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THE RADIATION OF CHARACIFORM FISHES AND THE LIMITS OF RESOLUTION OF MITOCHONDRIAL RIBOSOMAL DNA SEQUENCES

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Abstract.—Phylogenetic relationships among fishes from ostariophysan orders, characiform families, and serrasalmin genera (e.g., "piranhas") were examined using partial mitochondrial ribosomal DNA sequences of the 12S and 16S genes. Phylogenetic information content of these sequences was assessed at three levels of taxonomic inclusiveness by analyzing the patterns of nucleotide substitution using secondary structure models. Conserved and variable regions were identified, mapped onto the structural models, and compared at increasing levels of taxonomic divergence. In general, loop regions (unpaired) exhibited a higher level of variation than did stem regions (paired). A high proportion of compensatory substitutions was observed in stem regions in three data sets, suggesting strong selection to maintain the secondary structure. Saturation due to multiple substitutions was indicated by decreasing transition/transversion ratios and strong structural constraints on variation in comparisons among orders of Ostariophysi but was not obvious among familes of Characiformes and was not detected among serrasalmin genera. Reliable phylogenetic signal successfully reconstructed relationships among serrasalmin genera. However, aside from a few well-supported clades, relationships could not be reconstructed with confidence among characiform families and ostariophysan orders. The reciprocal monophyly of African and Neotropical characiform lineages was rejected (based on maximum likelihood ratio tests), and some support for previous hypotheses based on morphology was provided by the molecular data. The radiation of characiform fishes is discussed in a historical biogeographic context. [Biogeography; Characiformes; mitochondrial DNA; molecular phylogenetics; Ostariophysi; phylogenetic information; secondary structure.]

Characiform fishes provide a prime example of the complex evolutionary and biogeographical patterns often seen in tropical and subtropical faunas. They constitute a group of ecologically and morphologically diverse fish that live in rivers and lakes in Africa and the Neotropics. The vast array of trophic specializations found among characiforms is comparable to that of cichlids and includes detritivory (mud eating), herbivory, planktivory (plankton filtering), predation, fin and scale eating, and the notorious group predation of piranhas. Some species have peculiar morphological and physiological adaptations for survival in extreme hypoxic conditions commonly found in their floodplain environments (e.g., air breathing and special membranous extensions of the lips for gaseous exchange in the surface film of the water column). Other species ("hatchetfishes") have adaptations for "flight" up to several centimeters above water. Size range among characiforms is also remarkable: the largest species are predatory forms known to reach 130 cm in length and a weight of 38 kg (Géry, 1977; Sverlij and Espinach Ros, 1986), whereas the smallest, known as miniature species (Weitzman and Vari, 1988), have adults that do not exceed 26 mm in length (e.g., some "tetras," glandulocaudines, lebiasinids). Only a few groups exhibit parental care.

The order Characiformes is divided into 16 families (Greenwood et al., 1966) or 14 families (Géry, 1977) (see the Appendix); 4 of these families are endemic to Africa (ca. 200 species), and the rest are endemic to South and Central America (more than 1,200 species). The confinement of characiforms to exclusively freshwater habitats renders them closely linked to the dynamics of geological history and makes them an important model group for biogeographic studies (e.g., Myers, 1938). A major

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issue in characiform biogeography concerns the evolutionary consequence of drift-vicariant events caused by the separation of the African and South American continents, approximately 84–106 million years ago (Parrish, 1993; Pitman et al., 1993). The geographic distribution of Characiformes has been interpreted in a phylogenetic framework by Lundberg (1993), using a provisional hypothesis of relationships. Phylogenetic relationships among characiform lineages have been controversial (e.g., Weitzman and Fink, 1983; Uj, 1990; Buckup, 1991; Lucena, 1993; Vari, 1995), and so far have been addressed using morphological characters only (Fig. 1). Because African and Neotropical forms do not seem to form reciprocally monophyletic groups, Lundberg (1993) suggested that the origin of most major clades must have preceded the continental split, and as a consequence, their current distribution can only be explained by either unlikely marine dispersals or a remarkably disproportionate extinction of African taxa. To test current hypotheses of relationships as well as these biogeographic scenarios, a molecular phylogeny of the major groups of Characiformes is presented.

Most phylogenetic research using mitochondrial DNA (mtDNA) sequences of animals has been most successful at the population, species, and genus levels (reviewed by Wilson et al., 1985; Moritz et al., 1987; Avise et al., 1988; Meyer, 1994a, 1994b; Simon et al., 1994). MtDNA evolves at a high rate (e.g., Brown et al., 1982), and phylogenetic information is presumably lost over evolutionary time. However, Mindell and Honeycutt (1990) and Hillis and Dixon (1991) suggested that mitochondrial ribosomal genes (which are some of the most conserved in the mitochondrial genome) could be used as phylogenetic markers to resolve relationships among taxa that had diverged as long as 300 or 65 million years ago, respectively. Simon et al. (1994) concluded that the 12S and 16S ribosomal RNA (rRNA) genes are likely to be useful at the population level, where highly variable sites are not saturated with multiple substitutions, and at "deep" levels of divergence, where the more conserved sites contain useful phylogenetic information.

A recurring theme in all of these studies, and one of the most crucial issues in molecular systematics, is understanding what makes a molecule a suitable marker to trace organismal history. An understanding of the evolutionary behavior of molecular characters provides the basis for reliable phylogenetic analyses. The nucleotide substitution patterns (e.g., frequency of change, transition/transversion ratios, base compositional biases) might also be good indicators of the strengths and limitations of particular data sets. For some groups of organisms, highly corroborated phylogenies are available. These "known" phylogenies, which are derived from congruent hypotheses based on several independent data sets, may provide good benchmarks for empirical tests of candidate molecular markers (e.g., Friedlander et al., 1992, 1994; Graybeal, 1994). A molecule shown to harbor information that allows researchers to correctly arrive at these known phylogenies is deemed suitable for the study of other groups of taxa of comparable age of divergence. But at least two possible shortcomings of this approach have been noted: (1) nucleotide substitution rates may differ among taxa (e.g., Li and Tanimura, 1987), and (2) the time of the radiation of a particular group of interest might not necessarily be well known from the fossil record (Friedlander et al., 1992, 1994; Graybeal, 1994). Clearly, a gene shown to be a good marker because it provided data for the recovery of the phylogeny of a particular group of taxa may not be appropriate for other taxa.

For any molecular marker, the limit of phylogenetic resolution for recent divergences is easily defined by the lack of variable characters among taxa. At the other extreme, the limit of resolution for ancient divergences must be determined by using known phylogenies and looking for the degree of support that particular relationships receive (e.g., Smith, 1989) and/or by analyzing the pattern of molecular evolu-

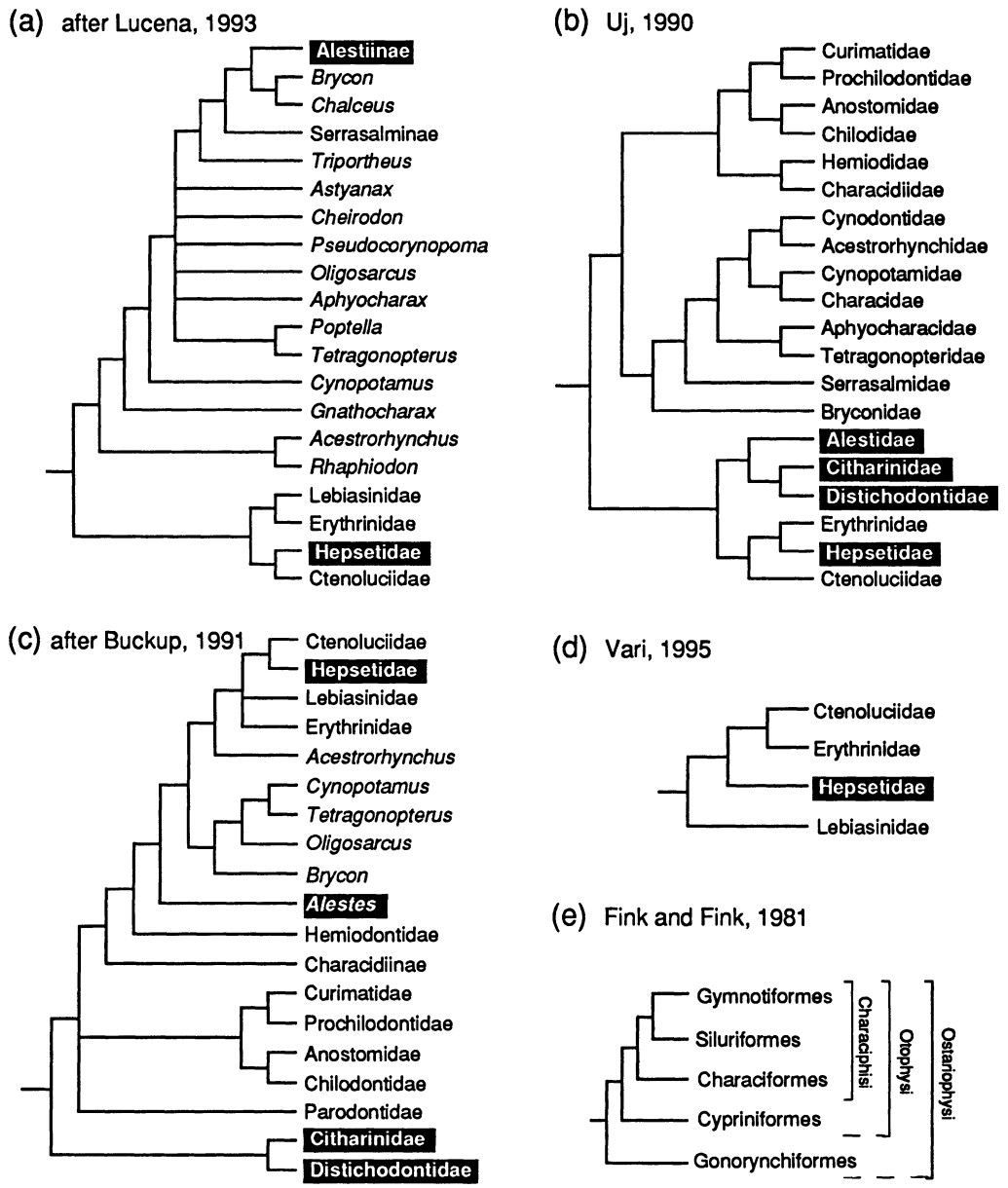

FIGURE 1. Phylogenetic hypothesis among ostariophysan and characiform taxa, based on morphological evidence. African taxa are enclosed in black boxes. (a) Modified from Lucena (1993). (b) From Uj (1990). (c) Modified from Buckup (1991). (d) From Vari (1995). (e) From Fink and Fink (1981).

tion. The latter alternative may constitute a complementary approach useful in the absence of preexisting information. We present an empirical study in which we used this approach to assess the limit of resolution of mitochondrial ribosomal DNA sequences for the phylogeny of characiform fishes. We examined in detail nucleotide substitution patterns for data sets with increasing levels of taxonomic inclusiveness, from closely related species and genera (within the subfamily Serrasalmi-

nae, which includes piranhas) to families (of Characiformes) and orders (of Ostariophysi, which includes Characiformes). Assuming that increasing taxonomic divergence translates into increasing divergence times, we describe the evolutionary behavior of the molecular characters, from the earliest stages of divergence, to determine at what taxonomic level they cease to contain reliable phylogenetic information.

