BMC
Evolutionary Biology

Evolutionary history of Otophysi (Teleostei), a major clade of the modern freshwater fishes: Pangaean origin and Mesozoic radiation

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Abstract

Background: Freshwater harbors approximately 12,000 fish species accounting for 43% of the diversity of all modern fish. A single ancestral lineage evolved into about two-thirds of this enormous biodiversity (≈ 7900 spp.) and is currently distributed throughout the world's continents except Antarctica. Despite such remarkable species diversity and ubiquity, the evolutionary history of this major freshwater fish clade, Otophysi, remains largely unexplored. To gain insight into the history of otophysan diversification, we constructed a timetree based on whole mitogenome sequences across 110 species representing 55 of the 64 families.

Results: Partitioned maximum likelihood analysis based on unambiguously aligned sequences (9923 bp) confidently recovered the monophyly of Otophysi and the two constituent subgroups (Cypriniformes and Characiphysi). The latter clade comprised three orders (Gymnotiformes, Characiformes, Siluriformes), and Gymnotiformes was sister to the latter two groups. One of the two suborders in Characiformes (Characoidei) was more closely related to Siluriformes than to its own suborder (Citharinoidei), rendering the characiforms paraphyletic. Although this novel relationship did not receive strong statistical support, it was supported by analyzing independent nuclear markers. A relaxed molecular clock Bayesian analysis of the divergence times and reconstruction of ancestral habitats on the timetree suggest a Pangaean origin and Mesozoic radiation of otophysans.

Conclusions: The present timetree demonstrates that survival of the ancestral lineages through the two consecutive mass extinctions on Pangaea, and subsequent radiations during the Jurassic through early Cretaceous shaped the modern familial diversity of otophysans. This evolutionary scenario is consistent with recent arguments based on biogeographic inferences and molecular divergence time estimates. No fossil otophysan, however, has been recorded before the Albian, the early Cretaceous 100-112 Ma, creating an over 100 million year time span without fossil evidence. This formidable ghost range partially reflects a genuine difference between the estimated ages of stem group origin (molecular divergence time) and crown group morphological diversification (fossil divergence time); the ghost range, however, would be filled with discoveries of older fossils that can be used as more reasonable time constraints as well as with developments of more realistic models that capture the rates of molecular sequences accurately.

Background

Although freshwater lakes and rivers occupy a small portion of the Earth's surface (0.8%) and hold a negligible amount of the total water on Earth (0.01%), these ecosystems support an extraordinarily high proportion of the world's biodiversity, consisting of at least 100,000 species or nearly 6% of all described species [1]. While this enormous biodiversity has been described through continued efforts by taxonomists, its origin and the history of the diversification on a global scale remain largely unexplored across diverse taxa. This is even true for well-studied taxa such as fishes, which account for the largest proportion of vertebrate diversity and is the taxon that controls the trophic structure of freshwater ecosystems [2].

Freshwater fishes are disproportionately species-rich. Of the currently recognized 27,977 fish species, 11,952 species (42.7%) occur exclusively in freshwater [3]

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(Figure 1). These freshwater fishes are polyphyletic, being found in 30 of the 40 orders from the bottom to the top of the ray-finned fish phylogenies [3]. A single ancestral lineage, however, diversified into approximately two-thirds of this enormous diversity (7943 spp.), and it is distributed throughout the world's continents except Antarctica [4]. This clade, Series Otophysi, comprises four primarily freshwater orders (Figure 1): Cypriniformes (minnows, carps, loaches, suckers), Characiformes (tetras, piranhas), Siluriformes (catfishes), and Gymnotiformes (electric eels). These otophysan fishes share modifications of the inner ear, gas bladder, and of the four or five anterior vertebrae and associated elements together called the Weberian apparatus [3]. In a broader phylogenetic context, otophysan fishes represent one of the clades in Subdivision Otocephala [5] along with the orders Gonorynchiformes (= Anotophysi) [6] and Alepocephaliformes [5,7]. Otocephala itself represents a sister clade of Subdivision Euteleostei [8] comprising numerous marine species, including those of Series Percomorpha [3,9,10].

Although recent molecular phylogenetic studies have increased our understanding of the interfamilial relationships within each of the four otophysan orders remarkably (Cypriniformes [11-18]; Characiformes [19,20]; Siluriformes [21,22]; Gymnotiformes [23,24]), few studies have specifically addressed the interordinal relationships of the otophysans. Following the advent of cladistic methods [25,26], Fink and Fink [27] proposed the first explicit hypothesis of the otophysan phylogenies (Figure 2A) based on examinations of 127 morphological characters. Subsequently Dimmick and Larson [28] corroborated this hypothesis based on the combined morphological and molecular data (nuclear and mitochondrial rDNA sequences), although the molecular data alone provided a different hypothesis (Figure 2B). The latter hypothesis is congruent with that reported by Saitoh et al. [4] who performed a maximum likelihood (ML) analysis using whole mitogenome sequences from major otophysan lineages. Monophyly of Characiformes, however, was not recovered in the two publications of Ortí and Meyer, who analyzed characiphysan phylogenies using nuclear ependymin [29] (Figure 2C) and mitochondrial rDNA sequences [19] (Figure 2D), which is also true for Peng et al. [30] and the results of maximum parsimony analysis reported by Saitoh et al. [4] but in a different manner (Figure 2E). Notably, more recent molecular phylogenetic studies based on both
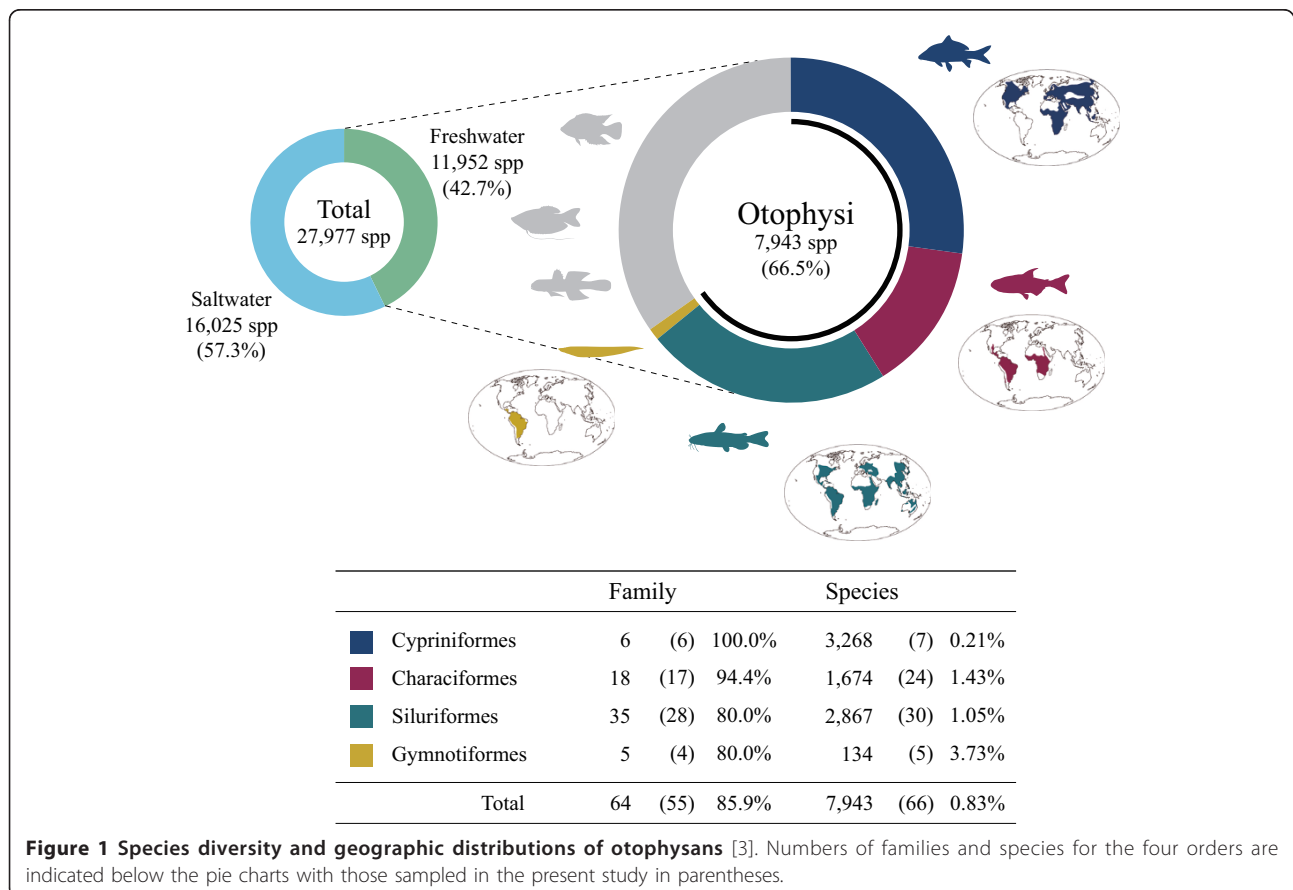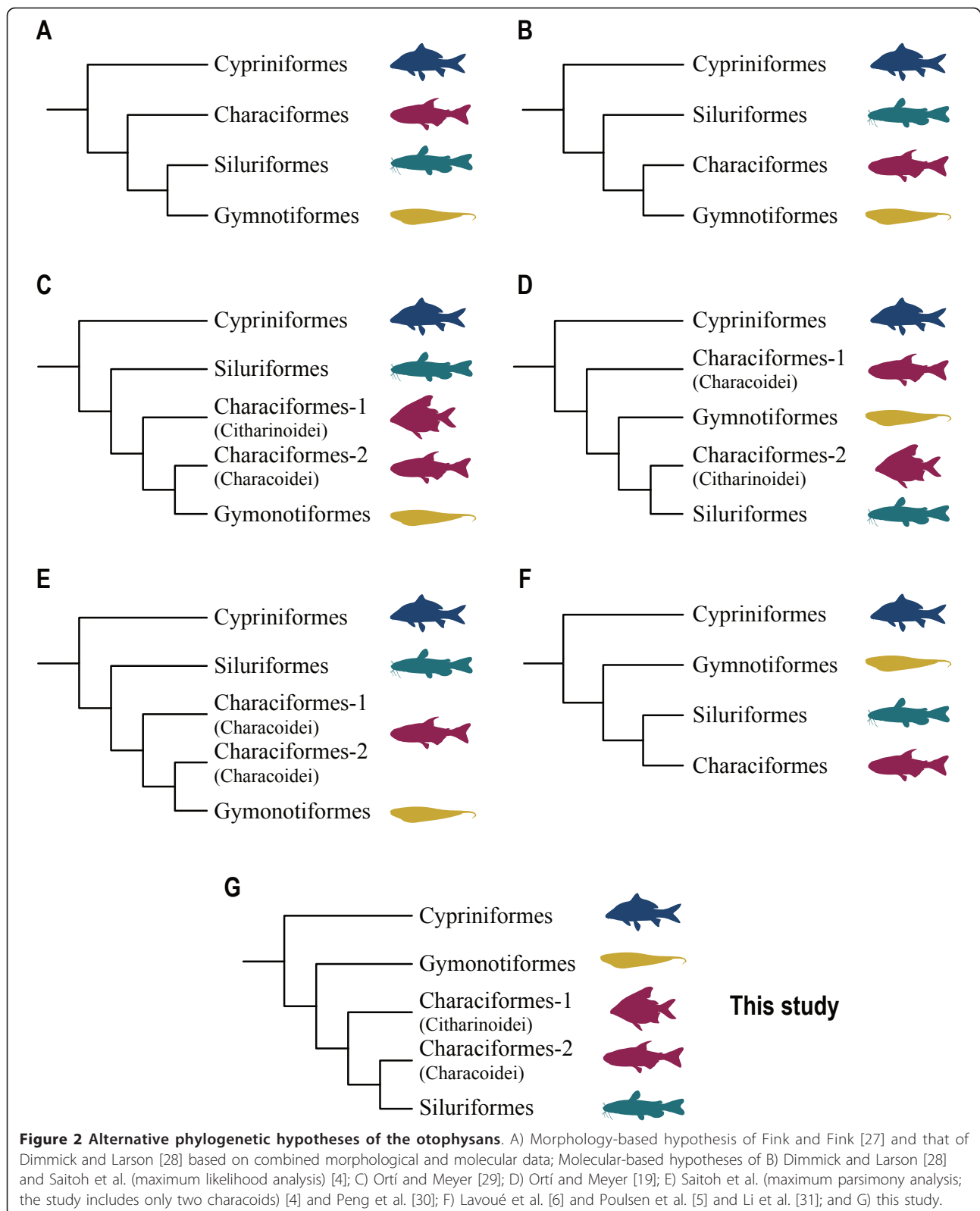

	Family			Species		
Cypriniformes	6	(6)	100.0%	3,268	(7)	0.21%
Characiformes	18	(17)	94.4%	1,674	(24)	1.43%
Siluriformes	35	(28)	80.0%	2,867	(30)	1.05%
Gymnotiformes	5	(4)	80.0%	134	(5)	3.73%
Total	64	(55)	85.9%	7,943	(66)	0.83%

Figure 1 Species diversity and geographic distributions of otophysans [3]. Numbers of families and species for the four orders are indicated below the pie charts with those sampled in the present study in parentheses.

