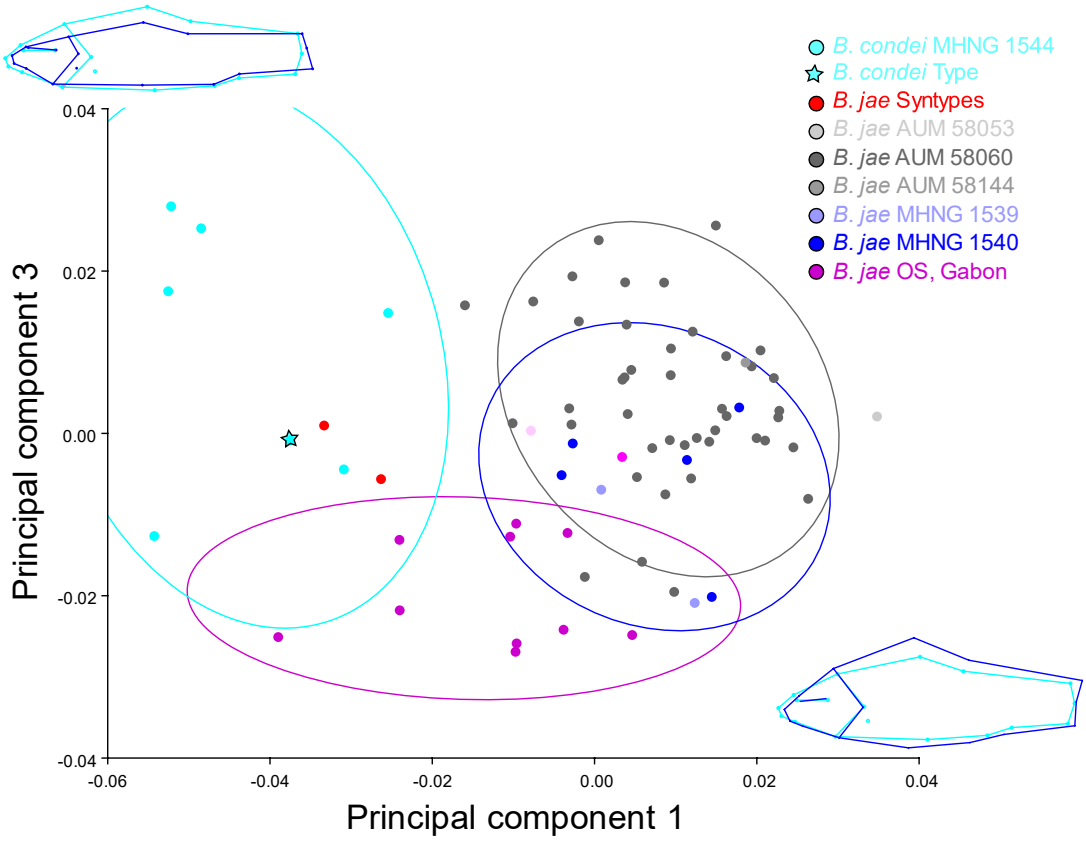

Figure 5. Principal Component Analysis (PCA) of *Baka jae* from Cameroon and Gabon. Specimens are colored by collection site. 95% confidence ellipses are overlain on the graph. Wireframes of principal axes are shown with light blue representing the consensus shape, and dark blue representing the most positive extreme of the axis.

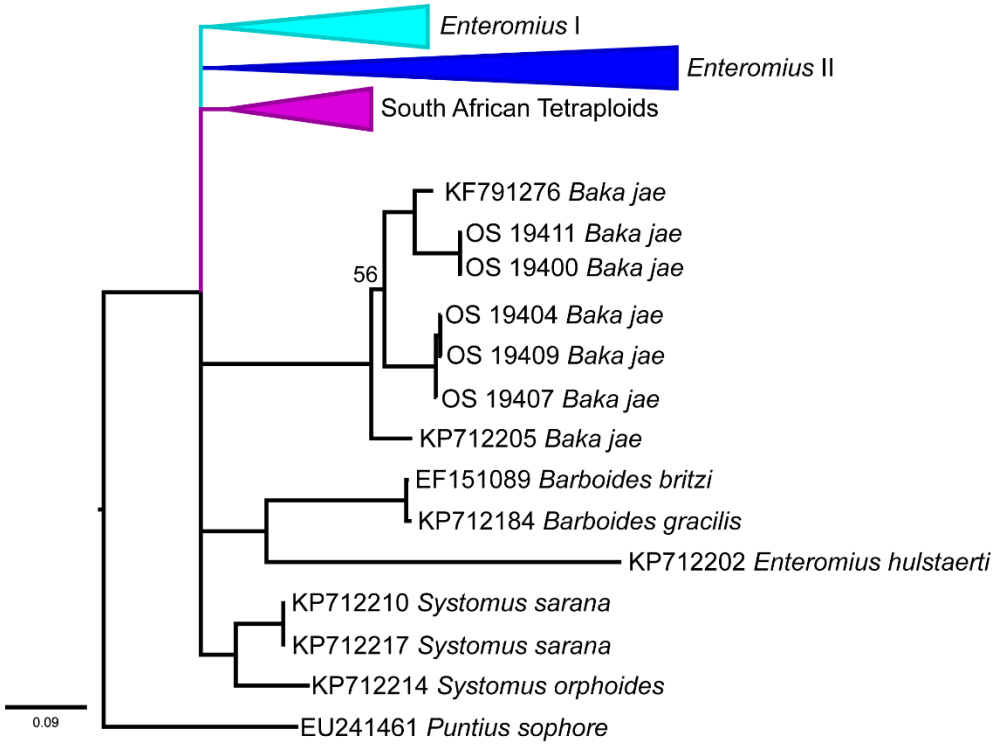

Figure 6. Fifty-percent majority rule consensus tree from Bayesian Inference of cytochrome b sequences. Each node represents a posterior probability >90% unless otherwise noted

Chapter 4 – Conservation Genetics of the Broadstripe Shiner, *Pteronotropis euryzonus*, an Endemic to the Middle Chattahoochee River

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Abstract

The Broadstripe Shiner, *Pteronotropis euryzonus* (Suttkus, 1955), is a freshwater minnow endemic to a small area that includes the tributaries of the middle and lower Chattahoochee River in Alabama and Georgia. Populations of *P. euryzonus* appear fragmented because individuals have not been detected in the main channel. This suggests limited dispersal potential and low gene flow between populations, and previous studies have suggested multiple forms of *P. euryzonus* in the Chattahoochee. A total of 125 samples of *P. euryzonus* were collected for genetic analysis from twenty-three sites in eleven tributaries of the Chattahoochee River, and museum specimens were used for morphometric analyses. The mitochondrial genes cytochrome c oxidase I (COI) and cytochrome b (cytb) were used to assess genetic structure of *P. euryzonus* throughout its range. Geometric morphometrics were used to quantify shape variation among populations. Results suggest the presence of three genetically distinct populations: Northern, Pataula, and Southern, which also exhibit distinct morphologies. These populations support previous hypotheses of multiple forms of *P. euryzonus* in the Chattahoochee River. The presence of three genetically and morphologically distinct populations has significant conservation implications for *P. euryzonus*, which is currently

listed as imperiled in both Alabama and Georgia. Three distinct populations, a restricted range, and recent disturbance to gene flow from local infrastructure may necessitate further protections to prevent extirpation.

Introduction

Many political borders are defined by rivers, and economic and conservation decisions on either side of these borders can have massive implications for those who depend on the watershed. As has been demonstrated in the Water Wars between Alabama, Florida, and Georgia as well as the implications of drought in California, Nevada and Arizona and many other instances worldwide, conservation and water management must proceed in a way which benefits either side of a political border as well as the downstream neighbors impacted by the decisions. Additionally, decisions regarding water use must balance economic considerations with the health of streams and the organisms that inhabit them. When species are confined to limited areas, the chances of imperilment by resource development are high. In order to fully understand ecosystem function and evolutionarily significant units within species of limited range, we must understand the genetic diversity throughout the system.

The Chattahoochee River originates in northern Georgia and later forms the border between the states of Georgia and Alabama before eventually flowing through the panhandle of Florida and entering the Gulf of Mexico in the Apalachicola Bay. The Broadstripe Shiner, *Pteronotropis euryzonus* (Suttkus, 1955), is a freshwater minnow endemic to the tributaries of the lower Chattahoochee River in Alabama and Georgia, north to Upatoi Creek and south to Cedar Creek (Figure 4-1). South of Cedar Creek, *P. euryzonus* is replaced by *Pteronotropis grandipinnis* (Boschung & Mayden, 2004; Page

& Burr, 2011). Because *P. euryzonus* is not known from the main channel of the Chattahoochee River, their populations appear disjunct, and it is unknown if there is genetic connectivity between the various locations in which the fish are found (Johnston & Evans, 2002; Suttkus, 1955)

Little is known regarding the biology of the Broadstripe Shiner, including its dispersal capabilities. *Pteronotropis euryzonus* has a range restricted to Lee County through Houston County in Alabama, and from Talbot County to Clay County in Georgia (Alabama Natural Heritage Program, 2014; Georgia Department of Natural Resources, 2013). Due to its restricted distribution, the Broadstripe Shiner is considered an imperiled species at the S2 rank in the states of Alabama and Georgia (Alabama Natural Heritage Program, 2014; Georgia Department of Natural Resources, 2013). In addition to its small range, *P. euryzonus* is highly susceptible to catchment disturbances via anthropogenic impacts (Maloney et al., 2006), has a shrinking range (Mayden & Allen, 2015), and populations have recently been physically separated due to the installation of the Walter F. George dam in the 1960s. With a limited distribution in the Chattahoochee River system, and its susceptibility to anthropogenic impacts, it is important to assess the historical and present connectivity between populations of *P. euryzonus* and predict where disturbance impacts may be most felt, so that conservation efforts across both states can be effective.

Morphological variation within *Pteronotropis euryzonus* has been recognized since its original description (Suttkus, 1955) in which two races of *P. euryzonus* were described. The first race is the Uchee River race encompassing all samples collected from the Uchee River in Alabama. The Lower Chattahoochee tributaries as defined by Suttkus

(1955) include Abbie Creek, Hannahatchee Creek, Hatchechubbee Creek, Hichitee Creek, Hodchodkee Creek, Omussee Creek, and Upatoi Creek. These races were mainly distinguishable by number of anal rays with the Lower Chattahoochee River race having a higher average of anal rays; however, the ray counts overlapped between Uchee Creek and Lower Chattahoochee races. Suttkus (1955) suggested clinal variation in the number of vertebrae in populations throughout the Chattahoochee River, but did not analyze the data statistically.

A later study suggested *Pteronotropis euryzonus* and *P. grandipinnis* occur sympatrically in the Chattahoochee (Gilbert, 1969), which may lead to hybrid individuals. The presence of a hybrid zone might explain variation in the Lower Chattahoochee race seen by Suttkus (1955); however, later examination of putative hybrids revealed that the specimens could be assigned to either *P. euryzonus* or *P. grandipinnis*, and thus no hybridization was observed (Suttkus & Mettee, 2001).

In a report made to the State of Alabama, Johnston and Evans (2002) used the mitochondrial marker cytochrome b to investigate population-level variation of *P. euryzonus*. The study reported two genetically distinct lineages of *P. euryzonus*, a northern clade and southern clade, which did not correspond to the original Uchee and Lower Chattahoochee races described by Suttkus (1955). The northern clade recovered by Johnston and Evans (2002) included tributaries in and north from Russell County, AL and Chattahoochee County, GA as well as Hog Creek in Clay County, GA. The southern clade included specimens caught from Henry and Houston Counties in AL, and Stewart and Randolf Counties in GA. Johnston and Evans (2002) proposed the genetic distinctiveness of the groups might be a result of a cline; however low sampling in the

intervening areas, and the apparent extirpation of populations of *P. euryzonus* in tributaries in Barbour County, AL, prevented explicit support for this hypothesis. Mayden and Allen (2015) examine the phylogenetics of *P. euryzonus* based on the mitochondrial marker ND2, and found genetic variability to be low in the study. However, Mayden and Allen included only specimens from Russell County, AL.

Understanding the patterns of connectivity between populations of *Pteronotropis euryzonus* is imperative, especially considering its range within the Chattahoochee River system falls between the political borders of Alabama and Georgia, two entities whose protection of the species is critical to its survival. If genetic connectivity between populations is present, but limited, clinal variation across the range would be expected. Conservation methods associated with clinal variation could include stocking from throughout the range of the shiner and efforts to protect habitats between populations. If these fish have limited dispersal capability, then one would expect to see an isolation-by-distance effect as samples are compared downstream. Isolated populations would be genetically distinct, and conservation efforts would best be implemented at the tributary level, determined by each state. The most geographically distant populations would be expected to be genetically distinct from one another, but share haplotypes with intermediate sampling areas. Evidence of a cline may also be found in morphological characteristics similar to patterns described by genetic cline.

This study explores the range and genetic connectivity of *Pteronotropis euryzonus* populations, focusing on the intermediate areas as described by Johnston and Evans (2002) where clinal variation may be present both genetically and morphologically. This study also tests whether the Chattahoochee River acts as a barrier for dispersal of *P.*

euryzonus populations and also discusses the effects of the Walter F. George Dam and Lake Eufaula on dispersal. The hypothesis of clinal variation within populations of *P. euryzonus* in tributaries to the Chattahoochee River is also tested with dense genetic sampling in areas of uncertainty as well as analysis of whole-body morphological variation.

