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Mitochondrial molecular clocks and the origin of the major Otocephalan clades (Pisces: Teleostei): A new insight

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Abstract

The Otocephala, a clade including ostariophysan and clupeomorph teleosts, represents about a quarter of total fish species diversity, with about 1000 genera and more than 7000 species. A series of recent papers have defended that the origin of this clade and of its major groups may be significantly older than the oldest fossils of each of these groups suggest. Some of these recent papers explicitly defend a Pangean origin for some otocephalan groups such as the Siluriformes or Cypriniformes. To know whether or not the otocephalans as a whole, and particularly the mainly freshwater, cosmopolitan otophysans could have originated before the splitting of the Pangean supercontinent is of extreme importance, since otophysan fishes are among the most useful animal groups for the determination of historical continental relationships. In the present work we examined divergence times for each major otocephalan group by an analysis of complete mtDNA sequences, in order to investigate if these divergence times support the hypotheses advanced in recent studies. The complete mtDNA sequences of nine representative non-otocephalan fish species and of twenty-one representative otocephalan species was compared. The present study is thus, among the studies dealing with molecular divergence times of teleosts, the one in which a greater number of otocephalan species are included. The divergence times obtained support that the major otocephalan groups had a much older origin than the oldest fossil records available for these groups suggest. The origin of the Otocephala is estimated as having occurred about 282 Mya, with the origin of the Otophysi being estimated at about 251 Mya.

Keywords: Divergence times; Relaxed molecular clock; Mitochondrial genome; Otocephala; Otophysi

1. Introduction

Teleosts are a modern group of fishes including more than 23,000 species (e.g., Nelson, 1994). As recently noted by Stiassny et al. (2004), there are more teleost species than all the other vertebrates combined. In the recent overview of Stiassny et al. (2004), the Teleostei are subdivided into three main groups: the Osteoglossomorpha (arowanas and relatives), the Elopomorpha (eels, tarpons, and notacanths), and the Clupeocephala (Otocephala and Euteleostei). The Otocephala, including the Ostariophysi and the Clupeomorpha, represents about one quarter of the total fish species diversity, with about 1000 genera and more than 7000 species (e.g., Nelson, 1994; Lecointre and Nelson, 1996; Berra, 2001; Stiassny et al., 2004). Ostariophysi comprises the Otophysi and Anotophysi and includes the great majority of all freshwater fishes (Fink and Fink, 1981, 1996; Nelson, 1994). The otophysan clade includes the Siluriformes (catfishes), Cypriniformes (carps, minnows, and relatives), Characiformes (piranhas, tetras, and relatives) and Gymnotiformes (electric eels); the anotophysan clade includes the

Abbreviations: ATPase 6 and 8, ATPase subunits 6 and 8; bp, base pair(s); COI–III, cytochrome c oxidase subunits I–III; cyt b, cytochrome b; Mya, million years ago; Myr, million years; ND1–6, 4L, NADH dehydrogenase subunits 1–6, 4L; PCR, polymerase chain reaction; tRNA, transfer RNA; 12S rRNA and 16S rRNA, 12S and 16S ribosomal RNA.

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Gonorynchiformes (milkfishes and relatives). In contrast to Ostariophysi, members of the superorder Clupeomorpha are mainly marine. The superorder contains the Eliimmichthyiformes (exclusively represented by fossil taxa) and the Clupeiformes (herrings, anchovies, and relatives). These latter are amongst the most economically important of all fish species, being heavily exploited by man for food.

The earliest representatives of living teleost lineages date to the Late Jurassic some 150 Mya, but, as noted by Arratia (2001), if definitions of the group are to include related fossil lineages, this date is pushed back into the Late Triassic–Early Jurassic (ca. 200–210 Mya). With respect to otocephalan fishes, which are the main focus of the present work, fossil records start to occur only from the Late Jurassic, ca. 150 Mya (see Arratia, 1997; Stiassny et al., 2004). As explained in a recent paper (Briggs, 2005; also see Kumazawa et al., 1999), this has lead most authors to consider that the origin of otocephalans, or at least of most of the otocephalan subgroups, has occurred after the final separation between Laurasia and Gondwana. As Briggs (2005) noted, although the otocephalan otophysans are primary freshwater fishes, their presence in all continents has often been explained in the literature by the occurrence of transcontinental marine migrations.

However, a series of recent papers have questioned this view, arguing that the origin of the otophysan clade and some of its subgroups may be significantly older than previously thought (e.g., Saitoh et al., 2003; Diogo, 2004; Filleul and Maisey, 2004; Briggs, 2005; Inoue et al., 2005). Some of these papers (Saitoh et al., 2003; Diogo, 2004; Briggs, 2005) explicitly defend a Pangean origin for the otophysans, and consequently, for the otocephalans. Inoue et al. (2005), in a study of the divergence time between two coelacanths, also analysed the divergence times for four otocephalan fishes using complete mitochondrial sequence data, obtaining an estimate of 201–239 Mya for the origin of the Otocephala. These authors used two different data sets in their analysis: data set # 1 resulted in an estimate of 201 Mya, whilst data set # 2 resulted in an estimate of 239 Mya. To know whether or not the otocephalans as a whole, and particularly the mainly freshwater, cosmopolitan otophysans could have originated before the final splitting of the Pangean supercontinent is of extreme importance since, as stressed by Briggs (2005: 287), "of all terrestrial animal groups, the otophysan fishes are probably the most useful for the determination of historical continental relationships".