Figure 2 Alternative phylogenetic hypotheses of the otophysans. A) Morphology-based hypothesis of Fink and Fink [27] and that of Dimmick and Larson [28] based on combined morphological and molecular data; Molecular-based hypotheses of B) Dimmick and Larson [28] and Saitoh et al. (maximum likelihood analysis) [4]; C) Ortí and Meyer [29]; D) Ortí and Meyer [19]; E) Saitoh et al. (maximum parsimony analysis; the study includes only two characoids) [4] and Peng et al. [30]; F) Lavoué et al. [6] and Poulsen et al. [5] and Li et al. [31]; and G) this study.

whole mitogenomes [5,6] and nuclear genes [31] have converged to an additional hypothesis (Figure 2F; but see [24]), although these studies did not specifically address the resolution of otophysan phylogenies. Thus, no consensus exists regarding the relationships among major otophysan lineages with the exception of the most basal position of Cypriniformes. Nevertheless, these molecular studies lacked sufficient taxonomic and/ or character sampling (Table 1) to resolve higher-level relationships among otophysan lineages, which exhibit enormous taxonomic diversity (Figure 1). Actually none of the previous studies sampled longer nucleotide sequences (e.g., >5000 bp) from more than three species across all of the four orders (Table 1).

In Otophysi, a great deal of attention has been paid to the biogeographic history that has shaped the current distribution patterns. Indeed, contrasting patterns in the geographic distributions of modern otophysans (Gondwanan vs. Laurasian vs. Pangaean distributions; Figure 1), the general acceptance of plate tectonics and continental drift as explanatory factors for dispersal across land connections and/or as a causal mechanism of vicariant speciation, and the presumed designation of the ostariophysans (Otophysi + Gonorynchiformes) as "primary freshwater fishes" (with dispersal across oceans being unlikely) together led many authors to propose evolutionary scenarios that attempt to identify a center of origin and dispersal routes through land connections based on various assumptions [4,12,27,32-37]. Reconstruction of the history of otophysan diversification (e.g., the history of the modern familial diversification), however, remains unchallenged, apparently because of poor representation in the fossil record before the Cenozoic period [24,38-41], a huge extant taxonomic diversity encompassing over 7943 species placed in 64

families and 1068 genera [3], and the absence of an adequate timescale for the phylogenies across major lineages (but see [30]). Indeed, in a review of the early radiation of teleosts, Arratia [42] stated "… the enormous radiation of some modern groups such as otophysans, atherinomorphs, perciforms, etc. is missing a historical framework."

To provide an overview of the history of modern otophysan diversification within the broad context of the evolutionary history of ray-finned fishes (Actinopterygii), we assembled whole mitochondrial genome (mitogenome) sequences from 66 otophysans (including 51 newly determined sequences), representing 55 of the 64 currently recognized families (86%; Figure 1). The 66 sequences were concatenated with those from 44 outgroup species for a total of 110 species and unambiguously aligned sequences (9923 bp excluding quickly saturated third codon positions) were subjected to phylogenetic analysis and a relaxed-clock Bayesian divergence time estimation. The resultant timetree suggests that the modern otophysan diversity has been shaped through the two consecutive mass extinction events on the Pangaea supercontinent and subsequent radiations during the Jurassic through the early Cretaceous.

Methods
Taxonomic sampling
In addition to the 54 fish species used in Azuma et al. [43], 66 otophysans were newly added in this study, making a total of 110 species analyzed (Table 2). The 54 outgroup species encompass the whole actinopterygians (ray-finned fishes) from the bottom to the top of the tree and the 66 otophysans include 55 of the 64 currently recognized families (Figure 1).

Table 1 A summary of character and taxon sampling in the previous molecular phylogenetic studies that include the four otophysan orders

Study	Figure 2	Marker[a]	Length (bp)	Cyp	Gym	Cha		Sil	Total
						Cith	Char		
Dimmick & Larson [28]	B	nc + mt rDNA	2477	2	2	1	1	3	9
Ortí & Meyer [29]	C	nc ependymin	588	3	2	1	12	4	22
Ortí & Meyer [19]	D	mt rDNA	870	2	3	2	11	4	22
Saitoh et al. [4]	B,E	mt genome	8096	7	2	0	2	2	13
Lavoué et al. [6]	F	mt genome	10395	7	2	0	2	2	13
Peng et al. [30]	E	mt genome	6198	8	2	0	2	5	17
Li et al. [31]	F	10 nc genes	7995	3	1	0	1	1	6
Poulsen et al. [5]	F	mt genome	11076	14	2	0	2	5	23
Alves-Gomes [24]	A	mt rDNA	701	15	15	2	13	15	60
This study	G	mt genome	9923	7	5	3	21	30	66
This study	G	10 nc genes	7995	3	2	1	2	2	10

Abbreviations for orders and suborders: Cyp = Cypriniformes; Gym = Gymnotiformes; Cha = Characiformes; Sil = Siluriformes; Cith = Citharinoidei; Char = Characoidei

[a] nc = nuclear; mt = mitochondrial.

Table 2 List of the species used in this study

Order/suborder/superfamily[a]	Family	Species[b]	Accession No.[c]
Outgroup			
Coelacanthiformes	Latimeriidae	*Latimeria menadoensis*	AP006858
Ceratodontiformes	Ceratodontidae	*Neoceratodus forsteri*	AJ584642
Polypteriformes	Polypteridae	*Polypterus ornatipinnis*	AP004351
		Polypterus senegalus	AP004352
		Erpetoichthys calabaricus	AP004350
Acipenseriformes	Acipenseridae	*Acipenser transmontanus*	AB042837
		Scaphirhynchus cf. *albus*	AP004354
	Polyodontidae	*Polyodon spathula*	AP004353
Lepisosteiformes	Lepisosteidae	*Lepisosteus oculatus*	AB042861
		Atractosteus spatula	AP004355
Amiiformes	Amiidae	*Amia calva*	AB042952
Hiodontiformes	Hiodontidae	*Hiodon alosoides*	AP004356
Osteoglossiformes	Osteoglossidae	*Osteoglossum bicirrhosum*	AB043025
		Pantodon buchholzi	AB043068
Albuliformes	Notacanthidae	*Notacanthus chemnitzi*	AP002975
Anguilliformes	Anguillidae	*Anguilla japonica*	AB038556
	Muraenidae	*Gymnothorax kidako*	AP002976
	Congridae	*Conger myriaster*	AB038381
Clupeiformes	Denticipitidae	*Denticeps clupeoides*	AP007276
	Engraulidae	*Engraulis japonicus*	AB040676
	Clupeidae	*Sardinops melanostictus*	AB032554
Gonorynchiformes	Chanidae	*Chanos chaos*	AB054133
	Gonorynchidae	*Gonorynchus greyi*	AB054134
	Kneriidae	*Kneria* sp.	AF007278
	Phractolaemidae	*Phractolaemus ansorgii*	AB070243
Alepocephaliformes	Platytroctidae	*Platytroctes apus*	AP004107
	Bathylaconidae	*Herwigia kreffti*	AP009582
	Alepocephalidae	*Alepocephalus tenebrosus*	AP004100
Salmoniformes	Salmonidae	*Salmo salar*	U12143
Esociformes	Esocidae	*Esox lucius*	AP004103
Polymixiiformes	Polymixiidae	*Polymixia japonica*	AB034826
Gadiformes	Gadidae	*Gadus morhua*	X99772
Perciformes	Cichlidae	*Oreochromis* sp.	AP009126
		Neolamprologus brichardi	AP006014
		Tropheus duboisi	AP006015
		Astronotus ocellatus	AP009127
		Paretroplus maculatus	AP009504
		Etroplus maculatus	AP009505
		Hypselecara temporalis	AP009506
		Ptychochromoides katria	AP009507
		Paratilapia polleni	AP009508
		Tylochromis polylepis	AP009509
Tetraodontiformes	Tetraodontidae	*Takifugu rubripes*	AJ421455
		Tetraodon nigroviridis	AP006046
Ingroup			
Cypriniformes			
Cyprinoidea	Cyprinidae	*Cyprinus carpio*	X61010
		Danio rerio	AC024175
	Psilorhynchidae	*Psilorhynchus homaloptera*	DQ026436
Cobitoidea	Gyrinocheilidae	*Gyrinocheilus aymonieri*	AB242164

Table 2 List of the species used in this study *(Continued)*

	Catostomidae	*Catostomus commersonii*	AB127394
	Cobitidae	*Cobitis striata*	AB054125
	Balitoridae	*Formosania lacustre*	M91245
Characiformes			
Citharinoidei	Distichodontidae	*Distichodus sexfasciatus*	AB070242**
		Ichthyborus sp.	AP011993**
	Citharinidae	*Citharinus congicus**	AP011985**
Characoidei	Parodontidae	*Parodon affinis*	AP011998**
	Curimatidae	*Curimatopsis evelynae*	AP011988**
	Anostomidae	*Leporinus affinis*	AP011994**
	Chilodontidae	*Chilodus punctatus*	AP011984**
	Crenuchidae	*Crenuchus spilurus*	AP011986**
	Hemiodontidae	*Hemiodopsis gracilis*	AP011990**
	Alestidae	*Micralestes* sp.	AP011996**
		Phenacogrammus interruptus	AB054129
	Gasteropelecidae	*Carnegiella strigata*	AP011983**
	Characidae	*Astyanax mexicanus*	AP011982**
		Chalceus macrolepidotus	AB054130
		Myleus sp.	AP011997**
		Paracheirodon innesi	AP011999**
		Pygocentrus nattereri	AP012000**
	Acestrorhynchidae	*Acestrorhynchus* sp.	AP011981**
	Cynodontidae	*Hydrolycus scomberoides*	AP011989**
	Erythrinidae	*Hoplias malabaricus*	AP011992**
	Lebiasinidae	*Lebiasina astrigata*	AP011995**
	Ctenoluciidae	*Boulengerella maculata*	AB070207
		*Ctenolucius hujeta**	AP011987**
	Hepsetidae	*Hepsetus odoe*	AP011991**
Siluriformes			
Diplomystoidea	Diplomystidae	*Diplomystes nahuelbutaensis*	AP012011**
Cetopsoidea	Cetopsidae	*Cetopsidium* sp.	AP012007**
		*Helogenes marmoratus**	AP012014**
Loricaroidea	Amphiliidae	*Amphilius* sp.	AP012002**
	Tricomycteridae	*Trichomycterus areolatus*	AP012026**
	Callichthyidae	*Corydoras rabauti*	AB054128
	Astroblepidae	*Astroblepus* sp.	AP012004**
	Loricariidae	*Pterygoplichthys disjunctivus*	AP012021**
Sisoroidea	Amblycipitidae	*Liobagrus reinii*	AP012015**
	Erethistidae	*Hara jerdoni*	AP012012**
	Aspredinidae	*Bunocephalus coracoideus*	AP012006**
Cranoglanoidea	Cranoglanididae	*Cranoglanis bouderius*	AY898626
Ictaluroidea	Ictaluridae	*Ictalurus punctatus*	AF482987
Doradoidea	Mochokidae	*Synodontis schoutedeni*	AP012023**
	Doradidae	*Amblydoras gonzalezi*	AP012001**
	Auchenipteridae	*Tatia pergiae*	AP012024**
		Tetranematichthys quadrifilis	AP012025**
Siluroidea	Siluridae	*Silurus asotus*	AP012022**
	Malapteruridae	*Malapterurus electricus*	AP012016**
	Auchenoglanididae	*Auchenoglanis occidentalis*	AP012005**
	Chacidae	*Chaca bankanensis*	AP012008**
	Plotosidae	*Plotosus japonicus*	AP012020**
	Clariidae	*Clarias* sp.	AP012010**