Materials and Methods

Fish Specimens and DNA Sequences

A total of 53 specimens representing all families of Characiformes (15 currently accepted, see the Appendix) and all orders of ostariophysan fishes were included in this study. Species identifications, localities of origin, museum collection numbers, GenBank accession numbers, and other relevant information are given in the Appendix. Fish tissues for DNA extraction were preserved in 70% ethanol. Genomic DNA was extracted from muscle tissue by Proteinase K/SDS dissolution and purified by phenol–chloroform extraction and ethanol precipitation (Maniatis et al., 1982; Kocher et al., 1989). The polymerase chain reaction (PCR; Saiki et al., 1988) was used to amplify segments of the small (12S) and large (16S) subunit RNA mitochondrial genes. Double-stranded amplifications were performed in 25-μl volumes containing 67 mM Tris (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM 2-mercaptoethanol, 0.5 mM of each dNTP, 1 μM of each primer, 10–1,000 ng genomic DNA, and 0.5 units of *Taq* Polymerase (Perkin/Elmer-Cetus). The following primers were used: for 12S, L1091 and H1478 (Kocher et al., 1989); and for 16S, 16Sar-L and 16Sbr-H (Palumbi et al., 1991). These primers amplify fragments of the 12S and 16S mitochondrial rRNA genes corresponding to positions 1091–1478 and 2510–3059 in the human mitochondrial genome, respectively (Anderson et al., 1981). Gel purification of double-stranded products was followed by generation of single-stranded DNA, obtained by asymmetric PCR using the protocol described by Kocher et al. (1989), for direct sequencing from both directions (Gyllensten and Erlich, 1988). Single-stranded DNA was concentrated and desalted in Millipore Ultrafree-MC filters and sequenced by the dideoxy chain-termination method (Sanger et al., 1977) with the limiting primer from the asymmetric PCR amplification, using a commercial kit (Sequenase 2, U.S. Biochemical).

Sequence Alignment and Phylogenetic Analyses

With the DNA sequences obtained in this study plus sequences of *Eigenmannia*, *Rhamphichthys*, and *Apteronotus* (Gymnotiformes), *Hypostomus*, *Cetopsis*, *Trichomycterus*, and *Malapterurus* (Siluriformes), and cyprinids (see Appendix for GenBank accession numbers), three major data sets representing different taxonomic levels were compiled: two were restricted to the characiforms, and the third included taxa from all five ostariophysan orders. These data sets were collected with the intention of testing five major hypotheses of characiform relationships (Vari, 1979, 1983, 1995; Buckup, 1991; Lucena, 1993) and one hypothesis on the relationships among all ostariophysan orders (Fink and Fink, 1981). The first data set contained 27 species, mostly from the family Characidae, following Lucena (1993) in taxon selection (Fig. 1a). The second characiform data set is more inclusive, containing 38 taxa representing all characiform families. All four families of African characiforms were included to test whether African and Neotropical groups form separate monophyletic groups. The third data set included 22 ostariophysan taxa.

DNA sequences were aligned using CLUSTAL W 1.5, with default settings (Thompson et al., 1994). For each data set, sequences from both ribosomal subunits were concatenated into a single file, and a single alignment was obtained. Additional alignments with different gap : change cost ratios were also performed to detect alignment-ambiguous sites, following Gatesy et al. (1994a), where a site is considered ambiguous when gap assignments are unstable, differing among alternative align-

ments. Default settings for CLUSTAL W are opening gap cost = 10, extending gap cost = 5; the alternative settings used were opening gap cost = 5, extending gap cost = 4, and opening gap cost = 20, extending gap cost = 8. Computer-generated alignments were then refined based on published secondary structure models described by Guttel et al. (1985), Dams et al. (1988), and Neefs et al. (1991) for the small subunit rRNA molecule and by Guttel and Fox (1988) and Guttel et al. (1993) for the large subunit rRNA molecule. From these models, a structure for the piranha rRNA sequences was previously derived (Ortí et al., 1996) and was used here to adjust the sequences to a secondary structure format. Alignment gaps were placed by CLUSTAL W in stem regions only rarely, and they were either left there and considered as "bulges" in the paired structure or moved to contiguous unpaired regions. These gaps were moved in agreement with our structural model only if the gap assignment within the stem disrupted several otherwise complementary base-pair interactions.

Phylogenetic inference was based on maximum parsimony using PAUP 3.1.1 (Swofford, 1993) and MacClade 3.0 (Maddison and Maddison, 1992), maximum likelihood (Felsenstein, 1981) using fastDNAml 1.0.8 (Olsen et al., 1994) and NUCML 2.2 (Adachi and Hasegawa, 1994), and neighbor joining (Saitou and Nei, 1987) using MEGA 1.0 (Kumar et al., 1993). Heuristic searches using PAUP were done by stepwise random addition of taxa, with at least 20 replications and TBR branch swapping with the MULPARS option in effect, and by collapsing zero-length branches. Overall consistency indices (consistency index [CI], Kluge and Farris, 1969; retention index [RI], Farris, 1989) were calculated as a measure of fit between the data and the reported topologies. Successive approximations, or a posteriori reweighting (Farris, 1969; Carpenter, 1988), was used to test the stability of resulting topologies and also to choose among several equally parsimonious trees (reweighting was done by maximizing the rescaled consistency index [RC]). Maximum likelihood searches were done using empirical base frequencies, with the "quick" and "global" options in effect, and by jumbling the input order of taxa until the same best tree was obtained at least three times. Neighbor-joining reconstructions were based on Kimura's two-parameter distances (Kimura, 1981). Different character weightings and inclusion schemes were used with these programs according to the analysis of the nucleotide substitution patterns and are discussed with the resulting topologies. Bootstrapping (Felsenstein, 1985) was used to estimate confidence in the results (100 pseudoreplications for parsimony, 500 pseudoreplications for neighbor joining).

Patterns of Nucleotide Change

Nucleotide substitution patterns and compensatory mutations in stems are described for the 27-taxon characiform data set and the 22-taxon ostariophysan data set only because they represent both extremes of taxonomic divergence sampled in this study. These patterns were compared with those of a previous study (Ortí et al., 1996) of a 33-taxon data set of piranhas and pacus (subfamily Serrasalminae). The computation of nucleotide substitutions among sequences was done by tracing the substitutions on the best tree obtained by the above methods rather than by pairwise comparisons (Fitch and Markowitz, 1970; but see Collins et al., 1994). This approach provides an estimate of the changes that occurred across the phylogeny under the assumption of maximum parsimony (Swofford and Maddison, 1992). Average changes of character state were computed using MacClade (Maddison and Maddison, 1992). Based on the secondary structure models, nucleotide composition and substitution rates for stem and loop regions were calculated separately, following the method outlined by Vawter and Brown (1993). Relative rates of change for each structural category and for each gene fragment were calculated with and without corrections for category size. Relative rates of each kind of nucleotide substitu-

TABLE 1. Summary of variable and phylogenetically informative sites by region based on the secondary structure models from the 12S and 16S rRNA genes. Values for the data set containing 22 ostariophysan (O) taxa and the data set containing 27 characiform (C) taxa are given. Data sets excluding alignment-ambiguous sites are also shown. Alignment gaps were treated as missing.

	No. aligned nucleotides		No. variable sites		No. informative sites	
Data	O	C	O	C	O	C
12S						
Stems	155	155	51	47	35	31
Loops	142	130	81	70	62	53
All	325	319	142	123	104	89
16S						
Stems	171	174	49	49	33	31
Loops	258	273	160	157	129	127
All	545	548	283	270	189	191
Ambiguous excluded	747	729	320	270	200	169
Total (12S + 16S)	870	867	425	393	293	280

tion (e.g., A ↔ C, A ↔ G, A ↔ T) for each structural category and for each gene fragment were corrected for base composition. Because loops, for example, are AC rich, more changes between A and C than between any other pair of nucleotides are expected to occur there. Correction factors were defined by adding the percent composition for the category of both bases involved in the change and dividing the sum by 50%. The observed number of changes was then divided by this factor, so that if there were compositional bias in favor of a pair of nucleotides, division by a number larger than unity would correct for this overrepresentation (Vawter and Brown, 1993).

For stem regions, a tally of the changes in paired nucleotides (single changes and double changes) that maintain and disrupt the pairing was used for comparison with expected values, following the method of Dixon and Hillis (1993).

RESULTS

DNA sequence fragments of approximately 340 bp and 530 bp were obtained for the 12S and 16S genes, respectively. These fragments were aligned to a secondary structure format following the piranha 12S and 16S models (Ortí et al., 1996), and stem and loop regions were identified. A synopsis of the sites assigned to each region and the observed variation for the 27-taxon characiform and 22-taxon ostariophysan data sets is shown in Table 1. For these two data sets, the alignments (12S and 16S combined) required a total of 18–25 and 14–27 alignment gaps per sequence, respectively. Indels (insertion/deletion events) represented between 1.6% and 3.1% of the aligned sequence length. Most indels were 1 bp in length, and maximum indel length was 4 bp and 9 bp for the characiform and ostariophysan data sets, respectively. Alignment-ambiguous sites (Gatesy et al., 1994a) were detected in loop regions and comprised 122 and 138 sites for the ostariophysan and characiform data sets, respectively. The aligned sequences (PAUP files) are available electronically at http://www.utexas.edu/ftp/depts/systbiol and were published previously (Ortí, 1995).

Sequence divergence among taxa for the 12S and 16S fragments combined is summarized in Table 2. Variation between os-

TABLE 2. Percent sequence divergence within and between taxa for the 12S and 16S fragments combined (ranges) for 22 ostariophysan taxa.

		Between			
Taxa	Within	2	3	4	5
1. Kneriidae	6.2	21.5–22.5	20.5–21.6	20.3–22.4	19.3–24.1
2. Cypriniformes	12.2	—	15.7–18.5	16.9–21.0	17.8–21.9
3. Siluriformes	11.3–14.0		—	15.1–19.5	11.4–20.0
4. Gymnotiformes	12.3–16.0			—	13.7–20.9
5. Characiformes	9.2–21.3				—

tariophysan orders ranged from 11.4% when *Distichodus* (Characiformes) and *Hypostomus* (Siluriformes) were compared to 24.1% for *Kneria* (Gonorhynchiformes) and *Nannostomus* (Characiformes). Mean sequence divergence among orders was >21% for comparisons between Gonorhynchiformes and the other ostariophysan orders and 17–18% among cyprinids, catfish, electric fish, and characiforms (otophysans, sensu Fink and Fink, 1981). Within Characiformes, the most divergent sequences (*Boulengerella* and *Aphyocharax*) were 21.3% different, but average sequence divergence among characiform taxa was 15–16%.