Materials and Methods

Sample Collection

Pteronotropis euryzonus was sampled from tributaries to the Chattahoochee River in Alabama and Georgia. These tributaries span the known range of the species with focused sampling effort in regions of uncertainty (e.g. Barbour County, AL). Specimens were captured using 1/8 inch mesh 10x6-foot seines and a Smith-Root backpack electrofisher. Fin clips were preserved in 95% ethanol. A total of 125 fin clips were taken from 22 collection localities (Figure 4-1) with a range from 1-22 individuals per site (Table 4-1). Each tributary was considered as a single population for a total of 11 subbasins. Voucher specimens were taken from previously undocumented localities and when mortality occurred due to handling. These specimens were anesthetized with MS-222, fixed in 10% formalin, and deposited into the Auburn University Natural History Museum, (AUM) in Auburn, Alabama (Table 4-2).

DNA Extraction, Amplification and Sequencing

Whole genomic DNA was extracted from fin clippings using an E.Z.N.A. Tissue DNA Kit (Omega BioTek Norcross, GA, USA). This study focused on two mitochondrial genes: cytochrome c oxidase subunit I (COI) and cytochrome b (cytb). Both genes were isolated by polymerase chain reaction (PCR) using previously developed and novel

primers and the following protocols. Amplification of COI used the following primers: Fish R2t1 (Ivanova, Zemlak, Hanner, & Hebert, 2007; Ward, Zemlak, Innes, Last, & Hebert, 2005) and VF2t1 (Ivanova et al., 2007; Ward et al., 2005). Amplification of cytb used L14724 (Irwin, Kocher, & Wilson, 1991) and a primer specifically developed for this investigation (H.Alt.Notropis, 5' CCTCGATCTTCGGATTACAAGAC 3'). The temperature profile for COI: initial denaturation of 94° C for 2 min, followed by 35 cycles at 94° C for 30 s, 52° C for 30 s, 72° C for 1 min, and a final extension of 72° C for 10 min. The temperature profile for cytb: initial denaturation of 94° C for 2 min, followed by 30 cycles of 94° C for 40 s, 52° C for 1 min, 72° C for 1 min 30 s, and a final extension of 72° C for 5 min. PCR products were size-verified on an 8% agarose gel through electrophoresis. PCR amplification did not work for both genes on all samples, although reactions were performed a second time for failed runs. Of all 125 fin clips, 104 successfully amplified for cytb and 114 successfully amplified for COI (Table 4-1). PCR purification and sample preparation for Sanger sequencing were performed at Beckman Coulter (Danvers, MA). Chromatographs from forward and reverse reads were assembled together, and assembled contiguous sequences were aligned and edited by eye using the program Geneious version 6.0.6 (http://www.geneious.com, Kearse et al., 2012). Due to inconsistencies in the sequencing process, the final alignments of COI and cytb did not include the entire gene.

Data Analysis

Haplotype networks were generated for COI and cytochrome b datasets using TCSv1.21 (Clement, Snell, Walker, Posada, & Crandall, 2002). This program estimates gene genealogies of the haplotypes present in the dataset through Statistical Parsimony

and outputs interconnected networks that include putatively missing haplotypes. Default settings were used including a 95% parsimonious branch connection limit.

Population-level genetic clustering and admixture was determined using the program BAPS6 (Corander & Marttinen, 2006; Corander, Marttinen, Sirén, & Tang, 2008; Corander & Tang, 2007). BAPS6 is a unique program that can accommodate the use of DNA sequences for population structure analysis without violating statistical assumptions due to the use of linked loci. A combined dataset was created using individuals for which both COI and cytb sequences were generated. Because the data were in DNA sequence form, they were first analyzed using the *Clustering with linked loci* option. An upper bound of 11 populations was given, based on the number of sampled tributaries to the Chattahoochee River. Results of the mixture analysis were then used as input in the population admixture analysis using *Admixture based on mixture clustering* option in BAPS6. This analysis determines the amount of genetic connectivity between previously recovered sub-populations. The minimum size of a population considered was set to one (Abbie Creek) and one reference individual was set for each population.

Phylogenetic analyses were performed separately on COI and cytb datasets, then again on a concatenated dataset. For each analysis, identical sequences of *Pteronotropis euryzonus* were excluded to limit the analysis to only unique haplotypes for each gene. Two outgroup taxa were included in the analysis: *P. hypselopterus* (COI: HQ579009; Cytb: HM224303) and *P. grandipinnis* (COI: KT334106; Cytb: KT335798). All individuals with both COI and cytb sequences were concatenated in Geneious v 6.0.6 (Biomatters), and unique haplotypes from the concatenated dataset were used in

phylogenetic analysis. Best-fit models of evolution for each gene and for the concatenated dataset were tested using jModelTest (Darriba, Taboada, Doallo, & Posada, 2012; Guindon & Gascuel, 2003). Bayesian Inference (BI) was run in MrBayes 3.2.2 on CIPRES Science Gateway (Miller et al., 2010; Ronquist et al., 2012). The BI analysis had two runs with four chains each for 10,000,000 generations and was sampled every 1,000 generations. A 25% burnin was calculated using the sump option and a 50% consensus tree was created.

The marker COI has been used for genetic barcoding of species, and accurate identification can be determined if the mean distance to the nearest neighbor is higher than the maximum intraspecific distance (April, Mayden, Hanner, & Bernatchez, 2011). To test whether populations of *Pteronotropis euryzonus* represent distinct species in the Chattahoochee River, individuals of *P. euryzonus* in the Northern + Pataula clade were grouped and their genetic distance compared to the southern individuals (Omussee, Foster, and Abbie creeks) using the Kimura 2-parameter model in MEGA v.6.08 (April et al., 2011; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

Geometric Morphometrics

Geometric morphometric analysis to quantify variation in whole body shape was performed on specimens vouchered at AUM (Table 4-2). Specimen totals by clade are Northern (43), Southern (15), Pataula (6); number of specimens was generally limited to five from each lot examined if there were greater than five specimens in the lot, and adults when possible. Specimens were photographed in lateral view with a 60 mm lens attached to a Nikon D5300 digital SLR camera. Photographs were digitized using TpsUtil 1.46 program (F. J. Rohlf, 2004) and landmarked using TpsDig 2.16 (F. James Rohlf,

2017). Eighteen homologous landmarks that describe biologically important shape variation in cypriniform fishes were used (Armbruster, 2012). The tps file was converted to an NTSPC file in TpsUtil and then analyzed in MorphoJ (Klingenberg, 2011). Landmarks were aligned with a Generalized Procrustes Analysis, the data were checked for outliers, a covariate matrix constructed, and Principal Components Analyses (PCA) and Canonical Variates Analyses (CVA) performed. Clades delimited by the phylogenies were used to assign specimens to groups in the CVA *a priori*. Permutation tests for pairwise distances were performed with 10,000 replications in the CVA to determine if the clades were significantly different from one another. Principal Components and Canonical Variates were plotted against standard length to determine if there was a relationship to size.

Results

Molecular Analyses COI

Sequence data were obtained from 114 individuals of *Pteronotropis euryzonus* for COI. The total fragment length of the gene was 544bp (GenBank Accession Nos: KT334013 - KT334127). The genetic distance among the ingroup was 0.3%. A total of 13 unique haplotypes were present in the dataset (Figure 4-2).

Haplotypes were highly conserved in the COI dataset as demonstrated by the two haplotype networks (Figure 4-2B). One network consists of an individual from Pataula Creek, while all other individuals cluster in a single network. Pataula Creek individuals (except for the single disconnect) share the same COI haplotype. Populations from Hog Creek in the Cemochechobee River to the north share the same haplotype or a single base pair change, and are designated as the Northern populations. Southern populations

(Omussee, Foster, and Abbie creeks), are separated from the northern cluster by two putatively missing haplotypes.

Bayesian Inference of the 13 unique haplotypes recovered from the COI dataset recovers a monophyletic *Pteronotropis euryzonus*. Relationships among the Northern, Pataula, and Southern populations were poorly supported; however, we recover a distinct southern clade, with 100% posterior probability support (Figure 4-2A).

Using the recovered phylogeny as a guide, we tested the genetic distances between the Northern + Pataula populations vs. the Southern populations to determine genetic diversity between the populations. The results of the genetic distance analysis using COI show higher distance to the nearest neighbor (Northern + Pataula to Southern) than within-group variation in the clusters. Each had 0.01% genetic distance within the group, and the genetic distance between Northern + Pataula and Southern groups was 0.07%, indicating these populations are genetically distinct from one another.

Molecular Analyses Cytb

Cytochrome b analyses included sequence data from 104 individuals of *Pteronotropis euryzonus* with a total length of 1082bp (Genbank Accession Nos: KT335721 - KT335824). The cytb dataset possessed 26 unique haplotypes, more than recovered with COI. The genetic distance among the ingroup was also higher with cytb than with COI with cytb genetic distance being 1.0%.

Two disconnected haplotype networks were recovered for the cytochrome b analysis (Figure 4-3B). These two networks have similar patterns to the COI haplotype analysis. We recover two clusters: a Northern and Southern cluster, with the Northern comprising Hog Creek subbasin and all other sampled tributaries to the north, excluding

Pataula Creek (Figure 4-1). The Southern cluster includes haplotypes from Omussee, Foster, and Abbie creeks as well as Pataula Creek. Although Pataula Creek haplotypes group with the Southern cluster, the haplotypes are separated by seven hypothetically missing haplotypes, and may be considered distinct.

Bayesian Inference of the 26 unique haplotypes resulted in monophyletic *Pteronotropis euryzonus* with three distinct clades: a northern clade containing Hog Creek and all tributaries north of Lake Eufaula, a unique Pataula clade, and a southern clade containing Omussee, Foster, and Abbie creeks (Figure 4-3A). Although each of the three clades is well-supported (≥99% posterior probability), the relationships among the clades are uncertain.

Concatenated Dataset

The concatenated dataset consisted of 82 individuals of *Pteronotropis euryzonus* with total of 1626bp. Population structure analysis estimated the number of populations (k) to be 4 (3 populations of *P. euryzonus* and 1 population of *P. grandipinnis*). Population admixture analysis, which examines the amount of gene flow between designated populations, recovered no statistically significant admixture between populations (Figure 4-4).

Thirty-four unique haplotypes were recovered in the concatenated dataset, and these were used to infer the phylogeny between the represented populations. Bayesian Inference of the concatenated dataset revealed three distinct clades, Northern, Pataula, and Southern, corroborating the results of the population structure and independent gene analyses. The combination of COI and cytb yielded a more robust phylogeny with each

clade well-supported (≥99% posterior probability), and places the Pataula clade sister to the southern clade with a 99% posterior probability (Figure 4-4).

Geometric Morphometrics

The PCA did not corroborate the geographic or phylogenetic patterns recovered in the molecular analysis. Members of each clade did not exhibit discrete lateral morphology. Principal Component 1 was largely correlated with body and head depth and head shape. Plotting PC1 vs. standard length yielded a significant positive relationship ($p=<0.0001$, $R^2=0.4506$) indicating that body and head depth increase with size, and does so similarly across the three populations. Further PCs did not describe meaningful variation in shape, and were not correlated with size.

The CVA separated the three clades (Figure 4-5). Pairwise differences in the permutation test of Procrustes distances among groups showed Northern and Southern to be significantly different ($p<0.0001$), Pataula and Southern significantly different ($p=0.0137$), but Northern and Pataula not significantly different ($p=0.6867$). P-values from permutation tests for Mahalanobis distances among groups recover all three populations as statistically significantly different from one another ($p=<0.0001$). Northern + Pataula differed from Southern on CV1, which is mainly body depth (Figure 4-5, inset), and Northern and Pataula differed on CV2, which is mainly subtle differences in head shape and dorsal-fin placement (Figure 4-5, inset).

Discussion

This study recovered three genetically distinct populations of *Pteronotropis euryzonus* in the Chattahoochee River: 1) a Northern population including Hog Creek and

all tributaries to the north except 2) Pataula Creek, which is recovered as a genetically distinct population, and 3) a Southern population which includes Omussee, Foster, and Abbie creeks. Morphologically, these three populations are differentiated; however, Pataula Creek is not statistically distinguishable from the Northern population in a permutation test of Procrustes differences in CV1 vs. CV2; however, a permutation test of univariate Mahalaobis distances on CV2 did provide significant differences. Although two forms of *P. euryzonus* in the Chattahoochee were hypothesized by Suttkus (1955), dissimilar patterns of variation are present in this analysis with Uchee Creek specimens clustering in both morphospace and genetics with specimens in the Northern population. This study confirms the report of Johnston and Evans (2002) that *P. euryzonus* in Omussee and Foster (and, by extension, Abbie) creeks are genetically divergent from other populations of *P. euryzonus* in the Chattahoochee River. Individuals from the southern populations are more genetically similar to one another than the northern populations in haplotype network, phylogenetic, and morphometric analyses.

This study also expands known extant localities of the Broadstripe Shiner. Johnston and Evans (2002) suggested that *P. euryzonus* was extirpated from Barbour County, AL; however, 22 specimens in this study were collected from Barbour Creek in June 2013. These individuals from Barbour County are genetically and morphologically assigned to the Northern population. Thus, the hypothesis that clinal variation exists in Barbour County populations can be rejected.