Table 2 List of the species used in this study *(Continued)*

	Heteropneustidae	*Heteropneustes fossilis*	AP012013**
Bagroidea	Claroteidae	*Chrysichthys* sp.	AP012009**
	Ariidae	*Sciades seemanni*	AP012003**
	Schilbeidae	*Pareutropius debauwi*	AP012017**
	Pangasiidae	*Pangasius larnaudii*	AP012018**
	Bagridae	*Pseudobagrus tokiensis*	AB054132
	Pimelodidae	*Pimelodus pictus*	AP012019**
Gymnotiformes			
Gymnotoidei	Gymnotidae	*Electrophorus electricus*	AP011978**
		*Gymnotus carapo**	AP011979**
Sternopygoidei	Rhamphichthyidae	*Gymnorhamphichthys* sp.	AP011980**
	Sternopygidae	*Eigenmannia* sp.	AB054131
	Apteronotidae	*Apteronotus albifrons*	AB054132

[a] Classifications follow [3] except for the recognition of Alepocephaliformes [5,7].
[b] Those species with single asterisks were also used for nuclear DNA sequence analysis.
[c] Those sequences with double asterisks were newly determined during this study.

Specimens and DNA extraction

A portion of epaxial musculature or pectoral fins (~0.25 g) from fresh specimens of each species was excised and the tissue was immediately preserved in 99.5% ethanol. Total genomic DNA from the ethanol-preserved tissue was extracted using DNeasy (Qiagen) and Aquapure genomic DNA isolation kit (Bio-Rad Laboratories, Inc.) in accordance with the respective manufacturer's protocols, or the standard phenol-chloroform method as described in Asahida et al. [44].

PCR and sequencing

Whole mitogenome sequences of the 51 otophysans (double asterisks in Table 2) were determined using a combination of long and short PCR methods developed by Miya and Nishida [45]. Briefly, the mitogenomes of the 51 otophysans in their entirety were amplified using a long PCR technique [46] in two or three reactions. Dilution of the long PCR products with TE buffer (1:10 to 100 depending on the concentration of the long PCR products) served as templates for subsequent short PCRs. Standard sets of fish-versatile primers (and species-specific primers if necessary) were used for short PCRs to amplify contiguous overlapping segments of the entire mitogenome for each otophysan species. The short PCR products were purified using the Exosap-IT enzyme (GE Healthcare Bio-Sciences Corp.) and subsequently sequenced with dye-labeled terminators (BigDye terminator ver. 1.1/3.1; Applied Biosystems) and the primers used in the short PCRs. Sequencing reactions were conducted according to the manufacturer's instructions, followed by electrophoresis on an ABI Prism 377, 3100, or 3130 DNA sequencer (Applied Biosystems). A list of PCR primers used in this study is available from MNa upon request.

In some cases when multiple bands were amplified during short PCRs, we conducted subcloning using MinElute (Qiagen), pGEM-T Easy Vector Systems (Promega), and Z-competent *E. coli* (ZYMO Research), in accordance with the manufactures' protocols. To avoid PCR errors, we sequenced eight clones for each fragment using SP6 and T7 primers.

Sequence editing and alignment

Mitogenome sequences from the 66 otophysans were concatenated with the pre-aligned sequences used in Azuma et al. [43] in FASTA format and subjected to multiple alignment using MAFFT ver. 6.707 [47]. The aligned sequences were imported into MacClade ver. 4.08 [48] and the resulting gaps in the aligned sequences were manually removed to correctly reproduce the alignment used by Azuma et al. [43]. All the resulting positions with gaps were removed, so the final data set consisted of 6904 positions from the first and second codon positions of the 12 protein-coding genes (excluding the ND6 gene because of its heterogeneous base composition and poor phylogenetic performance [49]), 1622 positions from the two rRNA genes, and 1397 positions from the 22 tRNA genes (total 9923 positions) (designated as 12_nRT_n: where 1, 2, R and T represent 1st codon position, 2nd codon position, rRNA gene and tRNA gene, respectively, and the subscript "n" denotes nucleotides). The third codon positions of the protein-coding genes were excluded from the data set because of the extremely high substitution rates (and the resulting multiple hits) and heterogeneous base composition as sources of systematic noise in phylogenetic analysis at this taxonomic level [49,50] and overestimation of divergence time [51,52]. The aligned sequences are available from

TreeBase with the following URL (http://purl.org/phylo/treebase/phylows/study/TB2:S11469).

To investigate the relationships within the otophysans, we also created an additional four data sets that treated 12 protein-coding genes differently. The first three data sets considered only transversional changes in the first and/or third codon positions by converting purine (A/G) and pyrimidine (C/T) nucleotides to A and C, respectively ($1_r2_nR_nT_n$, $12_n3_rRT_n$, $1_r2_n3_rRT_n$: where the subscript "r" denotes a modified RY-coding following Saitoh et al. [11]). The transitional changes in the first codon positions are somewhat saturated among distantly related taxa [49], and the first data set ($1_r2_nRT_n$) was expected to reduce phylogenetic noise from the original data set (12_nRT_n). The second data set ($12_n3_rRT_n$) added the RY-coded third codon positions to the original data set, which was expected to increase phylogenetic signals and was predominantly used to resolve interrelationships within the Cypriniformes [11,12], one of the major otophysan clades (Figure 1). The third data set ($1_r2_n3_rRT_n$) removed transitional changes in the first codon positions from the second data set. The last data set converted protein-coding genes into amino acids (designated as 123_aRT_n) to explore the utility of these sequences in resolving otophysan taxa. Only 66 otophysans plus 12 outgroup species (4 gonorynchiforms + 3 clupeiforms + 3 alepocephaliforms + 2 anguillifoms) were used in these additional data sets to minimize the computation time.

Phylogenetic analysis

Unambiguously aligned sequences were divided into three to five partitions depending on the data sets (three partitions in the 123_aRT_n data set, four partitions in the 12_nRT_n and $1_r2_nRT_n$ data sets, and five partitions in the $12_n3_rRT_n$ and $1_r2_n3_rRT_n$ data sets) and subjected to ML analysis. We used RAxML ver. 7.2.8 [53] because it is the only ML-based software that can handle large data sets with data partitioning. A general time reversible model (GTR) [54] with sites following a discrete gamma distribution (Γ) and some sites invariable (I) was selected as the best model of nucleotide sequence evolution by Modeltest ver. 3.7 [55] using the Akaike information criterion (AIC). For amino acid sequences, the MTREV model [56] with sites following a discrete gamma distribution (Γ) and some sites invariable (I) was used. We performed a rapid bootstrap (BS) analysis using this model (GTR + Γ + I) with 1000 replications (-f a option). This performs BS analysis using GTRCAT, which is a GTR approximation with optimization of individual per-site substitution rates and classification of these individual rates into a certain number of rate categories. After implementing the BS analysis, the program uses every fifth BS tree as a starting point for another

ML search using the GTR + Γ + I model of sequence evolution and saves the top 10 best-scoring ML trees (fast ML searches). Finally, RAxML calculates more correct likelihood scores (slow ML searches) for those 10 trees and puts BS probabilities (BSPs) on the best-scoring ML tree.

Evaluation of alternative hypotheses

We manually created the constrained tree topologies with reference to the alternative hypotheses using MacClade and then performed RAxML analysis with each constraint using the -g option. We conducted fast bootstrapping with 100 replicates as described above, and the resulting best-scoring ML tree was considered as the constrained ML tree. The constrained and unconstrained ML trees (best-scoring ML tree without constraint) were used to compute the per-site log likelihood scores for each tree using the -f g option in RAxML and the output was subjected to CONSEL [57] analysis to calculate statistical significance of the differences in likelihood scores. Probabilities of alternative phylogenetic hypotheses were calculated using the likelihood-based approximately unbiased (AU) test [58] as implemented in CONSEL v.0.1k [57]. The *P*-values from this test are calculated using the multi-scale bootstrap technique and are less biased than those of conventional methods [57] such as the BS probability (BSP) [59], the Kishino-Hasegawa (KH) test [60] and the Shimodaira-Hasegawa (SH) test [61].

Supplementary analysis using nuclear genes

To corroborate the novel relationships among major otophysan lineages obtained in this study (see below), we determined some of the putative single-copy nuclear gene sequences for the selected four otophysans (asterisks in Table 2) according to the methods described by Li et al. [31] (Table 3). We have added these sequences to the aligned data set used in Li et al. (available from http://www.treebase.org/treebase-web/home.html; newID: M3165) who studied actinopterygian phylogenies with 56 species (including three cypriniforms and three characiphysans), and the data set was subjected to partitioned ML analysis according to Kawahara et al. [62].

Divergence time estimation

A relaxed molecular clock Bayesian method implemented in the MCMCTREE program in PAML 4.4b [63] was used for dating analysis. We also attempted to use BEAST [64] for our data set, but MCMC samples failed to converge after 10^8 chains. The best-scoring ML tree from the 12_nRT_n data set was used for divergence time estimation. The ML estimates of branch lengths were obtained using BASEML and CODEML (in PAML) programs under the GTR + Γ_5 and MTREVF + Γ_5

Table 3 DNA accession numbers of the nuclear genes from the four characiphysans

Gene	*Citharinus congicus*	*Ctenolucius hujeta*	*Helogenes marmoratus*	*Gymnotus carapo*
zic1	AB605470	AB605476	———————	AB605488
myh6	———————	———————	———————	———————
PYR3	———————	———————	———————	———————
ptr	AB605467	AB605473	———————	AB605484
Tbr	AB605469	AB605471	AB605480	AB605487
ENC1	AB605465	AB605474	AB605477	AB605481
Glyt	———————	———————	———————	AB605482
SH3PX3	———————	AB605474	———————	AB605485
plagl2	AB605466	AB605472	AB605478	AB605483
sreb2	AB605468	AB605475	AB605479	AB605486

substitution models [54] for the 12_nRT_n and 123_a data sets, respectively, with the gamma prior set at 0.5. Two priors, the overall substitution rate (rgene gamma) and rate-drift parameter (sigma2 gamma), were set at G (1, 12.3) and G (1, 4.45) for the 12_nRT_n data set and G (1, 14.3) and G (1, 4.5) for the 123_a data set, respectively, using the strict molecular clock assumption with 445 Ma constraint to the divergence between Actinopterygii and Sarcopterygii (average of the upper and

lower constraints for the node between ray-finned and lobe-finned fish; see Table 4). The independent-rates (IR) model [65] was used to specify the prior of rates among internal nodes (clock = 2 in MCMCTREE). The IR model has been considered more appropriate in divergence time estimation than the autocorrelated-rates (AR) model in recent studies (see [66] and references therein), although additional analyses using AR model (clock = 3 in MCMCTREE) were also performed for

