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EVOLUTIONARY ORIGIN AND EARLY BIOGEOGRAPHY OF OTOPHYSAN FISHES (OSTARIOPHYSI: TELEOSTEI)

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The biogeography of the mega-diverse, freshwater, and globally distributed Otophysi has received considerable attention. This attraction largely stems from assumptions as to their ancient origin, the clade being almost exclusively freshwater, and their suitability as to explanations of trans-oceanic distributions. Despite multiple hypotheses explaining present-day distributions, problems remain, precluding more parsimonious explanations. Underlying previous hypotheses are alternative phylogenies for Otophysi, uncertainties as to temporal diversification and assumptions integral to various explanations. We reexamine the origin and early diversification of this clade based on a comprehensive time-calibrated, molecular-based phylogenetic analysis and event-based approaches for ancestral range inference of lineages. Our results do not corroborate current phylogenetic classifications of otophysans. We demonstrate Siluriformes are never sister to Gymnotiformes and Characiformes are most likely nonmonophyletic. Divergence time estimates specify a split between Cypriniformes and Characiphysi with the fragmentation of Pangea. The early diversification of characiphysans either predated, or was contemporary with, the separation of Africa and South America, and involved a combination of within- and between-continental divergence events for these lineages. The intercontinental diversification of siluroids and characoids postdated major intercontinental tectonic fragmentations (<90 Mya). Post-tectonic drift dispersal events are hypothesized to account for their current distribution patterns.

KEY WORDS: **Ancestral range, divergence times, nonmonophyly of the Characiformes, phylogeny, postdrift dispersal, vicariance.**

About 64% of the World's freshwater fish species belong to the Otophysi, by far the largest and most diverse group of primarily freshwater teleosteans (Nelson 2006). The Otophysi includes 9741 valid species (Eschmeyer and Fong 2012) with still several hundred undescribed species predicted to be discovered (Nelson 2006; Mayden et al. 2009). Species from this group are not only a major component of the modern ichthyofauna in their native habitats, but they also support important worldwide subsistence fisheries (Winfield and Nelson 1991) and are important in the aquarium trade (Collins et al. 2012). In addition to their fundamental importance in both ecosystems and human activities, these fishes also provide remarkable examples of species diversity and adaptive radiations, and have attracted a great deal of attention from biologists with different research foci, especially in evolutionary biology (Winfield and Nelson 1991; Mabee et al. 2007; Mayden et al. 2009; Chen and Mayden 2010; Pasco-Viel et al. 2010). These many varied attributes make Otophysi akin to a so-called "model group" for various studies. This is especially true given that a plethora of information exists on the biology, anatomy, physiology, and development of one "model organism," the zebrafish (*Danio rerio*), a species deeply embedded within this large clade of vertebrates (Mabee et al. 2007; Mayden et al. 2007; Schilling and Webb 2007).

Otophysan fishes are currently classified into four orders, Gymnotiformes (electric eels and relatives, 194 species), Siluriformes (catfishes, 3529 species), Characiformes (piranhas, tetras,

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etc., 1995 species), and Cypriniformes (minnows, carps, barbs, suckers, loaches, etc., 4023 species; Fink and Fink, 1996; number of valid species per order from Eschmeyer and Fong 2012). Otophysans are considered monophyletic and the sister group of the Gonorynchiformes (i.e., Anotophysi; milkfish and relatives, 37 species; Fink and Fink 1981, 1996; Lavoué et al. 2005). The Otophysi and Gonorynchiformes make up the Ostariophysi (Rosen and Greenwood 1970).

The origin and pattern of geographical diversification of Otophysi have been of particular interest for biogeographers for many years because these predominantly freshwater-inhabiting fishes (only two extant lineages from Siluriformes are secondarily adapted to marine habitats) are thought to be an excellent biogeographic model given their worldwide distributions (Fig. 1), ancient origin and hypothesized limited trans-marine dispersal abilities. Yet, many uncertainties exist as to the historical biogeography of the Otophysi; several alternative hypotheses exist regarding current distributions and underlying historical causes and means (Novacek and Marshall 1976; Briggs 1979; Gayet 1982; Lundberg 1993; Diogo 2004; Briggs 2005; Nakatani et al. 2011). This debate is largely the consequence of authors using different classifications (some not phylogenetic), different analytical methods to reconstruct ancestral area distributions, an insufficiently studied fossil record that was likewise largely overlooked by neontologists, and a necessarily complex history regarding the age of the Otophysi, concomitant with its vast geographical distribution.

The age of the Otophysi lineage has been deduced from interpretations of its fossil record (the oldest Otophysan known is "only" of Albian age, Lower Cretaceous; Filleul and Maisey 2004). More recent inferences from molecular dating provide radically different estimates, an origin as old as the Permian / Triassic (Peng et al. 2006; Nakatani et al. 2011). That the Otophysi is monophyletic and the great majority of extant otophysans are restricted to freshwater strongly suggests a freshwater origin for this group. A marine origin of the Otophysi with an early diversification through repeated marine / freshwater transitions has been hypothesized based on the presence of several marine / brackish forms in the earliest fossils assigned to this group (Gayet 1982; Patterson 1984; Cavin 1999). Different phylogenetic hypotheses have also been considered to discuss the early evolutionary history of the Otophysi: for example, the Rosen and Greenwood (1970) hypothesis identified Characiformes sister to Gymnotiformes, Fink and Fink (1981) argued for a sister-group relationship between Siluriformes and Gymnotiformes, and Nakatani et al. (2011) argued for a "Characiformes" (found to be paraphyletic) plus Siluriformes monophyletic group. All but the Rosen and Greenwood (1970)'s hypothesis corroborate the hypothesis for Cypriniformes sister to remaining Otophysi. In fact, a strict consensus tree derived from all previous hypotheses results in an unresolved hypothesis. Finally, the scenario proposed to explain disjunct geographical distributions of sister lineages of the Otophysi have relied on two different models (or assumptions): the first one (cladistic) favors vicariant events and widespread ancestral areas (Novacek and Marshall 1976; Lundberg 1993; Diogo 2004, Malabarba and Malabarba 2010) whereas the second favors dispersal events and regions of origin (Briggs 1979, 2005 Gayet 1982).

In this study we readdress the question of the biogeography of the Otophysi, first in providing a comprehensive phylogenetic hypothesis based on a character- and taxon-rich dataset (five nuclear gene loci, 4518 base pairs, 95 taxa). We then use a standard Bayesian method of divergence time reconstruction that incorporates a relaxed molecular clock and fossil-based calibration (Drummond et al. 2012) to provide a timescale for the origin and diversification of the Otophysi. We estimate the ancestral environment (marine / freshwater) of the Otophysi and its evolution through tree-based character reconstruction methods. Finally, we infer the early evolutionary history of the Otophysi using two ancestral range reconstruction models, the *dispersal–vicariance* model (Ronquist 1997) and the *dispersion—extinction–cladogenesis* model (Ree et al. 2005; Ree and Smith 2008).

Materials and Methods

TAXA AND CHARACTERS SAMPLINGS

Taxonomic sampling includes a large selection of teleosts including three osteoglossomorphs, four elopomorphs, and 85 samples from Clupeocephala. Within Clupeocephala, 70 taxa from major lineages of the Otocephala (i.e., 61 otophysans, three gonorynchiforms, four clupeiforms, and two alepocephaliforms) plus 15 euteleost representatives were chosen. The operational outgroups include the likely extant sister group of all living teleosts, the Holostei (*Amia calva*, Lepisosteidae; Grande 2010) plus a more distant taxon, *Polyodon spathula* (Acipenseriformes). Within the Otocephala, as often as possible, our taxon selection includes at least two distantly related representatives within a suborder and/or superfamily. Previous phylogenetic information taken into consideration for our sampling strategy within the Ostariophysi are from Sullivan et al. (2006) for the Siluriformes; Calcagnotto et al. (2005) and Oliveira et al. (2011) for the Characiformes; Chen and Mayden (2009), Chen et al. (2009), and Mayden and Chen (2010) for the Cypriniformes; and Lavoué et al. (2005) for the Gonorynchiformes. Our broad taxon and character sampling facilitates (1) a more accurate inference of the phylogenetic relationships among the major otocephalan and otophysan lineages; (2) test the monophyly of each order, suborder, and main supra-familial lineages; and (3) the reduced likelihood of any long-branch attraction artifact in bisecting possible long branches with a dense taxonomic sampling (Hillis et al. 2003).

