

# Relationships among characiform fishes inferred from analysis of nuclear and mitochondrial gene sequences

Daniela Calcagnotto<sup>a,b</sup>, Scott A. Schaefer<sup>a,\*</sup>, Rob DeSalle<sup>b</sup>

<sup>a</sup> *Division of Vertebrate Zoology, American Museum of Natural History, Central Park West at 79th St., New York, NY 10024-5192, USA*

<sup>b</sup> *Division of Invertebrate Zoology, American Museum of Natural History, Central Park West at 79th St., New York, NY 10024-5192, USA*

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## Abstract

Suprafamilial relationships among characiform fishes and implications for the taxonomy and biogeographic history of the Characiformes were investigated by parsimony analysis of four nuclear and two mitochondrial genes across 124 ingroup and 11 outgroup taxa. Simultaneous analysis of 3660 aligned base pairs from the mitochondrial 16S and cytochrome *b* genes and the nuclear recombination activating gene (RAG2), seven in absentia (*sia*), forkhead (*fkh*), and  $\alpha$ -tropomyosin (*trop*) gene loci confirmed the non-monophyly of the African and Neotropical assemblages and corroborated many suprafamilial groups proposed previously on the basis of morphological features. The African distichodontids plus citharinids were strongly supported as a monophyletic Citharinoidei that is the sistergroup to all other characiforms, which form a monophyletic Characoidei composed of two large clades. The first represents an assemblage of both African and Neotropical taxa, wherein a monophyletic African Alestidae is sister to a smaller clade comprised of the Neotropical families Ctenolucidae, Lebiasinidae, and the African Hepsetidae, with that assemblage sister to a strictly Neotropical clade comprised of the Crenuchidae and Erythrinidae. The second clade within the Characoidei is strictly Neotropical and includes all other Characiformes grouped into two well supported major clades. The first, corresponding to a traditional definition of the Characidae, is congruent with some groupings previously supported by morphological evidence. The second clade comprises a monophyletic Anostomoidea that is sister to a clade formed by the families Hemiodontidae, Parodontidae, and Serrasalminidae, with that assemblage, in turn, the sistergroup of the Cynodontidae. Serrasalminidae, traditionally regarded as a subfamily of Characidae, was recovered as the sistergroup of (Anostomoidea (Parodontidae + Hemiodontidae)) and the family Cynodontidae was recovered with strong support as the sistergroup to this assemblage. Our results reveal three instances of trans-continental sistergroup relationships and, in light of the fossil evidence, suggest that marine dispersal cannot be ruled out a priori and that a simple model of vicariance does not readily explain the biogeographic history of the characiform fishes.

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## 1. Introduction

Fishes of the Order Characiformes include the familiar piranhas, tetras, and tigerfishes and are among the most diverse freshwater fishes, with more than 1600 species (Daget and Gosse, 1984; Reis et al., 2003) distributed in

Africa, South and Central America, and southern North America. Their greatest diversity is found in the Neotropics (14 families and approximately 1460 species, versus 4 families and 208 species in Africa), where they dominate the ecologies of lowland freshwater drainage systems (Lowe-McConnell, 1975; Winemiller, 1996). Based on their current geographic distribution and the dramatic radiation of groups in the New World, characiforms have been regarded as prime biotic indicators of drift-vicariance associated with the opening of the southern Atlantic

\* Corresponding author. Fax: +1 212 769 5642.

E-mail address: [schaefer@amnh.org](mailto:schaefer@amnh.org) (S.A. Schaefer).

Ocean some 85–110 million years ago (Buckup, 1998; Gosline, 1944; Myers, 1967; Ortí and Meyer, 1997; Roberts, 1969). However, the occurrence of fossil forms in Europe (Cappetta et al., 1972; Cappetta and Thaler, 1974; Gaudant, 1979, 1980; Gayet, 1985; Taverne, 2003) and new insight into the phylogenetic relationships within certain characiform subgroups indicates that a simple vicariance model cannot readily explain the present distribution of characiforms in light of reciprocal non-monophyly of African and Neotropical groups and multiple instances of sistergroup relationships between clades now restricted to those continental faunas (Buckup, 1998; Gayet et al., 2003; Lundberg, 1993).

Characiform phylogeny and classification are not yet well resolved (see Vari, 1998; Weitzman and Fink, 1983, for reviews). Fink and Fink (1981) diagnosed the characiforms as monophyletic on the basis of seven morphological synapomorphies; these characters and their scheme of relationships for characiforms among the ostariophysan fishes have not been seriously challenged (see Fink and Fink, 1996). Within the order, however, hypotheses of relationships have been either patently non-phylogenetic (e.g., Géry, 1977; Greenwood et al., 1966; Poll and Gosse, 1982), based largely on morphological survey of single or limited character systems (e.g., Murray and Stewart, 2002; Roberts, 1969), or were restricted to various subsets of characiform taxa (e.g., Buckup, 1991; Lucena, 1993; Lucena and Menezes, 1998; Uj, 1990). Few African taxa have been included in the most recent studies of suprafamilial relationships and, with the exception of the revision of the Distichodontidae and Citharinidae by Vari (1979), African taxa have not received the same level of attention as have their Neotropical counterparts. Practical difficulties posed by the great morphological and  $\alpha$ -level taxonomic diversity of characiform fishes, coupled with numerous episodes of reductive and convergent evolution (Weitzman and Vari, 1988), have impeded assembly of comprehensive morphological datasets useful for assessing higher-level phylogenetic relationships within the group (Vari, 1998).

A number of characiform suprafamilial assemblages have been proposed and debated over the years, and five of these are now widely accepted on the basis of explicit statements of synapomorphy. Vari (1979) demonstrated a sistergroup relationship between the African families Distichodontidae and Citharinidae, a proposal that dates to Boulenger (1909). Fink and Fink (1981) placed the clade formed by these families as basal within Characiformes and this scheme has been corroborated in several subsequent studies (e.g., Buckup, 1998; Ortí and Meyer, 1997). The African genus *Hepsetus* was first proposed to be closely related to the Neotropical family Ctenolucidae by Roberts (1969), an idea subsequently supported by Lucena (1993) and Buckup (1998), whereas Vari (1995) proposed the Erythrinidae as sister to ctenolucids, with the clade formed by those families as

sister to hepsetids. Based on the shared presence of multicuspidate pharyngeal dentition, Roberts (1969, 1973) proposed the Neotropical families Anostomidae and Chilodontidae as closely related. This hypothesis was supported by additional synapomorphies and those families were placed as the sistergroup to a clade formed by the Neotropical families Prochilodontidae and Curimatidae in a more inclusive monophyletic assemblage by Vari (1983) and confirmed by Uj (1990) and Buckup (1991, 1998). Weitzman (1964) first proposed that *Acestrorhynchus* is more closely related to *Charax* and other Characidae than to other similarly predaceous taxa with conical teeth, such as *Ctenolucius* and *Boulengerella*, as was earlier proposed by Gregory and Conrad (1938). Buckup (1998) offered several synapomorphies for *Acestrorhynchus* as sister to an unresolved clade comprised of *Hoplias*, lebiasinids, and hepsetids plus ctenolucids, whereas Ortí and Meyer (1997) proposed *Acestrorhynchus* as sister to the African Alestidae, with that clade the sistergroup to a clade formed by ctenolucids plus the characid genus *Gnathocharax*. Lucena and Menezes, 1998, however, showed evidence for *Acestrorhynchus* as sister to the Cynodontidae. Finally, the so-called “African characids” (Greenwood et al., 1966; Nelson, 1994) are placed in the family Alestidae following Géry (1977). The monophyly of this assemblage has not been questioned; however, recent studies (e.g., Brewster, 1986; Vari, 1979) have challenged the continued recognition of the subfamily Hydrocyninae based on new concepts of relationships of *Hydrocynus* to subsets of the Alestinae.

In stark contrast to the rich history of characiform systematics based on analysis of morphological features, the only study to-date to apply nucleotide sequence data to the problem of characiform relationships is that of Ortí and Meyer (1997), who examined partial 12S and 16S mitochondrial ribosomal gene sequences across 38 taxa. That study offered support for some well-recognized clades and further proposed some novel hypotheses of relationship (e.g., *Acestrorhynchus* allied with alestids), but was limited by the relatively poor resolution afforded by those genes and by a limited sampling of taxa, particularly so for the African representatives (six species examined). Those authors attributed the lack of resolution obtained in their study to saturation of nucleotide substitution and low sequence divergence among characiform families relative to outgroup otophysans, suggesting that such deep clade divergences that presumably pre-date the 100+ million year old separation of Africa and South America (Pitman et al., 1993; Rabinowitz and LaBrecque, 1979) are not likely recoverable using these genes.

The purpose of this study was to test the previous hypotheses of suprafamilial phylogenetic relationships among characiform fishes. Our approach was to sequence multiple gene regions, including four nuclear and two mitochondrial genes, that involve broad substitution rate heterogeneity and to analyze these data

under the parsimony optimizing criterion, with hopes of achieving strong node support across multiple levels of taxon divergence (Chipindale and Wiens, 1994; Nixon and Carpenter, 1996). Second, our inclusion of 124 terminal and 11 outgroup taxa represents the most inclusive sampling of characiforms in any phylogenetic analysis to-date and affords a much greater chance of avoiding spurious results, such as the attraction of long branch segments (Kraus and Brown, 1998; Lecointre et al., 1993). The robust sampling of 59 African representatives in this study also represents the most inclusive analysis of African characiforms thus far, and allows us to evaluate previous statements for the number of cladogenic events involving African–South American sister-group pairs in the evolutionary history of Characiformes.