Table 4 Time constraints used for divergence time estimation

Node	Constraints	Calibration information
A	U 472	The minimum age for the basal split of bony fish based on the earliest known acanthodian remains from Late Ordovician [128]
	L 419	The †*Psarolepis* fossil (sarcopterygians) [129] from Ludlow (Silurian) [100]
B	U 419	The minimum age for the Sarcopterygii/Actinopterygii split
	L 392	The †*Moythomasia* fossil (actinopteran) from the Givetian/Eifelian boundary [100]
C	U 392	The minimum age for the Polypteriformes/Actinopteri split
	L 345	The †*Cosmoptychius* fossil (neopterygian or actinopteran) from Tournasian [100]
D	L 130	The †*Protopsephurus* fossil (Polyodontidae) from Hauterivian (Cretaceous) [100]
E	L 284	The †*Brachydegma* fossil (stem amiids) from Artinskian (Permian) [100]
F	L 136	The †*Yanbiania* fossil (Hiodontidae) from the Lower Cretaceous [100]
G	L 112	The †*Laeliichthys* fossil (Osteoglossidae) from the Aptian (Cretaceous) [130]
H	L 151	The †*Anaethalion*, †*Elopsomolos*, and †*Eoprotelops* fossil (Elopomorpha) from Kimmeridgian (Jurassic) [100]
I	L 94	The †*Lebonichthys* (Albulidae) fossil from the Cenomanian (Cretaceous) [130]
J	L 49	The *Conger* (Congridae) and *Anguilla* (Anguillidae) fossils from the Ypresian (Tertiary) [130]
K	L 146	The †*Tischlingerichthys* fossil (Ostariophysi) from Tithonian (Jurassic) [100]
L	L 56	The †*Knightia* fossil (Clupeidae) from the Thanetian (Paleogene) [130]
M	L 49	The †*Parabarbus* fossil (Cyprinidae) from the Ypresian (Paleogene) [130]
N	L 8	Gymnotiform fossil from the Late Miocene [131]
O	L 98	The *Santanichthys* (Characiformes) fossil from Albian (Cretaceous) [94]
P	L 74	The ariid fossil from Campanian (Cretaceous) [132]
Q	L 74	The †*Esteseox foxi* fossil (Esociformes) from the Campanian (Cretaceous) [133]
R	L 94	The †*Berycopsis* fossil (Polymixiidae) from the Cenomanian (Cretaceous) [130]
S	L 98	The tetraodontiform fossil from the Cenomanian [134]
T	L 32	The estimated divergence time between *Takifugu* and *Tetraodon* [135]
U	U 95 L 85	The upper and lower bounds of separation between Madagascar and Indian [104]
V	U 145 L 112	The upper and lower bounds of separation between Indo-Madagascar landmass and Gondwanaland [104]
W	U 120 L 100	The upper and lower bounds of separation between African and South American landmasses [104]

Maximum (U) and minimum (L) time constraints in million years ago (Ma) at nodes depicted in Additional file 1

comparison. The parameters of the birth-death process for tree generation with species sampling [67] were fixed at $\lambda = \mu = 1$ and $\rho = 0$, so that the priors are similar to those used in the previous mitogenomic studies [43,68] using MULTIDIVTIME [69]. A loose maximum bound for the root was set at <10.0 (= 1000 Ma).

The MCMCTREE program allows for minimum (lower) and maximum (upper) time constraints, and multiple calibration points have been argued to provide overall more realistic divergence time estimates [70]. Therefore we sought to obtain optimal phylogenetic coverage of calibration points across our tree, although we could set maximum constraints based on the fossil records only for the six nodes (Table 4). Other than these six nodes, 17 additional nodes were reasonably chosen to constraint their minimum ages only (total 29 time constraints for 23 nodes; Table 4). A hard and softbound version of the program (MCMCTREE-HS) was used, so that probabilities of the true divergence time falling outside the minimum bounds are zero, but small but not zero for the maximum bounds [71]. All time constraints are provided in units of 100 Ma (i.e., 1 = 100 Ma) because some of the model components in the Bayesian analysis are scale-variant [63]. The calibration nodes with minimal bound only were set as L ($t_{\min}$) and those with both minimal and maximal bounds were set as B ($t_{\min}$, $t_{\max}$). The former setting (L) assumes a heavy-tailed density (nearly a flat prior) based on a truncated Cauchy distribution of $p = 0.1$ and $c = 1$ as the default [63] ("standard minimum-age constraints" [72]). We did not manipulate the two shape parameters of the truncated Cauchy distribution because of insufficient information with which to specify meaningful prior distributions for most otophysan diversification times.

MCMC approximation with a burn-in period of 10,000 cycles was obtained, and every 50 cycles was taken to create a total of 10,000 samples. To diagnose possible failure of the Markov chains to converge to their stationary distribution, we performed two replicate MCMC runs with two different random seeds for each analysis. MCMC samples from the two runs were combined after checking the distributions of parameter values using Tracer 1.5 (available from http://tree.bio.ed.ac.uk/software/tracer/). The number of samples (20,000) was large enough to reach effective sample sizes (ESSs >200) for all parameters estimated in this study.

To evaluate the effects of topological uncertainties on divergence time estimation, we also conducted dating analyses with four of the 14 alternative topologies (four topologies with the second best P value, the two worst P values, plus a hypothesis advocated by Fink and Fink [27]).

Tracing character evolution

The ancestral habitat was reconstructed on the timetree under a ML optimality criterion using Mesquite ver. 2.71 [73]. The ML reconstruction methods found that the ancestral states maximizing the probability of the observed states would evolve under a stochastic model of evolution [74,75]. The Mk1 model ("Markov k-state 1 parameter model"), a k-state generalization of the Jukes-Cantor model that corresponds to Lewis' Mk model [76], was used to trace the character evolution. Two character states were assigned to the terminal node: saltwater (character state 0) and freshwater (state 1).

Results and Discussion
Genome organization

Complete L-strand nucleotide sequences from the mitogenomes of the 51 species newly determined during this study were deposited in the DNA Data Bank of Japan (DDBJ), European Molecular Biology Laboratory (EMBL), and GenBank (Table 2). The genome content of the 51 species included two rRNA, 22 tRNA, and 13 protein-coding genes, plus the putative control region, as found in other vertebrates. Their gene arrangements were identical to the typical gene order of vertebrates.

Interordinal/subordinal relationships

Partitioned ML analysis based on 110 whole mitogenome sequences ($12_n RT_n$ data set) resulted in a relatively well resolved tree, with approximately 70% of the internal branches supported by moderate to high (70-100%) BSPs (Figure 3). Otocephala was confidently recovered as a monophyletic group (BSP = 100%) and a clade containing two primarily marine orders (Clupeiformes and Alepocephaliformes) was sister to all other otocephalans. Gonorynchiformes was recovered as the sister group of Otophysi (BSP = 82%) and monophyly of the latter was confidently recovered (BSP = 100%; Figure 3), confirming the results of recent molecular studies [5,7,31]. Of the four orders within the otophysans, monophyletic Cypriniformes (BSP = 100%) was a sister to a clade containing the remaining three orders collectively called Characiphysi (BSP = 100%). Of the three characiphysan orders, Gymnotiformes and Siluriformes were confidently recovered as monophyletic groups (BSPs = 100%); however, one of the two suborders of Characiformes (Characoidei) was more closely related to Siluriformes (BSP = 79%) than to the other (Citharinoidei). Statistical support for a clade containing these three lineages (Citharinoidei, Characoidei, Siluriformes), however, was not strong (BSP = 67%).

Removal of transitional changes from the 1st codon positions ($1_r 2_n RT_n$ data set), addition of transversional changes from the 3rd codon positions ($12_n 3_r RT_n$ and $1_r 2_n 3_r RT_n$ data sets), or conversion of the
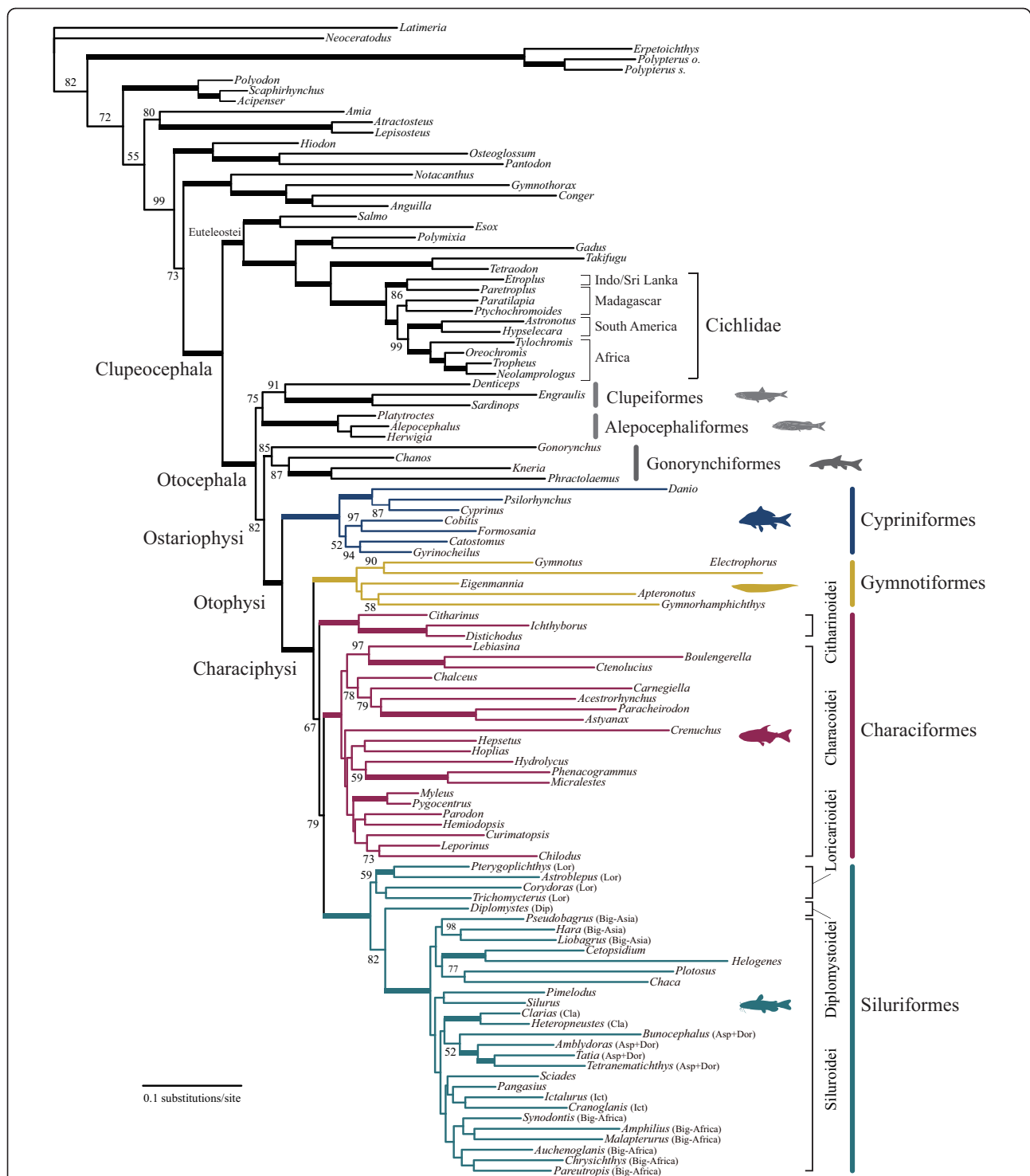
Figure 3 The best-scoring maximum-likelihood (ML) tree of the 66 otophysan and 44 outgroup species based on unambiguously aligned whole mitogenome sequences (12$_n$RT$_n$ data set; 9923 positions). Numerals beside internal branches indicate bootstrap probabilities (BSPs) of ≥50% based on 1000 replicates. Thick lines indicate those internal branches with 100% BSPs. Suprafamilial clades in Siluriformes reported by Sullivan et al. [21] are denoted as follows (abbreviations in parentheses): "Big Asia", "Claroidea" (Cla), "Aspredinidae + Doradoidea" (Asp + Dor), "Ictaluroidea" (Ict), and "Big Africa".

protein-coding genes into amino acid sequences (123_aRT_n) together supported the novel relationships, although they did not improve the statistical support (<50-67% BSPs for Citharinoidei + Characoidei + Siluriformes and 59-80% BSPs for Characoidei + Siluriformes) (Figure 4).