The main goal of the present study is to examine the times of origin of each major otocephalan group by an analysis of complete mitochondrial DNA (mtDNA) sequences, in order to investigate if these times support, or not, a rather old origin of these groups as defended in the recent papers discussed above. It should be stressed here, as by Bromham and Penny (2003), that lineage-specific variation in rate of molecular evolution can complicate molecular dating, because a calibration rate estimated from one lineage may not be an accurate representation of the rate in other lineages. Also, estimation of

Table 1
List of the 30 species used in the present study

Order	Family	Species	GenBank accession no.	References
Amiiformes	Amiidae	*Amia calva*	AB042952	Inoue et al., 2003
Osteoglossiformes	Osteoglossidae	*Osteoglossum bicirrhosum*	AB043025	Inoue et al., 2001a
Osteoglossiformes	Pantodontidae	*Pantodon buchholzi*	AB043068	Inoue et al., 2001a
Elopiformes	Elopidae	*Elops hawaiensis*	AB051070	Inoue et al., 2004
Anguilliformes	Anguillidae	*Anguilla anguilla*	AP007233	Minegishi et al., 2005
Albuliformes	Albulidae	*Albula glossodonta*	AP002973	Inoue et al., 2004
Clupeiformes	Clupeidae	*Sardinops melanostictus*	AB032554	Inoue et al., 2000
Clupeiformes	Engraulidae	*Engraulis japonicus*	AB040676	Inoue et al., 2001b
Gonorynchiformes	Chanidae	*Chanos chanos*	AB054133	Saitoh et al., 2003
Gonorynchiformes	Gonorynchidae	*Gonorynchus greyi*	AB054134	Saitoh et al., 2003
Cypriniformes	Balitoridae	*Crossostoma lacustre*	M91245	Tzeng et al., 1992
Cypriniformes	Balitoridae	*Lefua echigonia*	AB054126	Saitoh et al., 2003
Cypriniformes	Catostomidae	*Carpiodes carpio*	AY366087	Broughton et al. unpublished
Cypriniformes	Catostomidae	*Myxocyprinus asiaticus*	AY986503	This study
Cypriniformes	Cobitidae	*Cobitis striata*	AB054125	Saitoh et al., 2003
Cypriniformes	Cyprinidae	*Carassius auratus*	AB006953	Murakami et al., 1998
Cypriniformes	Cyprinidae	*Carassius carassius*	AY714387	Guo et al. unpublished
Cypriniformes	Cyprinidae	*Cyprinus carpio*	X61010	Chang et al., 1994
Characiformes	Alestiidae	*Phenacogrammus interruptus*	AB054129	Saitoh et al., 2003
Characiformes	Characidae	*Chalceus macrolepidotus*	AB054130	Saitoh et al., 2003
Gymnotiformes	Apteronotidae	*Apteronotus albifrons*	AB054132	Saitoh et al., 2003
Gymnotiformes	Eigenmanniidae	*sp.Eigenmannia*	AB054131	Saitoh et al., 2003
Siluriformes	Bagridae	*Pseudobagrus tokiensis*	AB054127	Saitoh et al., 2003
Siluriformes	Callichthyidae	*Corydoras rabauti*	AB054128	Saitoh et al., 2003
Siluriformes	Ictaluridae	*Ictalurus punctatus*	AF482987	Waldbieser et al., 2003
Siluriformes	Pangasiidae	*Pangasianodon gigas*	AY762971	Jondeung and Sangthong unpublished
Siluriformes	Cranoglanididae	*Cranoglanis bouderius*	AY989626	Peng et al. unpublished
Salmoniformes	Salmonidae	*Oncorhynchus mykiss*	L29771	Zardoya et al., 1995
Salmoniformes	Salmonidae	*Salmo salar*	U12143	Hurst et al. unpublished
Perciformes	Scombridae	*Scomber scombrus*	AB120717	Takashima et al. unpublished

divergence time is generally more difficult than reconstruction of a phylogenetic tree, because no gene would evolve at a constant rate (Glazko and Nei, 2003). Considering the effect of rate variation among a small number of genes, it is not surprising that the analyses based on partial mitochondrial gene sequences may sometimes be biased. As a genetic marker, animal mitochondrial DNA (mtDNA) has distinct characteristics, such as relatively fast evolutionary rate, lack of recombination, and maternal inheritance (e.g., Brown et al., 1979; Olivio et al., 1983). One way of reducing the problems associated with molecular clock dating is to compare long DNA sequence data (e.g., Inoue et al., 2001a, 2003; Yang and Yoder, 2003). Recent progress in molecular techniques has made it easier to obtain complete nucleotide sequences of fish mitochondrial genomes (e.g., Miya and Nishida, 1999). In this work, we will compare the complete mtDNA sequences of 9 representative non-otocephalan fish species and of 21 representative otocephalan species. One of these otocephalan species, *Myxocyprinus asiaticus* is a cypriniform for which the complete mtDNA sequence will be determined and documented for the first time in this work. The present study includes a greater number of otocephalan species than any other previous study dealing with molecular divergence times for teleostean fishes. It thus allows a more detailed discussion of the divergence times for the various groups of the otocephalan clade. Furthermore, instead of using solely a concatenated sequence or a multi-gene approach for dating divergence times, we also promote individual gene analysis. The present work therefore provides further insights into the evolutionary history of otocephalan fishes by examining mtDNA divergence estimates in view of the recently published studies on the origin and biogeography of otocephalans.