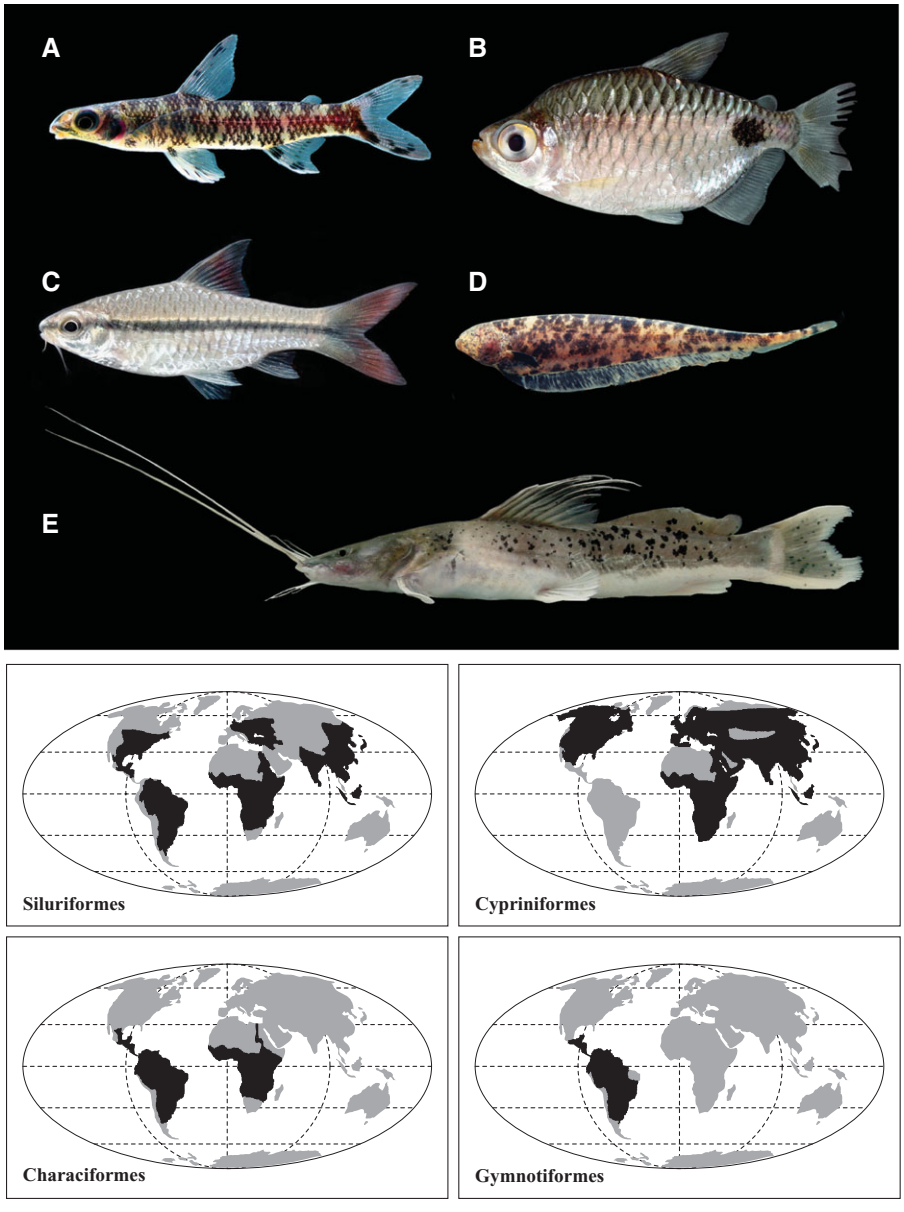


Figure 1. Photos of otophysan representatives (A: *Nannocharax* cf *gracilis* [Characiformes, Citharinoidei]; B: *Brachypetersius altus* [Characiformes, Characoidei]; C: *Barbus holotaenia* [Cypriniformes]; D: *Gymnotus* sp. [Gymnotiformes]; E: *Bagrus ubanguensis* [Siluriformes]). Present day geographical distributions of the four otophysan orders compiled from Berra (2007). Top-left, Siluriformes; top-right, Cypriniformes; bottom-left, Characiformes; bottom-right, Gymnotiformes.

The list of taxa examined in this study is provided in the table of electronic Supporting Information (Table S1), along with GenBank accession numbers of the corresponding nuclear gene sequences.

MOLECULAR LABORATORY WORK AND DNA DATA COLLECTION

The DNA sequences were generated from five phylogenetically informative nuclear gene markers (RAG1, recombination activation gene 1; RH, Rhodopsin; EGR1, 2B, and 3, early growth response protein genes 1, 2B, and 3; Chen et al. 2008). Some sequences used in this study were retrieved from GenBank or previously determined in Chen et al. (2008, 2009), Chen and Mayden (2009), and Mayden and Chen (2010). Protocols for collecting new DNA data follow those outlined in Chen et al. (2008). Primers used in this study were published by López et al. (2004) and Chen et al. (2007) for RAG1, and Chen et al. (2003, 2008) for Rhodopsin, EGR1, 2B, and 3. In addition, several newly designed primers were used in this study to obtain a nearly complete set of the nuclear sequences for this diverse set of 92 teleost taxa (only 16 over 460 sequences [= about

3.5%] are missing, see Table S1). The primer list is shown in Table S2.

Collected DNA sequences were edited using Se-Al v2.0a11 (available at http://tree.bio.ed.ac.uk/software/seal/). Compiled sequences were initially aligned with automatic multiple alignment program MUSCLE (Edgar 2004a, 2004b) using online server at http://www.ebi.ac.uk/Tools/muscle/index.html, then adjusted manually based on the inferred amino acid translation. Regions where large insertion/deletions segments (e.g., tandem repeats in EGR genes) showing high dissimilarity in sequence length, that may result in invalid assertions of homology were discarded from the phylogenetic analyses. The aligned sequence matrix of combined genes was 4920 bp. Because the proportion of missing data in analyses may be a potential source of inaccuracy in phylogenetic inference, we further trimmed a few positions located at the 5'- and 3'- extremities of certain gene fragments showing a large amount of missing data (roughly 10% of the total alignment length). Undetermined nucleotides at the extremities of these sequences resulted from the use of nested primers for PCR amplification and consequently the collection of shorter sequences. Our final alignment was 4518 pb, and contained only about 5.8% missing nucleotides from the total data; a text file containing our final alignment is available from the Dryad repository (doi:10.5061/dryad.62t87).

Descriptive statistics and test of homogeneity of base frequencies across taxa (conducted for each gene and codon position separately) using χ^2-tests were performed using PAUP* v.4.0b10 (Swofford 1999).

Phylogenetic analyses were carried out on six datasets, each based on the combination of a matrix (two in total) and a partition strategy (three in total). The first matrix, named "123_{ry}," includes the first, second, and only the transversions at the third codon positions (RY-coding scheme applied when tests of base composition stationarity reveal bias across taxa at variable sites or sites at a particular codon position, see Chen and Mayden 2009). The second matrix "123" includes the first, second, and third codon positions. The three partition strategies are: each of the three codon positions (regardless of individual genes) assigned to a partition (total: three partitions), each three codon position from each individual gene assigned to a partition (total: 15 partitions), and each gene (regardless of codon positions) assigned to a partition (total: five partitions).

MAXIMUM-LIKELIHOOD (ML) PHYLOGENETIC ANALYSES

Phylogenetic analyses were performed using the partitioned ML method as implemented in the RAxML-HPC (Stamatakis 2006) with its graphical interface raxmlGUI 0.93 (Silvestro and Michalak 2012) for the six different datasets. Heuristic searches were conducted under mix models of sequence evolution, which allow individual model parameters of nucleotide substitutions to be estimated independently for each partition in the analysis. A GTR + G + I model (with four discrete rate categories) was used for each partition; RAxML only provides GTR-related (Yang 1994) models of rate heterogeneity for nucleotide data (Stamatakis 2006). A thorough ML tree search was conducted through 100 distinct runs; the optimal tree was determined by comparison of the likelihood scores among the suboptimal trees obtained per run. To evaluate the robustness of internal branches of the ML tree, 1000 bootstrap replications (MLBS; Felsenstein 1985) were calculated for each data set under the same model of sequence evolution. Tree topology differences among the analyses were accessed visually.

We evaluated alternative topologies within the Characiphysi, including the hypothesis by Fink and Fink (1981), using the approximately unbiased (AU) test and the multiscale bootstrap technique as implemented in the program CONSEL v0.1j (Shimodaira and Hasegawa 2001). ML tree searches of the dataset 123_{ry} (partitioned in 15, see above) using constrained tree topologies were performed using RAxML and the GTR + G + I model. For each alternative phylogenetic hypothesis, the site-wise log-likelihoods for the constrained and unconstrained topologies were calculated with RAxML; *P*-values were calculated with CONSEL for each test.

BAYESIAN PHYLOGENETIC INFERENCE AND DIVERGENCE TIME ESTIMATION

Phylogenetic trees and divergence times and their 95% credibility intervals were simultaneously inferred using a partitioned Bayesian method incorporating a relaxed molecular clock, as implemented by BEAST v.1.7.4 (Drummond et al. 2012). BEAUTi was first used to create the XML input file. Given that the six different ML analyses provided mostly identical tree topologies and considering computation time constraints, we restricted our Bayesian analysis to the data matrix "123_{ry}" that was partitioned in three, according to the first and second codon positions and the transversions at the third codon positions. We selected the GTR + G + I model of sequence evolution for each of the three partitions, with parameters unlinked between partitions. In BEAUTi, we predefined 30 subsets of taxa for which the ages were constrained based on the fossil record information, as explained in the next section.

Two independent runs of 1×10^8 generations each were performed using BEAST. Each run was initiated from a user-starting time tree that we built in advance with BEAST using a simple HKY model of sequence evolution, no partitions, a strict molecular clock and a single prior age constraint for the root of the tree at 350 millions years ago (Mya). Trees and divergence time estimates were sampled once every 5000 generations, and each run's parameters were checked for convergence with the software Tracer v1.5. We determined the burn-in parts of each

run (10–20%) graphically; remaining tree samples from the two runs were then pooled into a combined file. TreeAnnotator v.1.7.4 (Drummond et al. 2012) was used to determine maximum clade credibility trees built from total tree samples, mean divergence times, and their 95% credibility intervals assigned to the nodes.

FOSSIL SELECTION AND CONSTRAINT DISTRIBUTIONS FOR NODE CALIBRATIONS

Based on our reading of the fossil record of the Neopterygii and Teleostei, we have selected 30 key fossils (described in the Appendix) from groups having rich and coherent fossil records to calibrate our chronogram. Our estimations of the molecular divergence times are also dependent on how the tree is calibrated with this selection. We used two different methods of calibration.

The first method of calibration is the most conservative. This method assumes that the fossil record provides a good estimate of diversification of Teleostei and selected fossils from groups having a rich fossil record provide ages for the origins of these groups. Prior age distributions of selected nodes follow an exponential distribution with a minimum age equal to the minimum age of the strata from which the oldest crown-group fossil was excavated and a maximum age (in the 95% credibility interval) equal to the maximum age of the strata from which was excavated either the oldest stem group fossil of this lineage or the oldest fossil of its sister lineage (Santini et al. 2009). However, to avoid circularity, the maximum ages of the nine fossil-based calibrated nodes within the Otophysi were relaxed as to the minimum age of the Otophysi.

The second explorative method is the less conservative. It assumes that the fossil record is too incomplete, providing only minimum ages for the origin of selected groups (to the exception of the root dating that is calibrated with maximum and minimum ages). In this case, prior age distributions of a selected node follow a uniform distribution in which the minimum age limit is equal to the minimum age of the strata from which the fossil was excavated and the maximum age is equal to the minimum age of the root of our tree.

ANCESTRAL RANGE ANALYSIS

Areas coding

Several studies have pointed out that the delimitation of geographical subareas is critical and somewhat controversial (reviewed in Crisci et al. 2003). We a priori used the following two criteria to explicitly delimitate five regions: (1) the continental marine coastal shores at the maximal fragmentation of the continents during the period 40–90 Mya; and (2) the current distribution of the main extant otophysan lineages. The five geographical regions are: South America (SA), North America (NA), sub-Saharan Africa (AF), temperate Eurasia (EA), and tropical and subtropical Southeast Asia (SEA), roughly corresponding to Indochina, Sundaland, and Myanmar/Bangladesh.

Ancestral range reconstruction methods

We used two different approaches to reconstruct the ancestral geographic ranges at nodes of the phylogenetic tree of the Otophysi: the Statistical *Dispersal–Vicariance* analysis as implemented in RASP (ex S-DIVA; Ronquist 1997; Nylander et al. 2008; Yu et al. 2010) and the model-based likelihood method *dispersal–extinction–cladogenesis* (DEC) using Lagrange (likelihood analysis of geographic range evolution; Ree et al. 2005; Ree and Smith 2008; Ree and Sanmartin 2009). Both methods stand on different assumptions to explain the current distributions of organisms: RASP is the statistical version of DIVA, relying on the maximum parsimony criterion for optimality in reconstructing ancestral ranges onto a phylogenetic tree while minimizing dispersal and extinction events. The parametric DEC model is a continuous time model for geographic range evolution in which dispersal events cause range expansions and extinction causes range reductions along phylogenetic branches. In addition, vicariant events is presumed to result in lineage divergence occurring within or between areas.