Although taxonomic sampling from otophysans in the nuclear data set was far sparser than that of the mitogenomic data set (10 spp. vs. 66 spp.), and data were missing in the original as well as newly added sequences (approximately 25% of the total positions; Table 3), partitioned ML analysis based on the 10 nuclear genes from the 60 species resulted in an identical tree topology to that of the mitogenomic tree regarding the relationships among the major otophysan lineages (Figure 5). Monophyly of Otophysi was confidently recovered at the apical position in Otocephala (BSP = 100%), with Cypriniformes being a sister to all other otophysans. Within the Characiphysi, Gymnotiformes was a sister to other characiphysans and monophyly of Characiformes plus Siluriformes was supported with 100% BSP (67% BSP in mitogenomic analysis; Figure 3), although the former was found to be paraphyletic, with Characoidei being more closely related to Siluriformes (BSP = 52%) than to Citharinoidei.

Paraphyletic characiforms have been repeatedly recovered in previous molecular studies (Figure 2C-E) but in different manners. For example, Ortí and Meyer [29] assembled nuclear ependymin sequences (588 bp) from 22 otophysans to explore the phylogenetic utility of this gene and found that characoids were more closely related to Gymnotiformes than to a citharinoid (*Distichodus*) (Figure 2C). To investigate the characiform radiation, Ortí and Meyer [19] used partial mitochondrial rDNA sequences (870 bp) as phylogenetic markers and observed that two citharinoids (*Distichodus* and *Citharinus*) were more closely related to Siluriformes than to characoids (Figure 2D). Although analyses of whole mitogenome sequences by Saitoh et al. [4] (maximum parsimony analysis of 8096 bp) and Peng et al. [30] (6198 bp) did not include citharinoids, one of the two characoids were more closely related to either Siluriformes or Gymnotiformes than to its own order (Figure 2E). These relationships, however, received weak statistical support (<50% BSPs) and alternative tree topologies could not be rejected [4,19,29].

Monophyly of Characiformes should be addressed in relation to the other two characiphysan orders. Accordingly, we evaluated the likelihoods of alternative hypotheses among four major characiphysan lineages (Gymnotiformes, Citharinoidei, Characoidei, Siluriformes) using the AU test (Table 5). Of the 15 possible tree topologies based on the mitogenomic data, AU tests confidently rejected only three that commonly

included the monophyly of Characoidei plus Gymnotiformes (P = 0.001-0.030; Table 5), with likelihoods of the other 12 topologies ranging from 0.051 to 0.702. Moreover, AU tests based on the nuclear data more confidently rejected 12 tree topologies (P = 0.000-0.006; Table 6). For either marker, however, AU tests could not reject all of those topologies that included monophyletic Characiformes, and this issue therefore remains unanswered even in the molecular phylogenetic context. Apparently the two deep-branching lineages in the characiforms (Citharinoidei and Characoidei) make it difficult to resolve their relationships with Siluriformes even with the large data sets used in this study. Also we expect that future uses of newly developed models accounting for site-specific modulations of the amino-acid replacement process, such as the CAT mixture model [77] implemented in PhyloBayes [78], may result in more clear resolution of the characiphysan phylogenies. Note that monophyletic Characiformes was well accepted and seemingly uncontroversial on a morphological basis [27,79-81].

Interfamilial relationships

Interfamilial relationships within each of the four major lineages are outside the scope of this study because taxonomic sampling is still sparse and requires at least several distantly related genera from the same family to reconstruct more reliable trees. Nevertheless, brief comparisons with previous studies are useful to direct future research.

In Cypriniformes, monophyly of one of the two superfamilies (Cyprinoidea) was consistently recovered, while another superfamily (Cobitoidea) has been variously recovered as mono- or paraphyletic depending on the data treatment (most notably that of the third codon position) even within some of the same studies [11-17]. An enigmatic *Psilorhynchus* (placed in its own family Psilorhynchidae) is nested within the cyprinid lineage (*Cyprinus* + *Danio*; Figure 3), rendering Cyprinidae paraphyletic as reported previously [15,17,18].

In Gymnotiformes, a sister group relationship between two gymnotids (*Electrophorus* and *Gymnotus*) was confidently recovered (BSP = 90%), but other portions of the tree were weakly supported (BSPs ≤58%; Figure 3) and do not warrant further comment. Such ambiguous interfamilial relationships have been reported in previous studies based on mitochondrial rDNA sequences [23,24].

In Citharinoidei, one of the two suborders of Characiformes, two distichodontid genera (*Distichodus* and *Ichthyborus*) formed a strongly supported monophyletic group (BSP = 100%), which was sister to a citharinid (*Citharinus*). These relationships have been consistently recovered in previous studies based on both morphological [79,82] and molecular [19,20,83] analyses. Within
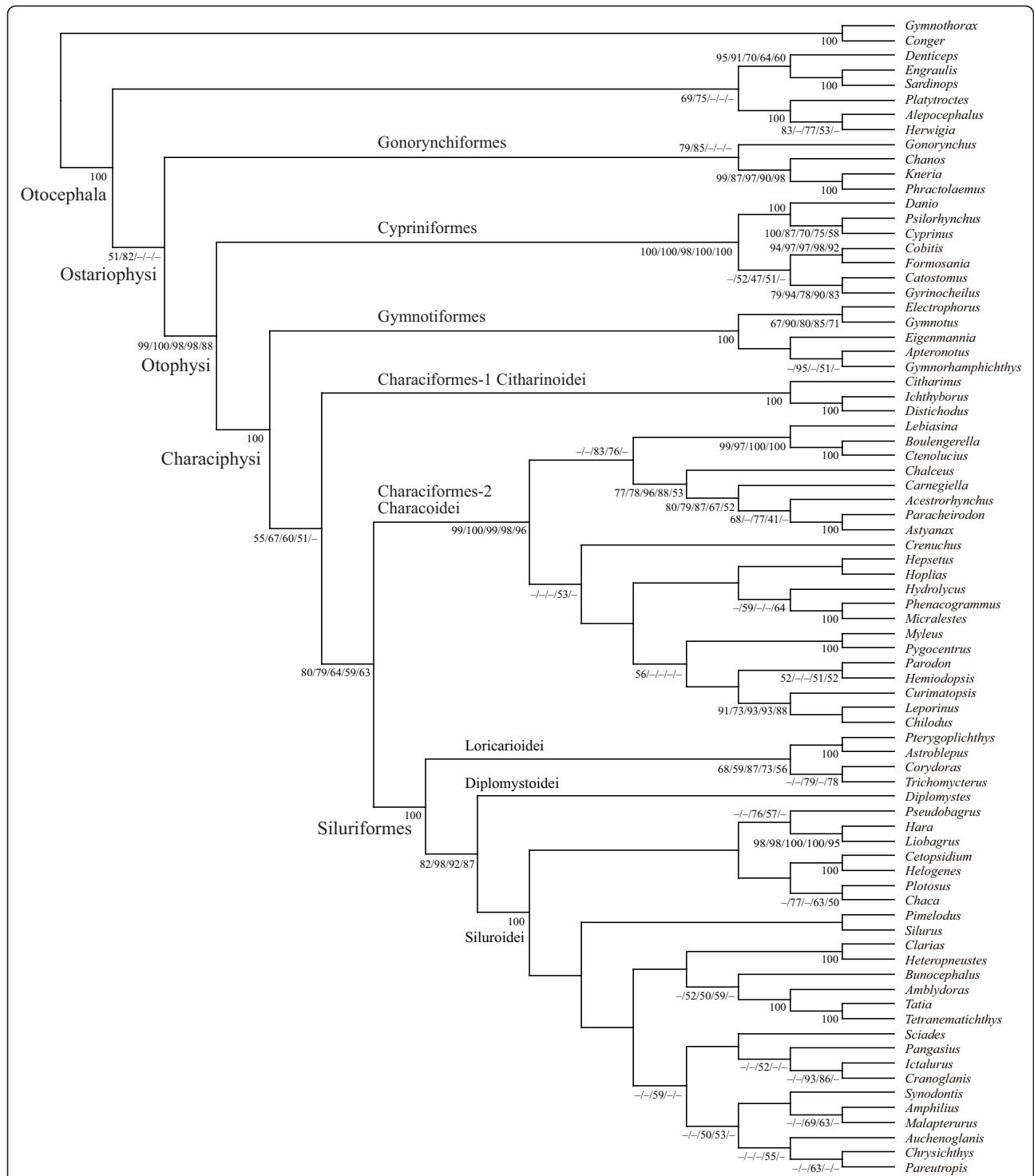
Figure 4 Bootstrap probabilities (BSPs) from RAxML analyses of the five different data sets that treated codon positions from the 12 protein-coding genes differently. The tree topology is derived from the RAxML analysis based on limited taxonomic sampling from the 12_nRT_n data set (all ingroup species + 12 outgroup species). Numerals beside internal branches indicate BSPs of ≥50% based on 1000 replicates (BSPs of $1_r2_nRT_n$/12_nRT_n/$1_r2_n3_rRT_n$/$12_n3_rRT_n$/123_aRT_n data sets from left to right). Single values denote the same BSPs obtained across the five data sets.
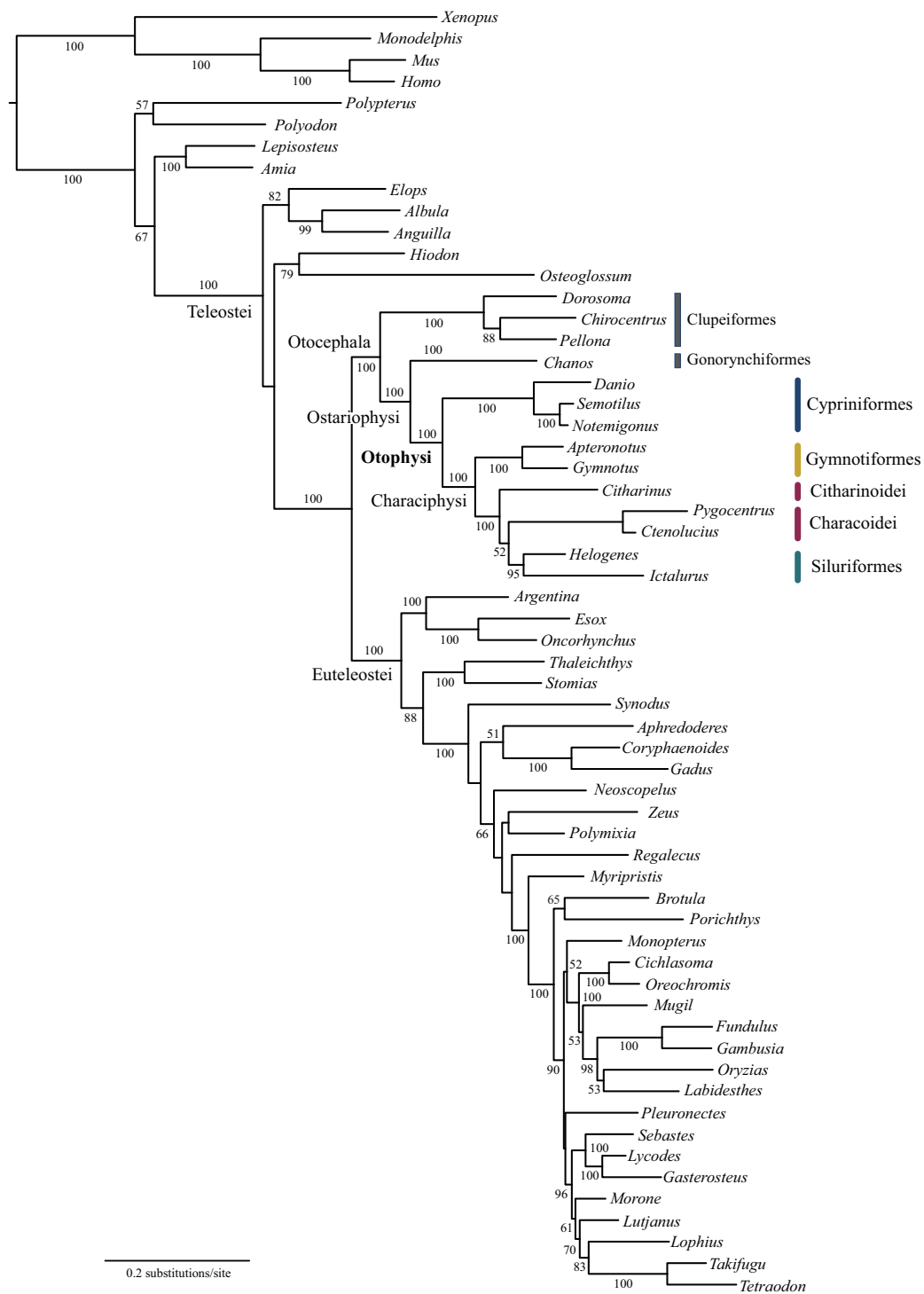
Figure 5 The best-scoring maximum-likelihood (ML) tree of the 10 otophysan and 50 outgroup species based on unambiguously aligned 10 nuclear gene sequences (8409 positions). Numerals beside internal branches indicate bootstrap probabilities (BSPs) of ≥50% based on 1000 replicates.