The genetically unique population of the Pataula Creek drainage is interesting because Hog Creek, which is south of Pataula Creek and the Walter F. George Dam, shares haplotypes with the remainder of the Northern populations. The presence of

Northern haplotypes in Hog Creek is not surprising, considering the specimens of Barbour County are also found to be in the Northern populations. Hog Creek may have been populated by Northern population individuals from Barbour County in the recent past, perhaps after the dam was installed. The small tributaries between Barbour County and the mouth of Pataula Creek may have historically been inhabited by *P. euryzonus*, but no longer have suitable habitats and are inundated with high, slack waters as an effect of the installation of the dam. Based on the rate of evolution of the mitochondrial genes in this study, it appears that the Patuala Creek population was diverging from the Northern population before dam construction. Although the dam may eventually have long-term effects on populations of *P. euryzonus,* the genetic differentiation recovered in this study suggests divergence before dam installment, and its presence can only accelerate the genetic isolation of the group.

The Northern population, Pataula Creek, and the Southern population of *P. euryzonus* are found in geologically different regions. Although all populations are found in the ecoregion III, Southeastern Plains (Griffith et al., 2001), a finer scale analysis of the region reveals geologic variation corresponding to the populations recovered in this analysis. The geology of streams between the Fall Line and the Walter F. George Dam is fairly homogenous, made of clay, mud and sand. The Walter F. George Dam was built at a geologic transition zone on the Clayton Formation (Stewart, 1973), which is a geologic feature of Pataula Creek. With a unique geology compared to northern tributaries, Pataula Creek populations of *P. euryzonus* may have diverged, with specific adaptations to the more alkaline properties of the Clayton Formation sediments. Downstream of the Clayton Formation, the geology shifts from clay, mud, and sand to limestone, a characteristic of

the Dougherty Plain (Griffith et al., 2001) creating a more alkaline habitat for the Southern populations to inhabit. Fewer tributaries to the Chattahoochee River are found in this region, and may add another layer of isolation for the Southern populations. The lack of tributaries in this small region represents an area where conservation efforts may focus on maintaining the genetic lineage of the southern populations as well as maintaining suitable habitats for additional populations to establish in intervening tributaries.

Clinal variation does not appear to exist across populations of *Pteronotropis euryzonus*. If Pataula were a remnant of a clade between Northern and Southern tributaries, we would predict that other intermediate streams (Barbour, Hog, and Abbie creeks) would have similar haplotypes; however, our genetic analyses recover Barbour and Hog creeks to be genetically similar to all other Northern tributaries, and Abbie Creek is genetically identical to individuals from the southern clade. Abbie Creek's shared genetics with Southern populations is based on a single individual from the tributary, and historical collections from this site suggest that *P. euryzonus* may not be completely established in Abbie Creek. If Abbie Creek is a reservoir for individuals migrating within the Chattahoochee River, the proximity of its mouth to Omussee and Foster creeks increases the likelihood of recruitment from those streams. With no shared haplotypes between the Northern, Southern, and Pataula populations, the hypothesis of a cline is rejected.

Increased genetic sampling of the species in these tributaries is needed to further clarify the relationships between the recovered groups. The markers used in this study, COI and cytb, are mitochondrial markers that exclusively track maternal lineages, and

evolve at a slower rate than some nuclear markers (i.e. microsatellites) that may give information on more recent gene flow. A future study employing rapidly evolving markers would aid in determining if uni- or bi-directional gene flow is happening between populations to assess dam effects post-construction.

The presence of three distinct populations of *Pteronotropis euryzonus* in the Chattahoochee River Basin demarcated by unique genetic lineages and supported by geometric morphometric analysis has conservation implications for the lower drainages of the Chattahoochee River for both Alabama and Georgia. The Northern, Pataula, and Southern populations appear to have been diverging before construction of the Walter F. George dam near Eufaula, AL.; however, the dam and its effects may now be acting as a barrier to upstream dispersal of *P. euryzonus*, a behavior indicated by the invasion of singletons into Abbie Creek from Omussee and Foster creeks. In the lower drainages, there are fewer tributaries for populations to temporarily inhabit and disperse through, an effect that will increase post-dam construction as dam effects such as changes to seasonal flow and temperature patterns as well as habitat degradation continue to take place downstream.

We also recognize Pataula Creek as a region that may already be experiencing impacts on its genetic diversity due to the same anthropogenic effects of the Walter F. George dam. Although the Pataula population was isolated genetically prior to dam construction, the dam and Lake Eufaula may increase this isolation by prohibiting colonization from nearby tributaries. The Pataula population should be specially monitored in Georgia to ensure that this genetically distinct lineage does not go extinct. Limited genetic differentiation is seen within clades, indicating that gene flow across the

Chattahoochee River had historically been extensive; however, gene flow within this region is likely decreasing with anthropogenic change.

The Chattahoochee River Basin is under further threat from dewatering. The states of Alabama, Georgia, and Florida have been battling over access to waters from the Chattahoochee for human development and biodiversity conservation. This study further underscores the importance of the middle Chattahoochee River Basin as a unique set of habitats that must be conserved and maintained to protect the aquatic organisms inhabiting them. Although populations of the Broadstripe Shiner have been proposed as being a single conservation concern (Johnston and Evans 2002), we find more genetic structuring and no evidence of clinal variation across the species range. Therefore, efforts to conserve populations of the Broadstripe Shiner in the Chattahoochee River should not focus on preserving one portion of the species' range since this would not necessarily preserve the genetic diversity present in the species. Based on our analyses, we suggest three conservation units are present within *P. euryzonus*: 1) the northern clade, representing the tributaries above the Walter F. George Dam, 3) Patuala Creek clade a set of unique haplotypes in Georgia, and 3) the southern clade, which includes the tributaries south of the Walter F. George Dam to Cedar Creek. Furthermore, we suggest future development in the Chattahoochee River basin recognize the need to preserve genetic diversity of the fishes of the region, and investigate the finer-scale patterns of gene flow across the range of *P. euryzonus*.

Conclusion

Locally, we recognize three evolutionarily significant units within the imperiled Broadstripe Shiner: the northern, southern, and Pataula clades. The southern and Pataula

clades are of particular importance as they are highly restricted and should be considered vulnerable (VU) under the IUCN categories (IUCN, 2012). The northern population is likely stable, but there has been substantial degradation of streams within its range and it should be considered near threatened (NT). Future changes to the Middle Chattahoochee, whether directly such as the Walter F. George Dam, or indirectly through additional impoundments or water diversions upstream, can greatly impact the available habitats and genetic connectivity of *Pteronotropis euryzonus*. We recommend continued monitoring of each of these populations to ensure their survival, and will continue to examine the populations to determine if separate species status should be applied to the populations.

The Chattahoochee River is symbolic of anthropogenic changes occurring throughout the world. It is experiencing the major stressors to aquatic life including urbanization, impoundment, dewatering, increased turbidity, deforestation, and intensive agriculture. In particular, dam construction for hydroelectric power has been on an increase world-wide, particularly in developing countries (Zarfl, Lumsdon, Berlekamp, Tydecks, & Tockner, 2014). Despite the ichthyofauna of the United States being among the best studied in the world, significant variation within a species with a very small range has been detected in this study. What had been a single species of concern, is now three separate populations of concern, one of which is known from a single small stream.

As environmental stressors increase worldwide, it is imperative that ecological impact statements include studies of genetic diversity within impacted species. There is considerable chance that even wide-ranging species contain cryptic diversity that can be detected with genetic techniques. Dams are often placed on geological features that

demarcate important geological barriers between populations, and the survival of species outside of impacted areas may not be enough if genetic diversity is not being conserved. Fishes, being the best studied of aquatic organisms (Abell et al., 2008), should particularly be examined in environmental impact statements, and they can have significant genetic diversity even within a restricted range. An integrated approach, as demonstrated here, using genetics, morphology, and biogeography should be used to distinguish evolutionary- and conservation-significant units.

Figures

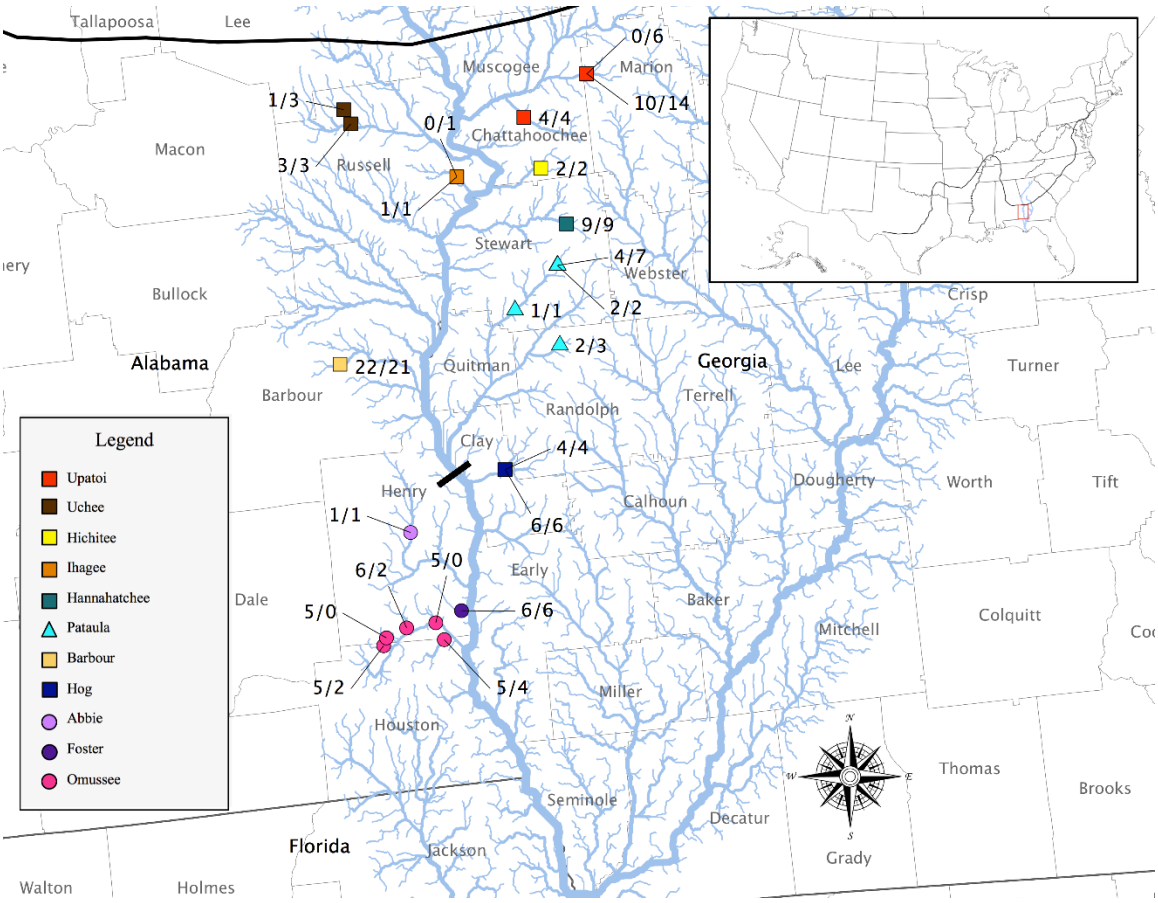

Figure 4-1. Map of collection localities in Alabama and Georgia tributaries to the Chattahoochee River. Each sampling locality is designated by a shape corresponding to the clades discussed in the text. Color represents the drainage as indicated in the legend. The Walter F. George dam near Eufaula, AL is indicated by a dash. County boundaries and names are indicated in grey. Numbers at each site indicate number of individuals sampled for COI and cytb analysis, respectively.

