MOLECULAR PHYLOGENETICS OF OTOPHYSAN FISHES: AFRICAN ALESTIDS (CHARACIFORMES: ALESTIDAE) AND CITHARINOIDS (CHARACIFORMES: CITHARINOIDEI), AFRO-ASIAN CHEDRINS (CYPRINIFORMES: CHEDRINI), AND NEOTROPICAL LORICARIINS (SILURIFORMES: LORICARIINAE) AS CASE STUDIES

By

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ABSTRACT

MOLECULAR PHYLOGENETICS OF OTOPHYSAN FISHES: AFRICAN ALESTIDS (CHARACIFORMES: ALESTIDAE) AND CITHARINOIDS (CHARACIFORMES: CITHARINOIDEI), AFRO-ASIAN CHEDRINS (CYPRINIFORMES: CHEDRINI), AND NEOTROPICAL LORICARIINS (SILURIFORMES: LORICARIINAE) AS CASE STUDIES

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Otophysan fishes (Ostariophysi: Otophysi) are members of a morphologically and ecologically diverse clade of teleosts that includes most freshwater species of fish, and comprises four major lineages classified in the orders Cypriniformes, Characiformes, Siluriformes, and Gymnotiformes, respectively. Partly because of their tremendous diversity, many groups of otophysan fishes remain poorly understood phylogenetically and in a state of taxonomic disarray. This is the case—to a greater or lesser extent—of African characiforms of the suborder Citharinoidei and the family Alestidae, Afro-Asian cypriniforms of the tribe Chedrini, and Neotropical siluriforms of the subfamily Loricariinae. To address the lack of robust, comprehensive, and/or up-to-date phylogenetic hypotheses for the aforementioned groups, this doctoral dissertation investigated their systematics and evolution through phylogenetic analyses of comparative DNA sequence data, including molecular-clock analyses that resulted in the first time-calibrated phylogenies ever proposed for both alestids and citharinoids (and characiforms for that matter). The molecular phylogenies arrived at herein represent the most comprehensive

hypotheses of relationships for each of the groups investigated. Although many of the relationships revealed by this study corroborated previous hypotheses based on morphological and/or molecular data, others are newly hypothesized or in conflict. Moreover, the results of this research revealed instances of para- and polyphyly in numerous nominal taxa (e.g., *Brycinus* [Alestidae], *Nannocharax* [Distichodontidae], *Raiamas* [Chedrini], *Lamontichthys* [Loricariinae]), prompting a reassessment of the taxonomies of the groups investigated. Information on the temporal context of alestid and citharinoid diversification was used to assess biogeographic hypotheses proposed to explain the Gondwanan distribution of characiforms. Likewise, the inferred chronograms shed some critical light on the historical processes that may have influenced diversification and biogeographic patterns in these and other groups of African freshwater fishes.

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INTRODUCTION

1. DISSERTATION BACKGROUND AND RATIONALE

Otophysan fishes (Series Otophysi; Rosen and Greenwood, 1970) are members of a morphologically and ecologically diverse clade of teleosts that comprises the orders Cypriniformes, Characiformes, Siluriformes, and Gymnotiformes (i.e., all non-gonorhynchiform ostariophysans) (Nelson, 2006, Wiley and Johnson, 2010) (Fig. 1). Otophysans include familiar species such as minnows, carps, piranhas, electric eels, and catfishes (Fig. 2), and with 9872 valid species (Eschmeyer and Fong, 2013) distributed throughout all continents except Australia and Antarctica (Berra, 2001; Lévêque et al., 2008), they account for the majority of freshwater fishes on Earth (Dudgeon et al., 2005) (Fig. 3). Members of this extremely speciose lineage are not only a major component of the modern continental ichthyofauna, but they are also economically important as many of them support worldwide commercial, subsistence, and recreational fisheries (Winfield and Nelson, 1991), while many others are popular among fishkeeping enthusiasts and therefore of commercial value in the aquarium fish trade (Collins et al., 2012).

While most otophysans are small to moderate in size, some of its members are among the largest species of freshwater fish, reaching up to a length of 3-4 m and a weight of 200-300 kg, such as in the siluriform species *Pangasianodon gigas* (Southeast Asian Mekong catfish) and *Silurus glanis* (the European Wels catfish), and the cypriniform species *Catlocarpio siamensis* (Indochinese giant barb). While not as large as the aforementioned species, the African tigerfish (*Hydrocynus goliath*), known to reach up to 1.5 m and 50 kg, and the electric eel (*Electrophorus electricus*), which grows to about 2 m and 20 kg, are the largest species in the orders Characiformes and Gymnotiformes, respectively. At the other end of the spectrum, reaching a maximum size of 10.3 mm, the cypriniform species *Paedocypris progenetica* is the smallest fish

(Kottelat et al., 2006) and second smallest vertebrate on Earth (Rittmeyer et al., 2012). Such extraordinary body size disparity, coupled with an equally wide-ranging morphological diversity, has undoubtedly contributed to the ecological success of otophysan fishes and their ability to colonize virtually any freshwater ecosystem.

As members of the Ostariophysi, otophysans possess the anatomical evolutionary novelty known as Weberian apparatus, a structure consisting of a modified swimbladder and series of modified anterior vertebrae and associated elements (i.e., neural arches, supraneurals, pleural ribs), which ultimately connects the swimbladder to the inner ear (Weber, 1820; Rosen and Greenwood, 1970). The mechanical linkage between the ear and the swimbladder provided by the Weberian apparatus provides a means to amplify sound waves that would otherwise be only slightly perceivable by the inner ear structure alone (Braun and Grande [2008] and references therein). The evolutionary success of ostariophysans has therefore been primarily attributed to the selective advantage in freshwater environments conferred by the possession of the Weberian apparatus. Furthermore, it appears that no other character shared among members of the ostariophysan clade could explain their ecological and evolutionary success (Chardon and Vandewalle, 1997).

Apart from the Weberian apparatus and other ostariophysan synapomorphies, members of the Otophysi are diagnosed by 13 anatomical synapomorphies (Wiley and Johnson, 2010), corresponding to 12 of the characters originally proposed by Fink and Fink (1981) (some subsequently modified by Fink et al. [1984] and Fink and Fink [1996]), plus a more recently proposed synapomorphy related to the development of the neural complex (Hoffman and Britz, 2006).

The monophyly of both ostariophysans and otophysans has been consistently supported by morphological data (Greenwood et al., 1966; Rosen and Greenwood, 1970; Rosen, 1973; Novacek and Marshall, 1976; Fink and Fink, 1981), traditional DNA sequence data (Ortí and Meyer, 1996; Li et al., 2008; Broughton, 2010; Nakatani et al., 2011; Near et al., 2012; Betancur-R et al., 2013; Broughton et al., 2013), mitogenomic data (Lavoué et al., 2005; Poulsen et al., 2009; Broughton, 2010—although see Saitoh et al., 2003), ultraconserved elements (UCE) data (Chakrabarty et al., 2013), and combined analysis of morphological and molecular characters (Dimick and Larson, 1996). Relationships among major otophysan lineages, however, have not reached the same level of consensus, and some molecular phylogenetic studies (based on both traditional and phylogenomic markers) have even cast doubt on the monophyly of the Characiformes (Ortí and Meyer, 1996; Nakatani et al., 2011; Chakrabarty et al., 2013; Chen et al., 2013) (Fig. 4). Hypotheses of characiform para/polyphyly based on molecular data are however weakly supported, while morphological evidence in favor of the monophyly of the Characiformes is strong, and includes seven osteological synapomorphies that remain unchallenged (Fink and Fink, 1981, 1996).

The prevailing conflict in the resolution of otophysan relationships using molecular data, coupled with the fact that the branches subtending the clades involved in these discrepancies are very short and weakly supported, appears to suggest that even large amounts of DNA sequence data (or the methods used to analyze these data) might not be suitable for robustly resolving the seemingly rapid series of cladogenetic events leading to the ancestral lineages that evolved into the major clades of ostariophysan fishes (Chakrabarty et al., 2013). Since the majority of these studies have failed to include a large sample of the enormous taxonomic diversity exhibited by

most otophysan lineages, insufficient taxon sampling may also explain the lack of consensus regarding otophysan relationships.

Besides the lack of accord regarding relationships among major otophysan lineages, the alpha-taxonomy and phylogenetic relationships of putative clades at shallower levels of divergence (e.g., families, genera) are poorly documented for the most diverse otophysan orders (i.e., Cypriniformes, Siluriformes, and Characiformes) (Vari and Malabarba, 1998). Even after a significant increase in the number of published phylogenies incorporating otophysan fishes over the past 10 years compared to previous decades, partly driven by recent NSF-funded large-scale initiatives aimed at improving our understanding of the diversity and evolutionary relationships of catfish (ACSI; Sabaj et al., 2003-2006) and cypriniforms (CToL: Mayden et al., 2004-2009; ACSI-2: Armbruster et al. 2010-2015), many infraordinal groups of otophysan fishes remain poorly understood phylogenetically and in a state of taxonomic disarray.

African characiforms such as citharinoids (suborder Citharinoidei) and alestids (family Alestidae), Afro-Asian cyprinids of the tribe Chedrini, and Neotropical catfishes of the subfamily Loricariinae, all epitomize—to a greater or lesser degree—the aforementioned lack of comprehensive and up-to-date taxonomic and phylogenetic treatments, and are therefore in pressing need for phylogeny-based classifications and revisionary studies. Accordingly, these groups have been chosen for the present doctoral dissertation as case studies to investigate the systematics of otophysan groups in need of in-depth phylogenetic research using modern analytical approaches and novel comparative data. The molecular phylogenetic studies that comprise the remaining chapters of this dissertation, some of which include rigorous molecular-clock analyses carried out to investigate timing of diversification and its historical biogeographic implications, resulted in the most complete and up-to-date hypotheses of relationships for the

groups investigated. Further research, however, will be necessary to shed light on some of the questions that the molecular data and/or analytical methods used herein failed to unambiguously answer. Likewise, future research (e.g., revisionary studies) will certainly be required to address some of the problematic taxonomies revealed by the phylogenetic studies presented here.

2. DISSERTATION LAYOUT

The systematics of African alestids (Characiformes: Alestidae) and citharinoids (Characiformes: Citharinoidea), Afro-Asian chedrins (Cypriniformes: Chedrini), and Neotropical loricariins (Siluriformes: Loricariinae) is the focus of this doctoral dissertation. In addition to this introductory chapter, there are four chapters, each of which deals exclusively with the systematics of one of the aforementioned groups of otophysan fishes.

In Chapter 2, relationships among members of the African characiform family Alestidae are investigated through phylogenetic analysis of a molecular dataset consisting of four markers (including both nuclear and mitochondrial genes) across a sample of 53 ingroup and 11 outgroup species (including members of several Neotropical lineages). Additionally, the temporal context of alestid diversification is investigated using a relaxed molecular clock (calibrated with fossil data) under a purely Bayesian framework. The taxonomic and biogeographic implications of the resultant time-scaled phylogeny are discussed.

In Chapter 3, the phylogenetic relationships of citharinoid fishes are investigated using a multi-locus dataset of seven genes sampled across 55 citharinoid species and the taxonomy of the group is discussed in light of the resultant topology. In addition, Bayesian molecular dating is used to estimate divergence times, with the molecular clock calibrated using node ages implied from both the fossil record and estimates from a time-calibrated molecular phylogeny of all ray-finned fishes. The temporal context suggested by the inferred time-scaled phylogeny is then used to test biogeographic hypotheses proposed to explain the Gondwanan distribution of characiform fishes, and to explore the influence of palaeogeographic processes in the diversification of citharinoid and other African fishes with similar distribution patterns.

Chapter 4 examines the phylogenetic relationships of Afro-Asian chedrin cyprinids by analyzing an expanded version (both in taxon and character sampling) of the molecular dataset used in a recent study investigating relationships among danionin cyprinids. By considerably increasing the sampling of African members of the tribe, this study provides the first opportunity to assess the validity of several genera of uncertain monophyly. The implications of the resultant phylogeny for the biogeographic history of chedrins are also discussed.

Lastly, Chapter 5 investigates the phylogenetic relationships among members of the Neotropical catfish subfamily Loricariinae using comparative DNA sequence data from four genes sampled from 56 species representing 21 loricariin genera. Instances of congruence and conflict with previous phylogenetic studies are indicated, the taxonomic implications discussed, and an updated classification scheme proposed.

Combined, chapters 2-5 constitute an important contribution to evolutionary biology in general and systematic ichthyology in particular, for they address the complex and somewhat neglected systematics of various clades of otophysan fishes in dire need of modern taxonomic and phylogenetic assessment. In addition to providing insight into the evolutionary relationships, temporal context of diversification, and biogeographic history of four remarkable clades of freshwater fishes, this dissertation contributes a significant addition of molecular data (over 2000 individual gene sequences from across 225 species [mostly nominal but also questionably nominal and/or undescribed] in five families and three orders), of voucher specimens and tissue samples (collected during the course of this dissertation in both Africa and South America), and taxon-specific primers designed for amplification and sequencing of some of the genes sampled for the phylogenetic analyses conducted as part of this doctoral research.

3. FIGURES

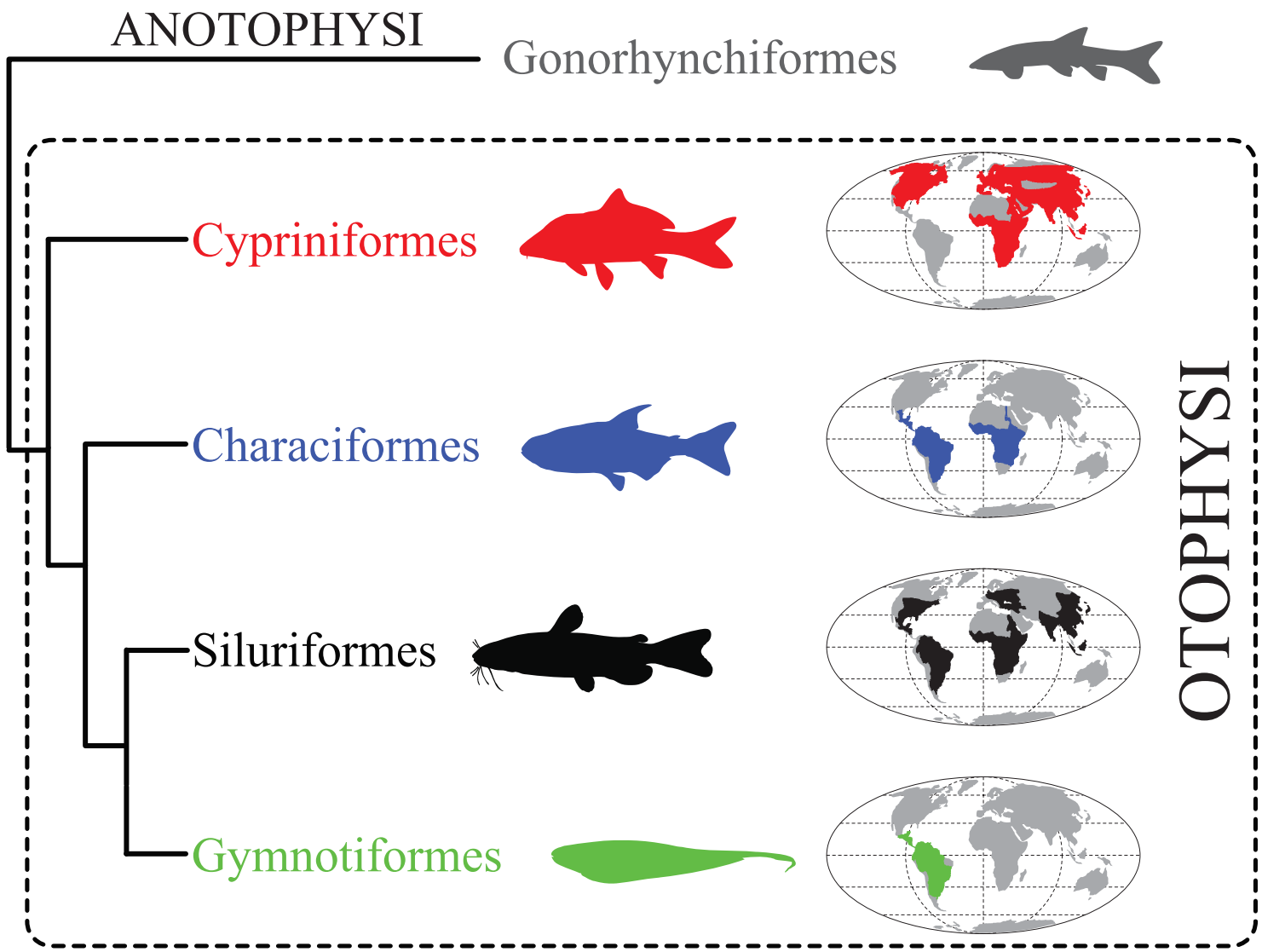


Figure 1. Phylogeny and present-day geographic distribution of major lineages (orders) of the superorder Ostariophysi (Fink and Fink, 1981; Berra, 2001).

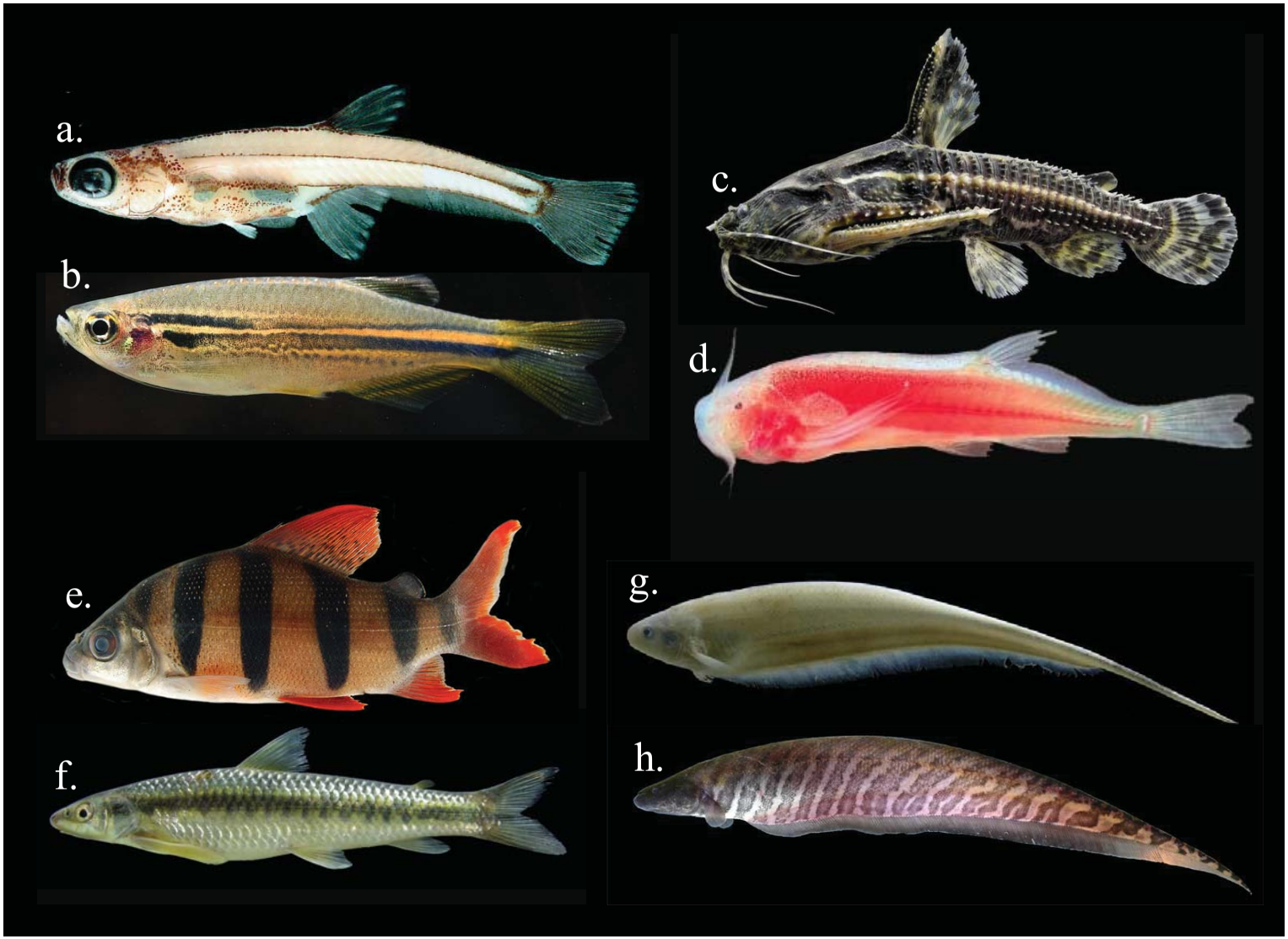
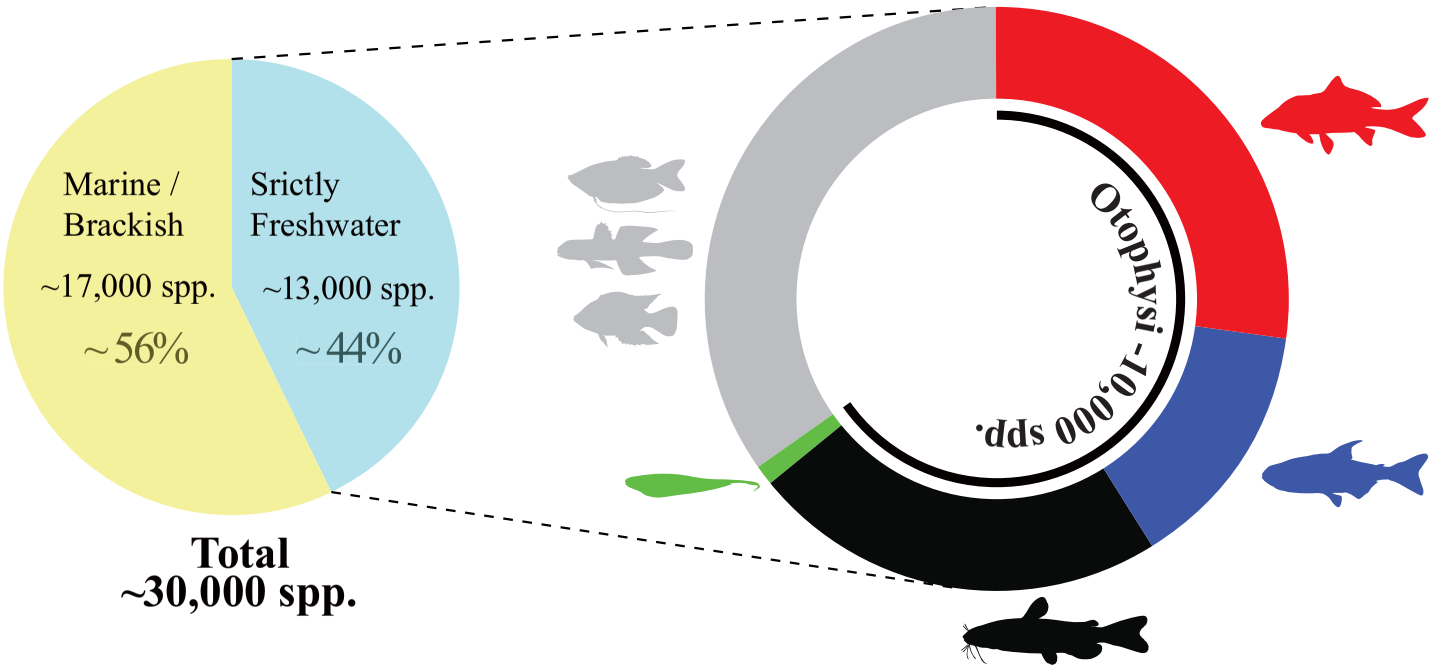


Figure 2. Morphological diversity of otophysan fishes as illustrated by the cypriniform species *Paedocypris progenetica* (a) and *Danio hikari* (b), the siluriform species *Acanthodoras spinosissimus* (c) and *Sarcoglanis simplex* (d), the characiform species *Distichodus sexfasciatus* (e) and *Apareiodon orinocensis* (f), and the gymnotiform species *Eigenmannia virescens* (g) and *Gymnotus carapo* (h).



Otophysi ~33% of all fish, ~77% of freshwater fish

	# Families	# Species
Cypriniformes	11	4075
Characiformes	23	2021
Siluriformes	37	3572
Gymnotiformes	5	204
Total	76	9872

Eschmeyer and Fong (2013)

Figure 3. Otophysan species richness in the context of the global diversity of fish (modified from Nakatani et al., 2011).

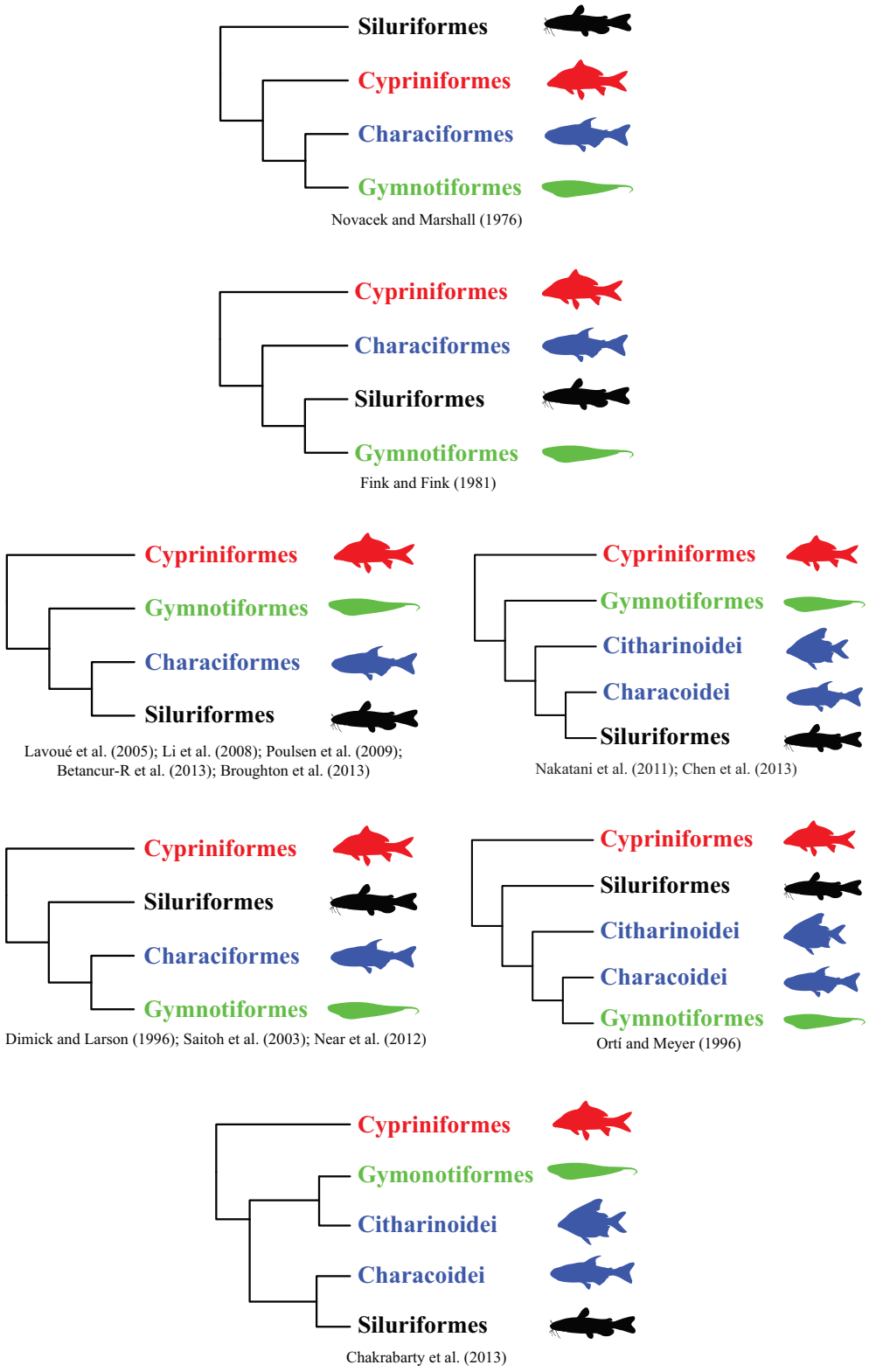


Figure 4. Conflicting hypotheses of relationships among major otophysian lineages.

CHAPTER 2

PHYLOGENETIC RELATIONSHIPS AND THE TEMPORAL CONTEXT FOR THE DIVERSIFICATION OF AFRICAN CHARACIFORM FISHES OF THE FAMILY ALESTIDAE (OSTARIOPHYSI: CHARACIFORMES): EVIDENCE FROM DNA SEQUENCE DATA

1. INTRODUCTION

Fishes of the order Characiformes are among the most diverse and abundant components of the Neotropical and African freshwater ichthyofaunas. Although by far the greatest diversity of the order occurs in the Neotropics, African freshwaters harbor more than 200 species currently arrayed in four families. With a total of 121 recognized species in 21 genera, and including well-known forms such as the giant African goliath tigerfish (*Hydrocynus goliath*) or the Congo tetra (*Phenocogrammus interruptus*) popular with aquarists, Alestidae is the most speciose of the African characiform families (Eschmeyer and Fong, 2010). While a few alestids are found in the lower Nile basin and in scattered localities in the horn of Africa, the family is primarily sub-Saharan where it occurs predominantly in lowland rivers, reaching highest diversity in the Congo River basin, Lower Guinea, and the coastal rivers of West Africa (Roberts, 1975; Zanata and Vari, 2005; Eschmeyer, 2010).

Alestidae was erected by Géry (1977) to include the African members of the family Characidae *sensu* Greenwood et al. (1966), originally classified into the subfamilies Hydrocyninae and Alestinae (Roberts, 1969). Alestid subfamilial taxonomy is widely recognized to be artificial, based on combinations of often broadly overlapping features, many of which are variable within and among species (Schaefer, 2007). The subfamily Hydrocyninae, for instance, was created to accommodate the exceptionally large-bodied tigerfishes of the genus *Hydrocynus*. The subfamily Alestinae (which includes all remaining alestid genera) was divided into Alestini and Petersiini to accommodate mid-sized and dwarf species, respectively (Roberts, 1969; Géry, 1995). Despite previous claims by Vari (1979: 342) suggesting that recognition of Hydrocyninae would render Alestinae non-monphyletic, Géry (1995) maintained Roberts' (1969) subfamilial classification.

In a study focusing on the higher-level relationships among characiform fishes, Ortí and Meyer (1997) presented for the first time preliminary evidence for the monophyly of the family. Their results, however, were based on a very limited taxon sampling that included only three alestid genera. Although Vari (1998) argued that previous studies (Roberts, 1969; Vari, 1979; Vari, 1995) provided morphological characters indicative of alestid monophyly, his conclusions were based on inductive generalizations rather than a list of synapomorphies resulting from a comprehensive cladistic analysis. Relationships among members of Alestidae were investigated further using phylogenetic methods by Murray and Stewart (2002), followed by the works of Hubert et al. (2005), Zanata and Vari (2005), and Calcagnotto et al. (2005). Although Murray and Stewart (2002) provided several synapomorphies supporting the monophyly of the family, their study focused on the relationships of the genera *Alestes* and *Brycinus*, and included limited sampling of alestid generic diversity. Likewise, the sampling of alestid taxa in the study of Hubert et al. (2005) consisting of only six genera. Such limited taxon sampling necessarily decreases phylogenetic accuracy (Hillis, 1998), rendering the results of those studies suspect at best. By contrast, the monumental contribution of Zanata and Vari (2005) included representatives of 19 alestid genera and surveyed a large number of characters from a wide variety of morphological systems, resulting in the most comprehensive compendium of alestid comparative anatomy and the ensuing phylogenetic hypothesis derived from that dataset. Similarly, the molecular phylogeny of Calcagnotto et al. (2005) –although primarily focused on suprafamilial relationships within the order Characiformes– included representatives of 14 alestid genera, and comparative data from two mitochondrial (*16S* and *cyt–b*) and four nuclear (*RAG2*, *sia*, *fkh*, and *trop*) genes. Both studies corroborated the monophyly of Alestidae, yet the recovered topologies were incongruous with each other (Fig. 1).

Based on both the age of the oldest fossil assignable to Alestidae and the time of the African/South American drift-vicariance event, Zanata and Vari (2005: 120, Fig. 44) proposed age estimates for higher-level clades within the family. Such estimates, however, are problematic for several reasons. First, use of palaeontological data alone very likely underestimates the ages of lineages, as the appearance of the oldest fossil is expected to postdate the origin of the clade it belongs to (Marshall, 1990). Second, use of a biogeographic event to estimate the age of the alestid clade is critically dependent on the phylogenetic accuracy of the topology, yet the position of the South American genus *Chalceus* with respect to alestids as hypothesized by Zanata and Vari (2005) might be an artifact of incomplete taxon sampling. This is because several lineages of Neotropical characins were not represented in their set of outgroup taxa. Third, even if the status of the Neotropical genus *Chalceus* as the most basal alestid is correct, it is not the only instance of an African/South American sister-group relationship across the phylogeny of the order (Ortí and Meyer, 1997; Buckup et al., 1998; Calcagnotto et al., 2005; Malabarba and Malabarba, 2010). Thus, there is no compelling reason to propose the breakup of Gondwana as the cause of the split between *Chalceus* and the alestid clade. As a result, the temporal context of alestid diversification as proposed by Zanata and Vari (2005) remains in need of critical evaluation. Reliably inferring the pattern and timing of cladogenetic events in the alestid phylogeny is therefore essential for an improved understanding of the evolutionary history of the family and its implications for the historical biogeography of characiform fishes.

Molecular-dating techniques, although still far from perfect, allow for estimation of the ages of clades by taking into account many of the uncertainties associated with converting genetic distances into time units (Rutschmann, 2006). In theory, dating the nodes of a phylogenetic tree from DNA sequence data requires a constant rate of substitution among lineages (i.e., a strict

molecular clock) and available fossil information (often treated as fixed) to calibrate the clock. Recently, however, so-called "relaxed-clock" methods (e.g., Sanderson, 1997; Rambaut and Bromham, 1998; Thorne et al., 1998; Drummond et al., 2006) allow departures from clock-like behavior. These present an enticing approach to divergence time estimation because molecular datasets rarely conform to a strict clock and such a lack of rate constancy has been regarded as the main obstacle for an accurate molecular dating of phylogenies (Smith and Peterson, 2002). In particular, the Bayesian relaxed-clock method of Drummond et al. (2006) excels in allowing users to employ probability distribution-based calibrations instead of fixed-age nodes, thus modeling some of the uncertainties associated with calibrating substitution rates from palaeontological data.

Although the works of Zanata and Vari (2005) and Calcagnotto et al. (2005) are hitherto the best studies addressing the monophyly and the phylogenetic relationships of Alestidae, they disagree on the placement of the Neotropical genus *Chalceus*, and derived discordant topologies. Therefore, a phylogenetic analysis of Alestidae based on independent evidence seems desirable, especially when a focused molecular phylogeny of the family has yet to be proposed. Furthermore, improved estimates for the absolute ages of clades in the alestid tree can be generated from fossil and DNA sequence data by means of molecular-dating techniques. Thus, the main objectives of the present study are to present the most comprehensive phylogeny of Alestidae based on molecular data and to date the nodes of the resultant phylogenetic tree. Accordingly, previous hypotheses of alestid interrelationships, the monophyletic status of the family and its genera, and the phylogenetic position of *Chalceus* are tested. By establishing the temporal context of alestid diversification, alternative biogeographic hypotheses explaining the Gondwanan distribution of the order Characiformes are explored.

2. MATERIALS AND METHODS

2.1. TAXON SAMPLING

Representatives of all valid genera, except *Petersius* and *Virilia* (for which tissue vouchers were unavailable) were sampled for phylogenetic analyses. Whenever possible, multiple species of each genus were included. Although multiple individuals were sequenced for most species, the analyses presented herein are based on a reduced dataset that includes DNA sequence data for only one individual per species. This was partially due to avoid analyses of an unnecessarily redundant and larger dataset, but mostly because it was a requirement for the Bayesian method of phylogeny and divergence time estimation employed in this study. Sequencing of multiple individuals per species, however, allowed for an improved control of sequence quality and contamination issues. Tissues were primarily obtained from specimens collected during field expeditions of the ongoing NSF-funded Biotic Surveys and Inventories Congo Project (http://research.amnh.org/vz/ichthyology/congo/index.html). Additional tissues were obtained from samples deposited in the Ambrose Monell Cryo Collection (AMCC) at the American Museum of Natural History (AMNH), augmented by donations from colleagues at the Cornell Museum of Vertebrates (USA), the Zoologische Staatssammlung München (Germany), and the South African Institute for Aquatic Biodiversity. Material examined (voucher specimens) and GenBank accession numbers for the gene sequences generated and included in this study are listed in Table 1. A total of 64 terminals (53 ingroup and 11 outgroup species) encompassed the taxonomic sampling for phylogenetic analyses. Outgroup choice was informed by previous higher-level phylogenetic hypotheses of characiform relationships (e.g., Buckup, 1998; Calcagnotto et al., 2005). Hence, outgroup taxa included representatives of seven characiform

families: the remaining three African families (i.e., Citharinidae, Distichodontidae, and Hepsetidae), and four of the Neotropical families historically regarded as closely related to alestids (i.e., Erythrinidae, Ctenoluciidae, Acestrorhynchidae, and Characidae).

2.2. MARKER SELECTION AND CHARACTER SAMPLING

Aiming to recover both deeper and more recent divergences, four protein-coding genes with markedly different rates of substitution were chosen for phylogenetic analyses. These consist of two nuclear (*SH3PX3* and *myh6*) and two mitochondrial (*COI* and *cyt–b*) markers, totaling more than 3000 bp. *SH3PX3* and *myh6* were proposed by Li et al. (2007) as promising markers with potential use in molecular systematics of actinopterygian fishes. These have been employed in empirical phylogenetic studies of Cypriniformes (Chen et al., 2008), Gasterosteiformes (Kawahara et al., 2009), Stomiiformes (DeVaney, 2008), Cyprinodontiformes (Meredith et al., 2010), and Perciformes (Li et al., 2010), among others. On the other hand, the mitochondrial markers *COI* and *cyt–b* have been consistently used in evolutionary studies across most animal phyla (e.g., Folmer et al., 1994; Kocher et al., 1989), and have proven useful in resolving phylogenetic relationships of characiform fishes (e.g., Calcagnotto et al., 2005; Javonillo et al., 2010).

2.3. DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

Total genomic DNA was extracted from tissues (muscle and fin clips), preserved in 95% EtOH and stored frozen, using DNEasy Tissue Extraction Kit (Qiagen) following the manufacturer's protocol. For each sample and gene, DNA amplification via Polymerase Chain Reaction (PCR) was preformed in a 25-μL volume containing one Ready-To-Go PCR bead (GE

Healthcare), 21 μL of PCR-grade water, 1 μL of each primer (10 μM), and 2 μL of genomic DNA. Double-stranded PCR products were purified using AMPure (Agencourt). Primer sequences and PCR profiles are listed in Table 2. Sequencing of each strand of amplified product was performed in a 5-μL volume containing 1μl of primer (3.2 μM), 0.75μl of BigDye® Ready Reaction Mix, 1μl of BigDye® buffer, and 2.25μl of PCR-grade water. Sequencing reactions consisted of a 2-minute initial denaturation at 95°C, followed by 35 cycles of denaturation at 95°C for 30s, annealing at 50°C for 60s, and extension at 72°C for 4 mins, followed by a 3-minute final extension at 72°C. For sequencing *COI*, however, annealing temperature was set at 45°C. All sequencing reactions were purified using CleanSEQ (Agencourt) and electrophoresed on an Applied Biosystems 3700 automated DNA sequencer at the AMNH Molecular Systematics Laboratories. Contig assemblage and sequence editing was performed using the software Geneious Pro v4.6.2.

2.4. PHYLOGENETIC ANALYSES

Phylogenetic analyses were carried out using both parsimony and model-based approaches. Prior to phylogeny estimation, nucleotide base correspondences ("primary homologies" *sensu* de Pinna, 1991) were identified by means of multiple sequence alignment (MSA). An alignment for each gene was performed using MUSCLE (Edgar, 2004) under default parameters, followed by concatenation of individual alignments. Calculation of the number of variable and parsimony informative sites was conducted using MEGA v4. (Tamura et al., 2007). When using parsimony as an optimality criterion, both non-additive optimization (Fitch, 1971) and direct optimization (Wheeler, 1996) were implemented in the programs TNT [Willi Hennig Society edition] (Goloboff et al., 2003; Goloboff et al., 2008) and POY v4.1. (Varón et al., 2008), respectively. A

dynamic homology approach (i.e., direct optimization) was applied with the expectation of finding more parsimonious phylogenetic hypotheses because in addition to minimizing substitutions and insertion–deletion events, nucleotide correspondences (i.e., putative homologies) themselves are chosen to minimize tree length (Wheeler, 2001; Wheeler et al., 2006). In all parsimony analyses gaps were treated as a fifth state and no cost for gap opening was specified. An indel/substitution cost ratio of 1:1 was set as the transformation cost matrix to be used in calculating the length of the tree. Tree searches were performed by building a set of Wagner trees (Farris, 1970) by random addition sequence of taxa (RAS), followed by tree bisection and reconnection (TBR) branch swapping (Swofford and Olsen, 1990), perturbation using the Parsimony Ratchet (Nixon, 1999), and tree fusing (Goloboff, 1999). Branches for which the minimum possible length was zero were collapsed. TNT analyses included 100 RAS. Tree searches in POY were executed using the *search* command, which performs as many builds, swaps, ratchets, and tree fusings as possible within the specified time (A. Varón, pers. comm.). POY analyses were carried out on the AMNH Parallel Computing Cluster over a period of 5 days, using 32 processors. Tree statistics for parsimony analyses included tree length (*L*), ensemble consistency index (CI; Kluge and Farris, 1969) and ensemble retention index (RI; Farris, 1989).

Maximum Likelihood [ML] (Felsenstein, 1981) and Bayesian (Rannala and Yang, 1996) analyses comprise the model-based approaches to phylogenetic inference used in this study. In order to accommodate the potential process heterogeneity that might occur among the different gene regions of our dataset, both ML and Bayesian analyses were conducted on the concatenated alignment partitioned into gene regions with parameters unlinked. Nucleotide substitution model selection for each gene partition was accomplished by means of both hierarchical likelihood ratio

tests (hLRTs) and the Akaike Information Criterion (AIC) using the program jModelTest (Posada, 2008). Acceptance/rejection of the null hypothesis in the hLRTs was based on a significance level of 0.05. Both indices determined that, for all gene partitions, the model that best described the evolution of the sequence data over the phylogeny was the GTR+I+Γ (see *Results* section). ML analyses were conducted with RAxML v7.0.4. (Stamatakis, 2006) through the Cyberinfrastructure for Phylogenetic Research (CIPRES) project web server (Miller et al., 2009). Bayesian analyses included the co-estimation of phylogeny and divergence dates, and were carried out in BEAST v1.4.8. (Drummond and Rambaut, 2007). Clade support was estimated by means of the bootstrap character resampling method (Felsenstein, 1985) for TNT and RAxML (Stamatakis et al. 2008) analyses, the Bremer Index (Goodman et al., 1982; Bremer, 1988) for POY analyses, and clade posteriors for BEAST analyses. Bootstrap support values were calculated based on 1000 pseudoreplicates.

2.5. DIVERGENCE TIME ESTIMATION

Prior to estimation of divergence times for the alestid phylogeny, a likelihood ratio test (LRT) was performed using the program PAUP* v4.0. (Swofford, 2003) in order to determine whether rates of nucleotide substitution in our dataset departed significantly from expectations under the assumption of a strict molecular clock. Results of the LRT indicated that a strict molecular clock was inappropriate to estimate dates of divergence in our dataset, so we relied on the Bayesian relaxed-clock method of Drummond et al. (2006) under the uncorrelated lognormal (UCLN) rate variation model with default parameters as implemented in the software BEAST. In the UCLN relaxed-clock model there is no correlation of rates on adjacent branches of the tree: the rate on each branch is drawn independently and identically from a lognormal distribution

described by mean and variance parameters (Drummond et al., 2006). Selection of this model was based on the idea that autocorrelated relaxed-clock models might be inappropriate under several conditions, for instance, when disparity in life-history traits among taxa contributes a substantial amount of variation in the inherited determinants of rates (Smith et al., 2010). In addition, empirical and simulation studies have shown that the UCLN model performs well both in terms of accuracy and power, even when the data were generated under alternative models (Drummond et al., 2006).

We assumed uniform priors for both the GTR+I+Γ and UCLN model parameters, and a Yule process prior for topology and divergence times. The Yule process implies a prior distribution under a pure-birth stochastic branching process model (i.e., speciation as random lineage splitting) and its use seems appropriate if priors on trees are expected to reflect the underlying physical process by which trees are generated (Velasco, 2008). Moreover, various authors have claimed that uniform priors on topologies introduce bias in favor of smaller and larger clades and against medium sized ones (Pickett and Randle, 2005; Goloboff and Pol, 2005; Yang, 2006), as well as a skewed distribution on tree shapes (Velasco, 2008). Likewise, labeled histories (i.e., topologies with a temporal ordering of the nodes) are not equally probable if all topologies are allowed the same prior probability (Velasco, 2008). A Yule process, instead, produces each labeled history with equal probability (Edwards, 1970). When implemented in BEAST, this prior assumes a constant speciation rate per lineage and has a single parameter (yule.birthRate) that represents the average net rate of lineage birth. Under this prior, branch lengths are expected to be exponentially distributed with a mean of $\text{yule.birthRate}^{-1}$ (Drummond et al., 2007).

Because fossils correctly placed in a phylogeny can only provide minimum age estimates for a particular lineage (Marshall 1990), estimates of substitution rates were calibrated using fossil information in the form of probabilistic priors rather than as fixed values. Specifically, a lognormally distributed prior (with a rigid lower bound, mean and standard deviation parameters) was chosen to accommodate some of the uncertainties associated with the use of fossil data to specify the ages of the internal nodes used as calibration points. This prior is generally regarded as the most appropriate for modeling palaeontological information (Hedges and Kumar 2004, Drummond et al., 2006) and its use implies that the actual cladogenetic event is most likely to have occurred at some time prior to the earliest appearance of the fossil (Ho, 2007). Absolute estimates of divergence times for the alestid phylogeny were calculated using the two oldest fossils unambiguously assignable to the family Alestidae as calibration points. These included isolated teeth from the Late Miocene (5-7 Mya) of Kenya and Zaire assignable to the most recent common ancestor (MRCA) of the genus *Hydrocynus* (Van Neer, 1992; Stewart, 1994, 2003), and from the Early Eocene (49–54.8 Mya) of the Iberian Peninsula assignable to the MRCA of the genera *Alestes*, *Brycinus* and *Bryconaethiops* (De la Peña Zarzuelo, 1996; Zanata and Vari, 2005: p 123). The mean and standard deviation parameters of the lognormal prior probability distribution associated with these calibrations were chosen so that most of the probability lies within the interval representing the age of the fossil, yet allowing the true age of divergence to extend much further back in time. The rigid minimum bounds were set as the lower limit of the fossil age interval (i.e., 5 and 49 Mya, respectively).

BEAST analyses were implemented using the Markov Chain Monte Carlo (MCMC) algorithm run for 20×10^6 generations, with a sampling frequency of 1000 generations and default proposal mechanisms. Convergence of the MCMC algorithm to a stationary distribution, and

thus the number of generations to be discarded as burn-in, was determined by examining trace plots of posterior probability vs. number of generations using Tracer v1.5 (Rambaut and Drummond, 2009). The maximum a posteriori (MAP) tree (Rannala and Yang, 1996), a chronogram indicating the mean ages of all nodes with their associated 95% highest posterior density (HPD) intervals, and the posterior probabilities of nodes were calculated from the set of post burn-in trees using TreeAnnotator v1.5.3 (Drummond and Rambaut, 2007).

3. RESULTS

3.1. NUCLEOTIDE SUBSTITUTION MODEL SELECTION

Summary of the results from the statistical selection of best-fit models performed in jModelTest is presented in Table 3. Both hLRTs and AIC established that the model that fit our data best for all gene partitions was the general time reversible (GTR; Rodriguez et al., 1990) with a proportion of invariable sites (I) and a gamma-distributed rate heterogeneity among sites (Γ).

3.2. NUCLEOTIDE HOMOLOGIES

A matrix corresponding to the hypotheses of primary homology as determined by MSA is presented in Appendix 1. The concatenated alignment included a total of 3271 characters, from which 1323 were variable, 9 indels, and 1145 parsimony informative. None of the indels was phylogenetically informative. Individual alignments for *COI*, *cyt–b*, *myh6* and *SH3PX3* consisted of 658, 994, 820, and 799 nucleotides, respectively.

3.3. ALESTID PHYLOGENY

Two equally most parsimonious trees of length 8344 were found during POY analyses. In contrast, cladogram search in TNT resulted in three equally most parsimonious trees of length 8358. The strict consensus trees of POY and TNT were identical in topology, except for the position of the least inclusive clade containing *Ctenolucius hujeta* and *Arnoldichthhys spilopterus*, which was recovered more basally by POY (Fig. 2). Similarly, the most optimal trees recovered by ML and Bayesian analyses were topologically equivalent (Fig. 3). Bootstrap

values in the TNT topology were notably low for several nodes. By contrast, most nodes in the POY topology were well supported, having Bremer values above 10. Similarly, most clades in the ML and Bayesian topologies were well supported (bootstraps >75% and clade posteriors >0.9) (Fig. 3). In general, the parsimony topology was concordant with that obtained by model-based methods. Both approaches recovered the African genera *Arnoldichthhys* and *Lepidarchus* nested well within the Neotropical members of the outgroup, thus rejecting the monophyly of the family as currently recognized (Alestidae *sensu lato*). Moreover, contrary to the hypothesis of Zanata and Vari (2005), the Neotropical genus *Chalceus* was resolved as more closely related to a subset of South American characins than to African alestids. All analyses recovered the remaining members of the ingroup (hereafter referred to as Alestidae *sensu stricto* [*s.s.*]) resolved into four major lineages (Clades A–D in Fig. 4). Resolution within each lineage, however, differed slightly between methods.

3.4. MONOPHYLY OF ALESTID GENERA

Due to taxon sampling limitations (i.e., availability of tissues), it was only possible to test monophyly for 10 of the 15 polytypic alestid genera. Overall, our results corroborated the monophyly of the genera *Bryconaethiops, Hydrocynus, Alestes, Nannopeterius, Micralestes,* and *Phenacogrammus*. The last-named, however, is not monophyletic according to the results of the parsimony analyses. This is because *P. aurantiacus* was found more closely related to *Clupeocharax schoutedeni* than to the other *Phenacogrammus* species included in this study (Fig. 2). On the other hand, we found no support for the monophyletic status of four currently recognized genera. *Brycinus* was recovered as polyphyletic with its members distributed in two separate clades: one at the base of the alestid tree and sister to all other alestids; the other

recovered as the sister group of *Bryconaethiops*. Similarly, the genera *Bathyaethiops*, *Alestopetersius*, and *Rhabdalestes* were recovered as paraphyletic given their placement with respect to members of the genera *Brachypetersius*, *Tricuspidalestes*, and *Hemigrammopetersius* respectively (Figs. 2-3).

3.5. DIVERGENCE TIME ESTIMATES

Estimates of divergence times for the alestid phylogeny (Fig. 5) indicate that the origin of the family Alestidae *s.s.* dates back to the Early Tertiary (54 Mya; 95% HPD interval = 63–49) and that most diversification occurred during the mid-Tertiary within a period of just 30 My (40–10 Mya). Furthermore, our analyses indicate that the split between the lineage leading to the MRCA of alestids and the lineage that includes all other characiforms –except citharinoids– occurred during the Late Cretaceous (78 Mya; 95% HPD interval = 99–59). The estimated age of the nodes used as calibration points did not exactly match the age of the fossils assigned to those clades, especially for the node representing the MRCA of the genus *Hydrocynus*, in which the estimated age (21 My, 95% HPD interval = 30–13) is considerably older than the 5-My minimum age based on fossil data. On the other hand, the estimated age of the MRCA of the genera *Alestes*, *Brycinus* and *Bryconaethiops* (54 My; 95% HPD interval = 63–49) did not differ significantly from that suggested by the fossil age (49 My). In addition to the timing of cladogenetic events within the Alestidae, our results indicate that the split between citharinoids and the lineage leading to the remaining characiforms must have occurred sometime between the mid- and Late Cretaceous (87 Mya; 95% HPD interval = 119–59). This latter result, however, should be viewed with caution given that our sampling of Neotropical characiform lineages is far from comprehensive and only two fossil calibrations –restricted to the alestid clade– were

included in the analyses.

4. DISCUSSION

4.1. MONOPHYLY AND INTERGENERIC RELATIONSHIPS OF THE ALESTIDAE

All optimality criteria resulted in a similar pattern of relationships, suggesting that the alestid phylogeny as inferred from our data is robust and not significantly affected by analytical method. However, in contrast to model-based approaches, parsimony analyses do not support a sister-group relationship between *Hydrocynus* and *Alestes* nor do they support a monophyletic *Phenacogrammus* (exclusive of *Clupeocharax)*. Additionally, results from different methods revealed a few subtle differences in the branching pattern among outgroup taxa (Fig. 4). Although our results indicate that continued inclusion of the genera *Arnoldichthhys* and *Lepidarchus* in Alestidae would render the family non-monophyletic, we cannot discount the possibility that the phylogenetic position of the diminutive West African *Lepidarchus* as revealed by our analyses is an artifact of missing data, given that none of the mitochondrial genes was successfully sequenced for this particular taxon. However, as most of the phylogenetic signal resolving intergeneric relationships (i.e., deeper divergences) is provided by less variable nuclear markers, we could anticipate that failure to include mitochondrial data would be problematic mostly for resolving divergences at less inclusive levels.