We pruned several biogeographically uninformative otophysan taxa (because their geographical distribution is the same as their sister group) and all non-otophysan taxa from the matrix, the Bayesian time tree (consensual chronogram; Fig. 3) and the time trees collection (as obtained from BEAST) to reduce the number of terminal leaves to 30. To assign terminal leaves (= taxa) to one of the preselected five regions, we have considered only their current distributions. Because some terminal leaves in our tree represent groups with a larger distribution, spanning two or more regions, we have to consider their ancestral distribution using the phylogenetic criteria (see Crisci et al. 2003). In only three cases, a multiregions coding was needed to reflect the uncertainty of their ancestral ranges. The ancestral range of the Siluroidei spans all regions (coding: All) because of its unresolved phylogeny (Sullivan et al. 2006); the same is true for the Catostomidae (coding: North America + Eurasia; Doosey et al. 2010; Chen and Mayden 2012), and for the clade containing gobionids, cultrins, tincids, acheilognathids, and tanichthyids (coding: all but South America; Chen et al. 2009; Mayden and Chen 2010).

We set up no restriction on the ancestral range area size in RASP and Lagrange; thus, ancestral range areas can span as many of the areas as possible. Default options of other parameters selected.

Results

GENES, SEQUENCE VARIATION, AND BASE COMPOSITION

A total of 4518 bp were aligned from the reduced (or trimmed) matrix for the exon regions of the five nuclear genes for 95 taxa (including three operational outgroups) sampled in this study.

Table 1. Individual genes characteristics.

	Gene 1 (RAG1)			Gene 2 (Rhodopsin)			Gene 3 (EGR1)		
Initial alignment [1]	1497			816			804		
Analyzed [1]	1452 (−45)			750 (−66)			726 (−78)		
Codon position	1	2	3	1	2	3	1	2	3
Parsimony informative sites	232	140	466	120	70	237	100	71	240
Empirical base frequency A/T/C/G	0.26/0.18/0.22/0.34	0.31/0.27/0.22/0.2	0.16/0.21/0.32/0.31	0.26/0.25/0.19/0.30	0.20/0.40/0.22/0.18	0.10/0.15/0.5/0.25	0.28/0.20/0.33/0.19	0.26/0.19/0.35/0.2	0.09/0.12/0.53/0.26
Base frequencies homogeneity	1	1	0.0000*	1	1	0.0000*	1	1	0.0000*
	Gene 4 (EGR2B)			Gene 5 (EGR3)			Total (Nuclear loci)		
Initial alignment	885			918			4920		
Analyzed	825 (−60)			765 (−153)			4518 (−402)		
Codon position	1	2	3	1	2	3	1	2	3
Parsimony informative sites	119	51	272	87	33	246	658	365	1461
Empirical base frequency A/T/C/G	0.25/0.23/0.33/0.19	0.23/0.20/0.37/0.2	0.13/0.14/0.47/0.26	0.26/0.17/0.34/0.23	0.30/0.20/0.30/0.20	0.12/0.13/0.48/0.27	0.26/0.20/0.27/0.27	0.31/0.27/0.22/0.20	0.12/0.16/0.44/0.28
Base frequencies homogeneity [2]	1	1	0.0000*	1	1	0.0000*	1	1	0.0000*

[1]Length of aligned DNA nucleotide sequences in base pair (bp).

[2]*P* value from χ^2-test of homogeneity of base frequencies across taxa. Asterisk sign indicates that the data are significantly rejected by χ^2-test.

Several indels were needed to adjust the sequence alignment of the RAG1 and EGR genes, but alignment was unambiguously achieved followed by triplet codes for amino acids, except for a small part of the 5' end region of the amplified fragment of EGR1 and 2B genes. This region contained a teleost-specific insert of ~20 to 50 residues of a serine/threonine-rich domain (Burmeister and Fernald 2005). The sequence length in the amplified fragment of EGR 1, 2B, and 3 genes from *Galaxias maculatus* was uniquely long because of taxon-specific inserts of amino acids (usually a single type) in tandem repeats from remote locations of the gene regions. The length of the aligned sequences and other descriptive statistics, as implemented in PAUP*, for each gene and total dataset are summarized in Table 1. Tests of base composition stationarity revealed that variable sites and sites at the third codon position in the sequences from all genes exhibited significant base composition bias across taxa.

PHYLOGENETIC RESULTS

The six different ML analyses of the concatenated five nuclear genes implementing equal weighting and partial RY coding procedures yielded mostly identical and strongly supported topologies for the main ostariophysan lineages and within the Otophysi. Nodal support evaluated by MLBS varied depending on analysis; equal weighting analyses provided generally higher bootstrap values. When topologies were different among the analyses, those differences were found in the relationships where nodal support was, in general, weak. The ML tree generated from the matrix 123_{ry} partitioned in 15 is presented herein in Figure 2. ML trees from other analyses can be found in the Figures S1–S5 of the supporting information.

From all ML analyses, four major monophyletic groups were found with strong supports (at least 94% of MLBS) in the Teleostei: Elopomorpha, Osteoglossomorpha, Euteleostei (minus the Alepocephaliformes), and Otocephala (including the Alepocephaliformes). Within the Otocephala, Clupeiformes, Alepocephaliformes, and the Ostariophysi sensu Rosen and Greenwood (1970) were each monophyletic (MLBS > 90%). Within our target group, the Ostariophysi, the Gonorynchiformes was the sister group of the Otophysi. The Otophysi was composed of two strongly supported monophyletic groups: the Cypriniformes (MLBS = 100%) and the Characiphysi (MLBS = 100%). Within the Characiphysi, two unexpected phylogenetic relationships were inferred: (1) the nonmonophyly of the Characiformes and (2) the nonmonophyly of the group Siluriformes plus Gymnotiformes (= Siluriphysi sensu Fink and Fink 1996 or Siluriformes sensu Fink and Fink 1981]. Sequence variation of the multiple nuclear

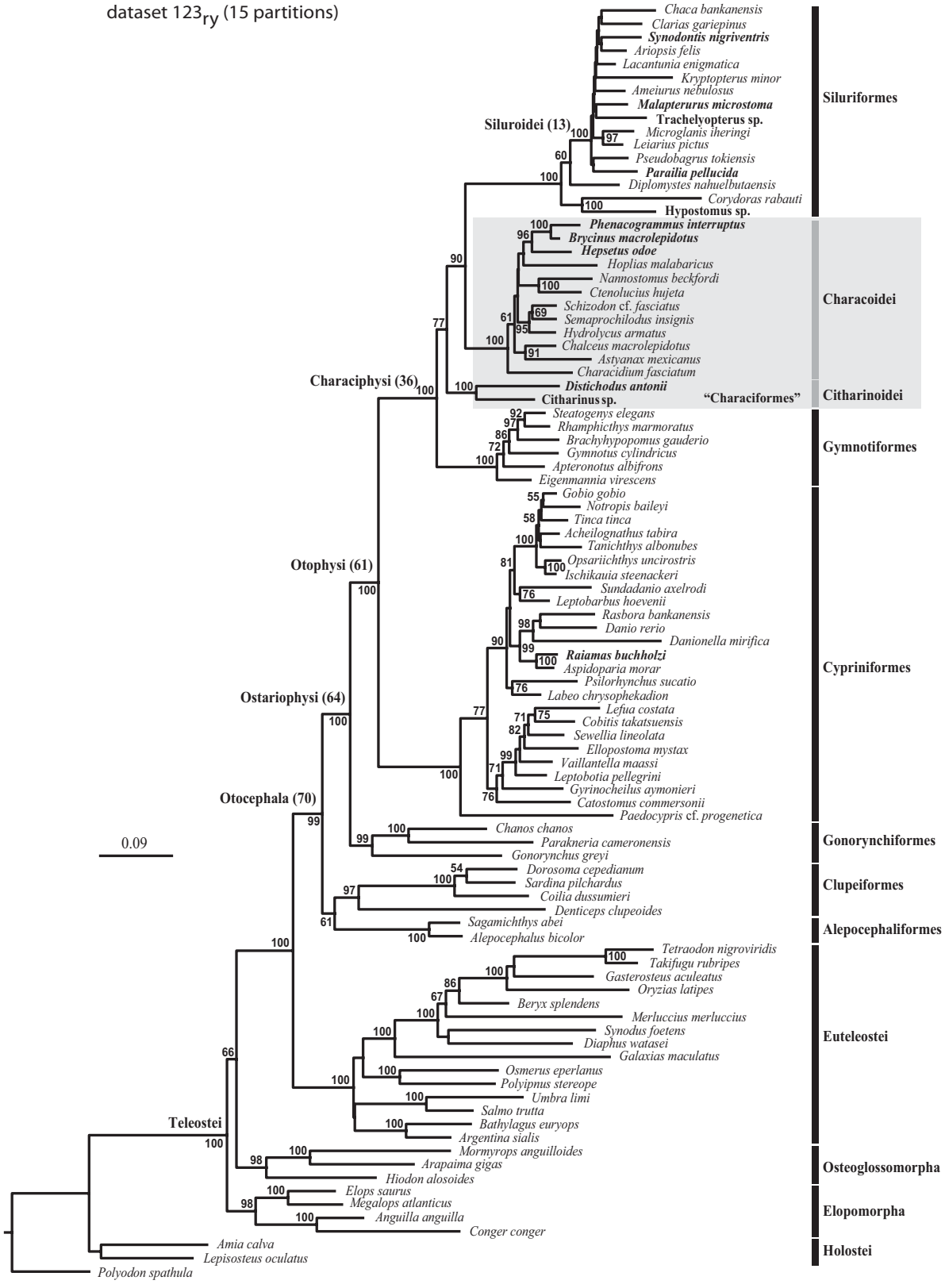


Figure 2. Best scoring maximum-likelihood tree of Teleostei obtained from the partitioned RAxML analysis of a five nuclear gene dataset, matrix "123_{ry}," which included an assignment of 15 partitions with respect to individual genes and codon positions. Branch lengths are proportional to the number of inferred substitutions. Numbers at nodes are bootstrap proportions (if ≥ 50%). Number of representatives examined in this study for the Otocephala, Ostariophysi, Otophysi, Characoidei, and Siluroidei are given in parenthesis after the corresponding taxon name. The tree is rooted with *Polyodon spathula*. African otophysans are highlighted in bold.

genes strongly supported a clade (MLBS > 85% from all analyses; mean = 93%) grouping the Siluriformes (= Siluroidei of Fink and Fink 1981) and the characiform suborder Characoidei. The second suborder of the Characiformes (Citharinoidei; species found only in Africa) was the sister group to the later clade (Figs. 2, S1–S5) and this relationship received moderate to high nodal support depending on the analyses evaluated by MLBS (70–98%; mean = 83%; Figs. 2, S1-S5). Although the AU test statistically rejected Fink and Fink (1981)'s hypothesis ($P = 0.031$), the phylogenetic hypothesis in which the monophyly of the Characiformes was constrained was not rejected ($P = 0.20$).