## 2. Materials and methods

### 2.1. Taxon sampling, DNA extraction, and sequencing

Tissue samples (muscle, fin or blood) from 124 characiform taxa, representing 12 of 14 Neotropical and all four African families, were obtained from field collections, gifts or donations, and from aquarium trade specimens (Appendix A). Four siluriform and six cypriniform

representatives were included as outgroups (Fink and Fink, 1981). DNA was extracted from tissues preserved in 95% EtOH or lysis buffer (Seutin et al., 1991) using DNEasy Tissue Extraction Kit (Qiagen). For tissues preserved in lysis buffer, we omitted the first step of the Qiagen protocol and 5 µl of proteinase-k was added directly to 200 µl of the lysed tissue. Double stranded DNA was synthesized for two mitochondrial genes, 16S and cytochrome *b* (Cyt *b*), for three nuclear genes, the recombination activating gene (RAG2), seven in absentia (*sia*) and forkhead (*fk*h), and also for intron 5 of the  $\alpha$ -tropomyosin (*trop*) by PCR in 25 µl reactions (2.5 µl 10 mM Tris–HCl + 15 mM MgCl<sub>2</sub> buffer; 1 µl each 10 mM primer; 2.5 µl dNTP 200 nM of each dinucleotide; 0.1 µl Ampliqaq or Ampliqaq Gold polymerase (Perkin-Elmer); 0.5 µl template DNA and 17.4 µl ddH<sub>2</sub>O). Sequences for mitochondrial and nuclear primers and PCR conditions are given in Tables 1 and 2, respectively. The PCR products were purified using the Qiaquick Purification kit (Qiagen) or the Array-It PCR Purification Kit with minor modifications. The amplicons were directly sequenced in both directions with the primers used for amplification, unless noted otherwise, using Big-Dye terminators (Perkin-Elmer) on a GeneAmp 9700 thermocycler. Unincorporated dyes were removed by ethanol precipitation and the products run on an

Table 1  
Sequences for primers used to amplify given gene fragments

Gene	Primer sequence <sup>a</sup>	Source
16S ar	ACG CCT GTT TAT CAA AAA CAT	Palumbi (1996)
16S br	CCG GTC TGA ACT CAG ATC ACG T	
Cyt <i>b</i> L 14841	AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA	Kocher et al. (1989)
Cyt <i>b</i> H 15915	AAC TGC CAG TCA TCT CCG GTT TAC AAG AC	Irwing et al. (1991)
TROP F	GAG TTG GAT CGG GCT CAG GA GCG	Friesen et al. (1999)
TROP R	CGG TCA GCC TCT TCA GCA ATG TGC TT	
<i>sia</i> /T3 <sup>b</sup>	ATT AAC CCT CAC TAA AGT CGA GTG CCC CGT GTG YTT YGA YTA	Lovejoy (1999)
<i>sia</i> /T7 <sup>b</sup>	AAT ACG ACT CAC TAT AGG AAG TGG AAG CCG AAG CAG SWY TGC ATC AT	
<i>fk</i> h/T3 <sup>b</sup>	ATT AAC CCT CAC TAA AGT CCC TAC TCC TAC ATC TCC CTG ATH ACN ATG	
<i>fk</i> h/T7 <sup>b</sup>	AAT ACG ACT CAC TAT AGC GCA GGT AGC AGC CGT TYT CRA ACA TRT	
RAG2 aF <sup>c</sup>	TTT GGR CAR AAG GGC TGG CC	
RAG2 bF <sup>c</sup>	GTR GAR TAG TAG GGC TCC CA	
RAG2 bR <sup>c</sup>	TGR TCC ARG CAG AAG TAC TT G	

<sup>a</sup> Listed from 5' to 3'.

<sup>b</sup> Incorporating T3 and T7 universal primers to automate the sequencing step.

<sup>c</sup> Different combinations used to amplify 1270 bp RAG2 fragment.

Table 2  
PCR conditions

Primer	Denaturation (95 °C)	Cycles	Extension (72 °C)
16S	10 min	35 × 95 °C/30 s, 48 °C/45 s, 72 °C/45 s	7 min
Cyt <i>b</i>	5 min	30 × 95 °C/30 s, 48 °C/45 s, 72 °C/90 s	7 min
<i>fk</i> h	5 min	30 × 95 °C/30 s, 55 °C/30 s, 72 °C/45 s	7 min
<i>sia</i>	5 min	30 × 95 °C/30 s, 60 °C/30 s, 72 °C/45 s	7 min
RAG2	5 min	30 × 95 °C/30 s, 48 °C/45 s, 72 °C/90 s	7 min
TROP	10 min <sup>a</sup>	30 × 95 °C/30 s, 60 °C/30 s, 72 °C/45 s	7 min

<sup>a</sup> Required Ampliqaq Gold.

Applied Biosystems 3700 DNA Analyzer. Whenever possible, samples from two individuals of each species were included. When heterozygotes were detected, mainly for  $\alpha$ -tropomyosin, the PCR products were cloned using the TOPO-TA cloning kit (Invitrogen). To avoid errors that could have been incorporated during cloning, five clones from each reaction were sequenced and DNA sequences of both strands were obtained using the Invitrogen's M13 forward and reverse sequencing primers. For some taxa, sequence data were not obtained for all partitions; in such cases, the data were coded as missing. Sequences were edited using the Sequencher software package Version 4.1 (Gene Codes).

## 2.2. Phylogenetic analyses

Sequences were aligned using Clustal X (Thompson et al., 1997) with default parameters for gap opening (=10) and extension costs (=0.2), and the alignments inspected by eye for any obvious misalignments. Aligned sequences were analyzed with PAUP\* 4.0b10 (Swofford, 2000) using maximum parsimony. Heuristic searches were performed with minimally 100 random taxon addition replicates and TBR branch swapping. All characters were unordered, all character transformations were equally weighted, and branches with maximum length of zero were collapsed. Gaps were treated as missing data, rather than as a fifth character state, so as to avoid presumptions concerning the nature and location of insertion/deletion events and subjective constraints on character state change. Tree searches also employed the Parsimony Ratchet method (Nixon, 1999), using a batch file generated by PAUPRat with the default parameters (Sikes and Lewis, 2001).

To examine the difference in phylogenetic signal between gene partitions, incongruence length differences (ILD) were calculated using the partition homogeneity test (Farris et al., 1994; Mickevich and Farris, 1981). Taxa with missing data in any single partition and uninformative characters were excluded; heuristic searches employed 100 replicates of one simple taxon addition and TBR branch swapping. Five categories of incongruence test were performed: 1, congruence of each gene partition with combined data; 2, between all possible pairwise gene combinations; 3, between nuclear partitions; 4, between nuclear and mitochondrial partitions; and 5, congruence among all partitions simultaneously.

Relative support for the internal nodes in the combined analysis of all gene partitions was estimated using bootstrap (B, Felsenstein, 1985) and Bremer support (BS, Bremer, 1988, 1994). Bootstrap values were estimated from 1000 replicates, each employing five random stepwise taxon addition sequences. For the Bremer support values, TreeRot (Sorensen, 1999) used a heuristic search on 20 random taxon addition replicates. TreeRot was also used to estimate partitioned branch support

(PBS, Baker and DeSalle, 1997; Baker et al., 1998) and the partitioned hidden branch support (PHBS, Gatesy et al., 1999). While branch support calculates the character support at a particular node and indicates the number of extra steps required for that particular node to collapse relative to the most parsimonious tree, hidden branch support represents the amount of support that arises at a particular node from combining the different partitions that is not evident in any single partition analysis. To that end, PBS and PHBS simply calculate the branch support and hidden branch support on a partition by partition basis. To estimate the PHBS, missing taxa and their nodes were excluded and the number of trees saved per replicate was limited to 1000.

## 3. Results

### 3.1. Character support dynamics

A total of 3660 nucleotide characters (lengths: 16S = 502 bp; Cyt *b* = 1014 bp; fkh = 273 bp; RAG2 = 1227 bp; sia = 447 bp; trop = 197 bp) were employed in a combined analysis. Of these, 1892 characters were variable and 1576 were parsimony informative. Primers for 16S, Cyt *b*, fkh, sia, and trop amplified a single fragment in all taxa. The trop fragments varied in length from 300 to 1200 bp and, because heterozygotes were detected among taxa having the larger intron, these fragments were cloned and five clones sequenced in both directions. When more than one specimen per species was sequenced, in some cases single nucleotide polymorphisms were detected. In these instances, a consensus sequence was used and the polymorphism coded according to the IUPAC-IUB ambiguity code.

All alignments were straightforward; however, because there were several long indels in 16S sequences, gap regions, presumably corresponding to the loops of the 16S secondary structure, were excluded from the analyses to avoid errors in positional homology (Gatesy et al., 1993). Insertions and deletions of one amino acid were detected within the RAG2 fragment. For example, some distichodontids (*Distichodus*, *Hemigrammocharax*, *Neolebias*, and *Ichthyborus*) show an insertion of one amino acid at position 251. All cyprinids included had a deletion of one amino acid at position 270.

Results of the partition homogeneity test of each partition versus the sum of the remaining five partitions indicated incongruence in all comparisons ( $P < 0.05$ ). Of the 14 pairwise comparisons among data partitions, only the 16S/Cyt *b* comparison showed congruence between datasets ( $P > 0.05$ ). The third category of incongruence test involving the comparison among nuclear partitions demonstrated significant incongruence ( $P < 0.05$ ). As expected, the nuclear dataset was incongruent with the mitochondrial data ( $P < 0.05$ ), most likely due to the

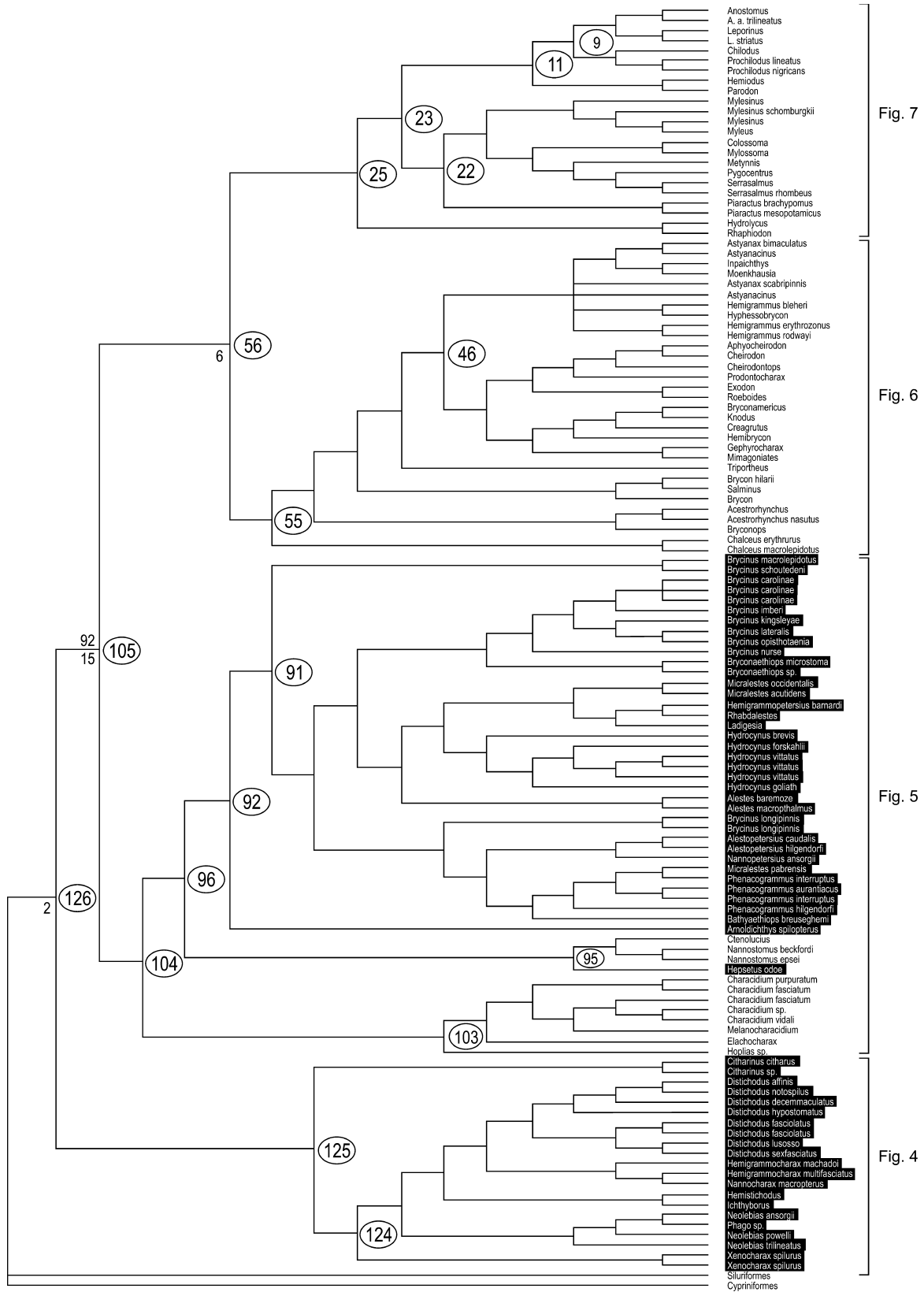


Fig. 1. Strict consensus of 18 equally most-parsimonious trees based on the combined analysis of six data partitions. African taxa are denoted in black. Clades designated at far right are examined in detail in subsequent figures, respectively. Nodes are numbered where referenced in the text; bootstrap ( $\geq 50\%$ ) and Bremer support values are shown above and below the node, respectively, for those nodes not represented in subsequent figures.