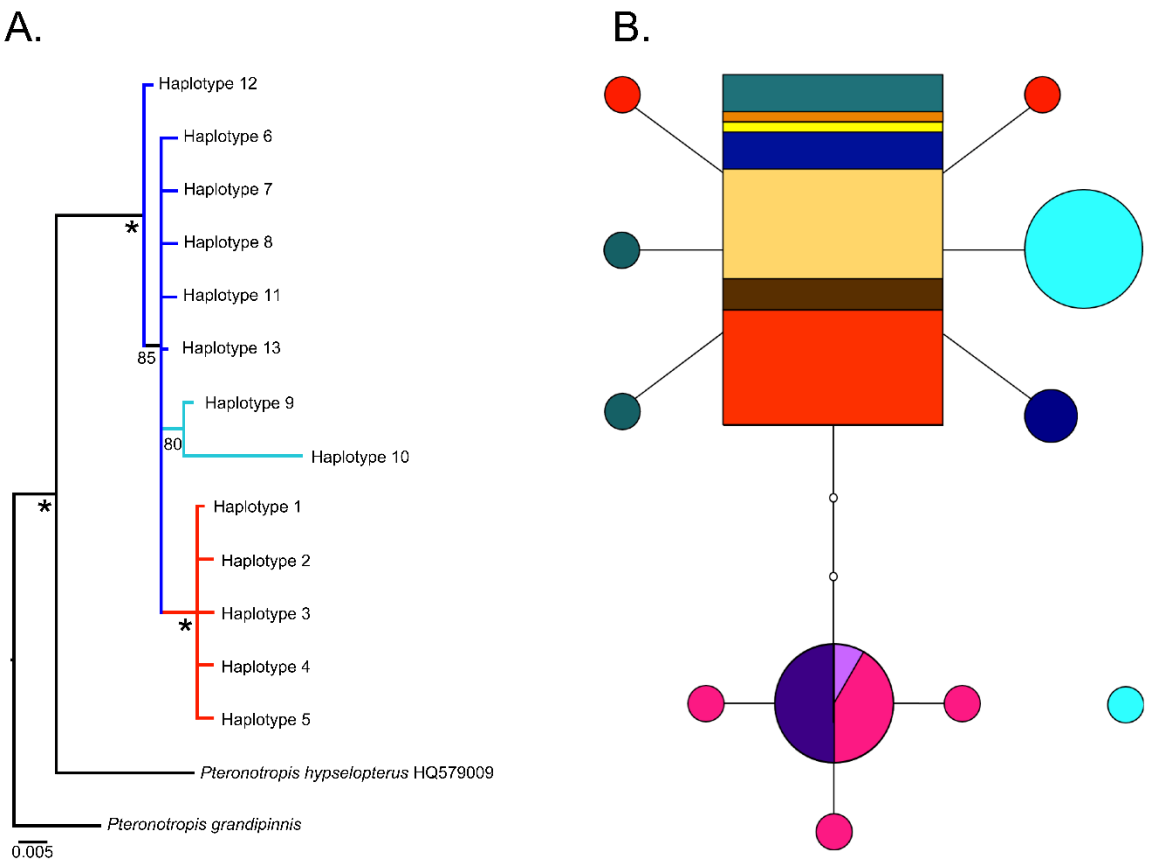

Figure 4-2. (A) Fifty-percent majority rule consensus tree from Bayesian analysis of 13 unique haplotypes of COI sequences. Values at nodes refer to posterior probabilities as a percentage, with asterisks indicating a posterior probability ≥90% (B) Haplotype network of COI across the 11 sampled tributaries. Each circle represents a unique haplotype, with circle size corresponding to frequency. A rectangle represents the inferred ancestral haplotype according to TCS analysis. Open circles represent hypothetically missing haplotypes. Coloration of haplotypes corresponds to tributary sampled as shown in Figure 1. COI analysis recovered two separate networks with all sampled individuals in one network and a single individual from Pataula Creek comprising a separate network.

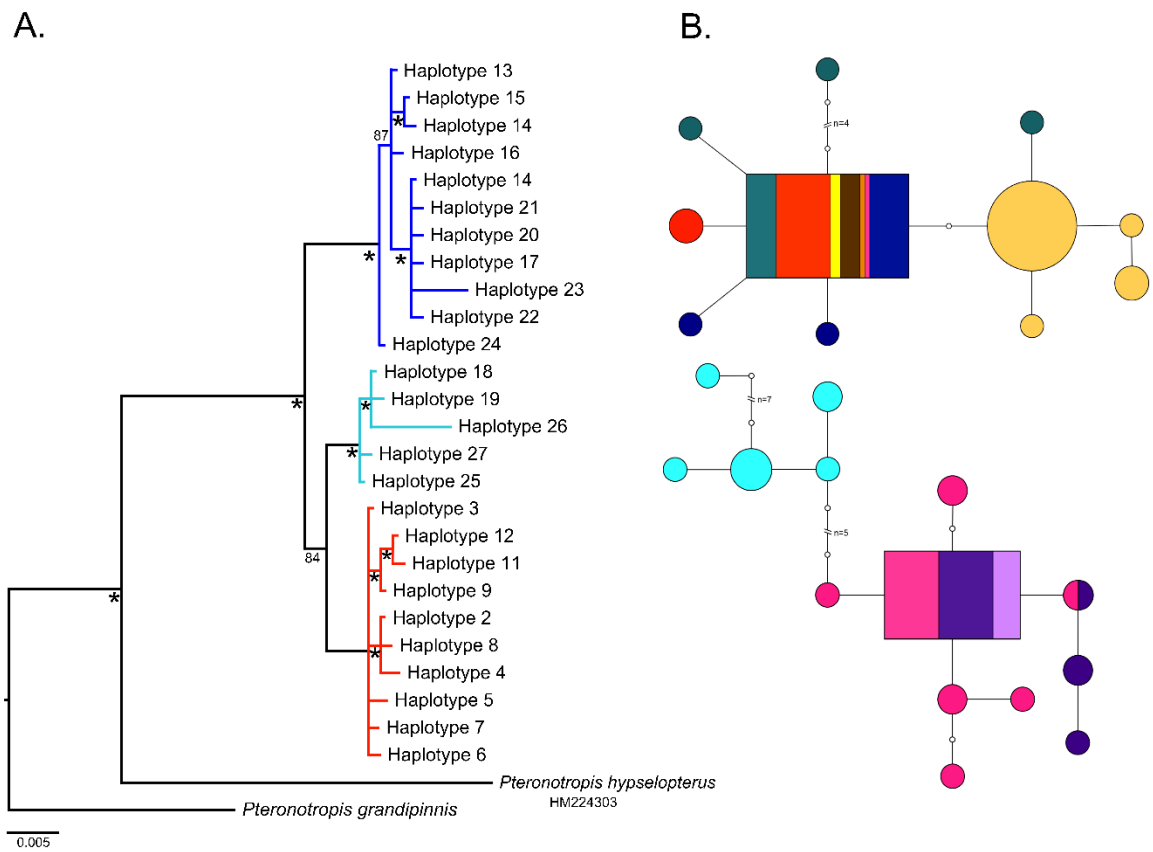

Figure 4-3. (A) Fifty-percent majority rule consensus tree from Bayesian analysis of 26 unique haplotypes of cytb sequences of *Pteronotropis euryzonus*. Values at nodes refer to posterior probabilities as a percentage. (B) Haplotype networks of cytb across the 11 sampled tributaries. Each circle represents a unique haplotype, with circle size corresponding to frequency. Rectangles represent the inferred ancestral haplotype according to the TCS analysis. Open circles represent hypothetically missing haplotypes, and numerous missing haplotypes are truncated by double lines and number of missing haplotypes removed are indicated. Coloration of haplotypes corresponds to tributary sampled as shown in Figure 1. Analysis recovered two separate networks: one with all tributaries north of Hog Creek and a Pataula Creek + southern tributaries network.

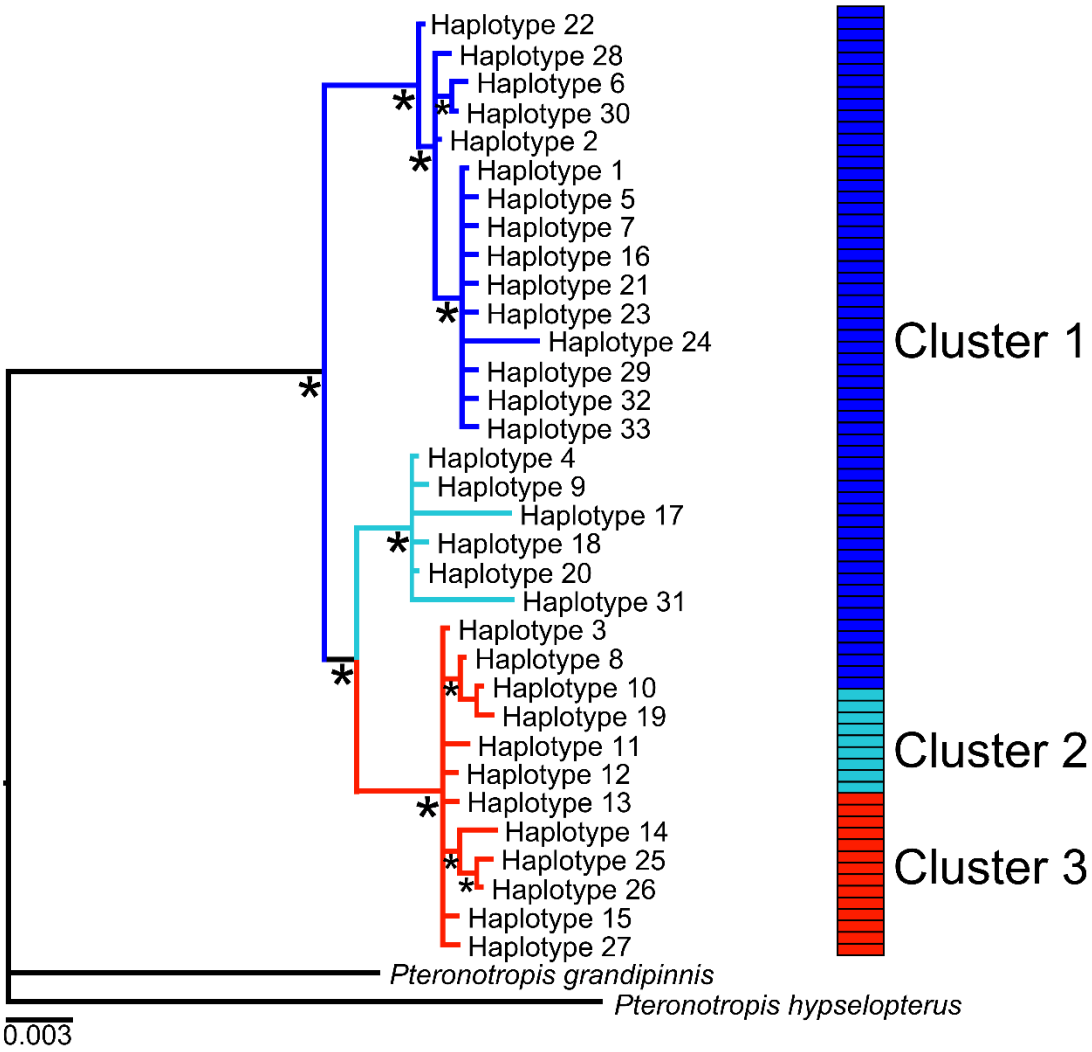

Figure 4-4. Fifty-percent majority rule consensus tree from Bayesian analysis of 34 unique haplotypes recovered from the concatenated dataset of COI and cytb sequences. Values at nodes refer to posterior probabilities as a percentage. Results from the BAPS6 admixture analysis are indicated by the colored bar. Colors correspond to recovered populations (K=3) and the assignment of individuals to each population. Each column represents an individual from the concatenated analysis.

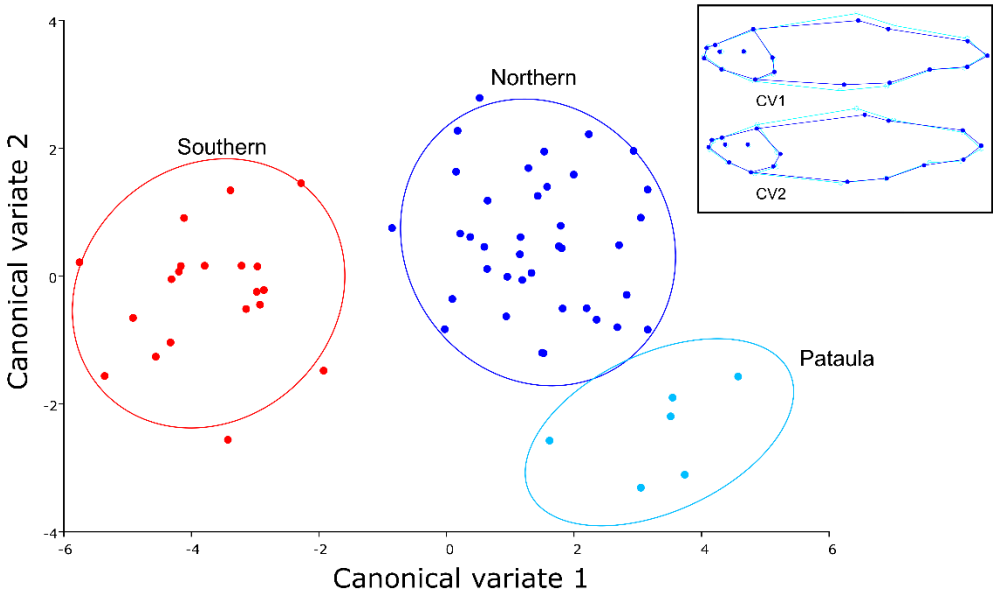

Figure 4-5. Canonical Variate Analysis of populations of *Pteronotropis euryzonus*. Northern, Pataula, and Southern populations were designated *a priori* for the analysis. Populations are enclosed by a 90% confidence ellipse, and are labeled with their *a priori* designation. Inset shows the wireframes of principal axes of variation in the Principle Components and Canonical Variate analyses. Light blue wireframe is the consensus and dark blue wireframe is the most positive extreme.

Tables

Table 4-1. Collection locations, GenBank Accession numbers, and unique haplotype identifiers for genetic specimens of *Pteronotropis euryzonus* used in this study.