DIVERGENCE TIME ESTIMATIONS

Our two time tree estimations relied heavily on how fossil-based constraints were applied (Figs. 3, S6; Table 2). As expected, applying a soft maximum limit through an exponential distribution in which the 95% credibility upper limit was equal to the maximum age of the strata where the oldest stem group fossil was excavated, provided the most conservative estimate with regard to the fossil record information (Fig. 3; Table 2). In this context, the age of the Otophysi was estimated to 153.1 Mya and the respective ages of the crown groups ranged from 71.0 to 117.7 Mya: Cypriniformes (117.7 Mya), Siluriformes (97.0 Mya), Gymnotiformes (70.9 Mya), Characoidei (93.1 Mya), and Citharinoidei (78.8 Mya; see Table 2 for 95% credibility intervals).

Our second reconstruction (Fig. S6; Table 2) provided older divergence time inferences. Here, the fossil selection was only used to specify minimum ages using a uniform distribution; maximum age being not constrained. Using this method divergence of Otophysi was estimated to 213.3 Mya, whereas ages of crown-group diversification ranged from 158.9 Mya for Cypriniformes, 116.9 Mya for Siluriformes, 88.4 Mya for Gymnotiformes, 113.2 Mya for Characoidei, and 106.3 Mya for Citharinoidei. Early divergence times within the Teleostei seem particularly old. Inferred ages of crown groups were estimated 304.7 Mya for Teleostei, 227.2 Mya for Osteoglossomorpha, 225.2 Mya for Elopomorpha, 250.1 Mya for Otocephala, and 272.3 Mya for Euteleostei. The inferred times of origins for these five groups well predate ages of their respective earliest fossils.

Which ever reconstruction is considered, 1 (Fig. 3) or 2 (Fig. S6), early diversification of the Otophysi predated the last stages of the separation of Africa and South America while diversification of the crown-group Siluroidei postdated the Gondwana fragmentation (Smith et al. 1994). The early diversification of the crown-group Characiphysi may have been concomitant with the fragmentation Gondwana but the age of the African characoid clade (Alestidae plus Hepsetidae) postdated this ancient event (Figs. 3, S6).

ANCESTRAL RANGE RECONSTRUCTION ANALYSIS

Ancestral range reconstruction analyses using Lagrange and RASP provided mostly similar and straightforward scenarios for the origin and early diversification of the Otophysi (Fig. 4). The most likely scenario derived included the following series of events. First, the inferred ancestral range for the Otophysi spanned a large area that included South America, Africa, and Southeast Asia. Second, the initial involved a basal split involving currently recognized clades Cypriniformes and Characiphysi. This may have been the result of the separation Southeast Asia (belonging to Laurasia at this time) and South America and Africa (Gondwana). Third, the ancestral range of the Characiphysi was restricted to Gondwana. Fourth, as most likely inferred the early diversification of the Characiphysi was a combination of speciation within- and between-areas (Gondwana), possibly mediated by vicariant events. Finally, the ancestral range of the most recent common ancestor to the crown-group Cypriniformes may have been restricted to Southeast Asia, from the Lower Cretaceous. Subsequent diversification of the Cypriniformes from this region to its current distribution in Eurasia, Africa, and North America would have been mediated via later dispersal, continental contacts during glacial periods, and events isolating one or more lineages identified in this order.

Although far less likely, but noteworthy to mention for completeness, both methods inferred (with lower probability) one other possible scenario: an ancestral range included only Southeast Asia and South America, and an early vicariant event may have occurred between both, followed by dispersal from South America to Africa either for the ancestor of the Characiphysi or, later, for the ancestor of the Citharinoidei plus Gymnotiformes.

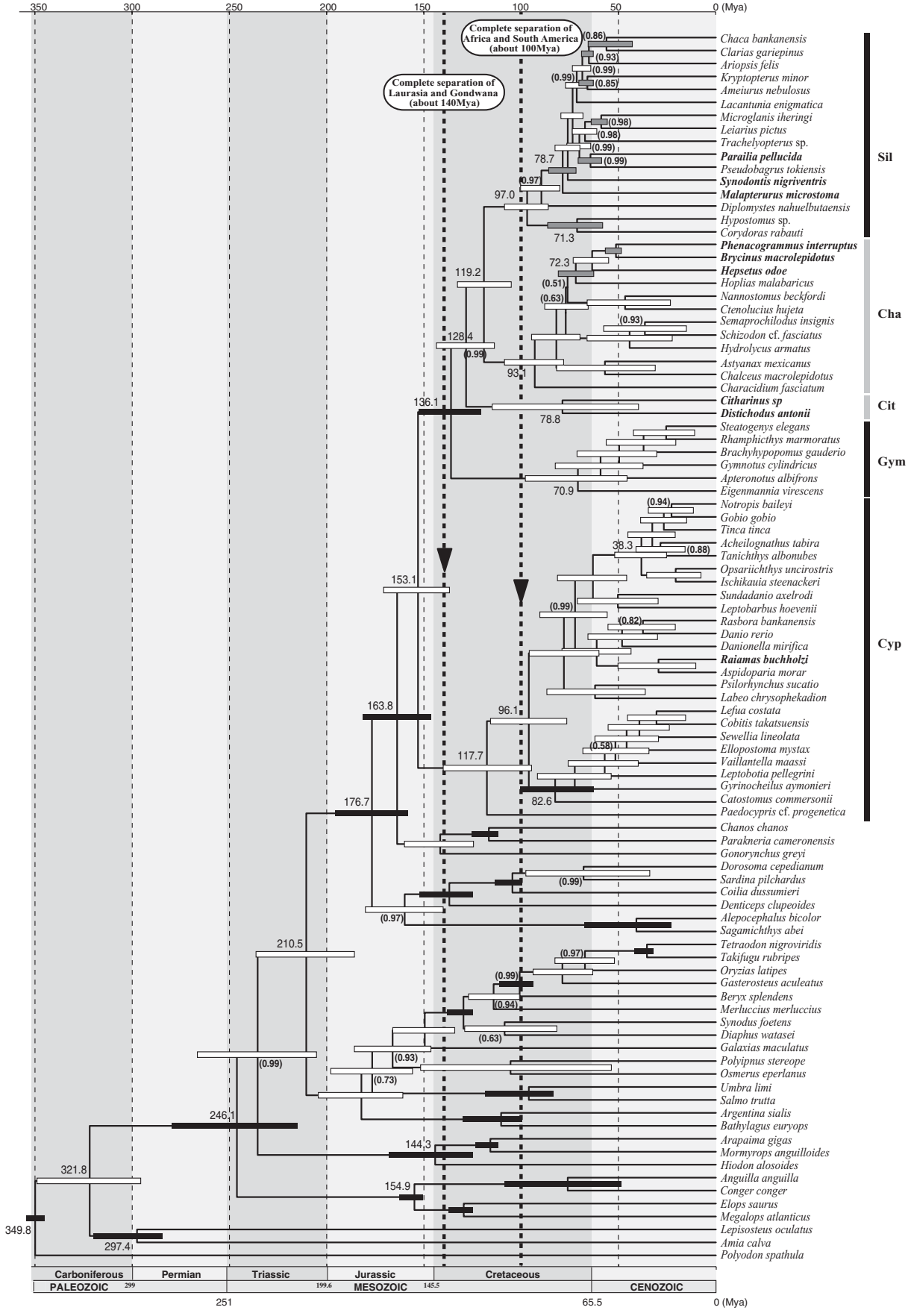
Importantly, regardless of our two inferred time scales (Figs. 3, S6), the diversification of the African characoid clade with its South American sister group and the diversification of the crown-group Siluroidei postdated fragmentation of both Africa and South America, and their current widespread and transoceanic distributions may be better explained by dispersal, perhaps via sporadically appeared land connections between different continents in the past (Fig. 4).

Discussion

Most of our inferred relationships within Teleostei (Fig. 2) are consistent with the backbone phylogenetic hypothesis based on morphology and recent modifications from molecular data (Nelson 2006; Chen and Mayden 2010; Near et al. 2012). However, our analyses of multiple nuclear genes using varied combinations of partitions did, in a few instances, provide moderate to strong support for unexpected phylogenetic relationships consistent across our different analyses and with previous studies. All these new findings deserve further discussion, and likewise

Table 2. Comparison of the ages of the Otophysi and several clades within the Otophysi. The ages were inferred by direct evidence (based on the oldest fossil assigned to the corresponding group) and indirect evidence (molecular-based estimations, this study and Nakatani et al. [2011]). Ages in million years before present (Mya).

	Fossil record	Exponential distribution (Fig 3.)	Uniform distribution (Fig. S6)	Nakatani et al. (2011)
Age of the crown-group Otophysi	99.6–112 Mya †*Santanichthys* Filleul and Maisey (2004)	153.1 Mya (95% CI = 146–181 Mya)	213.3 Mya (95% CI = 193–234 Mya)	248 Mya (95% CI = 227–268 Mya)
Age of the crown-group Cypriniformes	About 61 Mya Oldest catostomid Cavender (1991)	117.7 Mya (95% CI = 95–140 Mya)	158.9 Mya (95% CI = 129–191 Mya)	159 Mya (95% CI = 130–186 Mya)
Age of the crown-group Characiphysi	99.6–112 Mya †*Santanichthys* Filleul and Maisey (2004)	136.1 Mya (95% CI = 121–153 Mya)	183.5 Mya (95% CI = 161–206 Mya)	226 Mya (95% CI = 206–245 Mya)
Age of the crown-group Gymnotiformes	ca. 8–10 Mya †*Humboldtichys* Gayet and Meunier (1991)	71.0 Mya (95% CI = 46—98 Mya)	88.4 Mya (95% CI = 57–120 Mya)	189 Mya (95% CI = 166–212 Mya)
Age of the crown-group Citharinoidei	About 45 Mya †*Eocitharinus* Murray (2003)	78.8 Mya (95% CI = 40–115 Mya)	106.3 Mya (95% CI = 61–153 Mya)	160 Mya (95% CI = 124–190 Mya)
Age of the crown-group Characoidei plus Siluriformes	83.5–88.6 Mya Earliest catfish fossil Patterson (1993)	119.2 Mya (95% CI = 105–133 Mya)	154.8 Mya (95% CI = 133–177 Mya)	216 Mya (95% CI = 198–237 Mya)
Age of the crown-group Characoidei	About 75 Mya Unnamed fossil Newbrey et al (2009)	93.1 Mya (95% CI = 78–109 Mya)	113.2 Mya (95% CI = 92–136 Mya)	192 Mya (95% CI = 172–213 Mya)
Age of the crown-group Siluriformes	83.5–88.6 Mya Earliest catfish fossil Patterson (1993)	97.0 Mya (95% CI = 86–109 Mya)	116.9 Mya (95% CI = 99–135 Mya)	180 Mya (95% CI = 162–198 Mya)
Age of the crown-group Siluroidei	About 63 Mya Earliest fossils assigned to extant siluroid families Gayet and Otero (1999)	78.7 Mya (95% CI = 72–86 Mya)	86.2 Mya (95% CI = 75–98 Mya)	About 145 Mya (95% CI = not indicated)



continued investigation, but given the overall goal of this article, our discussion is restricted to the nonmonophyly of the group Siluriformes plus Gymnotiformes and the nonmonophyly of the Characiformes. These two inferred relationships have direct consequences on our biogeographic hypothesis.