different evolutionary dynamics of these genomes. When all six data sets were tested for congruence simultaneously, the results also point to significant incongruence ( $P < 0.05$ ). Although strong incongruence was detected among data partitions by ILD, our data nevertheless provide strong node support, much of that hidden, as indicated by the absence of negative partitioned branch support (Gates et al., 1999).

### 3.2. Tree statistics and nodal support

An unweighted total-evidence analysis of the data yielded 18 equally most parsimonious trees, each 15,351 steps in length, CI=0.207, RI=0.610. Twenty independent PAUPRat searches yielded the same 18 most parsimonious trees as the heuristic searches. The strict consensus of those trees is fully resolved (Fig. 1), except for two nodes involving relatively more terminal, lower-level portions of the tree: (1) an unresolved clade involving relationships among *Astyanax scabripinis*, *Astyanacinus*, and *Hemigrammus* species within Characidae, and (2) unresolved relationships among individuals of *Brycinus caroliniae* within Alestidae. Bremer support values varied from 0 to 49 across the entire tree. The overall contribution of each data partition to the topology (Fig. 2) revealed that all six partitions had positive net PBS scores. *Cyt b* made the largest contribution (375), followed by RAG2 (327), 16S (310), *sia* (145), *fkh* (143), and *trop* (23), respectively.

Because suprafamilial relationships are the main focus of this study, we examined node support in detail

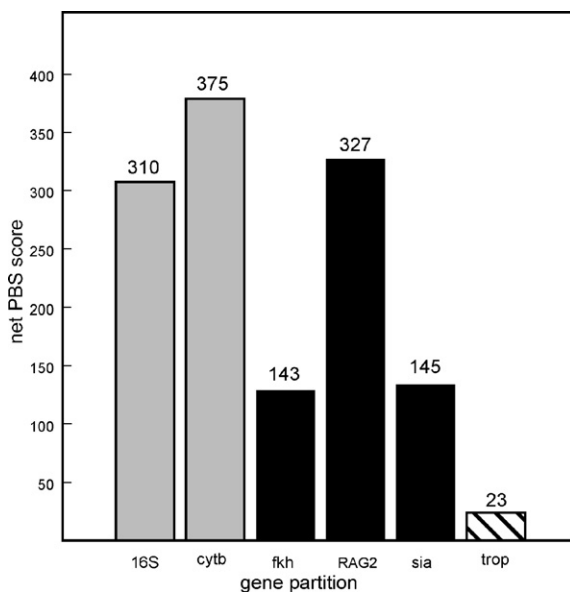


Fig. 2. Relative contribution of each gene partition to node support over all nodes of the strict consensus tree as revealed by the net partitioned branch support (PBS). Gray bars denote mitochondrial genes, black bars denote nuclear genes, and hatched bar denotes intron 5 of the tropomyosin gene.

for 17 of the 132 nodes in the strict consensus tree. Additional hidden support is present for 16 of these 17 nodes (Fig. 3). A PHBS value of zero for node 104 indicates neither hidden conflict or support. The *sia* and *trop* exon partitions uniformly showed no hidden support. Of particular interest is the high hidden support presented by the RAG2 partition. Nodes 124 (monophyly of Distichodontidae), 125 (Citharinidae + Distichodontidae), and 126 (Characiformes monophyly) involve more PHBS than the sum of PBS from all other partitions.

### 3.3. Tree topology

Our parsimony analysis confirmed previous findings regarding the non-monophyly of the African and Neotropical species assemblages (Fig. 1). The suborders Citharinoidei (clade 125, Fig. 1) and Characoidei (clade 105, Fig. 1) of [Buckup \(1998\)](#) were recovered with strong node support ( $B = 97$ ,  $BS = 12$  and  $B = 92$ ,  $BS = 15$ , respectively). Within the African Citharinoidei, the families Citharinidae and Distichodontidae were recovered as monophyletic (Fig. 4). Within Distichodontidae, *Xenocharax* was recovered in a basal position, the genus *Distichodus* was recovered as monophyletic with strong support ( $B = 93$ ,  $BS = 7$ ) and comprises two clades, one composed of *D. affinis*, *D. notospilus*, *D. decemaculatus*, and *D. hypostomatus*; and the other clade formed by *D. fasciolatus* sister to *D. sexfasciatus* and *D. lusosso*. Sister to *Distichodus*, we recovered a clade formed by *Hemigrammocharax* and *Nannocharax*.

Within Characoidei (node 105, Fig. 1), our hypothesis supports the existence of two major clades. The first clade comprises a monophyletic Alestidae (node 92; Fig. 5) as the sistergroup to a clade comprised of *Hepsetus* as sister to Ctenolucidae plus Lebiasinidae (clade 95; Figs. 1 and 5). That assemblage is the sistergroup to a clade formed by Crenuchidae plus Erythrinidae (clade 103; Figs. 1 and 5). This is the first proposal of a sistergroup relationship between Crenuchidae and Erythrinidae; however, support for this node involved low bootstrap ( $B < 50$ ) but moderate Bremer support ( $BS = 6$ ). Within Alestidae (clade 92), the position of *Arnoldichthys*, recovered as basal to all other alestids, is poorly supported ( $B < 50$ ,  $BS = 4$ ), whereas monophyly of all other Alestidae less *Arnoldichthys* was strongly supported ( $B = 100$ ,  $BS = 24$ ; Fig. 5). Our results corroborate previous findings on the non-monophyly of the tribe Petersiini and of genera such as *Brycinus* ([Murray and Stewart, 2002](#)). *Hydrocynus* is monophyletic and nested within the Alestidae with strong support ( $B = 73$ ,  $BS = 8$ ; Fig. 5), confirming previous observations that continued recognition of the family Hydrocynidae would render the Alestidae paraphyletic ([Buckup, 1998](#); [Ortí and Meyer, 1997](#)). Contra [Murray and Stewart \(2002\)](#), we did not recover a sistergroup relationship between *Hydrocynus* and *Alestes*, but rather find *Hydrocynus* more closely

Data set Nodes	16s	Cyt b	fkh	RAG2	sia	trop	TOTAL
9	0	0	1	5	0	0	6
10	0	1	0	5	0	0	6
11	0	0	1	2	0	0	3
22	1	0	0	0	0	0	1
23	0	1	0	0	0	0	1
25	0	1	0	0	0	0	1
55	2	0	1	0	0	0	3
56	0	1	0	0	0	0	1
92	1	0	1	0	0	0	2
95	0	0	1	0	0	0	1
96	0	1	0	0	0	0	1
103	0	0	1	0	0	0	1
104	0	0	0	0	0	0	0
105	0	2	1	9	0	0	12
124	3	1	0	42	0	0	46
125	3	0	0	42	0	0	45
126	0	0	0	71	0	0	71

Fig. 3. Sources of hidden partitioned branch support (PHBS) among data partitions for 17 nodes on the strict consensus tree. Nodes numbered as in Fig. 1; positive scores (white boxes) indicate net positive contribution; zero scores (gray boxes) indicate neutral contribution to overall support for a particular node in the strict consensus tree.

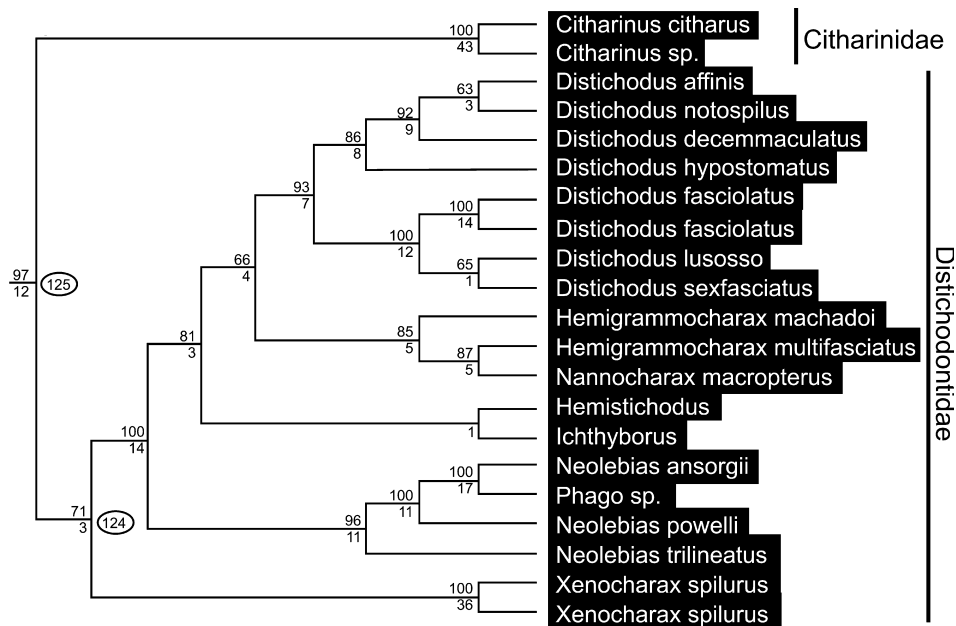


Fig. 4. Relationships among the African Citharinoidei (node 125, Fig. 1). Bootstrap ( $\geq 50\%$ ) and Bremer support values are shown above and below the each node, respectively.

related to a subset of dwarf alestids, with *Alestes* the sistergroup of that assemblage (following Ortí and Meyer, 1997).