Interestingly, this is the first time that such a hypothesis regarding the position of *Lepidarchus* has been proposed. Of the few studies that have investigated generic interrelationships of Alestidae, only the morphology-based analysis of Zanata and Vari (2005) included comparative data for this genus. In contrast to our results, they hypothesized a sister-group relationship between *Lepidarchus* and *Ladigesia* (Fig. 1), a relationship supported by eleven morphological synapomorphies (Zanata and Vari, 2005: p 113). Given the morphological

support for this species pair, our findings regarding the position of *Lepidarchus* should perhaps be viewed with caution because of the missing data issue. We note however, that most of the characters optimized as synapomorphic for the *Lepidarchus* + *Ladigesia* sister-pair by Zanata and Vari (2005) are reversals potentially homoplastically associated with developmental truncation at small size (true also for *Tricuspidalestes* which is also a diminutive species, and the proposed sister group to the *Lepidarchus* + *Ladigesia* pair). Hopefully, future analyses without missing data, and ultimately a comprehensive reevaluation of both molecular and morphological data in a total evidence context, will elucidate the actual pattern and taxonomic changes will be proposed if necessary.

Contrary to the unexpected placement of *Lepidarchus* in our study, the phylogenetic position of the genus *Arnoldichthhys* is perhaps not as surprising. Even though both Zanata and Vari (2005) and Calcagnotto et al. (2005) recovered this genus as basal to the Alestidae, the latter acknowledged that this result was indeed weakly supported. Despite attributing the lack of support to a long-branch attraction artifact, Calcagnotto et al. (2005: p 144) did not discount the possibility that the monophyly of Alestidae may be compromised by the problematic placement of *Arnoldichthys*. Zanata and Toledo-Piza (2004) and Zanata and Vari (2005) have noted the striking morphological resemblance between *Arnoldichthys* (a monotypic genus, endemic to the Niger Delta region of west Africa) and members of the Neotropical genus *Chalceus* (with five species distributed widely in South America) (Zanata and Toledo-Piza, 2004), and there is certainly no question that these two genera do bear a striking phenotypic similarity that is not shared with other African alestids. However, our analyses consistently recover the Neotropical taxon *Ctenolucius hujeta* as the sister group of *Arnoldichthhys*, and place *Chalceus* in a clade with the genera *Acestrorhynchus*, *Brycon*, and *Hemigrammus*. So despite the morphological

characters enumerated by Zanata and Vari (2005) supporting the placement of *Chaleus* and *Arnoldichthys* as sequential sister taxa to the remaining alestids, our analyses place *Chalceus* and *Arnoldichthys* well separated from the members of that clade. While our sampling of South American characins is far from comprehensive enough to allow us unequivocally to state that *Chalceus* is indeed more closely related to an assemblage that includes *Acestrorhynchus*, *Brycon*, and *Hemigrammus* or that *Arnoldichthys* is more closely related to the Ctenoluciidae than to any other characiforms, we find no support for their alignment with the remaining African taxa. In view of this conflict, we suggest it prudent to exclude both genera from the family in order to maintain a monophyletic Alestidae well supported by congruent molecular and morphological character data.

The phylogenetic analyses presented here recovered an Alestidae *s.s*. consisting of four major suprageneric assemblages represented by well-supported clades (A–D in Fig. 4). Except for the position of *Bryconalestes* and the genera not included in their study, Calcagnotto et al. (2005) recovered the same four major subfamilial clades, yet the branching pattern between, and resolution within each clade were not exactly as in the phylogeny proposed herein. Both studies strongly reject the monophyly of the subfamily Alestinae, as well as the monophyly of the tribes Alestini and Petersiini, and thus do not conform to the existing subfamilial and tribal classification (Roberts, 1969; Géry, 1995). Accordingly, we concur with Calcagnotto et al.'s (2005) claim that "continued recognition of subfamilies and tribes within Alestidae must be reconsidered". In the interest of promoting a phylogeny-based taxonomy, we suggest that the aforementioned suprageneric clades form a useful basis for future revisional studies.

Our results further corroborate some previous findings regarding intergeneric relationships at less inclusive levels within the alestid phylogeny. Namely, they support a close relationship

between *Hydrocynus* and *Alestes* (Brewster, 1986; Murray and Stewart, 2002; Hubert et al., 2005; Calcagnotto et al., 2005), a close relationship between *Ladigesia* and *Micralestes* (Géry, 1968; Calcagnotto et al., 2005), and a sister-group relationship between a subset of *Brycinus s.l.* and the genus *Bryconaethiops* (Murray and Stewart, 2002; Calcagnotto et al., 2005). These results are, however, in conflict with the morphology-based hypothesis proposed by Zanata and Vari (2005), suggesting that ultimate resolution of these discrepancies lie with an augmentation and reevaluation of both molecular and morphological data in the context of a total evidence analysis. Concern may be raised about not having combined our molecular data set with the morphological matrix of Zanata and Vari (2005) under a total evidence approach. There are, however, two main reasons behind our choice. First, the fact that our taxon sampling was substantially non-overlapping (especially at the species-level) with that of Zanata and Vari (2005), which would inevitably lead to considerable amounts of missing data. Second, and probably most importantly, the fact that at present we do not have access to all the comparative material necessary for a meaningful re-examination of characters and homology statements in order to understand and resolve potential conflicts. The focus of this study, as previously stated, was to reconstruct the most comprehensive phylogeny of the family based on independent evidence. Despite relying on DNA sequence data only, we believe that the molecular characters used in the present study proved to be quite informative, as indicated by the overall degree of phylogenetic resolution and high clade support values (Figs. 2 and 3). Although a combined analysis represents a major priority for future research (given the extensive disparity between the most comprehensive morphology- and DNA-based phylogenies to date), we consider that the results presented herein are a useful contribution to help direct revisionary studies in order to

facilitate a much-needed improvement of the alpha-taxonomy of the family (Stiassny and Schaefer, 2005; Schaefer, 2007).

4.2. MONOPHYLY OF ALESTID GENERA

Attempts at a phylogeny-based generic-level classification of Alestidae are generally recent, yet they remain conflicting and poorly supported by apomorphy-based diagnoses (Schaefer, 2007). Although a provisional scheme of alestid interrelationships was provided by Zanata and Vari (2005), the monophyletic status of most genera remains to be fully assessed. Overall, our results rejected the monophyly of *Brycinus*, *Rhabdalestes*, *Alestopetersius*, and *Bathyaethiops*. The polyphyly of *Brycinus*, a speciose and commercially important Pan-African genus, has been repeatedly suggested (Murray and Stewart, 2002; Hubert et al., 2005; Calcagnotto et al., 2005), all of who recognize the monophyly of the "macrolepidotus*"* species group of Paugy (1986); a grouping of 8 large-bodies species mainly distributed in Central Africa and which includes the type species of the genus (*B*. *macrolepidotus*). By corroborating this hypothesis, our results underscore the desirability of a future revisionary study of this economically important genus. Certainly, continued recognition of *Brycinus* as presently conceived should be reconsidered and efforts focused on revisional and phylogenetic studies to resolve the composition and relationships of the rump *Brycinus s.l.* Similarly, previous studies did not support the monophyly of *Rhabdalestes* (Hubert et al., 2005; Zanata and Vari, 2005) or *Alestopetersius* (Zanata and Vari, 2005), and a more encompassing study involving all nominal species within both genera must precede any change in the generic-level classification of these taxa. Likewise, although our results rejected the monophyly of the genus *Bathyaethiops* based on the phylogenetic position of

Brachypetersius altus, proposing taxonomic changes at this point is premature given the restricted sampling of *Bathyaethiops* and *Brachypetersius* species in our study.

In contrast to previous hypotheses (Hubert et al., 2005; Zanata and Vari, 2005), and despite the claim of Stiassny and Mamonekene (2007: p 20) that "the genus *Micralestes* lacks a rigorous phylogenetic diagnosis and as currently conceived encompasses a wide range of external morphological diversity and considerable anatomical variability", our results strongly support the monophyly of this genus. Nevertheless, our sampling of *Micralestes* species includes only about a third of those currently recognized, mostly from the lower Congo River. Conversely, the monophyly of the genera *Bryconaethiops*, *Hydrocynus*, *Nannopetersius*, and *Alestes* appears to be well established based on the results of this and previous studies (Zanata and Vari, 2005; Calcagnotto et al., 2005).

4.3. PHYLOGENETIC PLACEMENT OF THE GENUS CHALCEUS

The novel hypothesis of a sister-group relationship between the Neotropical genus *Chalceus* and the family Alestidae served as the basis for expanding the limits of the family (Zanata and Vari, 2005). However, according to the phylogenetic hypothesis presented herein, the genus *Chalceus* is more closely related to other Neotropical characins than to the members of the family Alestidae *s.s*. This finding is in agreement with previous hypotheses of higher-level characiform relationships (Lucena, 1993; Ortí and Meyer, 1997; Calcagnotto et al., 2005). Interestingly, if *Chalceus* is in fact a member of a strictly Neotropical clade, the remarkable similarities between this taxon and the genus *Arnoldichthys* (Zanata and Toledo-Piza, 2004) might be an indication that the latter is indeed more closely related to Neotropical characins than to alestids, as suggested by our data.

4.4. TIMESCALE OF ALESTID DIVERSIFICATION

Although the use of DNA sequence data to estimate the timing of evolutionary events is increasingly popular, this is the first study applying molecular-dating techniques to the estimation of absolute dates of divergence for a group of characiform fishes. Previous studies exploring the timing of origin and diversification of the order Characiformes (e.g., Lundberg, 1993; Lundberg, 1998; Malabarba and Malabarba, 2010) and the family Alestidae (Zanata and Vari, 2005) did so based solely on palaeontological evidence. Despite not conforming to the general conclusions of these previous studies, our results are nonetheless consistent with the existing characiform fossil record (Malabarba and Malabarba, 2010). Our hypothesis of an Early Tertiary origin of the family Alestidae *s.s.* —certainly much younger than suggested by Zanata and Vari (2005)— implies that the alestid radiation long postdates a Mesozoic fragmentation of Gondwana. Our age estimates for the origin of genera and higher-level clades within Alestidae are likewise far more recent than previously proposed and lead to the unanticipated conclusion that the origin and diversification of alestids took place in African waters.

Most contemporary river basins of Sub-Saharan Africa were formed only after the Late Cretaceous, when Central Africa emerged above sea level (Stankiewicz and de Wit, 2006). Interestingly, the timing of origin of the Alestidae *s.s*., as inferred from our analysis, broadly coincides with the development of the modern African river network and related ecosystems of the region. Similarly, while most diversification in the family took place throughout the mid-Tertiary (40–10 Mya), the highest rates of cladogenesis occurred during the Early Miocene (25-15 Mya), after the Congo was first captured by a western coastal river draining to the Atlantic following the uplift of the East African Highlands (Stankiewicz and de Wit, 2006). Although we have no evidence of a causal relationship between such geologic events and the diversification of

alestids, temporal correspondences like these are noteworthy given the current distribution patterns of the family.

The estimated ages of the nodes constrained by our fossil-based calibrations (nodes † in Fig. 5) are older than the ages of the fossils themselves. Such a discrepancy was particularly manifest for the node representing the MRCA of *Hydrocynus*, for which the estimated mean age (21 My; 95% HPD interval = 30–13) is about four times older than the minimum age suggested by the fossil itself (5 My). This mismatch between constrained and estimated node ages, demonstrates that fixed-age fossil calibrations may indeed be biased and that divergence times based solely on palaeontological data are often likely to underestimate true ages. Hence, the importance of using more refined methods that rely on probabilistic priors to calibrate molecular clocks.

4.5. CHARACIFORM BIOGEOGRAPHY

While the primary goal of this study was not to resolve the pattern and timing of characiform diversification, by including representatives of the most basal lineages (i.e., citharinoids) and several other families across the diversity of the order, the inferred chronogram provides new insights into the temporal context of characiform evolution and its biogeographic implications. The prevailing hypotheses explaining characiform biogeographic patterns (Fink and Fink, 1981; Lundberg, 1993; Buckup 1998; Malabarba and Malabarba, 2010) suggest that the disjunct distribution of the order be attributed to the African/South American drift-vicariance event and therefore the origin of characins necessarily precedes the mid-Cretaceous fragmentation of Gondwana. In contrast, our results indicate that the divergence between citharinoids and the clade that includes the remaining characiform taxa occurred during the Late Cretaceous (87 Mya; 95% HPD interval = 119–59). Thereby suggesting that the order

Characiformes most likely originated after Africa and South America had separated [*c.* 110 Mya] (McLoughlin, 2001). Considering that a barrier to intercontinental migration of freshwater fishes might have established much earlier than 115 Mya (Briggs, 2005), vicariance hypotheses seem even less likely. Our results are consistent with the existing fossil record, since the earliest characiform fossils known to date come from the Cenomanian (*c.* 95 Myr) of Sudan and Morocco (Werner, 1994; Dutheil, 1999) and otophysan fossils only extend back to the Albian stage of the mid-Cretaceous [*c.* 110 Mya] (Gayet and Meunier, 2003). Thus, based on current data, we suggest that diversification of the order might actually have commenced in Africa, shortly after the Gondwanan split. Under this scenario, the opening of the Atlantic Ocean does not provide an adequate explanation for the modern distribution of characiform fishes and therefore biogeographic hypotheses must recourse to dispersalist arguments.

Hypotheses involving marine dispersal of early characiform lineages have been proposed (Gayet, 1982; Filleul and Maisey, 2004; Otero et al., 2008), and although contentious in most cases (Malabarba and Malabarba, 2010), if a vicariance model does not readily explain current distributional patterns, dispersal scenarios should not be discounted simply because recent members of the order are intolerant of saltwater (Calcagnotto et al., 2005; p 147). In light of our limited sampling of Neotropical taxa, and the recurrent recognition of multiple African/South American sister-group relationships within the order (Ortí and Meyer, 1997; Buckup 1998; Calcagnotto et al., 2005), hypothesizing possible scenarios involving marine dispersal is premature. Even if the general conclusions of this study hold, it would require a comprehensive and robust phylogeny of the order –ideally with estimates divergence times– to properly explain the contemporary Gondwanan distribution of characiform fishes.

We emphasize however, a few caveats associated with the temporal context of characiform diversification as inferred from our analyses. First, although we included representatives of all four African families, our sampling of Neotropical lineages is far from comprehensive. In addition, after careful consideration of the available characiform fossil record we selected only two fossil calibration points, confined within the family Alestidae. This likely has implications for the accuracy of the estimated ages at deeper nodes in the phylogeny of the order. It is clear that inclusion of multiple calibration points has a strong impact on the overall accuracy of divergence time estimates (Near and Sanderson, 2004; Fulton and Strobeck, 2010), and that this is especially true for relaxed-clock methods, where multiple calibrations can act as landmark points detecting rate variation at multiple levels throughout the phylogeny (Benton and Donoghue, 2007). Hopefully, future studies investigating the timing of characiform diversification will use much larger datasets (in terms of both taxon and character sampling) and multiple, well-characterized, fossil-based calibration points across the phylogeny of the Characiformes.

Regardless of the accuracy of our estimates, the inclusion of temporal information is crucial to formulating and testing biogeographic hypotheses. Traditionally, vicariance biogeographers have explained the distribution of taxa based on phylogenetic patterns in conjunction with Earth history; specifically, by ascribing sister-group relationships to the emergence of a geographic barrier. Although vicariance hypotheses make sense in light of the congruence between phylogenetic and palaeogeographic patterns (Nelson and Platnick, 1981; Nelson and Rosen, 1981; Humphries and Parenti, 1999), they hold only if geological and cladogenetic events indeed match in time (Donoghue and Moore, 2003). The ultimate test for vicariance biogeographic hypotheses, as shown in this study, is provided by temporal information in the form of the

absolute ages of clades.

5. CONCLUSIONS

The molecular phylogeny presented herein did not corroborate the family Alestidae as monophyletic, with putative members scattered throughout the phylogeny of the order. Our results rejected the hypothesis that the genus *Chalceus* is the most basal alestid, for it was recovered well nested within a clade of Neotropical characins. Likewise, the genera *Arnoldichthhys* and *Lepidarchus* were found to be more closely related to members of strictly Neotropical lineages. Moreover, the resulting phylogeny revealed several instances of poly- and paraphyly among alestid genera, highlighting the necessity for future revisionary studies to better understand the alpha-taxonomy of the family. In accordance with one of the primary goals of phylogenetic systematics, which is to maintain a classification scheme were only monophyletic units are recognized, we propose a redefined Alestidae (i.e., Alestidae *sensu stricto*) that does not include the genera *Arnoldichthhys*, *Lepidarchus*, and *Chalceus*. The origin of the Alestidae *sensu stricto* dates back to the Early Tertiary (63–49 Mya), implying that the diversification of the family took place in African waters long after the Mesozoic fragmentation of Gondwana.

6. TABLES

Table 1. Taxa, voucher specimens and GenBank accession numbers for the gene sequences included in the analyses.

Taxon	Voucher	GenBank Accession Number			
		co1	***cyt–b***	***myh6***	***sh3px3***
OUTGROUP					
Citharinidae					
Citharinus gibbosus	AMNH 241039	TBA	TBA	TBA	TBA
Distichodontidae					
Distichodus fasciolatus	AMNH 240041	TBA	TBA	TBA	TBA
Distichodus kolleri	AMNH 249814	TBA	TBA	TBA	TBA
Hepsetidae					
Hepsetus odoe	AMNH 243489	TBA	TBA	TBA	TBA
Ctenoluciidae					
Ctenolucius hujeta	AMCC 102198	TBA	TBA	TBA	TBA
Erythrinidae					
Hoplias malabaricus	AMCC 110702	–	TBA	TBA	TBA
Acestrorhynchidae					
Acestrorhynchus sp.	AMCC 116689	TBA	TBA	TBA	TBA
Characidae					
Brycon sp.	AMCC 116693	TBA	TBA	TBA	TBA
Hemigrammus erythrozonus	AMCC 116707	TBA	–	TBA	TBA
Chalceus erythrurus	AMNH 233413	–	TBA	TBA	TBA
Chalceus macrolepidotus	AMCC 102150	TBA	TBA	TBA	TBA
INGROUP					
Alestidae					
Alestes baremoze	AMCC 102186	TBA	TBA	TBA	TBA
Alestes inferus	AMNH 242137	TBA	TBA	TBA	TBA
Alestes liebrechtsii	AMNH 246418	TBA	TBA	TBA	TBA
Alestes macrophtalmus	CU 91702	TBA	TBA	TBA	TBA
Alestopetersius caudalis	AMNH 242193	TBA	TBA	TBA	TBA
Alestopetersius compressus	AMNH 242455	TBA	TBA	TBA	TBA
Alestopetersius hilgendorfi	AMNH 242460	TBA	TBA	TBA	TBA
Alestopetersius nigropterus	AMNH 246321	TBA	TBA	TBA	TBA
Arnoldichthys spilopterus	AMCC 102151	TBA	TBA	TBA	TBA
Bathyaethiops caudomaculatus	CU 93170	TBA	TBA	TBA	TBA
Bathyaethiops greeni	CU 93147	TBA	TBA	TBA	TBA
Brachypetersius altus	AMNH 247407	TBA	TBA	TBA	TBA
Brycinus bimaculatus	AMNH 242467	TBA	TBA	TBA	TBA
Brycinus comptus	AMNH 244215	TBA	TBA	TBA	TBA
Brycinus fwaensis	ZSM 37775	TBA	–	TBA	TBA
Brycinus grandisquamis	ZSM 38246	TBA	TBA	TBA	TBA
Brycinus imberi	AMNH 245523	TBA	TBA	TBA	TBA
Brycinus kingsleyae	AMNH 247482	TBA	–	TBA	–
Brycinus lateralis	AMCC 102120	TBA	TBA	TBA	TBA
Brycinus macrolepidotus	AMNH 246621	TBA	TBA	TBA	TBA
Brycinus nurse	AMCC 116712	TBA	TBA	TBA	TBA
Brycinus opisthotaenia	AMNH 236470	–	TBA	TBA	–
Brycinus poptae	CU 93137	TBA	TBA	TBA	TBA
Brycinus sp. "Yaekama"	ZSM 39400	TBA	–	TBA	TBA

Brycinus cf. batesii	AMNH 249531	TBA	–	TBA	TBA
Bryconaethiops boulengeri	AMNH 243601	TBA	TBA	TBA	TBA
Bryconaethiops microstoma	AMNH 246417	TBA	TBA	TBA	TBA
Bryconaethiops sp."Mpozo"	AMNH 242188	TBA	TBA	TBA	TBA
Bryconaethiops yseuxi	AMNH 239455	TBA	TBA	TBA	TBA
Bryconalestes longipinnis	AMNH 249561	TBA	TBA	TBA	TBA
Clupeocharax schoutedeni	AMNH 242486	TBA	–	TBA	TBA
Duboisialestes tumbensis	AMNH 246659	TBA	TBA	TBA	TBA
Hemigrammopetersius barnardi	CU 93792	TBA	TBA	TBA	TBA
Hydrocynus brevis	AMCC 102185	TBA	TBA	TBA	TBA
Hydrocynus forskahlii	AMNH 249806	TBA	TBA	TBA	TBA
Hydrocynus goliath	AMNH 239463	TBA	TBA	TBA	TBA
Hydrocynus vittatus	AMNH 245529	TBA	TBA	TBA	TBA
Ladigesia roloffi	ZSM 39625	TBA	TBA	TBA	–
Lepidarchus adonis	ZSM 39626	–	–	TBA	TBA
Micralestes acutidens	AMNH 239476	TBA	TBA	TBA	TBA
Micralestes humilis	AMNH 246569	–	TBA	TBA	TBA
Micralestes lualubae	AMNH 239489	TBA	TBA	TBA	TBA
Micralestes occidentalis	ZSM 36182	TBA	TBA	TBA	–
Micralestes stormsi	AMNH 239483	TBA	–	TBA	TBA
Nannopetersius lamberti	ZSM 35562	TBA	–	TBA	TBA
Nannopetersius mutambuei	AMNH 246579	TBA	TBA	TBA	TBA
Phenacogrammus aurantiacus	ZSM 39495	TBA	TBA	TBA	TBA
Phenacogrammus interruptus	AMNH 233442	TBA	TBA	TBA	TBA
Phenacogrammus sp."Tshimbi"	ZSM 39307	TBA	–	TBA	TBA
Phenacogrammus taeniatus	ZSM 39279	TBA	–	TBA	TBA
Rhabdalestes maunensis	SAIAB 71928	TBA	–	TBA	TBA
Rhabdalestes septentrionalis	TM-503	TBA	–	TBA	TBA
Tricuspidalestes sp."Tshimbi"	ZSM 39324	TBA	–	TBA	TBA

*Institutional abbreviations: AMCC (Ambrose Monell CryoCollection, AMNH), AMNH (American Museum of Natural History), CU (Cornell University Museum of Vertebrates), SAIAB (South African Institute for Aquatic Biodiversity), ZSM (Zoologische Staatssammlung, Munich), TM (personnel collection of Timo Mortiz).

Table 2. Primers and PCR profiles for amplification of the genes used in this study.

Gene	Source	Primer	Primer Sequence[a]	PCR Thermal Profile[b]
co1	Folmer et al. (1994)	LCO1490	GGTCAACAAATCATAAAGATATTGG	(95°C/60s, 42°C/60s, 72°C/90s) x 35
		HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	
cyt-b	Kocher (1989)	L14841	AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA	(95°C/60s, 50°C/60s, 72°C/120s) x 35
	Irwin et al. (1991)	H15915	AACTGCCAGTCATCTCCGGTTTACAAGAC	
myh6	Li et al. (2007)	myh6_F459	CATMTTYTCCATCTCAGATAATGC	(98°C/10s, 53°C/30s, 72°C/45s) x 15 +
		myh6_R1325	ATTCTCACCACCATCCAGTTGAA	(98°C/10s, 51°C/30s, 72°C/45s) x 15
		myh6_F507[c]	GGAGAATCARTCKGTGCTCATCA	(98°C/10s, 62°C/30s, 72°C/45s) x 15 +
		myh6_R1322[c]	CTCACCACCATCCAGTTGAACAT	(98°C/10s, 60°C/30s, 72°C/45s) x 15
sh3px3	Li et al. (2007)	SH3PX3_F461	GTATGGTSGGCAGGAACYTGAA	(98°C/10s, 55°C/30s, 72°C/45s) x 15 +
		SH3PX3_R1303	CAAACAKCTCYCCGATGTTCTC	(98°C/10s, 53°C/30s, 72°C/45s) x 15
		SH3PX3_F532[c]	GACGTTCCCATGATGGCWAAAAT	(98°C/10s, 62°C/30s, 72°C/45s) x 15 +
		SH3PX3_R1299[c]	CATCTCYCCGATGTTCTCGTA	(98°C/10s, 60°C/30s, 72°C/45s) x 15

[a]Listed from 5' to 3'.
[b]Conditions for denaturation, annealing and extension steps for each cycle are listed in parenthesis, followed by the number of cycles. All reactions included a 5-minute initial denaturation at 95°C and a 7-minute final extension at 72°C.
[c]Primers used during a second (nested) PCR required for successful amplification.

Table 3. AIC values, likelihood function (-lnL), and likelihood ratio (LR) statistics with associated p-values for the null and alternative models for each gene partition.

Gene	Model	AIC	-lnL	LR	*P*-value
co1	GTR+I+ Γ	25451.3778	12421.6889	11.8318	0.018647
	HKY+I+ Γ	25455.2096	12427.6048		
cyt–b	GTR+I+Γ	34722.1206	17057.0603	73.036001	0
	HKY+I+ Γ	34782.2185	17093.5782		
myh6	GTR+I+Γ	22416.9149	5211.5098	4.617401	0.03165
	GTR+ Γ	22419.9031	5183.8185		
sh3px3	GTR+I+ Γ	11416.9149	5404.4574	26.5212	2.50E-05
	HKY+I+ Γ	11435.4361	5417.718		

7. FIGURES

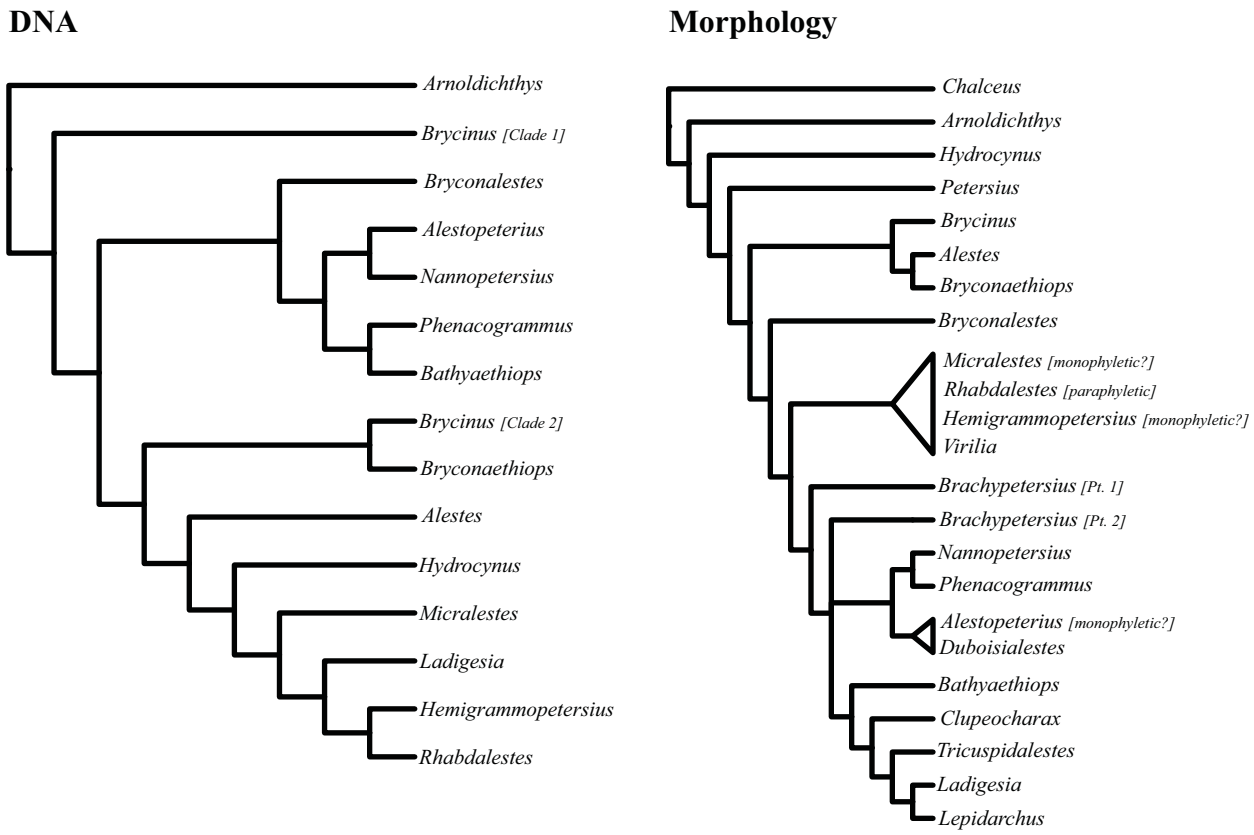


Figure 1. Previous hypotheses of alestid interrelationships based on molecular (Calcagnotto et al., 2005) and morphological data (Zanata and Vari, 2005).

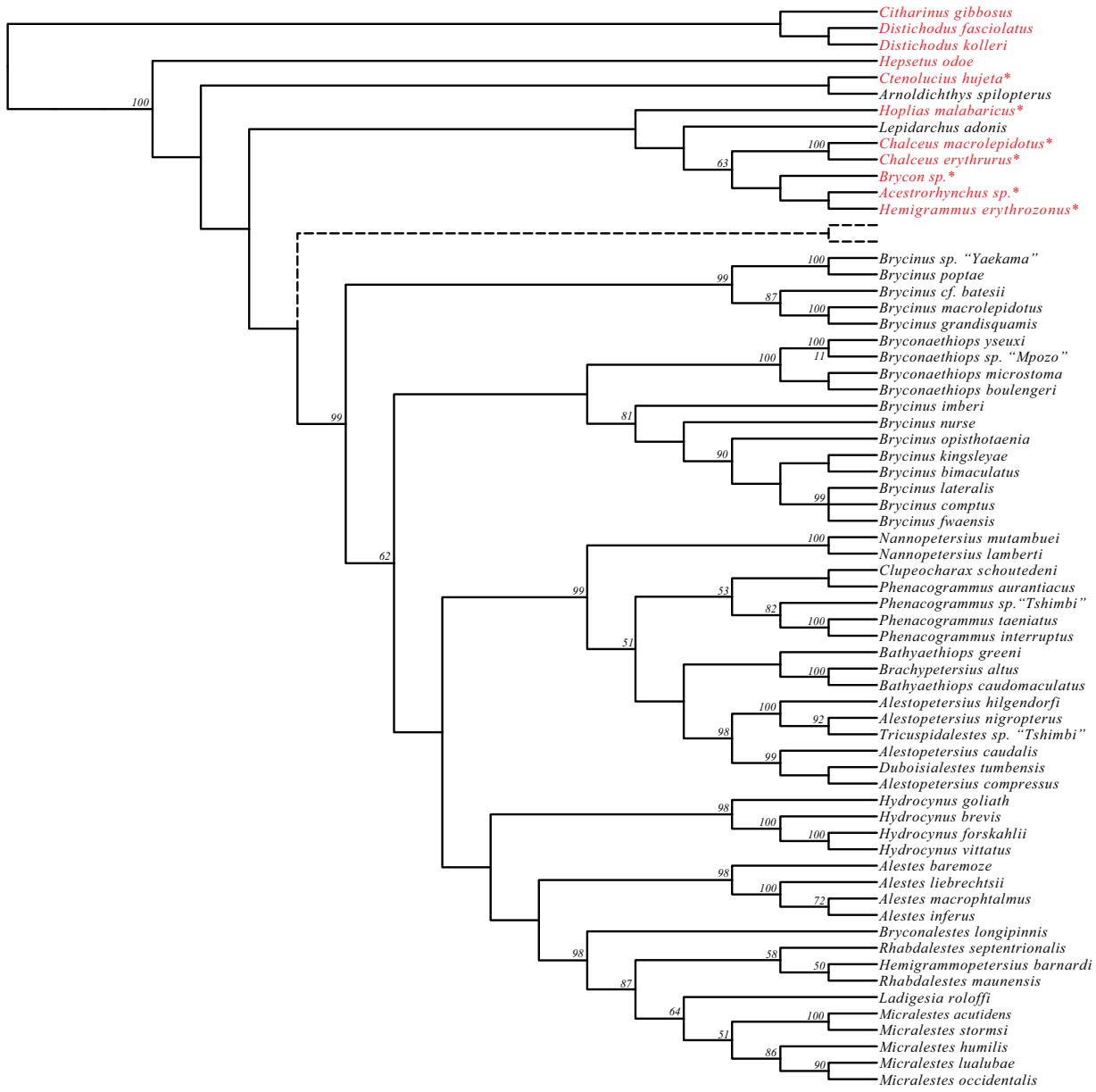


Figure 2. Phylogenetic relationships of the Alestidae as inferred by parsimony and represented by a strict consensus tree obtained with direct optimization (L=8344; CI=0.26 RI=0.52). Bremer and bootstrap (>50) support values are shown above and below branches, respectively. The position of the clade *Ctenolucius hujeta* + *Arnoldichthhys spilopterus* in the TNT topology (i.e., non-additive optimization) is represented by the dotted branch. Outgroup taxa in red. Neotropical members of the outgroup are labeled with an asterisk.

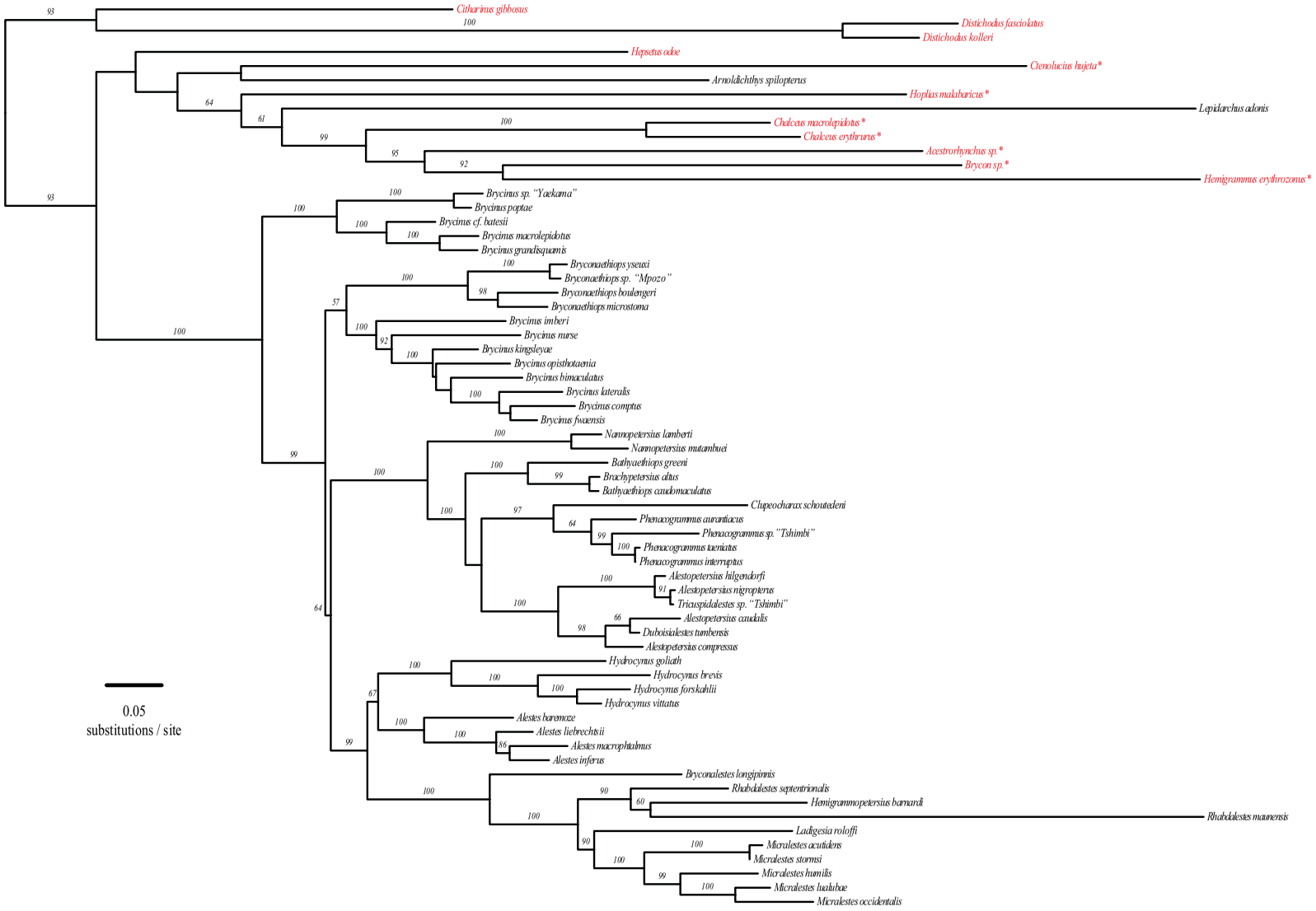


Figure 3. Phylogenetic relationships of the Alestidae as inferred by ML and Bayesian analyses, represented by the most optimal tree recovered by RAxML. Bootstrap values (>50%) and clade posteriors (>0.5) are shown above and below branches, respectively. Outgroup taxa in red. Neotropical members of the outgroup are labeled with an asterisk.

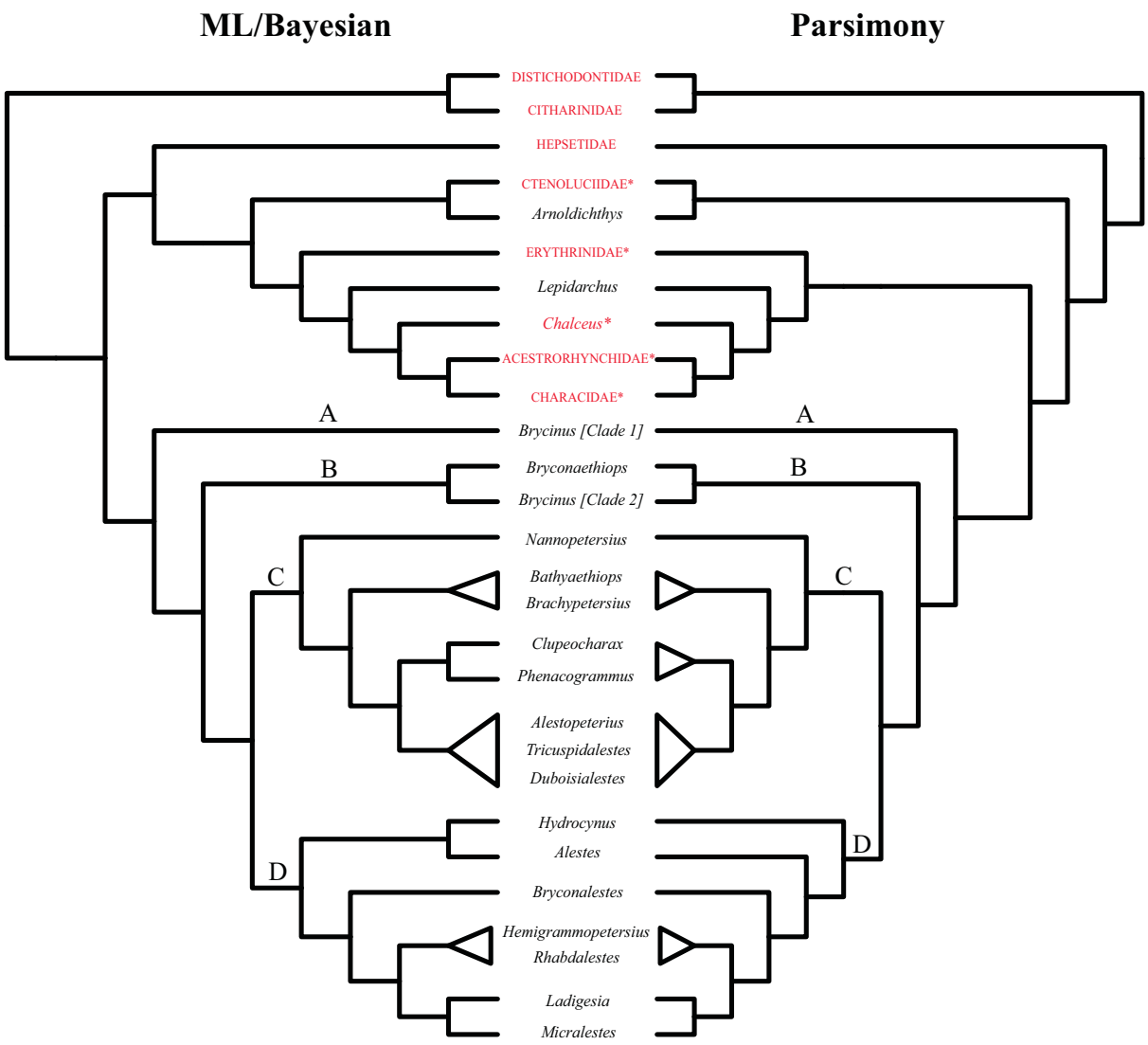


Figure 4. Summary trees representing intergeneric relationships within Alestidae as inferred by parsimony (right) and model-based approaches (left). Letters A-D indicate the main subfamilial clades. Outgroup taxa in red. Neotropical members of the outgroup are labeled with an asterisk.

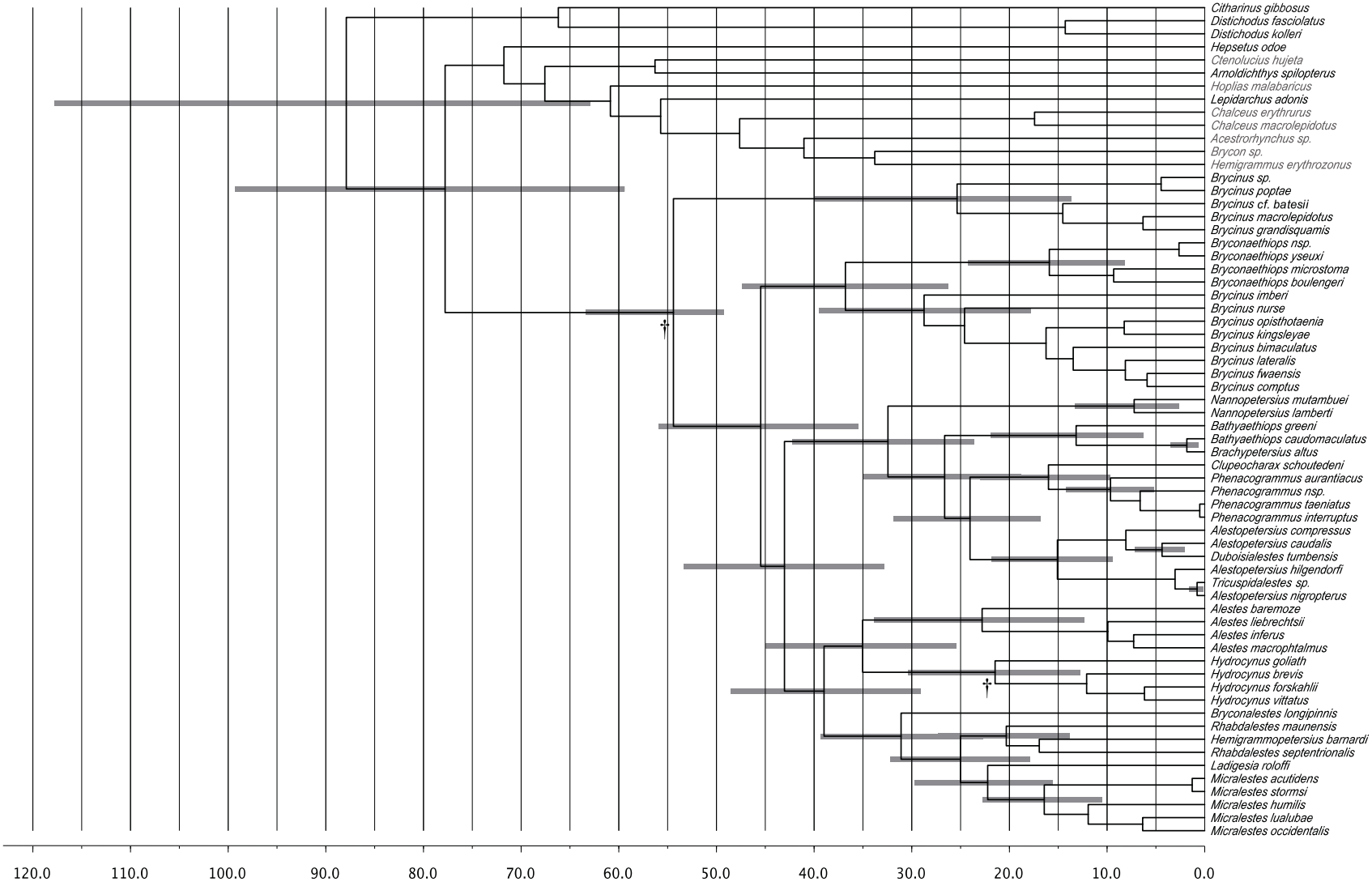


Figure 5. BEAST chronogram showing divergence time estimates for the alestid phylogeny represented by the mean ages of clades (nodes heights). Grey bars correspond to the 95% highest posterior density (HPD) intervals and are shown for intergeneric and higher-level divergences only. Timescale is in millions of years before present. Nodes used as calibration points are indicated by a dagger (†). African and Neotropical taxa names are in black and grey, respectively.

CHAPTER 3

ARE CHARACIFORM FISHES GONDWANAN IN ORIGIN? INSIGHTS FROM A TIME-SCALED MOLECULAR PHYLOGENY OF THE CITHARINOIDEI (OSTARIOPHYSI: CHARACIFORMES)

1. INTRODUCTION

Vicariance biogeography (Nelson and Platnick, 1980; Nelson and Platnick, 1981; Wiley, 1988) emerged in the late 1970's as an approach to explain distribution patterns of biotas by linking predictions of the, at the time, recently developed phylogenetic systematics (Hennig, 1966) and plate tectonics (Carey, 1959; Le Pichon, 1968) paradigms. The vicariance model proposes that large-scale plate-tectonic-driven geomorphological processes (e.g., orogenic uplift, continental drift) are sufficient to explain the disjunct distribution of sister lineages (Wiley, 1988). Despite the fact that vicariant scenarios only hold true if cladogenetic events and their hypothesized causal palaeogeographic processes are temporally congruent (Page, 1990; Donoghue and Moore, 2003; Crisp et al., 2011), most vicariance hypotheses are postulated without the benefit of information on the absolute timing of lineage divergences. For instance, on the basis of congruence between phylogenetic and continental break-up patterns, early biogeographic studies attributed disjunct occurrences of Gondwana-distributed taxa to vicariance due to continental drift (Brundin, 1965; Seberg, 1991; Linder and Crisp, 1995). Similarly, vicariance has traditionally been favored over marine dispersal to explain Gondwanan disjunctions in fish clades whose extant members are obligate freshwater species, such as lungfish (Dipnoi), bony-tongues (Osteoglossiformes), killifish (Cyprinodontiformes), cichlids (Cichlidae), leaffish (Nandidae), swamp eels (Synbranchidae), and characins and their allies (Characiformes) (Rosen, 1975; Lundberg, 1993; Lundberg et al., 2000; Maisey, 2000; Chakrabarty, 2004; Sparks and Smith, 2004). Although in these cases vicariance offers the most parsimonious interpretation given the available evidence, temporal discrepancies between the splitting of continentally disjunct lineages and the break-up of Gondwana should not be discarded a priori, and if discovered, these would falsify the posited vicariance scenarios.

Therefore, time-scaled phylogenies inferred from DNA sequence data using modern analytical methods of molecular dating afford a much-needed means of testing and/or refining such biogeographic hypotheses.

Fishes of the order Characiformes, found throughout much of the freshwaters of the Neotropics and the African continent, are the quintessential transoceanic clade whose present-day distribution has been primarily explained by means of vicariance hypotheses (Fink and Fink, 1981; Lundberg, 1993; Buckup, 1998; Calcagnotto et al., 2005; Malabarba and Malabarba, 2010). A feature common to all of these hypotheses holds that the Early Cretaceous opening of the South Atlantic Ocean is responsible for the disjunct distribution between African citharinoids (suborder Citharinoidei) and the remaining characiform radiation (most of which occurs in the Neotropics). Notwithstanding the growing popularity of molecular dating in phylogenetics, biogeographic hypotheses for the distribution of characiform fishes have barely been tested with temporal information from time-scaled molecular phylogenies. In fact, only the studies of Arroyave and Stiassny (2011) and Goodier et al. (2011) have implemented molecular clocks to investigate the timing of diversification in a clade of characiform fishes. These studies, nonetheless, were primarily focused on the African family Alestidae and the alestid genus *Hydrocynus*, respectively. Despite some caveats, the chronogram inferred by Arroyave and Stiassny (2011) suggests that the origins of characiforms might be too recent for the African/South American drift-vicariance event to adequately explain the split between citharinoids and its Afro-Neotropical sister clade. Under this novel biogeographic scenario, explaining the distribution of extant characiform lineages must recourse to dispersalist arguments.

To further advance knowledge of the chronological framework of characiform evolution, this study investigates the temporal context of citharinoid diversification using DNA sequence data and a Bayesian approach to divergence time estimation in a phylogenetic context. Because citharinoids constitute the sister lineage to the entire remaining Afro-Neotropical characiform radiation (Vari, 1979; Fink and Fink, 1981; Calcagnotto et al., 2005), a time-calibrated phylogeny of the Citharinoidei has potential to assess the adequacy of current vicariance hypotheses and thus to shed critical light on the early biogeographic history of characiform fishes. Additionally, a comparative examination of the inferred citharinoid evolutionary timescale and the timing of palaeogeological and palaeogeographic events on continental Africa (e.g., the development of contemporary riverine networks) may shed light on the historical processes influencing diversification in citharinoids fishes and other taxa with similar biogeographic patterns. This in turn will further inform a growing body of biogeographic scenarios proposed to explain current patterns of diversity in African freshwater fishes (Arroyave and Stiassny, 2011; Goodier et al., 2011; Day et al., 2013), most of which rest on the idea that phylogeographic patterns in continental ichthyofaunas are expected to reflect patterns of drainage isolation resulting from landscape evolution (Rüber et al., 2004; Albert et al., 2006; Lovejoy et al., 2010). Last but not least, given that the only comprehensive phylogenetic treatment of the Citharinoidei is a morphology-based analysis published over three decades ago (Vari, 1979), a molecular phylogeny of citharinoid fishes represents, in and of itself, an imperative endeavor and a significant contribution to the systematics of the poorly studied African ichthyofauna (Lundberg et al., 2000; Lévêque et al., 2008).

Diversity and historical overview of citharinoid systematics

Citharinoid fishes comprise two reciprocally monophyletic families: the Citharinidae, with eight species arrayed in three genera, and the much more speciose Distichodontidae, currently estimated at 96 species arrayed in 15 genera (Vari, 1979; Calcagnotto et al., 2005; Eschmeyer and Fong, 2013). Citharinids (commonly known as lutefishes), although not as morphologically and taxonomically diverse as distichodontids, are distributed throughout much of tropical Africa, and constitute an important component of the artisanal fisheries in the region (Weitzman and Vari, 1998). Similarly, members of the Distichodontidae occur throughout the freshwaters of much of sub-Saharan Africa and the Nile River basin. Despite their pan-African distribution, distichodontids are far from evenly spread across the continent, with species richness heavily concentrated in West-Central Africa and steeply attenuated with distance to the north, east and south. Likewise, levels of species endemism in distichodontids are centered in the Congo Basin, where, in addition to representation of all but one genus (the West-African and Nilo-Sudanic *Paradistichodus*), five genera are endemic (Fig. 1). While not as speciose as some other characiform families (such as the African Alestidae or the hyperdiverse Neotropical Characidae), distichodontids exhibit noteworthy morphological variation—particularly in jaw anatomy and dentition—that is reflected in diversified trophic ecologies, ranging from herbivory to carnivory, and including highly specialized ectoparasitic fin-eating behaviors (Roberts, 1990; Stiassny et al., 2013). Body size variation is equally noteworthy, with records of total length spanning from less than 2 cm (in certain *Neolebias spp.*) to over 80 cm (in large *Distichodus spp.*), and anecdotal reports suggesting that *D. nefasch* can reach over a meter in length. Based on a combination of aspects of jaw morphology and overall body size and shape, distichodontids have been traditionally divided into two evolutionary grades: micropredators and herbivorous species with variously modified jaws and highly variable body plans (as in *Neolebias* and *Distichodus*),

and carnivorous or ectoparasitic species with highly kinetic upper jaws, specialized dentition, and elongate bodies (as in *Eugnathichthys* and *Belonophago*) (Vari, 1979; Nelson, 2006).