THE FINK AND FINK HYPOTHESIS

The morphological work of Fink and Fink (1981) belongs in the category of works that profoundly and durably modified the perception of the systematics of ray-finned fishes. These authors addressed the question as to whether monophyly, within a phylogenetic framework, of each main lineage of ostariophysans should be retained and interrelationships of major clades based on a comparative survey of morphology. All proposed monophyletic groups were based on a series of synapomorphies. Fink and Fink (1981) reported an impressive list of 127 phylogenetically informative characters within the Ostariophysi; with only eight of these characters appearing to be homoplasic within the Otophysi. This thorough survey provided an unprecedented, well-supported hypothesis depicting the evolutionary relationships among the main ostariophysan lineages.

Fink and Fink (1981) listed their main phylogenetic findings in five points: (1) the Gonorynchiformes, Characiformes, Siluriformes, Cypriniformes, and Gymnotiformes are each monophyletic, supported by 8, 7, 17, 9, and 11 unique synapomorphies, respectively. (2) The Siluriformes and the Gymnotiformes are sister groups, supported by 23 synapomorphies. (3) This latter clade is sister to Characiformes, together forming the Characiphysi, supported by 15 synapomorphies. (4) The Characiphysi is the sister group of the Cypriniformes, forming the Otophysi (14 synapomorphies). Finally, (5) the Gonorynchiformes is the sister group of the Otophysi (14 synapomorphies), together forming the Ostariophysi. Fink and Fink (1996) updated their work in revising some of their precedent characters, but their conclusions stayed essentially the same. Our phylogenetic conclusions differ from the Fink and Fink's hypotheses in two areas discussed later.

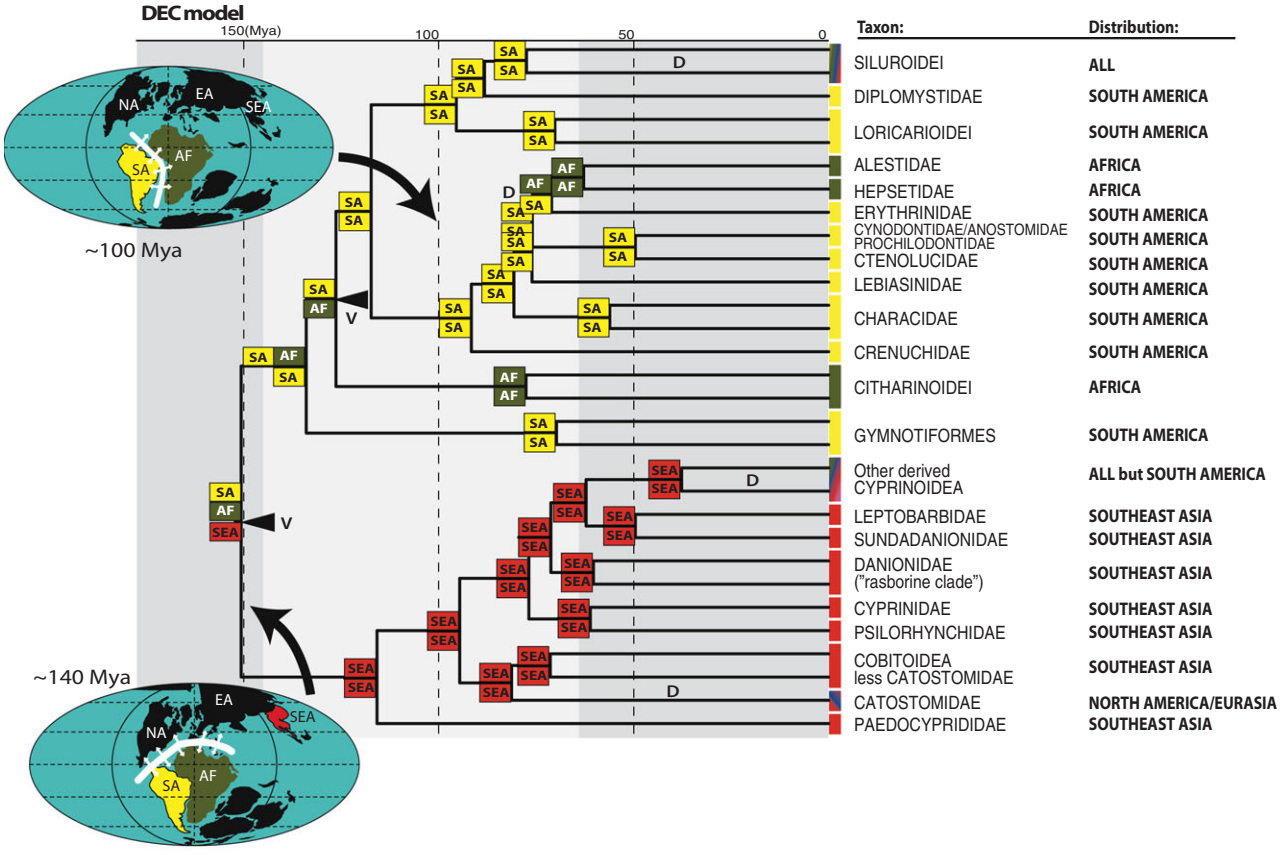
THE NONMONOPHYLY OF THE CHARACIFORMES

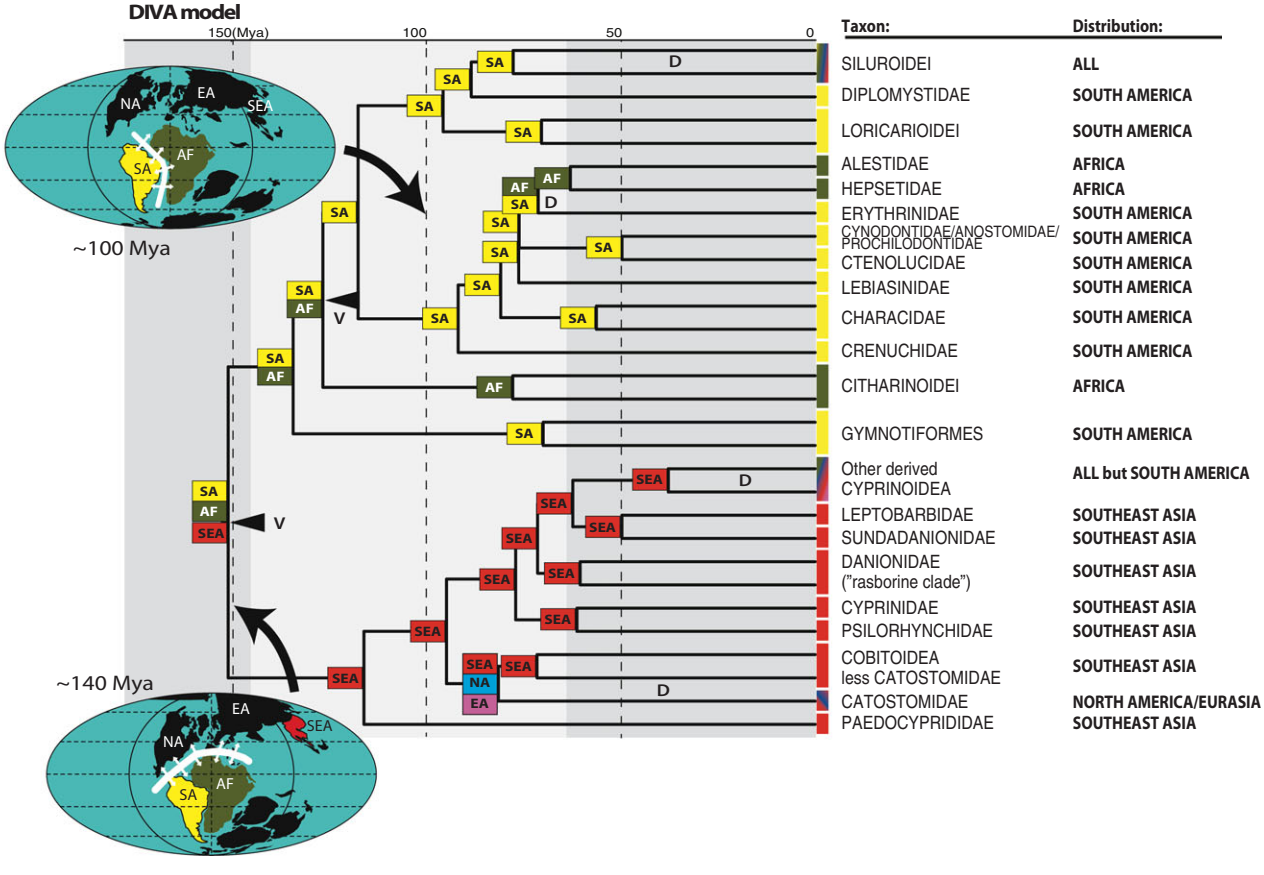
Although molecular data are congruent with morphological data in corroborating the monophyly of the Siluriformes (e.g., Sullivan et al. 2006; Nakatani et al. 2011), the Cypriniformes (e.g., Saitoh et al. 2006; Mayden and Chen 2011; Nakatani et al. 2011), and the Gymnotiformes (Nakatani et al. 2011), the monophyly of the Characiformes remains rather tenuous when representatives from both suborders, the Characoidei and the Citharinoidei, are included in molecular phylogenetic analyses with members of the above-identified monophyletic groups (Ortí and Meyer 1996, 1997; Nakatani et al. 2011). Only two studies have recovered the Characiformes as monophyletic (Dimmick and Larson 1996; Near et al. 2012).