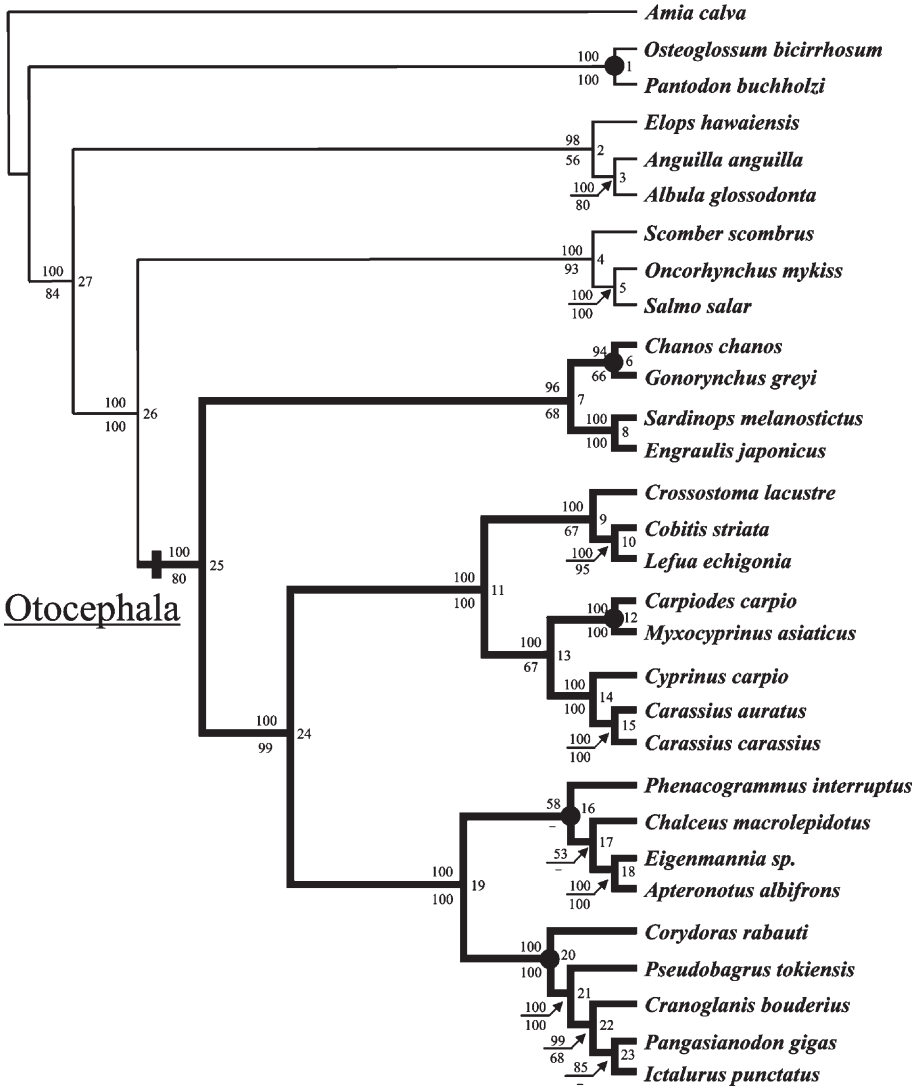

Fig. 1. 50% majority rule cladogram resulting from Bayesian analysis of 9 combined mitochondrial protein-coding genes sequences (without 3rd codon positions) based on a combined 1.5 million post burn-in generations under the GTR+I+G model of evolution. The black dots are nodes for which information from the fossil record was employed as calibrations and the numbers at the nodes indicate the nodes referred to in Table 4. Numbers on branches indicate posterior probabilities for Bayesian analysis (above), and bootstrap values for maximum likelihood analysis (below). Bootstrap values below 50% were indicated by dashes. Numbers at the nodes are estimated and calibrated nodes.

2. Materials and methods

2.1. Taxonomic sampling

Miya and Nishida (2000) recognised the importance of purposeful taxonomic sampling to increase phylogenetic accuracy in resolving higher-level relationships of teleosts and in estimating the divergence times between major teleostean clades. We have thus included 30 species in our analysis (Table 1): eight are representatives of the three major non-otocephalan teleost groups, i.e., Osteoglossomorpha, Elopomorpha and Euteleostei (see above); one is a representative outgroup of all teleosts, *Amia calva* (see Stiassny et al., 2004); twenty one are representatives of all the various otocephalan groups (Table 1, Figs. 1 and 2). The complete mitochondrial genome sequences of all these 30 species was downloaded from GenBank, with exception to that of *M. asiaticus*, which is documented for the first time in the present study. The specimens of *M. asiaticus* were obtained from the Yangtze River in Hubei province, China. The total genomic DNA was extracted from muscle tissue using a QIAamp tissue kit (Qiagen, Germany) following the manufacture's protocol, and was stored at 4 °C.

2.2. Long-PCR of the mitochondrial DNA genome and sequencing

The mitochondrial genome DNA of *M. asiaticus* was amplified in its entirety using a long PCR technique (Miya and