Structural Constraints on Variation

Sequence variation was similar among 16S and 12S sequences but was larger in loop than in stem regions (Fig. 2). For example, mean pairwise sequence divergence was only 7% in stems but was 22% in loops for the 27-taxon characiform data set. The relative frequency of change among genes and structural categories did not differ among comparisons of data sets comprising different levels of taxonomic rank. The same proportions were observed in comparisons among genera within the subfamily Serrasalminae as among orders of ostariophysans (Fig. 2, left panels). In contrast, the actual number of changes per site increased with increasing level of divergence, as expected. On average, the 12S and 16S sequences showed approximately 0.6 changes per site in comparisons among serrasalmins but >1.5 changes per site in comparisons among characiform families and ostariophysan orders. No significant increase in the number of changes per site was observed in comparisons among ostariophysan orders relative to comparisons among families within Characiformes (Fig. 2, right panels).

Transition substitutions were the most frequent changes observed in both genes, in all structural categories, and in all data sets (Figs. 3, 4). But among transitions, the proportion of A ↔ G and C ↔ T changes differed between structural classes. In both genes, C ↔ T transitions were the most

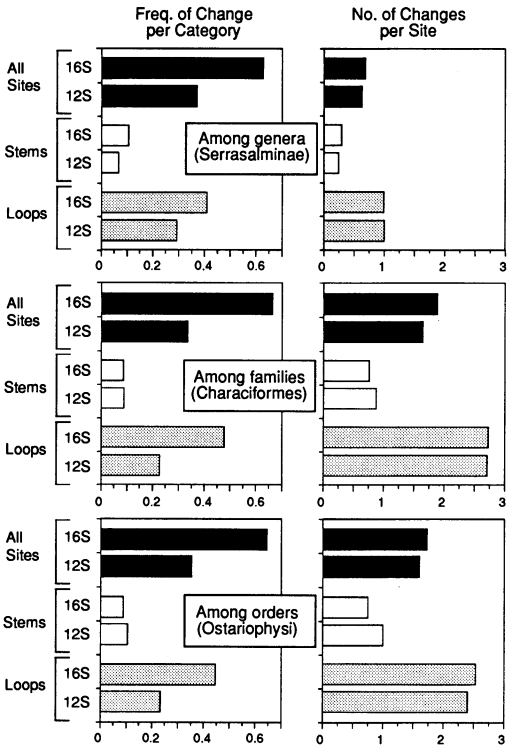

FIGURE 2. Distribution of variation among structural categories in the 12S and 16S sequences: changes among genera within the subfamily Serrasalminae (33 taxa, from Ortí et al., 1996), among families within the order Characiformes (27 taxa), and among orders within the superorder Ostariophysi (22 taxa). The relative frequency of change in the different genes and in stems and loops is the number of changes observed in the category divided by the total number of changes occurring at all sites. The amount of change per site in each category is the number of changes in the category divided by category size. All changes were reconstructed on the most-parsimonious trees (Figs. 8, 9) with MacClade.

common type of change in loop regions and overall, but A ↔ G transitions were more abundant among stem substitutions, especially in the 12S sequences. This bias in favor of A ↔ G transitions in 12S stems decreased with increasing sequence divergence. In comparisons among ostariophysan orders, the number of both kinds of transitions was approximately the same (Fig. 3, lower left panel). This decrease in A ↔ G bias was paralleled by an increase in double substitutions in stems (in both strands) as more distantly related sequenc-

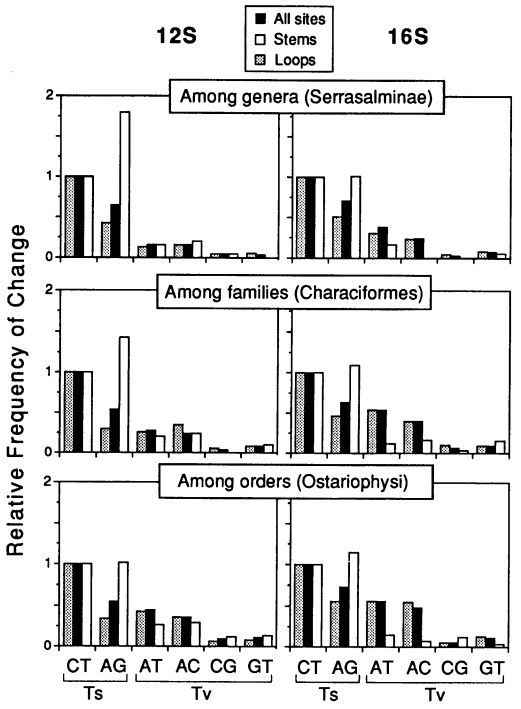

FIGURE 3. Relative frequencies of the different types of base change (corrected for base composition) in the 12S and 16S fish sequences. The average number of changes was reconstructed on the most-parsimonious trees using MacClade. For each gene and structural category, the rates are expressed as a proportion of the number of C ↔ T changes. Ts = transitions; Tv = transversions.

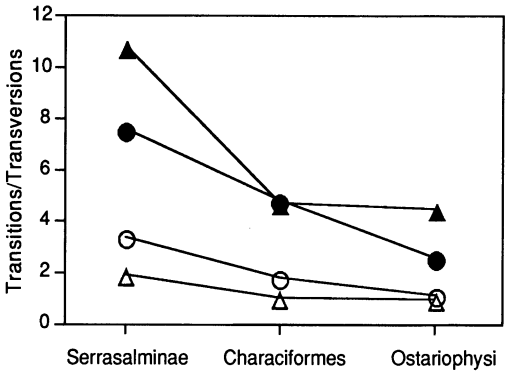

FIGURE 4. Transition/transversion ratios for changes reconstructed on the most-parsimonious tree for each of three fish data sets. Values for stem and loop regions for each gene fragment are shown separately. ▲ = 16S stems; ● = 12S stems; ○ = 12S loops; △ = 16S loops.

es are compared. In the 16S stem sequences, no such bias in favor of A ↔ G transitions was observed. Overall, transition bias was more pronounced in stem regions and in comparisons among recently diverged taxa (Fig. 4). The transition/transversion ratio was as high as 8–11 among serrasalmin stem sequences but dropped to 3–5 in comparisons among ostariophysans. With increasing taxonomic divergence the proportion of transversions increased, especially in loop regions (Figs. 3, 4), but there seemed to be no significant change in this proportion when going from characiform to ostariophysan comparisons. This leveling off of the transition/transversion ratio is more pronounced in the 16S sequences than in the 12S sequences (Fig. 4), suggesting that the 16S molecule might reach saturation levels sooner than the 12S molecule.

The most remarkable effect of structural constraints on level of variation among sequences can be seen in Figures 5 and 6. Regions within the sequences that are able to accept mutations were well defined for both genes. In comparisons among closely related serrasalmin genera, these regions were already well defined, and variation only accumulated within these regions in comparisons involving more distantly related taxa. This effect is more pronounced in 16S than in 12S because a few more variable regions were "recruited" in 12S, in comparisons among more distantly related taxa. Sequence variation seemed to reach saturation in comparisons beyond the family level; no obvious difference in sliding window profiles between the characiform and ostariophysan data sets could be detected (Figs. 5, 6, lower two panels). This observation, together with the leveling off of the transition/transversion ratio and the constancy in number of changes per site, strongly suggests saturation among sequences in the data set comparing ostariophysan taxa and to a lesser extent in the data set comparing characiform families.

Base composition for the different structural categories of the three 12S and 16S data sets is shown in Figure 7. Overall,

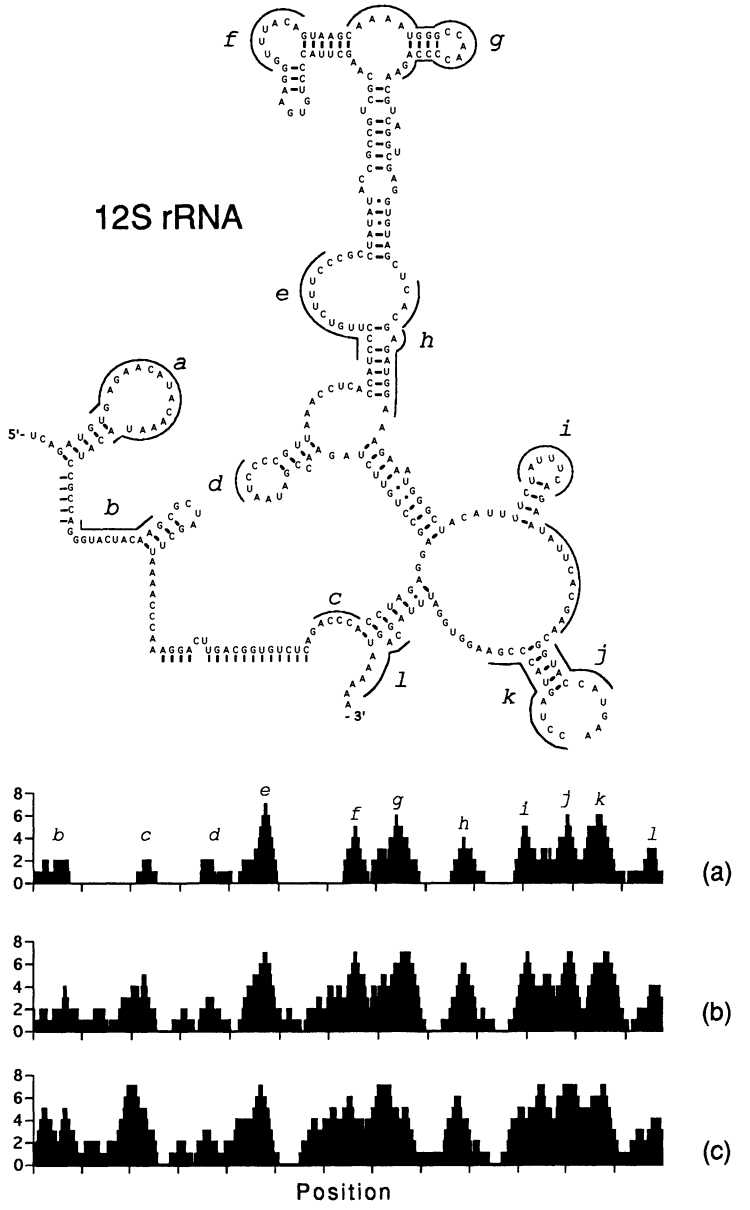

FIGURE 5. Secondary structure model for the piranha (*Pygocentrus nattereri*) 12S sequence (from Ortí et al., 1996). The three panels under the model show a sliding window analysis of variation (window size = 7, overlap = 1) for the serrasalmin (a), characiform (b), and ostariophysan (c) data sets. The number of variable sites in the window is plotted on the position along the sequence. Letters indicate the regions where variation among sequences is greatest.