Dimmick and Larson (1996) recovered a monophyletic Characiformes but included only one representative of each characiform suborder. However, when we realigned (using MUSCLE; Edgar 2004a, 2004b) and reanalyzed (using both parsimony and ML methods) their dataset that consisted of partial nuclear and mitochondrial rRNA genes, contrary their figure 5B showing a monophyletic Characiformes, we found the order Characiformes to be paraphyletic with respect to Gymnotiformes (results not shown).

Near et al. (2012) published a large-scale phylogenetic analysis for ray-finned fishes based on nine nuclear markers. Although most of the inferred relationships are in agreement with our phylogenetic hypothesis, these authors recovered the traditional opinion as to the monophyly of the Characiformes with strong support (i.e., Citharinoidei sister to Characoidei). This was an unexpected result given that their dataset shares eight genes with the nuclear dataset of Nakatani et al. (2011) and the latter authors could not corroborate the monophyly of the Characiformes. The taxonomic sampling by Near et al. (2012) included only one citharinoid species (*Distichodus maculatus*) from which DNA sequences were retrieved from the GenBank database (http://www.ncbi.nlm.nih.gov/; note that *Zic1* and *Glyt* sequences for this species were missing). Using the BLAST tool provided by NCBI, we found that for five nuclear markers, the *Distichodus maculatus* sequences are almost identical (>98% of similarity) to the sequences of the characoid *Ctenolucius hujeta* and more dissimilar to other citharinoid sequences (<91%). For only one gene (*tbr1*), the sequence of *Distichodus maculatus* is more similar (94%) to a citharinoid relative (*Citharinus congicus*) than to other characoid species. Therefore, we believe that

Figure 3. Phylogenetic chronogram of Teleostei based on a Bayesian relaxed clock approach (using BEAST v1.7.4), using the matrix "123_{ry}" partitioned in 3, and calibrated with 30 fossil-based constraints following exponential distributions (see text for details). *Polyodon spathula* is used to root the tree. Horizontal timescale is in million years before present (Mya). Black horizontal bars (indicating calibration constraints on the corresponding nodes), gray horizontal bars (indicating soft maximum ages calibration constraints on the corresponding nodes), and white horizontal bars at nodes are 95% age credibility intervals. Numbers given in parenthesis at nodes are Bayesian posterior probabilities if < 1. Times of the final separation of Laurasia and Gondwana (about 140 Mya) and the final separation of Africa and South America (about 100 Mya) are indicated with two vertical dotted lines. The Cypriniformes, Citharinoidei, Characoidei, Siluriformes, and Gymnotiformes are abbreviated as "Cyp," "Cit," "Cha," "Sil," and "Gym," respectively. African otophysans are highlighted in bold.





the phylogenetic position of the Citharinoidei in Near et al. (2012) warrants further testing and reidentification of specimens and the appropriate inclusion of multiple species that are unquestionably of both Citharinoidei and Characoidei.

In conclusion, thus far none of the multiple molecular studies provides convincing evidence to corroborate the hypothesized monophyly of Characiformes. However, there is a weak but recurrent phylogenetic signal supporting the nonmonophyly of Characiformes despite the morphological evidence (Fink and Fink 1981). The issues underlying the observed incongruence between morphological and molecular data and their phylogenetic evaluation indeed requires further evaluation, and likely more fine-scaled attention focused on hypothesized homologies within both data sets.

THE NONMONOPHYLY OF THE GROUP SILURIFORMES PLUS GYMNOTIFORMES

Our results consistently support the Siluriformes as the sister group of the Characoidei, not the Gymnotiformes as has been commonly accepted since Fink and Fink (1981). This is an unanticipated result considering that the monophyly of the Siluriformes plus Gymnotiformes received considerable morphological support (Fink and Fink 1981, 1996). In their updated work (Fink and Fink 1996), these authors discarded two of the original 23 synapomorphies (i.e., characters 74 and 100 in Fink and Fink 1981) and listed 16 additional synapomorphies from the electroreceptive system and the neuroanatomy (replacing their previous characters 119 and 120).

Nonetheless, despite this strong morphology-based support for their monophyly, a sister relationship between the Gymnotiformes and the Siluriformes has never been recovered by any molecular study. Notwithstanding the nonmonophyly of the Characiformes, most of the molecular phylogenetic studies including representatives of Siluriformes, Gymnotiformes, and Characiformes report a monophyletic group consisting of Siluriformes plus Characiformes sister to Gymnotiformes (Lavoué et al. 2005; DeVaney 2008; Li et al. 2008; Santini et al. 2009; Kawaguchi et al. 2010; Li et al. 2010; Nakatani et al. 2011; Lavoué et al. 2012). A few other studies have inferred the monophyly of the Gymnotiformes plus Characiformes (Dimmick and Larson 1996; Saitoh et al. 2003; Peng et al. 2006; Near et al. 2012).

The hypothesis about the nonsister group relationship between the Siluriformes and Gymnotiformes is challenging and requires a large number of homoplasic events of hypothesized homologous morphological features. Of all the characters supporting the monophyly of Siluriformes plus Gymnotiformes, the functional characters associated with the electroreceptive system seem to be the most irrefutable. Electroreception in teleost fishes is known in only two distantly related groups, the Notopteroidei (Osteoglossomorpha) and the Otophysi (Siluriformes and Gymnotiformes; Bullock and Heiligenberg 1986). If the Gymnotiformes is the sister group of the remaining Characiphysi and the Siluriformes is the sister of the Characoidei, it implies that either electroreception originated twice in Otophysi (once in the ancestor of the Siluriformes and once in the ancestor of the Gymnotiformes) or electroreception originated once in the ancestor of the Characiphysi and was subsequently lost or modified in the Characoidei and Citharinoidei.

We note that in the suborder Notopteroidei electroreceptive it is likely that the Asian Notopteridae secondarily lost electroreception (Lavoué and Sullivan 2004). Although the independent origins of electroreception is rare, therefore unlikely, its secondarily independent lost may explain the electroreception distribution both in the Notopteroidei and in Otophysi. Conversely, more detailed microanatomical evaluations might reveal differential origins and nonhomolog of the electroreception abilities of some Siluriformes and the Gymnotiformes; this can also be argued to be the case with respect to the "behavior" of molecular sequence data and differential analyses. Both warrant continued more detailed evaluations and additional specimen inclusion in analyses.

Figure 4. **Ancestral area reconstruction within the Otophysi using the DEC method as implemented in the software Lagrange (Ree et al. 2005; Top), and DIVA method as implemented in the software RASP (ex S-DIVA; Yu et al. (2010); Bottom). For the analysis with Lagrange, the simplified phylogram from the Figure 3 was used. At each node, each ancestral area is made either of the combination of two ranges inherited from each two descendant lineages (each placed above and below the corresponding node/branch) or a single range. For example, the inferred ancestral area of the most recent common ancestor of the clade (Citharinoidei [Siluriformes, Characoidei]) is made of "Africa" (lineage Citharinoidei, indicated below the branch) plus "South America" (lineage [Siluriformes, Characoidei], indicated above the branch). When two or more ancestral ranges at one node are inferred, only the most likely is shown. For the analysis with RASP, the BEAST collection trees and the simplified tree topology consensus of the Figure 3 were employed. At each node, the most likely inferred ancestral areas are drawn. The five subareas are as follow: South America (SA, code color: yellow), Africa (AF, green) North America (NA, blue) Southeast Asia (SEA, red), Eurasia (EA, pink). "*vicariant events*" are indicated with black arrowheads and "V"; "*postdrifting dispersion events*" are indicated with "D" but their precise dating is unknown. Paleaomap reconstructions at 140 and 100 Mya are shown to explain the first two vicariant events leading to the Cypriniformes and the Characiphysi lineages and to the Siluriformes plus Characoidei and the Citharinoidei lineages, respectively. The family-level classification of the Cypriniformes followed Mayden and Chen (2010); classifications of the Siluriformes and Characiformes followed Nelson (2006).**

DIVERGENCE TIME ESTIMATIONS AND CONGRUENCE WITH THE FOSSIL RECORD

The fossil record provides the only direct evidence for the presence of a group at a given geological period. In Table 2, we provide minimum ages for each main lineage of the Otophysi derived from their fossil records.

Our two divergence time reconstructions based on molecular sequence variation of five nuclear loci offer two different timeframes for the origin and diversification of the Otophysi. The first reconstruction (Fig. 3) suggests mostly compatible divergence times for the most ancient relationships with minimum divergence times provided by the fossil record (Table 2). This is not surprising given how we strongly calibrated this time tree. Our second divergence time reconstruction (Fig. S6) provides overall older estimates because maximum ages were not constrained. In this situation, some groups are hypothesized to be much older than their fossil records would predict. For example, the age of the crown-group Cypriniformes is inferred to 158.9 Mya, an estimate much more ancient than the fossil record of the Cypriniformes. If correct, such a result could be explained by the poor quality of some fish fossil records of some groups, specifically the Otophysi. For example, the poor quality of the fossil record of the cypriniforms has already been noted and attributed to unfavorable fossilization conditions due to moist paleo-climates where the fishes occurred and muddy substrates in these areas, that is Southeast Asia (Briggs 1979).

Whatever the accuracy of our two divergence time estimates, we hypothesize that they provide a range of possibilities from which future studies, based on more and better preserved fossils, can serve as important results and data in the development and polishing of molecular divergence time methods. Within this range of possibilities, we can assert that: (1) the origin of the Otophysi well predated the separation of Africa and South America and may also predate the early fragmentation of Pangea into Laurasia and Gondwana. (2) The early diversification of the Otophysi predated or was contemporary with the separation of Africa and South America whereas (3) the late diversification within each of the crown-groups Characoidei, Siluroidei, and Cyprinoidea postdated the separation of Africa and South America. Our age estimations within each of these three suborders do differ from those of Nakatani et al. (2011) wherein their analyses were based on mitochondrial genomic data (Table 2); however, our hypotheses are roughly similar to those of Arroyave and Stiassny (2011) for the Characoidei, Lundberg et al. (2007) for the Siluroidei, and Near et al. (2012) for both the Characoidei and the Siluroidei. The discrepancies among the above studies providing molecular dating estimates may be due to different methods and/or fossil calibrations used, and/or to the likely evolutionary rate differences between nuclear and mitochondrial genomes, all of which impact, to some degree, estimates of divergence times (Hurley et al. 2007). For example, it has recently been shown that mitochondrial genes tend to provide older but misleading age estimations at deeper nodes because of nucleotide saturation compressing or providing limited divergences and support of basal branches (Lukoschek et al. 2012). We identify these areas for much more concentrated efforts across morphological and molecular data and the algorithm variations impacting resulting hypotheses.