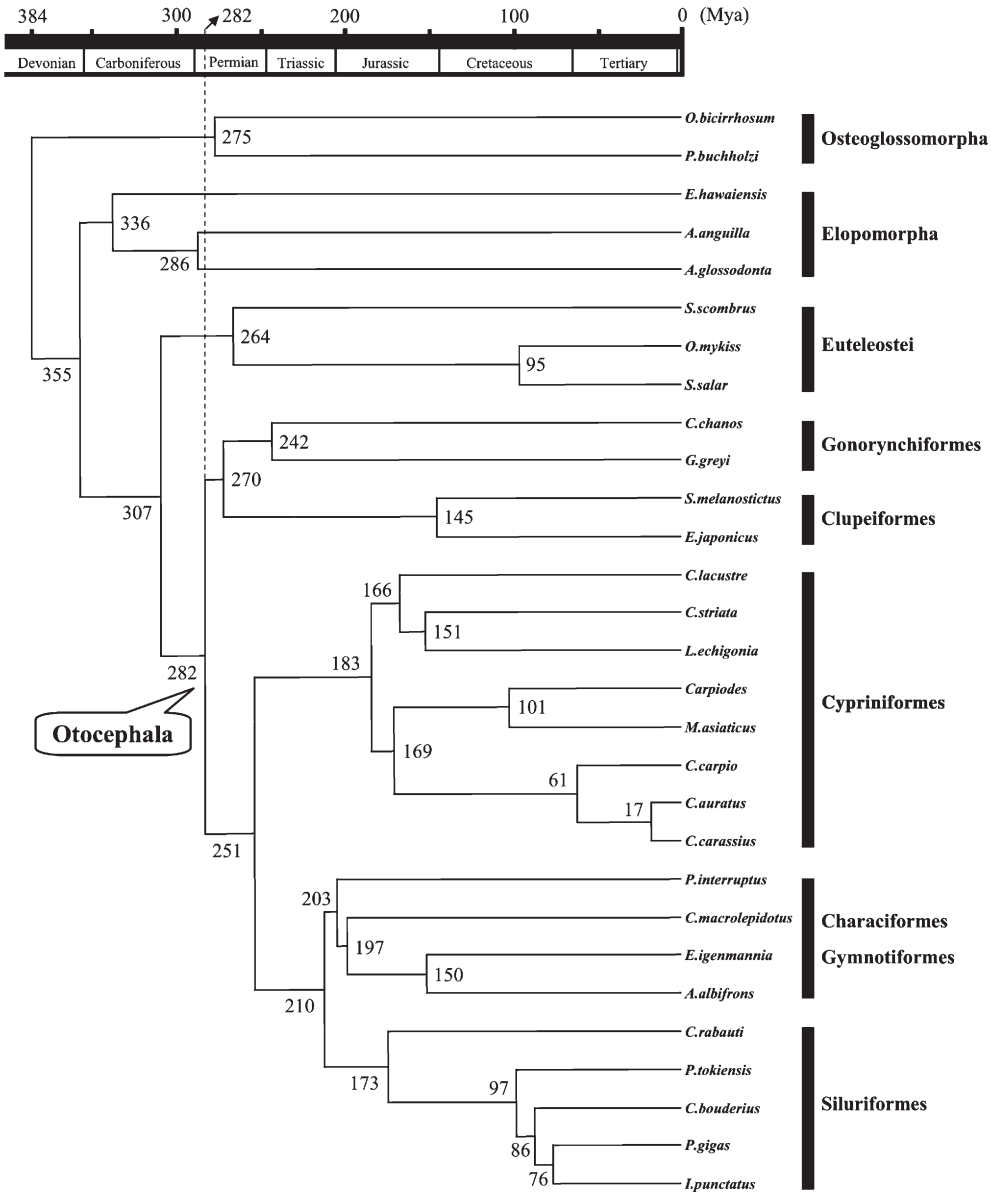

Fig. 2. Posterior distributions of the divergence times among teleostean fishes based on the partitioned Bayesian approach (Thorne and Kishino, 2002) for concatenated sequences from 9 protein-coding genes (total 6918 bp, without 3rd codon positions). The MULTIDIVTIME program was used to estimate divergence times in conjunction with the tree topology shown in Fig. 1. Numbers at the nodes indicate the estimated divergence time in Mya, of the corresponding nodes. A dash-dotted line indicates the posterior mean for divergence time in Mya for initial divergence for Otocephalan clades.

Nishida, 1999). The primers designed by Miya and Nishida (2000), and Inoue et al. (2001a) were used to amplify the total mitochondrial genome in two reactions. Long PCR was done in a PTC-100 programmable thermal controller (MJ Research, USA); reactions were carried out in 25 ul reaction volume containing 2.5 μl 10×LA PCR buffer II (Takara), 0.8 mM dNTPs, 2.5 mM $MgCl_2$, 0.5 μm each primer, 0.625 units LA Taq polymerase (Takara) and approximately 20 ng template DNA. The thermal cycle profile was: pre-denaturation at 94 °C for 2 min, and 30 cycles of denaturation at 98 °C for 10 s, annealing and extension combined at the same temperature (68 °C) for 16 min, and were electrophoresed on a 0.8% agarose gel (Promega, USA). The long PCR products were diluted in sterilised distilled water for subsequent use as PCR templates.

We used 22 different primers pairs that amplify contiguous, overlapping segments to amplify the entire mitochondrial genome of the fishes (Table 2). Eleven primer pairs were versatile, based on the complete mitochondrial genome of six bony fish species according to Miya and Nishida (2000). The others were special for *M. asiaticus*, being designed from the sequence that had been got from the versatile primers. Then PCR was done and reactions were carried out in 25 ul reaction volume containing 2.5 ul 10×PCR buffer (Takara), 0.4 mM dNTPs, 1.8 mM $MgCl_2$, 0.2 um each primer, 1 units Taq polymerase (Takara) and 1.0 ul long PCR products as template. The thermal cycle profile was: pre-denaturation at 94 °C for 2 min, and 30 cycles of denaturation at 94 °C for 15 s, annealing at 52 °C for 15 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min. PCR products were electrophoresed on 1.0% agarose gel (Promega). Double strand PCR purified products were subsequently used for direct cycle sequencing with dye-labelled terminators (ABI). PCR primers were used for sequencing. All sequencing reactions were performed according to the manufacturer's instructions. Labelled fragments were analysed on a model MegaBACE 1000 DNA sequencer (GE Healthcare Biosciences, USA).

2.3. Sequence analysis

We analysed the complete mitochondrial genome of the 30 species listed in Table 1. The sequences were aligned using Clustal X (Thompson et al., 1997) with default parameters, and alignments were inspected by eye for any obvious misalignments. Protein-coding, rRNA, and tRNA genes of *M. asiaticus* were identified by comparison with the corresponding known sequences of other carp taxa, including *carpio* (Broughton et al., unpublished), and *carpio* (Chang et al., 1994). Only protein coding gene sequences of the mitochondrial genome were used in this study. Excluding ND3, ND4L, and ATP8, due to their small size. The nucleotide alignment for ND6 was unreliable due to an unusual substitution pattern (Russo et al., 1996; Arnason et al., 2000; Yoder and Yang, 2000), and was thus also removed from the analysis. The "saturation" at the 3rd codon positions in the protein-coding genes alleviated for the analysis by simply excluding 3rd codon positions in the protein-coding genes (e.g., Inoue et al., 2005). Remaining 6918 bp of protein-coding gene sequences were used for subsequent analyses. Aligned sequence data in NEXUS format are available from one of us (Z. P.) upon request.