both gene fragments showed very similar base composition, with a slight overrepresentation of A and C. Stem and loop regions differed in their content of A and G but not in their content of T and C. There was no variation in average base composition among the different data sets, but the range of variation in base composition among taxa increased slightly with taxonomic divergence. However, no obvi-

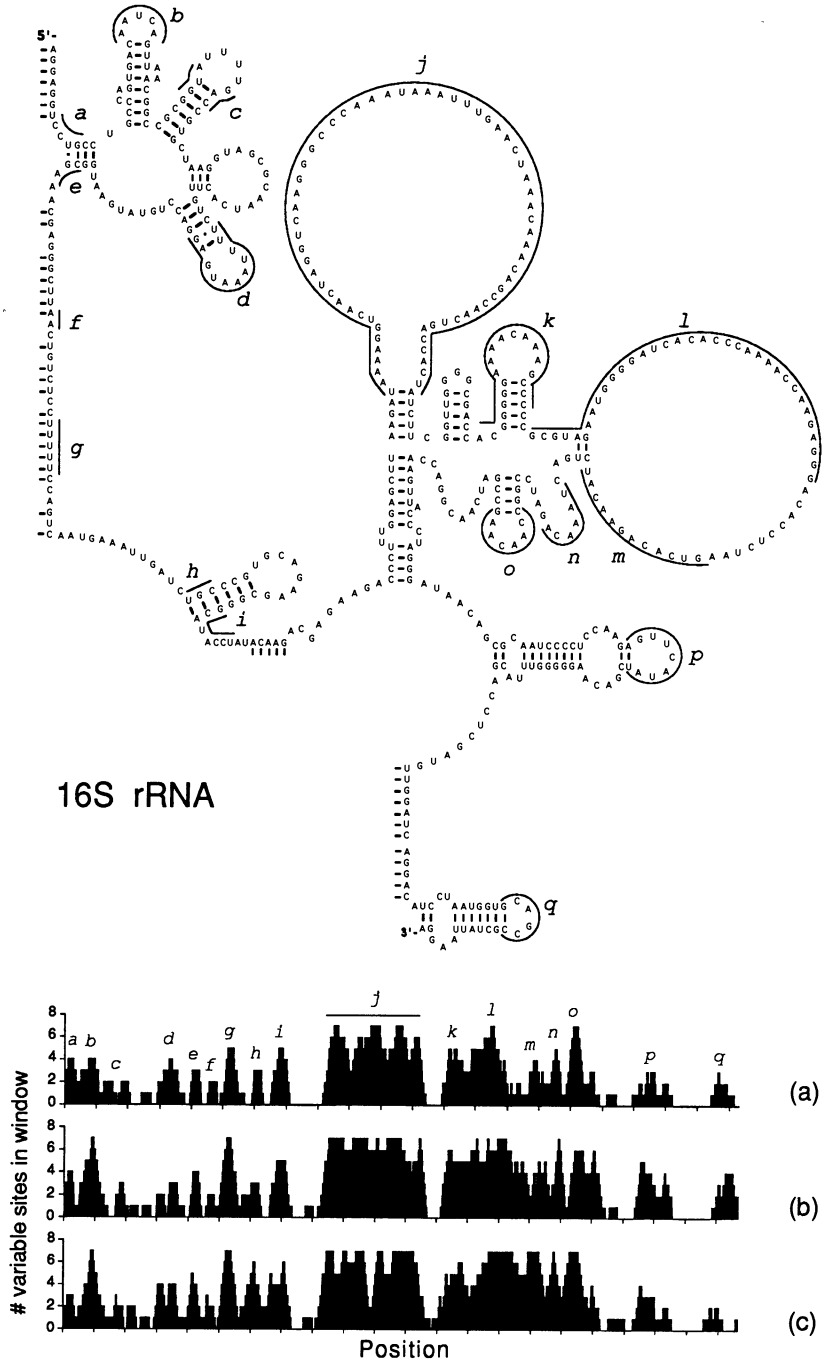

FIGURE 6. Secondary structure model for the piranha (*Pygocentrus nattereri*) 16S sequence (from Ortí et al., 1996). The three panels under the model show a sliding window analysis of variation (window size = 7, overlap = 1) for the serrasalmin (a), characiform (b), and ostariophysan (c) data sets. The number of variable sites in the window is plotted on the position along the sequence. Letters indicate the regions where variation among sequences is greatest.

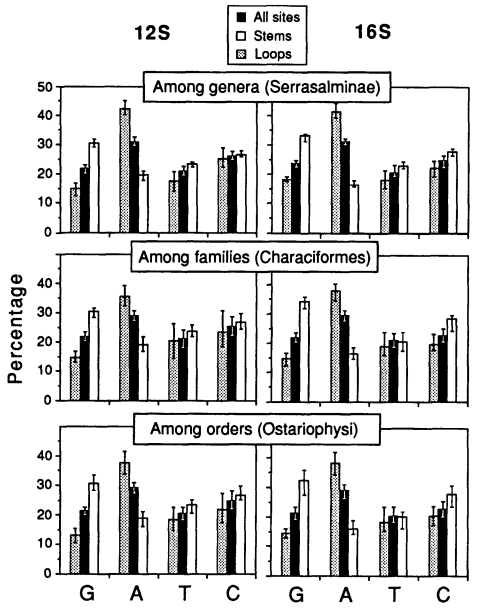

FIGURE 7. Base composition for stem, loops, and all sites for the 12S and 16S sequences in three fish data sets. Mean values and ranges are shown.

ous taxonomic bias in base composition was evident in any of the data sets.

Secondary Structure and Compensatory Changes

The secondary structure models proposed for the piranha sequences (Ortí et al., 1996) showed remarkable conservation across the ostariophysan and characiform taxa examined. The number of observed and expected compensatory substitutions (Dixon and Hillis, 1993) for both gene fragments and for both data sets is shown in Table 3. Most substitutions in stems did not disrupt base-pairing interactions, and there were significantly more compensatory mutations than expected by chance (χ^2 = 103.2 and 139.9 for single substitutions and χ^2 = 183.1 and 186.5 for double substitutions for the ostariophysan and characiform data sets, respectively; df = 1, $P < 0.001$). For the characiform data set, 192 of 243 substitutions in stem regions did not disrupt base-pairing interactions (only 50.5 nondisrupting mutations were expected by chance alone). Thus, 73.5% of all potential compensatory substitutions were realized (if all mutations were compensatory substitutions, this value would be 100%). For the ostariophysan data set, 68.3% of all potential compensatory substitutions were observed (194 of 259, of which only 54 were expected by chance). For both data sets analyzed, the proportion of potential compensatory substitutions was high, suggesting strong selection for maintaining secondary structure. A similar observation was reported for a comparison of the same gene fragments among serrasalmin genera (Ortí et al., 1996). However, sequence divergence among serrasalmins (piranhas and pacus) was maximally 8.9%, compared with the 11–24% divergence reported here. The percentage of all potential compensatory substitutions observed in stems was 73.6% for the serrasalmin data set (Ortí et al., 1996), almost identical to the values reported here.

TABLE 3. Substitutions observed in stem regions of the 12S and 16S sequences for the 22 ostariophysan (O) taxa and the 27 characiform (C) taxa.

Type of substitution	Expected		Observed	
	O	C	O	C
Single				
Base pairing to base pairing	11.6	11.1	44	48
Base pairing to non-base pairing	81.4	77.9	49	41
Double				
Base pairing to base pairing	21.2	19.7	75	72
Base pairing to non-base pairing	61.8	57.3	8	5

For both data sets most changes in stems occurred as pairs, rather than singly (when a base changed in one strand, there was a corresponding base change in the complementary strand). Single substitutions (regardless of whether or not they caused compensatory changes) comprised only 36% of all changes in stems (same value for both data sets), in contrast to 60.9% for the piranha data set (Ortí et al., 1996). Because U-G pairing is accepted in stems, single transitions (C ↔ T and A ↔ G) may not disrupt secondary structure. Transition bias is highest in stems for the serrasalmin data set (Fig. 4). As the level of divergence among taxa increases, so does the number

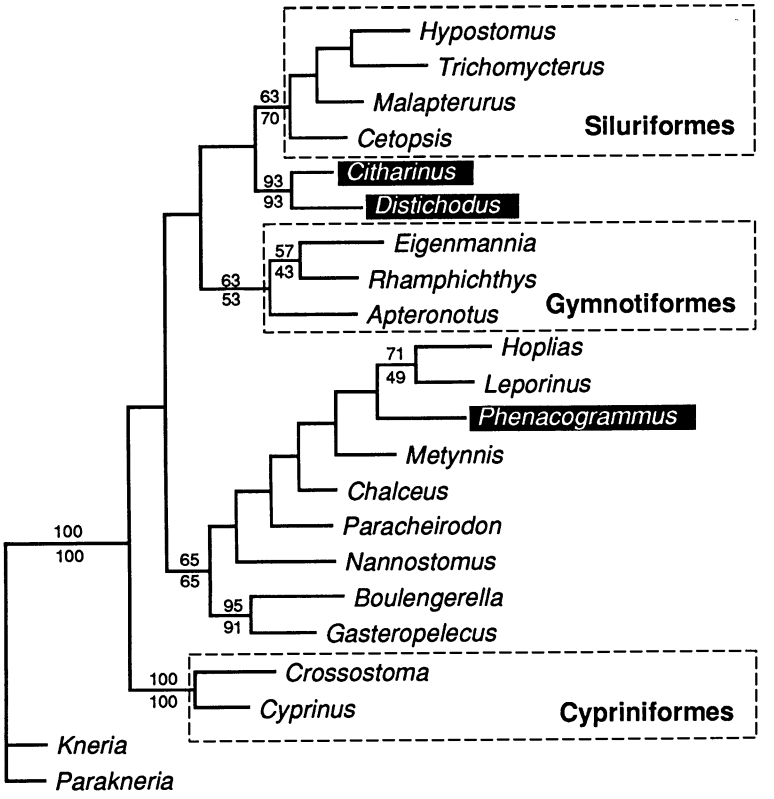

FIGURE 8. Shortest tree for the 22-taxon ostariophysan data set (all characters equally weighted, gaps treated as missing data). Gonorhynchiforms (*Kneria* and *Parakneria*) were treated as the outgroup. L = 1,460, CI = 0.429 (excluding uninformative characters), and RI = 0.384. Bootstrap support is shown only for those branches where at least one value was >50 (neighbor-joining bootstrap values above branches; parsimony values below branches). African characiform taxa are shown in black boxes.

of double changes and transversions in stems (Fig. 4), but the proportion of compensatory mutations remains at about the same level. Gatesy et al. (1994b) observed that the time lag for compensatory substitutions in the 12S and 16S genes of antelopes was rather small and suggested an increase in the rate of compensatory substitutions relative to other changes as a possible cause. The level of compensatory mutations observed for the mitochondrial 12S and 16S genes (close to 70%) was higher than that for the 28S gene (38%) reported by Dixon and Hillis (1993).