Latitude	Longitude	Tissue Voucher	GenBank Accession Number		Haplotype Number		
			Cyt b	COI	Cyt b	COI	Concat.
31.53469	-85.21379	AUF2609	KT335753	KT334052	3	2	3
31.89887	-85.34025	AUF1171	KT335791	KT334099	13	6	2
		AUF1172	KT335790	KT334098	14	6	6
		AUF1173	KT335789	KT334097	13	6	2
		AUF1174	KT335788	KT334096	13	6	2
		AUF1175	KT335787	-	13	-	-
		AUF1176	KT335786	KT334095	14	6	6
		AUF1179	KT335785	KT334094	13	6	2
		AUF1180	KT335784	KT334093	15	6	30
		AUF1181	KT335783	KT334092	16	6	28
		AUF1182	KT335782	KT334091	13	6	2
		AUF1183	KT335781	KT334090	13	6	2
		AUF1184	KT335780	KT334089	13	6	2
		AUF1185	KT335779	KT334088	13	6	2
		AUF1186	KT335778	KT334087	13	6	2
		AUF1187	KT335777	KT334086	13	6	2
		AUF1188	KT335776	KT334085	13	6	2
		AUF1811	KT335775	KT334084	13	6	2
		AUF1812	KT335774	KT334083	13	6	2
		AUF1813	KT335773	KT334082	13	6	2
		AUF1814	KT335772	KT334081	13	6	2
		AUF1815	KT335771	KT334080	13	6	2
		AUF1816	KT335770	KT334079	14	6	6
31.36097	-85.11121	AUF1134	KT335797	KT334105	11	2	2
		AUF1135	KT335796	KT334104	12	2	10
		AUF1136	KT335795	KT334103	12	2	10
		AUF1137	KT335794	KT334102	3	2	3
		AUF1138	KT335793	KT334101	3	2	3
		AUF1145	KT335792	KT334100	9	2	8
32.13435	-84.74280	AUF6448	KT335733	KT334028	22	6	16
		AUF6449	KT335732	KT334027	23	6	24
		AUF6450	KT335731	KT334026	24	6	22
		AUF6451	KT335730	KT334025	1	12	29
		AUF6452	KT335729	KT334024	1	6	1

Latitude	Longitude	Tissue Voucher	GenBank Accession Number		Haplotype Number		
			Cyt b	COI	Cyt b	COI	Concat.
		AUF6453	KT335728	KT334023	1	6	1
		AUF6455	KT335727	KT334022	1	6	1
		AUF6456	KT335726	KT334021	1	6	1
		AUF6457	KT335725	KT334020	1	13	33
32.25556	-84.79000	AUF1841	KT335758	KT334061	1	6	1
		AUF1842	KT335757	KT334060	1	6	1
31.64222	-84.96444	AUF1820	KT335769	KT334078	17	6	32
		AUF1821	KT335768	KT334077	1	6	1
		AUF1822	KT335767	KT334076	1	7	5
		AUF1823	KT335766	KT334075	1	6	1
		AUF1824	KT335765	KT334074	1	7	5
		AUF1825	KT335764	KT334073	1	6	1
31.64194	-84.96531	AUF6410	KT335752	KT334051	1	7	5
		AUF6411	KT335751	KT334050	20	6	23
		AUF6412	KT335750	KT334049	1	6	1
		AUF6413	KT335749	KT334048	1	6	1
32.25800	-84.99989	AUF1076	KT335799	KT334107	1	6	1
32.25861	-84.99972	AUF1846	-	KT334056	-	6	-
31.30505	-85.16143	AUF 1001	KT335824	KT334127	2	1	26
		AUF1005	KT335823	KT334126	3	2	3
		AUF1006	KT335822	KT334125	4	3	14
		AUF1007	KT335821	KT334124	3	2	3
		AUF1009	KT335820	-	3	-	-
31.30679	-85.31098	AUF1017	KT335819	KT334122	3	4	27
		AUF1018	KT335818	KT334121	3	2	3
		AUF1019	KT335817	KT334120	5	2	11
		AUF1020	KT335816	-	3	-	-
31.32241	-85.30189	AUF1037	KT335815	KT334119	6	2	15
		AUF1038	KT335814	KT334118	7	2	12
		AUF1039	KT335813	KT334117	5	2	11
		AUF1040	KT335812	KT334116	3	2	3
		AUF1041	KT335811	-	3	-	-
31.33824	-85.24996	AUF1054	KT335810	KT334115	3	2	3
		AUF1055	KT335809	KT334114	8	1	25
		AUF1056	KT335808	KT334113	3	2	3
		AUF1057	KT335807	KT334112	3	5	13
		AUF1058	KT335806	-	2	-	-

Latitude	Longitude	Tissue Voucher	GenBank Accession Number		Haplotype Number		
			Cyt b	COI	Cyt b	COI	Concat.
		AUF1059	KT335805	-	3	-	-
31.34206	-85.17735	AUF1068	KT335804	KT334111	3	2	3
		AUF1069	KT335803	KT334110	3	2	3
		AUF1070	KT335802	KT334109	3	2	3
		AUF1071	KT335801	KT334108	9	2	8
		AUF1072	KT335800	-	3	-	-
32.05111	-84.77750	AUF1836	KT335763	KT334066	18	9	4
		AUF1837	KT335762	KT334065	18	9	4
31.88583	-84.79444	AUF1843	KT335756	KT334059	19	9	18
		AUF1844	KT335755	KT334058	18	9	4
		AUF1845	-	KT334057	-	9	-
31.96898	-84.89438	AUF6414	KT335748	KT334047	18	10	31
32.05121	-84.77650	AUF6463	KT335724	KT334019	25	9	20
		AUF6464	KT335723	KT334018	26	9	17
		AUF6465	KT335722	KT334017	27	9	9
		AUF6466	-	KT334016	-	9	-
		AUF6467	-	KT334015	-	9	-
		AUF6470	-	KT334014	-	9	-
		AUF6471	KT335721	KT334013	27	9	9
32.39278	-85.24670	AUF1838	KT335761	KT334064	1	6	1
		AUF1839	KT335760	KT334063	1	6	1
		AUF1840	KT335759	KT334062	1	6	1
32.42370	-85.26002	AUF1850	KT335754	KT334055	1	6	1
		AUF1851	-	KT334054	-	6	-
		AUF1852	-	KT334053	-	6	-
32.43861	-84.64889	AUF1826	-	KT334072	-	6	-
		AUF1828	-	KT334071	-	8	-
		AUF1832	-	KT334070	-	6	-
		AUF1833	-	KT334069	-	6	-
		AUF1834	-	KT334068	-	6	-
		AUF1835	-	KT334067	-	6	-
32.36463	-84.81696	AUF6416	KT335747	KT334046	1	6	1
		AUF6417	KT335746	KT334045	1	6	1
		AUF6418	KT335745	KT334044	1	11	21
		AUF6419	KT335744	KT334043	1	6	1
32.43972	-84.64878	AUF6420	KT335743	KT334042	21	6	7
		AUF6421	KT335742	KT334041	1	6	1
		AUF6422	KT335741	KT334040	1	6	1

Latitude	Longitude	Tissue Voucher	GenBank Accession Number		Haplotype Number		
			Cyt b	COI	Cyt b	COI	Concat.
		AUF6423	KT335740	KT334039	1	6	1
		AUF6424	-	KT334038	-	6	-
		AUF6425	KT335739	KT334037	1	6	1
		AUF6426	KT335738	KT334036	21	6	7
		AUF6427	-	KT334035	-	6	-
		AUF6428	KT335737	KT334034	1	6	1
		AUF6430	-	KT334033	-	6	-
		AUF6431	-	KT334032	-	6	-
		AUF6432	-	KT334031	-	6	-
		AUF6433	-	KT334030	-	6	-
		AUF6434	-	KT334029	-	6	-
		AUF6436	KT335736	-	1	-	-
		AUF6442	KT335735	-	21	-	-
		AUF6443	KT335734	-	1	-	-

Table 4-2. All *Pteronotropis euryzonus* used in the morphometric portion of this study were from Auburn University Museum of Natural History collections (AUM). Data is organized by clades as recovered in the analysis.

Catalog Number	State	County	# Examined	Clade	Locality	Latitude	Longitude
AUM 677	Alabama	Russell	1	Northern	Igchee Cr., 15.8 mi N of Barbour-Russell Co.line on HWY 165	32.2581	-85.0011
AUM 14279	Alabama	Russell	5	Northern	Snake Cr., trib to Uchee Cr., 2.9 mi E of Marvyn, Hwy 80; T17N R27E S23SW	32.4400	-85.3153
AUM 14363	Alabama	Russell	5	Northern	Maringo Cr., trib to Uchee Cr., 3.1 mi W of Crawford, Hwy 80; T17N R28E S21W	32.4489	-85.2403
AUM 14804	Alabama	Russell	5	Northern	Adams Branch Cr., 4.7 airmi ESE Crawford, co rd.33	32.4061	-85.2933
AUM 30018	Alabama	Russell	2	Northern	Adams Branch Creek, Co.rd.33; SW of Crawford	32.4246	-85.2608
AUM 35209	Georgia	Clay	6	Northern	Hog Creek, ST 266, 2.8 mi ENE Fort Gaines	31.6422	-84.9644
AUM 35210	Georgia	Marion	5	Northern	Pine Knot Creek, 7 mi SSW Juniper St Rd 355	32.4386	-84.6489
AUM 37504	Alabama	Russell	1	Northern	Ihagee Creek, 4 mi N Holy Trinity, ST 165	32.2586	-84.9997
AUM 61228	Alabama	Russell	1	Northern	Ihagee Creek, upstream of highway 165, 2.5 miles N of Holy Trinity	32.2580	-84.9999
AUM 61340	Alabama	Barbour	5	Northern	Barbour Creek, at upstream of CR 79, near White Oak	31.8989	-85.3403
AUM 35206	Georgia	Randolph	2	Patuala	Little Pumpkin Creek, 8 mi N Cuthbert, ST 27	31.8858	-84.7944
AUM 35208	Georgia	Stewart	5	Patuala	Hodchodkee Creek, 2 mi E Lumpkin, ST 27	32.0511	-84.7775
AUM 14942	Alabama	Henry	5	Southern	Foster Cr., 4.8 airmi N of Columbia, Hwy 95	31.3611	-85.1114
AUM 14950	Alabama	Henry	1	Southern	Skipper Cr., trib to Abbie Cr., 2.5 airmi S of Abbeville	31.5361	-85.2533
AUM 61145	Alabama	Houston	5	Southern	Hurricane Creek, near Williams, upstream of CR 22	31.3051	-85.1614
AUM 61169	Alabama	Houston	5	Southern	White Branch, upstream of Country Garden Road [CR 41], 2 miles ENE of Kinsey	31.3068	-85.3110
AUM 61290	Alabama	Henry	5	Southern	Foster Creek, upstream of highway 95, 4.75 miles N of Columbia	31.3610	-85.1112

Chapter 5 – A Hotspot Atop: Rivers of the Guyana Highlands Hold High Diversity of Endemic Pencil Catfish (Teleostei: Ostariophysi: Siluriformes)

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Abstract

The Pakaraima Mountains are an ancient mountain range along the borders of Guyana, Brazil, and Venezuela. The high plateau is drained by multiple river systems in all directions. Although hypotheses have been presented for the biogeographic relationships of lowland rivers, the interconnectivity of rivers on the top of the plateau is unknown. With multiple complex rivers in a small, upland area we predicted a high level of endemism for stream fishes and complex biogeographic relationships. We explore this with the incredibly diverse pencil catfish genus *Trichomycterus*. Using collections from recent expeditions to the Pakaraima Mountains of Guyana, we amplified three mitochondrial (16S, COI, and cytb) and two nuclear markers (myh6 and RAG2). We constructed individual gene trees as well as a concatenated tree to determine the placement of these taxa within the *Trichomycterus* of the trans-Andean/Amazonian clade. Herein, we identify six endemic lineages of *Trichomycterus* from the highlands of the Pakaraima Mountains. Of the identified lineages, we find two species occupying multiple basins, suggesting that Pakaraima streams either maintain or had some degree of recent connectivity.

Introduction

The Pakaraima mountains run along the borders of Guyana, Brazil, and Venezuela. The streams there hold a high degree of endemism (Alofs, Liverpool, Taphorn, Bernard, & López-Fernández, 2014; Armbruster & Taphorn, 2011; Hardman, Page, Sabaj, Armbruster, & Knouft, 2002). The Pakaraimas (Figure 1) are drained to the north by the Mazaruni and Cuyuní rivers, to the east by the Potaro River (Essequibo River basin), to the southwest by the Ireng and Uraricoera rivers (Amazon River basin), and to the west and northwest by the Caroní River (Orinoco River basin). The mountains are the remains of Archaean and Proterozoic rocks whose lighter sediments have eroded to fill formerly lacustrine basins such as the Venezuelan Llanos and the Rupununi Savanna of Guyana (see Lujan & Armbruster, 2011, for review). This erosion has left behind a durable core that often has steep faces that the rivers run off of in spectacular waterfalls. Below the falls, the rivers often have some rapids complexes, but quickly reach lowland conditions (Lujan & Armbruster, 2011).

Current evidence for the relationships of the rivers draining the Pakaraima Mountains involves, in part, the development and subsequent fragmentation of a paleo-river drainage called the proto-Berbice (Lujan & Armbruster, 2011; Schaefer & do Vale, 1997; Sinha, 1968). The proto-Berbice contained what are now tributaries of the upper rio Branco (Amazon basin, including the Ireng), the upper Essequibo, the Berbice, and parts of the Courantyne and Orinoco. Meanwhile, the middle and lower Essequibo (including the Potaro/Kuribrong) likely joined the Mazaruni and Cuyuní near where the current mouths are. Slowly, the Amazon River has been capturing streams from the

proto-Berbice in an east-west manner. This pattern would suggest a similarity between the faunas of the Potaro/Kuribrong and the upper Mazaruni with the Ireng being more distantly related as it appears to have never been connected into the middle and lower Essequibo + Mazaruni.