HISTORICAL BIOGEOGRAPHY RECONSTRUCTION

Our integrative approach based on new lines of evidence—phylogeny and time—permit the reconstruction a hypothesis as to the biogeographic scenario for the origin and the early diversification of the Otophysi (Fig. 4):

(1) The Otophysi forms a monophyletic group within the Ostariophysi. The current ecological environment of the Otophysi strongly suggests a freshwater origin of its most recent common ancestor (see Nakatani et al. 2011; Fig. S7). The "marine origin" hypothesis of the Otophysi and their early diversification based on successive marine dispersions discussed by Gayet (1982) seems to us and to others (Fink et al. 1984; Patterson 1984) unlikely. Gayet (1982) found support for her hypothesis in the observation that most of the earliest otophysan fossils are marine. However, we argue the current uncertainties about the phylogenetic affinities of these fossils relative to the extant otophysan lineages, do not provide strong support for this hypothesis over the fact that most of the extant Otophysi are primarily freshwater species.

(2) The most recent common ancestor of the Otophysi may have had a Pangean origin with an ancestral distribution covering at least the South America/Africa/Southeast Asia regions. If correct, it is surprising that it did not occur in North America and Eurasia as well because current hypotheses contend that South America/Africa/Southeast Asia did not form a contiguous area at any time. A possible alternative hypothesis could be that the ancestor of the Otophysi actually occurred in a larger area including North America and Eurasia, at a time where climatic conditions were subtropical in the entire Laurasia. Later paleo-climatic fluctuations in North America and Eurasia, with a progressive decreasing of the temperatures through global cooling provoked the extinction of the tropical organisms living there. Low temperatures were not suitable for the survival of the tropical fishes like most of the current otophysan fishes. We note that present day taxa living in North America and Eurasia are the most terminal clades and are possibly only secondarily adapted for the lower temperatures at these temperate latitudes and they only recently invaded these two regions.

(3) Given the estimated ancient age of the Otophysi, the initial step into their diversification may have been linked to the

fragmentation of the Pangea (Fig. 4), with a northern lineage restricted to the Laurasia (leading to the Cypriniformes) and a southern lineage restricted to the Gondwana (leading to the Characiphysi).

(4) Although the diversification of the crown-group Cypriniformes took place later within the northern hemisphere (Saitoh et al. 2011), the early diversification of the Characiphysi predated (within-area speciation plus local dispersion/extinction) or was contemporary with the fragmentation of the South America and Africa in the Gondwana (vicariance; Fig. 4).

(5) Our timescale indicates that the diversification of the crown-groups Cyprinoidea, Siluroidei, and Characoidei possibly postdated any major continental fragmentations via tectonic movements, and thus they may be better accounted for by dispersal between areas. This hypothesis is in disagreement with the scenario proposed by Lundberg (1993), Diogo (2004), and Briggs (2005) who hypothesized that at least part of the diversification of the Characoidei and Siluroidei predated or was the consequence of the separation of South America and Africa (vicariant events).

Our scenario indicates that the respective ancestral areas of the Siluriformes and Characoidei were South America and these two groups reached Africa via multiple cross-continental dispersions from South America. In recent years, trans-Atlantic dispersal events postdating the final separation between Africa and South America have been hypothesized for a number of terrestrial and freshwater organisms (de Queiroz 2005; Lundberg et al. 2007; Vidal et al. 2008; Gamble et al. 2011).

One of the two possible hypotheses permitting freshwater fishes to invade other continents could be highly unlikely and untestable transmarine dispersals (Lundberg et al. 2007). The other possibility for the dispersal of early siluroids and characoids may be in association with temporary connections between continents but very early in their separations. For example, such connections between South America and North America occurred possibly in the Cretaceous at about 80 Mya (Pitman et al. 1993; Martin et al. 2005; Newbrey et al. 2009). Such a cross-continent dispersal route has been previously hypothesized for the early range expansion of some terrestrial vertebrates such as lizards, mammals, and dinosaurs (Estes 1983; Cifelli and Eaton 1987; Nydam 2002), and more recently characiform-like fishes (Newbrey et al. 2009). Newbrey et al. (2009) discovered a characiform-like fish fossil from the Cretaceous Formation in Alberta, Canada, a nowadays-temperate area where no living characiform fishes survives and no characiform fossils had previously been reported. If identified correctly, this implies the presence (at least during a short time span) of such tropical fishes living in more northern areas in the Late Cretaceous, a time of significantly warmer and more moist global climate than now, and suggests invasions of characiforms from South America to Northern America and then to Europe (Otero et al. 2008). Although whether the fossil discovered by Newbrey et al. (2009) can be well aligned to any living characiform lineage, especially any characoid lineage, is still questionable (B. Sidlauskas, pers. com.), the occurrence of several well-documented characoid fossils from the Early Eocene of Europe (Otero et al. 2008) provides additional supporting evidence, with characoid fishes, for post-Pangean connections between southern and northern continents, and the possibility of such an historically large terrestrial connection, opportunities for freshwater river connections and a route for these fishes.

A similar, although reversed, dispersal way has been hypothesized to explain the presence of the catfish *Lacantunia enigmatica* in Central America whereas its closest relatives are African (Lundberg et al. 2007). Global cooling after this time has been hypothesized to result in a mass extinction in the North, and the restriction of tropical fishes to inhabit southern North American, Central America, more southern areas in South Europe and tropical Africa. Mayden (1988) in discussion of the biogeographic origin of the eastern North American fish fauna proposed extensive extinction in more northern waterways during glaciation and possibly earlier rather than a northern fauna being "pushed" south to increase diversity in the southern faunas. The congruence of speciation patterns with preglacial drainage patterns supported the preglacial evolution of the fauna and no northern members of clades being "pushed" south in advance of glacial fronts.

Conclusion

Intercontinental relationships of terrestrial or freshwater organisms are often described as the result of paleo-continent fragmentations mediated by tectonics. However, recent studies incorporating molecular dating provide increasing evidence that diversification of several of these groups actually postdated major tectonic movements and dispersion is no longer perceived as a marginal process (de Queiroz 2005; Waters and Craw 2006; Lundberg et al. 2007; Vidal et al. 2008; Crisp et al. 2011; Klaus et al. 2011). In this study, we found strong evidence that the diversification of two freshwater fish groups, the Siluroidei and Characoidei, considered as prime examples in Africa–South America vicariance biogeography, actually postdated the final fragmentation between these two continents. Postfragmentation dispersal ways between Africa and South America remain uncertain; however, paleontological research may be particularly useful to reveal them.

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Appendix

Thirty selected key fossils used as calibration points for the divergence time estimations are from the following teleost groups. Ages of the geological stages are revised according to the stratigraphy chart of the International Commission on Stratigraphy (2009; available at http://www.stratigraphy.org/). The mean values when exponential distributions are considered (reconstruction 1) are indicated in brackets.

(1) *The tMRCA (time of the most recent common ancestor) of the clade* Amia calva *(Amiiformes) and the Lepisosteidae (Lepisosteiformes)* is set to a minimum of 284.4 millions of years ago (Mya), which corresponds to the minimum age of the geological stage Sakmarian (Permian) from which was excavated the oldest Amiiformes fossil known, †*Brachydegma* (Hurley et al. 2007). We used †*Cosmoptychius* (a neopterygian or actinopterian) known from the Tournasian (345.3–359.2 Mya; Inoue et al. 2009) to establish the soft upper bound age to 345.3 Mya [exponential distribution mean = 20].

(2) *tMRCA of the Elopomorpha*: The stem elopiform †*Anaethalion* is the earliest elopomorph fossil (Arratia 1987, 2000), from the Late Jurassic (Kimmeridgian, 150.8–155.6 Mya) and is also considered to be the oldest crown-group teleostean fossil. We used †*Anaethalion* to calibrate the age of the crown Elopomorpha with a minimum age of 150.8 Mya. According to Benton et al. (2009), 161.2 Mya should be considered as the soft maximum constraint because there is no crown-group teleost fossil discovered in the fossil rich localities of Oxfordian age (155.6–161.2 Mya) [exponential distribution mean = 3.5].

(3) *tMRCA of the Neoteleostei* is constrained by the oldest known aulopiform fossil, †*Atolvorator longipectoralis* (Gallo and Coelho 2008) that dates back to the Barremian (125–130 Mya; Davis and Fielitz, 2010). We used it to calibrate the minimum age of the crown-group Neoteleostei, in which the Aulopiformes belongs to, at 125 Mya. As Benton et al. (2009) stated, the oldest fossil record of the Euteleostei is represented with two stem-group euteleosts collected from the Tithonian stage (†*Leptolepides*, †*Orthogonikleithrus*). These two fossils provide a soft maximum age of 150.8 Mya. [exponential distribution mean = 8.5].

(4) *tMRCA of Osteoglossomorpha*: The first crown-group Osteoglossomorpha is †*Yanbiania* from the Barremian (125–130 Mya); it has been placed within the order Hiodontiformes (Zhang 2004; Wilson and Murray 2008). We used †*Yanbiania* to calibrate the age of the crown-group Osteoglossomorpha with a minimum age of 125 Mya. Santini et al. (2009) used the first crown-group Teleostei (the elopomorph †*Anaethalion*) to bracket the upper soft bound of the Osteoglossomorpha age to 155.6 Mya [exponential distribution mean = 10].

(5) *tMRCA of Otocephala*: The minimum age of the Otocephala is set to 149.8 Mya because of the oldest fossil known, †*Tischlingerichthys viohli*, which is a stem Ostariophysi from the Upper Jurassic (Early Tithonian, 149.8–150.8 Mya; Arratia 1997, 2000; Benton et al. 2009). According to Benton et al. (2009), 161.2 Mya should be considered as the soft maximum constraint because there is no crown-group teleost fossil discovered in the fossil rich localities of Oxfordian age (155.6–161.2 Mya) [exponential distribution mean = 3.8].

(6) *tMRCA of the clade* (Chanos, Parakneria): The oldest fossil assigned to the crown-group Gonorynchiformes is a species of *Chanos* (family Chanidae), †*Chanos leopoldi*, from the Aptian (112.0–126.0 Mya; Fara et al. 2010). We

therefore use this fossil to constraint the minimum tMRCA of the clade (*Chanos*, *Parakneria*) to 112 Mya. The oldest gonorynchiform fossil is †*Rubiesichthys gregalis* (Fara et al. 2010) collected from the Berriasian stage (145.5–140.2 Mya). This fossil was used to provide the soft maximum age (= 140.2 Mya). [exponential distribution mean = 9.5].

(7) *tMRCA of the African characoids*: Zanata and Vari (2005) discussed the identities and phylogenetic positions of most characiform fossils of the Old World. They concluded that the oldest fossils assignable to the Alestidae are of Early Eocene age (Ypresian, 48.6–55.8 Mya), such as †*Alestoides eocaenicus*. Therefore, we assigned a minimum age for the divergence Alestidae / Hepsetidae to 48.6 Mya. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 37.5].