Phylogenetic Relationships among Ostariophysan Orders

Gonorhynchiforms (family Kneridae) were used as outgroup taxa for the otophysans (Fink and Fink, 1981). Parsimony analysis of the ostariophysan 22-taxon data set resulted in a single most-parsimonious tree (length [L] = 1,460) when all characters were equally weighted and gaps were coded as missing data (Fig. 8). Monophyly of all orders except Characiformes was well supported. The clade formed by *Citharinus* and *Distichodus*, representing two African characiform families, grouped with catfishes rather than with the other characiforms. There were nine suboptimal trees a single step longer (L ≤ 1,461), a strict consensus of which showed no resolution among otophysan orders and the citharinid–distichodontid clade. Forcing characiform monophyly required only three extra-steps (L = 1,463). Bootstrap values <50 for all relationships among or-

ders indicate that resolution of deep branches in the tree is poor, and low consistency indices (CI = 0.429, RI = 0.384) indicate that the level of homoplasy in the data set is high. The tree resulting from neighbor-joining (NJ) analysis also grouped *Distichodus* and *Citharinus* with catfishes and placed Gymnotiformes as the sister group of Characiformes + Siluriformes. The same result was obtained with maximum likelihood (ML) analyses.

When the 123 alignment-ambiguous sites were excluded from the analysis, three equally most-parsimonious trees were obtained (L = 940, CI = 0.369, RI = 0.391). A strict consensus of these trees showed highly unlikely relationships, placing the cypriniforms nested within the catfish and the distichodontid–citharinid clade as the sister group of all other otophysans. When alignment-ambiguous sites were included in the analysis and only transversions were used, six equally parsimonious trees were obtained, leaving the deeper nodes unresolved and characiform monophyly unsupported. As suggested by the substitution pattern analysis, the sequences contained in this data set do not provide reliable information to solve this phylogeny with confidence.

Phylogenetic Relationships among Characiform Families: 27-taxon Data Set

The citharinid–distichodontid clade is the sister group of all characiform lineages (Fink and Fink, 1981; Ortí and Meyer, 1996) and was used as the outgroup for this data set. Parsimony analysis, giving all sites equal weight and treating alignment gaps as missing data, resulted in three shortest trees (L = 1,564, CI = 0.344, RI = 0.361). With a posteriori reweighting, one of the shortest trees was obtained (Fig. 9). However, bootstrap analysis only supported eight clades with values >50: (1) *Nannostomus* + *Pyrrhulina* (family Lebiasinidae), (2) *Hoplias* + *Hepsetus*, (3) *Boulengerella* + *Ctenolucius* (family Ctenoluciidae), (4) *Phenacogrammus* + *Hydrocynus* + *Alestes* (African subfamily Alestiinae) + *Acestrorhynchus*, (5) *Poptella* + *Oligosarcus* + *Astyanax*, (6) *Brycon* + *Salminus* (subfamily Bryconinae, in part), (7) *Pygocentrus* + *Colossoma* (subfamily Serrasalminae), and (8) *Citharinus* + *Distichodus*. NJ also supported clades 1–8 with high bootstrap values, but relationships among clades differed from those obtained with parsimony and were not supported by bootstrap analysis (Fig. 9). Additionally, NJ and parsimony bootstrap analysis both supported a clade formed by *Cynopotamus* and *Cheirodon* with values of 99 and 48, respectively (not shown in Fig. 9). The best ML tree (ln likelihood = −8829.15) was obtained in 6 of 10 "jumbled" replications using the fastDNAml program. It contained clades 1–8 and had a topology very similar to that shown in Figure 9.

When the 138 alignment-ambiguous sites were excluded from the analyses, parsimony yielded four shortest trees (L = 835, CI = 0.355, RI = 0.391). A strict consensus of these trees was mostly unresolved but contained clades 1–8 plus a clade formed by *Tetragonopterus* + *Chalceus*. The strict consensus tree also grouped serrasalmins (clade 7) with *Hepsetus* + *Hoplias* (clade 2) as sister groups and grouped this combined clade with lebiasinids (clade 1) + *Rhaphiodon*. A posteriori reweighting resulted in a single completely resolved tree with all the previously identified clades, but it was different in several respects from the tree shown in Figure 9. However, only clades 1–8 were supported by bootstrap analysis with values >50 (similar to those shown in Fig. 9). All other relationships received poor support. Similar results were obtained with NJ and ML analyses. For example, the best ML tree obtained when alignment-ambiguous sites were excluded (see Table 4) was only four steps longer than the most-parsimonious trees and differed in several ways from the topology shown in Figure 9. The only relationships among clades 1–8 suggested by all three methods, excluding the 138 alignment-ambiguous sites, were ((*Hoplias*, *Hepsetus*)(*Colossoma*, *Pygocentrus*)) and ((*Nannostomus*, *Pyrrhulina*)*Rhaphiodon*), both with very low bootstrap support.

The most conservative strategy for ob-

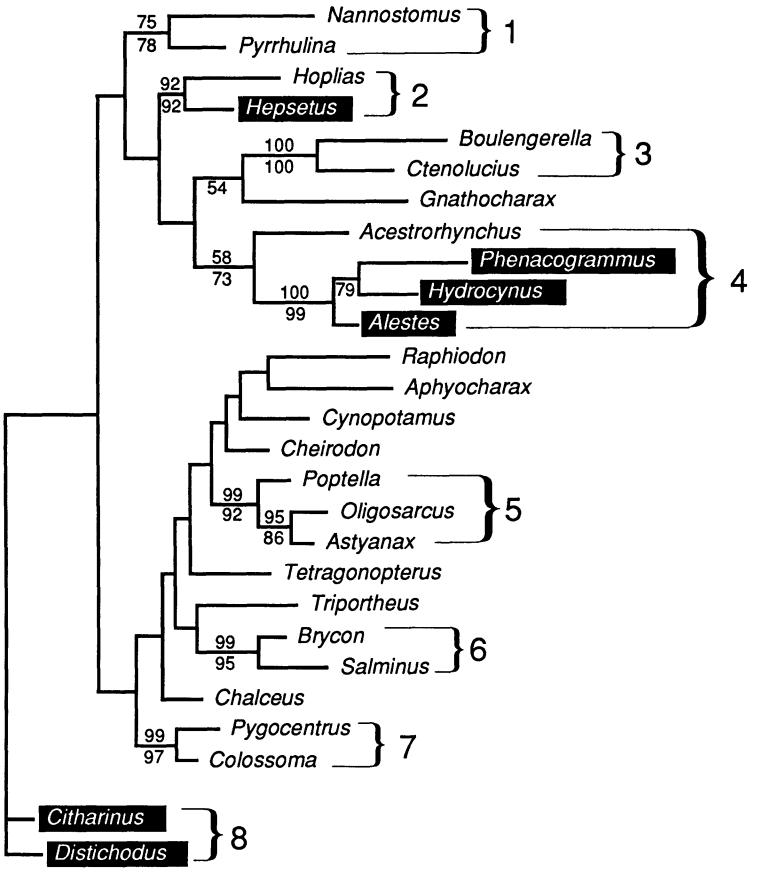

FIGURE 9. Characiform data set with 27 taxa. Shortest tree resulting from a posteriori character reweighting on the three equally parsimonious trees obtained when all sites were uniformly weighted (all characters used, gaps coded as missing). The reweighted tree had RC = 0.375. Bootstrap values above branches are from neighbor-joining analysis, and those below branches are from parsimony analyses. Branches without numbers had bootstrap values <50 in both analyses. Numbered braces highlight clades supported by bootstrap analyses. African taxa are shown in black boxes.

taining reliable phylogenetic information, according to the substitution pattern analysis described above, would be to eliminate saturated transitions and alignment-ambiguous sites (which are in the most quickly evolving regions within loops). Using this strategy, parsimony analysis resulted in 302 shortest trees. A posteriori reweighting of characters based on these trees yielded 15 equally parsimonious trees, a strict consensus of which is shown in Figure 10. Most of clades 1–8 are present in the strict consensus tree. However, Serrasalminae (clade 7), *Hoplias* + *Hepsetus* (clade 2), and Lebiasinidae (clade 1) were not supported in this analysis. NJ using only transversions gave a similar result, except that Serrasalminae was supported, but Lebiasinidae and *Hoplias* + *Hepsetus* were not. Parsimony and NJ analyses were also congruent in defining a clade formed by *Hoplias* and Ctenoluciidae (clade 3). However, bootstrap support for these groupings was very low (values not shown).

Although no single set of relationships was firmly supported by this data set (other than the components discussed above), it was used to test alternative topologies using the likelihood ratio test of Kishino

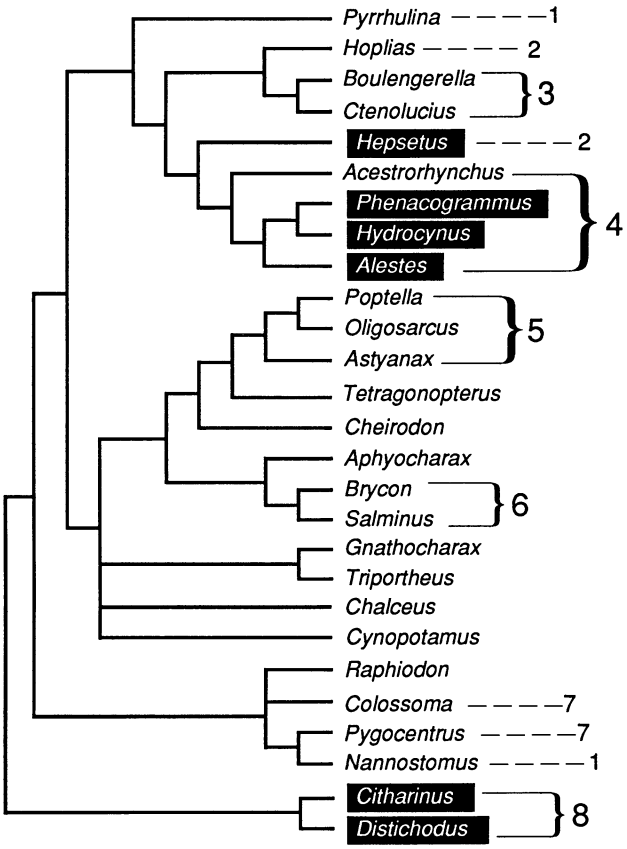

FIGURE 10. Characiform data set with 27 taxa. This strict consensus of 15 trees was obtained by a posteriori reweighting, excluding alignment-ambiguous sites and considering only transversions. Numbered braces identify clades also obtained in Figure 9, and dashed lines with numbers are clades shown in Figure 9 but not supported in this strict consensus. African taxa are shown in black boxes.