However, the upper courses of the rivers have not been explored biogeographically. The likely complex relationships of the upper courses of the rivers were suggested by the description of the crenuchid *Apareiodon agmatos*, and the loricariid taxa *Paulasquama callis*, *Neblinichthys brevibraccium*, and *N. echinasus* in the upper Mazaruni (Armbruster & Taphorn, 2011; Taphorn, Armbruster, López-Fernández, & Bernard, 2010; Taphorn, López-Fernández, & Bernard, 2008), all of which share affinities with the Orinoco River basin. Given the absence of these taxa in lowland streams, it is likely that these highland taxa were moving via stream capture or other events that connected these highland tributaries. Thus far, the relationships of the highland regions have been scarcely explored systematically. Lujan *et al*. (2018) found that *Paralithoxus bovallii* (Loricariidae) from the Ireng was more closely related to an undescribed species in the Courantyne than one from the lower Potaro, supporting the proto-Berbice hypothesis; however, *Paralithoxus* is not found elsewhere in the Pakaraima highlands. Lujan *et al*. (2019) found that *Corymbophanes* (Loricariidae), an upper Potaro/Kuribrong endemic, is sister to a new genus and species (*Yaluwak primus*) from the upper Ireng with the two clades separated by long branch lengths suggesting an ancient relationship.

Coupled with the lack of basic information on the fauna of the region, the area is also under extreme threat by gold and diamond mining with a strong potential of mining

eliminating species before they are even discovered (Alofs et al., 2014). In this study, we explore the potential interconnectedness of the high Pakaraima streams by examining the relationships of the pencil catfishes of the genus *Trichomycterus* in order to identify pertinent diversity and to uncover biogeographic patterns that could be duplicated in other Pakaraima organisms.

Trichomycteridae represents a diverse family of freshwater catfishes distributed across the Neotropics. Of the more than 300 recognized species (Fricke et al., n.d.), the majority of species (219) are found in the Trichomycterinae, which contains the genera *Bullockia*, *Cambeva*, *Eremophilus*, *Hatcheria*, *Ituglanis*, *Rhizosomichthys*, *Scleronema*, *Silvinichthys*, and *Trichomycterus*. Most of the diversity within Trichomycterinae can be attributed to *Trichomycterus*, with all other genera except *Ituglanis* (28 species), *Cambeva* (25 species), *Silvinichthys* (seven species), and *Scleronema* (three species) being monotypic (Fricke et al., 2018). While other genera exhibit apomorphic specializations, the lack of specializations unique to *Trichomycterus* has long made researchers suspect, and later confirm with molecular studies, the non-monophyly of the genus (Baskin, 1973, 2016; de Pinna, 2016; Ochoa *et al.*, 2017; Henschel *et al.*, 2018; Katz *et al.*, 2018).

The emerging phylogenetic pattern matches those of other similarly distributed fishes, such as doradid catfishes, characins, and armored catfishes, where distinct clades are geographically linked to a trans-Andean/Amazonian distribution or to south Atlantic coastal drainages (Katz et al., 2018; Ochoa et al., 2017; Ribeiro, 2006). Katz *et al*. (2018) attempted to solve some of the taxonomic problems of the Trichomycterinae by restricting *Trichomycterus* to a clade that

contained the type species (south Atlantic coastal drainages), describing *Cambeva* for a clade sister to *Scleronema*, a clade that is sister to *Trichomycterus sensu stricto*, and referring the Andean, Patagonian, Amazonian, and Guiana Shield species to "*Trichomycterus*" in quotation marks. "*Trichomycterus*" is paraphyletic and part of a clade that includes *Bullockia*, *Eremophlius sensu stricto*, and *Ituglanis*. Results were similar to those previously found in Ochoa *et al*. (2017). These patterns are not surprising, given the tectonic and geologic history of the continent that highlights the importance of the Guiana and Brazilian Shields as original uplands of South America, formation of the Andes, and uplift of the Eastern Cordillera (to name a few) with shaping the biogeography of Neotropical fishes (Lujan & Armbruster, 2011; Lundberg et al., 1998; Ribeiro, 2006). We will not be referring to *Trichomycterus* in quotation marks, and we refer our study species to genera as in Ochoa *et al*., (2017)

Trichomycterus are long, slender catfishes generally found only in swift waters. Such habitat, even in the mountains, is patchy, and we suspect that the fishes would be more likely to be isolated to drainages. Recent collections from this region have identified all specimens as *T. guianensis* (Eigenmann, 1912), but we noted significant differences in color and morphology in samples that we have made. Preliminary external visual examinations indicate the possibility for unrecognized diversity and perhaps misidentification of *T. guianensis* in the rivers of this region. The only other species recognized in the region is *T. conradi* (Eigenmann, 1912), and we have found some specimens from the Ireng and Kuribrong rivers that correspond to this species.

Recent studies have illuminated the need to identify unrecognized diversity within *Trichomycterus* and have highlighted the important role that geology and topography

play in contributing to that diversity (Katz et al., 2018; Ochoa et al., 2017; Unmack, Bennin, Habit, Victoriano, & Johnson, 2009). With our collections of seemingly multiple, diverse *Trichomycterus* species in the region, we 1) confirm the discovery of multiple endemic *Trichomycterus* species in the region using a multigene phylogeny, 2) examine the diversity and endemism of *Trichomycterus* in the Pakaraima Mountain region with respect to the unique geologic features that have likely influenced their genetic structure, and 3) provide clarification for the identification of *T. guianensis* and *T. conradi* based on morphology.

Materials and Methods

Taxon Sampling, DNA Extraction and Sequencing

Collections ranged across multiple years with research permits from Environmental Protection Agency of Guyana as follows, listed as year, reference number: 2011, 030510 BR 130; 2008, 300408 SP: 004; 2014, 040414 SP: 003; 2015, 123115 BR031; 2016, 012016 SP: 003. Fish were either collected with six-foot by ten-foot nylon coated seines with ⅛" mesh, or we joined fishing expeditions of the Patamona who used hiari, a root native to the area around the collection site and a natural source of rotenone. Collection sites were distributed across the Pakaraiama highlands (Figure 2, Table 1). After capture, fish were euthanized in a solution of tricaine methanesulfonate (MS-222) until no sign of respiration was observed for five minutes. Tissue samples were taken from the right pectoral fin or right axial musculature and placed into 1.5 ml vials containing RNALater or ethanol for preservation. Once tissue samples were taken, voucher specimens were fixed in a 3.7% formaldehyde solution for seven days, then rinsed in water for three days, and finally stored in 70% ethanol. Vouchers and tissue

samples were deposited in the AUM Fish Collection. Additional materials not collected by the authors were requested from the Royal Ontario Museum (ROM, Table 1).

Whole genomic DNA was extracted from tissues using either Chelex or an E.Z.N.A Tissue DNA Kit (Omega BioTek, Norcross, GA). The four genes 16S, COI, cytb, and RAG2 were amplified through 25µl polymerase chain reactions using the same primers as Ochoa *et al*. (2017). The 16S gene was amplified using the following primers and protocol: 16Sa-L and 16Sb-H (Palumbi & Baker, 1994) initial denaturation step of 180 s at 94°C then 30 cycles of denaturation (45 s at 95°C), annealing (30 s at 54°C), and extension (60 s at 68°C) followed by a final extension of 600 s at 68°C. The COI gene was amplified using the following primers and protocol: FishF1 and FishR1 (Ward et al., 2005) initial denaturation step of 180 s at 94°C then 30 cycles of denaturation (45 s at 94°C), annealing (30 s at 54°C), and extension (60 s at 68°C) followed by a final extension of 60 s at 68°C. The cytb gene was amplified using the following primers and protocol: L14841 and H1591 (Irwin et al., 1991; Kocher et al., 1989) initial denaturation step of 180 s at 94°C then 30 cycles of denaturation (45 s at 95°C), annealing (30 s at 54°C), and extension (60 s at 68°C) followed by a final extension of 60 s at 68°C. The RAG2 gene was amplified using a two-step protocol. The first reaction was performed using the touchdown protocol described in Lovejoy & Collette (2001) with RAG164F and RAG2R6 primers. The second PCR used 1.5µl of template from the first run and primers 176R and RAG2Ri (Oliveira et al., 2011) under the following conditions: initial denaturation step of 30 s at 95°C then 35 cycles of denaturation (30 s at 95°C), annealing (45 s at 56°C), and extension (90 s at 72°C). Primers used for PCR amplification were

also used for DNA sequencing for all genes, with 176R and RAG2Ri used for sequencing RAG2.

The products were visualized and size-verified on a 0.8% agarose gel. PCR purification, sample preparation, and Sanger sequencing were performed at GeneWiz (South Plainfield, NJ). Chromatographs from forward and reverse reads were imported into Geneious v. 10.2.3 (Kearse et al., 2012) for assembly. Assembled contiguous sequences were aligned using the MUSCLE algorithm (Edgar, 2004), and results were checked by eye. Due to length variation among sequences generated in this study and those of Ochoa *et al.* (2017), alignments were trimmed to the following lengths: 16S: 466; COI 522; cytb: 858; myh6: 543; and RAG2: 885. Each individual gene tree was analyzed with *Scleronema minutum* as an outgroup, while the concatenated dataset (3579 bp) included members from Ochoa *et al.*'s (2017) clades D1, D2, D3, and E with *S. minutum* as an outgroup. Data were exported both as individual alignments and as a concatenated dataset for phylogenetic analysis.

Phylogenetic Analysis

Best-fit models of evolution were tested using PARTITIONFINDER2 (Lanfear et al., 2017). Models were tested on individual gene trees partitioned by codon, and then on the concatenated dataset partitioned by gene. The resulting data blocks were then used in Bayesian Inference analysis. Bayesian Inference was performed using MrBayes v. 3.2.6 on XSEDE via CIPRES Science Gateway (Miller et al., 2010). Each dataset had 2 runs with 4 chains run for 15 million generations, sampling once every 1,000 generations. The parameters and trees

were summed in MrBayes v. 3.2.6 using the default 25% burn-in. The resulting 50% majority consensus rule phylogeny is reported. Convergence was further checked in Tracer v 1.7.1 with the requirement of an ESS value >200. A Maximum Likelihood analysis was also performed on the concatenated dataset. Branch supports were obtained with the ultrafast bootstrap (Hoang, Chernomor, von Haeseler, Minh, & Vinh, 2018) implemented in the IQ-TREE software (Nguyen, Schmidt, Von Haeseler, & Minh, 2015)

Results

The molecular analyses consistently recognized six species-level clades of Trichomycterus in the Pakaraimas: 1) *T. conradi* 2) *T. guianensis* 3) *T. cf. guianensis* (*sensu* Ochoa *et al.*, 2017), 4) Ireng spotted, 5) Potaro, elongate, and 6) Mazaruni, plain. Four gene trees were analyzed separately, then combined into a concatenated analysis. The first individual gene tree is cytochrome b (cytb, Figure 3A), which results in a well-supported clade (posterior probability >90%) of Pakaraima *Trichomycterus*. Members of true *Trichomycterus guianensis* are found sister to the Potaro, elongate form. This clade is sister to another well-supported clade (pp >90%) of *T. cf. guianensis* + Mazaruni, plain form. These are all sister to a single representative of *T. conradi*. This analysis did not include the Ireng, spotted form that is present in other analyses, due to difficulties in amplifying and successfully sequencing them.

The second gene tree generated from our data is based on COI (Figure 3B). This analysis again places *T. cf. guianensis* sister to the Mazaruni, plain form. In contrast to the cytb phylogeny, this clade is sister to *T. conradi*; however, this relationship is weakly supported (pp = 71%). The (*T. cf. guianensis* + Mazaruni, plain) *T. conradi* clade is sister to another clade consisting of the Ireng, spotted form and true *T. guianensis.* The

interrelationships among the clades are poorly supported (pp = 67%), but each recognized morphotype is well-supported (pp >90%) with the exception of the Ireng, spotted form (pp = 87%). Finally, the Potaro, elongate form is missing from this analysis due to difficulties in amplifying and successfully sequencing it. Overall, the COI tree is much less resolved than the other trees, with some nodes not reaching 90% posterior probability.

Ribosomal 16s data place *T. cf. guianensis* sister to the Mazaruni, plain form (Figure 3C). This clade is sister to *T. conradi*. As seen in the COI analysis, despite geographic proximity, the Ireng, spotted form is sister to true *T. guianensis* rather than *T. conradi*. The Potaro, elongate form is sister to the remaining members of the Pakaraima member clade.

Nuclear DNA analysis from the RAG2 data was the most divergent from the remainder of the data (Figure 3D). Again, *T. cf. guianensis* is recovered sister to the Mazaruni, plain form, but this is the only similarity with the other gene trees. The RAG2 data show true *T. guianensis* sister to the Ireng, spotted form. They form a clade sister to the Potaro, elongate form. The *T. conradi* is weakly monophyletic (pp = 56%), and its deeper relationships are unresolved due to a polytomy.

With gene tree heterogeneity rampant in this analysis, the four genes were concatenated and analyzed with the D1, D2, D3, and E clades from Ochoa *et al.* (2017, Figure 4). The Bayesian analysis resulted in two trees with equal likelihood. The two runs were separately examined and a rogue taxon, *Ituglanis parahybae*, was the cause. Because this taxon was not the focus of this study and because the topology was

otherwise unchanged, we present the 50% majority rule consensus tree. The Maximum Likelihood tree showed the same topology as the Bayesian 50% majority rule consensus tree and is thus not presented. The tree, with 24 individuals from our analysis, shows that all morphotypes we identified *a priori* are monophyletic. Two distinct clades compose Pakaraima *Trichomycterus*: *Trichomycterus cf. guianensis* + Mazaruni, plain form is sister to *T. conradi*. This clade is sister to another clade consisting of *Trichomycterus guianensis* + Ireng, spotted which is sister to the Potaro, elongate form. Each of these relationships are supported with >90% posterior probability, while deeper relationships remain unresolved.