The earliest taxonomic treatments of the Citharinoidei date to Eigenmann (1909) and Boulenger (1915), who divided the assemblage into five and three subfamilies, respectively. Like Eigenmann, Regan (1911) recognized five subfamilies, but with different generic composition and limits. Later, Gregory and Conrad (1938) expanded the subfamily Citharininae by including the distichodontid genera *Nannaethiops*, *Neolebias*, *Xenocharax* and *Hemistichodus*. In subsequent works, both Monod (1950) and Greenwood et al. (1966) recognized three subunits within citharinoids; the former, however, did not assign the genera *Neolebias*, *Nannaethiops*, *Xenocharax* and *Paradistichodus* to any of these subunits. In contrast, Greenwood et al. (1966) retained membership of Boulenger's subfamilial groupings while elevating them, for the first time, to familial taxonomic rank. More recently, Poll (1973) restricted citharinoids to the families Ichthyboridae and Citharinidae, placing all members of the Distichodontidae (*sensu* Greenwood et al.) into the latter family. Subsequently, Vari (1979) presented a phylogeny (Fig. 2) based on osteology and soft anatomy across a comprehensive taxon sampling that included representatives of all distichodontid genera and two of the three citharinid genera (*Citharinus* and *Citharidium*). It was not until Vari's study that a classification of citharinoid fishes claimed to reflect evolutionary relationships inferred using cladistic methodology. Prior to this landmark contribution, generic and suprageneric groupings within citharinoids were defined on the basis of plesiomorphic (or combinations of plesiomorphic and derived) characters that failed to define monophyletic groups. Overall, Vari's findings supported the hypothesis that citharinids and distichodontids are sister taxa, and constitute the sister clade to the remaining characiform radiation. Nevertheless, previous hypotheses of intergeneric relationships were not entirely

supported by his study, requiring a rearrangement of the suprageneric taxonomy of the group. Specifically, the Ichthyboridae (*sensu* Greenwood et al.) was sunk into the Distichodontidae, the genera *Congocharax* and *Dundocharax* were synonymized with *Neolebias*, and the genera *Gavialocharax* and *Phagoborus* were synonymized with *Ichthyborus*. Whereas the monophyly of *Distichodus*, *Nannocharax*, or *Hemigrammocharax* was not supported in his study, Vari refrained from making nomenclatural changes regarding those taxa pending analyses based on more comprehensive sampling of species within those genera.

Despite the fact that more than three decades have passed since the publication of Vari's phylogenetic treatment of the Citharinoidei, there has been no attempt at testing his results, either with molecular or novel morphological data. Therefore, this study is also aimed at providing a comprehensive molecular phylogeny for citharinoid fishes (with emphasis on the Distichodontidae) to further investigate intergeneric relationships and update the evolutionary framework that has laid the foundations for the current classification of the group.

Molecular dating considerations

Although molecular dating methods have proven fruitful in addressing manifold questions in phylogenetics and evolutionary biology (e.g., Kumar and Hedges, 1998; Wang et al., 1999), these methods require accurate estimates of substitution rates such that genetic distances among taxa can be reliably translated into absolute times of divergence (Rutschmann, 2006). In cases where an independently estimated substitution rate is unknown (which is mostly the case), the age of one or more internal nodes is needed to calibrate rates of molecular divergence. Such node ages are normally obtained from paleontological evidence, or when such material is unavailable, from dated biogeographic events and/or divergence-time estimates from previous studies. In a

recent study aimed at providing a timed-scaled phylogeny of all ray-finned fishes (Actinopterygii), Near et al. (2012) suggested that their inferred node ages may be used to calibrate molecular clocks for actinopterygian lineages at lower taxonomic levels (e.g., families) that lack a fossil record. Therefore, another objective of the present study is to assess the suitability of Near et al's divergence-time estimates as calibration data when dating phylogenies of actinopterygian fishes such as the Citharinoidei.

In principle, one of the virtues of Bayesian inference methods of molecular dating is that these can, to some extent, account for the inherent uncertainty of fossil-based calibrations by incorporating prior knowledge in the form of probability distributions. In practice, however, justification is rarely provided for values assigned to the parameters (technically known as *hyperparameters*) that describe the shape of probability density functions used as priors (Heath, 2012; Sauquet et al., 2012). This is particularly troubling given that hyperparameter choice—and therefore the shape of probabilistic priors—can have a major impact on divergence time estimation (Marshall, 2008; Heath, 2012; Warnock et al., 2012). Although a hierarchical Bayesian model recently proposed by Heath (2012) offers a promising avenue toward a less biased choice of hyperparameter values, the author herself acknowledged that modeling hyperparameter uncertainty with hyperpriors such as the Dirichlet distribution does not necessarily represent a biologically explicit approach (Heath, 2012: p. 806). Moreover, Heath's model is currently limited to exponentially distributed priors and its analytical implementation is not readily available in Bayesian molecular dating programs (e.g., BEAST). Given that a standard protocol to properly specify parameters of calibration priors has yet to be proposed, a final objective of this study is to empirically assess the impact of different calibration strategies (particularly the shape of calibration prior densities), as well as the impact of using non-

informative priors for the parameters of the clock, speciation, and substitution models on divergence-time estimates. Thus, the robustness of node ages in the presence of analytical uncertainty can be considered when discussing the biogeographic implications of our findings.

2. MATERIALS AND METHODS

2.1. TAXON SAMPLING

Ingroup taxa included representation of all valid citharinoid genera, with the exception of the monotypic *Citharidium*, *Citharinops*, and *Paraphago*, from which tissue samples were unavailable. Apart from *Paradistichodus* and *Mesoborus*, genera currently considered monotypic (Eschmeyer, 2013), and *Microstomatichthyoborus* (for which individuals of only one of two described species were available), all sampled genera were minimally represented by two species. The overall ingroup sampling thus consisted of 55 valid species (three of the Citharinidae and 52 of the Distichodontidae), thereby encompassing 37.5% and 54.2% of citharinid and distichodontid species diversity, respectively. Where available, multiple individuals per species were included, and sampling of multiple individuals of *Paradistichodus dimidiatus* and *Mesoborus crocodilus* allowed for testing the monophyly of these putatively monotypic genera. In addition to increasing geographic sampling, sequencing of multiple individuals per species allowed for an improved control of sequence quality and recognition of potential contamination issues.

Outgroup taxa comprised representatives of the families Ictaluridae (*Ictalurus punctatus*), and Cyprinidae (*Danio rerio*), both members of otophysan orders closely related to the Characiformes. Outgroup choice was informed by previous studies of characiform and ostariophysian relationships (Fink and Fink, 1981; Buckup, 1998; Calcagnotto et al., 2005; Near et al., 2012), all of which strongly support the monophyly of both the Characiformes and the Citharinoidei, as well as a sister-group relationship between citharinoids and a clade containing the remaining members of the order.

Tissues were obtained primarily from specimens collected during the NSF-funded Congo Project (http://research.amnh.org/vz/ichthyology/congo/index.html) and/or recent fieldwork in West and West-Central Africa. Fishes were collected and euthanized prior to preservation in accordance with recommended guidelines for the use of fishes in research (Nickum et al., 2004). Taxonomically verified vouchers are deposited in the American Museum of Natural History's ichthyological collection, available online at the museum's Vertebrate Zoology Collection Database (http://entheros.amnh.org/db/emuwebamnh/index.php). Additional tissues were obtained through donations from colleagues at the Cornell Museum of Vertebrates (USA), Texas A&M University—Corpus Christi (USA), and the Royal Ontario Museum (Canada). Species identity of all loaned tissue vouchers was confirmed, either by examination of loaned voucher specimens, or on taxonomic authority of the loaning institution.

Overall, DNA sequence data was obtained from a total of 121 individuals. Voucher catalog numbers and GenBank accession numbers for the gene sequences generated and included in this study are listed in Table 1.

2.2. MARKER SELECTION AND CHARACTER SAMPLING

Nuclear and mitochondrial genes, spanning a range of substitution rates, were sampled for phylogenetic analyses. In total, seven protein-coding genes/gene fragments comprise the comparative data of this study. Nuclear markers consisted of myosin-heavy polypeptide 6-cardiac muscle-alpha (*myh6*), SH3 and PX domain-containing 3-like protein (*sh3px3*), ectodermal-neural cortex 1 (*enc1*), and glycosyltransferase (*glyt*), all of which were originally proposed by Li et al. (2007) as promising markers for use in molecular systematics of actinopterygian fishes, and have since been successfully employed in empirical phylogenetic

studies of characiform fishes and other ostariophysians (Chen et al., 2008; Arroyave and Stiassny, 2011; Oliveira et al., 2011). Mitochondrial markers consisted of cytochrome c oxidase subunit 1 (*co1*), cytochrome b (*cyt-b*), and NADH dehydrogenase 2 (*nd2*), each of which has proven useful in resolving relationships of characiform fishes at multiple phylogenetic levels (Calcagnotto et al., 2005; Javonillo et al., 2010; Arroyave and Stiassny, 2011).

2.3. DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

General procedures for DNA extraction, amplification, and purification, along with primers and thermal profiles for sequencing *myh6*, *sh3px3*, and *co1* follow Arroyave and Stiassny (2011). Primer sequences and PCR profiles for *enc1*, *glyt*, *cyt-b*, and *nd2* are listed in Table 2. Distichodontid-specific primers for *cyt-b* and *nd2* were designed on conserved flanking regions for each fragment using Primer3 (Rozen and Skaletsky, 1999). Contig assemblage and sequence editing was performed using Geneious Pro version 5.6.5 (Biomatters, available from http://www.geneious.com/). IUPAC nucleotide ambiguity codes were used to represent heterozygous sites.

2.4. ALIGNMENT AND MODEL SELECTION

Each gene partition was aligned based on the translated amino acid sequence using the *Translation Align* algorithm under default parameters, as implemented in Geneious. The number of variable and parsimony-informative sites of the concatenated alignment was determined using MEGA 5 (Tamura et al., 2011). Nucleotide substitution model selection for each gene partition was accomplished by means of the Bayesian Information Criterion (BIC) as implemented in jModelTest (Posada, 2008) under the following likelihood settings: *Number of substitution*

schemes = 3; *Base frequencies* = +F; *Rate variation* = +I and +G with nCat = 4; and *Base tree for likelihood calculations* = Fixed BIONJ-JC, so that a total of 24 models were evaluated.

2.5. ASSESSMENT OF SUBSTITUTION SATURATION

Third codon positions of each gene partition were checked for substitution saturation using both Xia et al.'s (2003) test and saturation plots (i.e., observed number of transitions and transversions against corrected genetic distance for all pairwise comparisons among terminals), following the guidelines provided in Xia and Lemey (2009). In saturation plots, corrected genetic distances were calculated based on the best-fit substitution models previously determined by jModelTest, and trend lines were estimated using second-order polynomial curves fit to the data. Both approaches were implemented in DAMBE (Xia and Xie, 2001). Because of the limited number of available substitution models in DAMBE, corrected genetic distances were calculated using F84 as an alternative for HKY+I+G and GTR as an alternative for SYM+I+G and GTR+I+G. Saturation in a data partition was assumed when: a) The index of substitution saturation (I_{SS}) was either larger or not significantly smaller than the critical value ($I_{SS.C}$) and/or b) transversions outnumbered transitions in saturation plots.

2.6. PHYLOGENETIC RECONSTRUCTION

The concatenated alignment was analyzed using both statistical (model-based) and parsimony methods of phylogenetic inference. Statistical approaches to phylogeny estimation included both frequentist (likelihood) (Felsenstein, 1981) and Bayesian (posterior probability) (Rannala and Yang, 1996) inference methods. To accommodate potential process heterogeneity among gene regions, model-based analyses were conducted on the concatenated alignment

partitioned into gene regions with parameters unlinked. Likelihood analyses were carried out in RAxML version 7.2.8 Black Box (Stamatakis, 2006). Bayesian inference of phylogeny was carried out in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and implemented using the Markov Chain Monte Carlo algorithm (MCMC) run for 5×10^7 generations with a sampling period of 1000 generations, under default priors and default proposal mechanisms. A total of two independent runs—of four chains each—was performed. Convergence of the MCMC algorithm to a stationary distribution—and thus the number of generations to be discarded as burn-in—was determined by examination of trace plots of posterior probability vs. number of generations using Tracer (Rambaut and Drummond, 2007). Graphical exploration of MCMC runs was also achieved by plotting posterior probabilities of splits at selected increments over an MCMC run (cumulative function) using the web-based tool AWTY (Wilgenbusch et al., 2004; Nylander et al., 2008). Further assessment of MCMC convergence was undertaken by examination of the average standard deviation of split frequencies, with values << 0.01 taken as indicative of stationarity. Accordingly, 25% of MCMC samples were discarded as burn-in, and substitution model parameters were calculated from the remaining 75%. Likewise, branch lengths and posterior probabilities of nodes were calculated from the set of post burn-in trees using TreeAnnotator version 1.7.4 (Drummond et al., 2012) and summarized as a 50% majority rule consensus tree. Both RAxML and MrBayes analyses were implemented through the CIPRES Science Gateway V. 3.3 (Miller et al., 2010).

Parsimony analyses were carried out using TNT (Willi Hennig Society edition) (Goloboff et al., 2003; Goloboff et al., 2008) with gaps treated as fifth state, an indel-substitution cost ratio of 1, and no cost for gap opening. The heuristic tree search strategy in TNT included 1000 Wagner trees (Farris, 1970), tree bisection and reconnection (TBR) branch swapping (Swofford and

Olsen, 1990), perturbation using the Parsimony Ratchet (Nixon, 1999), and tree fusing (Goloboff, 1999). Branches with zero possible length were collapsed. Ensemble consistency index (CI) (Kluge and Farris, 1969) and ensemble retention index (RI) (Farris, 1989) were used as measures of homoplasy and synapomorphy, respectively.

In likelihood and parsimony analyses, nodal support was estimated by means of the bootstrap character resampling method (Felsenstein, 1985; Stamatakis et al., 2008) using 1000 pseudoreplicates, whereas in Bayesian analyses nodal support was assessed using clade posteriors. When detected, saturated positions were removed from the data so as to produce an alternative, more restricted dataset, which was analyzed in the same manner as the original dataset. All resultant phylogenies were rooted at *Danio rerio*.

2.7. BAYESIAN ESTIMATION OF DIVERGENCE TIMES

Node ages were estimated using the Bayesian relaxed-clock method of Drummond et al. (2006) under the uncorrelated lognormal (UCLN) rate variation model as implemented in BEAST version 1.7.4 (Drummond and Rambaut, 2007; Drummond et al., 2012). A Yule process prior for topology and divergence times was assumed. Justification for the use of both the UCLN relaxed-clock model and the Yule process prior is provided in Arroyave and Stiassny (2011). Both primary (i.e., fossil-based) and secondary (i.e., based on age estimates from previous molecular dating studies) calibrations were used to estimate substitution rates and eventually absolute times of divergence. Primary calibrations were incorporated using log-normally distributed priors based on the oldest fossils assignable to members of the suborder Citharinoidei; namely, Eocene (*ca*. 46 Ma) fossil remains corresponding to †*Eocitharinus macrognathus* (Murray, 2003) and Late Miocene (*ca*. 7.5 Ma) fossilized dentition attributable to

Distichodus (Stewart, 2003). †*Eocitharinus macrognathus* was described from a compression fossil of a partial skeleton (part and counterpart of the anterior two-thirds of the body) from the Eocene Mahenge site in north-central Tanzania, whose absolute age has been dated to 45-46 Ma (Harrison et al., 2001). While †*Eocitharinus macrognathus* exhibits several features that suggest a close association with the Citharinidae and Distichodontidae (e.g., prominent lateral ridge on the anterodorsal corner of the opercle, fused postcleithra 2+3), it lacks determinable synapomorphies of either family and has been thereby classified as Citharinoidei *incertae sedis* (Murray, 2003; Malabarba and Malabarba, 2010). Therefore, †*Eocitharinus macrognathus* was incorporated for calibration purposes as a stem member of the Citharinoidei. The other calibration fossil used corresponds to the oldest from a series of Neogene *Distichodus* remains. This *Distichodus* fossil consists of a single tooth recovered from Late Miocene fluvial strata of the Lower Nawata (7.44 ± 0.05 Ma) formation, Lothagam, Kenya (McDougall and Feibel, 1999; Stewart, 2001; Feibel, 2003; Stewart, 2003). *Distichodus* dentition recovered at Lothagam corresponds to a fish estimated to be of up to a meter long and therefore similar in size to certain extant species from the region (Stewart, 2003). Absolute age estimates of †*Eocitharinus macrognathus* and *Distichodus* fossils were used to calibrate the nodes (as minimum age constraints) corresponding to the most recent common ancestor (MRCA) of citharinoids (as a stem lineage) and the MRCA of the genus *Distichodus*, respectively (Table 3). Secondary calibrations were incorporated using normally distributed priors based on divergence-time estimates imported from a recent study on the timing of diversification of actinopterygian (ray-finned) fishes (Near et al., 2012); specifically, the estimated ages of the MRCA of the Otophysi (170 Ma; 95% HPD=185-155), and the MRCA of the Characiformes and Siluriformes (130 Ma; 95% HPD=140-120). The standard deviation (σ) parameter of the normal distribution associated

with these secondary calibrations was chosen so that 95% of the probability lay within the boundaries of the 95% HPD intervals recovered in (Near et al., 2012) (see Table 3).

In log-normally distributed calibration priors, the larger the mean (μ), the flatter the probability density function, and thereby the older the 95th percentile soft maximum bound for the age of the node. Accordingly, the robustness of inferred node ages to changes in the shape of log-normally distributed priors was assessed by conducting a series of analyses (in the manner of sensitivity analysis) where the standard deviation hyperparameter was fixed (σ=0.5) but μ was allowed to vary so that three alternative priors (arbitrarily assigned as "young", "intermediate", and "old") were considered at each calibration node (Fig. 3; Table 3). These alternative priors—and thus their corresponding μ—were formulated to be consistent with molecular clock- and fossil-based age estimates of clades bracketing each calibration node. Specifically, the proposed alternative priors for the age of the MRCA of the Citharinoidei node were devised in accordance with the estimated age of the MRCA of the Characiformes (*ca*. 90-100 Ma) (Arroyave and Stiassny, 2011; Near et al., 2012) and the stratigraphic distribution of fossils unambiguously assignable to the order (Malabarba and Malabarba, 2010). Similarly, the proposed alternative priors for the age of the MRCA of *Distichodus* were formulated to be compatible with paleontological evidence (Stewart, 2001; 2003) and the chronogram presented in (Arroyave and Stiassny, 2011). Moreover, because the distinctive character(s) that assign a fossil to a given taxon might have evolved along the stem lineage (Brady, 2011), an additional prior for the age of the MRCA of *Distichodus* in which the fossil was treated as a stem member (as opposed to crown member; the default option in BEAST) was considered.

The effect of using non-informative priors for the parameters of the UCLN relaxed clock model (μ and σ), the Yule speciation process (birth rate), and the model of molecular evolution

for each gene (substitution rates, base frequencies, gamma shape, and proportion of invariant sites), was similarly assessed by performing analyses using default vs. uniform (thus non-informative) priors. Additionally, the suitability of Near et al.'s (2012) inferred node ages as calibration information was explored by comparing the results of an analysis that included both primary and secondary calibrations with those from analyses using either only primary or only secondary calibrations. A detailed description of the analyses conducted to assess the impact of different calibration strategies on divergence-time estimates is presented in Table 4.

Each analysis listed in Table 4 consisted of two independent, identical runs, with a chain length of 5×10^7 generations (except for Analyses 2 and 10, which required 2×10^8 to reach convergence), a sampling period of 1000 generations, and default proposal mechanisms. Parameter files (.log) from the two independent runs were examined for convergence using Tracer. After ensuring that stationarity had been reached, tree files (.trees) of independent (yet identical) runs were combined into a single file using LogCombiner (Drummond et al., 2012), discarding 25% of samples as burn-in. The posterior sample of trees (post burn-in) contained in each combined tree file was then summarized using TreeAnnotator to produce a chronogram indicating posterior probabilities and mean ages of all nodes with their associated 95% highest posterior density (HPD) intervals.

3. RESULTS

3.1. SEQUENCE DATA SUMMARY STATISTICS AND SUBSTITUTION MODEL SELECTION

The concatenated alignment of all seven genes consisted of 5820 sites, of which 2599 were variable (including 18 indels) and 2234 parsimony-informative. A few instances of failed DNA amplification and/or sequencing resulted in ~4% of missing data. Summary statistics for each individual gene partition and the results of the statistical selection of best-fit models performed in jModelTest are presented in Table 5.

3.2. SUBSTITUTION SATURATION

Results of Xia et al's (2003) statistical test of saturation were ambivalent, ultimately dependent on the shape of tree topology. Assuming a perfectly symmetrical topology, third codon positions of all genes resulted in a calculated index of substitution saturation (I_{SS}) significantly lower than the critical value ($I_{SS.C}$), thus implying little saturation in the data. Under an assumption of a markedly asymmetrical topology, however, third positions of *co1*, *cyt-b*, and *nd2*, resulted in I_{SS} values significantly higher than their corresponding $I_{SS.C}$ values, thereby suggesting that these sites have experienced substantial saturation. Conversely, plots of observed number of transitions and transversions against corrected genetic distance (Fig. 4) indicated that only third codon positions of the mitochondrial genes *cyt-b* and *nd2* had reached substitution saturation, specifically at genetic distances of approximately 0.26 and 0.29 substitutions/site, respectively (Fig. 4b, 4f). This implies that about 14% of pairwise comparisons might be affected by saturation in third positions of *nd2*, whereas less than 3% of pairwise comparisons might be affected by saturation in third positions of *cyt-b*. Although *co1* comes near to experiencing

substitution saturation in third codon positions at a corrected genetic distance of about 0.27 substitutions/site, this has no practical implications since genetic distances of virtually all pairwise comparisons in the dataset fall below this value (Fig. 4a). Based on the results from both Xia et al's test and saturation plots, it seems reasonable to assume that only third positions of *nd2* might negatively affect (although most likely not substantially) phylogeny estimation due to substitution saturation. Therefore, in addition to the original matrix, an alternative dataset excluding these putatively saturated positions was analyzed, so that potential differences in topology after removal of saturated data could be considered when discussing citharinoid relationships.

3.3. CITHARINOID PHYLOGENY

Model-based analyses resulted in likelihood (i.e., RAxML most optimal tree) and Bayesian (i.e., MrBayes 50% majority rule consensus tree) phylogenies of almost identical topology, with similar clade support and relative branch lengths (Figs. 5 and 6). These phylogenies are mostly well supported and confirm the reciprocal monophyly between the Citharinidae and the Distichodontidae (Figs. 5 and 6). Parsimony analysis resulted in 12 equally most parsimonious trees of length 14047, of which a strict consensus is presented in Fig. 7. Most of the very few polytomies in the parsimony strict consensus involve within-species resolution (as expected), whereas only two instances involve failure to completely resolve within-genus relationships, namely in *Ichthyborus* and *Distichodus*. Monophyly of citharinids and distichodontids was likewise supported by the parsimony topology.

Both model-based and parsimony phylogenies recovered the genus *Xenocharax* as sister to all other distichodontids (node B; Figs. 5 and 6), and a clade consisting of the diminutive genera

Nannaethiops and *Neolebias* (although not reciprocally monophyletic—node D; Figs. 5 and 6) as sister to the remaining distichodontid radiation (node C; Figs. 5 and 6). Likewise, both approaches recovered a clade containing the reciprocally monophyletic *Ichthyborus* and *Hemistichodus* (node E; Figs. 5 and 6), and a strongly supported suprageneric clade (although not identically resolved) containing ectoparasitic fin-eating (*Belonophago*, *Eugnathichthys*, and *Phago*), ichthyophagous (*Mesoborus*), and micropredatory (*Microstomatichthyoborus*) genera (node G; Figs. 5 and 6). Most suprageneric clades in the model-based phylogenies (nodes B-J in Fig. 5 and B-I in Fig. 6) are well supported, with only two nodes (H, J; Fig. 5) exhibiting bootstrap values below 60 in the likelihood tree.

The parsimony tree differed with the model-based trees mainly in the placement of three clades, namely, the one containing the African darters of the genera *Hemigrammocharax* and *Nannocharax*, the one containing members of *Ichthyborus and Hemistichodus*, and the one containing individuals of the species *Paradistichodus dimidiatus.* Not surprisingly, the nodes involved are in most cases either weakly supported or collapsed (Figs. 5-7).

Overall, excluding *nd2* third positions did not result in major topological differences, and most of the abovementioned well-supported suprageneric clades (nodes B-J) were similarly recovered. Only the phylogenetic placement of the clades *Nannocharax+Hemigrammocharax* and *Ichthyborus+Hemistichodus* was influenced by removal of these allegedly saturated data. A comparative summary of the results of parsimony and model-based analyses, including those with *nd2* third positions removed, is presented in Fig. 8.

3.4. MONOPHYLY OF DISTICHODONTID GENERA

Except for the genus *Microstomatichthyoborus* for which tissues from only a single species were available, taxon sampling allowed for testing of generic monophyly in the Distichodontidae. In all trees, regardless of optimality criterion, monophyly of *Belonophago, Distichodus*, *Hemistichodus*, *Ichthyborus*, *Mesoborus, Nannaethiops,* and *Xenocharax* was corroborated (Figs. 5-7). Monophyly of *Eugnathichthys*, however, was confirmed in the likelihood and Bayesian trees (Figs. 5 and 6) but not in the parsimony tree (Fig. 7). By contrast, regardless of inference method applied, no support for the monophyly of *Hemigrammocharax*, *Nannocharax*, *Neolebias*, or *Phago* was found. Placement of *Nannaethiops* well nested within *Neolebias* rendered the latter paraphyletic. Similarly, sampled species of *Hemigrammocharax* were recovered nested within *Nannocharax*, rendering the latter paraphyletic. Perhaps more surprisingly, *Phago* was recovered as polyphyletic by all methods (although differently resolved).

3.5. TIMESCALE OF CITHARINOID DIVERSIFICATION

A time-scaled phylogeny of the Citharinoidei, inferred using both primary (based on "intermediate" priors) and secondary calibrations (Analysis 1; Table 4), resulted in a topology identical to that of the likelihood tree, and with comparable nodal support (i.e., most clades with posterior probabilities > 0.95) and relative branch lengths (Fig. 9). The chronogram indicates that, based on estimated mean node ages, the origins of the Citharinoidei and the Distichodontidae date to the Turonian (90.86 Ma; 95% HPD=110-73) and the Maastrichtian (66.9 Ma; 95% HPD=83-51) of the Late Cretaceous, respectively. However, most modern distichodontid genera, as well as the citharinid genus *Citharinus*, appear to have originated and diversified much more recently, mainly during the Neogene (23-2.6 Ma). For instance, the

youngest of the well-supported suprageneric clades recovered—represented by the MRCA of fin-eating distichodontids and allied genera (node G; Figs. 5 and 6)—dates to the Early Miocene (*ca.* 18 Ma).

Further, our results indicate that estimated ages of fossil-calibrated nodes are considerably older than the ages of the calibration fossils. Specifically, the inferred age for the node representing the MRCA of *Distichodus* (17.22 Ma; 95% HPD=23-12) is more than twice as old as the age of the fossil used to calibrate the node (*ca.* 7.5 Ma). Likewise, the inferred age of the node representing the MRCA of the Citharinoidei (90.86 Ma; 95% HPD=110-73) is almost twice as old as the age of the stem citharinoid fossil (†*Eocitharinus macrognathus*) used for calibration (*ca.* 46 Ma). By contrast, the inferred ages of the (external) nodes calibrated using Near et al's (2012) divergence-time estimates are very similar to the mean ages proposed in their actinopterygian chronogram. This is, 166.86 Ma [95% HPD=185-150] (vs. 170 Ma) for the node represented by the MRCA of the Otophysi, and 124.73 Ma [95% HPD=137-113] (vs. 130 Ma) for the node represented by the MRCA of characiforms and siluriforms.

Results of analyses aimed at assessing robustness of the inferred node ages to changes in calibration settings and parameter priors (Analyses 1-16) are presented in Table 6. By and large, changes to the shape of log-normally distributed priors did not result in substantially different divergence-time estimates. In fact, differential calibration priors for the node representing the MRCA of Citharinoidei (Analyses 1, 4, and 5) resulted in nearly identical estimates. While divergence times based on "intermediate" and "old" calibration priors for the node representing the MRCA of *Distichodus* were likewise very similar (Analyses 1 and 3), using a "young" calibration prior resulted in comparatively younger estimates (Analysis 2). On the other hand, exclusion of secondary calibrations (Analysis 7) had a sizeable impact on inferred node ages, and

resulted in estimates almost twice as young as in the control (i.e., Analysis 1). Conversely, exclusion of primary (i.e., fossil) calibrations resulted in the oldest estimates (Analysis 8). Treating the fossil used to calibrate the node representing the MRCA of *Distichodus* as a stem (as opposed to crown) member (Analysis 6) did not result in significantly different divergence-time estimates. Likewise, using uniform instead of default priors for the parameters of the UCLN relaxed clock model, Yule process, and substitution models (Analyses 9-16) resulted in negligible differences in the estimated distichodontid node ages.

4. DISCUSSION

4.1. SUBSTITUTION SATURATION

By and large, results from saturation tests and plots suggest that phylogenetic signal in the sampled genes is unlikely to be erased or confounded by substitution saturation. While *nd2* third positions show some signs of saturation (at relatively high levels of divergence), removal of these sites resulted in a similarly resolved topology, differing only in the placement of clades already weakly supported and sustained by particularly short branches. If third positions of *nd2* were indeed fully saturated, the resultant phylogenetic noise would be expected to affect primarily resolution at deeper divergences. However, this was not the case, as most nodes inferred with putatively saturated sites were almost identically resolved and supported. Therefore, it appears that either the presumed multiple substitutions have not occurred or any saturation-driven noise has been swamped by the phylogenetic signal in the remaining data.

4.2. CITHARINOID PHYLOGENY AND TAXONOMIC CONSIDERATIONS

Although similar in many respects, the model-based and parsimony topologies inferred in this study exhibit some noteworthy differences (Fig. 8). However, because of our preference for model-based phylogenetic inference methods over parsimony when dealing with molecular data, and the fact that both likelihood and Bayesian topologies were fully concordant, better resolved, and with higher support than the parsimony tree, the following discussion is based primarily on the likelihood topology. We note also that our model-based topologies are in strong accord with the morphology-based tree of Vari (1979), and thereby require considerably less invocation of homoplasy to explain the evolution of morphological traits than would the parsimony topology.

Monophyly of the suborder Citharinoidei and the family Distichodontidae has not been questioned in most phylogenetic treatments of the Characiformes and the Ostariophysi (Ortí and Meyer, 1997; Buckup, 1998; Calcagnotto et al., 2005). However, a recent study focused on characid interrelationships by Oliveira et al. (2011) recovered *Citharinus* nested within the Distichodontidae. Recognizing that this conclusion contradicted robust morphological and molecular evidence, Oliveira et al. (2011) stressed the need for further investigation of this finding with increased taxon and character sampling. The results presented here strongly support reciprocal monophyly between citharinids and distichodontids, and so provide additional evidence for the continued recognition of these taxa. The problematic finding of Oliveira et al. (2011) is most likely a result of limited sampling of citharinoids given the Neotropical focus of their study.

In addition to corroborating distichodontid monophyly, our results provide strong support for the recognition of various suprageneric assemblages represented by well-supported clades (nodes B-G; Figs. 5 and 6) and the intergeneric relationships entailed by the placement and composition of these clades. Results of the present study provide the first opportunity to test the hypothesis of distichodontid relationships arrived at by Vari (1979) and, although derived from a different type of data and with only partially overlapping taxon sampling, the findings presented here are in general agreement with Vari's morphology-based phylogeny, particularly with regard to the composition of the main suprageneric assemblages (Fig. 10). It is worth noting that Vari's study, being the first to apply cladistic methodology to the investigation of citharinoid relationships, predated the implementation of computer-assisted phylogenetic analyses. Therefore, no data matrix specifying character state distributions among sampled taxa was presented, and his resultant phylogeny is presumed to be derived from implementation of the

"Hennigian Argumentation" procedure (Hennig, 1966), which is not guaranteed to find the most parsimonious tree (Felsenstein, 1982; Lipscomb, 1998; Wiley and Lieberman, 2011). Notwithstanding, our results coincide with Vari's in large degree (Fig. 10), supporting: monophyly of both the Citharinoidei and the Distichodontidae (clade A), phylogenetic placement of *Xenocharax*, and a clade containing *Nannaethiops* and *Neolebias* (clade D), recognition of a clade containing *Nannocharax* and *Hemigrammocharax* (clade F), recognition of a clade containing *Hemistichodus, Ichthyborus*, *Microstomatichthyoborus, Mesoborus, Eugnatichthys, Phago* and *Belonophago* (clade J), and a subclade within that radiation containing *Microstomatichthyoborus, Mesoborus, Eugnatichthys, Phago* and *Belonophago* (clade G). Resolution within the abovementioned clades, however, differed between the two studies, and certain noteworthy novel relationships are suggested by our results.

Xenocharax is confirmed as the sister to the remaining distichodontid radiation and, whereas currently considered monotypic (Eschmeyer, 2013), our results provide support for the validity of a second species, *X. crassus*, a Congo-Basin endemic that has long been considered a synonym of *X. spilurus*, the type species of the genus (Daget, 1960). Anatomical examination of *X. crassus* specimens confirms this species to be morphologically distinct from *X. spilurus*. Therefore, *X. crassus* Pellegrin 1900 is recognized here as a valid species, pending a more detailed taxonomic treatment and formal resurrection.

Monophyly of a clade containing *Nannaethiops* and *Neolebias* (clade D) is strongly supported in this study. However, *Neolebias* is rendered paraphyletic by the placement of *Nannaethiops* species, a result that is equally strongly supported. This finding is not unanticipated given the comments of previous authors (Poll and Gosse, 1963; Vari, 1979). In light of our findings and the minimal anatomical evidence in support of the reciprocal

monophyly between these genera (Vari, 1979), we concur with Géry and Zarske (2003) in concluding that *Neolebias* should be considered a junior synonym of *Nannaethiops*.

Although placement of the genus *Paradistichodus* in our study is not strongly supported, model-based analyses converge on a finding of *Paradistichodus* as the sister group of *Distichodus*. This result conflicts with Vari's phylogeny, in which *Paradistichodus* is recovered more basally (Figs. 2 and 10) as a result of lacking a series of somewhat subjectively designated characters associated with increasingly kinetic oral jaws. While our molecular data do not provide sufficient evidence to allow for a conclusive assignment of *Paradistichodus*, reexamination of Vari's morphological data indicates that character states in *Paradistichodus* require recoding in many instances. Moreover, our own exploratory anatomical survey suggests that the placement of *Paradistichodus* within a clade containing members of *Hemigrammocharax*, *Nannocharax* and *Distichodus* (clade H) has morphological support.

Monophyly of the clade of African darters (*Nannocharax* and *Hemigrammocharax*; clade F) is well supported both by our results and Vari's. However, reciprocal monophyly between these two genera is strongly refuted by our study. While taxon sampling of *Hemigrammocharax* (only 3 of 9 species) and *Nannocharax* (only 12 of 25 species) was notably muted, the sampled *Hemigrammocharax* species were consistently nested within different subclades of *Nannocharax* (Figs. 5-7). Vari (1979: p. 332) questioned the reciprocal monophyly between *Hemigrammocharax* and *Nannocharax* noting that a single character discriminates between them (the presence of an incomplete lateral line in *Hemigrammocharax* vs. complete in *Nannocharax*). Roberts (1967) had previously suggested that the presence of an incomplete lateral line in *Hemigrammocharax* species was probably the result of multiple and independent reductions from the plesiomorphic condition (i.e., complete lateral line). However, Vari and Géry (1981: p.

1082) and Vari and Ferraris (2004: p. 557) argued that differences in the extent of lateral line poring between *Nannocharax* and *Hemigrammocharax* might be the result of ontogenetic variation instead, especially since the hypothesized apomorphic lateral line reduction is incongruent with the distribution of other hypothesized apomorphic characters. This is in agreement with the findings of Coenen and Teugels (1989), who showed that variation in lateral line length between some *Nannocharax* and *Hemigrammocharax* species exhibited a unimodal, instead of the expected bimodal distribution. This finding therefore contradicts the existence of the gap that purportedly distinguishes the two genera and further strengthens the idea that completeness of lateral line is not a character indicative of evolutionary relatedness.

Based on our results and the fact that currently only a single character of questionable diagnostic and phylogenetic value serves to distinguish *Nannocharax* from *Hemigrammocharax*, continued recognition of these genera is untenable and therefore, pending a more detailed revisional study of all valid species, should be synonymized.

A particularly significant finding of Vari's study was the recognition of a large monophyletic subgrouping of distichodontids comprising the African darters (*Nannocharax* and *Hemigrammocharax*) and the pan-African genus *Distichodus* (Fig. 2), which together constitute over 58% of distichodontid species diversity. Anatomical support for what Vari considered to be "a very distinctive unit within distichodontids" is compelling, and comprises nine osteological and myological synapomorphies, all considered trophic-related modifications facilitating a unique type of horizontal motion of the lower jaw (Vari, 1979). Our model-based phylogenies also retrieved this clade, albeit with the inclusion of *Paradistichodus* and weak nodal support (clade H). As noted previously, the placement of *Paradistichodus* within this assemblage is

supported by reexamination of Vari's anatomical data and by novel morphological characters currently under investigation.

Although Vari found compelling support for a clade containing *Hemigrammocharax*, *Nannocharax*, and *Distichodus*, he could locate no unambiguously synapomorphic characters uniting the five *Distichodus* species included in his study. In fact, he noted that aspects of neurocranial architecture in *Distichodus lusosso*, *D. niloticus* (=*D. nefasch*), and *D. fasciolatus* suggested a closer relationship of these species with African darters than with the other *Distichodus* species included in his study (i.e., *D. notospilus* and *D. brevipinnis*). This proposed phylogenetic pattern, if verified, would render *Distichodus* paraphyletic. Despite these observations, Vari refrained from making taxonomic or nomenclatural changes, urging instead for further study to determine the distribution of these and other derived characters among the numerous *Distichodus* species. Contrary to Vari's finding, *Distichodus* (16 of 23 species) was resolved as monophyletic by our data regardless of optimality criterion, yet with only moderate nodal support. Interestingly however, both parsimony (Fig. 7) and model-based phylogenies (Figs. 5 and 6) partition our sampling of *Distichodus* into two well-supported subclades, with *D. lusosso* and *D. fasciolatus* located in one, and *D. notospilus* in the other. However, the relatively low support for a monophyletic *Distichodus* in our data clearly indicates that this large genus of commercially important distichodontids is in need of further study employing both morphological and additional molecular data.

Our study provides strong support for a sister-group relationship between two ecomorphologically derived genera: *Ichthyborus* and *Hemistichodus.* This result was found regardless of optimality criterion (node E; Figs. 5-7), and with particularly strong support in the likelihood and Bayesian trees (bootstrap and posterior probability values of 100 and 1.0,

respectively). While Vari (1979) found no anatomical evidence for a close relationship between these two taxa (to the exclusion of other members of clade J), the robustness of this sister-group relationship—as recovered by DNA sequence data—predicts that further anatomical scrutiny of these taxa may reveal previously unrecognized synapomorphies uniting the two.

A particularly unexpected finding of the present study is the apparent polyphyly of the morphologically distinctive genus *Phago*. However, we view this result with reservation, and underscore that the branches separating *P. boulengeri* from *P. intermedius* and linking them with allied genera are weakly supported, as are most branches defining intergeneric relationships within this suprageneric clade (node G; Figs. 5 and 6). According to Vari (1979: p. 337), evidence for the monophyly of *Phago* consists of two uniquely derived characters, namely the presence of heavily ossified, thickened, vertically elongate scales, and anteroventrally-curved premaxillae overlaping the anterior ends of the dentaries. Our own examination of *Phago* voucher specimens confirms the species identity of the sampled taxa, supports Vari's conclusions regarding the anatomical evidence for the monophyly of this distinctive taxon, as well as the extensive morphological support for its sister-group relationship with the equally distinctive *Belonophago*. Therefore, while our results provide strong support for Vari's hypothesized clade consisting of the genera *Microstomatichthyoborus*, *Mesoborus*, *Eugnathichthys*, *Phago* and *Belonophago* (clade G), it appears that the phylogenetic signal in our data is unable to unambiguously resolve relationships within that assemblage.

4.3. ROBUSTNESS OF THE INFERRED NODE AGES TO CHANGES IN PARAMETER PRIORS AND CALIBRATION STRATEGIES

4.3.1. Impact of the shape of calibration prior densities

Of the analyses conducted to assess the impact of changes in the shape of log-normally distributed priors on divergence-time estimates (Analyses 1-5; Tables 4 and 6), only Analysis 2 (i.e., applying a "young" prior to the age of the MRCA of *Distichodus*) resulted in considerably different (younger) node ages. This result may be indicative of an interaction between prior informativeness and calibration node age, given that the proposed calibration priors for the *Distichodus* node were comparatively more informative than the corresponding priors for the citharinoid node (Fig. 3; a-c vs. e-g), and that "younger" priors at a given calibration node were likewise more informative than "older" ones (Fig. 3; d and h). If such an interaction does indeed exist, then the younger the calibration node the stronger the impact of using overly informative priors. This observation is in agreement with claims by previous authors suggesting that overly informative priors, particularly for younger fossils, may lead to biased underestimates of divergence times (Yang and Rannala, 2006; Heath, 2012). Similarly, our results agree with the suggestion that calibrations at deeper nodes are the most effective for obtaining precise—although not necessarily accurate—node age estimates (Hug and Roger, 2007; Ho and Phillips, 2009). In contrast with the findings of other empirical studies (Marshall, 2008; Sauquet et al., 2012; Warnock et al., 2012), our results indicate that divergence-time estimates are robust to (reasonable) changes in the soft maximum constraint of the fossil calibrations used. Therefore, conclusions from previous empirical studies may not necessarily be applicable to all molecular dating analyses, and instead, the impact of hyperparameter choice on divergence time estimation may depend on specific characteristics of the data and of the taxonomic group. Where the data is relatively uninformative, the prior is therefore likely to exert a greater influence on the posterior distribution of divergence times (Ho and Phillips, 2009).

4.3.2. Impact of calibration data type: primary vs. secondary calibrations

The analysis relying exclusively on primary (i.e., fossil-based) calibrations (Analysis 7; Tables 4 and 6) resulted in divergence-time estimates almost twice as young as in the control (i.e., Analysis 1). This result does not seem to be related to the adequacy (or lack thereof) of secondary calibrations (i.e., Near et al's [2012] dates), but rather to the impact of the absence of an explicit (i.e., user-specified) prior for the age of the root. More precisely, the exceptionally young divergence-time estimates in Analysis 7 are presumed to be an artifact of the markedly young root-age prior implied by the combined effects of the calibration priors on other internal nodes and the (Yule process) tree prior (Ho and Phillips, 2009). Conversely, the analysis relying exclusively on secondary calibrations (Analysis 8; Tables 4 and 6) resulted in the oldest estimated node ages. While 10-20 % older than in most analyses, these estimates are nonetheless more consistent with the control than those from Analysis 7, suggesting that Near et al's (2012) dates may indeed be a reasonable alternative to calibrate actinopterygian molecular clocks in the absence of fossil evidence. The results of these analyses also provide further support to the notion that, by detecting rate variation across different levels of divergence, multiple calibrations improve the accuracy of divergence-time estimates (Benton and Donoghue, 2007; Sauquet et al., 2012). Similarly, these results suggest that calibrating molecular clocks with deeper nodes, especially the root of the tree, produces more precise, and possibly more accurate, divergence-time estimates (Ho and Phillips, 2009).

4.3.3. Impact of non-informative priors on the parameters of the clock, speciation, and substitution models.

While most molecular-dating studies employing BEAST appear to use default priors for the parameters of the clock, speciation, and substitution models, we felt it important to explore the effects of using uniform priors instead. The fact that applying uniform priors on the parameters of the UCLN relaxed clock model, the Yule process, and the substitution models (Analyses 9-16; Tables 4 and 6) resulted in negligible differences in the estimated node ages, suggests that explicit knowledge about the parameters that describe these priors may not be necessary for arriving at reliable divergence-time estimates. Future molecular-dating studies, however, should assess the effect of using non-informative priors, for explicit knowledge about these processes is often lacking and the results presented here may be contingent to our dataset.

Given that at present there is no method for the objective formulation of priors that accurately summarize all the available evidence, the approach taken in this study (i.e., to assess the sensitivity of divergence-time estimates to variations in parameter priors and calibration strategies) offers a reasonable strategy to account for some of the uncertainties inherent to Bayesian approaches for dating molecular phylogenies. The overall robustness of citharinoid divergence-time estimates thus provides an inferred evolutionary timescale with a stronger sense of confidence than otherwise would have been the case.

4.4. TIMESCALE OF CITHARINOID DIVERSIFICATION AND IMPLICATIONS FOR CHARACIFORM BIOGEOGRAPHY

Despite advances in analytical methods of divergence time estimation using comparative DNA sequence data, and their increasingly widespread use in molecular phylogenetics, little is know about the tempo of characiform evolution beyond the information contained in its modest fossil record. As a result, most biogeographic hypotheses proposed to explain distribution

patterns of extant characiform lineages have been framed in accordance with temporal information derived from fossil evidence, yet fossils only provide minimum age estimates.

Unraveling the biogeographic history characiforms has been a challenging task, not only because of the lack of a comprehensive time-scaled phylogeny of the order, but also because of the multiple instances of continentally disjunct sister-group relationships, and the phylogenetic uncertainty regarding some of these divergences (Fink and Fink, 1981; Ortí and Meyer, 1997; Buckup, 1998; Calcagnotto et al., 2005; Arroyave and Stiassny, 2011). Although a variety of biogeographic hypotheses have been proposed—most of which may be testable with divergence-time estimates from molecular phylogenies, there is currently no widely accepted explanation for the distribution of extant characiform lineages.

Perhaps the most popular of such hypotheses is the one that attributes the unbalanced Afro-Neotropical distribution of the order to a vicariance model coupled with extinction of numerous lineages in Africa (Lundberg, 1993). Since any number of diversification scenarios invoking extinction may fit distribution patterns of extant taxa, testing the extinction component of this hypothesis is particularly problematic. Moreover, although in principle extinction may be verifiable (with fossils of extinct members), absence of evidence (i.e., fossils) does not imply evidence of absence (Maisey, 1993); extinction hypotheses are ultimately unfalsifiable. In any case, fossils assignable to Neotropical characiform lineages have not been found in Africa (Malabarba and Malabarba, 2010). Lastly, this particular vicariance model implies a homogeneous distribution of early characiform lineages across western Gondwana before the break-up. Such a biogeographic pattern, however, is seldom observed in modern continental ichthyofaunas, which are instead generally unevenly distributed across regions and drainage basins (Abell et al., 2008; Lévêque et al., 2008). A testable prediction of this hypothesis,

however, is that the ancestral lineages leading to modern characiforms must have diversified well before the African/South American drift-vicariance event, and therefore, based on geological estimates for these palaeogeographic event (McLoughlin, 2001; Briggs, 2005), the divergence between citharinoids and the remaining members of the order (i.e., the MRCA of extant characiforms) must date to at least 100 Ma.

Other attempts at explaining transcontinental sister-group relationships among characiforms have resorted to more complex vicariance models, such as that proposed by Maisey (2000). This model, while not exclusively devised to address characiform biogeography, suggests that transatlantic disjunctions are most likely the result of multiple (instead of a single) vicariant episodes, including earlier tectonic events that may have driven pre-drift intercontinental divergences of Early Cretaceous freshwater fishes in western Gondwana. Testing this scenario, however, would require a comprehensive time-scaled phylogeny of the entire order (currently unavailable) so that the absolute ages of all transcontinental divergences and the timing and geometry of western Gondwana break-up models can be confronted and assessed for congruence.

Various authors have invoked marine dispersal to explain characiform distributions, arguing that it should not be excluded a priori when a simple model of vicariance does not readily explain present-day distributions (Gayet, 1982; Filleul and Maisey, 2004; Calcagnotto et al., 2005; Otero et al., 2008; Arroyave and Stiassny, 2011). Although biogeographic hypotheses involving dispersal are generally regarded as untestable, we agree that marine dispersal should not be invoked unless vicariance hypotheses have been already falsified.

In the first study applying molecular-dating techniques to investigate the timing of diversification in a clade of characiform fishes, Arroyave and Stiassny (2011) presented a time-

scaled molecular phylogeny of the African family Alestidae that also included (as outgroup taxa) representatives of the remaining African families and of various Neotropical lineages. Although their estimated mean ages for the MRCA of the Characiformes and the MRCA of the Citharinoidei (*ca*. 87 and *ca*. 67 Ma, respectively) might be problematic given their limited sampling of Neotropical characiforms and the fact that fossil calibrations were restricted to the alestids clade, these ages, even if only fairly accurate, imply that the origins of characiform fishes most likely postdate the mid-Cretaceous break-up of western Gondwana and thereby their present-day distribution could not be attributed to any of the abovementioned vicariance hypotheses.

By contrast, the results presented here provide a considerably older age estimate for the origins of the citharinoid clade (*ca*. 90 Ma; 95% HPD=110-73), which was the focus of our study. Assuming the mean age estimate derived here is a reasonable approximation of the actual divergence time between citharinids and distichodontids, it follows that the origins of the Characiformes would necessarily be considerably older than suggested by Arroyave and Stiassny (2011) and as a result most likely older than the mid-Cretaceous break-up of Gondwana. Indeed, our results conform to available paleontological evidence, as the oldest characiform fossils date to the Cenomanian (*ca*. 95 Ma) (Werner, 1994; Dutheil, 1999; Malabarba and Malabarba, 2010) and thus, assuming temporal gaps in the fossil record, the MRCA of characiforms must have already been present in the Early Cretaceous. Several authors have argued that divergence-time estimates become more accurate as the number of reliable calibrations increases (Near and Sanderson, 2004; Fulton and Strobeck, 2010), particularly for relaxed-clock methods where multiple calibrations act as landmark points detecting rate variation at different levels of divergence (Benton and Donoghue, 2007). Therefore, given that Arroyave and Stiassny's

resultant chronogram was based only on two fossil alestid (ingroup) calibrations (Arroyave and Stiassny, 2011: Fig. 4), the estimated age for the origins of citharinoids presented here is deemed considerably more reliable. Additionally, the hypothesis of a Gondwanan origin for the Characiformes is further supported by the fact that our divergence-time estimates are robust to changes in calibration and analysis settings.

By reconciling molecular-clock and fossil-based estimates of clade ages, our results have noteworthy implications for understanding characiform historical biogeography. Namely, they provide independent temporal evidence in support for the hypothesis that attributes the disjunct distribution between African citharinoids and Neotropical characiforms to the mid-Cretaceous fragmentation of western Gondwana. Likewise, if the timing of divergence of African alestids and hepsetids from their respective Neotropical sister groups is correspondingly older than proposed by Arroyave and Stiassny (2011), then explaining the modern distribution of Characiformes would not necessitate invocation of post-drift dispersal (Filleul and Maisey, 2004; Calcagnotto et al., 2005), but simply an African/South American drift-vicariance event coupled with differential distribution patterns of primeval characiform lineages inhabiting Gondwana before the break-up (Malabarba and Malabarba, 2010).

While it is expected for divergence-time estimates based on log-normally distributed priors to be older than calibration fossils, our inferred node age for the MRCA of Citharinoidei is substantially older than its corresponding calibration fossil (Fig. 9). As recently noted by Near et al. (2012), explaining such large discrepancies requires invoking temporally large gaps in the fossil record. Interestingly, except for a few isolated teeth from the Late Cretaceous/Early Paleocene, there is indeed a relatively large gap (~40 Ma; Ypresian-Cenomanian) in the stratigraphic distribution of characiform fossils (Malabarba and Malabarba, 2010: Fig. 3) that

broadly corresponds to the difference between the molecular clock- and fossil-based citharinoid clade age estimates. While the taxonomic structure of the fossil record is largely shaped by sampling bias (Lloyd et al., 2012), such a gap in the stratigraphic distribution of characiform fossils might be real (either because of low levels of diversity in the early stages of characiform evolution and/or because of lack of suitable fossil deposits). Regardless, future paleontological research should be aimed at filling the gaps in the still meager characiform fossil record, since additional fossils would necessarily lead to more accurate and reliable molecular-dated phylogenies.

While the estimated mean age for the origin of the Distichodontidae proposed here (*ca*. 67 Ma) is older than that estimated for the Alestidae (*ca*. 54 Ma) (Arroyave and Stiassny, 2011), it is noteworthy that for both families the origins of modern genera (and most cladogenetic events) appear to have occurred between the Early Oligocene and the Late Miocene, during nearly the same time interval (*ca*. 30–10 Ma). The lower bound of this interval coincides with the Eocene-Oligocene tectonic uplift of eastern Africa (*ca*. 40-30 Ma), a geologic event that profoundly affected the geometry of contemporary African rivers (Beadle, 1974; Goudie, 2005) and initiated the development of the modern Congo Basin (Doucouré and de Wit, 2003; Stankiewicz and de Wit, 2006; Runge, 2008). The striking overlap in the current distribution patterns of alestids and distichodontids—both with highest species richness and endemism concentrated in the Congo Basin, coupled with the idea that diversification in these families was broadly concurrent with the early stages of development of the modern drainage of the Congo River, suggests that diversification and biogeographic patterns in alestids and distichodontids—and possibly numerous other groups of African freshwater fishes—may have been greatly influenced by the Neogene reconfiguration of drainage patterns in Central Africa.

We acknowledge that based on our results it is difficult to assume, let alone prove, a causal relationship between the geotectonic events that shaped the modern Central African drainage system and the diversification of alestids and distichodontids. Nevertheless, our discovery of spatio-temporal congruencies between cladogenetic and palaeohydrologic events conforms to a growing body of evidence indicating that diversification in African freshwater fishes was profoundly influenced by Oligocene and Miocene tectonism (Goodier et al., 2011; Schwarzer et al., 2012; Day et al., 2013). Future research aimed at detecting temporal shifts in diversification rates and phylogeographic signatures of drainage evolution may provide additional evidence to further test the influence of palaeogeographic processes (e.g., drainage basin isolation and recapture) on the evolution of these clades.

5. TABLES

Table 1. Taxa, voucher catalog numbers*, and GenBank accession numbers for the gene sequences included in the analyses.