and Hasegawa (1989). Table 4 shows parsimony tree lengths (from MacClade) and log likelihood values (computed with the program NUCML; Adachi and Hasegawa, 1994) for alternative topologies, including and excluding the alignment-ambiguous sites. Nine tree topologies were compared with this method (see Table 4) and were included in the "trees" block in the (electronically available) data file. In addition to testing different results obtained with different phylogenetic methods and inclusion sets, the main purpose of these comparisons was to evaluate a previous hypothesis (Lucena, 1993; Fig. 1a) and the phylogeny implied by a single viacariant event separating African and Neotropical lineages (i.e., monophyly of the Neotropical taxa). These alternative topologies were obtained by optimizing (with parsimony or with ML) while enforcing topological constraints. For example, the African taxa (*Hepsetus* and Alestiinae) were forced to a basal position, joining the *Citharinus* + *Distichodus* clade in the "Neotropical monophyly" constraint tree, and the best solution for a fully resolved tree was searched for using PAUP or NUCML. Similarly, the topology used to test Lucena's (1993) hypothesis was the best (ML or parsimony) tree among the 945 fully resolved trees that agree with the partially resolved topology shown in Figure 1a. When all sites were considered, the best parsimony tree (tree 1, Table 4; also shown in Fig. 9) was not significantly different from the

TABLE 4. Tree lengths for parsimony (PARS) analyses (from MacClade) and log likelihoods for maximum likelihood (ML) analyses (from NUCML) for alternative phylogenetic hypotheses using the characiform 27-taxon data set, including and excluding alignment-ambiguous sites. Tree topologies are as follows: (1) best parsimony "reweighted" tree using all sites (shown in Fig. 9); (2) best ML tree from fastDNAml, using all sites; (3) best ML tree from fastDNAml, excluding alignment-ambiguous sites; (4) best parsimony tree using all data, constraining monophyly of all Neotropical taxa; (5) best ML tree using all data, constraining monophyly of all Neotropical taxa; (6) best parsimony tree (equal to best ML tree) excluding alignment-ambiguous sites, constraining monophyly of all Neotropical taxa; (7) best ML tree using all sites, constraining the topology to satisfy Lucena's (1993) hypothesis (see Fig. 1a); (8) best parsimony "reweighted" tree, excluding alignment-ambiguous sites and transitions (TS) (the best ML tree of the 15 trees summarized in Fig. 10); (9) best ML tree excluding alignment-ambiguous sites, constraining the topology to satisfy Lucena's (1993) hypothesis (see Fig. 1a). Extra steps are counted with respect to the shortest tree in this table, for each inclusion set. ΔL_i is the difference in log likelihood between the best tree and tree i (SE = standard error). The best topologies for each method and inclusion set are underlined, and log likelihood values significantly worse than the best estimate have asterisks.

	Parsimony (tree lengths + extra steps)		Maximum likelihood			
			All sites		Ambiguous excluded	
Topology	All sites	Ambiguous excluded	Log likelihood	ΔL_i + SE	Log likelihood	ΔL_i ± SE
1. PARS all data	1,564	849 (+10)	−8870.2	−24.4 ± 23.0	−5455.2*	−51.3 ± 25.5
2. ML all data	1,568 (+4)	858 (+19)	−8845.9	0.0 ± 0.0	−5462.0*	−58.2 ± 23.1
3. ML amb. excluded	1,569 (+5)	839	−8865.1	−19.3 ± 28.1	−5403.9	0.0 ± 0.0
4. PARS all data, Neo. monophyly	1,590 (+26)	863 (+24)	−8931.2*	−85.3 ± 30.6	−5480.8*	−77.0 ± 25.5
5. ML all data, Neo. monophyly	1,593 (+29)	866 (+27)	−8896.9*	−51.1 ± 25.2	−5488.2*	−84.4 ± 30.9
6. PARS-ML amb. excluded, Neo. monophyly	1,606 (+42)	847 (+8)	−8966.8*	−120.9 ± 40.5	−5448.1	−44.2 ± 27.0
7. ML all data, Lucena	1,649 (+85)	901 (+62)	−9056.8*	−210.9 ± 42.9	−5604.8*	−200.9 ± 42.3
8. PARS amb. and TS excluded	1,655 (+91)	890 (+51)	−9033.1*	−187.3 ± 42.1	−5507.8*	−103.9 ± 36.2
9. ML amb. excluded, Lucena	1,660 (+96)	903 (+64)	−9072.3*	−226.5 ± 43.4	−5602.0*	−198.1 ± 42.8

best ML tree (tree 2, Table 4), but all other alternative topologies tested (trees 4–9) were significantly worse. Following Kishino and Hasegawa (1989), alternative topologies were considered significantly worse than the best ML tree when the 95% confidence interval of the log likelihood difference between them did not include zero. Significantly worse alternative topologies are shown with asterisks in Table 4 for both inclusion sets. Hypotheses of Neotropical monophyly and Lucena's (1993) hypothesis were not supported by the 12S and 16S data sets. Of all alternative topologies tested, only tree 6 (Table 4) was not rejected but was close to being significantly worse than the best ML tree (tree 3) when tested with the data set excluding alignment-ambiguous sites ($\Delta L_6 = -44.2 \pm 27$).

Phylogenetic Relationships among Characiform Families: 38-taxon Data Set

Parsimony analysis of the 38-taxon characiform data set, weighting all sites equally and treating alignment gaps as missing, resulted in eight shortest trees (L = 2,059, CI = 0.283, RI = 0.371), but their strict consensus is highly unresolved. Clades 1–8 (see Figs. 9, 10) are also present in the consensus, plus a few others shown in Figure 11. For simplicity, only results obtained when excluding alignment-ambiguous sites will be presented because all major differences between inclusion sets are not strongly supported.

Parsimony analysis excluding 140 alignment-ambiguous sites resulted in 14 shortest trees (L = 1,052, CI = 0.290, RI = 0.392), and with a posteriori reweighting a single completely resolved tree was obtained (Fig. 11). The same pattern as that for the 27-taxon data set emerges, but in addition to the previously described eight clades, four more clades are supported. Clade 9 (family Gasteropelecidae), clade 10 (*Prochilodus* + family Curimatidae), and clade 11 (subfamily Glandulocaudinae, family Characidae) are strongly supported by bootstrap analysis (both NJ and parsimony), and each constitutes a well-supported unit in terms of morphology. The best tree from ML searches (ln likelihood = −6604.3, using fastDNAml) was the same as 1 of the 14 shortest parsimony trees; all 14 shortest trees from parsimony had very similar log likelihoods and did not differ significantly from each other when tested simultaneously (Kishino and Hasegawa, 1989). NJ analysis supported the same components as parsimony (see NJ bootstrap values in Fig. 11) but resulted in a globally different topology overall, which required 28 extra steps for parsimony and had a significantly worse likelihood than the best tree.

Forcing the monophyly of Neotropical taxa resulted in seven equally parsimonious trees with 13 extra steps (L = 1,065). When these seven trees were tested against the best ML tree with NUCML, they all had significantly worse log likelihood values (not shown). Alternative hypotheses (Fig. 1) such as those from Buckup (1991) and Uj (1990) were not tested with this data set because they did not include all taxa used in this analysis.

DISCUSSION

Sequence Variation and the Limits of Phylogenetic Resolution

Most phylogenetic relationships among taxa were not resolved with confidence in this study using ribosomal mtDNA sequences. Lack of resolution might be attributed to saturation of nucleotide changes and/or to the mode of evolutionary diversification of the taxa involved. Comparisons of 12S and 16S sequences among characiform families showed a slightly lower level of mean sequence divergence (14.9%) than did comparisons among orders of otophysans (17.3%). Assuming rate constancy across all lineages, this observation could be taken as evidence for dating the origination of the major lineages of Characiformes very close to the origin of the otophysan orders (cypriniforms, catfishes, electric fishes). Alternatively, similar values of sequence divergence among lineages may reflect saturation at the DNA level, given the structural constraints on sequence varia-

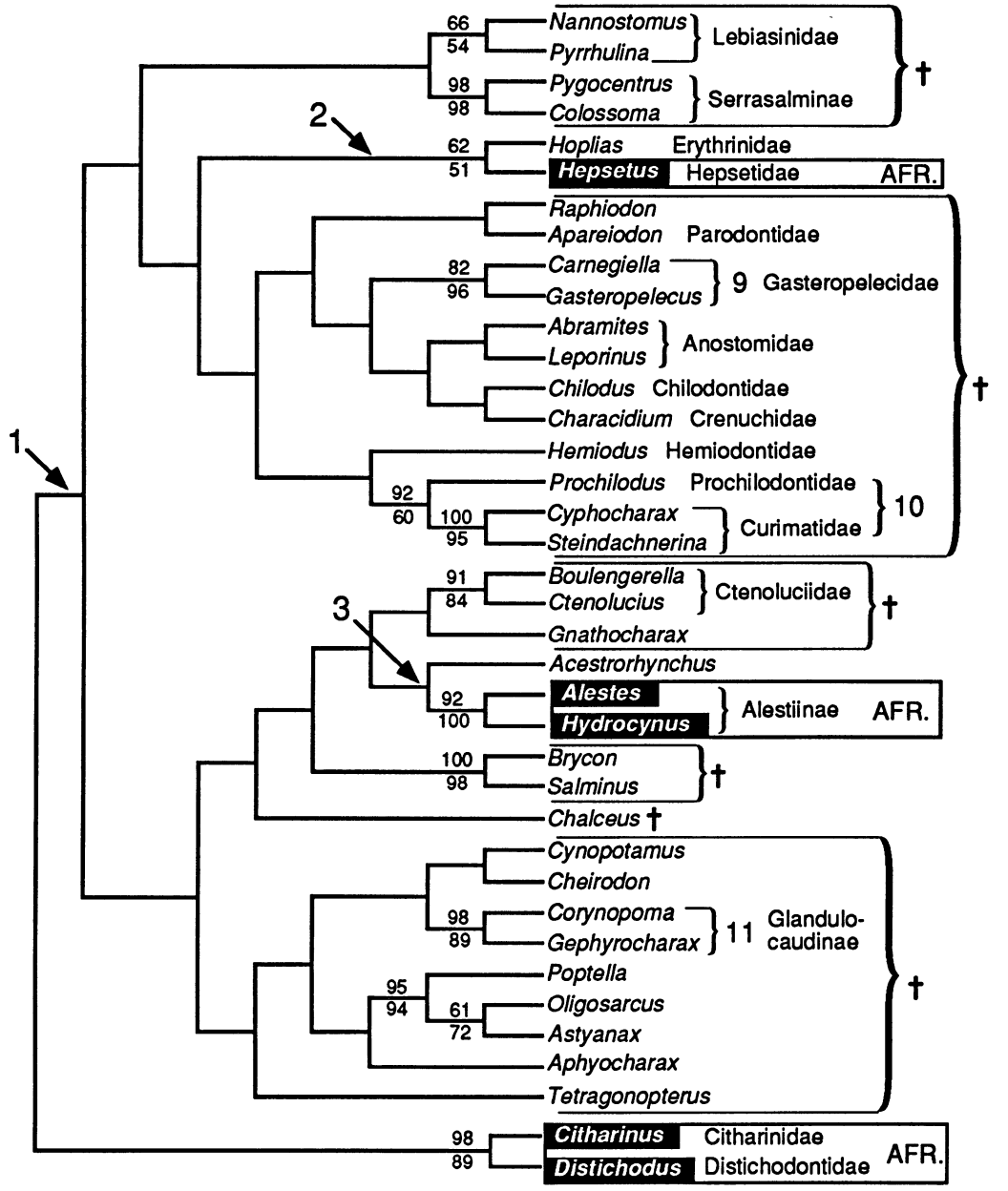

FIGURE 11. Characiform data set with 38 taxa. Shortest tree obtained with a posteriori reweighting on 14 equally parsimonious trees, when alignment-ambiguous sites were excluded. Numbers above and below branches are bootstrap values from neighbor joining and parsimony analyses, respectively (only values >50 are shown). Numbered braces (9–11) identify clades, other than those (1–8) shown in Figure 9, that are supported by bootstrap analyses. Family names are given next to each taxon, except for the family Characidae. For the Characidae, only subfamilial groupings supported by bootstrap analyses are indicated (Serrasalminae and Glandulocaudinae). Numbered arrows mark putative drift-vicariant events between African and Neotropical groups; † = clades postulated to have gone extinct in Africa. African taxa are shown in black boxes.