(8) *tMRCA of Bagridae (Siluroidei)*: The oldest bagrids (†*Eomacrones wilsoni*, †*Nigerium gadense*, and †*Nigerium wurnoënse*) are found in the Middle Paleocene series, providing a minimum age (58.7 Mya) for the MRCA of the clade (*Pseudobagrus*, *Parailia*; Gayet and Otero 1999). In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 34.5].

(9) *tMRCA of the clade* (Tetraodon, Takifugu, Gasterosteus, Oryzias, Beryx): Santini et al. (2009) used several beryciform fossils of the Cenomamian age (99.6–93.6 Mya), such as †*Hoplopteryx* sp. and †*Trachichthyoides* sp., to calibrate the age of the MRCA of the Acanthomorpha. We used these same fossils as grounds for setting the minimum age of the MRCA of the clade (*Tetraodon*, *Takifugu*, *Gasterosteus*, *Oryzias*, *Beryx*) to 93.6 Mya. We also followed Santini et al. (2009) to establish a soft maximum age of 122 Mya based on the earliest acanthomorph fossil ("otoliths") from the Early Aptian (Lower Cretaceous, 124–122 Mya) [exponential distribution mean = 9.5].

(10) *tMRCA of the Cobitoidei*: (61.1 Mya) The earliest cobitoid fossils from the family Catostomidae are possibly known from the Early Paleocene (Danian, 61.1–65.5; Cavender 1991; Briggs 2005; Liu and Chang 2009). Therefore, we constrained the minimum age of the Cobitoidei to 61.1 Mya. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 33.5].

(11) *tMRCA of the Characiphysi*: Filleul and Maisey (2004) redescribed †*Santanichthys diasii* from the Albian stage (99.6–112 Mya), identifying this fossil as a stem characiform species. As such, it represents the oldest known characiform and characiphysan fossil. The first siluriform fossil may be an undescribed species collected from Coniacian-Santonian (83.5–88.6 Mya) strata in Africa (Patterson 1993; Gayet and Otero 1999). We applied a minimum age of 99.6 Mya for the tMRAC of the Characiphysi. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 20.5].

(12) *tMRCA of the crown-group Siluroidei*: Several siluroid fossils begin to appear in Campanian stage (70.6–83.5 Mya) in South America (Gayet and Otero 1999). We used them to constraint the minimum age of the Siluroidei to 70.6 Mya. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 30.2].

(13) *tMRCA of the crown-group Loricarioidei*: The oldest Loricarioidei fossil is the callichthyid †*Corydoras revelatus* (Lundberg et al. 2007) from the Late Paleocene, that is, 58.2–58.5 Mya. This fossil provides a minimum age of 58.2 Mya for the crown-group Loricarioidei. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 34.5].

(14) *tMRCA of the Elopiformes*: According to Forey et al. (1996), the first crown-group elopiform fossil is †*Elopoides*, which is of Barremian age (about 125.0–130.0 Mya). Thus, we set the minimum age for the MRCA of the clade (*Elops*, *Megalops*) to 125 Mya. We used the first elopomorph †*Anaethalion* to bracket the upper soft bound of the Osteoglossomorpha age to 155.6 Mya [exponential distribution mean = 10].

(15) *tMRCA of the Ostariophysi*: The oldest fossil of the crown-group Ostariophysi is the stem gonorynchiform †*Rubiesichthys gregalis* (Fara et al. 2010) collected from the Berriasian stage (145.5–140.2 Mya). This fossil was used to provide the minimum age of the crown-group Ostariophysi (= 140.2 Mya) [exponential distribution mean = 7].

(16) *tMRCA of the clade* (Umbra, Salmo): The Early Campanian †*Estesesox foxi* (Wilson et al. 1992; Newbrey et al. 2008) is the earliest esocoid. It provided us with the minimum age constraint (83.5 Mya) for the MRCA of the clade (*Umbra*, *Salmo*). As Benton et al. (2009) stated, the oldest fossil record of the Euteleostei is represented with two stem-group euteleosts collected from the Tithonian stage (†*Leptolepides*, †*Orthogonikleithrus*). These two fossils provide a soft maximum age of 150.8 Mya [exponential distribution mean = 22.5].

(17) *tMRCA of the Teleostei*: The oldest fossil assigned to the crown-group Teleostei are †*Anaethalion* spp (Kimmeridgian, 150.8 Mya; Santini et al. 2009) The oldest stem teleost is †*Pholidophoretes salvus* (Pholidophoridae), from the Early Carnian (Triassic, 228.7–216.5 Mya; Santini

et al. 2009). Our prior assumed 150.8 Mya as the minimum age and 228.7 Mya for the upper bound [exponential distribution mean = 26].

(18) *tMRCA of the clade* (Takifugu, Tetraodon): †*Archaeotetraodon winterbottomi* is the first Tetraodontidae fossil known from the Oligocene and its age has been estimated to a minimum of 32.0 Mya. This fossil is used to calibrate the minimum age of the clade (*Takifugu, Tetraodon*; Carnevale and Tyler 2010). Santini et al. (2009) establish a soft maximum age of 50.0 Mya based on the stem tetraodontid †*Eotetraodon pygmaeus* known from the middle Ypresian of Monte Bolca, Italy (48.6–55.8 Mya) [exponential distribution mean = 6].

(19) *tMRCA of the clade* (Arapaima, Mormyrops): †*Laeliichthys australis* and †*Chandlerichthys* sp. (Li and Wilson 1996; Taverne 1998; Forey and Hilton 2010) are dated to the Aptian age (125–112 Mya) and represent the oldest fossils assigned to the subfamily Heterotidinae (= Arapaiminae). We constrained the tMRCA of the clade (*Arapaima*, *Mormyrops*) to a minimum age of 112 Mya. The genus †*Lycoptera* is the oldest stem-group osteoglossomorph dated to the Barremian age (130–125 Mya). It provides a soft maximum age of 130 Mya [exponential distribution mean = 6].

(20) *The root of the tree* is constraint to a minimum age of 345.3 Mya and a maximum of 359.2, which corresponds to the minimum and maximum ages of the strata of the first fossil of the lineage leading to the *Polyodon spathula*, †*Cosmoptychius* (a neopterygian or actinopterian) known from the Tournaisian (345.3–359.2 Mya; Inoue et al. 2009).

(21) *tMRCA of the crown-group Clupeoidei* (*i.e.*, Dorosoma, Sardina, Coilia): The earliest fossil assigned to the Clupeidae *ad interim* provided a minimum age for the crown-group Clupeoidei of 99.6 Mya (De Figueiredo 2009a). The soft maximum age is set to 125 Mya, which is the minimum age of the oldest stem clupeoid [exponential distribution mean = 10].

(22) *tMRCA of the crown-group Clupeiformes* (*i.e.,* Denticeps *plus* clupeoids): †*Pseudoellimma gallae* is considered as the first stem clupeoid (De Figueiredo 2009b). Therefore, it provided a minimum age of 125 Mya (Barremian) for the divergence between the Clupeoidei and Denticipitoidei. The soft maximum age is set to 161.2 Mya [exponential distribution mean = 12].

(23) *tMRCA of the crown-group Alepocephaliformes*: The oldest alepocephaliform fossil is the alepocephalid †*Carpathichthys polonicus* of Miocene age (Jerzmanska 1979), which provided with a minimum age of 23 Mya for the MRCA of the clade (*Alepocephalus*, *Platytroctes*) and the very soft maximum age is set to 140.2 Mya [exponential distribution mean = 46].

(24) *tMRCA of the clade* (Anguilla, Conger): Inoue et al. (2009), citing Patterson (1993), set the minimum age constraint for the divergence between *Conger* (Congridae) and *Anguilla* (Anguillidae) to 48.6 Mya (Ypresian). The soft maximum age constraint is set to 130 Mya that corresponds to the age of the oldest elopiform [exponential distribution mean = 37.5].

(25) *tMRCA of the clade* (Argentina, Bathylagus): The oldest fossil assigned to the family Argentinidae is †*Nybelinoides brevis* (consult Santini et al. 2009). Therefore, it provided a minimum age of 99.6 Mya for the divergence between the *Argentina* and *Bathylagus*. The soft maximum age constraint is set to 140.2 Mya [exponential distribution mean = 37.5].

(26) *tMRCA of the clade* (Leiarius, Microglanis): The oldest pimelodid fossil is from the Paleogene of South America (Gayet and Otero 1999). It provided a minimum age of 55.8 Mya for the divergence between *Leiarius* (Pimelodidae) and *Microglanis*. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 35].

(27) *tMRCA of the clade* (Ariopsis, Clarias, Chaca): The family Ariidae is known from the Early Paleogene (Gayet and Otero 1999). Therefore, it provided a minimum age of 63.0 Mya for the divergence between the family Ariidae and its sister group. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 32.5].

(28) *tMRCA of the clade* (Clarias, Chaca): The first fossil Clariidae is excavated from Oligeocene strata (Gayet and Otero 1999). It provided a minimum age of 40.4 Mya for the divergence between *Clarias* and *Chaca*. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 40.2].

(29) *tMRCA of the clade* (Amieurus, Kryptopterus): Based on its fossil record, the minimum age of the Ictaluridae is Early Paleocene (63.0 Mya; Gayet and Otero 1999). Therefore, it provides a minimum age for the divergence between the ictalurid *Amieurus* and *Kryptopterus*. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 32.5].

(30) *tMRCA of the stem group* (*Alestidae, Hepsetidae*): The sister group of Alestidae/Hepsetidae is *Hoplias*, which as a fossil record that extends back to the Late Cretaceous (Gayet et al. 2003). Therefore, it provides a minimum age for the stem group (Alestidae, Hepsetidae) to 65.5 Mya. In the exponential distribution reconstruction, we bracketed the soft maximum age to 140.2 Mya [exponential distribution mean = 31.7].

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Taxa included in this study and accession numbers of sequences in GenBank.

Table S2. PCR/Sequencing primer information.

Figures S1–S5. Best scoring maximum-likelihood tree of Teleostei obtained from the partitioned RAxML analysis of the five nuclear gene dataset from the matrix "123_{ry}" partitioned in 5 (Fig. S2) or 3 (Fig. S4), and the matrix "123" partitioned in 15 (Fig. S1), 5 (Fig. S3), and 3 (Fig. S5).

Figure S6. Phylogenetic chronogram of the Teleostei based on a Bayesian relaxed clock approach and calibrated with 30 fossil-based constraints following uniform distributions.

Figure S7. Reconstruction of the evolution of the ecological character within the Ostariophysi using likelihood optimization on the Bayesian topology (Fig. 3). "Freshwater" is indicated in white and "marine" in black. At each node, the relative probabilities of each state (sum = 1) are drawn using pie charts.