Table 5 Results from AU-tests among 15 alternative tree topologies of the four major lineages derived from analysis of whole mitogenome sequences

Tree[a]	ln *L*	Diff -ln *L*	*P* [b]
1(Gym, (Cit, (Cha, Sil)))	-237742.581	best	0.702
2(Gym, (Sil, (Cit, Cha)))	-237746.644	4.063	0.588
3((Cit, Gym), (Cha, Sil))	-237749.618	7.037	0.473
4(Gym, (Cha, (Cit, Sil)))	-237749.793	7.212	0.400
5(Cha, (Sil, (Cit, Gym)))	-237752.770	10.189	0.400
6(Cit, (Gym, (Cha, Sil)))	-237752.584	10.003	0.362
7(Cit, (Cha, (Sil, Gym)))	-237755.898	13.317	0.267
8(Cha, (Cit, (Sil, Gym)))	-237755.375	12.794	0.140
9((Cit, Cha), (Sil, Gym))	-237758.976	16.395	0.140
10(Cha, (Gym, (Cit, Sil)))	-237756.070	13.489	0.129
11(Sil, (Cha, (Cit, Gym)))	-237760.049	17.468	0.069
12(Sil, (Gym, (Cit, Cha)))	-237764.849	22.268	0.051
13(Cit, (Sil, (Cha, Gym)))	-237761.012	18.431	0.030*
14((Cha, Gym), (Cit, Sil))	-237765.117	22.536	0.009*
15(Sil, (Cit, (Cha, Gym)))	-237765.958	23.377	0.001*

[a] Gym: Gymnotiformes; Cit: Citharinoidei; Cha: Characoidei; Sil: Siluriformes.
[b] Statistically significant differences (≤ 0.05) denoted by asterisks.

Charachoidei, another suborder of Characiformes, basal relationships were poorly resolved and only four of the 19 internal branches that connect two species from the same families received the highest statistical support (100% BSPs; Figure 3). With the exception of such ambiguous basal relationships, the resulting phylogenies showed several similarities to those reported by Calcagnotto et al. [20], who analyzed four nuclear and two mitochondrial genes across 124 characiform taxa. In

Table 6 Results from AU-tests among 15 alternative tree topologies of the four major lineages derived from analysis of 10 nuclear gene sequences

Tree[a]	ln *L*	Diff -ln *L*	*P* [b]
1(Gym, (Cit, (Cha, Sil)))	-131608.5	best	0.723
2(Gym, (Cha, (Cit, Sil)))	-131610.3	1.758	0.447
3(Gym, (Sil, (Cit, Cha)))	-131612.0	3.495	0.200
4((Cit, Cha), (Sil, Gym))	-131649.9	41.379	0.006*
5(Cha, (Gym, (Cit, Sil)))	-131633.8	25.322	0.005*
6(Cit, (Gym, (Cha, Sil)))	-131644.2	35.689	0.004*
7((Cha, Gym), (Cit, Sil))	-131633.6	25.100	0.003*
8(Cha, (Sil, (Cit, Gym)))	-131657.8	49.233	0.002*
9(Sil, (Gym, (Cit, Cha)))	-131656.9	43.761	0.001*
10(Cha, (Cit, (Sil, Gym)))	-131656.9	45.503	0.001*
11(Cit, (Cha, (Sil, Gym)))	-131654.0	45.504	0.001*
12(Sil, (Cit, (Cha, Gym)))	-131656.9	48.413	0.001*
13(Cit, (Sil, (Cha, Gym)))	-131656.9	48.413	0.001*
14((Cit, Gym), (Cha, Sil))	-131644.2	35.662	0.000*
15(Sil, (Cha, (Cit, Gym)))	-131657.9	49.336	0.000*

[a] Gym: Gymnotiformes; Cit: Citharinoidei; Cha: Characoidei; Sil: Siluriformes.
[b] Statistically significant differences (≤ 0.05) denoted by asterisks.

particular, we confirmed that the two African lineages represented by Hepsetidae (*Hepsetus*) and Alestidae (*Phenacogrammus* and *Micralestes*) did not form a monophyletic group, but were nested in the same clade with strictly Neotropical species, such as *Hydrolycus* (Cynodontidae) and *Hoplias* (Erythrinidae) (Figure 3).

In Siluriformes, monophyly of the two currently recognized suborders (Loricaroidei, Siluroidei [21]) was confirmed and the Loricaroidei was recovered as sister to other clades, followed by the divergence of Diplomystoidei and Siluroidei (BSP = 82%; Figure 3). These basal divergences are fully congruent with the results of a recent molecular phylogenetic study by Sullivan et al. [21], who analyzed two nuclear genes across 110 catfish species representing 36 of 37 families. Thus, the two different lines of evidence support the most basal Loricaroidei in the siluriform phylogenies. Note that previous morphological studies have consistently argued the most basal Diplomystoidei ([41,84-89]), which was supported by a molecular study by Hardman [22], who used *Diplomystes mesembrinus* (Diplomystidae) as the only outgroup to root the tree. Within the Loricaroidei, a sister group relationship between *Pterygoplichthys* (Loricariidae) and *Astroblepus* (Astroblepidae) was strongly supported as in previous morphological and molecular studies [21,89]. As in the Characoidei, basal relationships in the Siluroidei were poorly resolved and only five of the 23 internal branches received high statistical support (≥95% BSPs; Figure 3). Nevertheless we recovered some of the suprafamilial clades reported by Sullivan et al. [21], and list them below with genera used in this study (Figure 3): "Big Asia" (*Pseudobagrus*, *Hara*, *Liobagrus*), "Claroidea (Cla)" (*Clarias*, *Heteropneustes*), "Aspredinidae + Doradoidea (Asp + Dor)" (*Bunocephalus*, *Amblydoras*, *Tatia*, *Tetranematichthys*), "Ictaluroidea (Ict)" (*Ictalurus*, *Cranoglanis*), and "Big Africa" (*Synodontis*, *Amphilius*, *Malapterurus*, *Auchenoglanis*, *Chrysichthys*, *Pareutropius*).

Estimation of divergence time

MCMCTREE analyses of the divergence times based on the two data sets (12_nRT_n and 123_a) with the assumption of independent rates (IR) across nodes yielded similar estimated node ages (see Additional file 1). Overall dating analysis based on the nucleotide data set (12_nRT_n) provided slightly older node ages (absolute differences in posterior means: 16.3 million years ± 21.5 SD) with consistently smaller 95% credible intervals (absolute differences: 30.3 million years ± 23.0 SD) than those from the amino acid data set (123_a). Consequently the 95% credible intervals from both data sets greatly overlapped with each other across all nodes. These tendencies held true for the estimated node ages from the most recent common ancestors (MRCAs) of the five

major otophysan lineages themselves (Cypriniformes, Gymnotiformes, Citharinoidei, Characoidei, Siluriformes) and those from MRCAs comprising subsets of these five lineages (total = nine nodes), with the absolute difference in posterior means of 17.8 million years ± 5.7 SD. Considering the long evolutionary history of actinopterygians (≈ 450 million years), the differences seem minor and the following description and discussion of the results were based on the nucleotide data set (12_nRT_n) for simplicity.

MCMC analysis based on the 12_nRT_n data set indicated that an ancestral lineage of the modern otophysans diverged from a stem ostariophysan during the end-Permian about 261 Ma with a 95% credible interval of 240-282 Ma (Figure 6). Basal otophysan divergences that produced common ancestors of the five modern lineages were estimated to occur in a relatively short time window during the Triassic (251-200 Ma): divergences between Cypriniformes and Characiphysi = 248 Ma (with a 95% credible interval of 227-268 Ma); Gymnotiformes and Characiformes + Siluriformes = 226 Ma (206-245 Ma); Citharinoidei and Characoidei + Siluriformes 220 Ma (201-240 Ma), Characoidei and Siluriformes 216 Ma (198-237 Ma). The origins of these ancestral lineages were followed by the onset of radiations in each lineage during the Jurassic (200-146 Ma), with the estimated ages of the most recent common ancestor (MRCA) of the modern Cypriniformes = 160 Ma (130-186 Ma); Gymnotiformes = 189 Ma (166-212 Ma); Citharinoidei = 160 Ma (124-190 Ma); Characoidei = 192 (172-213 Ma); and Siluriformes = 180 Ma (162-198 Ma). For the summary of MCMC samples for node ages and entire timetree including non-otophysans, see Additional files 2 and 3, respectively.

We confirmed that uncertainties in relationships among major characiphysan lineages (Gymnotiformes, Citharinoidei, Characoidei, Siluriformes) least affect divergence time estimates, with the largest difference being only 10 million years (Table 7). In addition, despite the use of the IR model across nodes using MCMCTREE in the present study, the resulting ages for selected nodes were very similar to those reported in previous studies employing the AR model using MULTIDIVTIME [43,68] (Table 8). When the AR model was used in MCMCTREE (clock = 3), differences in the resulting node ages were minor, with absolute differences in posterior means of 0.7 million years ± 16.4 SD (Additional file 1). Thus, the topological uncertainties and use of the different models of rate variations across nodes (and associated software) did not affect the divergence time estimates, at least in the present data set.

Our age estimates, however, are remarkably older than those indicated in previous reports based on the fossil record [43,68,90-92]. For example, the oldest

otocephalan fossil discovered to date was from the Late Jurassic 150 Ma [93], while our estimate was 265 Ma. The oldest otophysan fossil record dates back to the Albian (100-112 Ma) [94], while our estimate was 248 Ma. For all other major otophysan lineages, our molecular estimates (from 160 Ma in Cypriniformes to 220 Ma in Characiformes) are over 100 million years older than those of the fossil records used as minimum constraints (from 8 Ma in Gymnotiformes to 98 Ma in Characiformes; Table 4). Azuma et al. [43] also noted such large differences and plotted minimum time constraints based on the fossil record against molecular time estimates of the corresponding nodes. They found that four data points in the Paleozoic showed a fairly good 1:1 relationship, whereas other points in the Mesozoic were considerably below the line of a 1:1 relationship. They considered that these significant departures from the expected relationships for the Mesozoic fossils may reflect the fact that they do not really represent the oldest fossil for the corresponding lineages.

For such remarkably older molecular estimates, Benton and Ayala [51] noted four pervasive biases that may cause molecular dates to be too old: 1) the calibration dates that are too old based on previous molecular studies; 2) undetected rapidly evolving genes; 3) an ancestral polymorphism that was maintained through a long evolutionary period; and 4) asymmetric distributions of estimated times, with a constrained younger end but an unconstrained older end. As discussed by Azuma et al. [43], whose data set formed the basis of this study, the first factor is not the case in the present study because we did not use calibration dates based on previous molecular studies. The third factor would be applicable when the genomic regions used are under long-term balancing selection, but no mitochondrial genes have been reported to be under such selection. Neither the second factor nor the fourth factor are true in this study because quickly saturated third codon positions and the control region were excluded from the present analysis, and because each mitochondrial gene used here was tested and shown to perform well for dating vertebrate divergences [95]. We agree with the arguments of Azuma et al. [43] as well as those of Benton and Ayala [51], who stated that "careful choice of genes may be a more appropriate strategy (than the larger data strategy), with a focus on long and fast-evolving (yet alignable) sequences" for reliable dating. Whole mitogenome sequences seem to accommodate such requirements.