Discussion

Our results demonstrate the presence of multiple species of *Trichomycterus* in the Pakaraima Mountains of Guyana. Based on the concatenated analysis, there were two major clades, one consisting of *T. guianensis* and two undescribed species from the Potaro and Ireng Rivers (Ireng, spotted and Potaro, elongate), and the second clade including *T. conradi* and two undescribed species, one from Mazaruni River (Mazaruni, plain) and *T.* cf. *guianensis*. Both *T. guianensis* and *T. conradi* appear to be the rarer species in the region (based on collections), and *T. guianensis* is only in the upper Potaro River. Based on preliminary examination of the types and comparison with specimens we have collected, *Trichomycterus guianensis* is a deep bodied species with irregular blotches (Figure 4D). Ireng, spotted (Figure 4E) is the dominant species in the Ireng, and it is similar in morphology to *T. guianensis* and was recovered as sister to it. In morphology, the Ireng Spotted species is even shorter and deeper-bodied than *T. guianensis*. Sister to the clade of *T. guianensis* and Ireng, spotted is a very elongate,

almost entirely brown species from near Ayangana in the far upper Potaro with miniscule pelvic fins (= Potaro, elongate, Figure 4F). It was found in sluggish, swampy areas, which is habitat more indicative of *Ituglanis*. *Ituglanis* was diagnosed (Costa & Bockmann, 1993) by the presence of a very small supraoccipital foramen (vs. large), an anteriorly directed anterior process of the sphenotic (vs. anteroventral), and a concave mesial margin of the autopalatine (vs. almost straight). Potaro, elongate has a very large foramen on the supraoccipital and an anteroventrally directed anterior process of the sphenotic, but it does share a concave mesial border of the autopalatine with *Ituglanis* (JWA pers. obs.). In addition, *Ituglanis* has very few ribs, and a reduction in number to seven or fewer pairs was considered to be a synapomorphy for *Ituglanis* and a clade consisting of the subfamilies Tridentinae, Stegophilinae, Vandelliinae, Sarcoglanidinae, and Glanapteryginae (Costa and Bockman 1993; de Pinna and Keith 2003). Potaro, elongate has four pairs of ribs. Ochoa *et al*. (2017) found *Ituglanis* and *Trichomycterus* to be closely related and that *Ituglanis* is not sister to Tridentinae, Stegophilinae, Vandelliinae, Sarcoglanidinae, and Glanapteryginae, suggesting that rib number is homoplastic. Given the molecular phylogeny, the presence of synapomorphies of *Ituglanis* in Potaro, elongate would represent homoplasy, perhaps related to similar habitats, and we found Potaro, elongate to be related to other Pakaraima *Trichomycterus* in the molecular analysis. Potaro, elongate and Ireng, spotted are opposites in their morphology, the former being deep bodied and short and the latter being long and slender, suggesting a strong capacity for body rearrangement in the genus. The morphological variation of all these species

is currently under study by the authors and descriptions and diagnoses are underway.

The other major clade contains a widespread, elongate species with dark, small or large, regular spots (*Trichomycterus* cf. *guianensis*, Figure 4A). This species is found in the upper Potaro, Kuribrong, and Mazaruni rivers. Little geographic structure was present in the specimens examined, suggesting fairly recent movement between the basins. Sister to this species is a similar but unspotted species from the Mazaruni River (Mazaruni, plain, Figure 4B). Finally, a clade composed of the plain colored *T. conradi* (Figure 4C) from the Ireng and the lower Kuribrong is sister to the other two species.

Two species of *Trichomycterus* have been described from the upper Caroní, Orinoco River basin section of the Pakaraima Mountains: *T. celsae* Lasso and Provenzano 2002 and *T. lewi* Lasso and Provenzano 2002. Based on preliminary examination of specimens at AUM, *Trichomycterus celsae* is most similar to *T. conradi* and *T. lewi* may be the same species as *T. cf. guianensis*. Further morphological investigation is underway to determine species identity. Unfortunately, we do not have tissue samples from the Venezuelan species.

Biogeography of the Pakaraima Mountains

The biogeographic story that the species of *Trichomycterus* of the Pakaraimas tell is a complex one. *Trichomycterus* cf. *guianensis* appears to have moved between river systems relatively easily. Mazaruni samples are sister to those in the Kuribrong and Potaro rivers, but the Mazaruni samples are paraphyletic. Tributaries of the Mazaruni interdigitate with the Kuribrong and Potaro rivers, and species living as high in their drainages as *Trichomycterus* would be more likely to be able to move via river capture events where tributaries erode their divides and switch from one system to the next.

Anecdotal reports suggest that the upper courses of at least the Potaro and Kuribrong connect during particularly rainy times; flying over the area reveals numerous fissures that seem to run between the two rivers (JWA pers. obs.). These drainages also interdigitate with Caroní and Ireng tributaries. As mentioned above, *T. cf. guianensis* may be conspecific with *T. lewi* described from Venezuela; however, we found no similar species in the Ireng suggesting a limit to the distribution of the species.

The upper Caroní and the Ireng were once part of the proto-Berbice paleodrainage basin along with the upper Branco, upper Essequibo, Berbice, and Courantyne rivers while the Mazaruni was likely independent (Lujan and Armbruster, 2011). The Essequibo makes a westward bend near Massara and away from a nearby Berbice tributary (Gibbs & Barron, 1993), suggesting a likely point of demarcation between the upper Essequibo as part of the proto-Berbice and the lower Essequibo, which probably joined with the Mazaruni at the present mouth of the Essequibo. This would mean that the Potaro and Mazaruni were part of the same system and not part of the proto-Berbice. However, the mixing of Ireng, Potaro, and Mazaruni *Trichomycterus* in the phylogeny suggests that there likely existed faunal exchange between the proto-Berbice, Potaro, and Mazaruni rivers at least in the highlands prior to the breakup of the proto-Berbice during the Pliocene and Pleistocene, potentially leading to complex interrelationships between these basins. A similar finding was made in Lujan *et al*. (2019) who found that *Corymbophanes* was sister to a new genus and species from the Ireng (*Yaluwak primus*); however, the branch lengths were much longer than what was observed here. Further exploration into the relationships of *Trichomycterus* along with a molecular clock will likely lead to fascinating insights into the biogeography of the Pakaraima Mountains, but this further

insight will require extensive collecting in the difficult to explore Brazilian tributaries of the Pakaraima Mountains and further collecting in Venezuela, that is difficult now because of civil strife.

Although Ochoa et al. (2017) did perform a molecular dating analysis, only one of the two dates used for calibration was from a fossil, and that from only ~4.5 Mya. The other calibration point was based on an estimated age of the family from another study. The divergence date given by Ochoa et al. (2017) for *T. cf. guianensis* from a trans-Andean sister clade is 19.22 Mya. We felt that given the only fossil was recent but from a distantly related clade, the ancient origin of the family that was based not on fossils but ages from another study, and the likely large gaps in sampling between Pakaraima *Trichomycterus* and potentially related taxa that performing a molecular clock analysis here would be premature and potentially misleading.

Trichomycterus conradi appears to be a more lowland form found in the rapids below Kaieteur and Amaila Falls on the Potaro and Kuribrong, respectively, as well as in the Ireng. The shallow nodes between the Kuribrong and Ireng samples sequenced suggest that movement has been relatively recent. We were only able to obtain 16S sequences for a specimen of *T. cf. conradi* from the Maroni River of eastern Suriname, and it was sister to *T. conradi*. A similar distribution across the northern Guiana Shield was found for *Paralithoxus bovallii* from the Ireng River and hypothesized new species related to it in the Potaro, Courantyne, and Coppename rivers (Lujan *et al*. 2018). The distributions of *T. conradi* and *P. bovallii sensu lato* suggest interconnectivity across the Guiana Shield even for small fishes restricted to fast-flowing streams. Clearly, we are just

beginning to understand the complexities of the biogeography of the western Guiana Shield and the interconnectedness of it with the eastern portion of the shield.

Threats to Biodiversity in the Guyana Highlands

The Pakaraimas represent the cores of ancient mountains, which are among the main sources of gold and diamonds. Alofs *et al*. (2014) review some of the issues with gold mining in the upper Mazaruni River, and we have observed similar issues in the Kuribrong and Potaro Rivers as well. Large swaths of forest have been removed from around the rivers with the sediment pumped through sieves to extract gold and diamonds. Gold is removed with mercury amalgamation leading to high mercury levels in the water, fishes, and humans (Miller *et al*. 2003) and large swaths of forest replaced by denuded landscapes and toxic spoil ponds. On larger rivers like the lower Potaro, large dredging machines suck up sediment and process it directly in the river leaving behind piles of gravel in the river that alter the natural hydrology. Although Hardman *et al*. (2002) did not find significant differences between their study of the fishes of the Potaro River and Eigenmann (1912), certain species that had been present and common in Eigenmann's survey were absent 90 years later. Mol & Ouboter (2004) and Brosse, Grenouillet, Gevrey, Khazraie, & Tudesque (2011) found that the erosion related to gold mining has reduced fish diversity. As of our 2014 trip to the upper Kuribrong and 2016 trip to the Ireng, there was little impact to the rivers from mining; however, a recently completed road now provides easier access to the upper Kuribrong, and one small mine was observed in 2014. The lower Kuribrong has been heavily impacted, and after flying over the Potaro River in 2014, JWA can state that the Potaro looks less clear than it had during the 1998 expedition reported in Hardman *et al*. (2002).

Although depauperate in total numbers of species, the high degree of endemism makes the Pakaraima Mountains a particularly interesting hot spot of biodiversity. As expressed by Alofs *et al*. (2014) for the upper Mazaruni, the whole high plateau of the Pakaraimas supports an endemic fauna as is evidenced here. While there is some interconnectivity of the river systems, narrow endemic *Trichomycterus* are found in each of the rivers in this study. It is clear from studies like this one, López-Fernández et al. (2012), and Lujan et al. (2019) that the high Pakaraimas are acting as species generators, and the very factors that make the area difficult to colonize (like large waterfalls) also lead to genetic isolation. In *Trichomycterus* alone, we know of the six species presented here, *T. celsae*, *T. lewi*, and at least one more undescribed species from Venezuela in the Pakaraimas. Few areas of this size outside of the Pakaraimas have such high diversity of *Trichomycterus*. Conservation of the unique landscape of the Pakaraimas that has become part of our shared cultural heritage is important, and further studies on the unique fauna of the region are needed.

Figures

Figure 5-1. A topographical map of the Pakaraima highlands depicting the physiography of rivers. Major rivers are labelled along their flow. Country names are listed horizontally in all capital letters

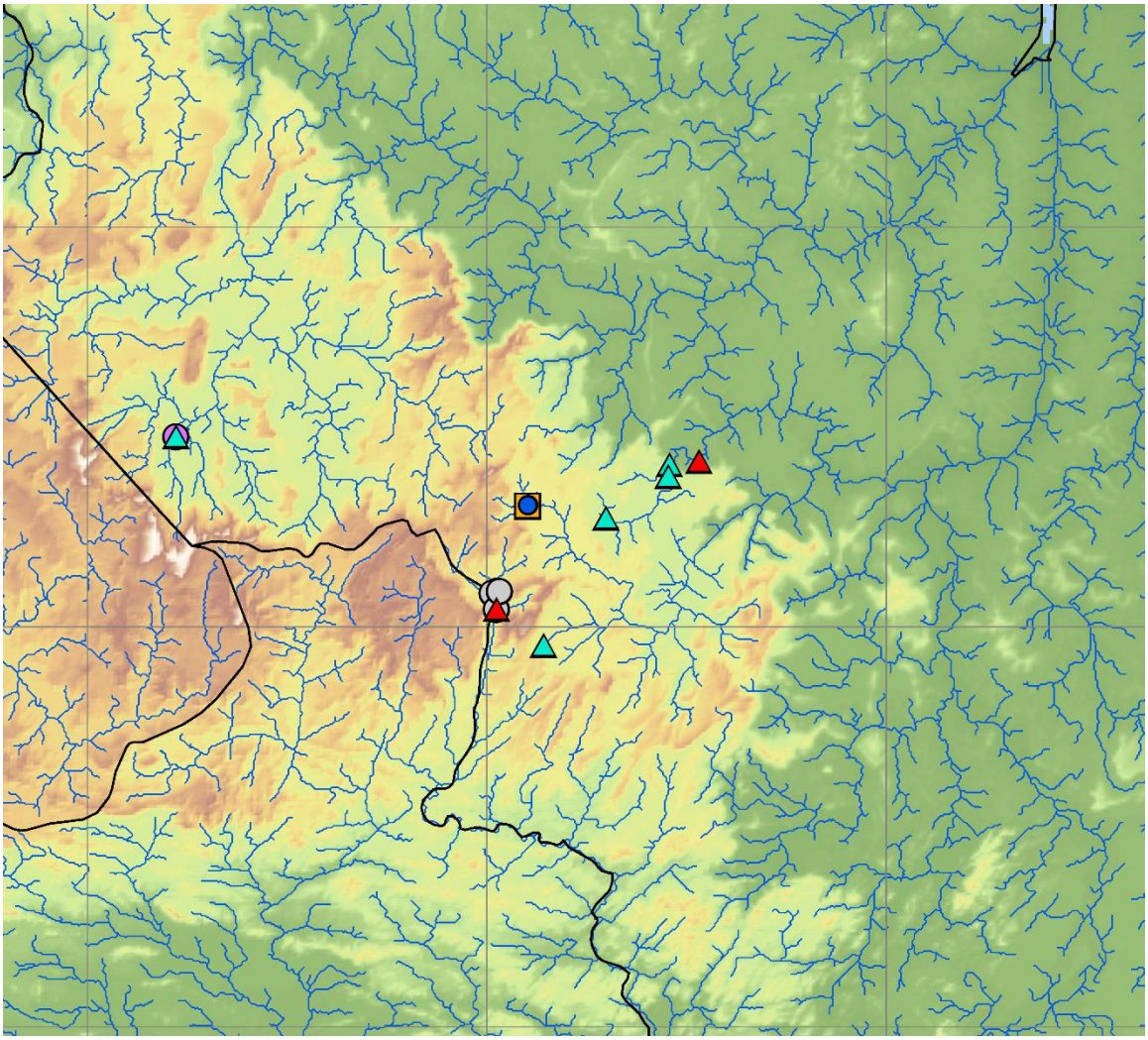

Figure 5-2. Collection localities for species of Trichomycterus found in this study. Color codes correspond to images in Figure 4 and are as follows: red triangles, T. conradi; blue circle, T. guianensis; teal triangles, T. cf. guianensis; purple circle, Mazaruni, plain form; orange square, Potaro elongate; gray circle, Ireng, spotted form.