Taxon	Voucher	GenBank Accession Number						
		co1	***cyt-b***	***enc1***	***glyt***	***myh6***	***nd2***	***sh3px3***
OUTGROUP								
Cyprinidae								
Danio rerio	N/A	NC_002333	NC_002333	EF032975	EF032988	EF032923	NC_002333	EF033001
Ictaluridae								
Ictalurus punctatus	N/A	AF482987	AF482987	EF032981	EF032994	EF032929	AF482987	EF033007
Citharinidae								
Citharinus citharus	AMNH 226441	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus citharus	AMNH 226441	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus congicus	AMNH 252692	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus congicus	AMNH 240019	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus gibbosus	AMNH 240020	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus gibbosus	AMNH 243512	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus gibbosus	AMNH 238209	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus sp.	CU 92980	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Citharinus sp.	CU 92980	TBA	TBA	TBA	TBA	TBA	TBA	TBA
INGROUP								
Distichodontidae								
Belonophago hutsebouti	AMNH 241850	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Belonophago hutsebouti	AMNH 241851	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Belonophago tinanti	AMNH 238286	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus affinis	AMNH 252633	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus affinis	AMNH 247062	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus antonii	CU 95832	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus antonii	AMNH 246450	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus atroventralis	AMNH 246956	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus atroventralis	AMNH 255281	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus decemmaculatus	AMNH 247931	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Distichodus decemmaculatus	AMNH 252263	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus engycephalus	CU 94663	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus engycephalus	AMNH 257169	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus engycephalus	AMNH 257704	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus fasciolatus	AMNH 240040	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus fasciolatus	AMNH 253304	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus hypostomatus	AMNH 249522	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus hypostomatus	AMNH 249522	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus kolleri	CU 93515	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus kolleri	AMNH 236538	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus lusosso	AMNH 247230	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus lusosso	AMNH 250310	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus maculatus	CU 91523	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus maculatus	CU 95265	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus mossambicus	AMNH 251295	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus mossambicus	AMNH 253393	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus noboli	AMNH C07-560	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus noboli	AMNH 247930	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus notospilus	AMNH 249523	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus notospilus	AMNH 249537	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus petersii	CU 93783	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus sexfasciatus	AMNH 251287	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus sexfasciatus	AMNH 240874	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus teugelsi	AMNH 253625	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Distichodus teugelsi	AMNH 253758	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Eugnathichthys macroterolepis	AMNH 253084	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Eugnathichthys macroterolepis	AMNH 245508	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Eugnathichthys sp.nov.	AMNH 249790	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Eugnathichthys sp.nov.	AMNH 246319	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hemigrammocharax multifasciatus	CU 91291	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hemigrammocharax ocellicauda	AMNH 241885	TBA	TBA	TBA	TBA	TBA	N/A	TBA

Hemigrammocharax ocellicauda	AMNH 247924	TBA	TBA	TBA	TBA	TBA	N/A	TBA
Hemigrammocharax ocellicauda	AMNH 241885	TBA	TBA	TBA	TBA	TBA	N/A	TBA
Hemigrammocharax uniocellatus	AMNH 252623	N/A	TBA	TBA	TBA	TBA	TBA	TBA
Hemigrammocharax uniocellatus	AMNH 252623	N/A	TBA	TBA	TBA	TBA	TBA	TBA
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Hemistichodus lootensi	AMNH 242506	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hemistichodus mesmaerksi	AMNH 252258	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Hemistichodus mesmaerksi	AMNH 242505	TBA	TBA	TBA	TBA	TBA	TBA	TBA
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Ichthyborus ornatus	AMNH 249768	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Ichthyborus ornatus	AMNH 238282	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Ichthyborus quadrilineatus	AMNH 257081	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Ichthyborus quadrilineatus	AMNH 257096	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Mesoborus crocodilus	AMNH 252426	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Mesoborus crocodilus	CU 91474	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Mesoborus crocodilus	AMNH 249770	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microstomatichthyoborus bashforddeani	CU 91515	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microstomatichthyoborus bashforddeani	AMNH 257161	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Microstomatichthyoborus bashforddeani	AMNH 257162	TBA	TBA	TBA	TBA	TBA	TBA	TBA
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Nannaethiops unitaeniatus	AMNH 249555	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannaethiops unitaeniatus	AMNH 249555	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax altus	AMNH 253852	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Nannocharax altus	AMNH 253991	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Nannocharax altus	AMNH 253854	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Nannocharax altus	AMNH 253854	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Nannocharax ansorgii	AMNH 257013	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax ansorgii	AMNH 257013	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax brevis	AMNH 253723	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax elongatus	AMNH 251111	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Nannocharax elongatus	AMNH 242261	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax fasciatus	AMNH 254206	TBA	N/A	TBA	TBA	TBA	TBA	N/A
Nannocharax fasciatus	AMNH 257016	TBA	TBA	N/A	TBA	TBA	TBA	TBA
Nannocharax fasciatus	AMNH 257016	TBA	TBA	N/A	TBA	TBA	TBA	TBA
Nannocharax hollyi	CU 91674	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Nannocharax occidentalis	AMNH 256973	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax occidentalis	AMNH 256973	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax parvus	AMNH 253853	TBA	N/A	TBA	TBA	TBA	TBA	TBA
Nannocharax procatopus	AMNH 251110	TBA	TBA	N/A	TBA	TBA	TBA	TBA
Nannocharax procatopus	AMNH 251110	TBA	TBA	N/A	TBA	TBA	TBA	TBA
Nannocharax procatopus	AMNH 243636	TBA	TBA	N/A	TBA	TBA	TBA	TBA
Nannocharax rubrolabiatus	CU 93458	N/A	TBA	TBA	TBA	TBA	TBA	TBA
Nannocharax schoutedeni	AMNH 241889	TBA	TBA	TBA	TBA	TBA	N/A	TBA
Nannocharax schoutedeni	AMNH 241890	TBA	TBA	TBA	TBA	TBA	N/A	TBA
Nannocharax sp. "So'o"	AMNH 241774	TBA	TBA	TBA	TBA	TBA	TBA	N/A
Neolebias ansorgii	CU 93512	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Neolebias ansorgii	AMNH 250442	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Neolebias ansorgii	AMNH 251003	TBA	TBA	TBA	TBA	TBA	TBA	TBA
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Neolebias gracilis	AMNH 241888	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Neolebias trewavasae	AMNH 249536	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Neolebias trewavasae	AMNH uncat.	TBA	TBA	N/A	N/A	TBA	TBA	TBA
Neolebias trilineatus	CU 91513	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Neolebias trilineatus	AMNH 247928	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Neolebias trilineatus	AMNH 247928	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Paradistichodus dimidiatus	AMNH 257747	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Paradistichodus dimidiatus	AMNH 257747	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Paradistichodus dimidiatus	AMNH 257765	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Paradistichodus dimidiatus	AMNH 257765	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Phago boulengeri	CU 91510	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Phago boulengeri	AMNH 240067	TBA	TBA	TBA	TBA	TBA	TBA	TBA

Phago boulengeri	AMNH 241663	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Phago intermedius	AMNH 252519	TBA	TBA	N/A	N/A	TBA	N/A	TBA
Phago intermedius	CU 92907	TBA	TBA	N/A	N/A	TBA	N/A	TBA
Xenocharax crassus	AMNH 249785	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Xenocharax crassus	CU 92908	TBA	TBA	TBA	TBA	TBA	TBA	TBA
Xenocharax spilurus	AMNH 253876	N/A	TBA	TBA	TBA	TBA	TBA	TBA
Xenocharax spilurus	AMNH 253910	N/A	TBA	TBA	TBA	TBA	TBA	TBA

*Institutional abbreviations: AMNH (American Museum of Natural History), CU (Cornell University Museum of Vertebrates).

Table 2. Primers and PCR profiles for amplification of *enc1*, *glyt*, *cyt-b*, and *nd2*.

Gene	Source	Primer	Primer Sequence[a]	PCR Thermal Profile[b]
enc1	Li et al. (2007)	ENC1_F85	5'-GACATGCTGGAGTTTCAGGA-3'	(98°C/20s, 57°C/30s, 72°C/45s) x 25 + (98°C/20s, 55°C/30s, 72°C/45s) x 10
		ENC1_R982	5'-ACTTGTTRGCMACTGGGTCAAA-3'	
		ENC1_F88[c]	5'-ATGCTGGAGTTTCAGGACAT-3'	(98°C/20s, 57°C/30s, 72°C/45s) x 25 + (98°C/20s, 55°C/30s, 72°C/45s) x 10
		ENC1_R975[c]	5'-AGCMACTGGGTCAAACTGCTC-3'	
glyt	Li et al. (2007)	Glyt_F559	5'-GGACTGTCMAAGATGACCACMT-3'	(98°C/20s, 57°C/30s, 72°C/45s) x 25 + (98°C/20s, 55°C/30s, 72°C/45s) x 10
		Glyt_R1562	5'-CCCAAGAGGTTCTTGTTRAAGAT-3'	
		Glyt_F577[c]	5'-ACATGGTACCAGTATGGCTTTGT-3'	(98°C/20s, 57°C/30s, 72°C/45s) x 25 + (98°C/20s, 55°C/30s, 72°C/45s) x 10
		Glyt_R1464[c]	5'-GTAAGGCATATASGTGTTCTCTCC-3'	
cyt-b	This study	*cyb*_Dist_f	ACAGGTCTTGGTTAGARTCCRGGYGGG	(95°C/60s, 58°C/60s, 72°C/120s) x 35
		*cyb*_Dist_r	CCGGATTACAAGACCGGCGCT	
nd2	This study	*nd2*_Dist_f	AGCTTTTGGGCCCATACCCCA	(95°C/60s, 58°C/60s, 72°C/120s) x 35
		*nd2*_Dist_r	AGGRACTAGGAGATTTTCACTCCTGCT	

[a]Listed from 5' to 3'.
[b]Conditions for denaturation, annealing and extension steps for each cycle are listed in parenthesis, followed by the number of cycles. All reactions included a 5-minute initial denaturation at 95°C and a 7-minute final extension at 72°C.
[c]Primers used during a second (nested) PCR, required for successful amplification; 1:20 dilution between rounds.

Table 3. Prior distributions and parameter settings of calibration nodes.

Node	Log-normal 95th percentile soft max. bound	BEAUti Settings
MRCA Otophysi[a]	n/a	Normal (*Initial Value*: 170; μ=170; σ=9.1)
MRCA Siluriformes+Characiformes[a]	n/a	Normal (μ=130; σ=6.1)
MRCA Citharinoidea[b]	"Young" = 52.83 Ma	LogNormal (μ=1.1; σ=0.5; offset: 46)
	"Intermediate" = 80.14 Ma	LogNormal (μ=2.7; σ=0.5; offset: 46)
	"Old" = 107.45 Ma	LogNormal (μ=3.3; σ=0.5; offset: 46)
MRCA *Distichodus*[c]	"Young" = 9.5 Ma	LogNormal (μ=0; σ=0.5; offset: 7.5)
	"Intermediate" = 17.54 Ma	LogNormal (μ=1.6; σ=0.5; offset: 7.5)
	"Old" = 25.58 Ma	LogNormal (μ=2.2; σ=0.5; offset: 7.5)

[a]Near et al. (2012)
[b]Murray (2003)
[c]Stewart (2003)

Table 4. Sensitivity analysis devised to explore the robustness of divergence-time estimates to changes in prior hyperparameters and analysis settings.

Analysis	MRCA Citharinoidea Prior	MRCA *Distichodus* Prior	Secondary Calibrations	UCLN, Yule, and Substitution Model Priors
1	Intermediate	Intermediate	Yes	Default
2	Intermediate	Young	Yes	Default
3	Intermediate	Old	Yes	Default
4	Young	Intermediate	Yes	Default
5	Old	Intermediate	Yes	Default
6	Intermediate	Intermediate[1]	Yes	Default
7	Intermediate	Intermediate	No[2]	Default
8	No	No	Yes	Default
9	Intermediate	Intermediate	Yes	Uniform
10	Intermediate	Young	Yes	Uniform
11	Intermediate	Old	Yes	Uniform
12	Young	Intermediate	Yes	Uniform
13	Old	Intermediate	Yes	Uniform
14	Intermediate	Intermediate[1]	Yes	Uniform
15	Intermediate	Intermediate	No[2]	Uniform
16	No	No	Yes	Uniform

[1]Calibrated as stem lineage
[2]Calibrated using implied tree prior

Table 5. Summary statistics for each individual gene partition and the results from the statistical selection of best-fit models.

Gene	OTUs coverage (%)	Alignment Length	Variable Sites (#)	Variable Sites (%)	Parsimony-informative Sites (#)	Parsimony-informative Sites (%)	jModelTest best-fit Model (BIC)
co1	96	657	268	40.8	260	39.5	HKY+I+G
cyt-b	92.5	999	495	49.5	459	46	HKY+I+G
enc1	93.4	825	283	34.3	231	28	SYM+I+G
glyt	97.5	843	377	44.7	299	35.5	K80+I+G
myh6	100	795	278	35	208	26.2	GTR+I+G
nd2	94.2	981	636	64.8	585	59.6	GTR+I+G
sh3px3	98.3	720	262	36.4	192	26.7	K80+I+G

Table 6. Estimated mean ages* and associated 95% HPD intervals of select nodes from the sensitivity analysis chronograms.

Analysis	Otophysi[1]	MRCA of Characiformes and Siluriformes[1]	Citharinoidea[2]	Distichodontidae	*Distichodus*[2]
1	166.86 (185-150)	124.73 (137-114)	90.86 (110-73)	66.90 (84-51)	17.22 (23-12)
2	158.59 (180-130)	122.27 (134-111)	76.92 (103-47)	54.86 (74-34)	11.68 (16-8)
3	166.33 (185-148)	125.93 (138-114)	94.23 (113-76)	69.96 (86-54)	18.85 (24-14)
4	166.17 (183-149)	121.89 (133-109)	90.18 (109-72)	66.19 (83-51)	16.96 (22-12)
5	166.33 (185-149)	126.07 (138-115)	92.11 (111-73)	67.88 (84-51)	17.39 (23-12)
6	165.78 (184-148)	124.92 (137-113)	91.46 (111-72)	67.35 (84-51)	17.21 (23-13)
7	68.48 (94-51)	60.32 (73-50)	46.78 (59-36)	35.38 (45-26)	10.82 (13-9)
8	167.1 (185-149)	130.58 (142-119)	99.08 (118-79)	74.63 (92-57)	21.87 (29-15)
9	166.37 (183-148)	125.01 (137-113)	91.31 (110-73)	67.55 (84-52)	17.4 (23-12)
10	158.25 (180-130)	122.31(135-111)	76.97 (103-47)	55.01 (74-34)	11.67 (16-8)
11	166.18 (186-148)	125.87 (137-114)	94.67 (114-76)	70.26 (88-54)	18.9 (25-13)
12	166.18 (183-149)	121.97 (134-110)	89.82 (108-71)	66.48 (82-50)	16.92 (22-12)
13	166.18 (184-149)	126.15 (138-115)	93 (112-74)	68.48 (85-53)	17.52 (23-12)
14	166.2 (185-149)	124.78 (137-113)	91.25 (110-72)	67.27 (83-50)	17.62 (23-13)
15	68.71 (93-50)	60.3 (73-50)	46.55 (59-36)	34.88 (45-26)	10.79 (13-8)
16	167.31 (185-149)	130.37 (142-119)	99.24 (118-79)	74.99 (92-58)	21.85 (29-15)

*Node ages in Ma.
[1]Primary-calibrated nodes
[2]Secondary-calibrated node

6. FIGURES

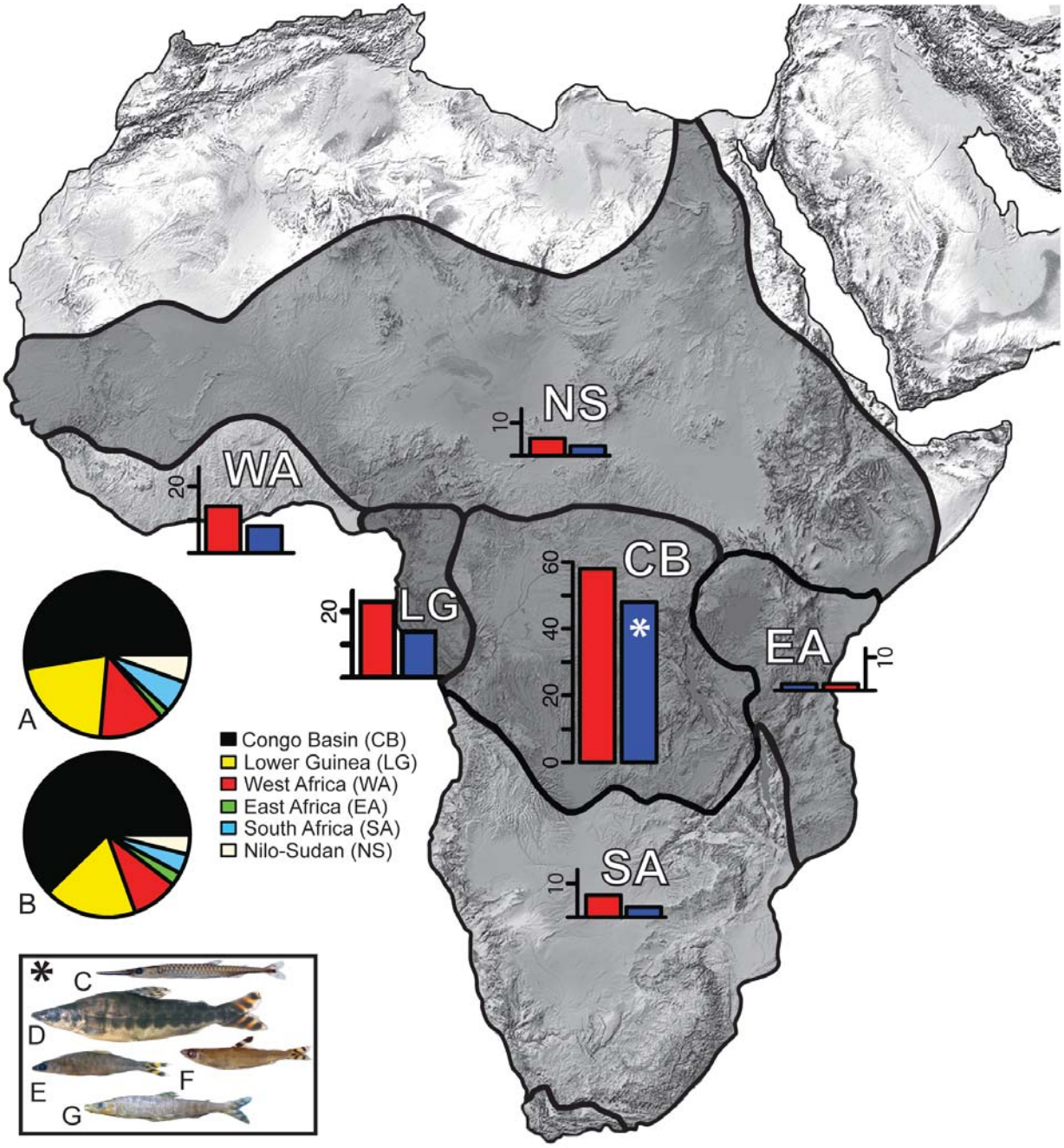

Figure 1. Distichodontid species diversity partitioned by geographic region. CB=Congo Basin, EA=East Africa, NS=Nilo-Sudan, LG=Lower Guinea, SA=South Africa, and WA=West Africa. Inset bar charts indicate number of species present (red) and number of species endemic (blue) to each region. Inset pie charts indicate species occurrences (A) and species endemism (B) across African regions. Inset box shows genera endemic to the Congo Basin: *Belonophago* (C), *Eugnathichthys* (D), *Microstomatichthyoborus* (E), *Hemistichodus* (F), and *Paraphago* (G).

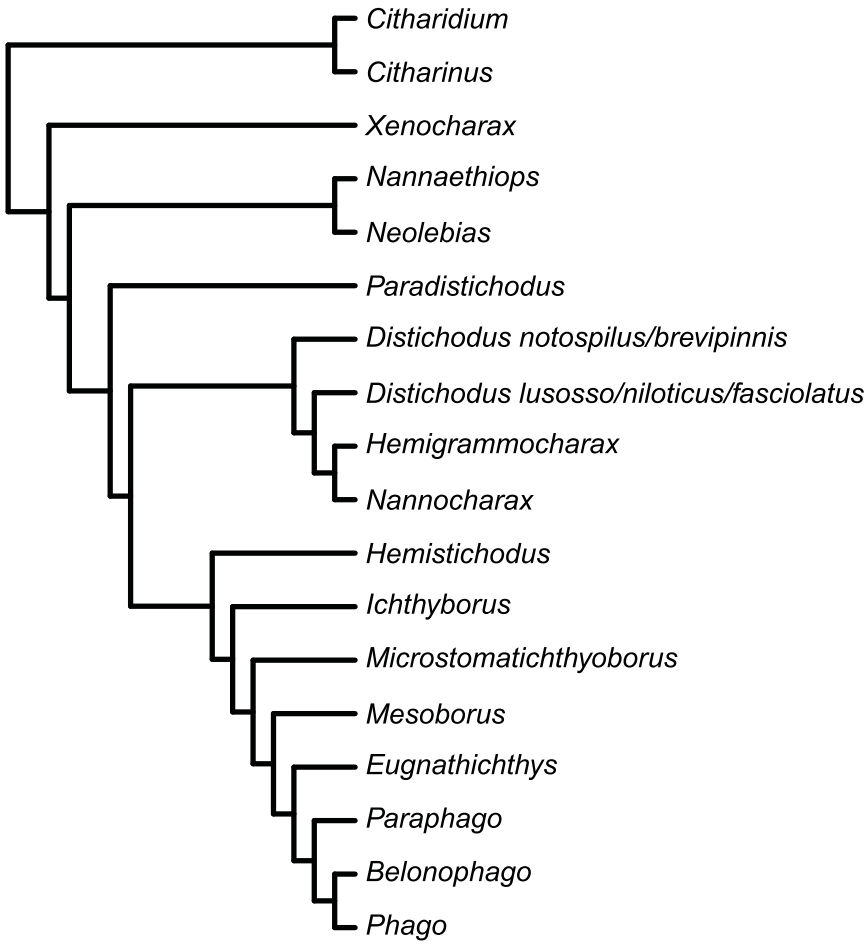

Figure 2. Hypothesis of distichodontid intergeneric relationships as proposed by Vari (1979).

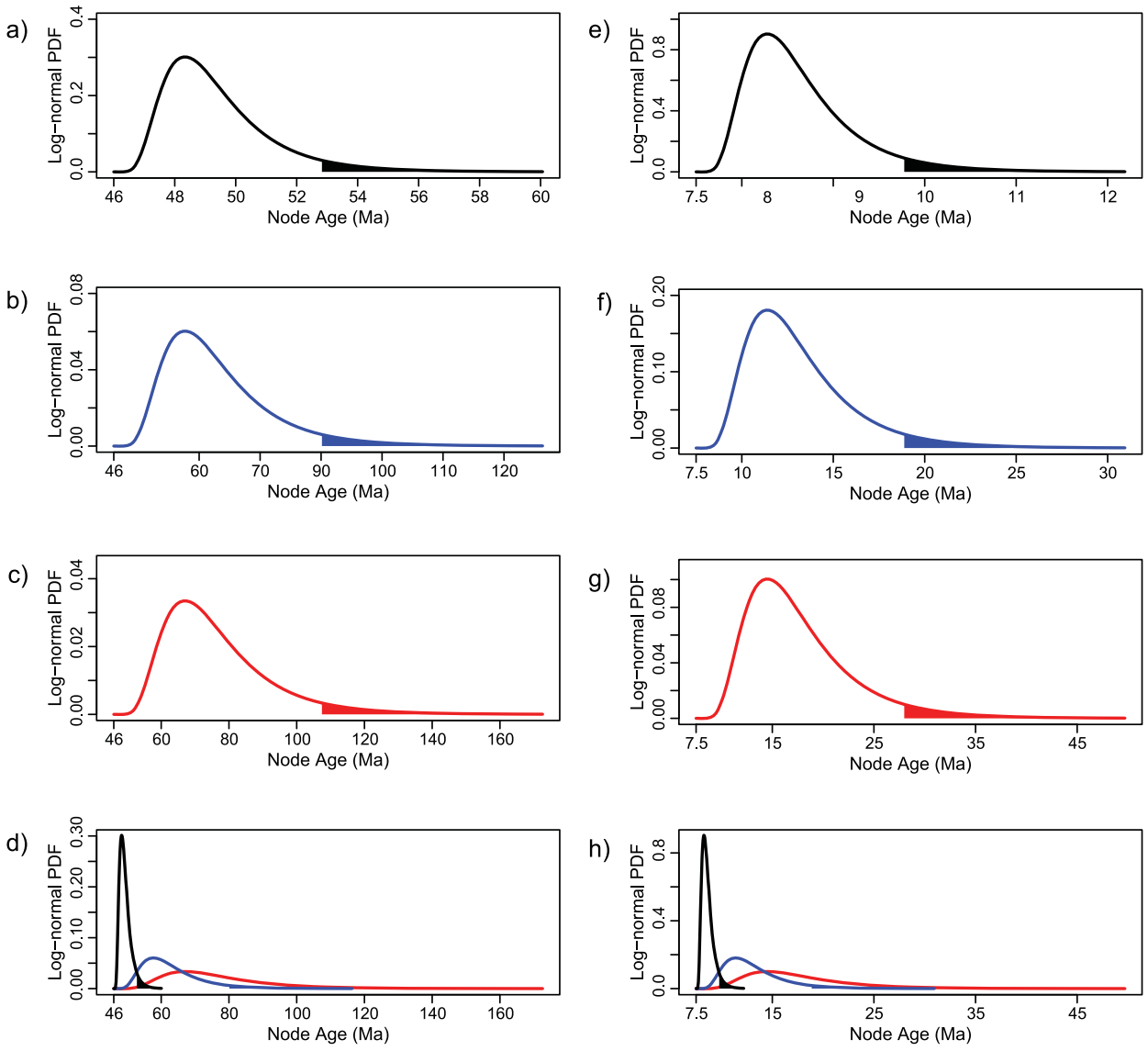

Figure 3. Alternative priors of fossil-calibrated nodes used in the sensitivity analysis. Log-normally distributed calibration priors for the age of the MRCA of Citharinoidei (a-d) and *Distichodus* (e-h) as plotted separately and differentially scaled (a-c, e-g), and combined and equally scaled (d, h). The lower limit of the x-axis interval defining the area shaded under the curves corresponds to the 95th percentile soft maximum bound of each calibration prior.

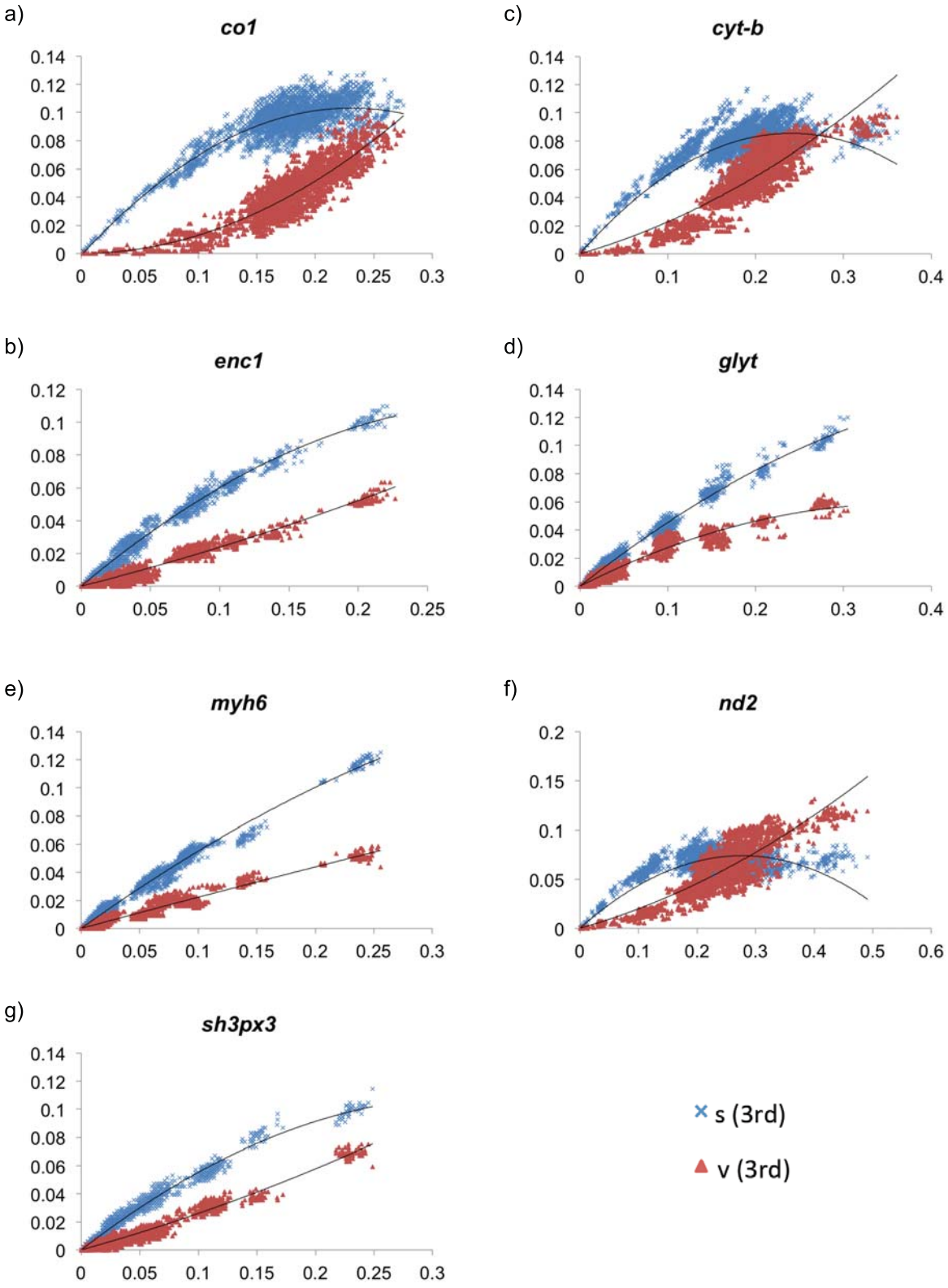

Figure 4. Saturation plots. Scatterplots of observed number of transitions and transversions against corrected genetic distance for third codon positions of each gene sampled in this study.

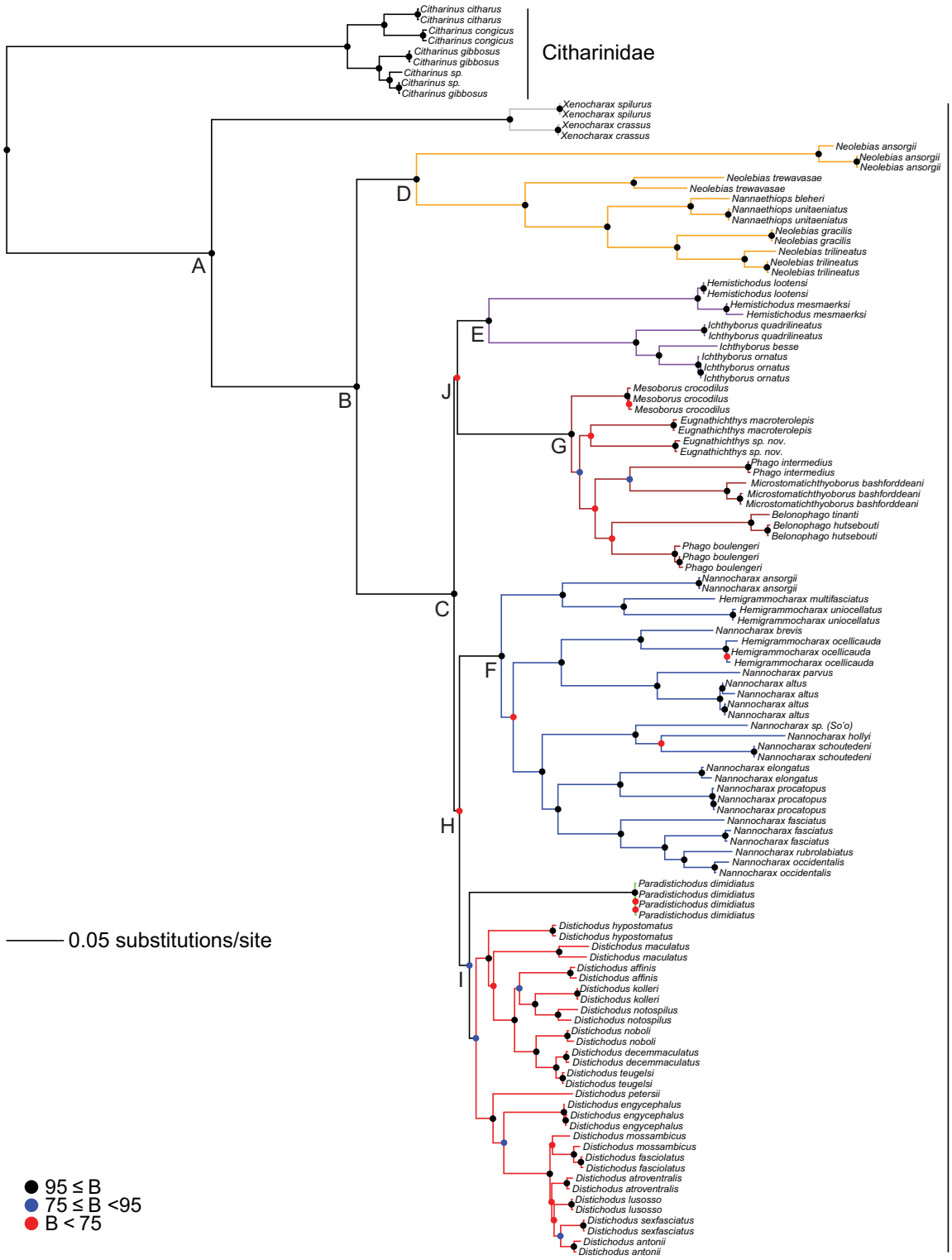

Figure 5. Phylogeny of the Citharinoidei as inferred by likelihood in RAxML. Letters A-J indicate major suprageneric clades. Colored circles on nodes indicate degree of support as determined by bootstrap values (B). Branches of select generic and suprageneric assemblages are differentially colored to indicate composition and configuration of distichodontid subclades discussed in the text. Long branches leading to the outgroup species *Danio rerio* (root) and *Ictalurus punctatus* are not shown.

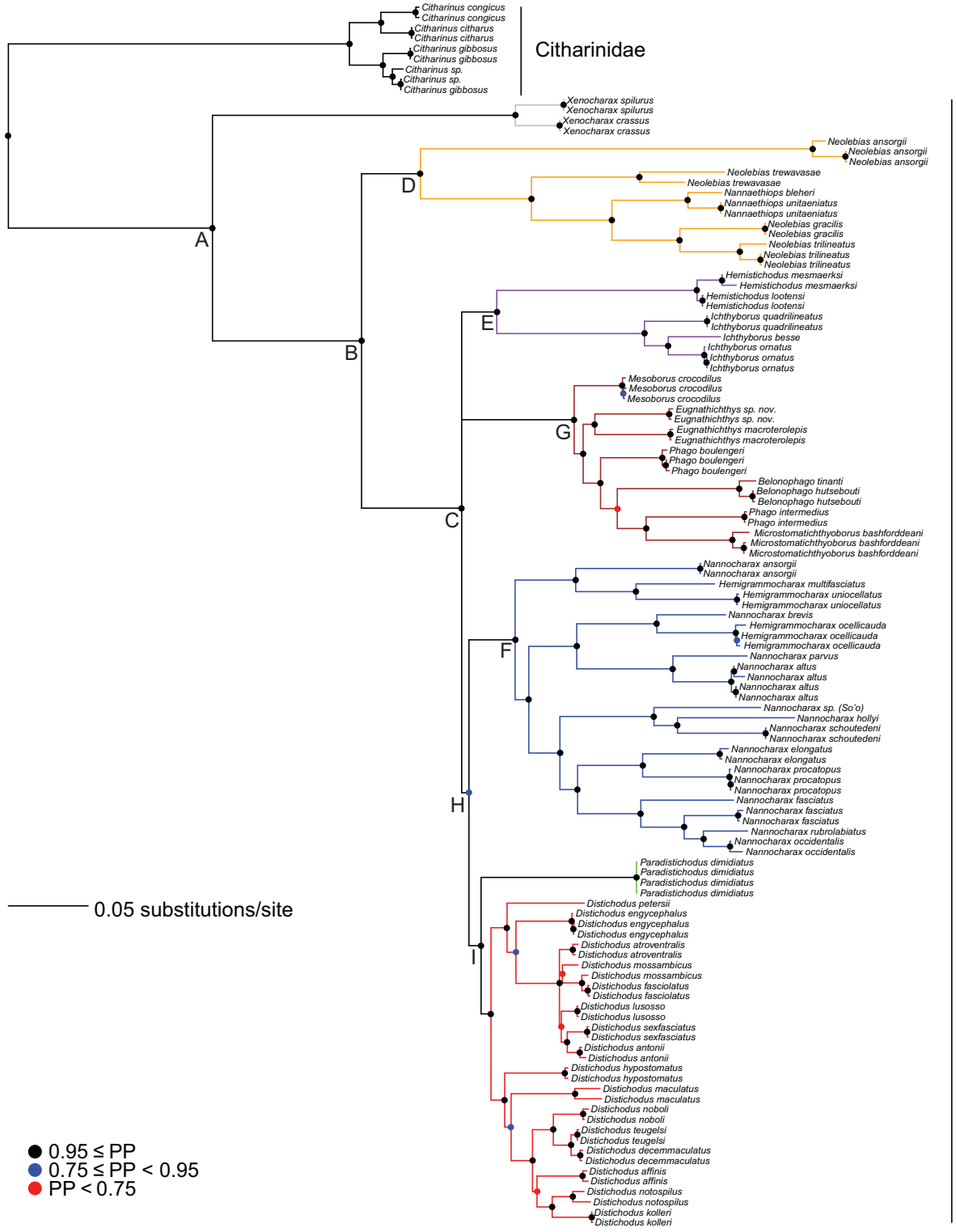

Figure 6. Phylogeny of the Citharinoidei as recovered by Bayesian inference in MrBayes. Colored circles on nodes indicate degree of support as determined by posterior probabilities (PP). Branches and terminals colored as in the RAxML tree (Fig. 5).

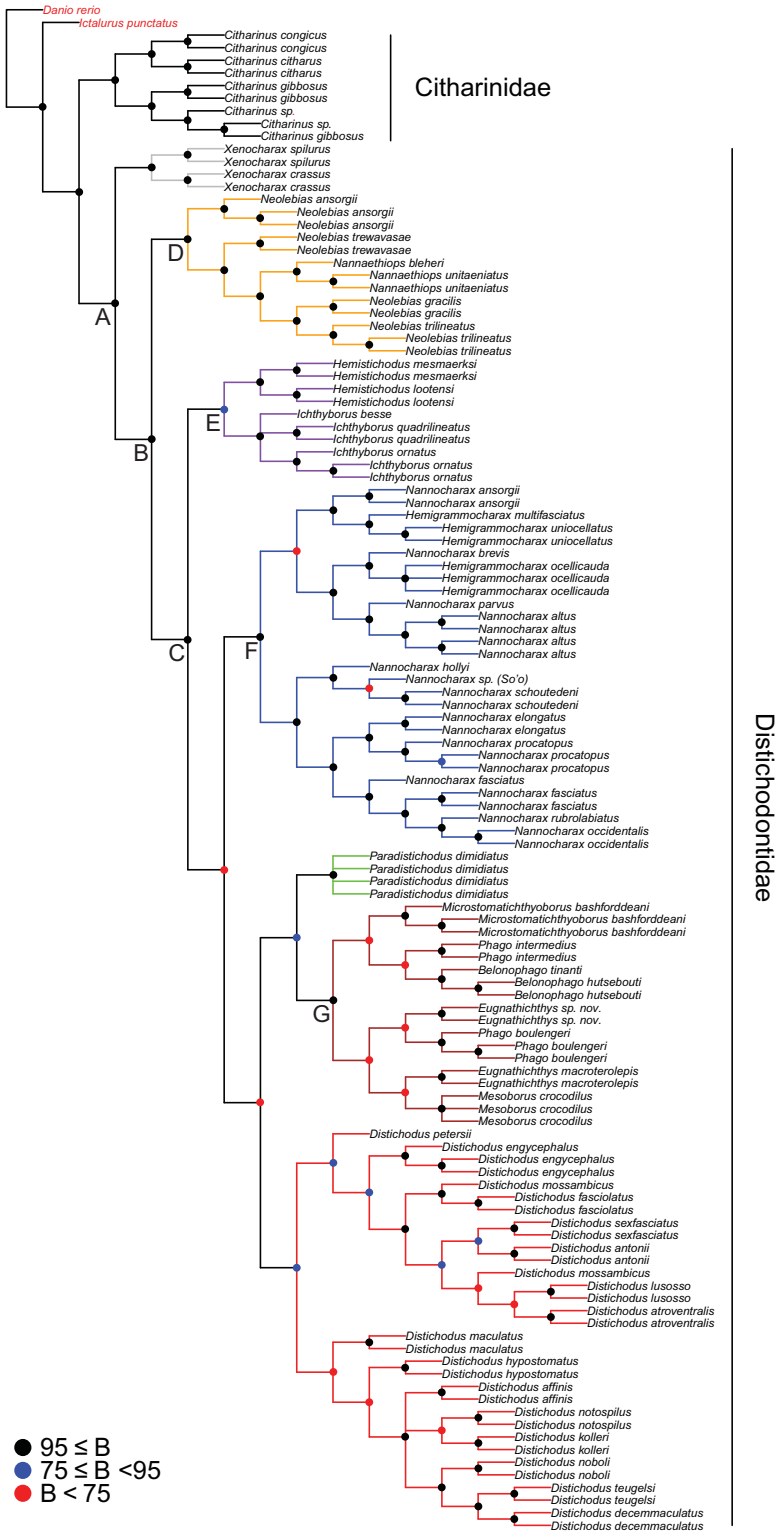

Figure 7. Phylogeny of the Citharinoidei as recovered by parsimony in TNT. The topology corresponds to the strict consensus of 12 equally most parsimonious trees (L=14047; CI=0.272; RI=0.765). Letters A-G indicate major suprageneric clades also recovered by model-based methods. Colored circles on nodes indicate degree of support as determined by bootstrap values (B). Branches and terminals colored as in the RAxML tree (Fig. 5). Outgroup taxa in red.

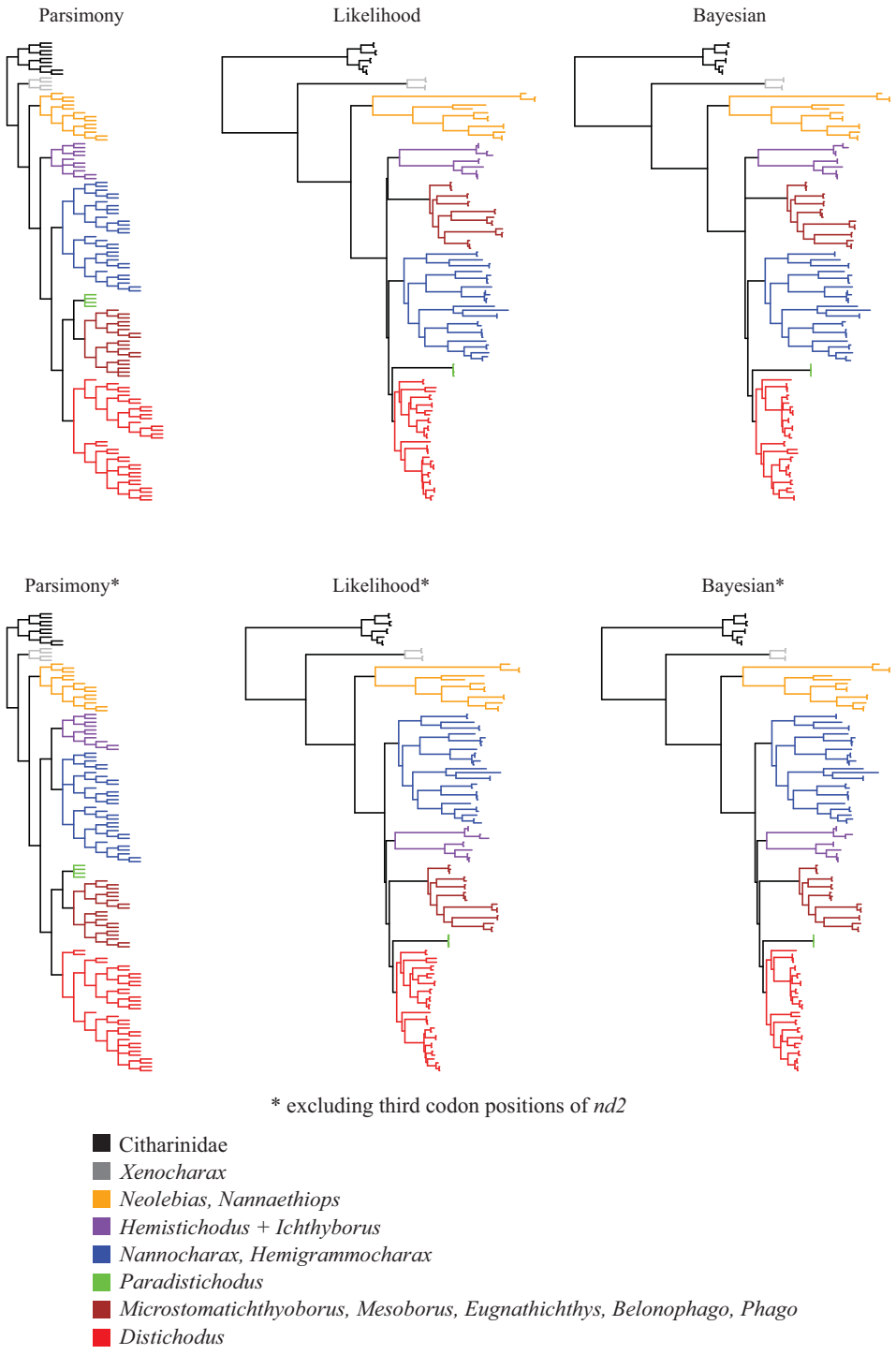

Figure 8. Comparative summary of citharinoid phylogenies inferred with and without putatively saturated sequence data. Parsimony and model-based phylogenies based on the original dataset (top) and a restricted dataset with *nd2* sequence data removed (bottom). Branches are differentially colored by generic/suprageneric subclade, of which the composition is indicated by the genus/genera matching the color of its branches.

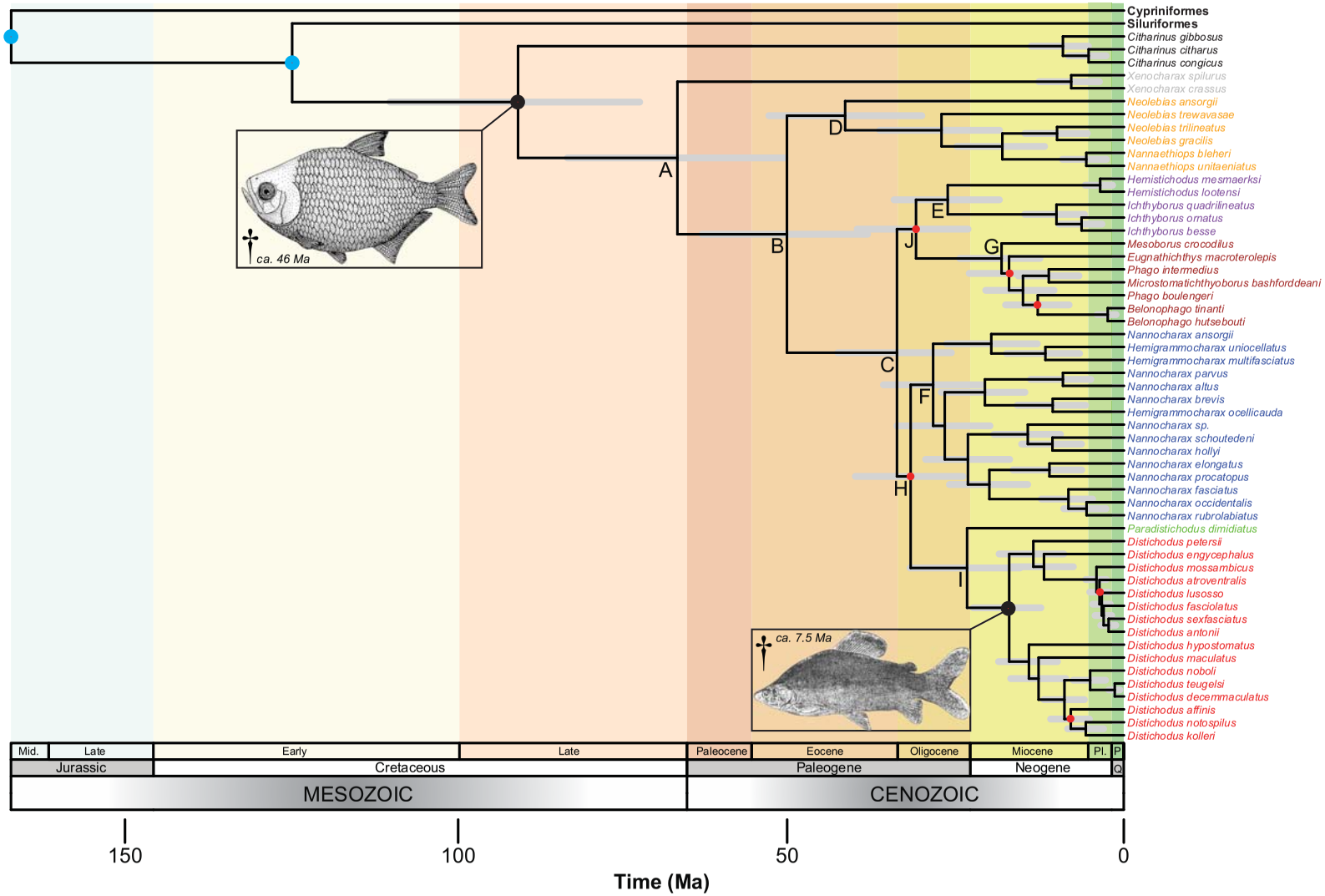

Figure 9. Time-scaled citharinoid phylogeny. This chronogram was inferred using both primary (with "intermediate" priors) and secondary calibrations (Analysis 1; Table 4). Primary calibration nodes are indicated by black dots and linked to a figure representing the fossil. Secondary calibration nodes are indicated by blue dots. Divergence-time estimates are represented by the mean ages of clades. Gray bars correspond to 95% highest posterior density (HPD) intervals of mean node ages. Terminal taxa are colored by generic/suprageneric clade membership, following the color scheme of Figs. 5-8. All nodes resulted in posterior probabilities (PP) larger than 0.95, except for those labeled in red, for which PP < 0.75.

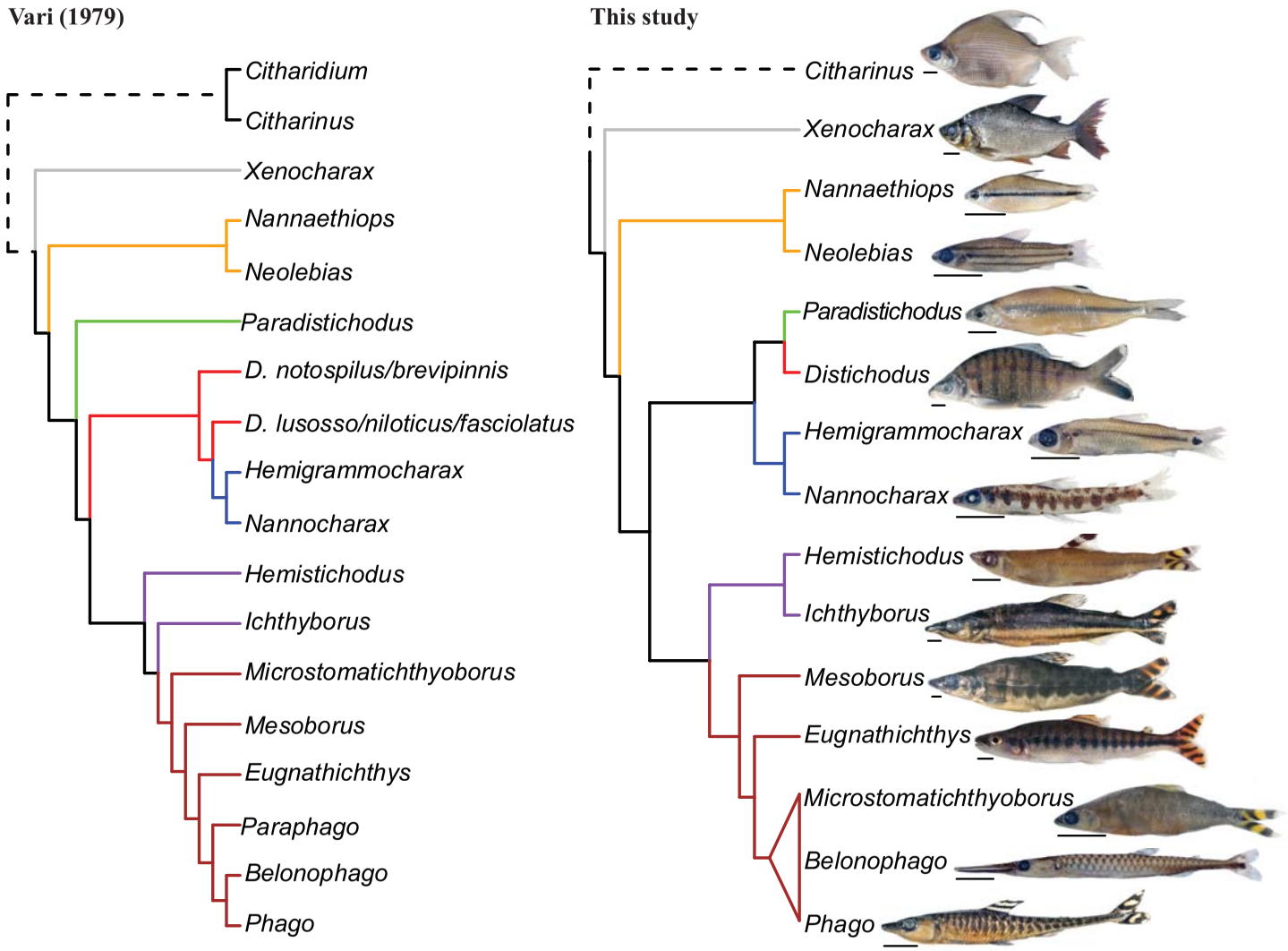

Figure 10. Comparison of citharinoid intergeneric relationships as inferred form anatomical (Vari, 1979) and molecular data (likelihood topology of this study).