2.4. Phylogenetic analysis

Phylogenetic relationships were inferred by probabilistic (maximum likelihood and Bayesian) method based on the 6918 bp of protein-coding gene sequences. Maximum likelihood (ML) trees were obtained using PHYML (Guindon and Gascuel, 2003) with 100 bootstrapped data sets. Bayesian inference was accomplished by using MrBayes, version3.0 (Ronquist and Huelsenbeck, 2003). Each Markov chain was started from a random tree and run for 10^6 generations with every 100th cycle sampled from the chain to assure independence of the samples. Model parameters were treated as unknown variables with uniform priors and were estimated as part of the analysis. We ran four chains simultaneously, three heated (temperature=0.5) and one cold, using Metropolis-coupled Markov chain Monte Carlo to enhance the mixing capabilities of the Markov chains. To check that stationarity had been reached, we monitored the fluctuating value of the likelihood and all the phylogenetic parameters graphically, and repeated each simulation two times starting from different random trees and then comparing means and variances for each model parameter. All sample points prior to reaching stationarity were

Table 2
PCR and sequencing primers for *M. asiaticus* designed from the complete mitochondrial genome of six bony fish species according to Miya and Nishida (2000)

Forward[a]	Sequence (5′ to 3′)[b]	Reverse[a]	Sequence (5′ to 3′)[b]
L709-12S	TAC ACA TGC AAG TCT CCG CA	H2009-16S	CCT AAG CAA CCA GCT ATA AC
L1969-16S	CGT CTC TGT GGC AAA AGA GTG G	H3058-16S	TCC GGT CTG AAC TCA GAT CAC GTA
L709-12S	TAC ACA TGC AAG TCT CCG CA	H3058-16S	TCC GGT CTG AAC TCA GAT CAC GTA
L3074-16S	CGA TTA AAG TCC TAC GTG ATC TGA GTT CAG	H5937-CO1	TGG GTG CCA ATG TCT TTG TG
L1969-16S	CGT CTC TGT GGC AAA AGA GTG G	S7-6-R4	AAA TGC GAT GGG CAG GGC
L4633-ND2	CAC CGC CCW CGA GCA GTT GA	H8319-Lys	CAC CWG TTT TTG GCT TAA AAG GC
L8329-Lys	AGC GTT GGC CTT TTA AGC	H10035-Gly	CTT TCC TTG GGK TTT AAC CAA G
L9655-CO3	GTA ACW TGG GCT CAT CAC AG	H13069-ND5	GTG CTG GAG TGK AGT AGG GC
L11424-ND4	TGA CTT CCW AAA GCC CAT GTA GA	H13727-ND5	GCG ATK ATG CTT CCT CAG GC
L12329-Leu	CTC TTG GTG CAA MTC CAA GT	H14080-ND5	AGG TAK GTT TTG ATT AKK CC
L13562-ND5M	TCT TAC CTA AAC GCC TGA GCC CT	H1065-12S	GGC ATA GTG GGG TAT CTA ATC CCA GTT TGT

[a] L and H denote heavy and light strands, respectively.
[b] Positions with mixed bases are labeled with their IUB codes: K=G/T; M=A/C; R=A/G; S=G/C; W=A/T; Y=C/T.

discarded as "burn in". Posterior probabilities for individual clades obtained from separate analyses were combined and summarised on a majority-rule consensus tree (Huelsenbeck et al., 2002). We used the hierarchical likelihood ration testing (hLRT) method implemented in ModelTest3.7 (Posada and Crandall, 1998) to select an appropriate model of evolution to base our maximum likelihood analysis and Bayesian inference on.

2.5. Molecular clocks

Divergence times were determined using a Bayesian approach that incorporates variation of rates of evolution among genes and among lineages (Kishino et al., 2001; Thorne and Kishino, 2002), which were estimated with MULTIDISTRIBUTE program (available from J. Thorne). This parametric approach relaxes the assumption of a strict molecular clock with a continuous autocorrelation of substitution rates across the phylogeny, and allows the use of several calibrations/time constraints. This approach involved two steps. First, ESTBRANCHES was run to estimates branch lengths from the data and a fixed tree topology using the F84+G model of sequence evolution. This allows rates to vary among sites following a discrete gamma distribution with four rate categories (Yang, 1994) along with their variance–covariance matrix. Parameters for the F84+G model were estimated using the BASEML program in PAML (Yang, 2000). Estimated parameters are presented in Table 3.

Next, the species used as an outgroup of all extant teleosts, *A. calva*, is pruned from the tree and MULTIDIVTIME is used to estimate the prior and posterior ages of branching events, their standard deviations, and the 95% credibility intervals via Markov chain Monte Carlo. The Markov chain was run for 100,000 generations and sampled every 100 generations after an initial burn-in period of 100,000 cycles. Prior gamma distributions on three parameters of the relaxed clock model were assumed and specified through the mean and SD of the root age, root rate, and rate autocorrelation; 450 Mya (SD=450 Mya) for the expected time between tips and root without node time constraints, 0.01 (SD=0.01) substitutions per site per million years for the rate at the root node, 0.4 (SD=0.4) for the parameter (v) that controls the degree of rate autocorrelation per Myr along the descending branches of the tree and 0.0 (SD=0.0) for v for a global molecular clock. To check for convergence of the MCMC, analyses were run from at least two different starting points.

Table 3
Parameters for the F84+G model estimated via maximum likelihood using PAML (Yang, 2000)

	Position	L	π_T	π_C	π_A	π_G	κ	α
Mitogenome	1	3459	0.21	0.27	0.27	0.25	2.43	0.28
Protein-coding genes	2	3459	0.40	0.28	0.18	0.14	1.07	0.16

L=number of nucleotides. π_T=empirical frequency of thymine. π_C=empirical frequency of cytosine. π_A=empirical frequency of adenine. π_G=empirical frequency of guanine. κ=transition/transversion parameter. α=shape parameter of the gamma distribution.

Table 4
Maximum (U) and minimum (L) constraints (Mya) and calibration information for nodes in Fig. 1

Node	Constraints	Calibration information
1	L 61	The osteoglossid fossil from Danian, Early Paleocene [a]
6	L 140	The chanid fossil from about 140 Mya (Poyato-Ariza, 1996)
12	L 60	The catostomid fossil from the Paleocene (Cavender, 1986)
16	L 99	The characiform fossils from the Cretaceous (Gayet, 1982)
20	L 56	The *Corydoras* fossil from the Late Palaeocene (Cockerell, 1925)
Tip-Root	U 450	The split between sarcopterygians and actinopterygians [b]

[a] Minima (L) are based on earliest occurrences in the fossil records (Benton, 1993).
[b] Calibration constrains are adopted from the reference of Hedges and Kumar (2003), and references therein.