The second major clade recovered within the Characoidae includes two well-supported subclades. The first (node 55; Figs. 1 and 6) includes taxa traditionally

grouped in the family Characidae (Géry, 1977); the second (node 25; Figs. 1 and 7) includes taxa placed in the Anostomoidea, Serrasalminae, and Cynodontidae. Within Characidae, we recovered some groupings previously supported by morphological evidence (but, see Malabarba, 1998 for caveats). For example, our results

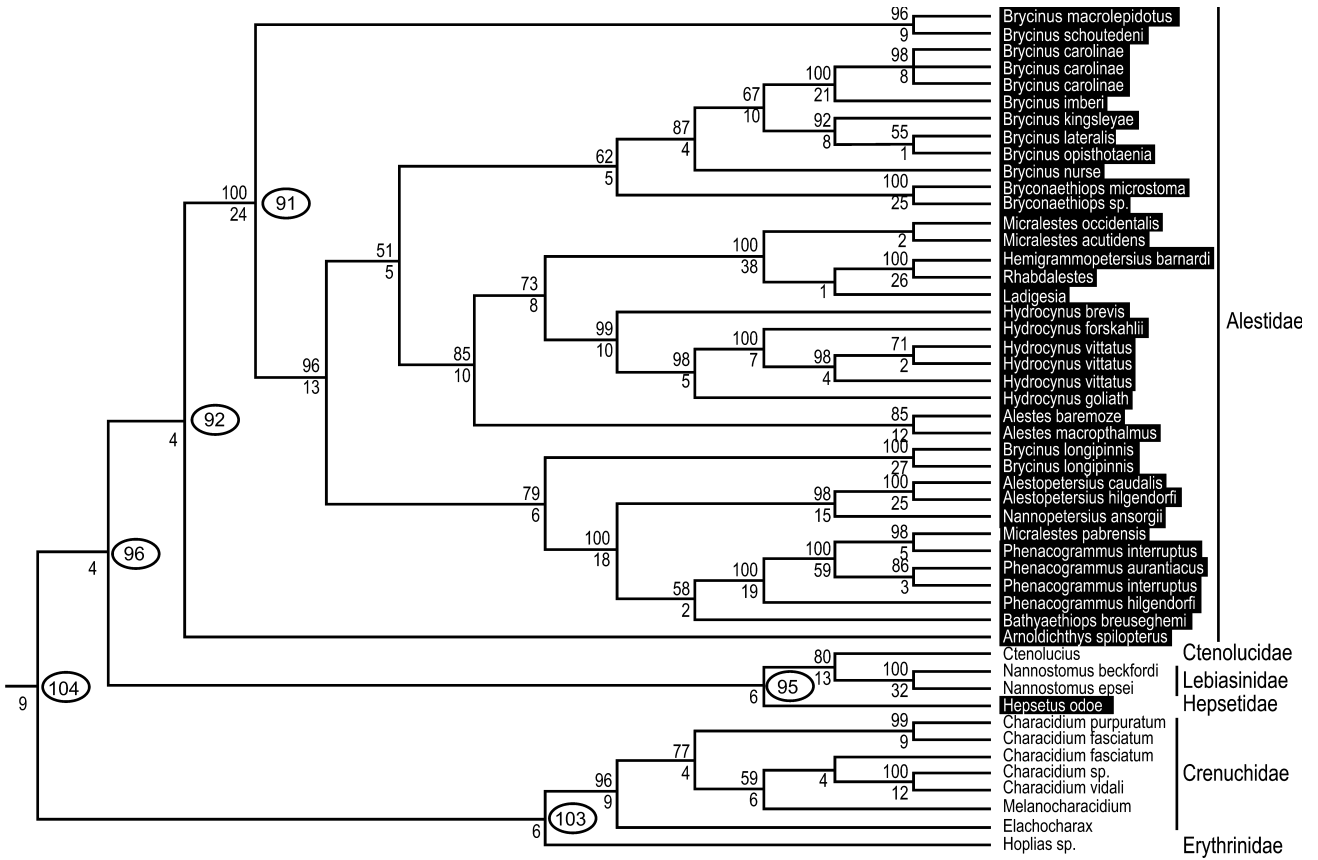


Fig. 5. Relationships among the Neotropical families Ctenolucidae, Lebiasinidae, and Erythrinidae and the African (taxa denoted in black) Alestidae and Hepsetidae (node 104, Fig. 1). Symbology as in Fig. 4.

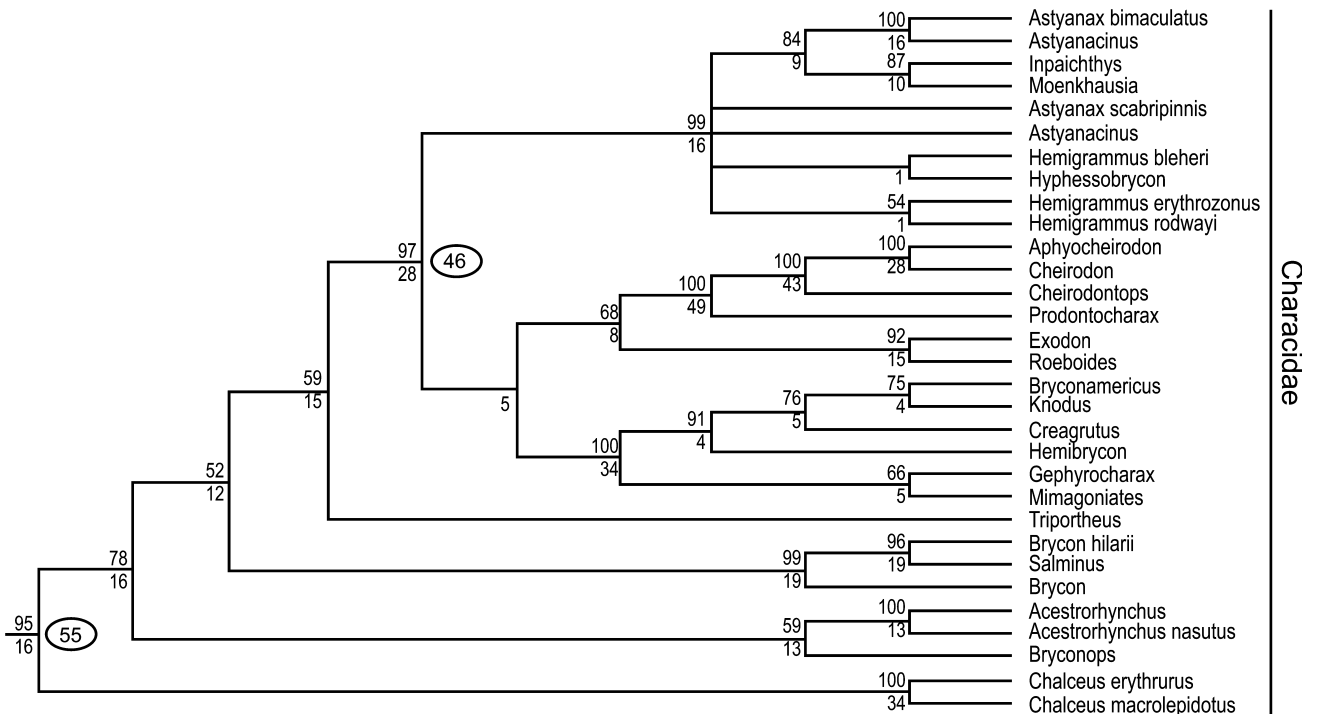


Fig. 6. Relationships among the Neotropical Characidae (node 55, Fig. 1). Symbology as in Fig. 4.

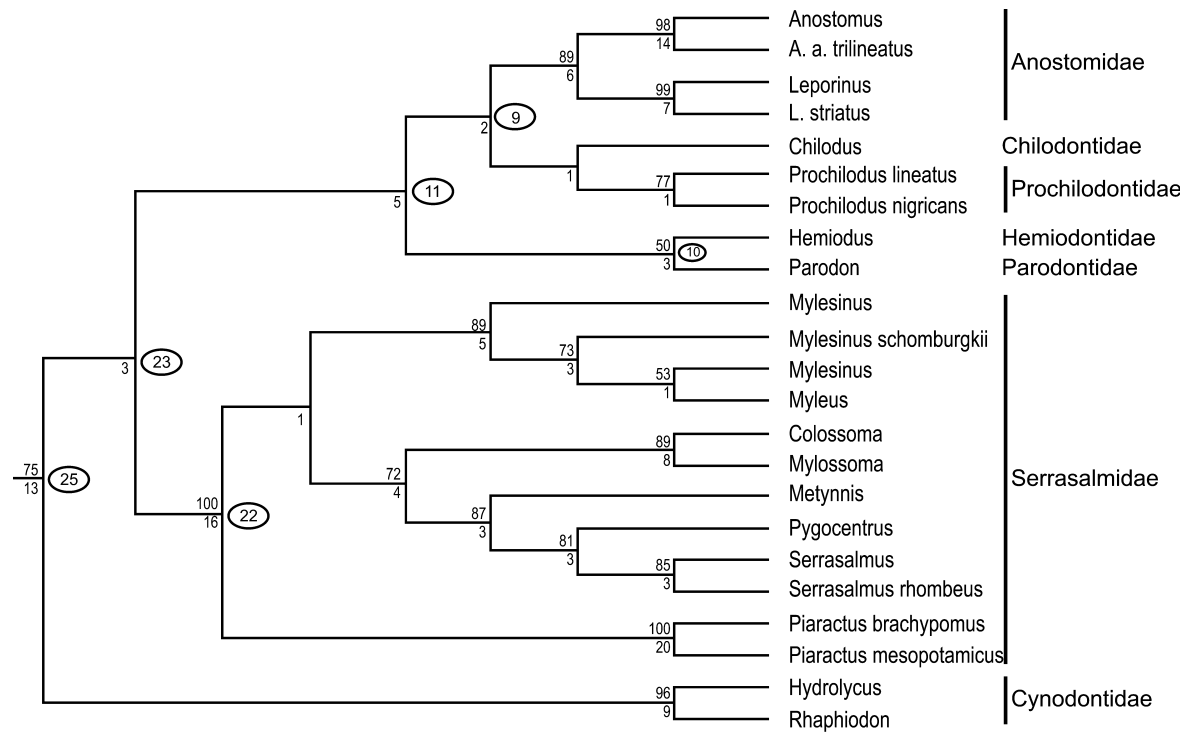


Fig. 7. Relationships among the Neotropical Anostomoidea (families Anostomidae, Chilodontidae, Prochilodontidae, Hemiodontidae, and Parodontidae), Serrasalminidae and Cynodontidae (node 25, Fig. 1). Symbology as in Fig. 4.

are congruent with: (1) monophyly of the subfamily Cheirodontinae, represented in our study by the genera *Cheirodon*, *Aphyocheirodon*, *Cheirodontops*, and *Prodon-tocharax*, (2) the monophyletic “clade A” of *Malabarba and Weitzman* (2003, fig. 11), represented by the genera *Bryconamericus*, *Knodus*, *Creagrutus*, *Hemibrycon*, *Gephyrocharax*, and *Mimagoniates*, (3) a sistergroup relationship between *Brycon* and *Salminus* (e.g., *Ortí and Meyer*, 1997), and (4) a non-monophyletic Tetragonopterinae (Fig. 6). Contrary to what has been suggested previously, we do not find a close relationship between *Triportheus*, *Brycon*, and *Salminus*. *Acestrorhynchus*, considered the sistergroup of Alestidae by *Ortí and Meyer* (1997) and the sistergroup of the Cynodontidae by *Lucena and Menezes* (1998), is nested within our characid clade. *Bryconops*, traditionally regarded as a member of the Tetragonopterinae within the Characidae (*Géry*, 1977), is supported in our study as the sistergroup of *Acestrorhynchus*. *Chalceus* is strongly supported as the sistergroup of all other so-called Characidae (Fig. 6, node 55).