Recently Peng et al. [30] and Saitoh et al. [12] estimated divergence times of Ostariophysi and Cypriniformes, respectively, based on whole mitogenome sequences. As expected from similarities in the data sets and priors used in the relaxed-molecular clock Bayesian analysis, their estimates generally agreed with those of
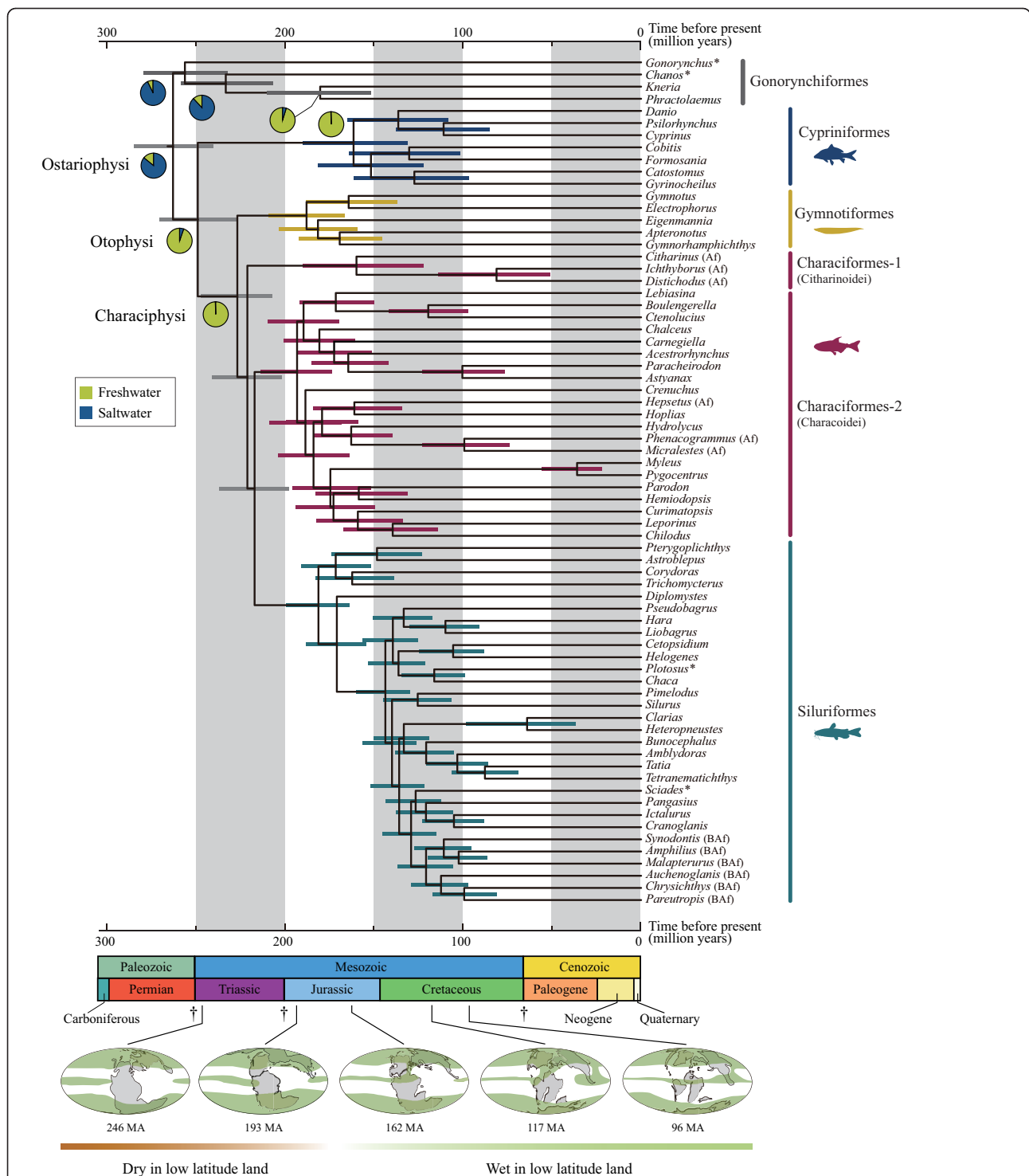
Figure 6 Timetree derived from the Bayesian relaxed-molecular clock method (only ostariophysan portions shown). Horizontal bars indicate 95% credible intervals of the divergence time estimates. ML reconstruction of ancestral habitats are indicated on selected nodes with pie charts showing the likelihoods for two character states (blue, freshwater; light green, saltwater). African characiforms and Big African clade in Siluriformes reported by Sullivan et al. [21] are denoted by "Af" and "BAf", respectively. All marine species are indicated by asterisks. Dagger symbols indicate the three big mass extinction events after 300 Ma. Paleocoastline maps [126] are shown below the timetree with moist zones schematically illustrated by green [127].

Table 7 Comparisons of the divergence time estimates (Ma) for selected nodes from alternative tree topologies

Node (MRCA)[a]	Topology 1 (P = 0.702)	Topology 2 (P = 0.588)	Topology 14 (P = 0.009)	Topology 15 (P = 0.001)	Topology 9 (P = 0.140)
Ostariophysi	261 (240-282)	261 (237-282)	260 (236-282)	261 (237-283)	259 (236-281)
Otophysi	248 (227-268)	246 (225-267)	245 (224-266)	245 (223-267)	245 (224-267)
Cypriniformes	159 (130-186)	160 (131-188)	160 (132-187)	159 (131-186)	160 (132-187)
Characiphysi	226 (206-245)	224 (203-244)	220 (200-239)	220 (201-242)	220 (200-242)
Gymnotiformes	189 (166-212)	184 (163-206)	179 (159-198)	180 (157-200)	180 (158-201)
Citharinoidei	160 (124-190)	152 (118-185)	155 (120-186)	154 (120-186)	153 (119-188)
Characoidei	192 (172-213)	190 (171-210)	187 (168-207)	187 (167-210)	188 (169-209)
Siluriformes	180 (162-198)	180 (162-197)	179 (161-197)	180 (162-197)	180 (162-197)

Four topologies with the two best (topologies 1 and 2) and worst (topologies 14 and 15) probabilities of AU tests in Table 3 plus that of Fink & Fink (topology 9) [27]. Upper and lower values are means and ranges of 95% posterior probabilities, respectively.
[a] Indicated by taxonomic names of the most recent common ancestor (MRCA)

our study (Table 9). More recently Santini et al. [96] analyzed divergence times for major fish lineages including those of Otophysi to investigate the effects of fish-specific whole-genome duplication events on the radiations of teleost fishes. Their analyses were based on the nuclear RAG1 gene sequences across 227 vertebrates downloaded from the database, and the phylogenetic relationships were reconstructed by constraining the monophyly of several groups to reflect generally accepted phylogenies. They used BEAST [64] to estimate divergence times under a model of uncorrelated but lognormally distributed rates, with soft upper (= maximum) bounds assigned to the prior distributions of 45 fossil calibrations using lognormal distributions. Note that their estimated ages are considerably younger than those in our study and those reported by Peng et al. [30] and Saitoh et al. [12] (Table 9) and thus require explanation.

As expected from the remarkably narrow 95% credible intervals in the estimated ages for Otocephala (149-153 Ma; Table 9), Santini et al. [96] set both minimum (149 Ma) and maximum (152 Ma) time constraints for the MRCA of Ostarioclupeomorpha (= Otocephala in this study). The upper bound (152 Ma) was chosen based on their strong belief that the MRCA of Otocephala was unlikely to be older than the oldest stem elopomorph (true eels and their relatives: †*Anaethalion*) from the late Kimmeridgian, Jurassic 152 Ma [42]. Accordingly,

they manipulated three priors of the lognormal distribution (offset = 149; mean = 0.1; SD = 0.6), which allowed only 5% of the MCMC samples to exceed the soft upper bound (152 Ma). This approach is called "phylogenetic bracketing" [97], which obtains not only minimum, but also maximum constraints on the timing of a branching event using the dates of the preceding and subsequent branching episodes [98,99]. Donoghue and Benton [97] argued that phylogenetic bracketing may be problematic because it assumes that the branching events above and below a calibration more reliably capture the timing of the branching event in question than the estimated date of the calibration itself. For other clades within the Otocephala, Santini et al. [96] also set relatively narrow time constraints: Ostariophysi (125-149 Ma), Characiformes (68-100 Ma), and Siluriformes (73-83.5 Ma). Accordingly, their resulting estimates (Table 9) fit very well with the fossil records and little chance exists (<5%) that the MRCA of Otocephala is more than 152 Ma.

We recognize that the approach in Santini et al. [96] is superior in that it can lend more credence to the fossil record than the standard minimum-age constraints. Nevertheless, we did not provide upper (maximum) bounds for these nodes or manipulate two parameters of the truncated Cauchy distributions (= lognormal distributions in Santini et al. [96]) because insufficient information exists with which to specify meaningful prior distributions for most otophysan diversification

Table 8 Comparisons of divergence time estimates (Ma) for basal actinopterygian nodes between the present and previous mitogenomic studies

Node (MRCA)[a]	This study (Topology 1)	Setiamarga *et al.* [68]	Azuma *et al.* [43]
Osteichthyes	445 (419-472)	428 (419-442)	429 (417-449)
Actinopterygii	425 (401-449)	411 (398-419)	415 (400-434)
Actinopteri	380 (360-395)	383 (368-392)	384 (370-392)
Neopterygii	362 (339-381)	364 (346-378)	365 (348-378)
Teleostei	334 (312-357)	332 (312-350)	331 (312-348)
Clupeocephala	293 (271-315)	289 (269-310)	288 (268-307)

[a] Indicated by taxonomic names of the most recent common ancestor (MRCA)

Table 9 Comparisons of divergence time estimates (Ma) for basal actinopterygian nodes between the present and previous mitogenomic studies

Node (MRCA)[a]	This study (Topology 1)	Peng et al. [30]	Saitoh et al. [12]	Santini et al. [96]
Otocephala	265 (243-286)	279 (264-293)	251 (230-273)	151 (149-153)
Ostariophysi	261 (240-282)	—————	239 (218-260)	128 (125-134)
Anotophysi	254 (232-276)	242 (——)	228 (206-250)	—————
Otophysi	248 (227-268)	251 (——)	220 (198-241)	—————
Cypriniformes	160 (130-186)	183 (——)	156 (136-176)	92 (56-123)
Characiphysi	226 (206-245)	210 (——)	185 (164-207)[b]	—————
Gymnotiformes	189 (166-212)	150 (——)	—————	—————
Characiformes	220 (201-240)	203 (——)	160 (139-183)[b]	80 (68-84)
Siluriformes	180 (162-198)	173 (——)	125 (104-148)[b]	88 (77-98)

[a] Indicated by taxonomic names of the most recent common ancestor (MRCA)

[b] Including only two characoid characiforms plus two siluriforms, which are likely to provide underestimation of the node ages owing to insufficient taxonomic sampling.

times. Thus, the remarkable gaps between estimated ages from the two studies mostly reflect differences in priors on node ages (as the time constraints) rather than differences in software (MCMCTREE vs. BEAST) or data (mitogenomes vs. nuclear genes).

Some recent studies have argued that mitochondrial genes are likely to yield older node ages than nuclear genes. For example, Hurley et al. [100] investigated the relationships and divergence times of the basal actinopterygians (including teleosts) using four nuclear genes and whole mitogenomes. A relaxed molecular clock Bayesian analysis based on these two data sets provided node ages for the most recent common ancestor of teleosts with posterior means of 219 Ma (181-265 Ma) and 246 Ma (206-292 Ma) depending orthologues in the nuclear genes (only the former estimate shown in their figure 4) and 296 Ma (268-326 Ma) in the mitochondrial genome (data taken from their supplementary tables 3 and 7). Despite the overlap between these two results (95% credible intervals), Hurley et al. [100] concluded that the mitochondrial estimate of the most recent common ancestor of teleosts was 50-100 million years older than that of the nuclear genes. Based on this comparison, they further argued that the discrepancy between their nuclear and mitochondrial estimates may have been due to evolutionary rate differences between these two genomes. Note that their nuclear estimation did not include any species from the osteoglossomorphs (the putative most primitive teleosts; see Figure 3), which is likely to result in underestimation of the crown node age of teleosts with insufficient taxon sampling.