The MULTIDIVTIME program allows for both minimum and maximum fossil constraints. Whereas minima are often based on earliest occurrences in the fossil record, maxima are intrinsically more difficult to estimate. So we used the estimated divergence time between sarcopterygians and actinopterygians (450 Mya) based on the results of Hedges and Kumar (2003) for the upper time of tip-root (Table 4, Fig. 1). Accordingly, we used the one maximum and five minimum constraints from fossil records for the divergence time estimation (Table 4, Fig. 1). We analyzed the nine protein-coding genes (with 3rd codon positions) individually and the concatenated sequences for all these protein-coding genes (without 3rd codon positions), the different codon positions were always treated as different partitions, with their heterogeneity being taken into account (e.g., Table 3).

2.6. Simulation studies

As described by Douzery et al. (2004), simulation studies were conducted to evaluate the ability of the global and relaxed clock approaches to accurately estimate divergence times when rates do correlate or not along tree branches. We generated 10 matrices of 30 taxa and 6918 bp nucleotide under SEQ-GEN (Rambaut and Grassly, 1997) by using the General-Time-Reversible (GTR) nucleotide substitution model. This model assumes a symmetric substitution matrix as well as for unequal frequencies of the four nucleotides (base frequencies)—the 0.24, 0.29, 0.18, and 0.29 base frequencies for A, T, G, and C, respectively—for a gamma distribution parameter (α=0.69) for rate heterogeneity among site, and for proportion of invariable sites (i=0.51). Each branch length was set equal to the product of its rate (i.e., the average of the rates at the nodes that begin and end it) by its time duration (i.e., the age difference between its beginning and ending nodes) as measured by MULTIDIVTIME. For each of these 10 simulated data sets, we applied the six calibration constraints and computed the Bayesian divergence times as described above. Moreover, a second set of 10 complete matrices was generated under a global clock model. The same topology, base frequencies,

gamma parameter, and distribution of node times were used, but a single (constant) substitution rate of 0.01 per sites per Myr was enforced for branch length entries. Autocorrelated-rate and constant-rate matrices were analysed under ESTBRANCHES. Then, MCMC runs were conducted under MULTIDIVTIME to estimate divergence times, assuming either rate autocorrelation along branches (mean and SD for $v \neq 0$) or a perfect clock (mean and SD for $v=0$).

3. Results

3.1. Features of the M. asiaticus mitochondrial genome

The complete L-strand nucleotide sequence of *M. asiaticus* has been registered in GenBank under the accession number AY986503. The total length of the *M. asiaticus* mitochondrial genome was 16,623 bp. The organisation of *M. asiaticus* mitochondrial genome followed that of generalised teleostean and vertebrate mitochondrial genomes. It consists of 13 protein-coding, 22 tRNA, two rRNA genes, and a control region. Most genes of this species were encoded on the H-strand, except for the ND6 and eight tRNA genes. All genes were of similar length to those in other bony fishes. The gene order was identical to that so far obtained in many other vertebrates: the protein-coding genes start with the codons ATG or GTG, and stop with codons TAA, TAG, or a T in the 1st position of a putative stop codon.

3.2. Divergence times estimate

Both maximum likelihood analysis and Bayesian inference obtained identical tree topologies under the GTR+I+G model of evolution with some nodal supporting value difference as shown in Fig. 1. Monophyly of Otocephala received 100% posterior probability and 80% bootstrap support. Monophyly of Otophysi received 100% posterior probability and 99% bootstrap support (Fig. 1).

As explained in the Material and Methods Section, divergence times for the nodes in the tree (Fig. 2) were estimated using a Bayesian relaxed molecular clock method. The partitioned Bayesian approach estimated the divergence time for Otocephala divergence at 281.5 Mya with the 95% credibility interval of 196.5–343.3 Mya, for concatenated mitogenome protein-coding gene sequence data set (without 3rd codon positions). The mean time estimates also varied little when comparisons were made of individual gene sequences (263.9–317.2 Mya) (Table 5). The results were similar to the results of 200.8 Mya for data set # 1 and 239.0 Mya for data set # 2 in Inoue et al. (2005). The divergence time for the tree root (divergence between the Osteoglossomorpha and the remaining teleost groups) is at 384.1 Mya with the 95% credibility interval of 272.6–447.2 Mya for the concatenated sequence data set. It is 346.5–390.4 Mya for individual sequence data sets (Table 5). These results were also similar to the results of 284.7 Mya for data set # 1 and 333.8 Mya for data set # 2 in Inoue et al. (2005). As expected, the concatenated gene credibility interval was considerably narrower than individual gene Bayesian estimations made by the same method (Table 5) (Thorne and Kishino, 2002; Schrago and Russo, 2003).

For the sake of comparison, we have also shown the divergence times of the other teleostean fishes used in this study (Table 6). Among these, the *Osteoglossum–Pantodon* split is of special interest because it has been intensively studied (see, e.g., Kumazawa and Nishida, 2000). Concatenated sequences assuming a relaxed clock rendered time estimates for this split at around 275.1 Mya, which is in agreement with that of Kumazawa and Nishida (2000). The global clock approach however dates the split at about 304.6 Mya (with the 95% credibility interval ranging from 231.5 to 371.9 Mya), which is somewhat older than the 259 Mya proposed by these latter authors.

Table 6
Mean and 95% credibility interval of divergence times (in Mya) for selected teleostean lineages using concatenated sequences

Lineage[a]	Relaxed clock	Global clock
Osteoglossum–Pantodon (1)	275.1 (186.2, 343.5)	304.6 (231.5, 371.9)
Carpiodes–Myxocyprinus (12)	101.1 (65.0, 141.2)	72.1 (57.9, 90.4)
Ictalurus–(*Pangasianodon* +*Cranoglanis*) (22)	86.3 (55.4, 120.1)	130.9 (99.4, 161.3)
Carassius (15)	17.0 (8.7, 29.5)	17.6 (11.2, 24.7)

[a] Numbers in parentheses indicate the node numbers in Fig. 1.