The second clade within Characoidea (node 25, Figs. 1 and 7) includes taxa belonging to the superfamily Anostomoidea (*Buckup*, 1998) and the families Serrasalminidae and Cynodontidae. Anostomids were weakly recovered as the sistergroup of chilodontids plus prochilodontids (node 9; Fig. 7), with that assemblage the sistergroup to a clade formed by the families Hemiodontidae and Parodontidae (clade 11; Fig. 7). Serrasalminidae

(clade 22; Fig. 7) was strongly supported as monophyletic ( $B = 100$ ,  $BS = 16$ ) and recovered as the sistergroup of the Anostomoidea; however, without strong node support ( $B < 50$ ,  $BS = 3$ ). Finally, the Cynodontidae was strongly supported as the sistergroup to the Anostomoidea plus Serrasalminidae ( $B = 75$ ,  $BS = 13$ ).

## 4. Discussion

### 4.1. Congruence with previous morphological studies

*Weitzman* (1962) described the Characiformes (his Characidae) as “an extreme case of evolutionary radiation and adaptation.” The immense morphological and taxonomic diversity within the group makes any attempt to resolve their phylogenetic relationships, based solely on morphological characters, an incredibly difficult task. Although we agree that the utility of morphological characters is indisputable (*Jenner*, 2004) and that it is necessary to have familiarity with the objects of study that transcends mere description of its genotype (*Weitzman and Malabarba*, 1998), application of molecular phylogenetics to the study of characiform systematics offers the potential for independent evaluation of the evidence supporting previous statements of relationships and a rapid assessment of the major patterns of relationships. The volume of morphological data assembled for characiform fishes to-date is enormous and undergoing

review and re-analysis. Our results offer the most inclusive sampling of taxa and largest assemblage of phylogenetically informative characters in any analysis of characiform relationships thus far and are surprisingly congruent with previous results based solely on morphological data, more so than the results of Ortí and Meyer (1997), the only other application of nucleotide sequence data to characiform phylogeny.

#### 4.1.1. African distichodontids and citharinids

A close relationship between the African families Citharinidae and Distichodontidae was suggested long ago (Boulenger, 1909; Gregory and Conrad, 1938; Regan, 1911) and Vari (1979), based mostly on osteological characters, confirmed that they are monophyletic, once the Ichthyboridae of Greenwood et al. (1966) was subsumed within the Distichodontidae. We confirm the monophyly of Citharinidae plus Distichodontidae (clade 125; Figs. 1 and 4) and further note that *Ichthyborus* and *Phago* (taxa formerly placed in Ichthyboridae) are both well-nested within Distichodontidae (Fig. 4). In our analysis, however, relationships among genera within Citharinoidea are not uniformly well-resolved and several groupings are doubtful (e.g., *Phago* not closely related to *Ichthyborus*, but rather sister to *Neolebias*). Representatives of the former two genera specialize in feeding on the fins of other fishes (Géry, 1977), whereas *Neolebias* is a diminutive feeder on small crustaceans and insects. On the other hand, we did recover a clade composed of *Distichodus*, *Nannocharax*, and *Hemigrammocharax* that was previously recognized on the basis of nine synapomorphic characters seemingly related to specializations involving horizontal movement of the lower jaw (Vari, 1979). That author further suggested that some *Distichodus* species (e.g., *D. lusosso*, *D. niloticus*, and *D. fasciolatus*) were more closely related to *Hemigrammocharax* and/or *Nannocharax* than to other *Distichodus* species. We found strong support for a clade of *Distichodus* species that includes two of the aforementioned three species (Fig. 4). Our results lend further support to the notion that *Xenocharax* is the basal-most distichodontid (Fink and Fink, 1981; Vari, 1979).

#### 4.1.2. African alestids

The remaining “African characins” have been recognized as a monophyletic subfamily of Characidae (i.e., Alestinae—Ortí and Meyer, 1997; Paugy, 1986) or as a separate family (i.e., Alestidae—Géry, 1977; Buckup, 1998; Alestiidae—Eschmeyer, 2002). As evidence of monophyly, Roberts (1969) cited the presence of a bony pedicle on the posterior part of the premaxilla that is absent in Neotropical characiforms and Vari (1979, 1995) cited the presence of a third posttemporal fossa and caudal-fin stays as additional synapomorphies shared exclusively by the Alestidae. These observations were taken as indicative of the monophyly of Alestidae,

but were not obtained via studies employing broad taxonomic sampling. Our results, based on the inclusion of 37 alestid species as terminal taxa, provide strong confirmation for a monophyletic Alestidae despite the problematic placement of *Arnoldichthys*, the latter a result that was weakly supported in our analysis and which has not been previously suggested. *Arnoldichthys* shares a similar head and body configuration with other alestids, yet possesses some unusual morphological differences, such as seven (vs. 3–4) teeth in the inner row of the premaxilla and irregularly shaped scales above the lateral line resembling the apparently convergent condition observed in the Neotropical characid genus *Chalceus* (Géry, 1977). The poor resolution and questionable placement of *Arnoldichthys* at this basal position within the Alestidae may reflect an artifact of long branch attraction, perhaps a result of extensive extinction of alestids in Africa, as has been suggested by Lundberg (1993) and Gayet et al. (2003).

Our results demonstrate that continued recognition of subfamilies and tribes within Alestidae must be reconsidered. For example, our results disagree with previous hypotheses regarding the placement of the large predaceous African tigerfish, *Hydrocynus*. Roberts (1966, 1969) first suggested that *Hydrocynus* is closely related to *Alestes*, while Brewster (1986) argued for a close relationship between *Hydrocynus* and a restricted subset of *Alestes* species (i.e., *A. dentex*, *A. baremoze*, *A. macrophthalmus*, *A. stuhlmanni*, and *A. liebrechtsii*). Although our study did not include an extensive sampling of *Alestes* species, our results show support for the unexpected placement of *Hydrocynus* nested within a clade comprised of taxa currently placed in the patently non-monophyletic tribe Petersiini (Paugy, 1990; Poll, 1967), the so-called “dwarf alestids.” We are not aware of morphological support for this relationship. The tribe Alestini contains the genera *Alestes*, *Brycinus*, and *Bryconaethiops* and is distinguished from members of the tribe Petersiini on the basis of the shared presence of derived molariform inner premaxillary teeth. Members of Petersiini are recognized by two features of dubious diagnostic value: small size, and absence of molariform premaxillary dentition, the latter a plesiomorphic condition for characiforms. Apart from monophyly of Hydrocyninae, our results (Fig. 5) do not support continued recognition of tribes within Alestinae as presently constituted. In particular, *Bryconaethiops* is more closely related to a subset of *Brycinus* species than to *Alestes*, *Brycinus* is not monophyletic (Murray and Stewart, 2002), and Alestinae is rendered paraphyletic by continued recognition of Hydrocyninae. Taxa traditionally placed in the tribe Petersiini fall into two well-supported clades: one comprised of *Micralestes*, *Rhabdalestes*, and *Ladigesia* (including species with and without inner mandibular teeth), and the second comprised of *Phenacogrammus* and allied genera. These two clades are

relatively well separated from one another and each is more closely related to members of other tribes or subfamilies. A revised taxonomy for Alestidae represents a major priority for future research.

The relationship of Alestidae to other characiforms has remained problematic because most studies conducted to-date have not directly addressed the question via adequate sampling inclusive of both African and South American taxa. For example, in the two most complete studies of higher-level characiform relationships published thus far, Ortí and Meyer (1997) sampled from six African species and Buckup (1998) included representatives of five African taxa. Our analysis of 32 alestid taxa recovered a clade formed by the Neotropical taxa *Ctenolucius*, *Nannostomus*, and the African *Hepsetus* as the sister group of Alestidae (Fig. 5). This is the first time that such a relationship has been proposed. Monophyly of an assemblage composed of the families Ctenolucidae, Lebiasinidae, Erythrinidae, and the monotypic African Hepsetidae has been advocated by Vari (1995) and formally recognized as the Erythrinioidea by Buckup (1998). In Vari's hypothesis, Ctenolucidae is the sister group of Erythrinidae and this clade is sister to Hepsetidae and Lebiasinidae. In Buckup's (1998: Fig. 6) strict consensus cladogram, Ctenolucidae and Hepsetidae are more closely related to one another and their relationships with Erythrinidae and Lebiasinidae are not resolved. In our hypothesis (Figs. 1 and 5), erythrinids are included in a clade with the Crenuchidae, with that assemblage forming the sistergroup to Alestidae plus the remaining "erythrinoid" taxa. In such a hypothesis, the recognition of a monophyletic Erythrinioidea that includes Ctenolucidae, Lebiasinidae, and Hepsetidae would further require the inclusion of Crenuchidae and Alestidae. Support for clades 103 (Crenuchidae + Erythrinidae) and 104 ((Crenuchidae + Erythrinidae) (Ctenolucidae + Lebiasinidae + Hepsetidae)) Alestidae) involve low bootstrap values but high BS and PHBS values (Figs. 1, 3, and 5). Interestingly, the novel hypothesis of a close relationship between Crenuchidae and certain African characiforms was first suggested by Buckup (1998) in his review of the Characidiinae, where Crenuchidae (Crenuchinae + Characidiinae) was proposed as the sistergroup of a large clade composed of Neotropical and African (*Alestes*) taxa.