More recently, Brandley et al. [52] investigated intercontinental dispersal of *Plestiodon* (*Eumeces*) lizards based on analysis of a single mitochondrial gene (ND1) plus seven nuclear genes. They performed a relaxed molecular clock Bayesian divergence time estimation using unpartitioned and partitioned data sets across

these genes and found that extreme saturation obscured the underlying rate of evolution in the mitochondrial gene, resulting in overestimation of the divergence times. Such overestimation in the mitochondrial gene was most pronounced in the unpartitioned data set and less so in the two partitioned data sets. Brandley et al. [52] agreed with Phillips [101], who demonstrated that genes or gene partitions that evolve at extremely high rates may accumulate so many hidden substitutions, making it difficult to estimate the underlying process that created the data. Although their arguments are convincing, comparisons made by Brandley et al. [52] were based on a single mitochondrial gene, including third codon positions. We used whole mitochondrial genomes with various substitution rates across genes [102], excluded quickly saturated third codon positions entirely, and partitioned the data sets.

Also the estimated ages may be too old simply because of the entire absence of a fossil record [100]. The absence of fossils, however, should not be taken as evidence of absence, as discussed extensively by Diogo [41]. In an article on the early radiation of teleosts, Arratia [42] stated "... we know almost nothing concerning the fossil record of most otophysans, of most living perciforms, atheriniforms, etc." Thus, to assume a lack of otophysan fossil record during the Mesozoic is natural if the group existed as stem groups during this period.

As Brown et al. [72] convincingly argued, although ample room for improvement exists on both sides of the "rock-clock" divide (e.g., accounting for ghost lineages in the fossil record and developing more realistic models of rate evolution for molecular genetic sequences), the consistent and conspicuous disagreement between these two sources of data more likely reflects a genuine difference between estimated ages of 1) stem group origins and 2) crown group morphological diversification, respectively.

Comments on trans-Atlantic clades

Although this study was not intended to address questions regarding historical biogeography of the otophysans, divergence times of some of the transcontinental sister group relationships, particularly those between Africa and South America in Characiformes and Siluriformes, warrant brief comments regarding their origins.

Based on a hypothesis presented by Backup (unpublished thesis [103]), Lundberg [37] examined the implications of applying a strict vicariance scenario to the three transcontinental sister group pairs in Characiformes and noted that most of the diversification among characiforms would have occurred prior to the continental breakup (i.e., on Gondwana). Our timetree corroborates this hypothesis because all of our divergence time estimates of the three transcontinental pairs (Citharinoidei vs. Characoidei = 220 Ma; two alestids *Micralestes* + *Phenacogrammus* vs. *Hydrolycus* = 162 Ma; *Hepsetus* vs. *Hoplias* = 160 Ma) fall well before the Gondwanan separation (100-120 Ma) [104]. Note, however, that recent discoveries of marine or brackish characiforms (*Santanichthys* [94] and *Sorbinicharax* [105]) from the Cretaceous challenge such a simple vicariance hypothesis [106].

Recently Lundberg et al. [107] examined the relationships of a highly distinct freshwater catfish, *Lacantunia enigmatica* (Lacantuniidae) from Chipas, Mexico, and found that *Lacantunia* was derived from within a multifamily clade of African freshwater catfishes ("Big Africa" in Sullivan et al. [21]; see Figures 3, 6). Based on a maximum-age constraint of 144 Ma for the stem of the siluriform lineage (= siluriform + gymnotiform node) and seven additional minimum-age constraints for the internal nodes, they estimated the divergence time of *Lacantunia* using a relaxed-molecular clock method implemented in MULTIDIVTIME [69] and r8s [108]. The resulting divergence estimates ranged from 83 to 87 Ma depending on the partitioning schemes with 95% credible intervals of 75-94 Ma. These ages fall after the separation of Africa and South America, and Lundberg et al. [107] proposed a new scenario based on an ancient intercontinental passage for explaining disjunct distributions of relictually endemic *Lacantunia* and its African sister clade. Also note that they acknowledge that their choice of 144 Ma as the maximum-age constraint is arbitrary, but conservatively informed by the fossil record of actinopterygians. Although we did not include *Lacantunia* in our analyses, the onset of diversification of the Big African clade is estimated to be 129 Ma (113-144 Ma) and divergence of *Lacantunia* from its stem should be much older. Thus our estimated ages did not require an ancient intercontinental passage as hypothesized by Lundberg et al. [107].

History of diversification

ML reconstruction of the ancestral habitats on the timetree suggests that a shift from character state 0 (saltwater) to 1 (freshwater) occurred between the crown node of Ostariophysi (P_0 = 0.859; P_1 = 0.142) and that of Otophysi (P_0 = 0.055; P_1 = 0.945), corresponding to the end-Permian (263-249 Ma) (Figure 6). Moreover, a similar habitat shift was also recovered in Gonorynchiformes, a sister clade of Otophysi (Figure 6). Based on these ancestral habitat reconstructions and the divergence time estimates with reference to Earth's history, we identified the following five global turning points in the history of otophysan diversification. Note, however, that the true ancestral habitats and dynamics of diversification cannot be inferred from time-calibrated phylogenies of the extant lineages alone [109]; one need to include the fossil record to fully understand the ancestral habitats and diversity dynamics of the otophysans.

First, the common ancestor of Otophysi presumably entered freshwater around 263-249 Ma. This period corresponds to the end-Permian when the largest mass extinction in Earth's history occurred, wiping out 96% of all species [110]. The end-Permian mass extinction has been associated with a massive release of carbon gases into the atmosphere, causing a global greenhouse effect and abrupt climate warming [111]. During the same time, the oceans are believed to have become anoxic worldwide [112,113] and to have contained free hydrogen sulfide [114]. These environmental perturbations greatly altered the marine ecosystems [115] and may have driven fish extinctions. Note that freshwater contains much higher levels of dissolved oxygen than saltwater for a given atmospheric concentration, and this difference can affect the biology of the organisms [116]. The possible habitat shift (from salt- to freshwater) in the common ancestor of Otophysi should have a causal relationship with the survival of this lineage across the end-Permian. To our knowledge, however, no comparable examples of habitat shift in other groups of organisms exist that independently support this evolutionary scenario.

Second, the five major otophysan lineages (corresponding to common ancestors of the present-day orders or suborders) have successively originated from an ancestral lineage of the otophysans in a short time window during the Triassic (251-200 Ma). During this period all continents remained united as the supercontinent Pangaea, with a climate characterized by globally warm temperatures, extreme seasonality, and high aridity over much of the inland region [117]. However, the climate was more moderate around the edges of the supercontinent. The regions around Pangaea had sufficient rainfall to produce

vigorous forests along riverbanks [118], which possibly facilitated stepwise divergences of the five lineages on Pangaea. Note that the Pangaea was cut almost in half along the east-west axis by a huge embayment called the Tethys Seaway (Figure 6). Over time, as the Tethys Seaway expanded, it may have led to the first vicariant divergence between the common ancestors of Cypriniformes and Characiphysi as suggested by Saitoh *et al.* [4,12].

Third, these five major otophysan lineages survived the end-Triassic mass extinction, which was one of the five largest extinctions in Earth's history in which 80% of all species became extinct [117]. Widespread magmatic activity of the Central Atlantic Magmatic Province (CAMP) has been suggested to have caused this catastrophic event, and repeated release of SO_2 gas, heavy metal emissions, and darkening were the main environmental stressors [119]. Relatively long stem branches from the common ancestors of the five major lineages across the Triassic-Jurassic boundary suggest profound effects of the mass extinction and associated environmental perturbations on the patterns of otophysan diversification.

Fourth, extant otophysan lineages began to diversify in each of the five clades during the Jurassic and through the early Cretaceous (200-100 Ma) (Figure 6), providing a framework for the modern otophysan diversity. The onset of diversification depends on the clade (193-160 Ma), although all fell in the Jurassic (200-146 Ma). Note that familial diversification of the South American gymnotiforms, characoids, and loricarioids was recovered much earlier than that of cypriniforms, citharinoids and siluroids (Figure 6). The Jurassic was a time of particularly swift change due to the Pangaean breakup and the resulting development of new oceans and a gentle tropical climate over the formerly arid interiors of the supercontinent [118]. Such environmental changes likely yielded numerous novel habitats, facilitating otophysan diversification throughout the Mesozoic until about 100 Ma.

Finally these modern otophysan lineages established during the Mesozoic survived the third mass extinction event at the end of the Cretaceous (Figure 6). Such "mass survival" of the modern lineages across the Cretaceous-Paleogene boundary has been noted for birds and mammals based on molecular evidence [120].

The present timetree suggests a Pangaean origin and Mesozoic radiations of the modern otophysans. This evolutionary scenario is in good agreement with recent biogeographic inferences. For example Diogo [41] broadly surveyed the higher-level phylogeny, biogeographic distribution, physiology, and ecology of catfishes, and suggested that these fishes originated at a time when some Pangaean connections still existed between Laurasia and Gondwana. In addition, Briggs [33] examined the phylogeny and geographic distribution patterns of ostariophysan fishes and proposed the Late Jurassic (160-150 Ma) origins of the cypriniforms and siluriforms, which are highly congruent with the estimated node ages for the most recent common ancestor of Cypriniformes (160 Ma) and Siluriformes (180 Ma) in this study.

Of course the evolutionary scenario presented here just represents a testable hypothesis and should be viewed with caution because the fossil record provides no direct evidence. Although Mesozoic freshwater deposits are geographically and stratigraphically patchy and the freshwater fishes from most of these deposits have not been fully documented across all continents [121-123], available information suggests that major components of the freshwater fishes were basal actinopterygians (non-teleost ray-finned fishes), chondrichthyans (sharks, rays, chimaeras) and sarcopterygians (lungfishes and coelacanths) [121-124], and few freshwater teleosts (e.g., the Late Triassic *Jiangilichthys*) are referable to extinct "pholidophoriforms," phylogenetically located outside the modern teleosts [125]. Moreover the fossil record suggests that the modern teleostean lineages did not diversify until the Late Jurassic about 150 Ma [125]. Thus, acceptance of the present evolutionary scenario requires the origin and survival of the ghost lineage from the Triassic through Late Jurassic for over 100 million years.

Conclusions
The timetree presented here indicates that survival of the ancestral lineages through the two consecutive mass extinctions on Pangaea and subsequent radiations during the Jurassic and through the early Cretaceous shaped the modern familial diversity of otophysans. The Pangaean origin and Mesozoic radiations of the modern otophysans are consistent with recent arguments based on biogeographic inferences [40,41] and molecular divergence time estimates [37,40]. No fossil otophysan, however, has been recorded before the Albian, the early Cretaceous 100-112 Ma [42], creating an over 100 million year time span without fossil evidence. This extremely large ghost range partially reflects a genuine difference between the estimated ages of stem group origin (molecular divergence time) and crown group morphological diversification (fossil divergence time) [72]; the ghost range, however, may be filled with future discoveries of older fossils that can be used as more reasonable time constraints as well as with the development of more realistic models that accurately capture the divergence rates of molecular sequences.

Additional material

Additional file 1: A cladogram showing all nodes with < 50% BSPs in Figure 3 collapsed to polytomy.

Additional file 2: Summary of MCMC samples for node ages (means and 95% credible intervals) in the independent-rates (IR) and autocorrelated-rates (AR) analyses using MCMCTREE-HS [63]. Results from the two data sets (12_nRT_n and 123_a) are shown separately. For node numbers, see tree at the end of this file.

Additional file 3: Timetree derived from the Bayesian relaxed-molecular clock method (non-ostariophysan portions also shown). Upper (maximum) and lower (minimum) time constraints used in this study are shown by arrowheads with corresponding nodes connected by dotted lines. All marine species are indicated by asterisks.

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Authors' contributions

MNa, MM, KM and MNi designed this study. MNa and KS mainly collected the specimens and carried out the molecular work. MNa and MM analyzed the data. MM drafted the original manuscript and MNa, KM, KS and MNi contributed to its improvement. All authors read and approved the final manuscript.

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