tion. The higher average divergence observed in comparisons between gonorhynchiforms and otophysans (21.1%) suggests that the divergence values among otophysans might be close to but not yet at complete saturation. However, maximum divergence values among characiform families, otophysan orders, and ostariophysan orders were essentially all the same (21.3%, 21.9%, and 24%, respectively; Fig. 12), indicating that saturation is a problem beyond the family level.

The same conclusion may be predicted by inspecting molecular variation at other gene loci. Comparing amino acid sequences of the ependymin gene among ostariophysans, Ortí and Meyer (1996) reported larger sequence divergences in comparisons among ostariophysan orders than within Characiformes. For example, amino acid sequence divergence between *Distichodus* and the other characiforms (ca. 22%) was slightly less than divergence between characiforms and electric fish (25%) and than that between characiforms and cyprinids (27%). But ependymin amino acid sequence divergences between characiforms and catfishes and between cyprinids and electric fishes were >34%. Furthermore, distances among characiform taxa sampled in that study, other than *Distichodus*, were <15%. Ependymin DNA sequence comparisons show the same trend, in sharp contrast with 12S and 16S data (Fig. 12).

Sliding window analyses of variation (Figs. 5, 6), transition/transversion ratios (Fig. 4), and the amount of change per site in different data sets (Fig. 2) all indicate that beyond the family level, multiple changes per site are to be expected in the 12S and 16S mtDNA sequences. Whether saturation plagues these sequences at the family level is less apparent but is suggested by the differences in sequence divergence. Low consistency indices of the phylogenetic trees obtained for the different data sets indicate a high level of homoplasy at every phylogenetic level. For example, the consistency indices were 0.50, 0.34, and 0.43 for the serrasalmin (33 taxa), characiform (27 taxa), and ostariophysan (22 taxa) data sets, respectively.

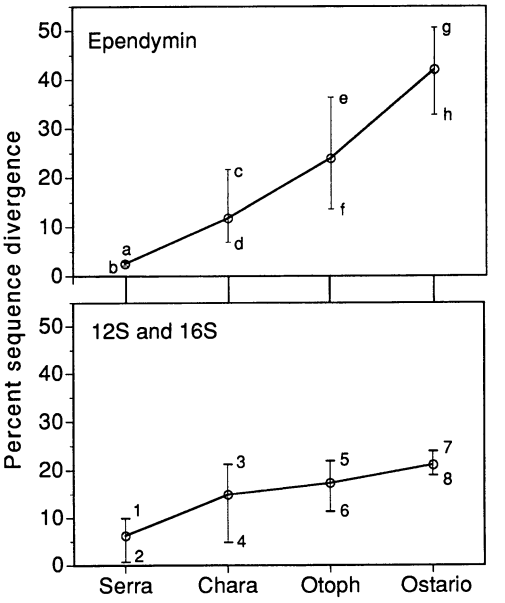

FIGURE 12. Mean (range) percentage of DNA sequence divergence (uncorrected) in the 12S and 16S fragments and in the ependymin gene (see Ortí and Meyer, 1996) for pairwise comparisons between taxa. Serra = only comparisons among genera within the Serrasalminae (12S and 16S) or comparisons among closely related species in the family Characidae (ependymin); Chara = only comparisons among taxa from different characiform families; Otoph = only comparisons among taxa from different otophysan orders (Characiformes, Gymnotiformes, Siluriformes, Cypriniformes); Ostario = only comparisons between gonorhynchiforms and taxa from all otophysan orders (12S and 16S) or comparisons between salmoniforms and otophysans (ependymin). For ependymin sequences, the most and least divergent pairs of taxa in each taxonomic assemblage are indicated by letters: a = *Alestes–Phenacogrammus* (subfamily Alestiinae); b = *Paracheirodon–Gymnocorymbus* (in subfamilies Cheirodontinae and Tetragonopterinae, respectively); c = *Phenacogrammus–Distichodus*; d = *Metynnis–Nannobrycon*; e = *Cyprinus–Schilbe*; f = *Paracheirodon–Pimelodus*; g = *Schilbe–Salmo*; h = *Boulengerella–Esox*. For the mtDNA data sets, the most and least divergent pairs are indicated by numbers: 1 = *Acnodon–Metynnis*; 2 = *Myleus–Mylesinus*; 3 = *Gnathocharax–Hoplias*; 4 = *Prochilodus–Cyphocharax*; 5 = *Crossostoma–Boulengerella*; 6 = *Hypostomus–Distichodus*; 7 = *Kneria–Nannostomus*; 8 = *Kneria–Citharinus*.

Mindell and Honeycutt (1990) and Hillis and Dixon (1991) suggested that mitochondrial ribosomal genes could resolve phylogenetic relationships among taxa that

had diverged as long as 300 or 65 million years ago, respectively. The oldest unequivocal gonorhynchiform fossils date from the early Cretaceous (Patterson, 1975, 1984), and the earliest otophysan fossils are late Cretaceous catfishes and characiforms (reviewed by Lundberg, 1993, 1996). Thus, the otophysan stem group probably originated before the separation of Africa and South America (Lundberg, 1993), dated at 84–106 million years ago (Parrish, 1993; Pitman et al., 1993). Fossils do not provide detailed evidence on the sequence of origins of the main otophysan and characiform lineages but suggest a window of application for the 12S and 16S molecular markers closer to 100 than to 300 million years.

Because of these limitations of the ribosomal DNA sequences for comparisons among characiform families, only a few hypotheses of relationships among Characiformes could be established with confidence: clades 1–11 (Figs. 9–11), of which only three propose interfamilial (or subfamilial) sister-group relationships in addition to the citharinid–distichodontid clade. A close relationship of Prochilodontidae and Curimatidae was proposed by Vari (1983) and confirmed by the molecular data (clade 10, Fig. 11). Within the Characidae, the systematic position of *Oligosarcus* (subfamily Acestrorhynchinae) close to *Astyanax* (subfamily Tetragonopterinae) and *Poptella* (subfamily Stethaprioninae) was strongly supported by the molecular data. But a close relationship of *Astyanax* with *Tetragonopterus* was not supported. *Oligosarcus* was traditionally placed with *Acestrorhynchus*, but Buckup (1991), Lucena (1993), and P. Petry (pers. comm.) found evidence for a closer relationship of *Oligosarcus* with tetragonopterins (Fig. 1) than with *Acestrorhynchus*. Lucena (1993) proposed a close relationship of *Poptella* with *Tetragonopterus* but not with *Astyanax* (Fig. 1). The third component supported by the molecular data is formed by *Hepsetus* and *Hoplias* (clade 2, Figs. 9, 10), members of African and South American families Hepsetidae and Erythrinidae, respectively.

African–South American Relationships: Phylogenies from Molecules and Morphology

Phylogenetic analyses of DNA sequences (this study; Ortí and Meyer, 1996) suggest three African–South American sister-group relationships, in agreement with previous studies using morphological characters (Buckup, 1991; Lucena, 1993; Vari, 1995). Hypotheses of the monophyly of Neotropical taxa were rejected by the mtDNA sequences. The African distichodontid–citharinid clade is the putative sister group to all other characiforms, but in the molecular studies, monophyly of Characiformes was not well supported (this clade was, in some analyses, placed with catfish, which occur in Africa, America, and Asia, or with gymnotids, which are restricted to the Neotropics). However, low resolution at this deep phylogenetic level seems more likely to result from the limit of resolution of the molecular markers used, given that monophyly of the order Characiformes is firmly established on morphological grounds (e.g., Fink and Fink, 1981). A second sister-group relationship is that of the African pike-characiform *Hepsetus* and the Neotropical family Erythrinidae, genus *Hoplias* (Figs. 9, 11; also supported by Uj, 1990). Both of these taxa are ambush predators that live in backwaters among the aquatic vegetation and exhibit nest building and parental care behaviors, unusual among characiforms (Roberts, 1972). Although this hypothesis seems well supported by the molecular data, ctenolucids and erythrinids (both Neotropical groups) or ctenolucids alone were proposed as the sister group of *Hepsetus*, based on morphology (Fig. 1; Buckup, 1991; Lucena, 1993; Vari, 1995). The third clade with a trans-Atlantic sister-group relationship includes the African subfamily Alestiinae and the Neotropical genus *Acestrorhynchus* (Figs. 9–11). Relationships of Alestiinae and *Acestrorhynchus* with Neotropical characids are controversial (Fig. 1), and no agreement may be reached regarding the systematic position of these two taxa based on previous hypotheses (Uj, 1990; Buckup, 1991; Lucena,

1993). However, no one has proposed a close relationship between alestins and *Acestrorhynchus*.

Mean percentages of sequence divergence (uncorrected, 12S and 16S genes) between the African taxa and their corresponding Neotropical sister groups were 16.2% for *Distichodus* + *Citharinus*, 11.2% for *Hepsetus*, and 15.1% for Alestiinae. Divergence between *Hepsetus* and ctenolucids (putative sister groups according to morphological studies) was 16.6%. These values are within the same range of divergence values recorded among the other families of Characiformes, below the 21–24% saturation value (Fig. 12), suggesting that most lineages (families) of characiform fishes had originated before the vicariant event separating African and Neotropical taxa, approximately 100 million years ago. If Characiformes experienced a rapid evolutionary radiation comparable to that of cichlid fishes in East African lakes (e.g., Greenwood, 1984; Meyer et al., 1990; Meyer, 1993), but 100 million years ago, resolution of phylogenetic relationships among the major lineages is not expected to be easily obtained. Poor resolution of relationships among characiform taxa using phylogenetic analyses of ependymin DNA sequences (Ortí and Meyer, 1996) and conflicting phylogenetic hypotheses from morphological data seem to agree with this prediction.