#### 4.1.3. Neotropical characids

Géry (1977) characterized the subfamily Tetragonopterinae as an ecologically successful group of characins; however, there is no evidence supporting the monophyly of this subfamily or of the majority of its more speciose genera, such as *Astyanax* and *Hemigrammus* (Weitzman and Malabarba, 1998). Our results corroborate non-monophyly of Tetragonopterinae, but point to the likelihood that monophyletic subsets of tetras will be recovered in future analyses, such as indicated by the

clade recovered in our study formed by *Astyanax*, *Astyanacinus*, *Moenkhausia*, *Inpaichthys*, *Hemigrammus*, and *Hyphessobrycon* (Fig. 6) that includes species with five or more teeth in the inner series of the premaxilla. A second monophyletic subset of genera currently regarded as members of the Tetragonopterinae was recovered in our analysis and includes *Bryconamericus*, *Knodus*, *Creagrutus*, and *Hemibrycon* that constitutes the sister group to the Glandulocaudinae genera *Gephyrocharax* and *Mimagoniates* (Fig. 6), a hypothesis congruent with the "clade A" proposed by Malabarba and Weitzman (2003). It is also noteworthy that *Bryconops*, the only tetragonopterine genus to possess a supraorbital bone (a condition plesiomorphic for Characiformes—Malabarba and Weitzman, 2003; Weitzman and Malabarba, 1998), is placed relatively basal in Characidae (Fig. 6). The clade that includes *Cheirodon* corroborates a monophyletic Cheirodontinae as one of the best resolved within Characidae (Malabarba, 1998), diagnosed by presence of a pseudotympanum, a structure characterized by absence of muscle tissue at the anterior portion of the swim bladder whose function is believed to be related to more efficient sound transmission. A similar structure occurs in two Characinae genera, *Roeboides* and *Charax*. However, in the absence of other characters supporting a close relationship between these genera, Malabarba and Lucena (1995) and Malabarba (1998) considered the pseudotympanum to be independently derived in these taxa. Ortí and Meyer (1997), using mitochondrial nucleotide characters, also recovered a close relationship between Cheirodontinae and *Cynopotamus* (Characinae). We recovered the same grouping of Cheirodontinae and Characinae (the latter subfamily here represented by *Roeboides* and *Exodon*). *Exodon* was included by Géry (1977) in Characinae, but Menezes and Roberts in Roberts (1970) and Howes (1976) found this genus to be more closely related to Tetragonopterinae based on cranial morphology. Although at present *Exodon* is considered *incertae sedis* in Characidae, our results may indicate homology of the pseudotympanum in these cheirodontin and characin species.

Our results also do not corroborate either of the previous hypotheses for the relationships of Acestrorhynchinae, which regard *Acestrorhynchus* as being closely related to *Brycon*, *Salminus*, and *Triporthes* based, in part, on the shared presence of a supraorbital bone (Weitzman and Malabarba, 1998). Further, these hypotheses do not propose a close relationship between these taxa and *Bryconops* (which also shares the bone) as observed in this study. Those authors questioned the utility of this presumably plesiomorphic character and our results confirm that interpretation. We also note with interest the basal position of the genus *Chalceus* (node 55; Fig. 6), represented by two species in our study, relative to all other characids. Historically, *Chalceus* has been regarded as closely related to *Brycon*

(e.g., Lucena, 1993; Weitzman and Malabarba, 1998; but see Howes, 1982). Based on mitochondrial sequence data, Ortí and Meyer (1997) also recovered *Chalceus* in a basal position within a characid subclade. However, our results differ from those authors in finding strong node support for the inclusion of *Chalceus* in a clade that excludes the Alestidae.

Early classifications included the cynodontines (*Hydrolycus*, *Rhaphiodon*, and *Cynodon*) as a subfamily of the Characidae (Greenwood et al., 1966), whereas we resolved *Hydrolycus* and *Rhaphiodon* (Cynodontidae) as the sister clade of a large assemblage formed by the Anostomoidea, Hemiodontidae, Parodontidae, and Serrasalminidae. Tantalizing morphological support for this relationship was offered by Toledo-Piza (2000) in terms of putatively homologous ventral processes on the mesethmoid of cynodontines and anostomoids (i.e., Prochilodontidae (*Prochilodus rubrotaeniatus*), Chilodontidae (*Caenotropus maculosus*), Anostomidae (*Laemolyta tainiata*), and Hemiodontidae (*Hemiodus* sp.); absent in Curimatidae). These processes are also absent in *Acestorhynchus* and in species of the subfamily Roestinae, taxa previously considered to be most closely related to cynodontids (Lucena, 1993; Lucena and Menezes, 1998). Further arguments against a close relationship between cynodontids and roestines are based on the presence of derived fin hooks in roestines and several representatives of the Characidae (Malabarba and Weitzman, 2003). The absence of roestine representatives in our analysis precluded an evaluation of the these hypothesis.

The piranhas and pacus (Serrasalminae or Serrasalminidae) constitute a distinctive assemblage of 14 genera (Machado-Allison, 1983) endemic to the Neotropics and widely distributed in South America. Machado-Allison (1983) was the first to conduct a cladistic analysis of the group based on morphology and recognized two major lineages, the pacu and piranha clades. Ortí et al. (1996), in the first phylogenetic study of the group to use nucleotide sequence data, placed the pacu genus *Acnodon* as sister to a clade formed by the piranhas and recovered a clade comprising the fruit-eating pacu genera *Mylossoma*, *Colossoma*, and *Piaractus* in a basal position. Our results are largely inconsistent with both previous studies. We recovered a well-supported monophyletic Serrasalminidae (node 22; Figs. 1 and 7) and a piranha clade (*Metynnis*, *Pygocentrus*, and *Serrasalmus*) nested within a subset of the pacus. Using morphological characters, Zanata (2000) recovered an unresolved *Serrasalmus/Colossoma* clade placed outside Characidae; both results argue for separate family status for the piranhas (contra Buckup, 1998). However, the scheme of relationships within the family should be interpreted with caution because an analysis at that level of taxonomic detail was beyond the scope of our study.

Finally, our study is congruent with previous results that recognize Anostomoidea, a large assemblage of Neo-

tropical characiforms comprised of the families Curimatidae, Prochilodontidae, Chilodontidae, and Anostomidae (Boulenger, 1904; Géry, 1977; Greenwood et al., 1966; Gregory and Conrad, 1938; Günther, 1864; Regan, 1911; Roberts, 1973; Vari, 1983). Vari (1983) presented synapomorphies for ((Curimatidae + Prochilodontidae) (Anostomidae + Chilodontidae)), whereas we recovered Anostomidae as the sister group of a clade formed by Chilodontidae and Prochilodontidae. However, the absence of Curimatidae in our study precludes further comparisons. Sister to Anostomoidea, we recovered a clade formed by the families Hemiodontidae and Parodontidae. Roberts (1974) and Langeani (1998) noted similarities between hemiodontids and parodontids; however, their findings were not sufficient to corroborate a hypothesis of sister group relationship between these two families. Rather, synapomorphies presented by Buckup (1998) and Langeani (1998) point to a close relationship of Hemiodontidae to a large clade formed by several Neotropical groups and the African Alestidae. Ortí and Meyer (1997) also resolved *Hemiodus* as sister to a Prochilodontidae/Curimatidae clade. In our analysis, the relationship between Hemiodontidae + Parodontidae clade and Anostomoidea has no bootstrap support; however, it shows moderate Bremer support (BS = 5; Fig. 7) and additional hidden support (Fig. 3).

#### 4.2. Trans-continental relationships

Our results agree with those of Buckup (1991, 1998) and Ortí and Meyer (1997) regarding the occurrence of three instances of African/South American sistergroup relationships among characiform fishes (Fig. 8). The African taxa involved in these three trans-continental sistergroup pairs are the same under each of the three phylogenetic hypotheses: the citharinoid clade, the alestid clade, and the hepsetid clade. This observation is not particularly noteworthy because these three clades encompass the entire diversity of characiforms in Africa at present. However, the Neotropical taxa represented in each of these trans-continental sistergroup associations are different under each hypothesis. The first sistergroup pair common to the three phylogenetic hypotheses (Fig. 8, node 1) involves the monophyletic unit formed by the two African families Citharinidae and Distichodontidae as sister to all other Characiformes, a relationship well supported by both morphological and molecular data (Buckup, 1991, 1998; Ortí and Meyer, 1997; Vari, 1979). The second relationship (Fig. 8, node 2) involves the African family Hepsetidae as sister to a clade formed by either the Neotropical families Erythrinidae (Fig. 8A, Ortí and Meyer, 1997), to the Ctenolucidae (Fig. 8B, Buckup, 1991, 1998; Vari, 1995), or to the Lebiasinidae plus Ctenolucidae (Fig. 8C, this study). Note that this sistergroup pair occupies a relatively terminal position under all three hypotheses. The third involves the

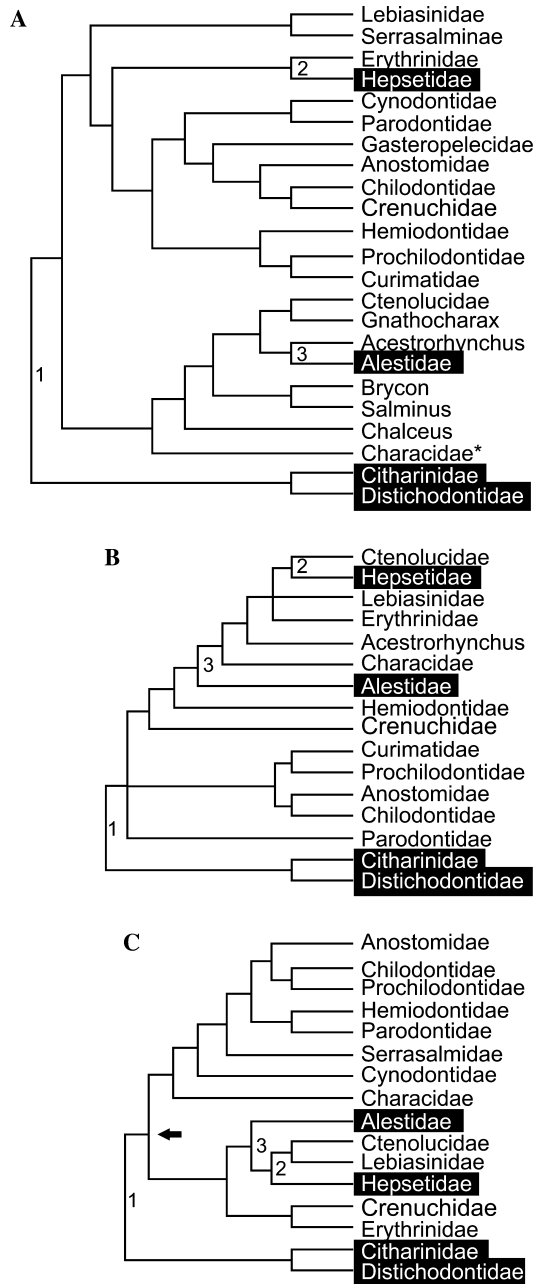


Fig. 8. Occurrence of trans-continental sistergroup relationships among characiform fishes implied by the results of A, Ortí and Meyer (1997); B, Buckup (1998); and C, this study. Numerals correspond with nodes that involve a sistergroup pair comprised of African and Neotropical taxa; arrow denotes divergence event corresponding with node 105 (Fig. 1) discussed in text; African taxa denoted in black; asterisk denotes Characidae as defined by Weitzman and Malabarba (1998).

African family Alestidae (Fig. 8, node 3). Ortí and Meyer's (1997) cladogram presents the only hypothesis of the three wherein the African Alestidae forms the sistergroup of, and is the sole African lineage nested entirely within, a strictly Neotropical clade (Fig. 8A). In both Buckup's (1998; Fig. 8B) and our results (Fig. 8C), alestids are nested within a larger assemblage that includes

both African and Neotropical taxa. Interestingly, both of the latter analyses find both alestids and hepsetids (African) included within a clade that also involves the Neotropical erythrinoids.