CHAPTER 4

MOLECULAR PHYLOGENY OF THE CHEDRINI (CYPRINIDAE: DANIONINAE), WITH EMPHASIS ON THE AFRICAN MEMBERS OF THE TRIBE

1. INTRODUCTION

Members of the tribe Chedrini (subtribe Chedrina *sensu* Liao et al., 2011a; 2012) are a speciose (>100 spp.) and monophyletic assemblage of cyprinid fishes (Cypriniformes: Cyprinidae) distributed in the freshwaters of both Africa and Asia (Fig. 1) (Tang et al., 2010; Liao et al., 2011a; Liao et al., 2011b, Liao et al., 2012; Eschmeyer, 2013). This widespread group of small- to medium-sized fishes constitutes one of the three major clades that make up the cyprinid subfamily Danioninae, according to recent phylogenetic studies (Liu and Chen 2003; Rüber et al. 2007; Wang et al. 2007; Conway et al. 2008; Chen and Mayden, 2009; Fang et al., 2009; Tang et al., 2010; Liao et al. 2011a; Liao et al. 2011b). The Danioninae is notable among other groups of fishes because it includes the species *Danio rerio*, one of the most important vertebrate model organisms in studies of developmental biology, toxicology, and functional genomics, among others (Ekker, 2000; Nagel, 2002; Meyer et al., 2003; Spitsbergen and Kent, 2003). Similarly, danionins are noteworthy because the smallest fish and second smallest vertebrate known to science, the species *Paedocypris progenetica*, is also a member of the subfamily.

Although currently estimated at 318 species (Eschmeyer and Fong, 2013), the taxonomic diversity of the Danioninae is unsettled and in need of revision for many genera (Tang et al., 2010). Despite the recent proliferation of phylogenetic studies including members of the Danioninae, most of these investigations have arrived at somewhat different conclusions regarding the limits and composition of the subfamily and its constituent lineages. A molecular phylogeny resulting from the most comprehensive of those studies (Tang et al., 2010; 4117 bp, 270 terminal taxa) revealed that putative members of the Danioninae *sensu lato* did not form a monophyletic group but were instead scattered throughout the Cyprinidae. Accordingly, Tang et

al. (2010: p. 14) redefined the generic composition of the subfamily and restricted the Danioninae to only 28 of the 43 genera recognized as valid at the time. However, the phylogenetic placement of eight putative danionin genera, including three that had been previously associated with members of the Chedrini (i.e., *Bengala* (=*Megarasbora*), *Engraulicypris*, and *Rastrineobola*), was not assessed by Tang et al. (2010) due to the incompleteness of their taxon sampling.

Tang et al.'s (2010) phylogeny resolved chedrins as sister to the remaining danionin radiation, a clade consisting of the reciprocally monophyletic tribes Danionini and Rasborini (Fig. 2). Based on their findings, Tang et al. (2010: p. 14, Table 3) proposed a revised classification of the Danioninae in which chedrins were assigned tribal status and restricted to 13 genera (14 counting *Mesobola*, which appears to be mistakenly omitted from their list of chedrin genera). In contrast, the morphology-based phylogeny of Liao et al. (2011a), although generally congruent with Tang et al.'s (2010) regarding the limits and composition of the three major danionin subclades, was resolved with the tribe Rasborini sister to a clade (therein referred to as the tribe Danionini) subdivided into the reciprocally monophyletic subtribes Chedrina and Danionina (Fig. 3). The findings of Liao et al. (2011a), with both *Bengala* (=*Megarasbora*) and *Esomus* nested within members of the Chedrina (Fig. 3), resulted in the expansion of the chedrin clade originally proposed by Tang et al. (2010). More recently, Liao et al. (2012) provided molecular phylogenetic evidence (Fig. 4) supporting the inclusion of the exclusively African *Engraulicypris* and *Rastrineobola* in the Chedrini, increasing to 18 the number of valid chedrin genera (Table 1).

Traditionally classified as the Bariliinae Regan 1922 and referred to as "bariliin(e)s" in early systematic treatments of the Cyprinidae (e.g., Roberts, 1975; Howes, 1979, 1980, 1983, 1991;

Fink and Fink, 1981; Cavender and Coburn, 1992), members of the Chedrini have received the least attention among danionins in the most recent phylogenetic studies of the subfamily, partly due to taxon sampling incompleteness—especially of African genera.

Prior to the work of Tang et al. (2010), none of the systematic studies including members of the Danioninae had sampled for phylogenetic analyses more than four chedrin genera (Table 2). Most of those previous studies, however, agreed in resolving the sampled chedrin genera within a clade consisting of members of the Danioninae *sensu stricto*. Notwithstanding the limited number of systematic studies including a comprehensive representation of chedrin genera, both molecular (Tang et al., 2010; Liao et al., 2012) and morphological (Liao et al., 2011a) studies have presented evidence in support of the monophyly of this Afro-Asian clade of danionin cyprinids.

According to Liao et al.'s (2011a) morphology-based phylogenetic analysis, the monophyly of chedrins is supported by the shared derived condition of having a postcleithrum that is either greatly reduced or absent, and when present positioned perpendicular to the pleural ribs. The genera *Chelaethiops*, *Mesobola* and *Esomus* represent the extreme condition in which the postcleithrum has been completely lost (Liao et al., 2011a: p. 228). Besides revealing a monophyletic Chedrini, the phylogeny of Tang et al. (2010) strongly supports a clade of exclusively African species containing several genera of uncertain monophyly (e.g., *Opsarius, Raiamas, Salmostoma*) (Fig. 4). This finding was broadly corroborated by Liao et al. (2011a) and further explored by Liao et al. (2012) (Fig. 5).

Despite the recent progress in the systematics of the group, chedrins are in the greatest need of revisionary work among the danionins; several chedrin genera appear to be non-monophyletic, and generic assignment of species remains problematical and often unsupported by character

data. This is particularly the case for genera with widespread distributions and/or high species-level diversity (e.g., *Barilius*, *Raiamas*, *Opsaridium*). Moreover, resolution of the phylogenetic relationships among chedrins has been hindered by the limited sampling of African members of the tribe. Thus, the purpose of the present study is to further investigate Tang et al.'s (2010) and Liao et al.'s (2011a, 2012) phylogenetic hypotheses by analyzing a considerably expanded version—both in taxon and character sampling—of Tang et al.'s (2010) data matrix. By increasing the sampling of African chedrins with respect to previous works, the present study is also aimed at assessing generic limits in genera of questionable monophyly, such as the morphologically similar *Leptocypris*, *Raiamas*, and *Opsaridium*.

2. METHODS

2.1. TAXON AND CHARACTER SAMPLING

Ingroup taxa included representation of all valid chedrin genera, with the exception of *Bengala* (monotypic) and *Esomus*, both restricted to Asian freshwaters. Despite the lack of consensus as to its position in the phylogeny of the Danioninae, *Esomus* was herein treated as a member if the Chedrini following Liao et al. (2011a). Except for *Cabdio, Mesobola, Nematabramis,* and *Neobola*, all sampled polytypic genera were minimally represented by two nominal species. Similarly, all sampled monotypic genera (i.e., *Engraulicypris*, *Malayochela*, *Rastrineobola*, and *Securicula*) were represented by at least two individuals. Where available, several individuals per species were included. In addition to increasing geographic coverage, sequencing of multiple individuals per species allowed for an improved control of sequence quality and recognition of potential contamination issues. Overall, 155 individuals, representing 50 valid plus eight undescribed or questionably nominal ingroup (=chedrin) species, were sampled for phylogenetic analyses. African chedrins (including the Afro-Asian genus *Raiamas*), the emphasis of this study, were represented by *ca.* 60% of their species-level diversity. Tissues were obtained primarily from specimens collected during the NSF-funded Congo Project (http://research.amnh.org/vz/ichthyology/congo/index.html) and recent fieldwork in West and West-Central Africa. Additional tissues were obtained through donations from colleagues at the Swedish Museum of Natural History (Naturhistoriska Riksmuseet; NRM), The South African Institute for Aquatic Biodiversity (SAIAB), and The Bavarian State Collection of Zoology (Zoologische Staatssammlung München; ZSM).

The comparative molecular data of the present study consisted of six protein-coding genes/gene fragments, including both nuclear and mitochondrial markers. Nuclear markers comprised the genes myosin-heavy polypeptide 6-cardiac muscle-alpha (*myh6*), SH3 and PX domain-containing 3-like protein (*sh3px3*), recombination-activating gene 1 (*rag1*), and rhodopsin (*rho*). Mitochondrial markers comprised the genes cytochrome c oxidase subunit 1 (*co1*) and cytochrome b (*cyt-b*). DNA extraction, amplification, and purification procedures, along with primers and PCR thermal profiles for sequencing *co1*, *myh6*, and *sh3px3* are described in Arroyave and Stiassny (2011). Chedrin-specific primers for *cyt-b* and *rag1* were designed on conserved flanking regions for each fragment using Primer3 (Rozen and Skaletsky, 1999) and are listed (with their corresponding PCR thermal profiles) in Table 3. Primers and PCR thermal profiles for amplification and sequencing of *rho* are listed in Tang et al. (2010). DNA sequence data for *co1*, *cyt-b*, *rag1*, and *rho* from 45 of the 156 terminal taxa analyzed herein were generated by Tang et al. (2010) and retrieved from the GenBank database. Sequences for the remaining 111 taxa were generated during this study. Contig assemblage and sequence editing was performed using Geneious Pro version 5.6.5 (Biomatters, available from http://www.geneious.com/). IUPAC nucleotide ambiguity codes were used to represent heterozygous sites. Voucher catalog numbers and GenBank accession numbers for the gene sequences generated and included in this study are listed in Table 4.

2.2. MODEL SELECTION AND ASSESSMENT OF SUBSTITUTION SATURATION

Individual sequences were aligned by gene based on the translated amino acid sequence using the *Translation Align* algorithm under default parameters as implemented in Geneious. The best-fit substitution models for each gene partition was selected from among 24 models using the

Bayesian Information Criterion (BIC) as implemented in jModelTest (Posada, 2008) under the following likelihood settings: *Number of substitution schemes* = 3; *Base frequencies* = +F; *Rate variation* = +I and +G with nCat = 4; and *Base tree for likelihood calculations* = Fixed BIONJ-JC.

Substitution saturation in third codon positions was assessed via saturation plots and the test by Xia et al. (2003), both implemented in the software DAMBE (Xia and Xie, 2001) following the guidelines provided in (Xia and Lemey, 2009). In saturation plots, corrected genetic distances were calculated using the substitution models previously selected by jModelTest or the best-approximating models available in DAMBE; trend lines were estimated using second-order polynomial curves fit to the data.

2.3. PHYLOGENETIC ANALYSES

Concatenation of individual gene alignments was carried out using the software 2matrix.pl (Salinas and Little, 2012). The number of variable and parsimony-informative sites of the concatenated alignment was determined using MEGA 5 (Tamura et al., 2011). The concatenated alignment was analyzed using Maximum Likelihood [ML] (Felsenstein, 1981) in RAxML version 7.2.8 Black Box (Stamatakis, 2006) and Bayesian Inference [BI] (Rannala and Yang, 1996) in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). In RAxML analyses, likelihood of final tree was evaluated and optimized under the GTR+I+G model, and individual alpha-shape parameters, GTR-rates, and empirical base frequencies were estimated and optimized for each partition. MrBayes analyses were implemented using the Markov Chain Monte Carlo algorithm (MCMC) run twice (each time using four chains) for 3×10^7 generations, with a sampling period of 1000 generations, under

default priors and proposal mechanisms. MCMC convergence was assessed by examining trace plots of posterior probability vs. number of generations in Tracer (Rambaut and Drummond, 2007) and by plotting posterior probabilities of splits at selected increments over an MCMC run (cumulative function) using the web-based tool AWTY (Nylander et al., 2008; Wilgenbusch et al., 2004). Further assessment of MCMC convergence was undertaken by examination of the average standard deviation of split frequencies, with values << 0.01 taken as indicative of stationarity. Pre-convergence MCMC samples were discarded as burn-in, and substitution model parameters were calculated from the remaining samples. Likewise, branch lengths and posterior probabilities of nodes were calculated from the set of post burn-in trees using TreeAnnotator version 1.7.4 (Drummond et al., 2012), and summarized as a 50% majority-rule consensus tree. Both ML and BI analyses were conducted on the concatenated alignment partitioned into gene regions with parameters unlinked (to accommodate potential process heterogeneity among gene regions) and implemented through the CIPRES Science Gateway V. 3.3 (Miller et al., 2010). The phylogeny was rooted at *Danio rerio* (the sole outgroup taxon) and drawn in a ladderized configuration.

3. RESULTS AND DISCUSSION

3.1. DATASET STATISTICS, BEST-FIT SUBSTITUTION MODELS, AND SATURATION TESTS

The concatenated alignment consisted of 5380 sites (~14% missing data), of which 1894 were variable and 1552 parsimony-informative. Tissue samples from the majority of Asian chedrins were unavailable for DNA extraction and sequencing, which resulted in most of the missing data corresponding to *myh6* and *sh3px3* sequences of Asian taxa. Asian chedrins, however, were fully represented in the data matrix by *co1*, *cyt-b*, *rag1*, and *rho* sequences already available in GenBank (Table 1). The best-fit substitution models for the sequenced genes were: GTR+I+G for *co1* and *cyt-b*, K80+I+G for *myh6*, *sh3px3*, and *rag1*, and HKY+I+G for *rho*. Both saturation plots and Xia et al's (2003) statistical tests imply little to no saturation in the molecular dataset of this study. Transversions did not outnumbered transitions in third positions of any of the sampled genes. Similarly, the index of substitution saturation (ISS) was significantly smaller than the critical value (ISS.C) in all sampled genes (p-value << 0.0001).

3.2. CHEDRIN PHYLOGENY

Maximum Likelihood (ML) estimation of phylogeny resulted in a tree with a likelihood score (ln*L*) of −56306.04 (Fig. 6). Bayesian inference (BI) of phylogeny resulted in a 50% majority-rule consensus tree identical in topology to the ML except for a few polytomies at shallow nodes. Nodal support and relative branch lengths in the BI phylogeny were comparable to those of the ML tree. A summarized phylogeny, focused on chedrin intergeneric relationships as inferred herein, is presented in Fig. 7.

The results of this study constitute the most comprehensive hypothesis of interrelationships among members of the tribe Chedrini, and thus they provide an in-depth phylogenetic framework (particularly for African chedrins) to test the monophyly of chedrin genera and revise the patently disarrayed taxonomy of the group. The phylogeny corroborates the notion that Asian members of the Chedrini constitute a paraphyletic assemblage (Tang et al., 2010; Liao et al. 2012), herein resolved into seven major lineages represented by distinct generic and suprageneric clades (clades 1-7; Figs. 6, 7). African chedrins, on the other hand, were herein strongly supported as a monophyletic group, sister to a clade consisting of Asian members of the manifestly polyphyletic genus *Raiamas*. The resultant phylogeny, which included the largest sampling of African chedrins to date, likewise confirms previous findings regarding the monophyly of both African chedrins (Tang et al., 2010; Liao et al. 2012) and Asian *Raiamas* (Liao et al. 2012).

Chedrins were resolved herein with the genera *Nematabramis* and *Malayochela* forming a clade (clade 1; Figs. 6, 7) sister to the remaining members of the tribe. This result, including a sister-group relationship between *Nematabramis* and *Malayochela* (both endemic to the Malay Archipelago), was similarly found by previous authors using molecular data (Britz et al., 2009; Fang et al., 2009; Tang et al., 2010; Liao et al. 2012). The species *Opsarius koratensis* was herein resolved as the sister taxon of *Luciosoma* (clade 2; Figs. 6, 7), instead of the sister group of all chedrins other than *Nematabramis* and *Malayochela*, as found by Tang et al. (2010). Although moderate, nodal support for a sister-group relationship between *O. koratensis* and *Luciosoma* is stronger than for the phylogenetic pattern suggested by Tang et al. (2010). Notwithstanding the differences regarding the phylogenetic placement of *O. koratensis* with respect to *Luciosoma*, the results of the present study provide further support for the inclusion of

Luciosoma within the Chedrini (Roberts, 1989; Liao et al., 2010; Liao et al., 2011a), thus contradicting previous studies that suggested a closer association of this genus with *Rasbora* and other non-chedrin danionins (Howes, 1984; 1991).

A clade containing members of the genera *Cabdio* (=*Aspidoparia*), *Salmostoma* (=*Salmophasia*; herein resolved as paraphyletic), and *Securicula* (clade 3, Figs. 6, 7) was strongly supported by the molecular data of the present study. Although differently resolved, a *Cabdio*-*Salmostoma*-*Securicula* clade was also found in previous molecular phylogenies of the group (Tang et al., 2010; Liao et al. 2012). The affinities between *Securicula* and *Salmostoma* had been previously noted on the basis of morphological data (Howes, 1979), even to the extent of placing the former in the synonymy of the latter (Bănărescu, 1968). The paraphyly of *Salmostoma* with respect to *Securicula*, as consistently found in the most recent molecular phylogenetic studies of the group, strongly supports synonymization of these genera to maintain generic monophyly. Nevertheless, *Salmostoma* and *Securicula* are morphologically distinct, and putative synapomorphies for a clade consisting of both genera are lacking at this time. Therefore, each genus name is here maintained pending further investigation aimed at discovering morphological characters in support of their synonymization. As noted by Tang et al. (2010), *Salmostoma* would have priority if it were to be synonymized with *Securicula*.

The remaining lineages of Asian chedrins in the resultant phylogeny include both generic and suprageneric clades whose limits and composition reveal widespread instances of non-monophyly, such as in the genera *Barilius*, *Opsarius*, and *Raiamas* (Fig. 7). The non-monophyly of *Barilius* had been previously suggested by Howes (1983), who considered that species of the genus were in fact members of two distinct, non-reciprocally monophyletic groups: one including the species *B. barila* (type species of the genus), *B. evezardi*, *B. modestus*, and *B.*

vagra, and another containing all of the remaining species of the genus. Following Howes (1983), Tang et al. (2010) treated the species in the former group as members of *Barilius sensu stricto*, and those in the latter group as members of *Opsarius*. A detail account of the convoluted taxonomic history of *Barilius* can be found in Tang et al. (2010: p. 15).

Barilius was herein represented by six species, two of which are representatives of *Barilius sensu stricto* (*B*. cf. *barila*, *B. vagra*), and the remaining four including both nominal (*B. bernatziki*, *B. mesopotamicus*) and unidentified or questionably nominal species (*B*. sp. "Bangladesh", and *B*. sp. "Thailand"). The genus was resolved as polyphyletic, with some of its members forming a distinct, moderately supported clade (clade 4; Figs. 6, 7) sister to a large Afro-Asian chedrin subclade, and the remaining sampled species (i.e., *Barilius* sp. "Thailand" and *Barilius bernatziki*) well nested within an otherwise exclusively *Opsarius* clade (clade 5; Figs. 6, 7). Although this is the first study failing to support the monophyly of *Barilius* on the basis of comparative DNA sequence data, it should be noted that it is the one with the largest sampling of *Barilius* species and therefore the best suited for testing the monophyly of the genus. As previously found by Liao et al. (2011b), the resultant phylogeny supports the recognition of the Middle Eastern species *Barilius mesopotamicus* as a member of *Barilius.* Conversely, the results of the present study imply that *Barilius* sp. "Thailand" and *Barilius bernatziki* should be synonymized with *Opsarius*. The available molecular-based phylogenetic evidence (Tang et al., 2010; Liao et al., 2011b; Liao et al., 2012), including the findings of this study, suggests that the name *Barilius* should be retained for the clade that includes members of *Barilius sensu stricto* and closely related species (clade 4; Figs. 6, 7).

Similarly, the findings of this and previous molecular phylogenetic studies (Tang et al., 2010; Liao et al., 2011b; Liao et al., 2012) strongly indicate that the genus *Opsarius* is

polyphyletic. As in the phylogeny of Tang et al. (2010), putative members of *Opsarius* were resolved herein forming three distinct and distantly related lineages: one represented by *O. koratensis* (resolved here as the sister group to *Luciosoma*), another consisting of the species *O. bakeri* and *O. canarensis* (clade 6; Figs. 6, 7), and a third consisting of the rump *Opsarius* sampled species plus *Barilius* sp. "Thailand" and *Barilius bernatziki* (clade 5; Figs. 6, 7). Given that the phylogenetic placement of the type species of *Opsarius* (*O. tileo*) remains uncertain (it was herein unavailable for DNA sequencing), it is not possible at this time to establish which of these three clades should bear the name *Opsarius*. Notwithstanding, the results of the present study constitute further evidence in support of the notion that the composition and limits of both *Barilius* and *Opsarius* are ill-defined and in need of additional investigation. A phylogenetic analysis with a much larger sampling of putative *Barilius* and *Opsarius* species is therefore needed to lay the foundations for a focused revisionary study aimed at resolving the intricate taxonomy of the group(s).

Like *Opsarius*, the Afro-Asian predatory genus *Raiamas* (the sole chedrin nominal taxon with a transoceanic distribution) was herein resolved as polyphyletic, with Asian members of the genus (i.e., *R. bola* and *R. guttatus*) forming a clade (clade 7; Figs. 6, 7) sister to the remaining—and exclusively African—chedrin radiation. African *Raiamas* were likewise resolved as polyphyletic, with the species *Leptocypris taiaensis* nested within an otherwise exclusively *Raiamas* subclade, and the species *Raiamas salmolucius* nested within an otherwise exclusively *Opsaridium* clade. This result is broadly congruent with previous studies (Tang et al., 2010; Liao et al. 2012), and calls for a complete and in-depth reassessment of *Raiamas* taxonomy.

The history of classification of the genus *Raiamas*, as that of many other chedrin genera, is complex and reflects a problematic taxonomy and unstable nomenclature. The genus was

established for the South Asian species *R. bola* (Jordan, 1919), and further expanded by Howes (1980) to include an additional Asian species (*R. guttatus*) and 15 African species formerly placed in either *Barilius* or *Opsaridium*. Subsequently, however, five of these African species were either synonymized (with other *Raiamas* species) or reassigned to *Leptocypris* (Lévêque and Daget 1984, Lévêque, 1990; Stiassny et al., 2006), while four species were added to the list by either discovery of undescribed diversity or generic reassignment (from *Opsaridium*) (Howes and Teugels 1989; Stiassny et al. 2006). More recently, the molecular phylogeny of Tang et al. (2010), although lacking in sampling of African chedrins, suggested for the first time that *Raiamas*, as currently defined, most likely represented a polyphyletic assemblage. Precisely because of their limited sampling of African taxa, Tang et al. (2010) urged for additional phylogenetic studies with increased coverage of African *Raiamas* in order to draw definite conclusions regarding the limits and composition of the genus. With a taxon sampling that includes 10 of the 16 currently valid *Raiamas* species (Liao et al. 2012), the present study constitutes the most comprehensive test of *Raiamas* monophyly and thus provides a phylogenetic framework necessary to revise the taxonomy of the group.

In order to maintain generic monophyly and a classification that accurately reflects phylogenetic relationship, the genus name *Raiamas* needs to be restricted to the clade consisting of the Asian species *R. bola* and *R. guttatus,* since the former is the type species of the genus (Liao et al., 2012). Howes (1980) had indeed suggested that Asian *Raiamas* were morphologically distinct from their African congeners. Nonetheless, he considered that the osteological characters proposed to distinguish African from Asian *Raiamas* probably reflected a serial trend of cranial elongation rather than a phylogenetic disjunction, and therefore retained a single transoceanic genus. Similarly, the presence of maxillary barbels, another character

proposed by Howes (1980) to distinguish Asian from African *Raiamas*, appears to correspond to the plesiomorphic condition rather than a potential synapomorphy for Asian members of the genus (Cockerell, 1923; Liao et al. 2012). More recently, however, Liao et al. (2012) provided five novel apomorphies that do diagnose Asian *Raiamas* as phylogenetically distinct from the African species. Although Tang et al. (2010) questioned the monophyly of Asian *Raiamas* (resolving its members as a paraphyletic group because of the placement of *Opsarius bakeri* and *O. canarensis*), the results of both Liao et al. (2012) and the present study reveal a sister-group relationship between *R. bola* and *R. guttatus* (although not strongly supported), and therefore the restriction of *Raiamas* to the Asian species of the genus.

The remaining rump *Raiamas*, exclusively African in distribution, was herein found broadly scattered throughout most of the African chedrin clade (Fig. 7). A first portion of African *Raiamas* (i.e., *R. batesii*, *R. nigeriensis*, *R. senegalensis*) was retrieved forming a strongly supported clade (sister to the remaining African chedrin radiation) also containing the species *Leptocypris taiaensis* (clade 8; Figs. 6, 7). Indeed, Stiassny et al. (2006) had previously noted that *R. batesii* and *R. senegalensis* appeared to be distinct (at least morphologically) from other African *Raiamas*. The inferred position of *L. taiaensis*, well nested within this partial *Raiamas* clade, represents phylogenetic evidence for the placement of this species in *Raiamas*. A second portion of African *Raiamas*, represented by the species *R. steindachneri* (clade 9; Figs. 6, 7), was herein resolved as sister to all African chedrins except those in the clade consisting of *L. taiaensis* and a partial *Raiamas* (clade 8; Figs. 6, 7). Nodal support for the placement of *R. steindachneri* is weak, and therefore it is possible that this species is in fact a member of the *R. batesii* clade. A third portion of African *Raiamas* was herein resolved as a strongly supported clade consisting of the species *R. buchholzi*, *R. christyi*, and *R. kheeli* (clade 12; Figs. 6, 7).

Because species in this clade are distinguished from all other chedrins by the shared apomorphic presence of a large precaudal spot (Liao et al. 2012), the monophyly and distinctiveness of this supraspecific assemblage appears to be supported by both molecular and morphological evidence. The fourth and last portion of African *Raiamas* sampled in this study is represented by the species *Raiamas salmolucius*, which was found well nested within an otherwise exclusively *Opsaridium* clade (clade 13; Figs. 6, 7). This finding, also discovered by Liao et al. (2012), constitutes strong evidence for the placement of *R. salmolucius* in *Opsaridium*. Incidentally, the recent reassignment of *Raiamas weeksii* to *Leptocypris* and of *Opsaridium christyi* to *Raiamas* by Stiassny et al. (2006) is supported by the results of the present study.

In addition to the generic reassignments suggested above, a taxonomy conforming to the findings of this study would require establishing new genus names for each of the three distinct partial *Raiamas* clades (i.e., clades 8, 9, 12; Figs. 6, 7). However, since all newly proposed names must be accompanied by diagnoses in order to fulfill the requirement of availability (ICZN: Articles 10-20), and the discovery of anatomical synapomorphies and/or diagnoses for the abovementioned subsets of African *Raiamas* was beyond the scope of this investigation, the genus name *Raiamas* is herein retained pending morphological studies aimed at diagnosing these clades. Future research should also be directed at reassessing the ambiguous generic diagnoses for the morphologically similar *Leptocypris*, *Opsaridium*, and *Raiamas* (Stiassny et al., 2006).

The results presented herein also failed to support the monophyly of the genus *Leptocypris*. In addition to placing *L. taiaensis* in *Raiamas*, the resultant phylogeny entails the placement of *Neobola bottegoi* in *Leptocypris* so as to maintain generic monophyly (clade 10; Figs. 6, 7). It should be noted that the phylogenetic position of *Neobola* as inferred here, nested within an otherwise exclusively *Leptocypris* clade, is surprising. *Neobola* and *Leptocypris* are easily

distinguished from each other by the position of the dorsal-fin origin (anterior to the anal-fin origin in *Leptocypris*), a supposedly phylogenetically conserved character (Liao, pers. comm.). Although the non-monophyly of *Leptocypris* is a novel finding, the sampling of *Leptocypris* diversity of the present study (5 of the 9 valid species, plus one putatively undescribed species) is the most comprehensive of all published phylogenetic analyses of the group, and therefore it provides the strongest test of its monophyly. Discovery of a prominent, dorsally directed, ethmoid process on the palatine head in *Neobola bottegoi* would further support its placement in *Leptocypris*, as this character is considered to represent the sole putative synapomorphy for the genus *Leptocypris* (Stiassny et al. 2006).

On the other hand, the resultant phylogeny failed to recover the "Neoboline" group of Howes (1984: p. 172) (i.e., a clade consisting of the genera *Chelaethiops*, *Neobola*, *Mesobola* and *Rastrineobola*). Instead, *Neobola*, as previously noted, was inferred nested within members of *Leptocypris*, while the remaining neobolines were resolved forming a clade with the morphologically distinct genus *Engraulicypris* (clade 11; Figs. 6, 7). Due to taxon sampling limitations, *Chelaethiops* was the only polytypic neoboline genus tested for monophyly. This study, which sampled three of the five valid species in the genus, strongly supports the monophyletic status of *Chelaethiops* (Fig. 6).

3.3. BIOGEOGRAPHIC CONSIDERATIONS

The monophyly and derived phylogenetic placement of African members of the Chedrini suggests that the diversification of the tribe started in Asian freshwaters and that the lineage leading to the African chedrin radiation must have dispersed from Asia into the African continent, unless chedrins had already diversified by the time when the fragmentation of East

Gondwana led to the split between modern-day Somalia and the Indian subcontinent, a palaeogeographic process possibly initiating as early as *ca.* 160 Ma according to recent tectonic models (Zahirovic et al. 2012; Gibbons et al., 2013). While divergence time estimates for deeper nodes in the phylogeny of ostariophysian fishes (Near et al. 2012; Broughton et al., 2013) suggest that it is very unlikely that chedrins would have already diversified by the late Jurassic, a focused time-scaled phylogeny of the Chedrini would provide a more direct and conclusive means of testing such biogeographic scenario. If the origins and diversification of chedrins were corroborated as younger than the fragmentation of East Gondwana, then current biogeographic patterns would have to be explained by westward dispersal from Asia, coupled with extinction of select chedrin lineages. In fact, the distribution gap at the Arabian Peninsula has been hypothesized to be a result from a recent desertification in the region (Otero, 2001; Liao et al. 2011b).

Beside danionins, the only other cyprinid subfamily with an Afro-Asian disjunct distribution is the Cyprininae, for which Durand et al. (2002) suggested a similar dispersal pattern as the one proposed herein for chedrins, with the Middle East as an interchange area rather than a center of origin. Likewise, a westward intercontinental dispersal model has been proposed for the cyprinid genus *Garra* (Colli et al., 2009), the tribe Labeonini (Tang et al., 2009), and the catfish family Clariidae (Agnese and Teugels, 2005), all fish clades with a geographic distribution similar to that of chedrins.

4. TABLES

Table 1. Chedrin genera currently recognized as valid according to Tang et al. (2010) and Liao et al. (2011a), their continent-level distribution, number of valid species, and number of sampled species in the present study.

Genus	Continent	Valid Species*	Sampled Species
Barilius Hamilton 1822	Asia	30[a]	6[b, c]
Bengala Gray 1833	Asia	1	0
Cabdio[1] Hamilton 1822	Asia	2	1
Chelaethiops Boulenger 1899	Africa	5	3
Engraulicypris Günther 1894	Africa	1	1
Esomus[3] Swainson 1839	Asia	12	0
Leptocypris Boulenger 1900	Africa	9	7[c]
Luciosoma Bleeker 1855	Asia	5	3
Malayochela Bănărescu 1968	Asia	1	1
Mesobola Howes 1984	Africa	3	1
Nematabramis Boulenger 1894	Asia	4	1
Neobola Vinciguerra 1895	Africa	4	1
Opsaridium Peters 1854	Africa	13	9[c]
Opsarius McClelland 1839	Asia	5[a]	9[b, c]
Raiamas Jordan 1919	Africa, Asia	14	10
Rastrineobola Fowler 1936	Africa	1	1
Salmostoma[2] Swainson 1839	Asia	13	3[c]
Securicula Günther 1868	Asia	1	1
Total		**124**	**58**

*According to Eschmeyer (2013)
[1]Senior synonym of *Aspidoparia*
[2]Senior synonym of *Salmophasia*
[3]Although its phylogenetic placement in the phylogeny of the Danioninae is contentious, *Esomus* is herein treated as a member of the Chedrini following Liao et al. (2011a).
[a]*sensu lato*
[b]*sensu stricto*
[c]Including undescribed or questionably nominal species

Table 2. Chedrin genera sampled for phylogenetic analysis in previous systematic studies of the Danioninae.

Study	Chedrin genera sampled for phylogenetic analyses
Mayden et al. (2007)	*Opsaridium*
Rüber et al. (2007)	*Chela* [=*Malayochela*], *Luciosoma*, and *Nematabramis*
Conway et al. (2008)	*Barilius* [=*Opsarius*], *Luciosoma*, and *Opsaridium*
Mayden et al. (2008)	*Aspidoparia* [=*Cabdio*], *Barilius* [=*Opsarius*], *Luciosoma,* and *Opsaridium*
Britz et al. (2009)	*Chela* [=*Malayochela*], *Luciosoma*, and *Nematabramis*

Table 3. Primers and PCR profiles for amplification of *cyt-b* and *rag1*.

Gene	Source	Primer Sequence[a]	PCR Thermal Profile[b]
cyt-b	This study	GCCTACGRAAAACMCACCCGC[*] AGCTCATTCAAGYGCTTTRTTTTCT[**]	(95°C/60s, 53°C/60s, 72°C/120s) x 35
rag1	This study	CCAACCCCTGCACACTCTACGT[*] TCAAACGTTTTGGACTGCCTTGCATT[**]	(95°C/60s, 58°C/60s, 72°C/120s) x 35

[a]Listed from 5' to 3'.
[b]Conditions for denaturation, annealing and extension steps for each cycle are listed in parenthesis, followed by the number of cycles. All reactions included a 5-minute initial denaturation at 95°C and a 10-minute final extension at 72°C.
[*]Forward primer
[**]Reverse primer

Table 4. Taxa, voucher catalog and tissue numbers*, and GenBank accession numbers for the gene sequences generated and analyzed in this study.

Species	Voucher	Tissue/Source	Locality (Country, River/Region)	GenBank Accession Number					
				co1	*cyt-b*	*myh6*	*sh3px3*	*rag1*	*rho*
Danio rerio (outgroup)	N/A	GenBank	N/A	AC024175	AC024175	TBA	TBA	U71093	L11014
Barilius bernatziki	NRM 52526	uncataloged	Unknown (aquarium specimen)	TBA	N/A	TBA	TBA	TBA	TBA
Barilius cf. *barila*	CBM-ZF-11313	GenBank	Unknown (aquarium specimen)	HM224138	HM224257	N/A	N/A	HM224020	HM223900
Barilius mesopotamicus	NRM 60683	uncataloged	Turkey, Tigris	TBA	TBA	TBA	TBA	N/A	N/A
Barilius mesopotamicus	uncataloged	uncataloged	Turkey, Tigris	TBA	TBA	TBA	TBA	TBA	TBA
Barilius mesopotamicus	uncataloged	uncataloged	Turkey, Tigris	TBA	TBA	TBA	TBA	TBA	TBA
Barilius sp. "Bangladesh"	UAIC uncataloged	GenBank	Bangladesh	HM224139	HM224258	N/A	N/A	HM224021	N/A
Barilius sp. "Thailand"	NRM 55248	uncataloged	Thailand, Chao Phraya	TBA	N/A	TBA	TBA	N/A	TBA
Barilius vagra	UAIC 14179.07	GenBank	Unknown (aquarium specimen)	HM224140	HM224259	N/A	N/A	HM224022	HM223901
Cabdio morar	CBM-ZF-11391	GenBank	Unknown (aquarium specimen)	AP011335	AP011335	N/A	N/A	EU711105	FJ531343
Cabdio morar	NRM 57195	uncataloged	Myanmar (aquarium specimen)	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops bibie	SUD-119	62-6118	Sudan	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops bibie	SUD-07/040	62-6129	Sudan	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops bibie	SUD-07/038	62-6131	Sudan	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops bibie	BMNH 2006.3.7.2	GenBank	Sudan	HM224141	HM224260	N/A	N/A	HM224023	HM223902
Chelaethiops bibie	BMNH 2006.3.7.3	GenBank	Burkina Faso	HM224142	HM224261	N/A	N/A	HM224024	N/A
Chelaethiops congicus	AMNH 251146	75-7474	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops congicus	AMNH 251116	74-7396	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops congicus	AMNH 250566	72-7111	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops congicus	AMNH 250727	72-7128	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops congicus	AMNH 255284	72-7141	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops congicus	AMNH 255284	72-7142	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops elongatus	AMNH C09-348	68-6797	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops elongatus	AMNH 247817	55-5489	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Chelaethiops elongatus	AMNH 241005	31-3021	DRC, Congo	TBA	TBA	TBA	TBA	N/A	TBA
Chelaethiops elongatus	Uli Kis 2008-052	62-6138	Unknown	TBA	TBA	TBA	TBA	TBA	TBA

Engraulicypris sardella	AMNH 248841	57-5628	Lake Malawi	TBA	TBA	TBA	TBA	TBA	TBA
Engraulicypris sardella	AMNH 248841	57-5629	Lake Malawi	TBA	TBA	TBA	TBA	TBA	TBA
Engraulicypris sardella	AMNH 248841	57-5630	Lake Malawi	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris lujae	AMNH 240473	24-2361	DRC, Congo (at Pool Malebo)	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris lujae	AMNH 256400	107-10681	DRC, Kwilu	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris lujae	AMNH 252663	80-7993	DRC, Kasai	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris lujae	AMNH 247312	46-4567	DRC, Congo	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris modestus	AMNH 240469	24-2363	DRC, Congo (at Pool Malebo)	TBA	TBA	TBA	TBA	TBA	N/A
Leptocypris modestus	AMNH 250551	68-6778	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris modestus	AMNH 251744	AMCC 196815	DRC, Congo	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris cf. modestus	AMNH 252481	AMCC 197373	DRC, Kwilu	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris niloticus	ZSM 07-264	61-6063	Sudan	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris niloticus	ZSM 07-268	61-6064	Sudan	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris niloticus	CBM-ZF-11419	GenBank	Unknown (aquarium specimen)	AP011428	AP011428	N/A	N/A	HM224052	HM223933
Leptocypris niloticus	BMNH 2006.3.7.5	GenBank	Burkina Faso	HM224175	HM224294	N/A	N/A	HM224051	N/A
Leptocypris niloticus	UAIC uncataloged	GenBank	Ethiopia	HM224174	HM224293	N/A	N/A	HM224050	HM223932
Leptocypris sp."Kwilu"	AMNH 252482	AMCC 197353	DRC, Kwilu	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris sp."Kwilu"	AMNH 252482	AMCC 197363	DRC, Kwilu	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris taiaensis	AMNH 259413	AMCC 213023	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris taiaensis	AMNH 259413	AMCC 213024	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris weeksii	AMNH 240471	24-2364	DRC, Congo (at Pool Malebo)	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris weeksii	AMNH 250552	68-6779	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris weeksii	AMNH 251750	AMCC 196738	DRC, Congo	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris weeksii	AMNH 254903	96-9511	DRC, Mayi Ndombe	TBA	TBA	TBA	TBA	TBA	TBA
Leptocypris weynsii	AMNH 252664	081-8006	DRC, Kasai	N/A	TBA	TBA	TBA	TBA	TBA
Luciosoma bleekeri	CBM-ZF-11202	GenBank	Cambodia, Kandal	AP011399	AP011399	N/A	N/A	HM224053	HM223934
Luciosoma pellegrinii	uncataloged	uncataloged	Unknown	TBA	TBA	TBA	TBA	TBA	TBA
Luciosoma setigerum	CBM-ZF-11273	GenBank	Unknown (aquarium specimen)	AP011423	AP011423	N/A	N/A	EU292704	FJ531352
Malayochela maassi	NRM 50167	GenBank	N/A	FJ753486	EF151098	N/A	N/A	FJ753522	EU241379
Malayochela maassi	NRM 50167	uncataloged	Unknown (aquarium specimen)	TBA	TBA	TBA	TBA	TBA	TBA
Mesobola brevianalis	SAIAB 66270	GenBank	Swaziland, Lubombo	HM224176	HM224295	N/A	N/A	HM224054	HM223935

Nematabramis steindachneri	BMNH uncataloged	GenBank	N/A	FJ753496	EF151106	N/A	N/A	FJ753532	N/A
Neobola bottegoi	uncataloged	GenBank	Ethiopia, Sheikh Hussein	HM224178	HM224296	N/A	N/A	HM224056	HM223936
Neobola bottegoi	CTOL02623	78-7745	Unknown	TBA	N/A	TBA	TBA	TBA	TBA
Opsaridium boweni	AMNH 243665	38-3735	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium boweni	AMNH 247413	54-5383	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium boweni	AMNH 253471	80-7970	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium boweni	AMNH 251140	74-7383	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium microcephalum	AMNH 256988	112-11161	Lake Malawi	TBA	TBA	TBA	TBA	N/A	N/A
Opsaridium microlepis	AMNH 256989	112-11162	Lake Malawi	TBA	N/A	TBA	TBA	TBA	TBA
Opsaridium peringueyi	SAIAB 66263	GenBank	Swaziland, Manzini	HM224192	HM224311	TBA	N/A	HM224072	HM223954
Opsaridium sp. "Boumba"	ZSM 39044	73-7287	Cameroon, Boumba	N/A	N/A	TBA	TBA	TBA	TBA
Opsaridium sp. "Itimbiri"	ZSM 39378	74-7314	DRC, Itimbiri	TBA	N/A	TBA	TBA	TBA	TBA
Opsaridium sp. "Kasai"	AMNH 253198	079-7872	DRC, Kasai	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium sp. "Kasai"	AMNH 253198	79-7870	DRC, Kasai	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium sp. "Kasai"	AMNH 253198	79-7871	DRC, Kasai	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium sp. "Kasai"	AMNH 253299	079-7851	DRC, Kasai	TBA	TBA	TBA	TBA	N/A	N/A
Opsaridium sp. "Kasai"	AMNH 253299	079-7859	DRC, Kasai	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium ubangiense	AMNH 253926	88-8745	DRC, Kouilou-Niari	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium ubangiense	AMNH 253894	uncataloged	Republic of the Congo	TBA	N/A	TBA	TBA	TBA	TBA
Opsaridium ubangiense	AMNH 253894	uncataloged	Republic of the Congo	TBA	N/A	TBA	TBA	TBA	TBA
Opsaridium ubangiense	AMNH 249521	63-6205	Cameroon, Bitande	TBA	N/A	TBA	TBA	TBA	TBA
Opsaridium ubangiense	AMNH 253895	088-8709	Republic of the Congo, Passi Passi	TBA	TBA	TBA	TBA	TBA	TBA
Opsaridium ubangiense	N/A	GenBank	Gabon	HM224193	HM224312	N/A	N/A	HM224073	HM223956
Opsaridium zambezense	SAIAB 66692	GenBank	Botswana, Ngamiland	HM224194	N/A	TBA	N/A	N/A	N/A
Opsarius bakeri	CBM-ZF-11327	GenBank	Unknown (aquarium specimen)	HM224197	HM224315	N/A	N/A	HM224076	HM223959
Opsarius barnoides	CAS 222964	GenBank	China, Yunnan	HM224199	HM224317	N/A	N/A	HM224078	HM223961
Opsarius barnoides	CAS uncataloged	GenBank	Myanmar	HM224198	HM224316	N/A	N/A	HM224077	HM223960
Opsarius barnoides	NRM 58418	uncataloged	Myanmar, Mekong	TBA	N/A	TBA	TBA	TBA	TBA
Opsarius bendelisis	CBM-ZF-11272	GenBank	Unknown (aquarium specimen)	AP011433	AP011433	N/A	N/A	EU292693	FJ531346
Opsarius canarensis	CBM-ZF-11287	GenBank	Unknown (aquarium specimen)	HM224200	HM224318	N/A	N/A	HM224079	HM223962
Opsarius caudiocellatus	CAS 223036	GenBank	China, Yunnan	HM224201	HM224319	N/A	N/A	HM224080	HM223963

Opsarius koratensis	UAIC 14182.11	GenBank	Thailand, Nan	HM224204	N/A	N/A	N/A	HM224083	HM223966
Opsarius koratensis	CBM-ZF-11529	GenBank	Thailand, Chiang Mai	HM224205	HM224322	N/A	N/A	N/A	HM223967
Opsarius pulchellus	UAIC 14180.60	GenBank	Unknown (aquarium specimen)	HM224206	HM224323	N/A	N/A	HM224084	HM223968
Opsarius pulchellus	CBM-ZF-11532	GenBank	Thailand, Mae Kachan	HM224207	HM224324	N/A	N/A	HM224085	HM223969
Opsarius cf. *shacra*	CBM-ZF-11297	GenBank	Unknown (aquarium specimen)	HM224203	HM224321	N/A	N/A	HM224082	HM223965
Opsarius sp. "Myanmar"	NRM 58599	uncataloged	Myanmar, Salween	TBA	TBA	TBA	TBA	TBA	TBA
Opsarius sp. "Myanmar"	NRM 59451	uncataloged	Myanmar, Chindwin	TBA	N/A	TBA	TBA	TBA	TBA
Opsarius sp. "Myanmar"	NRM 59302	uncataloged	Myanmar, Chindwin	TBA	N/A	TBA	TBA	TBA	TBA
Opsarius sp. "Myanmar"	BMNH uncataloged	GenBank	Myanmar	HM224208	HM224325	N/A	N/A	HM224086	HM223970
Raiamas batesii	AMNH 249530	63-6226	Cameroon, Nyong	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas batesii	AMNH 249530	63-6227	Cameroon, Nyong	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas batesii	AMNH 249530	63-6228	Cameroon, Nyong	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas bola	CBM-ZF-11680	GenBank	Nepal, Sapta Koshi	HM224212	HM224329	N/A	N/A	HM224089	HM223974
Raiamas buchholzi	AMNH 249520	63-6202	Cameroon, Bitande	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas buchholzi	AMNH 249520	63-6203	Cameroon, Bitande	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas buchholzi	AMNH 249520	63-6204	Cameroon, Bitande	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas buchholzi	N/A	GenBank	Gabon, Makokou	HM224213	HM224330	N/A	N/A	HM224090	HM223975
Raiamas christyi	AMNH 240453	21-2066	DRC, Congo	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	AMNH 249771	65-6406	DRC, Lomako	N/A	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	AMNH 251817	AMCC 196965	DRC, Ngampoku	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	C10-47	AMCC 196755	DRC, Ngampoku	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	C10-44	AMCC 196752	DRC, Ngampoku	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	ZSM 39483	74-7318	DRC, Itimbiri	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	ZSM 37546	74-7321	DRC, Tshopo	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	AMNH 250553	72-7106	DRC, Nsele	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	AMNH 249788	65-6426	DRC, Lomako	N/A	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	AMNH 246955	42-4200	DRC, Congo	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas christyi	AMNH 240465	24-2365	DRC, Congo (at Pool Malebo)	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas cf. *christyi*	ZSM 37685	73-7295	DRC, Kwango	N/A	TBA	TBA	TBA	TBA	TBA
Raiamas cf. *christyi*	ZSM 37818	73-7296	DRC, Kwango	N/A	TBA	TBA	TBA	TBA	TBA
Raiamas cf. *christyi*	ZSM 39439	74-7312	DRC, Itimbiri	TBA	TBA	TBA	TBA	TBA	TBA

Raiamas guttatus	CBM-ZF-11566	GenBank	Myanmar	HM224214	HM224331	N/A	N/A	HM224091	HM223976
Raiamas guttatus	CBM-ZF-11363	GenBank	Unknown (aquarium specimen)	AP011222	AP011222	N/A	N/A	HM224092	HM223977
Raiamas guttatus	NRM 59315	uncataloged	Myanmar, Irrawaddy River	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas guttatus	NRM 44785	uncataloged	Unknown (aquarium specimen)	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas guttatus	BMNH uncataloged	GenBank	Myanmar, Gyobingauk	HM224217	HM224334	N/A	N/A	HM224095	HM223980
Raiamas kheeli	AMNH 246577	40-3979	DRC, Inkisi	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas kheeli	AMNH 246577	40-3982	DRC, Inkisi	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas kheeli	AMNH 246577	40-3984	DRC, Inkisi	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas kheeli	AMNH 247101	40-3990	DRC, Inkisi	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas nigeriensis	AMNH 256972	109-10878	Guinea, Niger	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas nigeriensis	AMNH 256972	109-10879	Guinea, Niger	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas salmolucius	AMNH 251113	74-7391	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas salmolucius	AMNH 251147	75-7476	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas salmolucius	AMNH 251213	76-7538	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas salmolucius	AMNH 251316	76-7551	DRC, Lulua	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas salmolucius	AMNH 247956	33-3293	DRC, Kwilu	TBA	N/A	TBA	TBA	TBA	TBA
Raiamas cf. *salmolucius*	AMNH 246467	41-4003	DRC, Ngongo	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas senegalensis	AMNH 254144	091-9065	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas senegalensis	BMNH 2006.3.7.4	GenBank	Burkina Faso, Ouessa	HM224215	HM224332	N/A	N/A	HM224093	HM223978
Raiamas senegalensis	UAIC 14302.02	GenBank	Guinea, Kouroussa	HM224216	HM224333	N/A	N/A	HM224094	HM223979
Raiamas senegalensis	uncataloged	uncataloged	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas senegalensis	AMNH 257745	110-10961	Guinea, Niger	TBA	TBA	TBA	TBA	N/A	N/A
Raiamas steindachneri	AMNH 257064	113-11256	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas steindachneri	AMNH 257122	113-11278	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas steindachneri	AMNH 257114	113-11286	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Raiamas steindachneri	AMNH 257127	113-11298	Guinea	TBA	TBA	TBA	TBA	TBA	TBA
Rastrineobola argentea	AMNH 256386	57-5636	Tanzania, Lake Victoria	TBA	TBA	TBA	TBA	TBA	TBA
Rastrineobola argentea	AMNH 256386	57-5637	Tanzania, Lake Victoria	TBA	TBA	TBA	TBA	TBA	TBA
Rastrineobola argentea	AMNH 256386	57-5638	Tanzania, Lake Victoria	TBA	TBA	TBA	TBA	TBA	TBA
Salmostoma bacaila	UAIC uncataloged	GenBank	Bangladesh, Sylhet	HM224244	HM224375	N/A	N/A	HM224129	HM224011
Salmostoma bacaila	CBM-ZF-11516	GenBank	India, West Bengal	AP011223	AP011223	N/A	N/A	HM224128	HM224010

Salmostoma bacaila	UAIC uncataloged	GenBank	Nepal	HM224245	HM224376	N/A	N/A	HM224130	HM224012
Salmostoma phulo	CBM-ZF-11673	GenBank	India, West Bengal	HM224248	HM224379	N/A	N/A	HM224133	HM224013
Salmostoma sp. "Myanmar"	NRM 59342	uncataloged	Myanmar, Irrawaddy	TBA	N/A	TBA	TBA	TBA	TBA
Salmostoma sp. "Myanmar"	BMNH uncataloged	GenBank	Myanmar, Pyay	HM224249	HM224380	N/A	N/A	HM224134	HM224014
Securicula gora	UAIC uncataloged	GenBank	Bangladesh, Kongsho	HM224250	HM224381	N/A	N/A	HM224135	HM224015
Securicula gora	UAIC uncataloged	GenBank	Bangladesh, Kongsho	HM224251	HM224382	N/A	N/A	N/A	HM224016

*Institutional abbreviations: AMNH, American Museum of Natural History; BMNH, British Museum (Natural History); CAS, California Academy of Sciences; CBM-ZF, Natural History Museum and Institute, Chiba; NRM, Naturhistoriska Riksmuseet; SAIAB, South African Institute for Aquatic Biodiversity; STL, Saint Louis University; UAIC, University of Alabama Ichthyological Collection. Tissue samples are deposited and cataloged in the Ichthyology collection of the AMNH unless otherwise noted.