Analyzing Buckup's (1991) phylogenetic hypothesis in a biogeographic context, Lundberg (1993) also arrived at the conclusion that the major groups of characiforms had originated before the African–South American vicariant event (although the proposed African–South American sister-group relationships differed). He also raised the important question of why more characiform subgroups now endemic to the Neotropics do not have close relatives in the African fauna. Assuming a strict vicariant view and no dispersal of characiforms across the widening Atlantic ocean, the present biogeographic distribution implies a remarkably uneven rate of extinction among African characiforms (Lundberg, 1993). If the topology shown in Figure 11 is taken at face value, and the nodes separating *Hepsetus* and alesines from their Neotropical sister taxa are assumed to reflect drift-vicariant events, the extinction of at least six major lineages of characiforms in Africa is implied (see Fig. 11).

Although the fossil record of Characiformes is not very useful for testing the above scenario, intriguing fossils have been described by Greenwood and Howes (1975) and Stewart (1994). Teeth and skulls of Miocene to Lower Pleistocene age were assigned to now-extinct characiform fishes (*Sindacharax lepersonnei* and *S. deserti*), apparently widespread in northern and eastern Africa. These teeth are more similar to the teeth of modern serrasalmins such as *Colossoma* and *Piaractus* than to those of any African characiform fish (Greenwood and Howes, 1975; Stewart, 1994). Serrasalmins form a well-supported monophyletic taxon endemic to South America (Machado-Allison, 1982; clade 7, Figs. 9, 11) and include herbivorous forms such as *Colossoma* and *Piaractus*, considered the primitive sister group to the more derived predatory piranhas (e.g., *Pygocentrus*; Ortí et al., 1996). The systematic position of serrasalmins within Characiformes could not be resolved with confidence in the present study (Figs. 9–11), and no close relationship of serrasalmins with other Neotropical characids is suggested. South American serrasalmin fossils indicate that forms similar to *Colossoma* had differentiated by at least 13 million years ago (Lundberg et al., 1986; Lundberg, 1996). Considering that serrasalmins are exclusively freshwater fishes, if *Sindacharax* really belongs to the serrasalmin clade, the origin of serrasalmins would have to be unequivocally placed before the African–South American continental split (>84 million years ago), in agreement with conclusions from DNA sequence divergences. *Sindacharax* would also provide an example of extirpation in Africa of one trans-South Atlantic clade (Lundberg, 1993). Fossil fishes from the Miocene fauna of the Magdalena Basin in Colombia provide a good example for extirpation of serrasalmins from a formerly

diverse Amazon–Orinoco fauna (Lundberg et al., 1986). The depauperate fauna of the present Magdalena River does not include *Colossoma* and piranha species, and local extinction due to tectonism and climatic changes during the Cenozoic has been suggested to explain the loss of diversity (Lundberg et al., 1986; Lundberg and Chernoff, 1992). Similar geological and climatic processes might have affected a previously characiform-rich African fauna and may be invoked to explain why only three lineages of characiforms are found there at present. Paleocene tectonic movements of the African plate and post-Miocene aridification affected the African continent more severely than they did South America and might have caused the well-known paucity of the tropical African flora (Goldblatt, 1993).

Two alternative and somewhat complementary hypotheses are also plausible. Extinction of characiform lineages in Africa could also have resulted from competition with other fish groups that invaded that continent after the Gondwanan fracture. For example, notopterids, mormyriforms, knerids, and cypriniforms are freshwater fishes present in Africa but not in South America, and cyprinids such as *Barbus* and *Labeo* may have entered Africa from Asia during the late Miocene (Stewart, 1994). Another scenario assumes that members of a clade (or single species that gave rise to the clade later) may have been restricted to a small part of the Gondwanan landmass and may have been carried off *in toto* when the continent broke up. This assumption would reduce the number of extinction events among characiform lineages needed to explain their modern geographic distribution.

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APPENDIX
SPECIMENS EXAMINED

Information provided includes locality of origin (when known, otherwise fish are from commercial sources; AFR = African taxa), G.O. (GO) collection numbers, GenBank (GB) accession numbers for the 12S and 16S sequences, and Museum catalog numbers for voucher specimens deposited in museum collections: USNM = U.S. Museum of Natural History; INPA = Instituto Nacional de Pesquisas da Amazonia. Characiform family-level classification follows that of Greenwood et al. (1966), with a few exceptions: the family Cynodontidae is included as a tribe in the family Characidae, as suggested by Howes (1976); the ichthyborids are included within the family Distichodontidae, following Vari (1979); and the characid subfamilies Characidiinae and Crenuchinae are grouped in the new family Crenuchidae (Buckup, 1991). As a consequence, 15 characiform families are recognized.

Order Characiformes

1. Family Hepsetidae (AFR)
   Hepsetus odoe (GO 126). GB: U33852, U33992.
2. Family Citharinidae (AFR)
   Citharinus congicus (R. Malagarasi, Tanzania; GO 195). GB: U33826, U33993.
3. Family Distichodontidae (AFR)
   Distichodus sp. (GO 72). GB: U33827, U33994.
4. Family Crenuchidae
   Characidium sp. (NE Brazil, USNM 318101). GB: U33828, U34030.
5. Family Characidae
   Subfamily Alestiinae (AFR)
   Alestes sp. (R. Malagarasi, Tanzania; GO 147). GB: U33829, U33995.
   Phenacogrammus sp. (GO 49). GB: U33830, U33996.
   Hydrocyon sp. (R. Malagarasi, Tanzania; GO 127). GB: U33960, U33997.
   Subfamily Characinae
   Tribe Characini
   Cynopotamus sp. (R. Uruguay, Salto Grande, Argentina; USNM 325689). GB: U33961, U33998.
   Gnathocharax steindachneri (GO 123). GB: U33589, U33624.
   Tribe Acestrorhynchini
   Acestrorhynchus sp. (GO 76). GB: U33962, U33999.
   Oligosarcus sp. (R. Uruguay, Salto Grande, Argentina; USNM 235690). GB: U33963, U34000.
   Subfamily Raphiodontinae
   Rhaphiodon vulpinus (R. Uruguay, Salto Grande, Argentina; GO 124). GB: U33964, U34001.
   Subfamily Bryconinae
   Tribe Salminini
   Salminus sp. (R. de la Plata, Buenos Aires, Argentina; GO 1B). GB: U33965, U34002.
   Tribe Bryconini
   Brycon sp. (R. Paraná, Bella Vista, Argentina; USNM 326005). GB: U33966, U34003.
   Chalceus macrolepidotus (GO 40). GB: U33587, U33622.
   Tribe Triportheini
   Triportheus paranensis (R. Paraná, Bella Vista, Argentina; GO 109). GB: U33588, U33623.
   Subfamily Aphyocharacinae
   Aphyocharax sp. (GO 93). GB: U33968, U34005.
   Subfamily Glandulocaudinae
   Corynopoma riisei (cultured stock from R. Manzanares, Venezuela; GO 73). GB: U33969, U34006.
   Gephyrocharax sp. (GO 122). GB: U33970, U34007.
   Subfamily Stethaprioninae
   Poptella sp. (GO 174). GB: U33971, U34008.
   Subfamily Tetragonopterinae
   Astyanax fasciatus (GO 65). GB: U33972, U34009.
   Tetragonopterus sp. (R. Paraná, Bella Vista, Argentina; GO 108). GB: U33973, U34010.
   Subfamily Cheirodontinae
   Cheirodon sp. (R. Paraná, Bella Vista, Argentina, USNM 325676). GB: U33974, U34011.
   Paracheirodon innesi (GO 43). GB: U33975, U34012.
   Subfamily Serrasalminae
   Pygocentrus nattereri (R. Solimoes, Ilha da Marchantaria, AM, Brazil; INPA 10143). GB: U33558, U33590.
   Colossoma macropomum (R. Solimoes, Ilha da Marchantaria, AM, Brazil; INPA 10149). GB: U33581, U33616.
6. Family Erythrinidae
   Hoplias malabaricus (R. Uruguay, Salto Grande, Argentina; GO 113). GB: U33976, U34013.
7. Family Ctenoluciidae
   Ctenolucius sp. (GO 67). GB: U33977, U34014.
   Boulengerella maculata (GO 66). GB: U33978, U34015.
8. Family Lebiasinidae

Nannostomus sp. (GO 120). GB: U33979, U34016.
Pyrrhulina sp. (R. Paraná, Bella Vista, Argentina, USNM 325675). GB: U33980, U34017.

9. Family Hemiodontidae
Hemiodus sp. (GO 191). GB: U33981, U34018.
10. Family Parodontidae
Apareiodon affinis (R. Paraná, Bella Vista, Argentina; GO 156). GB: U33982, U34019.
11. Family Gasteropelecidae
Carnegiella sp. (GO 95). GB: U33983, U34020.
Gasteropelecus sp. (GO 44). GB: U33984, U34021.
12. Family Curimatidae
Cyphocharax gilberti (NE Brazil; USNM 318079). GB: U33985, U34022.
Steindachnerina sp. (R. Uruguay, Salto Grande, Argentina; USNM 325691). GB: U33986, U34023.
13. Family Prochilodontidae
Prochilodus lineatus (R. de la Plata, Buenos Aires, Argentina; GO B1). GB: U33987, U34034.
14. Family Anostomidae
Abramites sp. (GO 77). GB: U33988, U34025.
Leporinus obtusidens (R. Paraguay, Asunción, Paraguay; GO 133). GB: U34031, U34026.
15. Family Chilodontidae
Chilodus sp. (GO 172). GB: 33989, U34027.

Order Gymnotiformes

Family Eigenmaniidae
Eigenmannia sp. GB: U15269, U15245 (from Alves-Gomes et al., 1995).
Family Rhamphichthyidae
Rhamphichthys sp. GB: U15257, U15233 (from Alves-Gomes et al., 1995).
Family Apteronotidae
Apteronotus albifrons. GB: U15275, U15226 (from Alves-Gomes et al., 1995).

Order Siluriformes

Family Loricariidae
Hypostomus sp. GB: U15263, U15239 (from Alves-Gomes et al., 1995).
Family Cetopsidae
Cetopsis sp. GB: U15272, U15248 (from Alves-Gomes et al., 1995).
Family Trichomycteridae
Trichomycterus sp. GB: U15251, U15227 (from Alves-Gomes et al., 1995).
Family Malapteruridae
Malapterurus sp. GB: U15261, U15237 (from Alves-Gomes et al., 1995).

Order Cypriniformes

Family Cyprinidae
Cyprinus carpio. GB: X61010.
Family Gastromyzontidae
Crossostoma lacustre. GB: M91245.

Order Gonorhynchiformes

Family Kneridae
Kneria sp. (R. Malagarasi, Tanzania; GO 194). GB: U33990, U34028.
Parakneria sp. (R. Malagarasi, Tanzania; GO 193). GB: U33991, U34029.