There is now broad consensus that the earliest diversification of characiform fishes pre-dated the separation of South America and Africa in the Late Cretaceous (Buckup, 1998; Bussing, 1985; Filleul and Maisey, 2004; Fink and Fink, 1981; Gayet et al., 2003; Lundberg, 1993) and that the Neotropical freshwater fish fauna has been essentially modern in taxic composition since the mid-Miocene (Lundberg, 1998; Weitzman and Weitzman, 1982). Based on Buckup's (1991) initial hypothesis of characiform relationships, Lundberg (1993) examined the implications of applying a vicariance scenario to the three trans-continental sistergroup pairs represented in that cladogram, with focus on the hepsetid/ctenolucid clade that occupies a terminal position in Buckup's (1991) and all more recent cladograms. Assuming a correspondence of this node with the vicariance event associated with the opening of the Southern Atlantic Ocean, and disallowing dispersal of taxa across a marine barrier, Lundberg (1993) pointed out that (1) most of the diversification among characiforms would have occurred prior to continent separation, and (2) a remarkably lopsided amount of extinction of characiforms in Africa would be required. The hepsetid/ctenolucid sistergroup pair was chosen for this consideration by Lundberg (1993), and similarly by Buckup (1998), because the two alternative vicariance scenarios involving trans-continental sistergroup pairs both posit older divergence events, coupled with more post-drift marine dispersal events, to explain the occurrence in Africa of representatives of predominantly Neotropical clades. The avoidance of, or bias against, post-drift marine dispersal of characiform fishes (see Gosline, 1944; Myers, 1938) stems from the observation that characiforms are an exclusively freshwater group, part of the larger radiation of otophysan fishes that are also predominantly freshwater, whose members are physiologically vulnerable to saltwater. However, our hypothesis of characiform relationships, coupled with the knowledge that certain early fossil otophysan taxa were marine (e.g., *Chanoidea*—Patterson, 1984) or occurred in brackish waters (e.g., *Santanichthys*—Filleul and Maisey, 2004), suggests that marine dispersal in the early history of characiform fishes cannot be ruled out simply because surviving members of the clade are at present intolerant of saltwater (Sparks and Smith, 2005). For example, if we were to assume that the divergence event represented by node 105 (denoted by arrow, Fig. 8C) corresponds with the vicariance event associated with the Late Cretaceous separation of Africa and South America, then only three instances of homoplasy would be required to explain the present continental association of characiform taxa: (1) dispersal of ctenolucids/lebiasinids to

South America; (2) dispersal of crenuchids/erythrinids to South America; (3) extinction of citharinoids in South America. Although available evidence does not point toward preference for a correspondence between any of these nodes and the presumed vicariance event, we note that node 105 in our phylogenetic hypothesis (Fig. 1) involves the most inclusive assemblage of characiform lineages of any node involving a trans-continental sistergroup pair, while at the same time invoking the fewest instances of incongruence in explaining the present-day distribution of characiforms relative to previous phylogenetic results.

It is now broadly accepted that the separation of Africa and South America cannot be regarded as a single event, but rather as a series of events that spanned more than 100 MY, and thus allowing for the possibility of multiple vicariance and dispersal events (Lundberg et al., 1998; Maisey, 1993, 2000, and references therein). Popoff (1988) suggested that three smaller plates, rather than a single plate, formed the South American Plate. Movement among these plates could have led to ephemeral secondary connections between the two continents during the early period of the opening of the South Atlantic (Maisey, 1993; Szatmari et al., 1987). The extensive endemic radiation of characids in the Neotropics may be a result of rapid diversification following a founder-event in the absence of competition (i.e., no cypriniforms in South America; Géry, 1977) and/or the opening of new habitats and expanded drainage evolution following the onset of Andean uplift in the Miocene (Lundberg et al., 1998). An endemic Neotropical radiation of characiforms is refuted if it turns out that the African fossil taxon *Sindacharax* (Greenwood and Howes, 1975; Stewart, 1994) belongs to the Serrasalminae as originally

proposed. Regardless, given an extended timeframe for continental separation and the marine and/or brackish occurrence for early otophysan and characiform fossil taxa, it now seems clear that hypotheses invoking marine dispersal cannot be excluded a priori, and that a simple model of vicariance does not readily explain the biogeographic history of the characiform fishes.

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### Appendix A

Material examined, voucher specimens, and GenBank accession numbers for gene sequences analyzed in this study

Taxon	Voucher	GenBank Accession number					
		16S	Cyt <i>b</i>	fhk	RAG2	sia	trop
<b>CHARACIFORMES</b>							
<b>Anostomidae</b>							
<i>Anostomus</i> sp.	AMNH233421	AY787954	AY791352	AY817286		AY790012	AY817179
<i>Anostomus a. trilineatus</i>	AMNH233414	AY787965	AY791362	AY817297		AY790024	AY817187
<i>Leporinus</i> sp.		AY788044	AY791416	AY817370	AY804095	AY790102	AY817252
<i>Leporinus striatus</i>	AMNH233300	AY788045		AY817371	AY804096	AY790103	AY817253
<b>Chilodontidae</b>							
<i>Chilodus punctatus</i>	AMNH233424	AY787997		AY817325		AY790056	AY817215
<b>Prochilodontidae</b>							
<i>Prochilodus lineatus</i>				AY817397	AY804117		AY817275
<i>Prochilodus nigricans</i>	AMNH233305	AY788075	AY791437	AY817400	AY804120	AY790133	AY817278
<b>Hemiodontidae</b>							
<i>Hemiodus gracilis</i>	AMNH233427	AY788027	AY791405	AY817353	AY804084	AY790086	AY817240
<b>Parodontidae</b>							
<i>Parodon</i> sp.	AMNH233323	AY788065	AY791427	AY817390	AY804110	AY790123	AY817269

## Appendix A (continued)

Taxon	Voucher	GenBank Accession number					
		16S	Cyt <i>b</i>	fhk	RAG2	sia	trop
<b>Serrasalminae</b>							
<i>Colossoma macropomum</i>		AY788000	AY791386	AY817328	AY804061	AY790059	AY817218
<i>Mylesinus</i> sp.		AY788056		AY817381		AY790114	AY817263
<i>Mylesinus schomburgkii</i>		AY787971	AY791367	AY817303	AY804035	AY790030	AY817192
<i>Myleus</i> sp.	MZUSP58511	AY788057	AY791422	AY817382	AY790115	AY817264	
<i>Myleus schomburgkii</i>	MZUSP58505	AY788055	AY817380	AY804105	AY790113	AY817262	
<i>Mylossoma</i> sp.		AY788049	AY817375	AY804100	AY790107	AY817257	
<i>Metynnys</i> sp.	MZUSP85843	AY788050		AY817376		AY790108	AY817258
<i>Piaractus brachypomus</i>	MZUSP85849	AY788067	AY791429	AY817392	AY804112	AY790125	AY817271
<i>Piaractus mesopotamicus</i>		AY788073	AY791435	AY817398	AY804118	AY790131	AY817276
<i>Pygocentrus nattereri</i>		AY788074	AY791436	AY817399	AY804119	AY790132	AY817277
<i>Serrasalmus</i> sp.		AY788079		AY817404	AY804123	AY790136	AY817281
<i>Serrasalmus rhombeus</i>	MZUSP58506	AY788081		AY817406		AY790138	
<b>Cynodontidae</b>							
<i>Hydrolycus pectoralis</i>		AY788033		AY817359	AY804088	AY790091	AY817244
<i>Rhaphiodon vulpinus</i>		AY788078		AY817403			
<b>Characidae</b>							
<i>Acestrorhynchus</i> sp.		AY787956	AY791353	AY817288	AY804026	AY790014	AY817181
<i>Acestrorhynchus</i> cf. <i>nasutus</i>	AMNH233420			AY817289		AY790015	AY817182
<i>Aphyocheirodon</i> sp.	AMNH233206	AY787966	AY791363	AY817298	AY804031	AY790025	
<i>Astyanacinus</i> sp.	AMNH233246	AY787969	AY791365	AY817301	AY804033	AY790028	AY817190
<i>Astyanacinus</i> sp.	AMNH233243	AY787987		AY817317	AY804051	AY790046	AY817209
<i>Astyanax bimaculatus</i>		AY787955		AY817287	AY804025	AY790013	AY817180
<i>Astyanax scabripinis</i>		AY787967		AY817299		AY790026	AY817188
<i>Brycon</i> sp.		AY787982		AY817312	AY804046	AY790041	AY817204
<i>Brycon hiliarii</i>		AY787976	AY791370	AY817307	AY804040	AY790035	AY817198
<i>Bryconamericus</i> sp.	AMNH233340	AY787984	AY791375	AY817314	AY804048	AY790043	AY817206
<i>Bryconoops</i> sp.		AY787985	AY791376	AY817315	AY804049	AY790044	AY817207
<i>Chalceus macrolepidotus</i>	AMNH233404	AY787999	AY791385	AY817327	AY804060	AY790058	AY817217
<i>Chalceus erythrusus</i>	AMNH233413	AY787990	AY791379	AY817320	AY804053	AY790049	AY817211
<i>Cheirodon</i> sp.	AMNH233214	AY787995	AY791382	AY817324	AY804057	AY790054	
<i>Cheirodontops</i> sp.	AMNH233215	AY787996	AY791383		AY804058	AY790055	
<i>Creagrutus</i> sp.	AMNH233216	AY788001			AY804062	AY790060	AY817219
<i>Exodon paradoxus</i>	AMNH233426	AY788013	AY791397	AY817340	AY804072	AY790072	AY817227
<i>Gephyrocharax</i> sp.	AMNH233275	AY788014	AY791398	AY817341	AY804073	AY790073	AY817228
<i>Hemibrycon</i> cf. <i>beni</i>	AMNH233328	AY788020	AY791402	AY817346	AY804079	AY790079	AY817234
<i>Hemigrammus bleheri</i>	AMNH233395	AY788017		AY817343	AY804076	AY790076	AY817231
<i>Hemigrammus erythrozonus</i>	AMNH233396	AY788023		AY817349	AY804081	AY790082	AY817236
<i>Hemigrammus rodwayi</i>	AMNH233397	AY788034		AY817360	AY804089	AY790092	AY817245
<i>Hyphessobrycon eques</i>	AMNH233428	AY788022		AY817348	AY804080	AY790081	AY817235
<i>Inpaichthys kerri</i>	AMNH233400	AY788039		AY817365	AY804093	AY790097	AY817248
<i>Knodus</i> sp.	AMNH233223	AY788041	AY791414	AY817367	AY804094	AY790099	AY817249
<i>Moenkhausia sanctaphilomenae</i>	AMNH233419	AY788054			AY804104	AY790112	AY817261
<i>Mimagoniates</i> sp.	AMNH233402	AY788051	AY791420	AY817377	AY804101	AY790109	AY817259
<i>Prodontocharax</i> sp.	AMNH233236	AY788064	AY791426	AY817389	AY804109	AY790122	
<i>Roebooides</i> sp.	AMNH233430	AY787994	AY791381	AY817323	AY804056	AY790053	AY817214
<i>Salminus maxillosus</i>		AY788080	AY791438	AY817405	AY804124	AY790137	AY817282
<i>Triportheus angulatus</i>	AMNH233403	AY788082		AY817407	AY804125	AY790139	AY817283
<b>Ctenolucidae</b>							
<i>Ctenolucius hujeta</i>	AMNH233412	AY787998	AY791384	AY817326	AY804059	AY790057	AY817216
<b>Lebiasinidae</b>							
<i>Nannostomus beckfordi</i>	AMNH233406	AY788059		AY817384		AY790117	AY817265
<i>Nannostomus espei</i>	AMNH233407	AY788062		AY817387		AY790120	AY817267
<b>Crenuchidae</b>							
<i>Characidium</i> sp.	MNRJ12843	AY787993				AY790052	
<i>Characidium fasciatum</i>	AMNH233235	AY787991		AY817321	AY804054	AY790050	AY817212
<i>Characidium fasciatum</i>	AMNH233251	AY787992	AY791380	AY817322	AY804055	AY790051	AY817213
<i>Characidium purpuratum</i>	AMNH233314	AY787988		AY817318	AY804052	AY790047	AY817210
<i>Characidium vidali</i>	MNRJ12838	AY788003	AY791388	AY817330	AY804064	AY790062	AY817221
<i>Elachocharax</i> sp.	AMNH233425	AY788032		AY817358			AY817243
<i>Melanocharacidium</i> sp.	AMNH233321	AY788083	AY791439	AY817408	AY804126	AY790140	AY817284