5. FIGURES

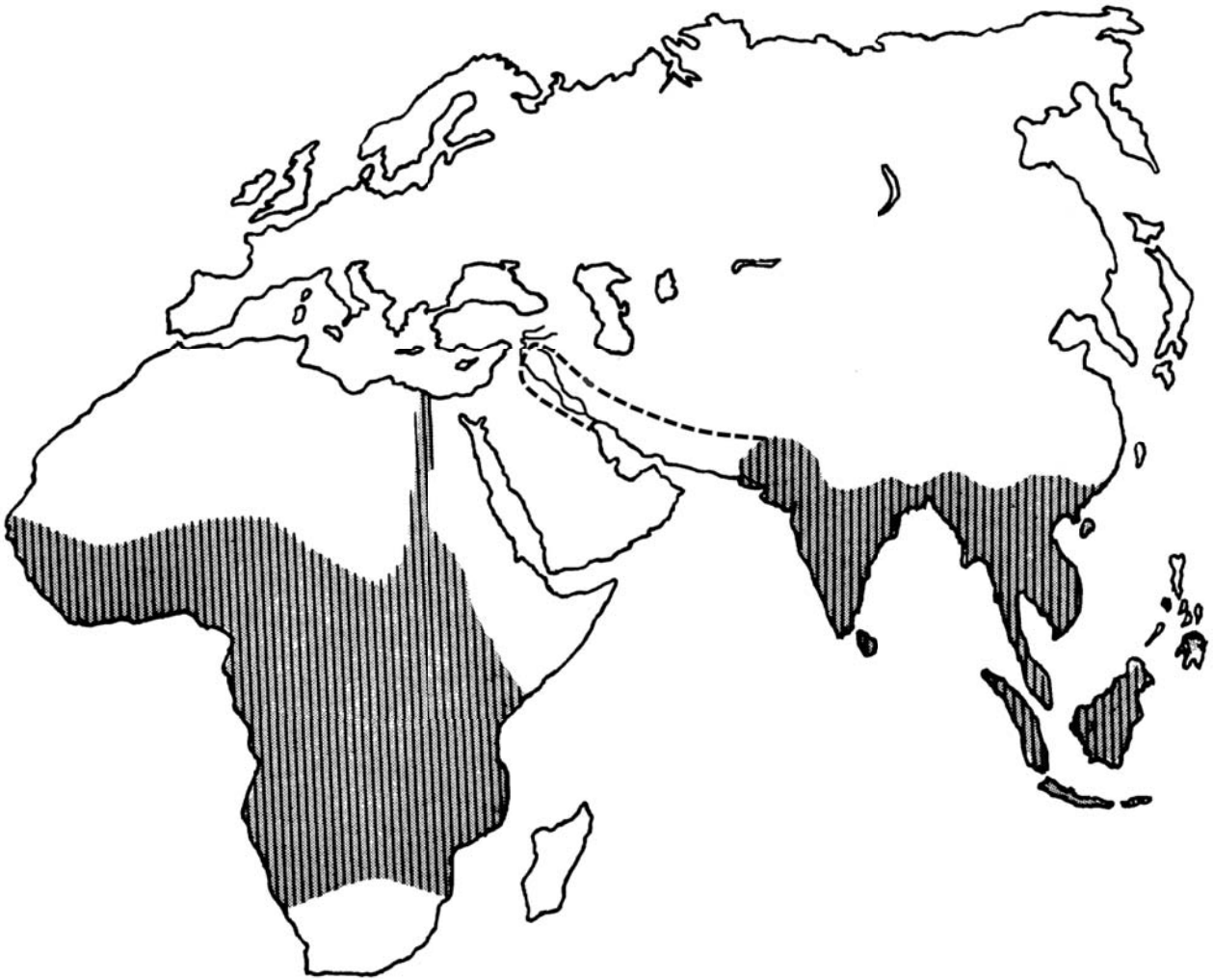

Figure 1. Geographic distribution of the tribe Chedrini (modified from Liao et al., 2011b). Shaded areas indicate regions where most chedrin genera are currently distributed, whereas the area delimited by the dashed line indicates occurrence of only two chedrin species: *Barilius mesopotamicus* (endemic to the Middle East) and *Cabdio morar* (also distributed from India to South East Asia). Inclusion of *B. mesopotamicus* in the Chedrini (Liao et al., 2011b) indeed resulted in a considerable westward expansion in the distribution of Asian chedrins from that originally proposed by Howes (1991).

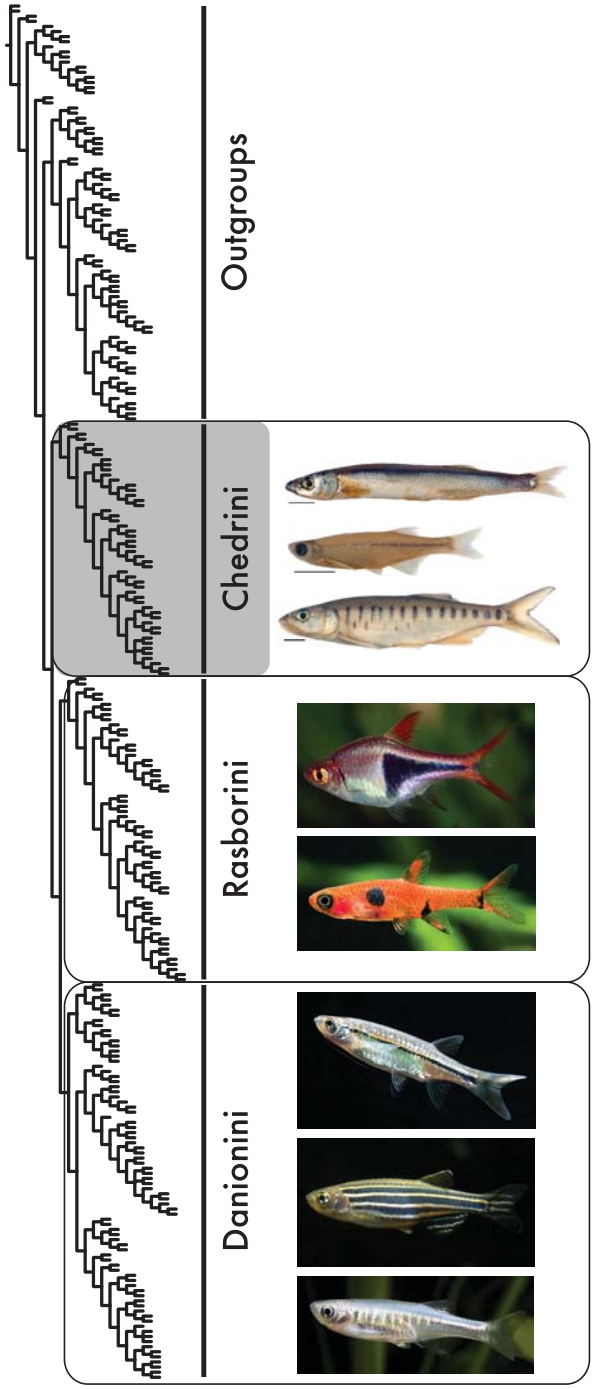

Figure 2. Relationships among the three major suprageneric clades of the Danioninae (assigned to tribal taxonomic rank) according to the most comprehensive molecular phylogeny of the subfamily (modified from Tang et al., 2010).

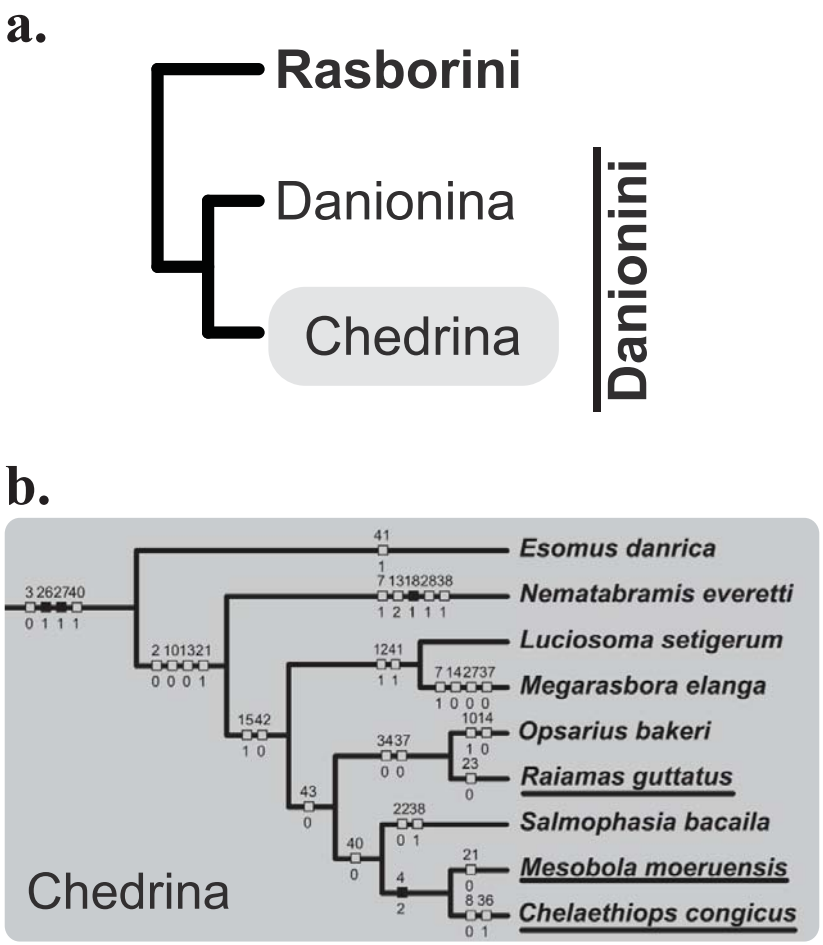

Figure 3. Relationships among the three major suprageneric clades of the Danioninae (a) and within the subtribe Chedrina (b) according to the most recent morphology-based phylogeny of the subfamily (modified from Liao et al., 2011a). Morphological synapomorphies supporting the monophyly of chedrins: (1) postcleithrum greatly reduced or absent [character 26 (state 1)], (2) postcleithrum approximately normal to abdominal ribs [character 27 (state 1)], (3) Baudelot's ligament extending to the supracleithrum [character 3 (state 0)] and (4) presence of rostral barbels [character 40 (state 1)]. The former two are unambiguous synapomorphies.

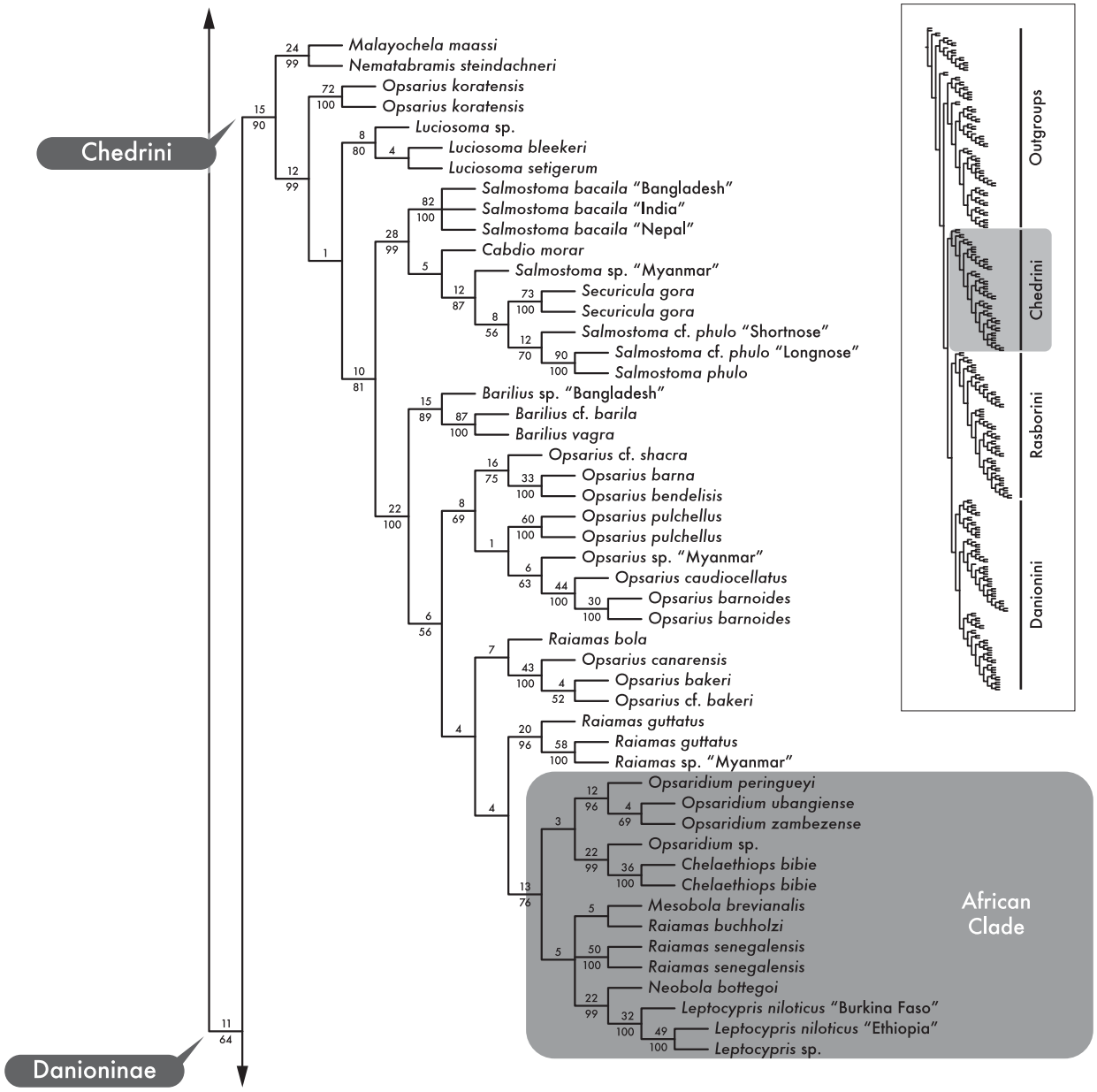

Figure 4. Phylogeny of the Chedrini highlighting the clade formed by African members of the tribe (reprinted from Tang et al., 2010).

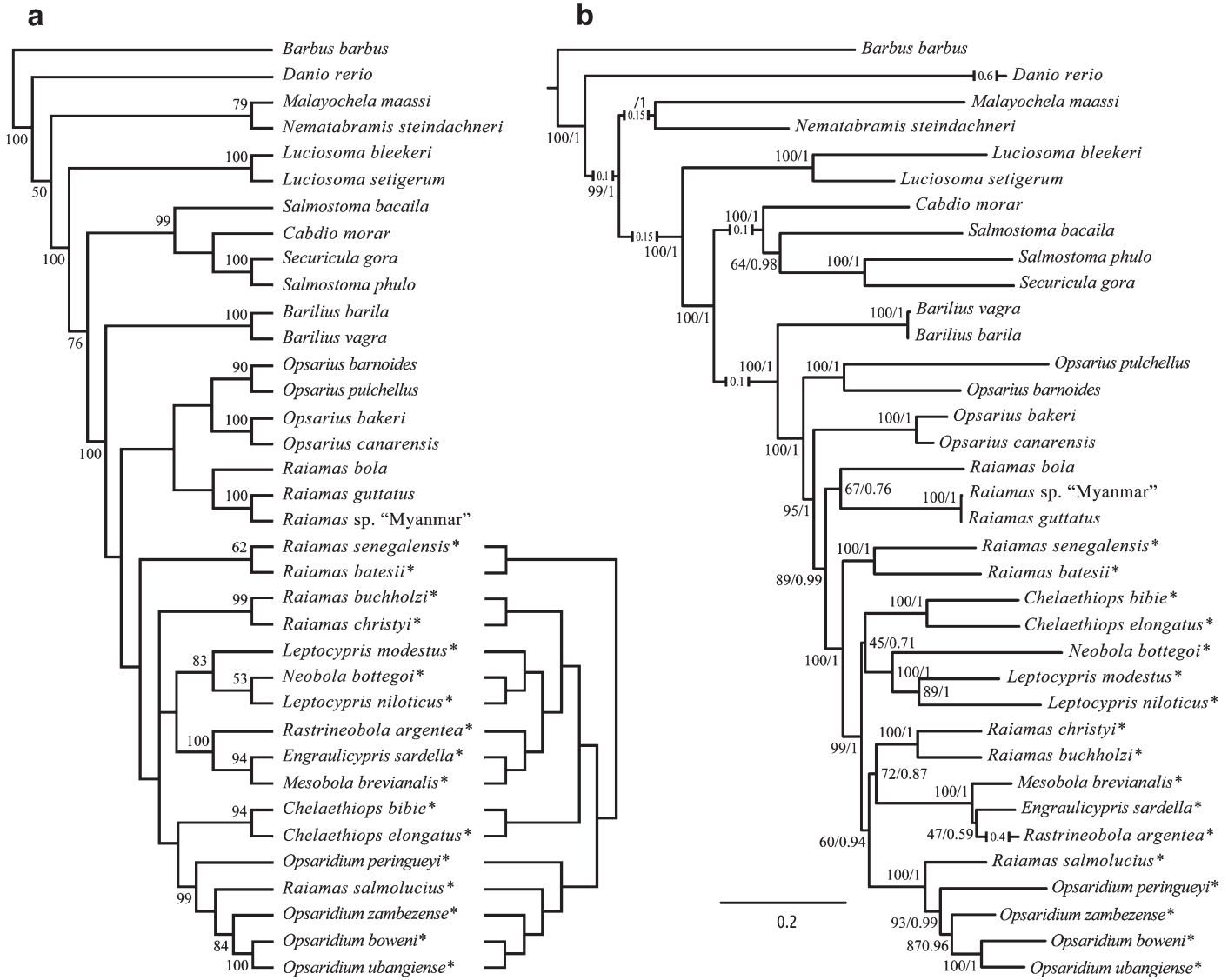

Figure 5. Phylogeny of the Chedrini (reprinted from Liao et al., 2012) as inferred using parsimony (a), and model-based methods (b).

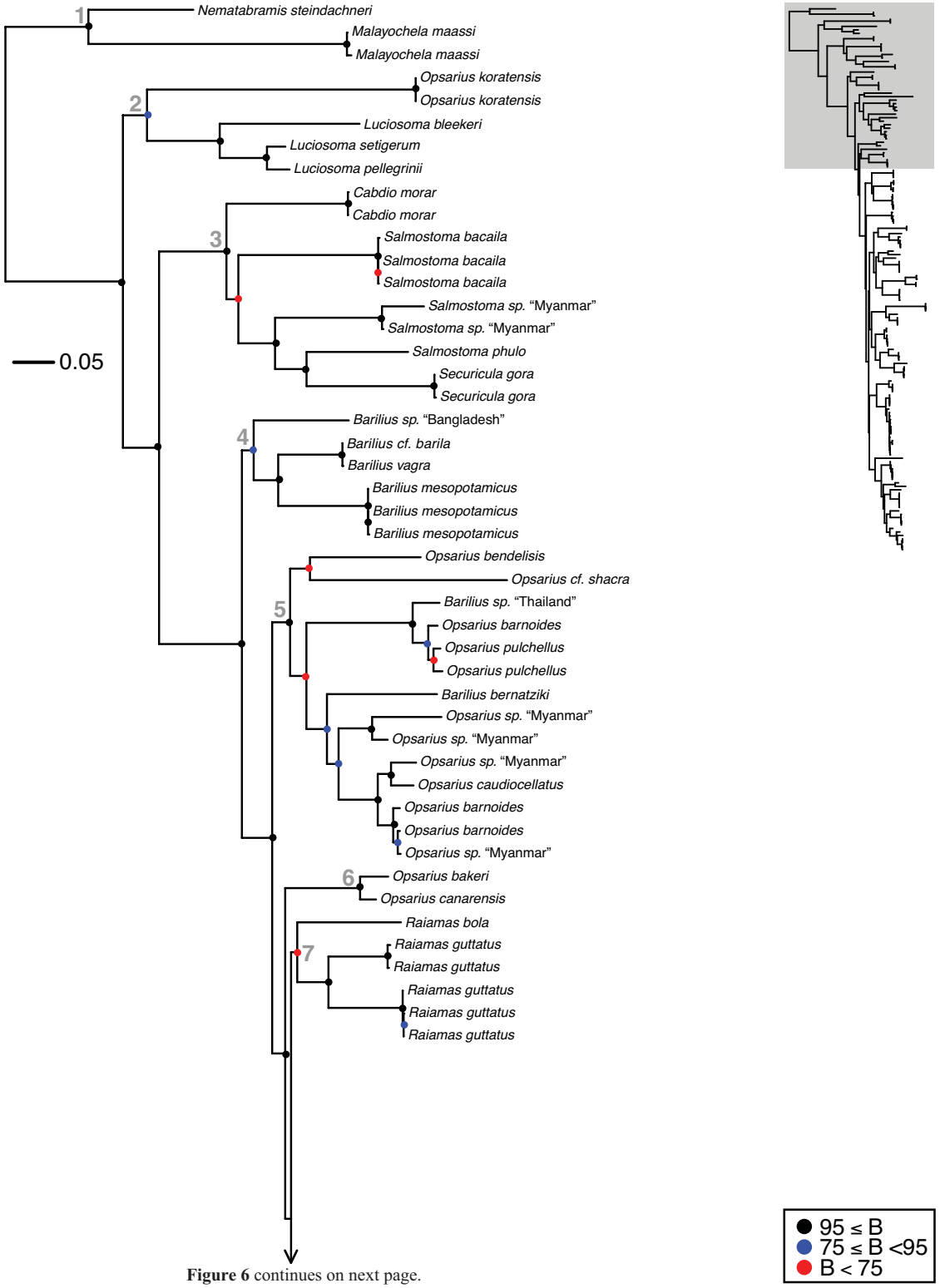

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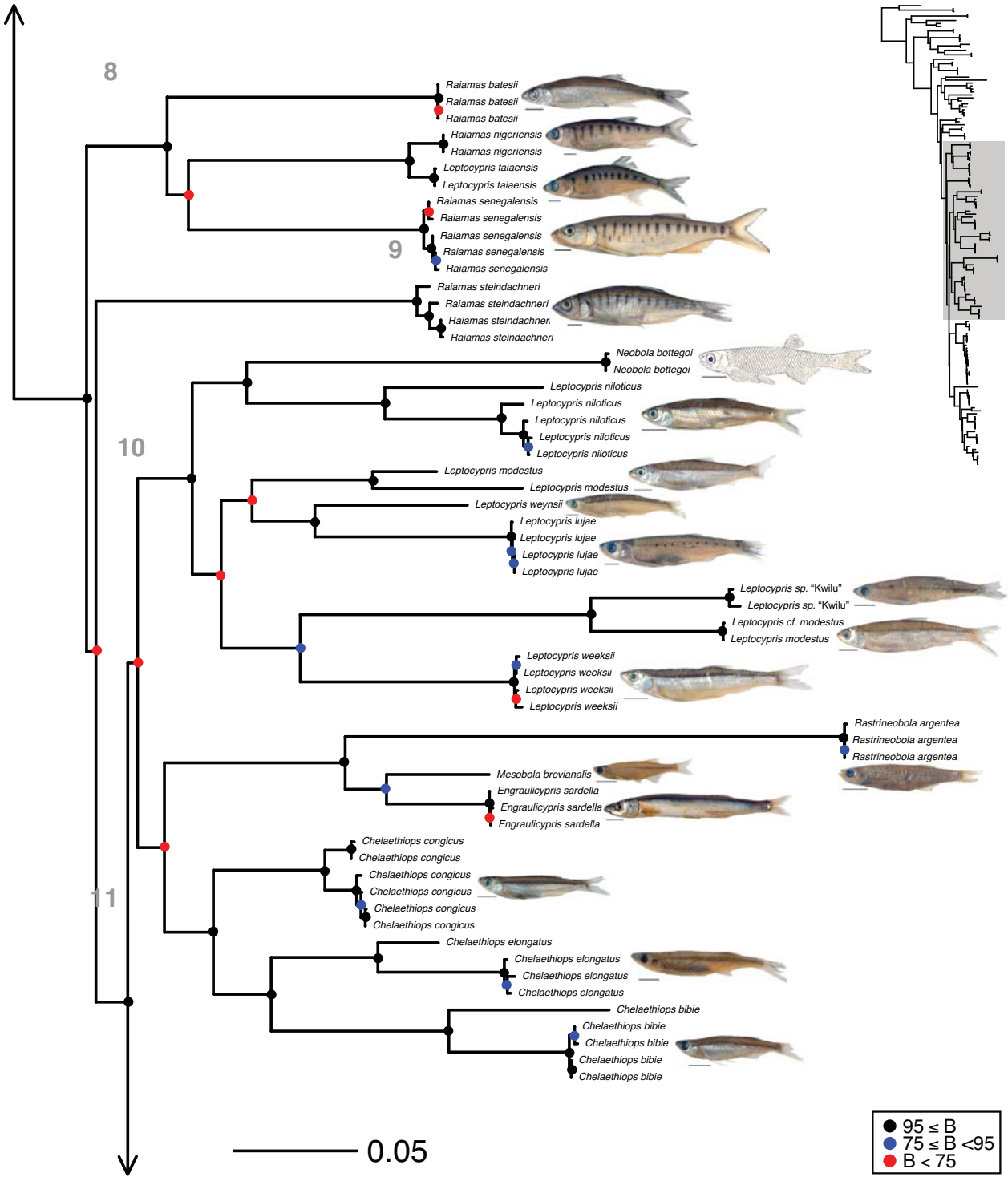
Figure 6. Phylogeny of the Chedrini inferred from the molecular dataset of this study using Maximum Likelihood. Colored circles on nodes indicate degree of support as determined by bootstrap values (B).

Figure 6 continued.



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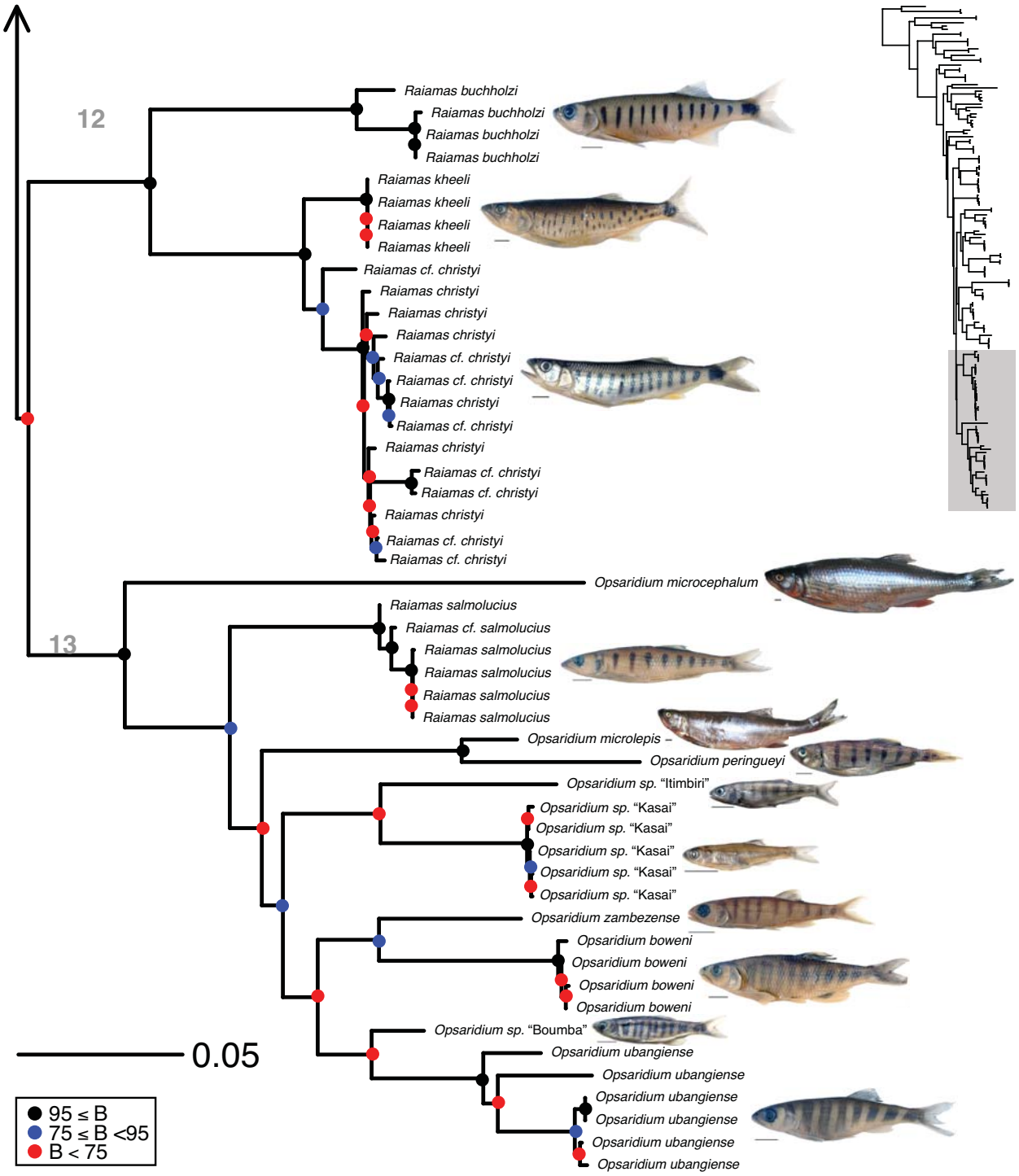

Figure 6 continued.

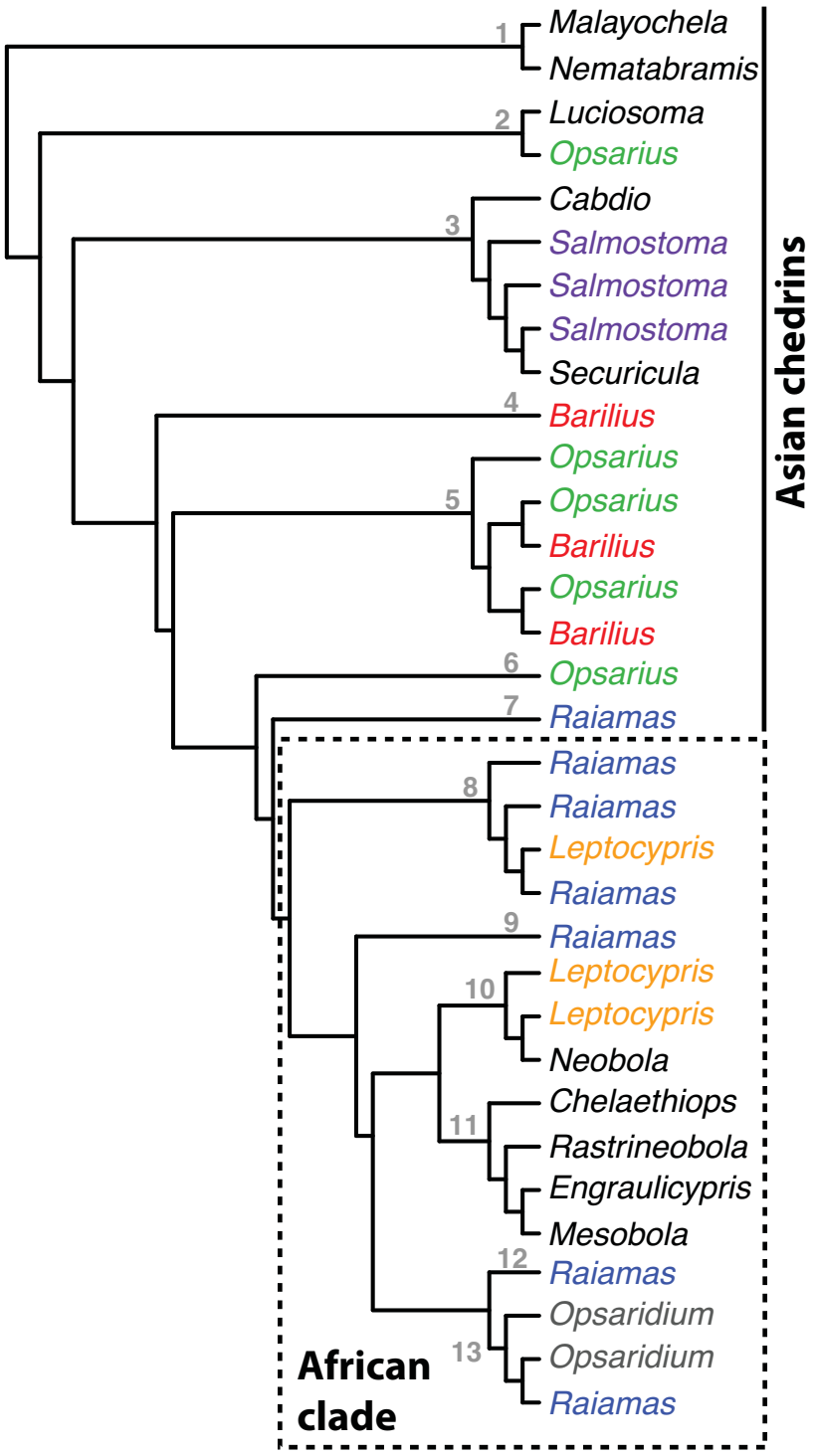

Figure 7. Chedrin intergeneric relationships as implied by the phylogeny presented in Fig. 6. Taxa names in color correspond to non-monophyletic genera. Numbers are used to indicate major generic and suprageneric clades discussed in the text.

CHAPTER 5

SYSTEMATICS OF NEOTROPICAL CATFISHES OF THE SUBFAMILY LORICARIINAE (SILURIFORMES: LORICARIIDAE) BASED ON PHYLOGENETIC ANALYSIS OF DNA SEQUENCE DATA

1. INTRODUCTION

Neotropical freshwaters harbor one of the most diverse yet poorly known ichthyofaunas in the World (Vari and Malabarba, 1998; Reis et al., 2003). Not surprisingly, very little is known about the evolutionary processes responsible for the production and maintenance of such staggering diversity. Catfishes (order Siluriformes), with more than 1600 species occurring in the freshwaters of Central and South America (Ferraris and Reis, 2005), constitute a major component of the megadiverse Neotropical ichthyofauna. Despite recent initiatives for a complete inventory of global catfish diversity (Sabaj et al., 2003-2006), the alpha-taxonomy and evolutionary relationships among catfishes remain poorly understood.

The family Loricariidae (armoured suckermouth catfishes), a strictly Neotropical clade containing over 850 species classified into six subfamilies (Eschmeyer and Fong, 2013), is by far the most speciose of all catfish families, and one of the most diverse and ubiquitous components of the Neotropical ichthyofauna (Schaefer, 1987; Schaefer, 1998; Armbruster, 2004; Reis et al., 2006; Ferraris, 2007, Schaefer and Provenzano, 2008). Nearly 200 species of loricariids have been described in the past 10 years (Eschmeyer and Fong, 2013), and it is expected that numerous more will continue to be described with increased exploration of freshwater ecosystems in Neotropical regions (Lundberg et al., 2000). The tremendous taxonomic, morphological, ecological, and geographic diversity exhibited by loricariid catfishes has undoubtedly challenged Neotropical ichthyologists and, to some extent, hampered efforts to produce robust phylogenetic hypotheses and their ensuing classification schemes.

With 234 currently valid species (Eschmeyer and Fong, 2013; Vera-Alcaraz et al. 2012) arrayed in 32 genera (Covain and Fisch-Muller, 2007; Covain et al., 2008; Fichberg and Chamon, 2008; Rodriguez et al. 2011; Covain et al., 2012) (Table 1), the Loricariinae constitutes

the second-most diverse subfamilial clade of loricariid catfishes, surpassed only by the Hypostominae. Besides the typical loricariid features (i.e., bony dermal plates covering most of the body, specialized mouth with lips modified into a sucking disc), members of the Loricariinae can be recognized by a long and depressed caudal peduncle and the absence of an adipose fin. Furthermore, loricariins exhibit noteworthy variation in body shape, size, dentition, and lip morphology, often enhanced by sexual dimorphism (Reis and Pereira, 2000; Covain and Fisch-Muller, 2007) (Fig. 1).

The earliest comprehensive taxonomic and phylogenetic treatments of the family Loricariidae either suggested or corroborated the monophyly of the Loricariinae (Isbrücker, 1980; Howes, 1983b; Schaefer, 1987). Although primarily derived from phenetic assessment of morphological variation, the classification scheme of Isbrücker (1980) recognized 27 loricariin genera arrayed in four tribes: Harttiini, Loricariini, Farlowellini, and Acestridinii. The latter, however, was subsequently removed from the Loricariinae by Nijssen and Isbrücker (1987), who placed the genus *Acestridium* within the subfamily Hypoptopomatinae. This proposal was later supported by the first phylogenetic analysis of the Hypoptopomatinae (Schaefer, 1991).

The scheme of Isbrücker (1980) and subsequent rearrangements served as the basis for the first focused phylogenetic study of the Loricariinae (Rapp Py-Daniel, 1997). Through analysis of osteological and external morphological variation, Rapp Py-Daniel (1997) corroborated the monophyly of the Loricariinae and recovered a topology consisting of two reciprocally monophyletic groups (Fig. 2) corresponding fairly well to the tribes Harttiini and Loricariini proposed by Isbrücker (1980). Her findings, however, necessitated redefinition of the tribe Harttiini, which was accordingly expanded to incorporate members of the former Farlowellini. Although the work of Rapp Py-Daniel (1997) is the most comprehensive attempt at resolving

loricariin intergeneric relationships to date (her taxon sampling consisted of 60 species in 22 genera; Table 1), 9 of the 31 genera already described at the time were not included in her study (i.e., *Brochiloricaria*, *Dasyloricaria*, *Harttiella*, *Ixinandria*, *Metaloricaria*, *Paraloricaria*, *Pterosturisoma*, *Pyxiloricaria*, *Ricola*). The genus *Cteniloricaria*, however, was represented in her study by the species *Harttia fowleri*, herein considered an objective synonym of *Cteniloricaria fowleri* (Covain and Fisch-Muller, 2007; Covain et al., 2012).

A significant increase in the generic diversity of the Loricariinae took place after the publication of Isbrücker et al. (2001), in which four new genera were described. Two of these, *Quiritixys* and *Proloricaria*, were described based on specimens previously assigned to the genera *Harttia* and *Loricaria*, respectively. *Quiritixys*, however, was subsequently synonymized with *Harttia* (Ferraris, 2007; Covain and Fisch-Muller, 2007). Similarly, claiming lack of diagnostic features*, Proloricaria* was later synonymized with *Loricaria* (Covain and Fisch-Muller, 2007); a taxonomic decision nonetheless disregarded by both Ferraris (2007) and Eschmeyer (2013). The remainder of the newly described genera in Isbrücker et al. (2001) resulted from taxonomic reassessment of the genus *Rineloricaria*, in which four phenetic groupings were recognized on the basis of external morphology. To accommodate these groups, the authors rediagnosed *Rineloricaria*, revalidated *Hemiloricaria*, and created two new genera: *Fonchiiichthys* and *Leliella*. Contrary to the rearrangement proposed by Isbrücker et al. (2001), Covain and Fisch-Muller (2007) treated *Hemiloricaria*, *Fonchiiichthys* and *Leliella* as junior synonyms of *Rineloricaria*. Although Ferraris (2007) agreed with Covain and Fisch-Muller (2007) in treating *Leliella* as a junior synonym of *Rineloricaria heteroptera* Isbrücker and Nijssen 1976, he maintained *Fonchiiichthys, Hemiloricaria* and *Rineloricaria* (*sensu stricto*) as valid. Likewise, Rodriguez and Reis (2008) argued for the distinctiveness and validity of

Hemiloricaria and *Rineloricaria* on the basis of morphological variation and geographic distribution patterns. Fichberg and Chamon (2008), however, found this distinction problematic, particularly for Amazonian species, and therefore suggested maintaining *Hemiloricaria* in synonymy with *Rineloricaria* pending a more focused revisional study of the group.

Based on multivariate and hierarchical analyses of external morphological variation, Covain and Fisch-Muller (2007) proposed a classification scheme for the Loricariinae in which 31 genera were recognized as valid (Fig. 3). Except for maintaining *Cteniloricaria* in synonymy with *Harttia*—as suggested by Rapp Py-Daniel (1997) and Rapp Py-Daniel and Oliveira (2001), this genus-level taxonomy was in complete agreement with Ferraris (2003, 2007). More recently, however, Covain et al. (2012) revalidated *Cteniloricaria* on the basis of genetic, morphological and ecological data. The classification of Covain and Fisch-Muller (2007), while not phylogenetically-based, recognized the previously proposed tribes Harttiini and Loricariini (Isbrücker, 1980; Rapp Py-Daniel, 1997) and further segregated the constituent genera of the Loricariini into four morphological groups (i.e., the Pseudohemiodon-, Loricaria-, Rineloricaria-, and Loricariichthys-group).

Attempts at using molecular data to elucidate loricariin relationships are scarce and limited both in taxonomic and character sampling. In the first comprehensive molecular phylogeny of the Loricariidae, Montoya-Burgos et al. (1998) examined, to some extent, the phylogenetic relationships within the Loricariinae (Fig. 4). Although based on a limited sampling of loricariin genera (~30%), the hypothesis of loricariid relationships presented by Montoya-Burgos et al. (1998) corroborated the monophyly of the subfamily Loricariinae. More recently, Covain et al. (2008) presented the first molecular phylogeny aimed at resolving loricariin intergeneric relationships (Fig. 5). Their study, however, included less than half of the genera recognized as

valid at the time (i.e., 14 out of 31) and relied on two mitochondrial markers (partial fragments of 12S and 16S) only. In the latest effort to resolve loricariin intergeneric relationships, Rodriguez et al. (2011) described a new genus (*Fonchiloricaria*) and investigated its placement in the phylogeny of the Loricariinae using an expanded version of Covain et al.'s (2008) dataset that included an additional marker (the nuclear gene *F-reticulon4*) but no additional taxa other than the newly described genus and two species of *Spatuloricaria* (Fig 6). Because of its reliance on Covain et al.'s (2008) taxon sampling, the study of Rodriguez et al. (2011) likewise failed to include a substantial fraction of loricariin genera (Table 1). Such an incomplete taxon sampling ultimately undermines the explanatory power of the hypothesis of relationships arrived at by Rodriguez et al. (2011).

Whereas the monophyly of the Loricariinae has been consistently supported by phylogenetic studies using morphological (Howes, 1983b; Schaefer, 1987; Rapp Py-Daniel, 1997; Armbruster, 2004) and molecular data (Montoya-Burgos, 1998; Covain et al., 2008; Rodriguez et al., 2011), loricariin intergeneric relationships have yet to be fully resolved, and the monophyletic status of most genera remains to be tested. This study is therefore aimed at providing the most taxonomically encompassing molecular phylogeny of the Loricariinae to date. The inferred phylogeny will, in turn, provide an opportunity to test with independent evidence previous hypotheses of loricariin relationships and, if necessary, update the classification of the group.

2. METHODS

2.1. TAXON AND CHARACTER SAMPLING

Ingroup taxa included representatives of 56 loricariin species distributed in 21 of the 32 genera herein recognized as valid (Tables 1, 2). The taxon sampling of this study therefore includes ~66% and ~24% of loricariin generic and species diversity, respectively. Despite the lack of consensus as to the sister-group of the Loricariinae, the most comprehensive morphology-based higher-level phylogenetic analyses of the Loricariidae (Howes, 1983b; Schaefer, 1987) recognized affinities and a close relationship between loricariins and the subfamilies Hypostominae and Hypoptopomatinae. Accordingly, outgroup taxa comprised representatives of these subfamilies (Table 2), with *Ancistrus macropthalmus* (Hypostominae) as the root.

Tissue samples were primarily obtained from specimens collected during expeditions of the NSF-funded All Catfish Species Inventory (ACSI) (http://silurus.acnatsci.org). Additional tissues were obtained through donations from colleagues at Universidad Nacional Mayor de San Marcos (Peru), Universidade de São Paulo (Brazil), Muséum d'histoire naturelle de la Ville de Genève (Switzerland), and Universidad del Tolima (Colombia). Fishes were collected and euthanized prior to preservation in accordance with established guidelines for the use of fishes in research (Nickum et al. 2004).

Taxonomically verified vouchers are mostly deposited in the Ichthyology collection of the Academy of Natural Sciences of Drexel University, Philadelphia (ANSP), fully accessible and searchable online at http://clade.ansp.org/ichthyology/FTIP/search.php. Species identity of

voucher specimens was determined and confirmed either by examination of loaned vouchers or on taxonomic authority of the loaning institution.

Comparative molecular data consisted of four protein-coding genes/gene fragments, comprising the nuclear myosin-heavy polypeptide 6-cardiac muscle-alpha (*myh6*), and the mitochondrial cytochrome c oxidase subunit 1 (*co1*), cytochrome b (*cyt-b*), and NADH dehydrogenase 2 (*nd2*). DNA extraction, amplification, and purification procedures, along with primers and PCR thermal profiles for sequencing *co1*, *cyt-b*, and *myh6* are described in Arroyave and Stiassny (2011). Primers and PCR thermal profiles for amplification and sequencing of *nd2* are listed in Arroyave et al. (submitted). DNA sequence data was obtained from a total of 117 individuals. Contig assemblage and sequence editing was performed using Geneious Pro version 5.6.5 (Biomatters, available from http://www.geneious.com/). IUPAC nucleotide ambiguity codes were used to represent heterozygous sites. Voucher catalog numbers and GenBank accession numbers for the gene sequences generated and utilized in this study are listed in Table 2.

2.2. MODEL SELECTION AND ASSESSMENT OF SUBSTITUTION SATURATION

For each gene, individual sequences were aligned based on the translated amino acid sequence using the *Translation Align* algorithm under default parameters as implemented in Geneious, and the best-fit substitution model was selected from among 24 models using the Bayesian Information Criterion (BIC) as implemented in jModelTest (Posada, 2008) under the following likelihood settings: *Number of substitution schemes* = 3; *Base frequencies* = +F; *Rate variation* = +I and +G with nCat = 4; and *Base tree for likelihood calculations* = Fixed BIONJ-JC.

Substitution saturation in third codon positions was assessed via saturation plots and the test by Xia et al. (2003), both implemented in the software DAMBE (Xia and Xie, 2001) following the guidelines provided in (Xia and Lemey, 2009). In saturation plots, corrected genetic distances were calculated using the substitution models previously selected by jModelTest or the best-approximating models available in DAMBE, and trend lines were estimated using second-order polynomial curves fit to the data.

2.3. PHYLOGENETIC ANALYSES

Prior to phylogenetic analyses, individual gene alignments were concatenated using the software 2matrix.pl (Salinas and Little, 2012). The number of variable and parsimony-informative sites of the concatenated alignment was determined using MEGA 5 (Tamura et al., 2011). The concatenated alignment was analyzed using Maximum Likelihood [ML] (Felsenstein, 1981), Bayesian Inference [BI] (Rannala and Yang, 1996), and Maximum Parsimony [MP] (Farris, 1970; Fitch, 1971) methods of phylogeny reconstruction. ML and BI analyses were conducted on the concatenated alignment partitioned into gene regions with parameters unlinked to accommodate potential process heterogeneity among gene regions. ML analyses were carried out in RAxML version 7.2.8 Black Box (Stamatakis, 2006). BI analyses were carried out in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003) and implemented using the Markov Chain Monte Carlo algorithm (MCMC) run twice (each time using four chains) for 2×10^7 generations, with a sampling period of 1000 generations, under default priors and proposal mechanisms. MCMC convergence was assessed by examining trace plots of posterior probability vs. number of generations in Tracer (Rambaut and Drummond, 2007) and by plotting posterior probabilities of splits at selected increments over an MCMC run

(cumulative function) using the web-based tool AWTY (Nylander et al., 2008; Wilgenbusch et al., 2004). Further assessment of MCMC convergence was undertaken by examination of the average standard deviation of split frequencies, with values << 0.01 taken as indicative of stationarity. Pre-convergence MCMC samples were discarded as burn-in, and substitution model parameters were calculated from the remaining samples. Likewise, branch lengths and posterior probabilities of nodes were calculated from the set of post burn-in trees using TreeAnnotator version 1.7.4 (Drummond et al., 2012), and summarized as a 50% majority-rule consensus tree. Both ML and BI analyses were implemented through the CIPRES Science Gateway V. 3.3 (Miller et al., 2010). MP analyses were carried out in TNT (Willi Hennig Society edition) (Goloboff et al. 2003; 2008) with gaps treated as fifth state, an indel-substitution cost ratio of 1, no cost for gap opening, 1000 starting trees, tree-searching algorithms including TBR branch swapping (Swofford and Olsen, 1990), Ratchet (Nixon, 1999), sectorial searches, tree-drifting, and tree-fusing (Goloboff, 1999), and collapsing branches of minimum length equal to zero. Ensemble consistency index [CI] (Kluge, 1969) and ensemble retention index [RI] (Farris, 1989) were used as measures of homoplasy and synapomorphy, respectively. Clade support was measured with bootstrap values (based on 1000 pseudoreplicates) in ML and MP analyses, and with clade posteriors in BI analyses. All resultant phylogenies were drawn in a ladderized configuration.

3. RESULTS

3.1. DATASET STATISTICS, BEST-FIT SUBSTITUTION MODELS, AND SATURATION TESTS

The concatenated alignment consisted of 3471 sites, of which 1656 were variable (including 9 indels) and 1493 parsimony-informative. Successful amplification and sequencing of targeted genes across sampled taxa resulted in almost no missing data (<2%) (Table 2). The HKY+I+G and GTR+G substitution models were determined to be the best fit for the mitochondrial (*co1*, *cyt-b*, *nd2*) and nuclear (*myh6*) genes, respectively. Because of the limited number of available substitution models in DAMBE, corrected genetic distances (d) in saturation plots were calculated using F84 and GTR as alternatives for HKY+I+G and GTR+G, respectively. Transversions outnumbered transitions only in third positions of *nd2* at corrected genetic distances larger than 0.27 substitutions/site (Fig. 7). This result, however, is assumed to have no practical implications given that genetic distances of almost all pairwise comparisons fall below this value. On the other hand, the index of substitution saturation (I_{SS}) was significantly smaller than the critical value ($I_{SS.C}$) in all sampled genes (p-value << 0.0001). Therefore, both saturation plots and Xia et al's (2003) statistical tests imply little to no saturation in the molecular dataset of this study.

3.2. PHYLOGENETIC ANALYSES

Maximum Likelihood (ML) estimation of phylogeny resulted in a tree with a likelihood score (ln$L$) of −51640.48 and moderately supported overall (Fig. 8). Bayesian inference (BI) of phylogeny resulted in a 50% majority-rule consensus tree that, while not fully resolved, was very similar to the ML tree in topology, relative branch lengths, and nodal support (Fig. 9). The ML

and BI trees differed primarily in the phylogenetic placement of the genera *Lamontichthys*, *Pseudohemiodon*, and *Rhadinoloricaria* (neither of which was recovered as monophyletic), and in the resolution of a clade—mostly unresolved in the BI tree—containing the genera *Furcodontichthys*, *Hemiodontichthys*, *Loricariichthys Pseudoloricaria*, and *Reganella*. Implementation of the maximum parsimony (MP) optimality criterion resulted in six equally most parsimonious trees of length 10891, of which a strict consensus (hereafter referred to as the MP tree) is presented in Fig. 10. As expected, model-based phylogenies were much more topologically congruent with each other than any of them with the MP tree (Fig. 11). Indeed, most suprageneric clades in the ML tree were shared with the BI tree, but not with the MP tree (clades 1-16, Fig. 11). Overall, five suprageneric clades—all with moderate to strong nodal support—were recovered independent of the method of phylogenetic inference (clades 1, 4, 5, 12, and 13; Fig. 11). Relationships within most of these clades, however, differed among trees. By recovering the tribes Harttiini and Loricariini as reciprocally monophyletic, the MP tree was the only to conform to the current tribal classification. Most of the discrepancies between model-based and parsimony topologies (particularly regarding intergeneric relationships) were associated with weakly supported nodes. Despite the instances of conflict between methods, the molecular dataset of this study strongly supported the monophyly of both the subfamily Loricariinae and the tribe Loricariini (clade 5; Fig. 11) regardless of optimality criterion. The taxon sampling of this study allowed for testing monophyly in nine (*Cteniloricaria*, *Farlowella*, *Loricaria*, *Lamontichthys*, *Loricariichthys*, *Pseudohemiodon*, *Rineloricaria*, *Spatuloricaria*, *Sturisoma*) of the 18 polytypic loricariin genera. Except for *Lamontichthys* and

Pseudohemiodon[1], all of the abovementioned genera were strongly recovered as monophyletic regardless of analytical method (Figs. 8-10).

[1] *Pseudohemiodon* was nevertheless represented herein by only one valid species (*P. apithanos*) and two putatively undescribed species (R. Covain, pers. comm.).

4. DISCUSSION

The present work represents the most inclusive molecular-based phylogenetic study of the Loricariinae to date, and the first to include DNA sequence data from the genera *Apistoloricaria*, *Furcodontichthys*, *Pseudoloricaria*, *Reganella*, and *Rhadinoloricaria*. Of the genera not sampled here, only *Aposturisoma*, *Dentectus*, *Fonchiiloricaria*, and *Sturisomatichthys* (most of which are monotypic) have been included in previous phylogenetic studies of the group (Rapp Py-Daniel, 1997; Covain et al., 2008; Rodriguez et al., 2011). The genera *Brochiloricaria*, *Harttiella*, *Ixinandria*, *Paraloricaria*, *Pterosturisoma*, *Pyxiloricaria*, and *Ricola*, conversely, have never been incorporated in a phylogenetic analysis of the Loricariinae. The results of the present study therefore provide a comprehensive phylogenetic framework to assess previous hypotheses of intergeneric relationships and the adequacy of the current taxonomy of the group.

Under the assumption that model-based phylogenetic inference methods are better suited to handle molecular data than parsimony (primarily due to substitution saturation and long-branch attraction artifacts), and the fact that the model-based phylogenies of this study are comparatively more congruent with the morphological phylogeny of Rapp Py-Daniel (1997) (and thereby require considerably less invocation of homoplasy to explain the evolution of morphological traits than would the MP phylogeny), the following discussion primarily relies on the resultant ML and BI trees, unless otherwise noted.

4.1. LORICARIIN INTERGENERIC RELATIONSHIPS: CONGRUENCE AND CONFLICT WITH PREVIOUS STUDIES

The phylogenetic studies of Schaefer (1991) and Rapp Py-Daniel (1997) reformed the phenetic classification of Isbrücker (1980), resulting in the dichotomization of the Loricariinae

into the tribes Harttiini and Loricariini. The monophyly of the Harttiini (*sensu* Rapp Py-Daniel, 1997), however, has not been supported by any of the molecular phylogenies of the group (Montoya-Burgos et al., 1998; Covain et al., 2008; Rodriguez et al., 2011), including the model-based trees of the present study (Figs. 8, 9). Although the MP tree presented herein (Fig. 10) agreed with Rapp Py-Daniel's (1997; Figs. 48-51) on the reciprocal monophyly of the tribes Harttiini and Loricariini, support for a monophyletic Harttiini is weak in both phylogenies. The available evidence therefore suggests that the Harttiini is most likely a paraphyletic assemblage. As a result, the morphological synapomorphies proposed by Rapp Py-Daniel (1997) to diagnose this suprageneric grouping (e.g., larger number of mandibular teeth [>50], bony plates on the upper lip, second basibranchial vestigial) would have to either represent the plesiomorphic condition or correspond to characters with a complex variation and distribution among both loricariids and loricariins. The latter possibility is indeed supported by the fact that most of these characters were fairly homoplastic ($ci < 0.5$) (Rapp Py-Daniel, 1997: p. 118). The paraphyly of the Harttiini, as previously implied by molecular data, ultimately led to Covain et al. (2008) to restrict the Harttiini to the genus *Harttia*. The findings of the present study, however, necessitate the redefinition of this tribe to include the genus *Cteniloricaria*. Recognition of *Cteniloricaria* as a member of the Harttiini is likewise supported by the sister-group relationship between the species *Harttia* (=*Cteniloricaria*) *fowleri* and a clade containing members of the genus *Harttia*, as recovered with morphological data (Rapp Py-Daniel, 1997: p. 209), and more recently by the findings of a taxonomic study of the Harttiini from the Guianas (Covain et al., 2012).