Table 5
Mean and 95% credibility interval of divergence time (in Mya) for Otocephala and the tree root estimates for individual protein-coding genes and for concatenated sequences without 3rd codon positions

Gene	Otocephala[a]		Root[a]	
	Relaxed clock	Global clock	Relaxed clock	Global clock
ATP6	317.2 (218.6, 404.0)	338.5 (248.2, 412.3)	390.4 (283.0, 447.8)	401.9 (307.1, 448.3)
COI	282.8 (195.8, 378.7)	312.1 (235.5, 389.5)	354.9 (241.8, 444.7)	392.9 (299.0, 447.6)
COII	265.2 (177.6, 369.4)	272.8 (181.5, 376.7)	358.4 (235.6, 445.6)	362.1 (239.4, 446.0)
COIII	284.1 (188.3, 384.0)	294.7 (199.4, 389.6)	357.8 (236.0, 445.5)	370.8 (251.1, 446.6)
Cyt b	285.4 (197.7, 379.0)	289.4 (290.5, 374.5)	367.3 (254.2, 446.2)	388.1 (285.5, 447.5)
ND1	272.0 (179.3, 376.7)	291.5 (194.2, 387.5)	346.5 (225.6, 444.4)	362.8 (242.1, 445.9)
ND2	292.7 (194.1, 391.7)	317.4 (215.1, 403.9)	359.7 (239.5, 445.6)	373.9 (256.6, 446.8)
ND4	263.9 (183.3, 358.8)	294.5 (199.6, 384.5)	366.1 (252.9, 445.9)	368.8 (252.1, 446.4)
ND5	274.1 (192.0, 367.7)	311.3 (211.9, 400.2)	370.1 (258.3, 446.3)	366.8 (250.8, 446.2)
Concatenated	281.5 (196.5, 343.3)	294.0 (228.4, 350.0)	384.1 (272.6, 447.2)	384.6 (299.6, 446.8)

[a] Relaxed clock and global clock stand for estimating divergence time of individual genes and concatenated sequences using Bayesian and constant rate analyses.

Table 7
Mean and 95% credibility interval of relaxed and global clock divergence time estimates (in Mya) for Otocephala and the tree root

	Clock	Simulations [a]	
		Variable rates	Constant rate
Otocephala	Relaxed	279.0 (264.2, 293.3)	193.6 (164.0, 265.4)
	Global	333.6 (323.5, 343.8)	256.7 (176.0, 324.9)
Root	Relaxed	446.9 (438.9, 449.9)	265.4 (224.0, 363.9)
	Global	448.1 (443.6, 449.9)	351.5 (241.1, 444.2)

[a] Divergence times were computed on 6918-bp-long sequences for 30 taxa, simulated under variable (autocorrelated) or constant rates of evolution. Expected divergence times are 281.5 Mya for Otocephala and 384.1 Mya for the tree root.

3.3. Global vs. relaxed clock estimates

To evaluate the accuracy and precision of dates estimated under linear versus autocorrelated rate extrapolations beyond calibrations, we conducted simulations. Table 7 recapitulates the age estimates of two selected nodes, Otocephala and the tree root, calculated under either global or relaxed clock assumptions onto data simulated with or without a constant rate of evolution. Relaxed clock timings were accurate, although slightly deeper than estimated on constant-rate data. They were less precise, as attested by 95% credibility interval width relative to global clock estimates, probably because of the greater number of parameters involved (Aris-Brosou and Yang, 2003). However, constant-rate extrapolations conducted with all other conditions being equal yield less accurate results (i.e., 1.01–1.20 deeper divergence times when actual rates do vary). The simulations indicate that a linear extrapolation on rate-variable data may overestimate divergence times, whereas the autocorrelation model of substitution-rate evolution obviously underestimates ages on clocklike data (Table 7).

4. Discussion

4.1. Evolutionary relationships among the major Otocephalan clades

The phylogenetic scenario illustrated in the cladogram obtained in the present work (Figs. 1 and 2) is highly congruent with that described in the extensive overview of Stiassny et al. (2004) on the relationships among the major gnathostome and teleost clades. The Osteoglossomorpha and Elopomorpha appear as the most basal teleost groups (Fig. 2). The 21 otocephalan species examined are grouped in a single clade, which is the sister-group of the Euteleostei. Within the Otocephala, the otophysans, i.e., Cypriniformes, Gymnotiformes, Characiformes and Siluriformes, are also grouped together, with each of these orders also appearing as monophyletic (Fig. 2). Also, the cypriniforms appear as the sister-group of the clade formed by gymnotiforms, characiforms and siluriforms, as proposed by Fink and Fink (1981, 1996).