(continued on next page)

## Appendix A (continued)

Taxon	Voucher	GenBank Accession number					
		16S	Cyt <i>b</i>	fhk	RAG2	sia	trop
Erythrinidae							
<i>Hoplias</i> sp.		AY788031	AY791409	AY817357	AY804087	AY790090	AY817242
Alestidae							
<i>Alestes baremoze</i>	AMNH 226451	AY787963	AY791360	AY817295	AY804029	AY790022	AY817185
<i>Alestes macrophthalmus</i>		AY787964	AY791361	AY817296	AY804030	AY790023	AY817186
<i>Alestopetersius caudalis</i>		AY788019	AY791401	AY817345	AY804078	AY790078	AY817233
<i>Alestopetersius hilgendorfi</i>	AMNH233438	AY788070	AY791432	AY817394	AY804114	AY790128	AY817273
<i>Arnoldichthys spilopterus</i>	AMNH233399	AY787968	AY791364	AY817300	AY804032	AY790027	AY817189
<i>Bathyaethiops breuseghemi</i>	AMNH233422	AY788068	AY791430	AY817393	AY804113	AY790126	AY817272
<i>Brycinus carolinae</i>	RUSI65125	AY787960	AY791357	AY817292		AY790019	
<i>Brycinus carolinae</i>	RUSI65136	AY787962	AY791359	AY817294	AY804028	AY790021	
<i>Brycinus carolinae</i>	AMNH233628	AY787981	AY791373	AY817311	AY804045	AY790040	AY817203
<i>Brycinus schoutedeni</i>		AY787986	AY791377	AY817316	AY804050	AY790045	AY817208
<i>Brycinus imberi</i>	RUSI61488	AY787972	AY791368		AY804036	AY790031	AY817194
<i>Brycinus kingsleyae</i>	AMNH231534	AY787973		AY817304	AY804037	AY790032	AY817195
<i>Brycinus lateralis</i>		AY787974	AY791369	AY817305	AY804038	AY790033	AY817196
<i>Brycinus longipinnis</i>	AMNH233405	AY787975		AY817306	AY804039	AY790034	AY817197
<i>Brycinus longipinnis</i>	AMNH230817	AY787980		AY817310	AY804044	AY790039	AY817202
<i>Brycinus macrolepidotus</i>	RUSI65121	AY787959	AY791356	AY817291		AY790018	AY817184
<i>Brycinus nurse</i>	AMNH233415	AY787970	AY791366	AY817302	AY804034	AY790029	AY817191
<i>Brycinus opistotaenia</i>		AY787978	AY791372	AY817309	AY804042	AY790037	AY817200
<i>Bryconaeithiops</i> sp.		AY787983	AY791374	AY817313	AY804047	AY790042	AY817205
<i>Bryconaeithiops microstoma</i>	AMNH233390	AY787977	AY791371	AY817308	AY804041	AY790036	AY817199
<i>Hemigrammopetersius barnardi</i>		AY788016		AY817342	AY804075	AY790075	AY817230
<i>Hydrocynus brevis</i>	AMNH22644	AY788018	AY791400	AY817344	AY804077	AY790077	AY817232
<i>Hydrocynus forskalli</i>		AY788024	AY791403	AY817350	AY804082	AY790083	AY817237
<i>Hydrocynus vittatus</i>	AMNH233623	AY788026	AY791404	AY817352	AY804083	AY790085	AY817239
<i>Hydrocynus vittatus</i>	RUSI65127	AY788036	AY791410	AY817362	AY804091	AY790094	AY817247
<i>Hydrocynus vittatus</i>	RUSI61489	AY788035		AY817361	AY804090	AY790093	AY817246
<i>Hydrocynus goliath</i>	AMNH233631	AY788025		AY817351		AY790084	AY817238
<i>Ladigesia rolffi</i>	AMNH233394	AY788046	AY791417	AY817372	AY804097	AY790104	AY817254
<i>Micralestes occidentalis</i>	RUSI65135	AY787961	AY791358	AY817293	AY804027	AY790020	
<i>Micralestes acutidens</i>	RUSI614871	AY788047	AY791418	AY817373	AY804098	AY790105	AY817255
<i>Nanopetersius ansorgii</i>		AY788048	AY791419	AY817374	AY804099	AY790106	AY817256
<i>Micralestes</i> cf. <i>pabrensis</i>	AMNH233417	AY788002	AY791387	AY817329	AY804063	AY790061	AY817220
<i>Phenacogrammus interruptus</i>	AMNH233442	AY788052	AY791421	AY817378	AY804102	AY790110	AY817260
<i>Phenacogrammus interruptus</i>	AMNH233444	AY788072	AY791434	AY817396	AY804116	AY790130	AY817274
<i>Phenacogrammus aurantiacus</i>	AMNH233441	AY788066	AY791428	AY817391	AY804111	AY790124	AY817270
<i>Phenacogrammus hilgendorfi</i>	AMNH233445	AY788071	AY791433	AY817395	AY804115	AY790129	
<i>Rhabdalestes maunensis</i>		AY788077		AY817402	AY804122	AY790135	AY817280
Hepsetidae							
<i>Hepsetus odoe</i>	AMNH231495	AY788030	AY791408	AY817356	AY804086	AY790089	AY817241
Citharinidae							
<i>Citharinus citharus</i>	AMNH226441	AY787989	AY791378	AY817319		AY790048	
<i>Citharinus</i> sp.	RUSI65184	AY788037	AY791411	AY817363		AY790095	
Distichodontidae							
<i>Distichodus affinis</i>	AMNH233391	AY788004	AY791389	AY817331		AY790063	AY817222
<i>Distichodus notospilus</i>	AMNH231537	AY788010	AY791395	AY817337	AY804069	AY790069	AY817224
<i>Distichodus decemmaculatus</i>	AMNH233392	AY788005	AY791390	AY817332	AY804065	AY790064	AY817223
<i>Distichodus hypostomatus</i>		AY788006	AY791391	AY817333	AY804066	AY790065	
<i>Distichodus fasciolatus</i>	RUSI65159	AY788007	AY791392	AY817334	AY804067	AY790066	
<i>Distichodus fasciolatus</i>	AMNH233624	AY788008	AY791393	AY817335		AY790067	
<i>Distichodus lusosso</i>	AMNH233437	AY788009	AY791394	AY817336	AY804068	AY790068	
<i>Distichodus sexfasciatus</i>	AMNH233393	AY788012	AY791396	AY817339	AY804071	AY790071	AY817226
<i>Hemigrammocharax machadoi</i>	RUSI61221	AY788028	AY791406	AY817354		AY790087	
<i>Hemigrammocharax multifasciatus</i>	RUSI63497	AY788029	AY791407	AY817355	AY804085	AY790088	
<i>Hemistichodus vaillanti</i>		AY788021		AY817347		AY790080	
<i>Ichthyborus</i> sp.	AMNH233626	AY788038	AY791412	AY817364	AY804092	AY790096	
<i>Nannocharax macropterus</i>		AY788060		AY817385		AY790118	AY817266
<i>Neolebias ansorgii</i>		AY788058	AY791423	AY817383	AY804106	AY790116	
<i>Neolebias powelli</i>	AMNH233408	AY788061	AY791424	AY817386	AY804107	AY790119	
<i>Neolebias trilineatus</i>	AMNH233439	AY788063	AY791425	AY817388	AY804108	AY790121	AY817268

## Appendix A (continued)

Taxon	Voucher	GenBank Accession number					
		16S	Cyt <i>b</i>	fhk	RAG2	sia	trop
<i>Phago</i> sp.	AMNH233627	AY788069	AY791431			AY790127	
<i>Xenocharax</i> sp.	AMNH231548	AY788084	AY791440	AY817409		AY790141	
<i>Xenocharax spilurus</i>		AY788085	AY791441	AY817410		AY790142	
CYPRINIFORMES							
Cobitidae							
<i>Botia</i> sp.	AMNH233431	AY787979			AY804043	AY790038	AY817201
<i>Misgurnus</i> sp.	AMNH233435	AY788053		AY817379	AY804103	AY790111	
Cyprinidae							
<i>Danio rerio</i>	AMNH233432	AY788011		AY817338	AY804070	AY790070	AY817225
<i>Puntius tetrazona</i>	AMNH233436	AY788076		AY817401	AY804121	AY790134	AY817279
<i>Labeo lineatus</i>	AMNH233625	AY788042		AY817368		AY790100	AY817250
<i>Labeo sorex</i>	AMNH233629	AY788043	AY791415	AY817369		AY790101	AY817251
Gyrinocheilidae							
<i>Gyrinocheilus</i> sp.	AMNH233433	AY788015	AY791399		AY804074	AY790074	AY817229
SILURIFORMES							
Loricariidae							
<i>Ancistrus</i> sp.	AMNH233339	AY787958	AY791354	AY817290		AY790016	AY817183
Bagridae							
<i>Chrysichthys</i> sp.	AMNH233630	AY787957	AY791355			AY790017	AY817193
Heptapteridae							
<i>Pimelodella</i> sp.	AMNH233447	AY787953	AY791351	AY817285		AY790011	AY817178
Ictaluridae							
<i>Ictalurus punctatus</i>	AMNH233448	AY788040	AY791413	AY817366		AY790098	

Institution abbreviations as listed in Leviton et al. (1985).

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