Early classification schemes of the Loricariinae assigned the genus *Metaloricaria* to the tribe Harttiini on the basis of very few characters of questionable phylogenetic value (Isbrücker, 1980; Isbrücker and Nijssen, 1982). Since *Metaloricaria* was not included in the taxon sampling

of Rapp Py-Daniel (1997), its placement in the phylogeny of the Loricariinae has yet to be assessed with morphological data. On the other hand, the molecular phylogenies of Covain et al. (2008) and Rodriguez et al. (2011), as well as the model-based trees of this study, consistently recover *Metaloricaria*—although weakly supported—outside of the Harttiini (*sensu stricto*) clade (Figs. 5-6, 8-9). Notwithstanding this lack of support, the available evidence warrants the exclusion of *Metaloricaria* from the tribe Harttiini pending a more comprehensive phylogenetic study based on both morphological and molecular data. The inferred position of *Metaloricaria*, outside of the Harttiini and sister to the remaining loricariin radiation, however, somehow reflects the unique combination of morphological characters that diagnose this genus, including those that distinguish it from members traditionally assigned to the Harttiini (e.g., fewer teeth and caudal-fin rays) and those seemingly autapomorphic (e.g., horseshoe-shaped lower lip, the presence of three very distinctive buccal papillae). Although Rapp Py-Daniel (1997) did not include *Metaloricaria* in her phylogenetic analyses, she expressed her disagreement with its inclusion in the Harttiini because of its reduced premaxilla and the clear differences in lip structure and dentition between this genus and other members of the Harttiini. Based on a series of characters either inconclusive or conflicting with the assignment of *Metaloricaria* to the Loricariini, she suggested that *Metaloricaria*, if a member of the Loricariini, would have to be classified apart from any subtribal clade. Interestingly, the results of the present study imply a very similar taxonomic arrangement (Fig. 11), only that the tribe Loricariini (*sensu* Rapp Py-Daniel, 1997) would have to be expanded to accommodate *Lamontichthys*, *Farlowella*, and *Sturisoma*, genera traditionally assigned to the Harttiini. Such an expanded Loricariini has indeed been proposed by Covain et al. (2008), and further divided into the subtribes Farlowellina and Loricariina, so as to fit the taxonomic implications of their molecular phylogeny. Except for the

phylogenetic placement of *Metaloricaria*, the findings of this study are fully congruent with the composition and reciprocal monophyly of the Farlowellina and Loricariina as defined by Covain et al. (2008). Because a classification scheme of the Loricariinae conforming to the findings of this study would require removal of *Metaloricaria* from the Loricariina (*sensu* Covain et al., 2008), a new subtribe, Metaloricariina, is proposed herein to accommodate members of *Metaloricaria*.

Notwithstanding the differences between the parsimony and model-based methods with respect to the phylogenetic placement and resolution of a clade containing members of *Lamontichthys*, *Farlowella*, and *Sturisoma* (Fig. 11), nodal support for this suprageneric assemblage was consistently high (Figs. 8-10). Therefore, the molecular data of this study provide further support for the recognition of the subtribe Farlowellina as proposed by Covain et al. (2008) to accommodate members of the Harttiiini (*sensu* Rapp Py-Daniel, 1997) other than *Harttia*. Morphological synapomorphies for the Farlowellina, however, have yet to be proposed, and additional data and improved taxon sampling may be required to unambiguously resolve relationships among members of this clade.

A restricted version of the Loricariina *sensu* Covain et al. (2008) (clade 5; Fig. 11) was herein resolved with *Dasyloricaria* as the sister of a clade containing the remaining members of the group. This pattern was strongly supported in both the ML and BI trees, and congruent with the findings of Covain et al. (2008) and Rodriguez et al. (2011). Morphological evidence for the abovementioned sister-group relationship, however, has yet to be discovered. Although previous authors (Isbrücker, 1980; Rapp Py-Daniel, 1997) suggested a close association between *Dasyloricaria* and *Spatuloricaria* based on external anatomical similarities (in head shape, lip

structure, dentition, pattern of abdominal plating), the available molecular evidence does support such a relationship.

A phylogenetic pattern similarly recovered both in previous molecular studies (Covain et al., 2008; Rodriguez et al., 2011) and the present work (although only with moderate support), is the sister-group relationship between the genus *Rineloricaria* and a clade containing members of the *Spatuloricaria-Hemiodontichthys* clade of Rapp Py-Daniel (1997: p. 101) (clade 7; Fig. 11). The *Spatuloricaria-Hemiodontichthys* clade is morphologically diagnosed by a series of anatomical synapomorphies (mostly osteological), of which a reduced dentary is the only one unique and unreversed [*ci* = 1] (Rapp Py-Daniel, 1997: p. 101). The molecular data of this study provide evidence for the monophyly of this clade, with moderate to strong nodal support (Figs. 8, 9). Moreover, all published molecular phylogenies of the group, in addition to the model-based trees of the present study, are congruent with morphology in the resolution of this clade into two reciprocally monophyletic subunits: the *Spatuloricaria-Rhadinoloricaria* and *Hemiodontichthys-Pseudoloricaria* subclades of Rapp Py-Daniel (1997: p. 101).

Based on their resultant phylogeny, Covain et al. (2008: p. 991) further subdivided members of the *Spatuloricaria-Rhadinoloricaria* subclade into the so-called "*Loricaria*-" and "*Pseudohemiodon*-" groups, while assigning members of the *Hemiodontichthys-Pseudoloricaria* subclade to the "*Loricariichthys*-group". The abovementioned suprageneric groups, plus an additional assemblage comprising the genera *Dasyloricaria*, *Ixinandria*, *Rineloricaria*, and *Spatuloricaria* (the so-called "*Rineloricaria*-group"), had been previously proposed by Covain and Fisch-Muller (2007) to classify—according to phenetic similarities in external morphology—genera traditionally assigned to the tribe Loricariini. The findings of the present study provide only partial support for the recognition of these suprageneric groups. According to

the inferred model-based phylogenies, for the "*Loricariichthys*-" and "*Pseudohemiodon*-" groups to represent monophyletic assemblages, the monotypic genus *Reganella* would have to be transferred from the latter to the former. These slightly modified "*Loricariichthys*-" and "*Pseudohemiodon*-" groups are represented by clades 8 and 13 (Fig. 11), respectively, and are congruent with the morphology-based phylogeny of Rapp Py-Daniel (1997). Anatomical evidence in support for the monophyly of the "*Loricariichthys*-group" including *Reganella* (referred to as the *Limatulichthys*-*Hemiodontichthys* clade in Rapp Py-Daniel, 1997: p. 112), however, is restricted to two osteological synapomorphies (mesethmoid tip enveloped by dermal plates, first basibranchial elongate), both fairly homoplastic ($ci < 0.35$). Conversely, the monophyly of the "*Pseudohemiodon*-group" excluding *Reganella* (referred to as the subtribe Planiloricariina in Rapp Py-Daniel, 1997: p. 105), is supported by 15 morphological synapomorphies, some of which appear to be related to the extreme skull and body depression characteristic of members of this clade (Fig. 1). Regardless of the unbalanced extent of morphological support, both of these suprageneric groups (clades 8 and 13; Fig. 11) have been consistently and independently recovered as monophyletic using DNA sequence data, and therefore warrant subtribal status. Accordingly, the names Loricariichthyna and Planiloricariina are proposed herein to accommodate members of the clades 8 and 13 (Fig. 11), respectively.

The taxon sampling of the present study did not allow testing the monophyly of the "*Loricaria*-group" of Covain and Fisch-Muller (2007), yet the inferred phylogenetic placement of *Loricaria* (sister to a clade containing members of the Planiloricariina) is congruent with previous molecular phylogenetic studies (Montoya-Burgos et al., 1998; Covain et al., 2008; Rodriguez et al., 2011) and the morphology-based phylogeny of Rapp Py-Daniel (1997). Because none of the genera accompanying *Loricaria* in the "*Loricaria*-group" (i.e.,

Brochiloricaria, *Paraloricaria*, *Ricola*) have ever been included in a phylogenetic analysis of the subfamily, the adequacy of this group for classification purposes remains to be assessed. Nevertheless, based on similarities in lip structure, dentition, abdominal plating, and sexual dimorphism, it is reasonable to assume a close relationship between members of the "*Loricaria*-group" of Covain and Fisch-Muller (2007). Such an assemblage is herein elevated to subtribal status under the name Loricariina pending a more inclusive phylogenetic analysis. The composition of this newly defined Loricariina would be only partially overlapping with the Loricariina originally proposed by Isbrücker (1980), which also included genera now assigned (based on phylogenetic evidence) to the subtribe Planiloricariina (i.e., *Crossoloricaria*, *Pseudohemiodon*, and *Rhadinoloricaria*).

In contrast to the partial correspondence between the "*Loricariichthys*-" and "*Pseudohemiodon*-" groups of Covain and Fisch-Muller (2007) and the phylogenies inferred in the present study, the "*Rineloricaria*-group" (which includes the genera *Dasyloricaria*, *Rineloricaria*, *Spatuloricaria*) was unambiguously recovered as polyphyletic regardless of analytical method. The polyphyly of the "*Rineloricaria*-group" had indeed been previously recovered with morphological (Rapp Py-Daniel, 1997) and molecular (Rodriguez et al., 2011) data. Given that this suprageneric assemblage clearly does not reflect phylogenetic relationships, it should not be used in classification schemes of the Loricariinae. Alternatively, the subtribal names Dasyloricariina, Rineloricariina, and Spatuloricariina are proposed herein to represent the lineages associated with the genera *Dasyloricaria*, *Rineloricaria*, and *Spatuloricaria*, respectively. This taxonomic arrangement is congruent with the available molecular phylogenies of the group, particularly that of Rodriguez et al. (2011).

To reconcile the taxonomy of the Loricariinae with the available phylogenetic evidence (both phenotypic and genetic), including the results of the present study, an updated classification scheme for the group, consisting of 10 monophyletic suprageneric taxa at different levels of universality (including tribal and subtribal ranks), is proposed herein (Table 3; Fig. 12). It is noted, however, that as any other phylogenetic hypothesis, this classification is provisional and contingent to the results of a more comprehensive phylogenetic analysis of the group, ideally combining comparative anatomical and molecular data (a study yet to be done).

4.2. MONOPHYLY OF LORICARIIN GENERA

With the exception of *Lamontichthys, Sturisoma,* and *Sturisomatichthys*, Rapp Py-Daniel (1997) tested the monophyletic status of all sampled polytypic genera, finding no support for the monophyly of *Crossoloricaria* and *Rineloricaria*. Her taxonomic sampling within several of the polytypic genera (e.g., *Spatuloricaria, Loricaria, Loricariichthys, Farlowella*), however, was limited and unlikely to provide a robust assessment of monophyly.

Except for the genera *Farlowella* (Retzer and Page, 1996), *Lamontichthys* (Paixão and Toledo-Piza, 2009), *Loricariichthys* (Reis and Pereira, 2000; Paixão, 2012) and *Sturisoma* (Ghazzi, 2003), the monophyly of polytypic loricariin genera has not been assessed with morphological data beyond the study of Rapp Py-Daniel (1997). On the other hand, because of the limited subset of loricariin taxa sampled in the molecular phylogenetic studies of Covain et al. (2008) and Rodriguez et al. (2011), up until the present study, the monophyly of most loricariin genera had yet to be tested with DNA sequence data.

Results from this investigation support the monophyletic status of the polytypic genera *Cteniloricaria*, *Farlowella*, *Loricaria*, *Loricariichthys*, *Rineloricaria*, *Spatuloricaria*, and

Sturisoma. Although the genus *Limatulichthys* is currently monotypic, it was represented in this study by the species *L. griseus* and three putatively undescribed species from Brazil. The inferred phylogenies recovered all four species forming a strongly supported clade (Figs. 8-10) sister to the monotypic genus *Pseudoloricaria*. Therefore, description of these undescribed species under the genus name *Limatulichthys* would be congruent with the available phylogenetic evidence and would render the genus polytypic. Similarly, the current monotypic genus *Rhadinoloricaria* was herein represented by *R. macromystax* and a supposedly undescribed species (ANSP 185044; *Rhadinoloricaria sp. nov.* in Table 2) from the Orinoco River in Venezuela (R. Covain, pers. comm.). The inferred phylogenies, however, did not recover these species as sister taxa, but forming a polyphyletic group instead (Figs. 8-10). Description of this putatively new loricariin species under the genus name *Rhadinoloricaria* would therefore conflict with the results of the present study.

The molecular dataset generated in this study offered the first opportunity to test the monophyly of *Cteniloricaria*, a genus described by Isbrücker and Nijssen (in Isbrücker, 1979) to accommodate species morphologically similar to *Harttia* but with a more slender body, and with differences in caudal fin shape and degree of abdominal plating. Although *Cteniloricaria* has been synonymized with *Harttia* by previous authors (Rapp Py-Daniel, 1997; Rapp Py-Daniel and Oliveira, 2001), others (Covain and Fisch-Muller, 2007; Covain et al., 2012) have contended that arguments for such a taxonomic decision are unfounded because *Cteniloricaria platystoma*, the type species of the genus, was not included in any of the comparative studies that formed the basis for synonymizing these two genera. Furthermore, according to Covain et al. (2012), the taxonomic distinction between members of *Cteniloricaria* and *Harttia* is further supported by genetic, morphological, and ecological differences between the two. As revealed by the

phylogenies presented herein (Figs. 8-10), *Cteniloricaria* and *Harttia* represent closely related yet independent evolutionary lineages; a phylogenetic pattern that provides further evidence for the recognition of both genera. Despite the fact that *Cteniloricaria* is defined on the basis of combination of characters present in other genera as opposed to uniquely diagnostic traits (Rapp Py-Daniel, 1997), the available phylogenetic evidence strongly supports the monophyly and validity of this taxon.

The genus *Farlowella*, diagnosed by 16 anatomical synapomorphies (Rapp Py-Daniel, 1997: p. 123), is the second-most diverse and perhaps the most morphologically distinct of all loricariin genera (Fig. 1). Precisely because of its uniquely elongate cylindrical body, produced snout, and placement of dorsal fin (opposite to anal fin), the monophyly of this genus had never been brought into question until the molecular phylogeny of Covain et al. (2008) recovered it as paraphyletic (although with weak nodal support and acknowledging the unexpectedness of this finding). The results of the present study, however, based on a much larger sampling of species, consistently and strongly corroborate the monophyly of the genus *Farlowella* (Figs. 8-10).

Evidence for the monophyly of *Loricaria* is strong, and besides the molecular phylogenies presented here, it includes morphological characters related to dentition and lip structure first pointed out by Isbrücker (1981), and a series of primarily osteological synapomorphies related to palatine, pharyngeal plates, and pelvic fin structure more recently discovered by Rapp Py-Daniel (1997). Similarly, the monophyly of the genus *Loricariichthys* is strongly supported by molecular data (Covain et al., 2008; Rodriguez et al., 2011; this study), and by a series of anatomical synapomorphies mostly related to the structure of hyoid and branchial arches (Rapp Py-Daniel, 1997) and lip morphology (Reis and Pereira, 2000), as well as by five unambiguous

morphological synapomorphies discovered during a recent revisional and phylogenetic treatment of the genus (Paixão, 2012).

Perhaps one of the most complex taxonomic histories of any loricariin genus is that of *Rineloricaria*, some of whose members have at once been designated under various other generic names, including *Hemiloricaria*, *Fochiiichthys*, and *Leliella*. With 65 currently valid species, *Rineloricaria* is by far the most speciose genus of the Loricariinae, and although lacking autapomorphies, it can be diagnosed by a unique combination of characters related to dentition, abdominal plating, lip morphology, and coloration (Fichberg and Chamon, 2008). While the study of Rapp Py-Daniel (1997) failed to support the monophyly of *Rineloricaria*, the available molecular phylogenies, including the results from the present study, strongly support monophyly of the genus. Given the extent of species diversity in *Rineloricaria*, however, a focused revisionary and phylogenetic study with a much broader taxon sampling of *Rineloricaria* species is ultimately necessary to unequivocally test the monophyly of this genus.

Spatuloricaria, another genus whose monophyly is supported by the results of the present study, was described by Schultz (1944) to accommodate a species similar to members of the genus *Loricaria*, but having bilobed spoon-shaped (spatulate) teeth; hence the genus name (Schultz, 1944). Not surprisingly, more than three-quarters of the species currently assigned to *Spatuloricaria* were originally described as *Loricaria*. In addition to the presence of derived oral dentition, Schultz (1944) regarded features of lip morphology, predorsal and abdominal plate patterns, fin shape, and sexually dimorphic traits as also being distinctive of the genus. The external anatomy of *Spatuloricaria* shows few uniquely derived traits; instead, the genus can be diagnosed by a unique combination of characters such as rounded papillose lips with inner rictal barbels, few and bilobed spoon-shaped teeth, abdominal cover consisting of minute disjointed

plates, and a predorsal keel (Covain and Fisch-Muller, 2007). Because most species of *Spatuloricaria* were described on the basis of vague morphological diagnoses, species limits in the genus are questionable. In fact, preliminary results from a revisionary study of the genus currently in progress (I. Fichberg, pers. comm.) indicate that only six of the eleven described species are valid, and that five new species are yet to be described, including two of the four undescribed species sampled for phylogenetic analyses herein (i.e., *Spatuloricaria sp. nov.* "Xingu/Iriri, Brazil" and *Spatuloricaria sp. nov.* "Ireng, Guyana"). The monophyly of *Spatuloricaria* was first corroborated by Rapp Py-Daniel (1997) based on a series of morphological synapomorphies that included the presence of internal rictal barbels, abdominal plates reduced to patches of odontodes, rounded hyoid arch cross-section, parapophyses of the 4th vertebra reaching the pterotic supracleithrum border, two lateral and symmetric arrector dorsalis flanges, a keel-shaped ridge (carina) on the supraoccipital scutes, antero-medial metapterygoid crest, and a preopercle sutured to dermal plates via dorsal ridge. Of these synapomorphies, however, only the presence of internal rictal barbels is uniquely derived and unreversed according to her hypothesis of loricariin relationships. Prior to the present study, the publication of Rodriguez et al. (2011) was the only molecular phylogeny to include members of *Spatuloricaria*, and although incorporating only two species, these were recovered as sister taxa, thus not rejecting with the monophyly of the genus. In contrast to the taxon sampling of Rodriguez et al. (2011), the present study included 18 individuals representing eight species of *Spatuloricaria*; the largest number of species (and individuals) of the genus ever sampled in a phylogenetic study of the Loricariinae. Accordingly, the results presented herein provide the strongest test and corroboration for the monophyly of *Spatuloricaria*, and the most comprehensive hypothesis of relationships proposed for the genus.

As in *Loricaria* and *Loricariichthys*, the monophyly of *Sturisoma* is supported by the findings of this study, of previous molecular phylogenetic analyses (Covain et al., 2008; Rodriguez et al., 2011), and of previous morphology-based revisionary (Ghazzi, 2003) and phylogenetic studies (Rapp Py-Daniel, 1997). Morphological synapomorphies for the genus, however, are few and highly homoplastic (Rapp Py-Daniel, 1997: p. 121), and the available phylogenetic hypotheses are based on a very limited sampling of *Sturisoma* species. Because *Sturisoma* appears to be morphologically ill defined (Rapp Py-Daniel, 1997), a phylogenetic analysis of the Loricariinae with a larger representation of the species diversity of the genus (currently estimated at 15) may be needed for a more robust assessment of monophyly.

Lamontichthys and *Pseudohemiodon* were the only genera whose monophyly was not supported by the results of the present study. The monophyly of *Lamontichthys*, not previously tested in the context of a phylogenetic analysis of the Loricariinae, is herein rejected because the sampled species, *L. stibaros* and *L. filamentosus* (which differ primarily in meristic counts and morphometric measurements, and the presence of fin filamentous extensions in the latter), were not recovered as sister taxa, irrespective of analytical method. This phylogenetic pattern, however, was not strongly supported in any of the resultant phylogenies, and therefore, additional makers and/or an increased sampling of *Lamontichthys* species may be necessary to more confidently assess the monophyly of this genus with DNA sequence data. Although her analyses included comparative morphological data only from the species *L. filamentosus*, Rapp Py-Daniel (1997: p. 124) reported five osteological synapomorphies for the genus, all of which were fairly homoplastic ($ci<0.4$). Conversely, the study of Paixão and Toledo-Piza (2009) corroborated the monophyly of *Lamontichthys* on the basis of six morphological

synapomorphies, including five newly proposed uniquely derived and unreversed osteological characters.

Pseudohemiodon, a genus not previously sampled for phylogenetic analyses with DNA sequence data, is herein recovered as paraphyletic. This result, however, does not necessarily falsify the monophyly of *Pseudohemiodon*, because it was based on a taxon sampling that included only one of the seven currently valid species (*P. apithanos*); the other two sampled species are putatively undescribed (R. Covain, pers. comm). What the findings of the present study suggest instead, is that these supposedly new species (i.e., *Pseudohemiodon sp. nov.* "Amazonas, Peru" and *Pseudohemiodon sp. nov.* "Napo, Peru") may in fact correspond to a closely related lineage that should be described under a different genus name. The description of this new taxon, however, will necessitate a more detailed and thorough anatomical assessment of voucher and associated specimens in order to identify morphological synapomorphies or a unique combination of characters that differentiate it from all other genera of the Loricariinae. *Pseudohemiodon* was recovered as monophyletic by Rapp Py-Daniel (1997), albeit with weak nodal support and based on synapomorphies highly homoplastic (ci<0.2). A definite conclusion on the monophyletic status of *Pseudohemiodon* therefore remains to be established. On the other hand, although *Planiloricaria* was originally described as a subgenus of *Pseudohemiodon (*Isbruker, 1971), the available phylogenetic evidence (including the findings of the present study) supports their current taxonomic distinction and generic rank.

A sister group relationship between *Rhadinoloricaria macromystax* and a clade consisting of the reciprocally monophyletic *Crossoloricaria* and *Apistoloricaria* was revealed (with moderate to strong nodal support) by the results of this study. Rapp Py-Daniel (1997: p. 110) similarly recovered these three genera forming a monophyletic group supported by seven morphological

synapomorphies. Her phylogeny, however, did not resolve relationships among these genera as in the present study, but with members of *Crossoloricaria* forming a paraphyletic assemblage with respect to both *Apistoloricaria* and *Rhadinoloricaria* (Rapp Py-Daniel, 1997: p. 209). Because of this pattern of relationships, coupled with the lack of unambiguous synapomorphies for any of these genera, Rapp Py-Daniel (1997: p. 138) suggested the synonymization of *Apistoloricaria* and *Crossoloricaria* with *Rhadinoloricaria* (the oldest available name). While this taxonomic recommendation is not necessarily in conflict with the findings of this study, the topology and branch lengths of the model-based phylogenies presented herein suggest that all three genera may in fact represent independent evolutionary lineages and thus should be recognized as such in a classification scheme of the Loricariinae. Therefore, the validity of *Apistoloricaria*, *Crossoloricaria*, and *Rhadinoloricaria* is maintained here.

5. TABLES

Table 1. Valid loricariin genera, number of valid species per genus, and number of species sampled in the most comprehensive phylogenetic studies of the Loricariinae.

Valid Genera[1]	Valid Species[2]	Sampled Species		
		Rapp Py-Daniel (1997)	Rodriguez et al. (2011)[3]	This Study
Apistoloricaria Isbrücker & Nijssen 1986	4	1	0	1
Aposturisoma Isbrücker, Britski, Nijssen & Ortega 1983	1	1	0	0
Brochiloricaria Isbrücker & Nijssen 1979	2	0	0	0
Crossoloricaria Isbrücker 1979	5	4*	1	1
Cteniloricaria Isbrücker & Nijssen 1979	2	1**	0	2
Dasyloricaria Isbrücker & Nijssen 1979	5	0	1	1
Dentectus Martín Salazar, Isbrücker & Nijssen 1982	1	1	0	0
Farlowella Eigenmann & Eigenmann 1889	26	2*	2	6
Fonchiiloricaria Rodriguez, Ortega & Covain 2012	1	0	1	0
Furcodontichthys Rapp Py-Daniel 1981	1	1	0	1
Harttia Steindachner 1877	22	8*	1	1
Harttiella Boeseman 1971	7	0	0	0
Hemiodontichthys Bleeker 1862	1	1	1	1
Ixinandria Isbrücker & Nijssen 1979	1	0	0	0
Lamontichthys Miranda Ribeiro 1939	6	1	1	2
Limatulichthys Isbrücker & Nijssen 1979	1	1	1	4*
Loricaria Linnaeus 1758	16	5*	2	4*
Loricariichthys Bleeker 1862	18	3	2	4*
Metaloricaria Isbrücker 1975	2	0	1	1
Paraloricaria Isbrücker 1979	3	0	0	0
Planiloricaria Isbrücker 1971	1	1	1	1
Pseudohemiodon Bleeker 1862	7	6*	0	3*
Pseudoloricaria Bleeker 1862	1	1***	0	1
Pterosturisoma Isbrücker & Nijssen 1978	1	0	0	0
Pyxiloricaria Isbrücker & Nijssen 1984	1	0	0	0
Reganella Eigenmann 1905	1	1	0	1
Rhadinoloricaria Isbrücker & Nijssen 1974	1	1*	0	2*
Ricola Isbrücker & Nijssen 1978	1	0	0	0
Rineloricaria Bleeker 1862	65	15*	3*	10*
Spatuloricaria Schultz 1944	11	2*	2*	7*
Sturisoma Swainson 1838	15	1*	2	2*
Sturisomatichthys Isbrücker & Nijssen 1979	4	1	1	0
Total: 32	**234**	**60*** **	**23**	**56**

[1] Following Covain and Fisch-Muller (2007), Covain et al. (2008), Fichberg and Chamon (2008), Rodriguez et al. (2011), and Covain et al. (2012).
[2] In accordance with Eschmeyer and Fong (2013).
[3] Identical taxon sampling to that of Covain et al. (2008), except for the inclusion of a newly described species and genus (*Fonchiiloricaria nanodon*) and two species of *Spatuloricaria*.
*Counting unidentified ("*sp.*") and/or undescribed ("*sp. nov.*") species.
**Represented by *Harttia fowleri*, a species name herein considered an objective synonym of *Cteniloricaria fowleri*.
***Without considering *Pseudoloricaria cf. punctata*, an invalid species name.

Table 2. Taxa, voucher catalog numbers*, and GenBank accession numbers for the gene sequences generated and analyzed in this study.

Taxon	Voucher	Locality (Country, River/Drainage)	GenBank Accession Number			
			co1	*cyt-b*	*nd2*	*myh6*
OUTGROUP						
Hypostominae						
Ancistrus macropthalmus	ANSP 185296	Venezuela, Orinoco	TBA	TBA	TBA	TBA
Pseudacanthicus leopardus	ANSP 179613	Guyana, Rupununi	TBA	TBA	TBA	TBA
Hypoptopomatinae						
Acestridium martini	ANSP 182901	Venezuela, Guapuchi	TBA	TBA	N/A	TBA
Hisonotus maculipinnis	ANSP 180680	Argentina, Paraná	TBA	TBA	TBA	TBA
Hypoptopoma guianense	ANSP 180667	Guyana, Simoni	TBA	TBA	TBA	TBA
INGROUP						
Loricariinae						
Apistoloricaria ommation	ANSP 182331	Peru, Amazonas	TBA	TBA	TBA	TBA
Apistoloricaria ommation	ANSP 182331	Peru, Amazonas	TBA	TBA	TBA	TBA
Crossoloricaria bahuaja	ANSP 180559	Peru, Madre de Dios	TBA	TBA	TBA	TBA
Crossoloricaria bahuaja	ANSP 180793	Peru, Manuripe	TBA	TBA	TBA	TBA
Crossoloricaria bahuaja	ANSP 180793	Peru, Manuripe	TBA	TBA	TBA	TBA
Crossoloricaria bahuaja	MUSM uncataloged	Peru, Yanamayo	TBA	TBA	TBA	TBA
Cteniloricaria maculata	ANSP 187330	Suriname, Lawa	TBA	TBA	TBA	TBA
Cteniloricaria maculata	ANSP 189122	Suriname, Litanie	TBA	TBA	TBA	TBA
Cteniloricaria platystoma	ANSP 182390	Guyana, Essequibo	TBA	TBA	TBA	TBA
Cteniloricaria platystoma	ANSP 182390	Guyana, Essequibo	TBA	TBA	TBA	TBA
Dasyloricaria cf. seminuda	CZUT-IC uncataloged	Colombia, Sogamoso	TBA	TBA	TBA	TBA
Dasyloricaria cf. seminuda	CZUT-IC uncataloged	Colombia, Sogamoso	TBA	TBA	TBA	TBA
Dasyloricaria cf. seminuda	CZUT-IC uncataloged	Colombia, Sogamoso	TBA	TBA	TBA	TBA
Dasyloricaria cf. seminuda	CZUT-IC uncataloged	Colombia, Sogamoso	TBA	TBA	TBA	TBA
Farlowella hahni	ANSP 181010	Argentina, Paraná	TBA	TBA	TBA	TBA
Farlowella nattereri	ANSP 179764	Guyana, Essequibo	TBA	TBA	TBA	TBA
Farlowella nattereri	ANSP 179766	Guyana, Simoni	TBA	TBA	TBA	TBA
Farlowella nattereri	MUSM ???	Peru, Madre de Dios	TBA	TBA	TBA	TBA
Farlowella nattereri	ANSP 180548	Peru, Manuripe	TBA	TBA	TBA	TBA
Farlowella nattereri	ANSP 180548	Peru, Manuripe	TBA	TBA	TBA	TBA
Farlowella nattereri	ANSP 182550	Peru, Nanay	TBA	TBA	TBA	TBA
Farlowella oxyrryncha	ANSP 179765	Guyana, Yuora	TBA	TBA	TBA	TBA
Farlowella oxyrryncha	ANSP 179765	Guyana, Yuora	TBA	TBA	TBA	TBA
Farlowella platorynchus	ANSP 179841	Peru, Itaya	TBA	TBA	TBA	TBA
Farlowella platorynchus	ANSP 178394	Peru, Yanayacu	TBA	TBA	TBA	TBA
Farlowella rugosa	ANSP 179768	Guyana, Simoni	TBA	TBA	TBA	TBA
Farlowella smithi	ANSP 180541	Peru, Manuripe	TBA	TBA	TBA	TBA
Furcodontichthys novaesi	ANSP 193314	Brazi, Tapajos	TBA	TBA	TBA	TBA
Harttia guianensis	ANSP 187328	Suriname, Lawa	TBA	TBA	TBA	TBA
Harttia guianensis	ANSP 187328	Suriname, Lawa	TBA	TBA	TBA	TBA
Hemiodontichthys acipenserinus	ANSP 182700	Peru, Itaya	TBA	TBA	TBA	TBA
Lamontichthys filamentosus	ANSP 181093	Peru, Amazonas	TBA	TBA	TBA	TBA
Lamontichthys filamentosus	ANSP 181100	Peru, Amazonas	TBA	TBA	TBA	TBA
Lamontichthys stibaros	ANSP 180635	Peru, Inambari	TBA	TBA	TBA	TBA
Limatulichthys griseus	ANSP 182351	Guyana, Essequibo	TBA	TBA	TBA	TBA
Limatulichthys griseus	ANSP 182707	Peru, Itaya	TBA	TBA	TBA	TBA
Limatulichthys sp. nov.	ANSP 193313	Brazi, Tapajos	TBA	TBA	TBA	TBA

Limatulichthys sp. nov.	ANSP 193312	Brazi, Xingu	TBA	TBA	TBA	TBA
Limatulichthys sp. nov.	ANSP 199647	Brazil, Iriri	TBA	TBA	TBA	TBA
Limatulichthys sp. nov.	ANSP 199647	Brazil, Iriri	TBA	TBA	TBA	TBA
Loricaria clavipinna	ANSP 178472	Peru, Itaya	TBA	TBA	TBA	TBA
Loricaria clavipinna	ANSP 182607	Peru, Nanay	TBA	TBA	TBA	TBA
Loricaria holmbergi	ANSP 182408	Argentina, Paraná	TBA	TBA	TBA	TBA
Loricaria simillima	ANSP 180498	Peru, Inambari	TBA	TBA	TBA	TBA
Loricaria simillima	ANSP 180509	Peru, Madre de Dios	TBA	TBA	TBA	TBA
Loricaria simillima	ANSP 180508	Peru, Manuripe	TBA	TBA	TBA	TBA
Loricaria simillima	ANSP 180508	Peru, Manuripe	TBA	TBA	TBA	TBA
Loricaria simillima	AUM uncataloged	Peru, Tahuamanu	TBA	TBA	TBA	TBA
Loricaria sp.	ANSP 182364	Guyana, Yuora	TBA	TBA	TBA	TBA
Loricaria sp.	ANSP 182364	Guyana, Yuora	TBA	TBA	TBA	TBA
Loricariichthys labialis	ANSP 181013	Argentina, Paraná	TBA	TBA	TBA	TBA
Loricariichthys microdon	ANSP 182371	Guyana, Rupununi	TBA	TBA	TBA	TBA
Loricariichthys microdon	ANSP 182379	Guyana, Rupununi	TBA	TBA	TBA	TBA
Loricariichthys platymetopon	ANSP 181009	Argentina, Paraná	TBA	TBA	TBA	TBA
Loricariichthys platymetopon	ANSP 182409	Argentina, Paraná	TBA	TBA	TBA	TBA
Loricariichthys sp.	ANSP 182568	Peru, Nanay	TBA	TBA	TBA	TBA
Metaloricaria paucidens	ANSP 187325	Suriname, Lawa	TBA	N/A	TBA	TBA
Metaloricaria paucidens	ANSP 187325	Suriname, Lawa	TBA	N/A	TBA	TBA
Metaloricaria paucidens	ANSP 187325	Suriname, Lawa	TBA	N/A	TBA	TBA
Planiloricaria cryptodon	ANSP 182304	Peru, Amazonas	TBA	TBA	TBA	TBA
Planiloricaria cryptodon	ANSP 182735	Peru, Amazonas	TBA	TBA	TBA	TBA
Pseudohemiodon apithanos	ANSP 178115	Peru, Amazonas	TBA	N/A	TBA	TBA
Pseudohemiodon sp. nov.	ANSP 182751	Peru, Amazonas	TBA	TBA	TBA	TBA
Pseudohemiodon sp. nov.	INHS 52730	Peru, Amazonas	TBA	TBA	TBA	TBA
Pseudohemiodon sp. nov.	SIUC uncataloged	Peru, Napo	TBA	TBA	TBA	TBA
Pseudoloricaria laeviuscula	MZUSP 110817	Brazil	TBA	TBA	TBA	TBA
Reganella depressa	ANSP 193316	Brazi, Tapajos	TBA	TBA	TBA	TBA
Reganella depressa	ANSP 193315	Brazi, Xingu	TBA	TBA	TBA	TBA
Rhadinoloricaria sp. nov.	ANSP 185044	Venezuela, Orinoco	TBA	TBA	TBA	TBA
Rhadinoloricaria macromystax	ANSP 182349	Guyana, Ireng	TBA	N/A	TBA	TBA
Rineloricaria fallax	ANSP 182363	Guyana, Rupununi	TBA	TBA	TBA	TBA
Rineloricaria fallax	ANSP 182381	Guyana, Rupununi	TBA	TBA	TBA	TBA
Rineloricaria formosa	ANSP 185291	Venezuela, Orinoco	TBA	TBA	TBA	TBA
Rineloricaria cf. formosa	AUM 40561?	Venezuela, Orinoco	TBA	TBA	TBA	TBA
Rineloricaria cf. formosa	AUM 40561?	Venezuela, Orinoco	TBA	TBA	TBA	TBA
Rineloricaria lanceolata	ANSP 182387	Guyana, Simoni	N/A	TBA	TBA	TBA
Rineloricaria lanceolata	ANSP 182382	Guyana, Yuora	N/A	TBA	TBA	TBA
Rineloricaria lanceolata	MUSM 39306	Peru (Southeast)	TBA	TBA	TBA	TBA
Rineloricaria lanceolata	ANSP 180684	Peru, Madre de Dios	TBA	TBA	TBA	TBA
Rineloricaria cf. lanceolata	INHS 52029	Peru, Amazonas	TBA	TBA	TBA	TBA
Rineloricaria parva	ANSP 182407	Argentina, Paraná	TBA	TBA	TBA	TBA
Rineloricaria parva	ANSP 182407	Argentina, Paraná	TBA	TBA	TBA	TBA
Rineloricaria cf. phoxocephala	ANSP 182695	Peru, Itaya	TBA	TBA	TBA	TBA
Rineloricaria platyura	ANSP 182376	Guyana, Ireng	TBA	TBA	TBA	TBA
Rineloricaria platyura	ANSP 182374	Guyana, Simoni	TBA	TBA	TBA	TBA
Rineloricaria sp.	ANSP 182368	Guyana, Essequibo	TBA	TBA	TBA	TBA
Rineloricaria sp.	MUSM 33819	Peru (Southeast)	TBA	TBA	TBA	TBA
Rineloricaria sp.	MUSM uncataloged	Peru, Yanamayo	TBA	TBA	TBA	TBA
Rineloricaria sp.	ANSP 191026	Venezuela, Orinoco	TBA	TBA	TBA	TBA
Spatuloricaria aff. caquetae	MHNG 2710.050	Peru, Huallaga	TBA	TBA	TBA	TBA
Spatuloricaria evansii	LBP 11507	Brazil, Araguaia	TBA	TBA	TBA	TBA

Spatuloricaria evansii	LBP 16145	Brazil, Araguaia	TBA	TBA	TBA	TBA
Spatuloricaria evansii	ANSP 180789	Peru, Urubamba	TBA	TBA	TBA	TBA
Spatuloricaria evansii	ANSP 180789	Peru, Urubamba	TBA	TBA	TBA	TBA
Spatuloricaria evansii	ANSP 180486	Peru, Yanatili	TBA	TBA	TBA	TBA
Spatuloricaria gymnogaster	IAVH uncataloged	Colombia, Magdalena	TBA	TBA	TBA	TBA
Spatuloricaria gymnogaster	IAVH uncataloged	Colombia, Magdalena	TBA	TBA	TBA	TBA
Spatuloricaria gymnogaster	IAVH uncataloged	Colombia, Magdalena	TBA	TBA	TBA	TBA
Spatuloricaria gymnogaster	uncataloged	Colombia, Sogamoso	TBA	TBA	TBA	TBA
Spatuloricaria gymnogaster	uncataloged	Colombia, Sogamoso	TBA	TBA	TBA	TBA
Spatuloricaria sp. nov.	INPA	Brazi, Xingu	TBA	TBA	TBA	TBA
Spatuloricaria sp. nov.	INPA	Brazil, Iriri	TBA	TBA	TBA	TBA
Spatuloricaria sp. nov.	ANSP 193005	Brazil, Iriri	TBA	TBA	TBA	TBA
Spatuloricaria sp. nov.	ANSP 182372	Guyana, Ireng	TBA	TBA	TBA	TBA
Spatuloricaria sp. nov.	AUM 35797	Guyana, Ireng	TBA	TBA	TBA	TBA
Spatuloricaria sp. nov.	MHNG 2677.071	Peru, Nanay	TBA	TBA	TBA	TBA
Spatuloricaria sp. nov.	ANSP 185303	Venezuela, Orinoco	TBA	TBA	TBA	TBA
Sturisoma nigrirostrum	ANSP 178322	Peru, Amazonas	TBA	TBA	TBA	TBA
Sturisoma nigrirostrum	ANSP 182587	Peru, Nanay	TBA	TBA	TBA	TBA
Sturisoma sp.	ANSP 182398	Guyana, Rupununi	TBA	TBA	TBA	TBA
Sturisoma sp.	ANSP 182397	Guyana, Yuora	TBA	TBA	TBA	TBA

*Institutional abbreviations [*sensu* Sabaj (2010)]: ANSP (Academy of Natural Sciences, Philadelphia, PA, USA), AUM (Auburn University Natural History Museum, Auburn, AL, USA), CZUT-IC (Colección Zoológica, Facultad de Ciencias, Universidad del Tolima, Ictiología, Ibagué, Colombia), IAVH (Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Villa de Leyva, Colombia), INHS (Illinois Natural History Survey, University of Illinois, Champaign, IL, USA), INPA (Instituto Nacional de Pesquisas da Amazônia, Manaus, Brazil), LBP (Laboratório de Biologia e Genética de Peixes, Departamento de Morfologia da Universidade Estadual Paulista, Botucatu, São Paulo, Brazil), MHNG (Muséum d'histoire naturelle, Genève, Switzerland), MUSM (Museo de Historia Natural "Javier Prado" de la Universidad Nacional Mayor de San Marcos, Lima, Peru), MZUSP (Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil).

Table 3. A proposed classification scheme for the Loricariinae, primarily based on the results of this study, but also considering some of the findings from previous phylogenetic and taxonomic studies of the subfamily.

- Subfamily Loricariinae
  - Tribe Harttiini
    - *Harttia*
    - *Harttiella*
    - *Cteniloricaria*
  - Tribe Loricariini
    - Subtribe Metaloricariina
      - *Metaloricaria*
    - Subtribe Farlowellina
      - *Aposturisoma*[1]
      - *Farlowella*
      - *Lamontichthys*
      - *Pterosturisoma*[1]
      - *Sturisoma*
      - *Sturisomatichthys*[1]
    - Subtribe Dasyloricariina
      - *Dasyloricaria*
    - Subtribe Rineloricariina
      - *Rineloricaria*
      - *Ixinandria*[1, 2]
      - *Fonchiiloricaria*[1]
    - Subtribe Loricariichthyna
      - *Furcodontichthys*
      - *Hemiodontichthys*
      - *Loricariichthys*
      - *Limatulichthys*
      - *Pseudoloricaria*
      - *Reganella*
    - Subtribe Spatuloricariina
      - *Spatuloricaria*
    - Subtribe Loricariina
      - *Brochiloricaria*[1]
      - *Loricaria*
      - *Paraloricaria*[1]
      - *Ricola*[1]
    - Subtribe Planiloricariina
      - *Apistoloricaria*
      - *Crossoloricaria*
      - *Dentectus*[1]
      - *Planiloricaria*
      - *Pseudohemiodon*
      - *Pyxiloricaria*[1]
      - *Rhadinoloricaria*

[1] Genera not included in the present study, and for which the proposed taxonomic placement is therefore based on previous phylogenetic and/or taxonomic studies.

6. FIGURES

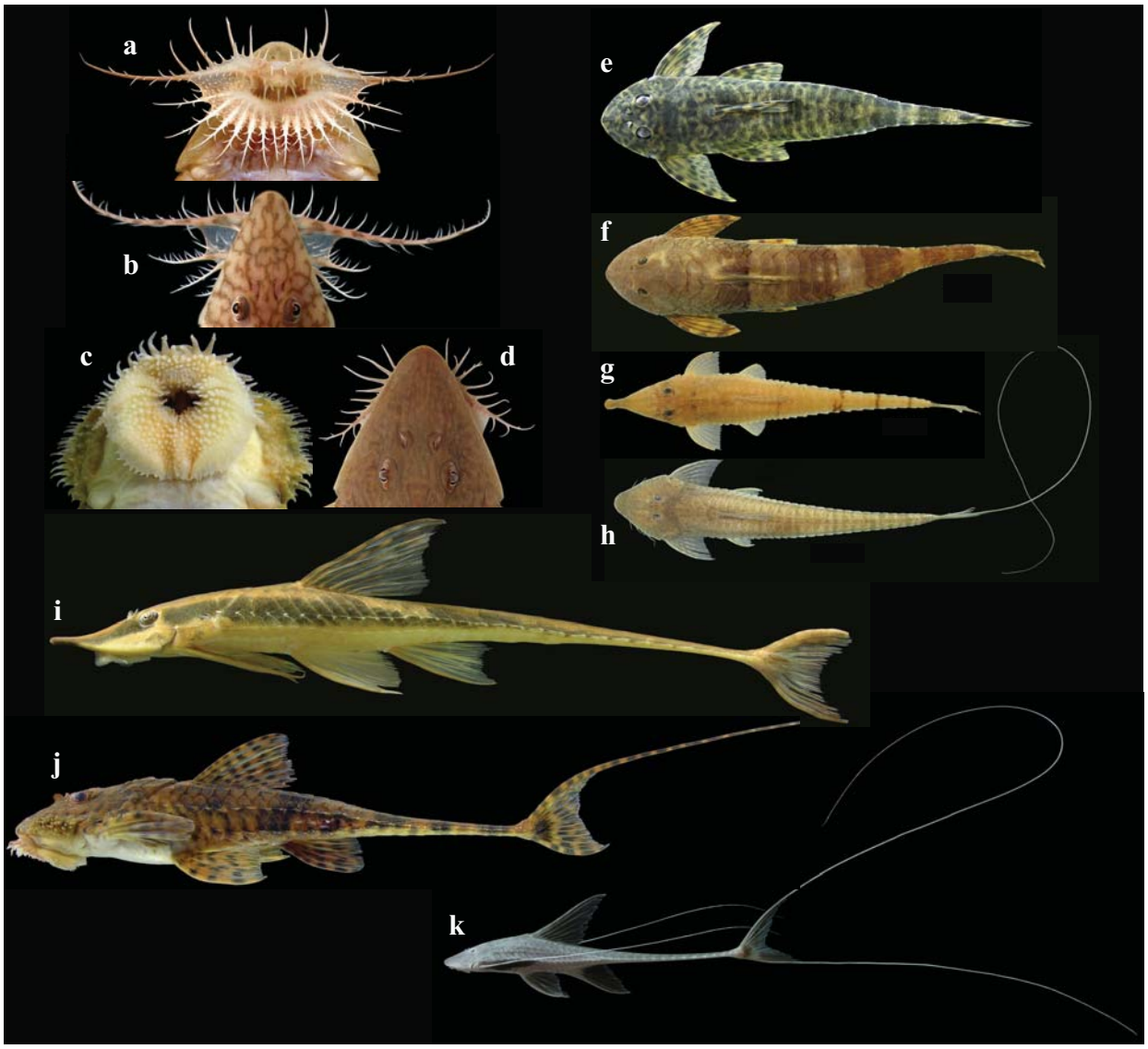

Figure 1. Morphological diversity in loricariin catfishes. Variation in lip morphology as seen in: *Crossoloricaria* (a), *Rhadinoloricaria* (b), *Spatuloricaria* (c), *Loricaria* (d). Variation in overall body shape as seen in: *Harttia* (e), *Fonchiiloricaria* (f), *Hemiodontichthys* (g), *Pseudohemiodon* (h), *Sturisoma* (i), *Spatuloricaria* (j), *Lamontichthys* (k).

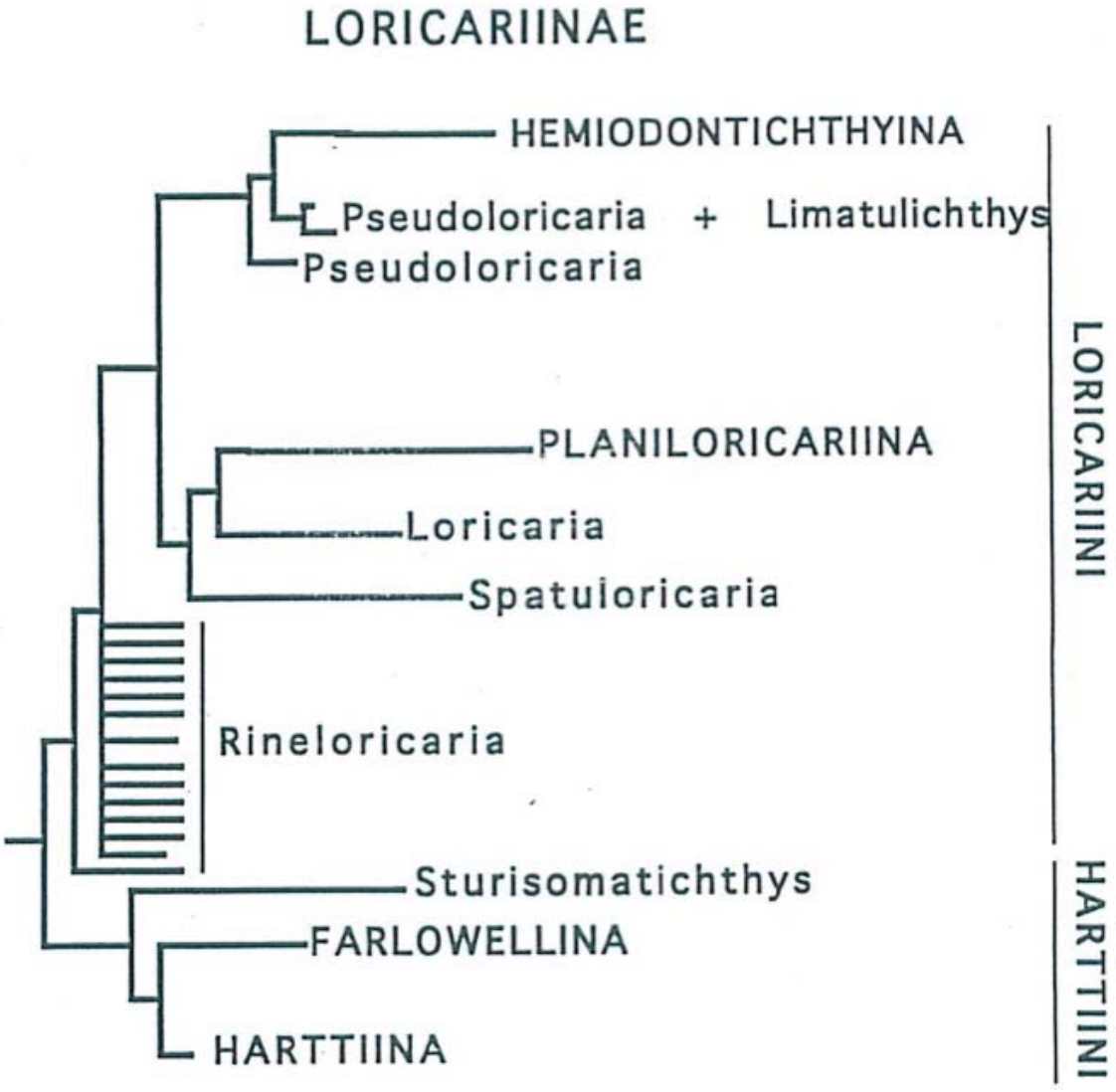

Figure 2. Hypothesis of loricariin intergeneric relationships as inferred from anatomical comparative data. Reprinted from Rapp Py-Daniel (1997).

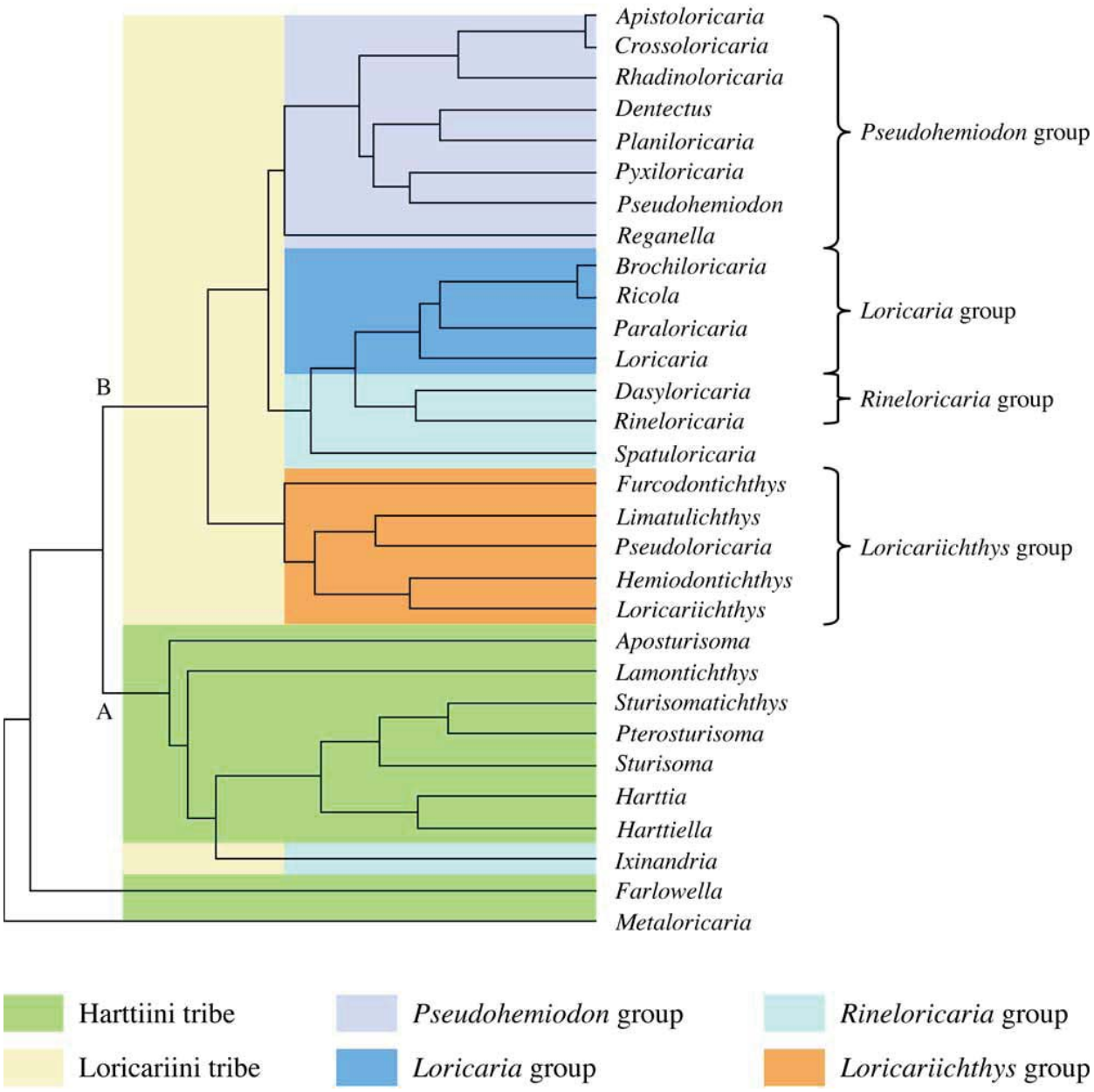

Figure 3. Classification scheme for the Loricariinae based on cluster analysis (UPGMA dendrogram) of external morphological variation. Reprinted from Covain and Fisch-Muller (2007).