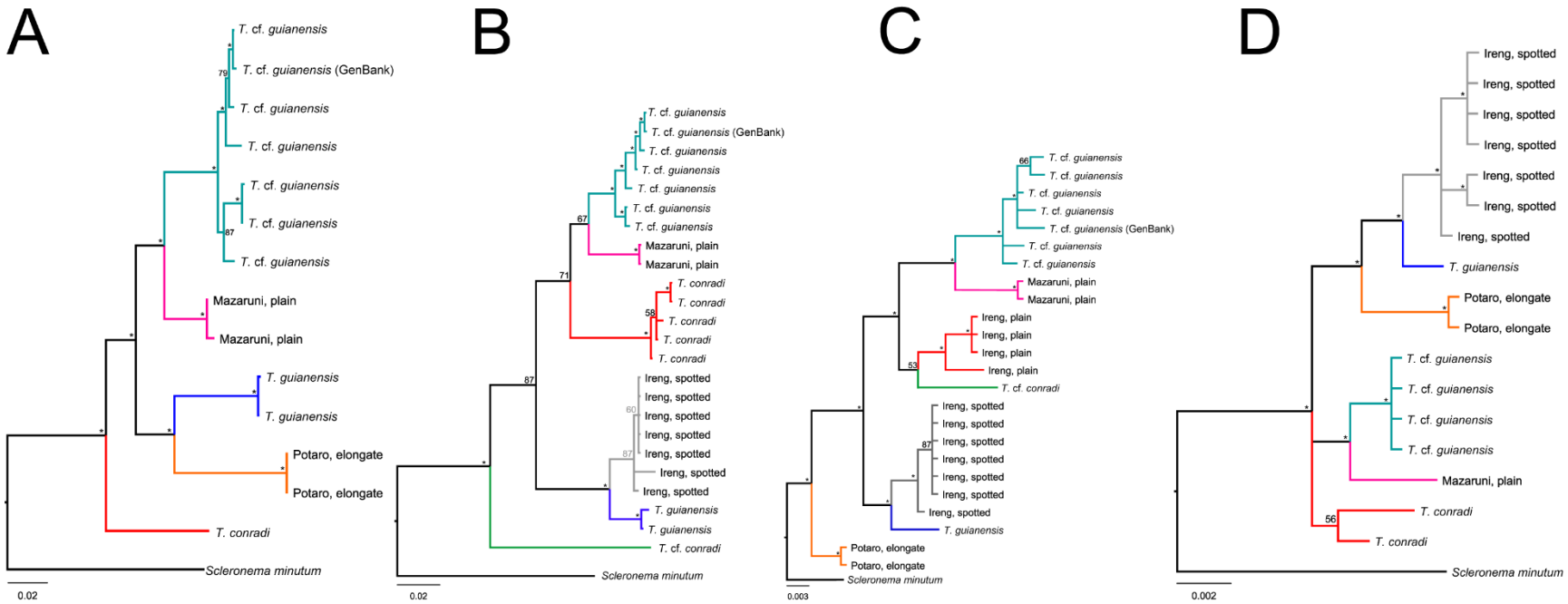

Figure 5-3. Fifty per cent majority rule consensus tree from Bayesian inference of each gene analyzed in this study. Cytochrome b (A), COI (B), 16S (C), and RAG1 (D). Nodes labelled with an asterisk (*) indicate posterior probabilities >90%. Values less than 90% are written on the trees. Branches are colored to match localities as seen in Figure 1. Tip labels correspond to individuals as denoted in Table 1.

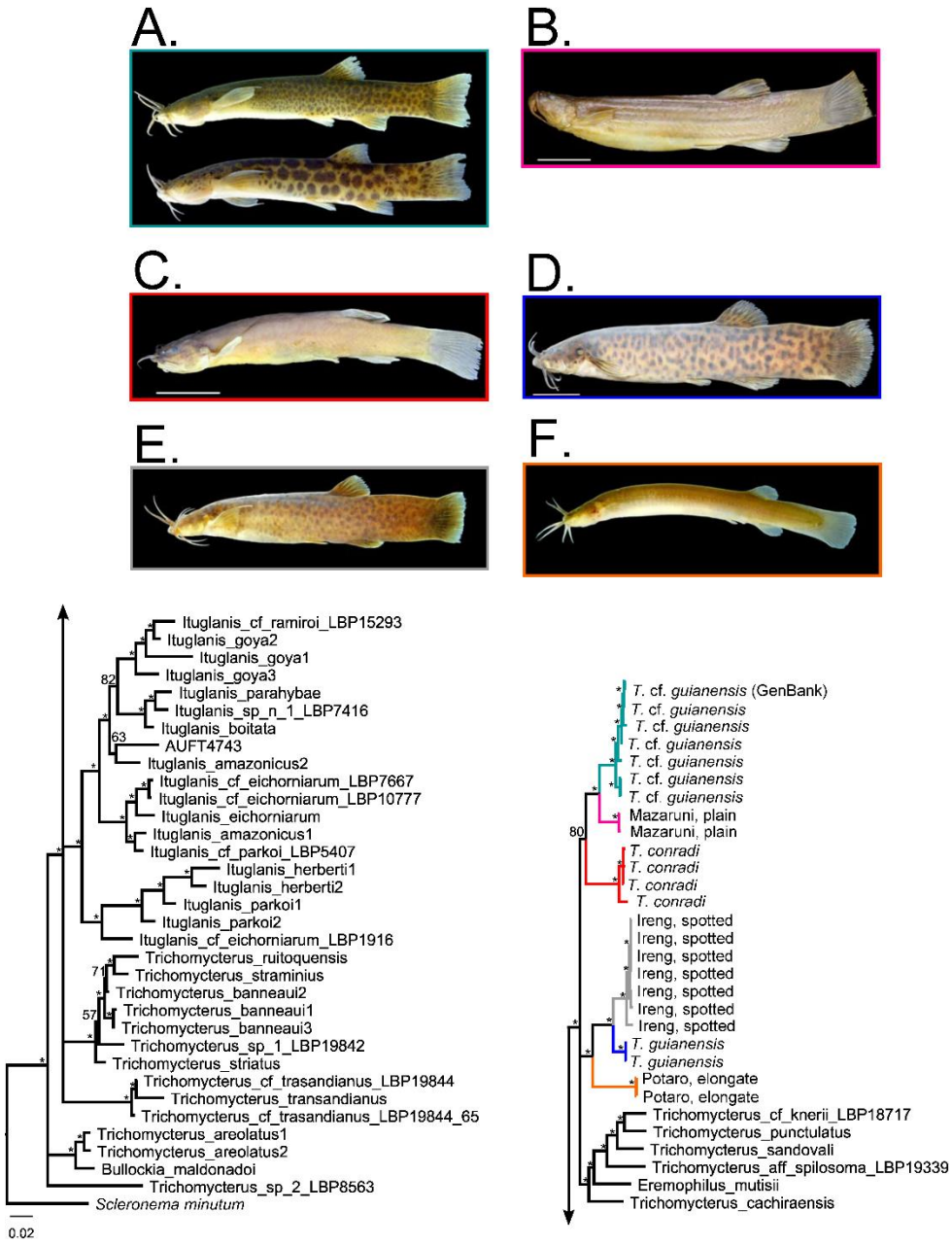

Figure 5-4. Fifty per cent majority rule consensus tree from Bayesian inference of concatenated sequences. Nodes labelled with an asterisk (*) indicate posterior probabilities >90%. Values less than 90% are written on the trees. Branches are colored to match localities as seen in Figure 1. Tip labels correspond to individuals as denoted in Table 1. These sequences are combined with the D1, D2, D3, and E clades from Ochoa et al (2017). Arrows connect disconnected branches in the phylogeny. Outlines of the photographs of specimens correspond to clade color and symbol color in Figure 1. A. T. cf. guianensis. B. Mazaruni, plain form. C. T. conradi. D. true T. guianensis. E. Ireng, spotted form. F. Potaro, elongate form.

Tables

Table 5-1. Collection information for *Trichomycterus* species used in this study. GenBank Accession numbers are provided for each gene and individual.

Tissue Catalog	Species ID	Voucher Number	Latitude	Longitude	16S	COI	Cytb	RAG2	Drainage
AUFT10166	Ireng, spotted	67129	5.08955	-59.97514	MT025525	MT017634	-	MT017607	Ireng River drainage
AUFT10168	Ireng, spotted	67129	5.08955	-59.97514	MT025526	MT017635	-	MT017608	Ireng River drainage
AUFT10169	Ireng, spotted	67129	5.08955	-59.97514	MT025527	MT017636	-	MT017609	Ireng River drainage
AUFT10170	Ireng, spotted	67129	5.08955	-59.97514	MT025528	MT017637	-	MT017610	Ireng River drainage
AUFT10212	*T. conradi*	67138	5.04398	-59.97717	MT025529	MT017638	-	MT017604	Ireng River drainage
AUFT10213	*T. conradi*	67138	5.04398	-59.97717	MT025530	MT017639	-	-	Ireng River drainage
AUFT10234	Ireng, spotted	67154	5.08388	-59.98762	MT025531	MT017640	-	MT017611	Ireng River drainage
AUFT10276	Ireng, spotted	67179	5.04398	-59.97717	MT025532	MT017641	-	MT017612	Ireng River drainage
AUFT10294	*T. conradi*	67194	5.08867	-59.96952	MT025533	MT017642	-	-	Ireng River drainage
AUFT10310	Ireng, spotted	67172	5.08955	-59.97514	MT025534	MT017643	-	MT017613	Ireng River drainage
AUFT2110	*T. guianensis*	63677	5.30181	-59.89838	MT025520	MT017630	MT017624	-	Potaro River drainage
AUFT2186	*T. cf. guianensis*	62902	5.40532	-59.5439	MT025521	MT017631	MT017617	MT017603	Potaro River drainage
AUFT4743	*T. cf. conradi*	51758	4.767118	-54.56462	MT025522	MT017632	-	-	Maroni River drainage

Tissue Catalog	Species ID	Voucher Number	Latitude	Longitude	16S	COI	Cytb	RAG2	Drainage
AUFT6563	*T. guianensis*	62932	5.30181	-59.89838	-	MT017633	MT017625	MT017606	Potaro River drainage
AUFT6596	Potaro, elongate	62949	5.304	-59.89819	MT025523	-	MT017628	MT017615	Potaro River drainage
AUFT6597	Potaro, elongate	62949	5.304	-59.89819	MT025524	-	MT017629	MT017616	Potaro River drainage
ROMT06183	Mazaruni, plain	83791	5.4755	-60.77967	MT025535	MT017644	MT017626	MT017614	Mazaruni River drainage
ROMT06184	Mazaruni, plain	83791	5.4755	-60.77967	MT025536	MT017645	MT017627	-	Mazaruni River drainage
ROMT06185	*T. cf. guianensis*	83790	5.4755	-60.77967	MT025537	MT017646	MT017618	-	Mazaruni River drainage
ROMT06186	*T. cf. guianensis*	83790	5.4755	-60.77967	MT025538	MT017647	MT017619	-	Mazaruni River drainage
ROMT12696	*T. cf. guianensis*	89932	4.95407	-59.85882	MT025539	MT017648	MT017620	MT017600	Potaro River drainage
ROMT15527	*T. cf. guianensis*	91392	5.272085861	-59.7026908	MT025540	MT017649	MT017621	MT017601	Potaro River drainage
ROMT15575	*T. cf. guianensis*	91500	5.3759978	-59.5472803	MT025541	MT017650	MT017622	MT017602	Potaro River drainage
ROMT15595	*T. conradi*	91436	5.413958782	-59.470252	MT025542	MT017651	MT017623	MT017605	Potaro River drainage

Chapter 6 References

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