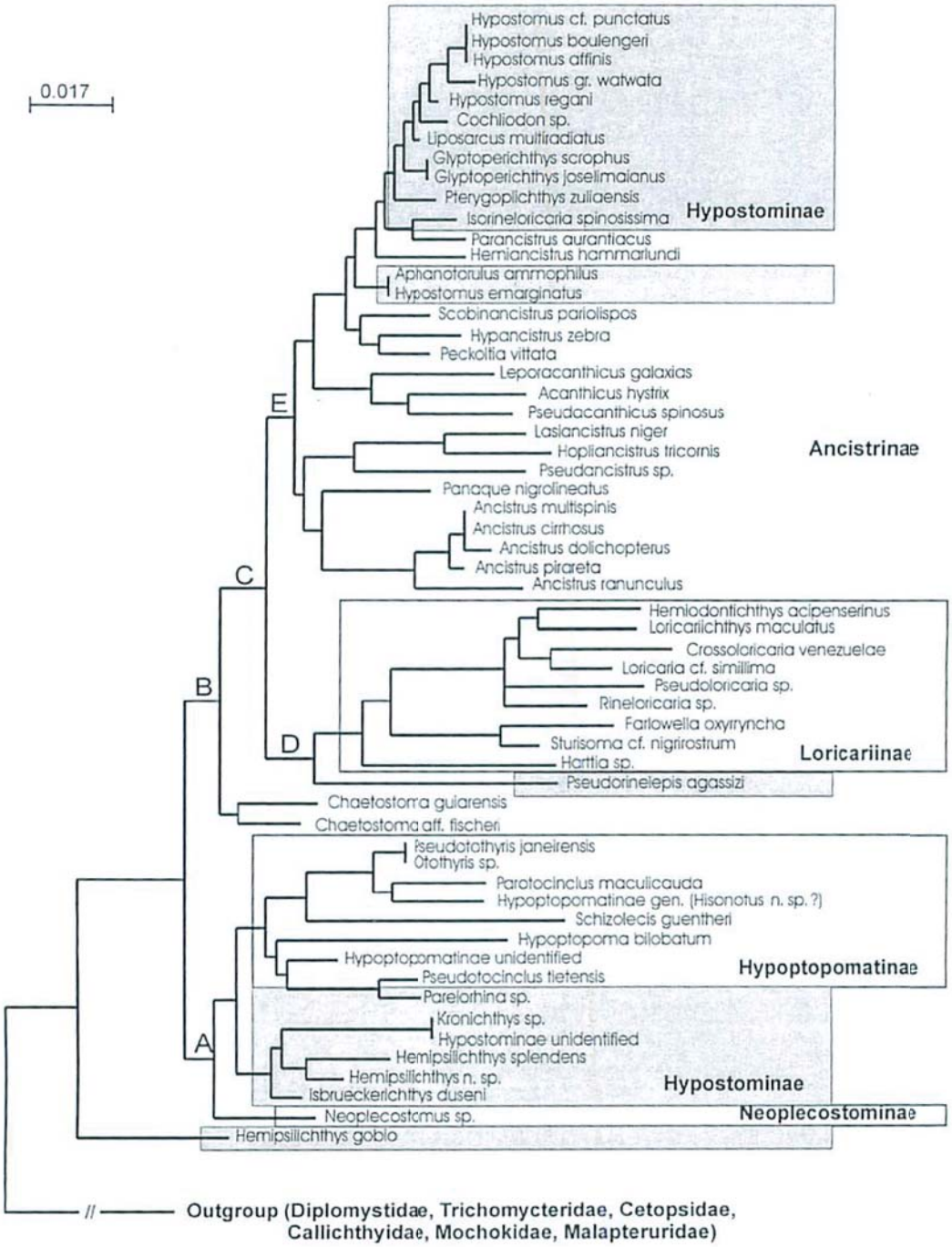

Figure 4. Loricariin relationships in the context of the first comprehensive molecular phylogeny of the Loricariidae. Reprinted from Montoya Burgos et al. (1998).

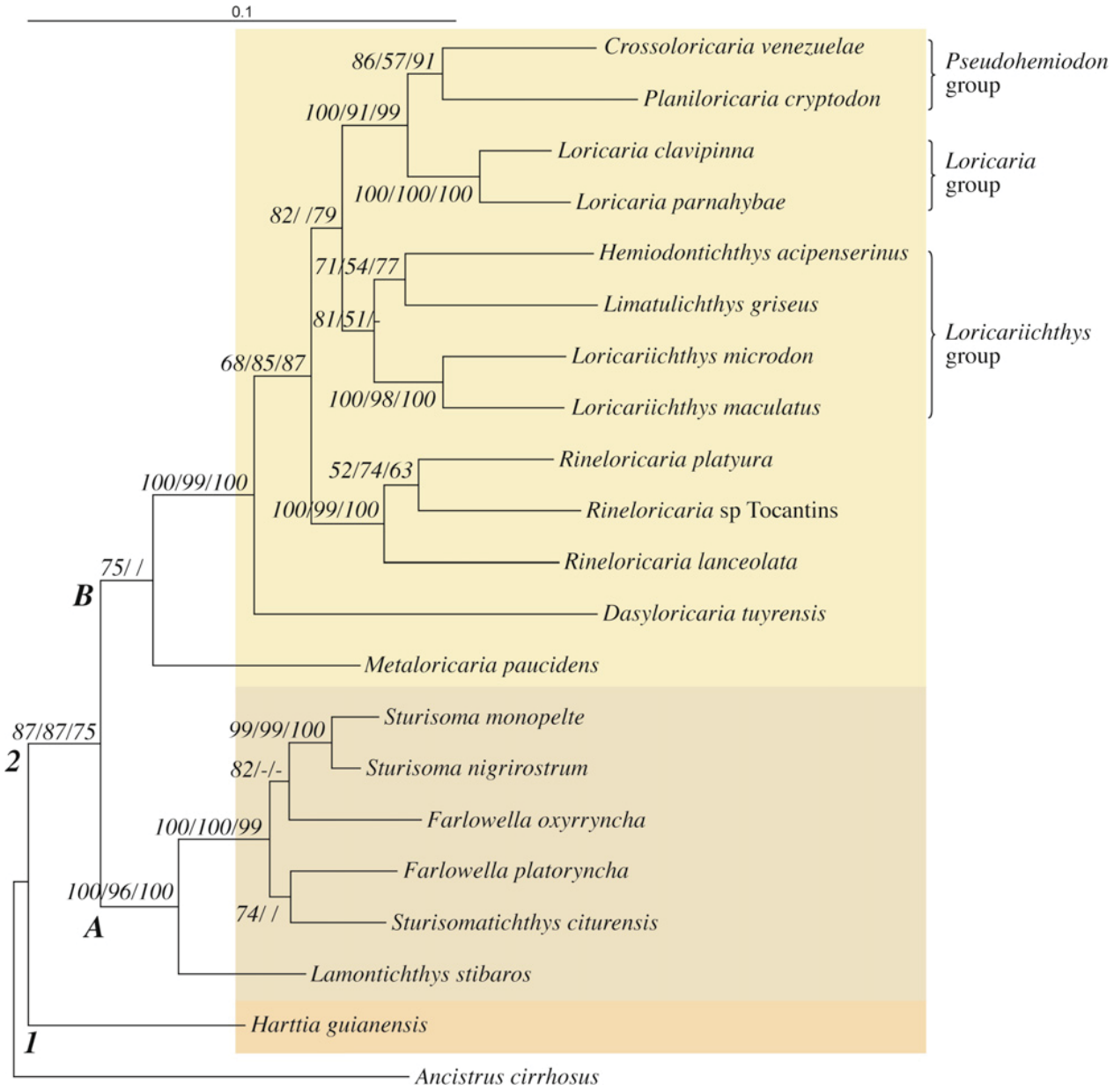

Figure 5. Hypothesis of loricariin intergeneric relationships as inferred from comparative DNA sequence data. Reprinted from Covain et al. (2008).

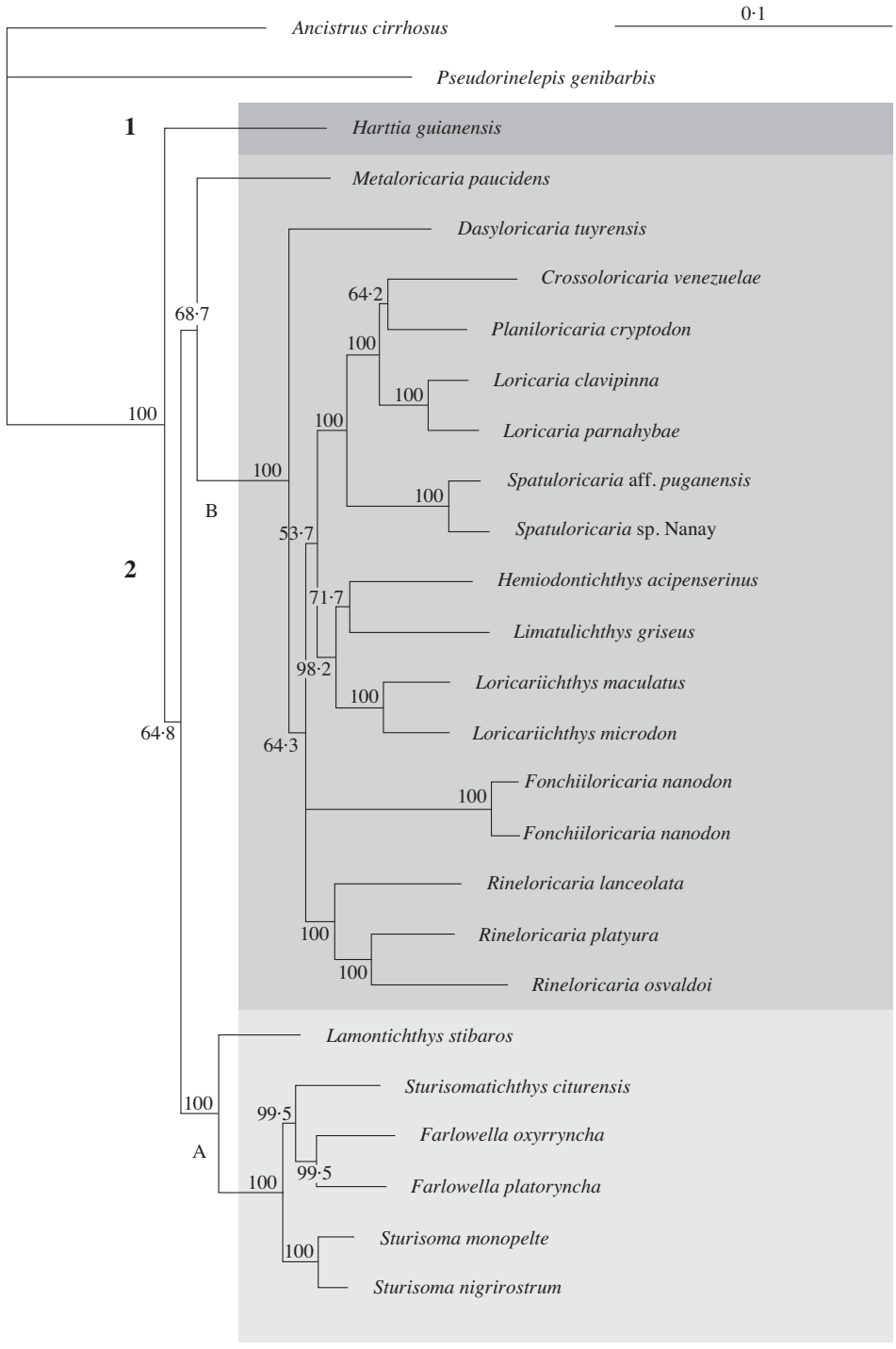

Figure 6. Molecular phylogeny of the Loricariinae as inferred from an expanded version of Covain et al.'s (2008) dataset. Reprinted from Rodriguez et al. (2011).

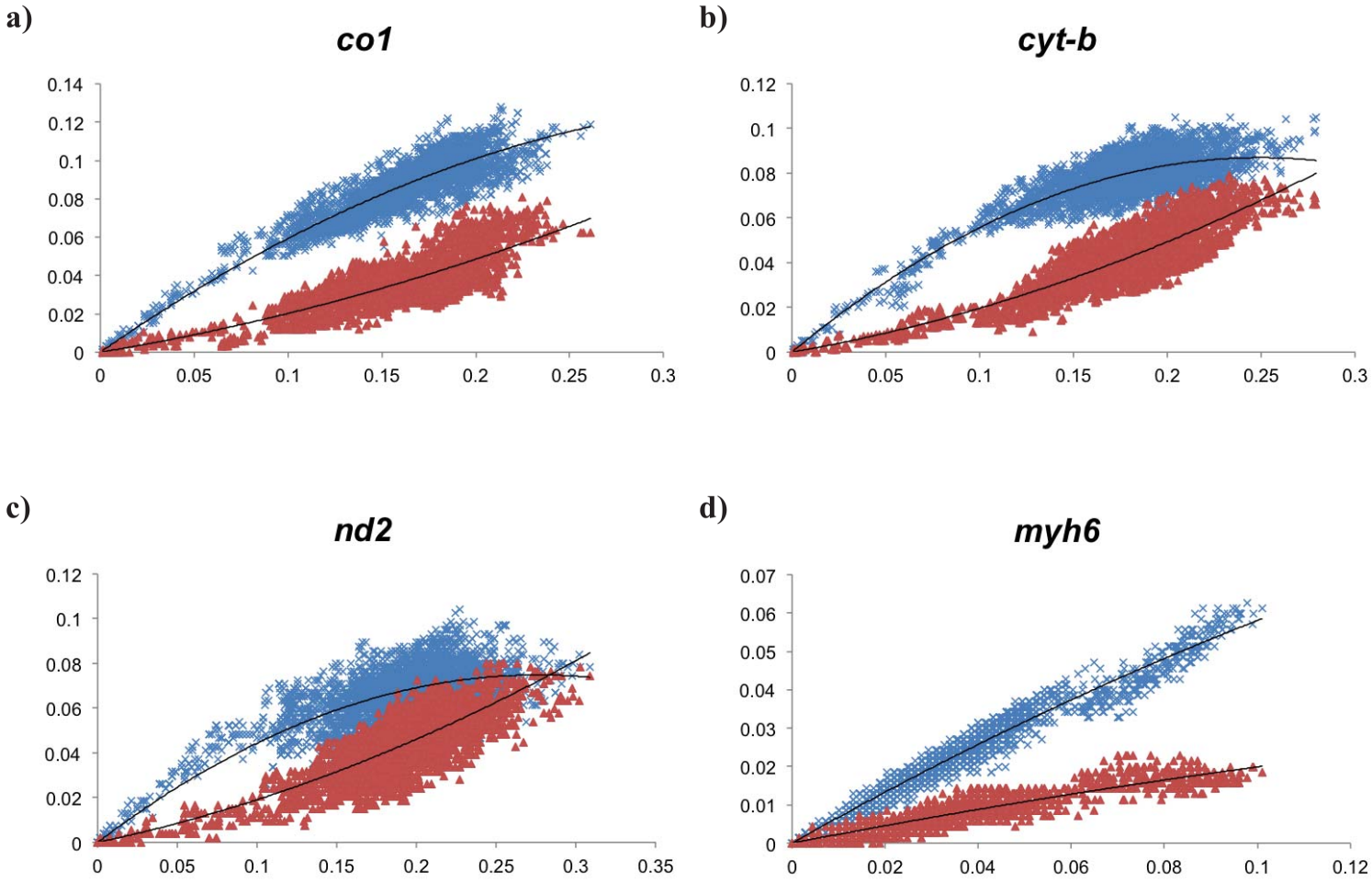

Figure 7. Scatterplots of observed number of transitions (×) and transversions (Δ) (x-axis) against corrected geneticdistance (y-axis) for third codon positions of each gene sampled in this study.

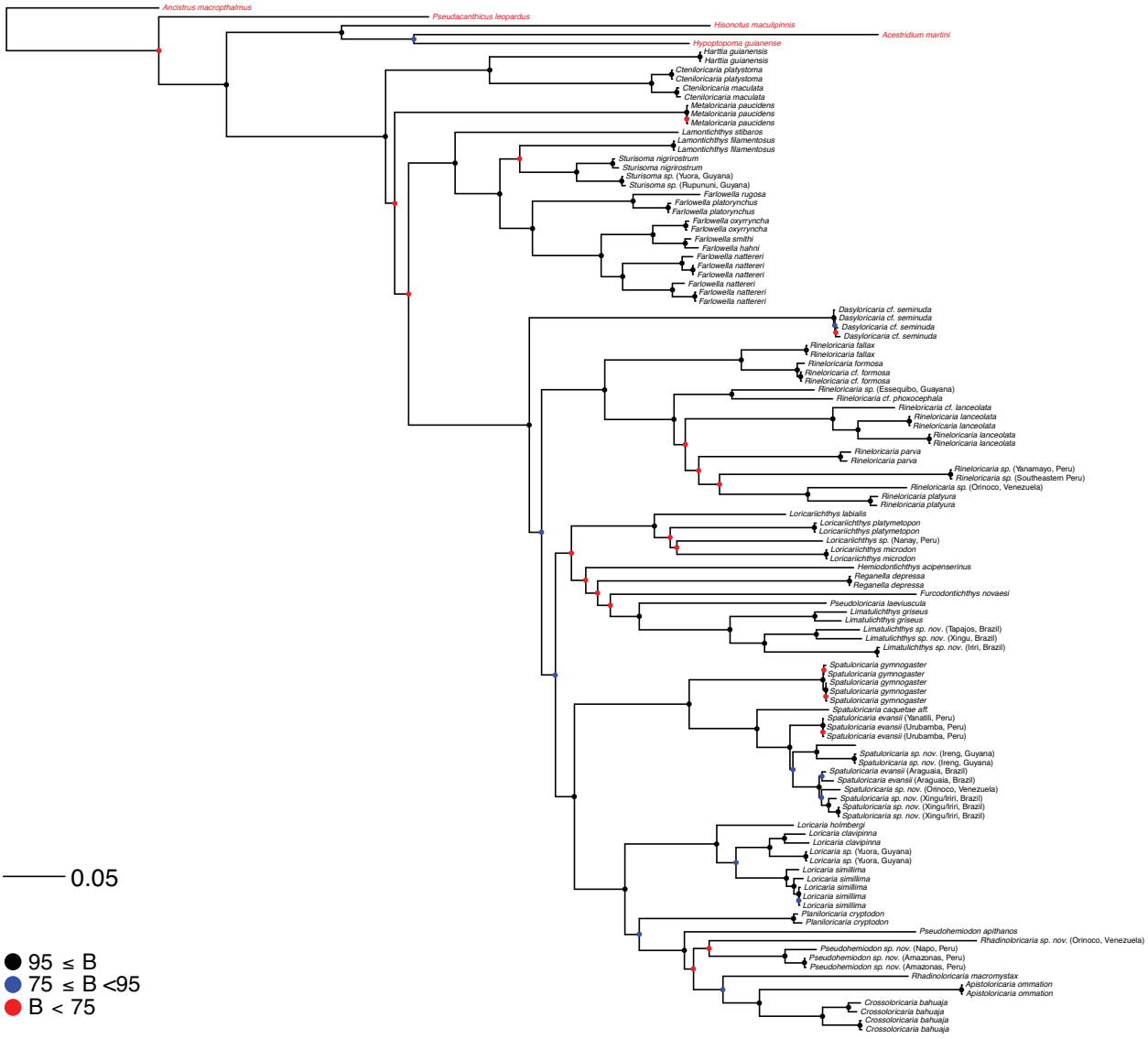

Figure 8. Phylogeny of the Loricariinae inferred from the molecular dataset of this study using Maximum Likelihood. Colored circles on nodes indicate degree of support as determined by bootstrap values (B).

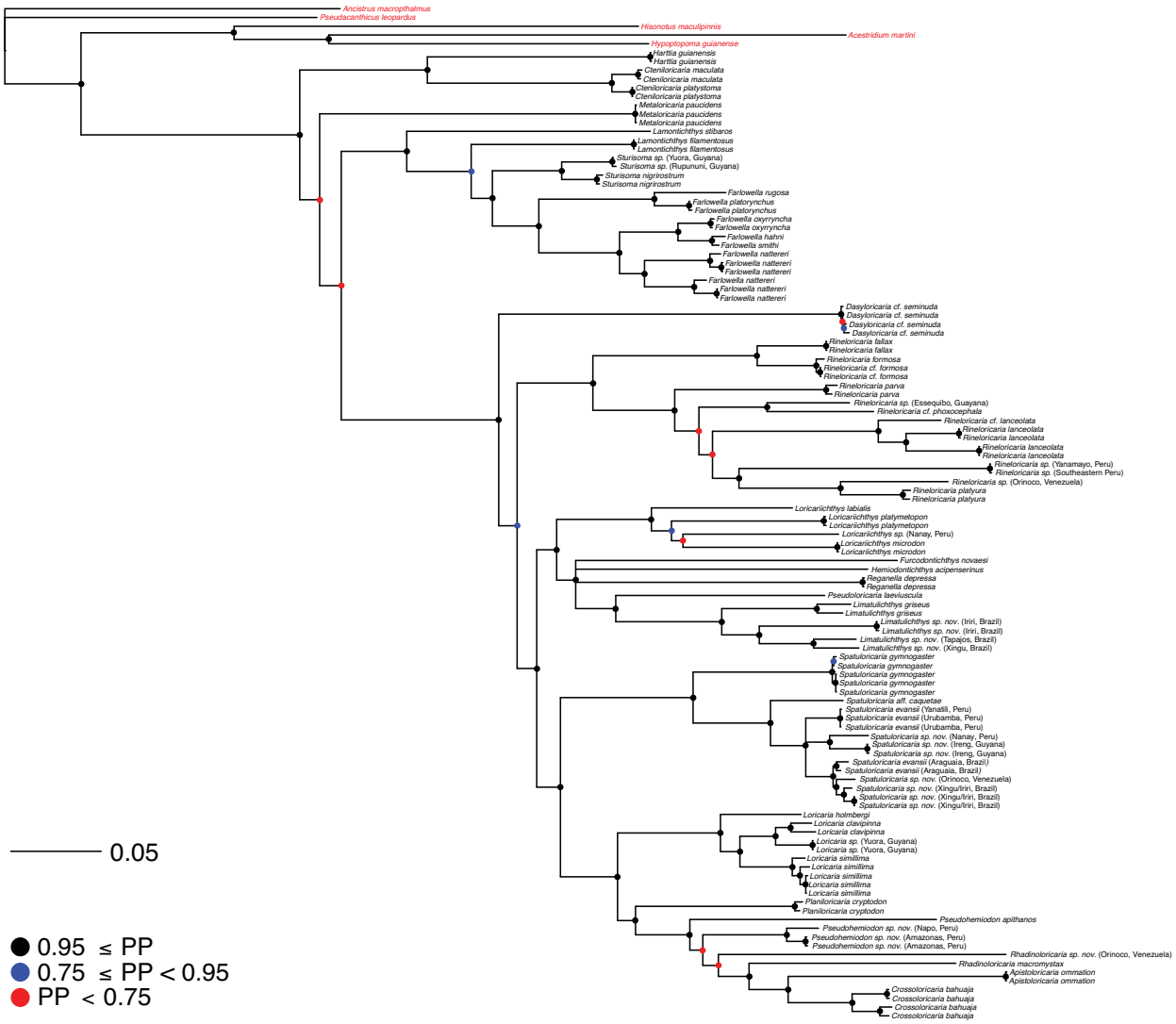

Figure 9. Phylogeny of the Loricariinae inferred from the molecular dataset of this study using MrBayes (BI). Colored circles on nodes indicate degree of support as determined by posterior probabilities (PP).

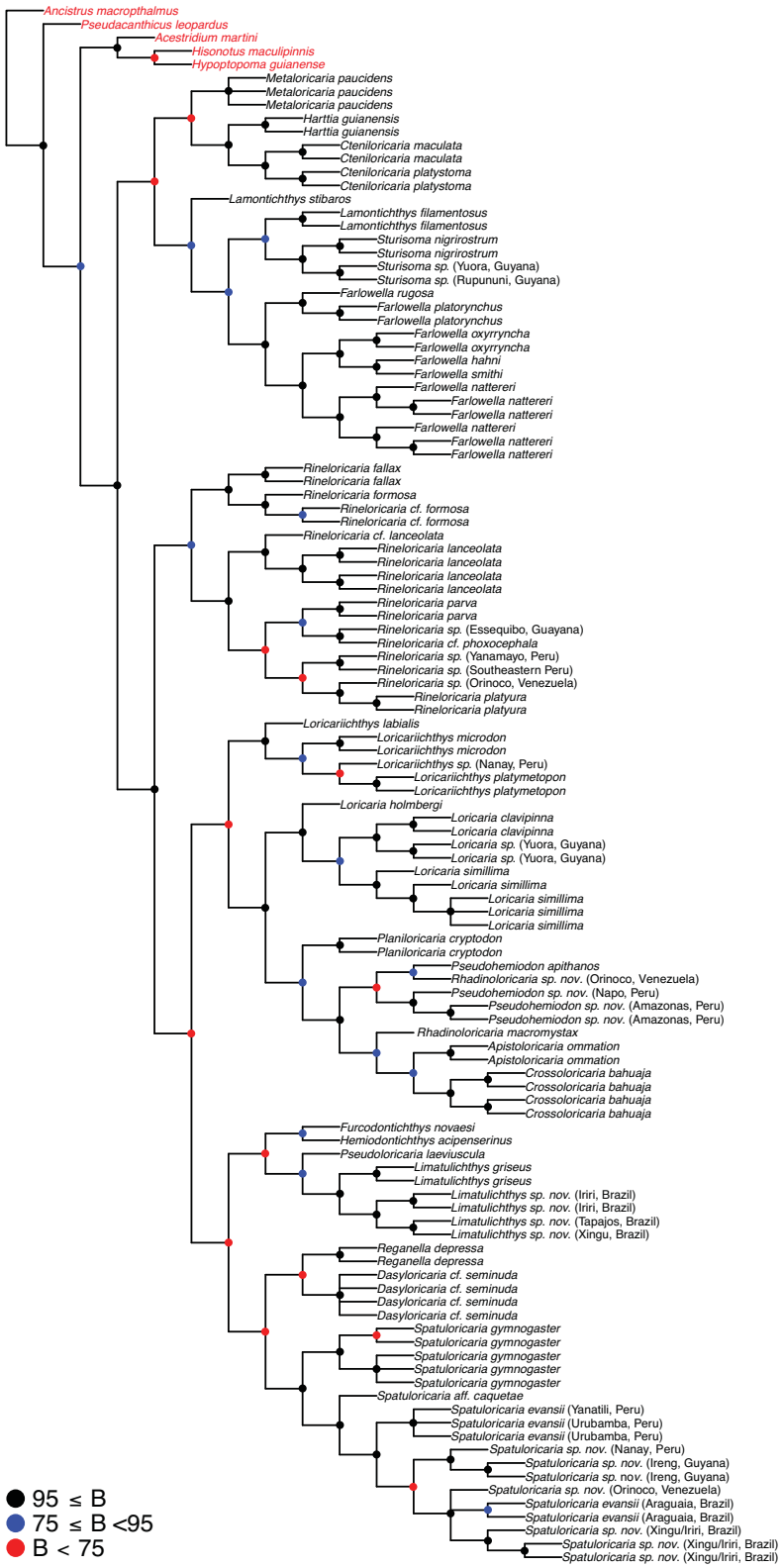

Figure 10. Phylogeny of the Loricariinae inferred from the molecular dataset of this study using parsimony (MP). Colored circles on nodes indicate degree of support as determined by bootstrap values (B).

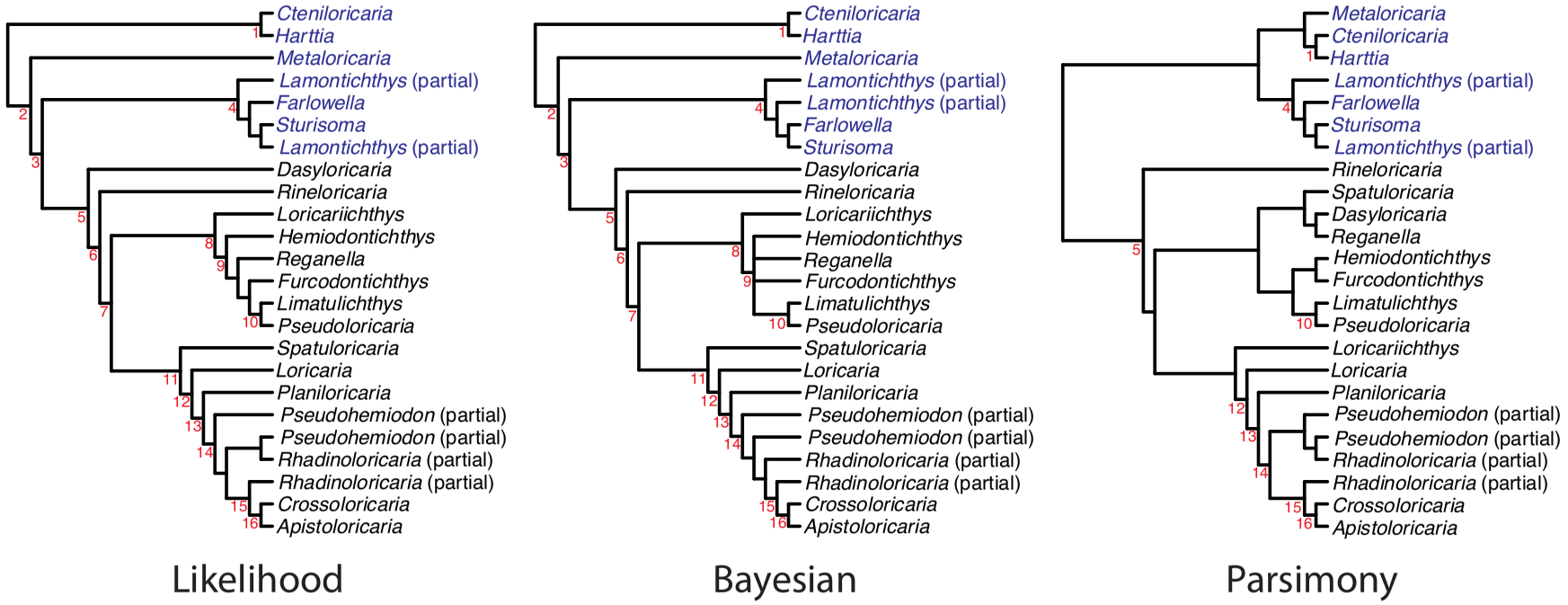

Figure 11. Loricariin intergeneric relationships as inferred from the molecular dataset of this study, contingent on the method of phylogenetic inference. Terminal taxa traditionally assigned to the tribes Harttiini and Loricariini are colored in blue and black, respectively. Suprageneric clades revealed by at least two inference methods are labeled with numbers in red.

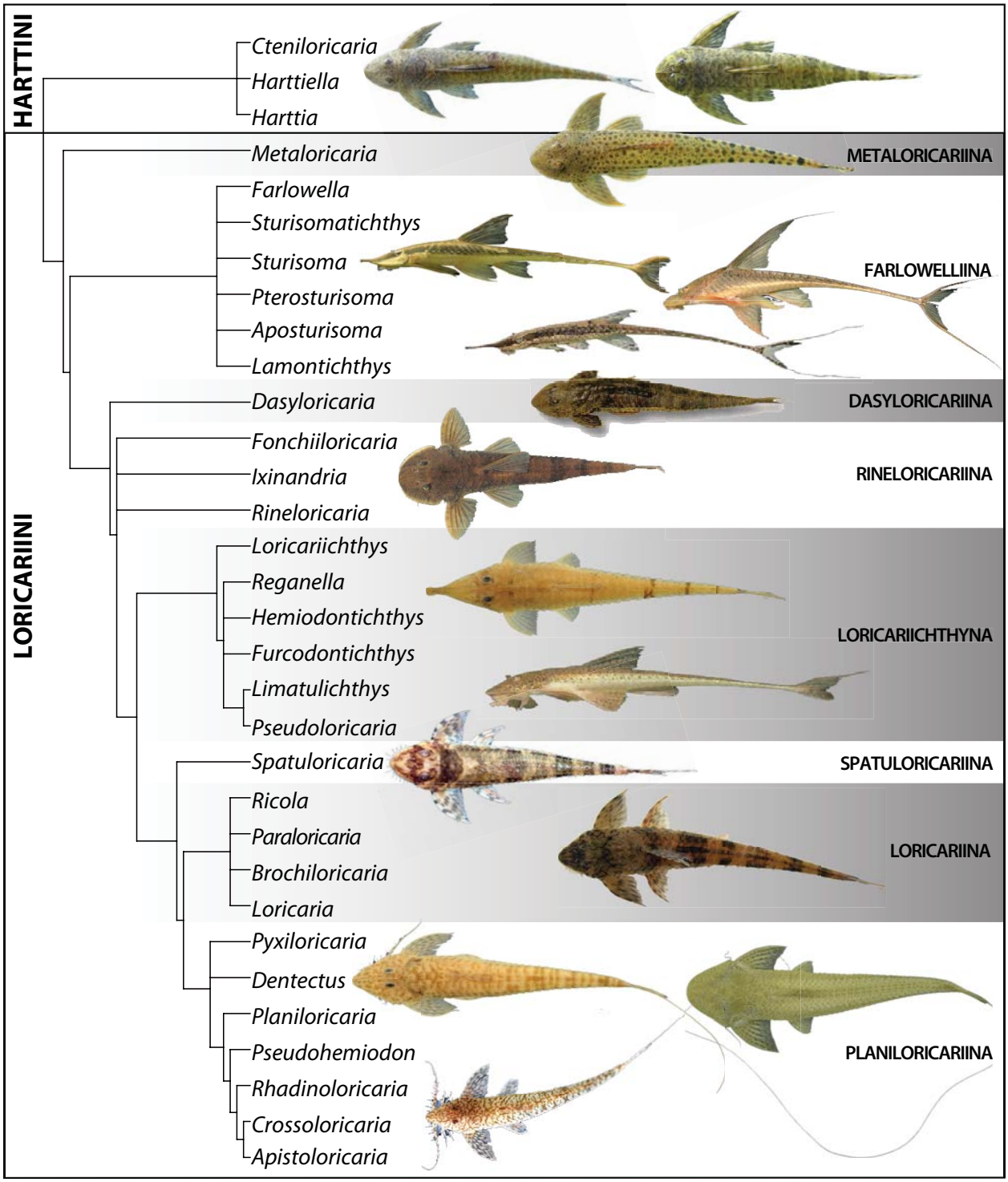

Figure 12. Hypothetical reconstruction of loricariin intergeneric relationships and proposed taxonomic ranks for suprageneric clades based on the available phylogenetic evidence, with emphasis on the model-based trees of the present study.

CHAPTER 6

CONCLUSIONS

1. INTRODUCTION

This concluding chapter offers a synthesis of the research presented in empirical chapters 2-5, which dealt with the systematics of four distinct groups of otophysian fishes: African characiforms of the family Alestidae and the suborder Citharinoidei, Afro-Asian cypriniforms of the tribe Chedrini, and Neotropical siluriforms of the subfamily Loricariinae.

The rationale for embarking on molecular phylogenetic studies of these four different lineages of otophysan fishes was mainly two-fold. First, otophysans as a whole are an ecologically and economically important group of fishes that, partly due to their extreme diversity (they comprise ~33% of all described fish species), remain poorly known in many aspects of their biology, including accurate estimates of the total number of species, and how these species are related to each other evolutionarily. Secondly, all four groups of otophysan fishes targeted for phylogenetic assessment in this dissertation represent taxa for which robust hypotheses of relationships were either lacking or in need of reappraisal with novel and independent data.

Accordingly, the main objective of this doctoral dissertation (and ultimately the common thread running through chapters 2-5) was to infer the evolutionary relationships between members of each of the abovementioned otophysan clades through phylogenetic analysis of comparative DNA sequence data. The resultant phylogenies provide an empirical basis for producing/updating classification schemes that reflect evolutionary history, and therefore for assessing the adequacy of current classifications and the identification of subgroups in critical need of future taxonomic research (e.g., patently non-monophyletic supraspecific assemblages). Likewise, the hypotheses of relationships arrived at in this doctoral dissertation provide a necessary framework for future evolutionary studies that employ phylogenetic comparative

methods such as the reconstruction of ancestral character states or the testing of adaptive radiations and key innovations hypotheses, among many others.

1.1. AFRICAN ALESTIDS

The Alestidae, with 121 species arrayed in 21 genera, is the most speciose of the four African-endemic characiform families. Although alestids are distributed throughout most of Africa, the majority of their diversity is centered in the Congo basin, where well-known species such as the giant African goliath tigerfish (*Hydrocynus goliath*) and the Congo tetra (*Phenocogrammus interruptus*) are found. Prior to the morphology-based phylogenetic study of Zanata and Vari (2005), the taxonomy of the family had been mostly artificial (i.e., not reflecting evolutionary relationships), with generic and suprageneric taxa of questionable monophyly and ambiguously diagnosed (Schaefer, 2007). Although a couple of studies had previously investigated alestid relationships (i.e., Murray and Stewart, 2002; Hubert et al., 2005), both suffered from considerably incomplete taxon sampling. In their monumental contribution to alestid systematics, Zanata and Vari (2005) proposed the novel—and somewhat unexpected—hypothesis that the exclusively Neotropical genus *Chalceus* is the sister taxon of the entire alestid radiation. This finding prompted them to include *Chalceus* in the Alestidae—making it the only transatlantic characiform family—and to attribute the split between *Chalceus* and the remainder alestids to vicariance driven by the Cretaceous fragmentation of Western Gondwana. Given the paucity of comprehensive hypotheses of relationships, and the lack of a focused molecular phylogeny of the family, a phylogenetic study of alestids based on DNA sequence data was both timely and desirable. Accordingly, the chapter of this doctoral dissertation on alestid systematics was primarily aimed at testing previous hypotheses of intergeneric relationships, the

monophyletic status of the family and its genera, and the phylogenetic placement of the genus *Chalceus*.

1.2. AFRICAN CITHARINOIDS

The suborder Citharinoidei comprises the African-endemic and reciprocally monophyletic families Citharinidae and Distichodontidae. Like alestids, both citharinoid families are distributed throughout much of tropical Africa, with species richness heavily concentrated in the freshwaters of West-Central Africa. Also, as in the case of alestids, citharinoids had yet to be comprehensively investigated phylogenetically using comparative DNA sequence data. For citharinoids, however, the situation was more critical, since only a single study—conducted more than 30 years ago—had investigated citharinoid relationships using cladistic methods (i.e., Vari, 1979). While the work of Vari (1979) remains the most thorough compendium of citharinoid comparative anatomy and its ensuing phylogenetic hypothesis, his conclusions had yet to be tested with novel and independent data. Because citharinoid relationships had not been examined beyond the morphology-based phylogeny of Vari (1979), a comprehensive molecular phylogeny was necessary to further investigate the systematics of citharinoids and to test the limits and composition of generic and suprageneric assemblages in which species of the suborder are classified.

1.3. AFRO-ASIAN CHEDRINS

The tribe Chedrini (subtribe Chedrina *sensu* Liao et al., 2011a; 2012), one of the three major clades that form the species-rich cyprinid subfamily Danioninae, is a very speciose taxon that comprises over a hundred species classified in 18 genera (Liao et al., 2012). While the other two

tribal clades of the Danioninae (i.e., Danionini and Rasborini) are restricted to South and Southeast Asia, chedrins have a transoceanic distribution with representation in both Asian and African freshwaters, which makes them an interesting group from a historical biogeographic perspective. The current taxonomy of chedrins, however, does not reflect evolutionary relationships, and is therefore in critical need of reassessment. Numerous genera have problematic diagnoses and/or appear to be non-monophyletic. The most recent and comprehensive phylogenetic studies of the Danioninae (Tang et al., 2010; Liao et al., 2011a) strongly support the monophyly of the Chedrini and also suggest that African members of the tribe form a monophyletic group. However, despite recent efforts to elucidate phylogenetic relationships in danionin cyprinids, a comprehensive hypothesis of chedrin relationships had yet to be proposed. This is partially because, prior to the present study, African members of the tribe had not been thoroughly sampled for phylogenetic analysis. One of the objectives of this doctoral dissertation was then to investigate the systematics of chedrin cyprinids by analyzing a considerably expanded version of Tang et al.'s (2010) DNA sequence data matrix so as to produce the largest, most comprehensive phylogeny of the tribe, with particular emphasis on African chedrins.

1.4. NEOTROPICAL LORICARIINS

The Loricariinae, with 234 species arrayed in 32 genera, is the second-most diverse subfamily of Neotropical catfishes of the family Loricariidae (Eschmeyer and Fong, 2013; Vera-Alcaraz et al. 2012). Among the groups of otophysan fishes investigated in this doctoral dissertation, the Loricariinae is probably the one with the most phylogenetic studies, which include both morphology- (Rapp Py-Daniel, 1997) and molecular-based (Montoya-Burgos,

1998; Covain et al., 2008; Rodriguez et al., 2011) analyses. However, despite the various efforts at resolving loricariin relationships, a comprehensive phylogeny of the Loricariinae has yet to be proposed, and the monophyletic status of most genera remains to be tested. Previous phylogenetic studies, particularly those using molecular data (Montoya-Burgos, 1998; Covain et al., 2008; Rodriguez et al., 2011), suffered from very incomplete taxon sampling. The chapter of this dissertation on loricariin systematics was therefore aimed at inferring the most taxonomically encompassing molecular phylogeny of the subfamily, one commensurate with the hypothesis of relationships proposed by Rapp Py-Daniel (1997) on the basis of morphological character data.

1.5. HISTORICAL BIOGEOGRAPHY AND THE TEMPORAL CONTEXT OF CHARACIFORM EVOLUTION

Fishes of the order Characiformes are a diverse and economically important clade of otophysans whose extant members are exclusively found in the freshwaters of Africa and the Neotropics. While their transatlantic distribution has been primarily attributed to the Early Cretaceous fragmentation of western Gondwana (Lundberg, 1993), prior to this doctoral dissertation vicariance had not been tested with temporal information beyond that contained in their meager fossil record. In fact, despite the growing popularity and refinement of analytical methods to infer time-scaled phylogenies from DNA sequence data, molecular clocks had never been used to investigate the timing of diversification in a group of characiform fishes. Such lack of time-scaled molecular phylogenies prompted one of the major components of this dissertation research: the implementation of molecular dating analyses to determine the temporal context of diversification in both alestids and citharinoids so as to shed some critical light on the poorly

known early biogeographic history of characiform fishes. Since members of the Citharinoidei constitute the sister lineage to the entire remaining Afro-Neotropical characiform radiation (Vari, 1979; Fink and Fink, 1981; Fink and Fink, 1996; Buckup, 1998; Calcagnotto et al., 2005; Near et al., 2012), information on the pattern and timing of citharinoid diversification was purposely used to test biogeographic hypotheses proposed to explain the Gondwanan distribution exhibited by extant members of the order.

2. SYNTHESIS OF EMPIRICAL FINDINGS

The main empirical findings of this doctoral dissertation are chapter specific. This section provides a synthesis of each chapter and its findings, including a general overview of the materials and methods used to answer the chapter-specific research questions stated in the preceding section.

2.1. SYSTEMATICS OF THE ALESTIDAE

Phylogenetic relationships within the family Alestidae were investigated using parsimony, maximum likelihood, and Bayesian approaches based on a molecular dataset that included both nuclear and mitochondrial markers. Multiple representatives of all but two of the recognized alestid genera were included, which allowed for testing previous hypotheses of intergeneric relationships and the monophyly of several genera. The phylogenetic position of the Neotropical genus *Chalceus* with respect to the family Alestidae was also examined. In order to understand the temporal context of alestid diversification, Bayesian methods of divergence time estimation using fossil data in the form of calibration priors were used to date the nodes of the phylogenetic tree. The resulting phylogeny rejected the monophyly of the family as currently recognized (Alestidae *sensu lato*) and revealed several instances of poly- and paraphyly among genera. The genus *Chalceus* was recovered well nested within Neotropical characiforms, thus rejecting the hypothesis that this taxon is sister to the remaining alestid radiation. The estimated mean divergence time for the alestid clade (Alestidae *sensu stricto*) was 54 Ma (95% HPD=63-49). The temporal context of alestid diversification as inferred from the molecular data set of this study is incongruent with the hypothesis proposed by Zanata and Vari (2005) that the origin of the family Alestidae predates the African-South American Drift-Vicariance event.

2.2. SYSTEMATICS OF THE CITHARINOIDEI

The systematics of citharinoid characiform fishes, with emphasis on the family Distichodontidae, was investigated by analyzing a multilocus dataset using parsimony and model-based methods of phylogenetic inference. The comparative DNA sequence data of this study consisted of seven protein-coding genes, comprising both mitochondrial and nuclear markers sampled for all valid citharinoid genera, with the exception of the monotypic *Citharidium*, *Citharinops*, and *Paraphago*, from which tissue samples were unavailable. In addition to inferring phylogenetic relationships, the temporal context of citharinoid diversification was investigated using a purely Bayesian approach to divergence time estimation, and variation in divergence-time estimates resulting from different calibration strategies was explored via sensitivity analysis. The resulting phylogenies corroborate the monophyly of the Citharinoidei, the Distichodontidae, and most distichodontid genera. Likewise, they provide support for the recognition of morphologically distinct suprageneric assemblages represented by well-supported clades. The present study provided the first opportunity to test the hypothesis of citharinoid relationships arrived at by Vari (1979) and, although derived from a different type of data and with only partially overlapping taxon sampling, the findings of this study are in general agreement with Vari's morphology-based phylogeny. The inferred chronogram is robust to changes in calibration priors and indicates that the origins of citharinoids and distichodontids date back to the Late- (ca. 90 Ma) and Mid-Cretaceous (ca. 67 Ma), respectively. Most modern distichodontid genera, however, appear to have originated and diversified much more recently, mainly during the Miocene. By reconciling molecular-clock- with fossil-based estimates for the origins of the Characiformes, these results provide further support for the hypothesis that attributes the disjunct distribution of the order to the mid-Cretaceous fragmentation of

Gondwana. Moreover, the striking overlap in tempo of diversification and biogeographic patterns between distichodontids and the African-endemic family Alestidae, suggests that their evolutionary histories were strongly and similarly influenced by Miocene geotectonic events that modified the landscape and produced the drainage pattern of Central Africa seen today.

2.3. SYSTEMATICS OF THE CHEDRINI

The systematics of Afro-Asian danionin cyprinids of the tribe Chedrini was investigated through phylogenetic analysis of a molecular dataset consisting of six protein-coding genes/gene fragments, including both nuclear and mitochondrial markers, sampled for all valid chedrin genera with the exception of *Bengala* (monotypic) and *Esomus*. Methods of phylogeny reconstruction comprised Maximum Likelihood and Bayesian Inference. The resultant phylogeny corroborated the notion that Asian members of the Chedrini constitute a paraphyletic assemblage (Tang et al., 2010; Liao et al. 2012), whereas African chedrins form a monophyletic group. The monophyly and derived phylogenetic placement of African members of the Chedrini suggests that the diversification of the tribe started in Asian freshwaters and that the lineage leading to the African chedrin radiation must have dispersed from Asia into the African continent, unless the unlikely event that chedrins had already diversified by the time when the fragmentation of East Gondwana led to the split between modern-day Somalia and the Indian subcontinent. Several instances of generic non-monophyly, such as in the Asian genera *Barilius* and *Opsarius*, the African genera *Leptocypris* and *Opsaridium*, and the Afro-Asian genus *Raiamas*, were revealed by the hypothesis of relationships arrived at in this study. In order to maintain a classification that accurately reflects phylogenetic relationships only taxon names of

monophyletic groups must be retained and therefore the taxonomy of these putatively non-monophyletic genera needs to be revised.

2.4. SYSTEMATICS OF THE LORICARIINAE

The systematics of suckermouth armoured catfishes of the subfamily Loricariinae was investigated via phylogenetic analysis of comparative DNA sequence data from 56 loricariin species representing 21 of the 32 currently valid genera. While not necessarily a comprehensive representation of loricariin generic diversity, this is the largest taxon sampling of any molecular phylogenetic study to date, and ultimately a commendable effort considering that a large number of loricariin genera are monotypic and/or geographically restricted. The molecular dataset of this study (four protein-coding genes, including both ncDNA and mtDNA, and totaling 3471 bp) was analyzed using both parsimony and model-based methods of phylogenetic inference. By recovering the tribes Harttiini and Loricariini as reciprocally monophyletic, the parsimony tree was the only to conform to the current tribal classification. Most of the discrepancies between parsimony and model-based topologies (particularly regarding intergeneric relationships) were associated with weakly supported nodes. Despite the instances of conflict between methods, the molecular dataset of this study strongly supported the monophyly of both the subfamily Loricariinae and the tribe Loricariini, regardless of optimality criterion. Although derived from only a partially overlapping taxon sampling, some of the results of this study (e.g., monophyly of the Loricariini, paraphyly of the Harttiini) are in general agreement with the most recently published molecular phylogenies of the group. Similarly, the findings of the present study are broadly congruent with the sole morphology-based phylogeny of the subfamily. The taxon sampling of this study allowed for testing monophyly in nine (*Cteniloricaria*, *Farlowella*,

Loricaria, *Lamontichthys*, *Loricariichthys*, *Pseudohemiodon*, *Rineloricaria*, *Spatuloricaria*, *Sturisoma*) of the 18 polytypic loricariin genera. Except for *Lamontichthys* and *Pseudohemiodon*, all of the aforementioned genera were strongly recovered as monophyletic regardless of analytical method. Whereas the results of this investigation offer a fundamental phylogenetic framework for testing previous hypotheses of relationships and assessing the adequacy of current classifications, expanded taxonomic coverage is necessary to provide a complete and more accurate picture of loricariin evolution. Likewise, the analysis of a combined morphological and molecular dataset may provide a more resolved and better-supported hypothesis of loricariin relationships.

3. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Whereas the systematics of alestids, chedrins, and loricariins had been previously investigated using molecules, the research on citharinoid systematics presented herein represents the first attempt at resolving phylogenetic relationships in this group of characiform fishes based on analysis of comparative DNA sequence data. Regardless of the scope and coverage of previous phylogenetic studies, this doctoral dissertation resulted in the most comprehensive hypotheses of relationships for the groups of otophysan fishes investigated. In addition to providing a phylogenetic framework needed to improve and clarify the taxonomy of the groups, the results of this research deliver a much clearer picture of their evolutionary histories with direct implications for future studies on the evolution of these particular taxa. However, much work remains to be done to fully document and classify the diversity of these groups, and to completely resolve the phylogenetic relationships among their constituent members. Although many of the relationships revealed by this study corroborated previous hypotheses based on morphological and/or molecular data, others are newly hypothesized or in conflict. As evidenced by the results of this study, all four groups of otophysan fishes investigated have, to a greater or lesser degree, taxonomies that do not fully reflect evolutionary relationships. The numerous instances of poly- and paraphyly in nominal taxa revealed by this doctoral research highlight the importance of phylogenetic analyses and underscore the necessity for future revisionary studies in order to maintain classification schemes where only monophyletic units are recognized. Examples of genera in critical need of taxonomic reassessment as revealed by the findings of this doctoral research include the alestid genus *Brycinus* (clearly polyphyletic), the distichodontid genus *Nannocharax* (paraphyletic with respect to *Nannaethiops*), the chedrin genus *Raiamas*

(clearly polyphyletic), and the loricariin genus *Lamontichthys*. Future revisional studies aimed at developing comprehensive monographic syntheses with predictive classification schemes that reflect evolutionary history may benefit from the integration of traditional morphology-based taxonomy with molecular phylogenetics, phylogeography, and ecological approaches to species delimitation. Similarly, because none of the groups of otophysan fishes targeted for phylogenetic analysis in this dissertation have been investigated under a total-evidence approach, future studies may benefit from the simultaneous analysis of morphological and molecular comparative data so as to account for all the available evidence.

The present study also resulted in the first time-scaled phylogenies ever proposed for a group of characiform fishes, and therefore it constitutes a significant contribution to our understanding of the temporal context of characiform evolution and of the historical biogeography of this transoceanic clade of otophysan fishes. Although the results of this work suggest that characiforms are Gondwanan in origin and therefore vicariance is the best hypothesis to explain the transatlantic distribution of members of the order, the multiple instances of continentally disjunct sister-group relationships and the phylogenetic uncertainty regarding some of these divergences make it very challenging to fully understand the biogeographic history of the Characiformes. Therefore, future research should be aimed at inferring a comprehensive time-scaled phylogeny for the entire characiform radiation in order to reconstruct a more accurate picture of the evolutionary history of characiform fishes in space and time.

While some of the findings of the present work suggest that diversification and biogeographic patterns in alestids and distichodontids may have been greatly influenced by the Neogene reconfiguration of drainage patterns in Central Africa, future research aimed at

detecting temporal shifts in diversification rates and phylogeographic signatures of drainage evolution are necessary to test the influence of these palaeogeographic processes on the evolution of these clades.

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