The three points in which the cladogram illustrated in Figs. 1 and 2 differ from the more consensual scenario presented in the recent overview of Stiassny et al. (2004) are: 1) the Gonorynchiformes appear as the sister-group of the Clupeomorpha, and not of the Otophysi; 2) the Characiformes appear as non-monophyletic, with *Chalceus macrolepidotus* being more closely related to the gymnotiform species examined than to *Phenacogrammus interruptus*; 3) the Gymnotiformes therefore do not appear as the sister-group of Siluriformes (Figs. 1 and 2). However, as stressed by Stiassny et al. (2004), there are indeed still many controversies concerning the intrarelationships of the Otocephala, and the position of the Gonorynchiformes within the Otocephala and of the Gymnotiformes within the Otophysi are precisely two of these controversial topics. In fact, although gonorynchiforms have often been considered as the sister-group of Otophysi, recent studies have suggested that they could in reality be more related to the Clupeomorpha than to otophysans, as suggested in the present study (e.g., Ishiguro et al., 2002; Saitoh et al., 2003; Inoue et al., 2004). Also, as recently stressed by Diogo (2004), the phylogenetic position of the Gymnotiformes within the Otophysi is still far from being clear. Fink and Fink (1981, 1996) suggested, on the basis of a morphological phylogenetic analysis, that the Gymnotiformes and the Siluriformes form a monophyletic clade, the Characiformes being the sister-group of this clade and the cypriniforms being the most basal otophysans. This scheme has, since then, been accepted by many authors. However, most of the studies dealing with otophysan phylogeny that have been published after the works of Fink and Fink (1981, 1996) have contradicted such a siluriform–gymnotiform sister-group relationship. For example, the independent analysis promoted by Dimmick and Larson (1996) based on molecular characters, without including the morphological characters of Fink and Fink, suggested that the sister-group of the Siluriformes is a clade formed by Gymnotiformes + Characiformes, as precisely suggested in the present study. Such a scenario was also supported by Orti's (1997) analysis of the first and second codon positions of the ependymin gene, as well as by the analysis of Saitoh et al. (2003) based on mitochondrial genome. Thus, the phylogenetic results of the present study supporting a clade gonorynchiforms + clupeomorphs and a clade gymnotiforms + characiforms (Figs. 1 and 2) are, in fact, not really so surprising. They are, instead, in agreement with the results of other studies that have been published in recent years. And we also tested the alternative hypotheses using Kishino–Hasegawa (KH) test (Kishino and Hasegawa, 1989) and Shimodaira–Hasegawa (SH) test (Shimodaira and Hasegawa, 1999). The results show that the hypothesis presented and discussed in the present study still appears as the strongest one, although there is no significant difference in P value (results not shown).

4.2. Divergence time estimates using molecular rates

Which method, the global or relaxed clock better describes the current data? Estimating a time scale by the mean of age extrapolation at a deep time scale may lead to biased estimates. Whereas interpolation of date estimates is certainly less biased because evolutionary rates are bounded by zero, extrapolation could conduct to rates increasing on average when moving far back in time. Two elements suggest that our analyses have not

been affected by such a trend (see also Douzery et al., 2004). First, we plotted the posterior evolutionary rate of each node against its mean posterior age estimate as measured on our concatenated data set (Fig. 3). No trend toward a rate increase far back in time is observed. And from the plot, we can conclude that the evolutionary rate of the first codon position (0.05) evolved slightly faster than that of the second (0.02) codon positions (Fig. 3). Second, simulation studies (Table 7) indicate that, at least in the specific case of the present study, the autocorrelated rates model does not underestimate divergence times on relaxed clock sequences although it may underestimate divergence time on clocklike sequences. Therefore, in the cladogram given in Fig. 2 of the present work, we have chosen to indicate the divergence times obtained from the relaxed-clock method.

4.3. Age estimates and the fossil records

As pointed out in recent papers such as Saitoh et al. (2003), Diogo (2004), Inoue et al. (2005) and Briggs (2005), there is often a significant difference between the times of divergence of major teleostean groups obtained from molecular clocks and those indicated by the oldest fossil record of these groups. This is also with the divergence times obtained in the present work, as can be seen in Fig. 2. For the Otocephala, for example, we obtained an estimate (using the relaxed method: see above) of about 282 Mya. The oldest otocephalan fossil discovered so far is from the Late Jurassic, ca. 150 Mya (Arratia, 1997). The Otophysi appear as 251 Mya, while the oldest otophysan fossil discovered to date is from the Albian (ca. 98–112 Mya) (see Filleul and Maisey, 2004). The group formed by gonorynchiforms and clupeomorphs, which appears as the sister-group of the Otophysi, is estimated to be 270 Mya, while the oldest fossil record of this group is about 140 Mya (see Poyato-Ariza, 1996). Within the Otophysi, the estimate for cypriniforms is 183 Mya; the oldest cypriniform fossil discovered to date is from the Paleocene (ca. 54–65 Mya) (see Cavender, 1986). For gymnotiforms, the estimate obtained is 150 Mya and the oldest record fossil of the group is from the Miocene (ca. 5–23 Mya) (see Gayet and Meunier, 1991). For the group including Characiformes and Gymnotiformes, the estimate obtained is 203 Mya. According to Filleul and Maisey (2004), the oldest characiform record is from the Albian (ca. 98–112 Mya). The oldest siluriform fossil know is about 72–75 Mya (see Gayet and Meunier, 2003), the estimate obtained in the present work being 157 Mya (Fig. 2).

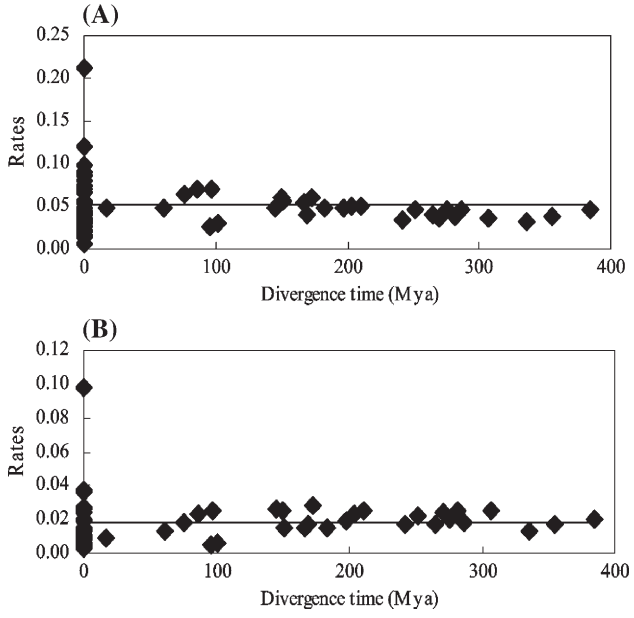

Fig. 3. Relationship between node rates and node times for (A) first, and (B) second codon positions. For each node of the chronogram, the substitution rate has been plotted against the estimated divergence times based on a relaxed clock. The mean of each rate is indicated by continuous horizontal lines.

According to Diogo (2004, 2005) and Briggs (2005), there are strong reasons to think that the appearance of most otocephalan groups may well have occurred effectively much before the direct evidence provided by the oldest fossil of these groups. Diogo (2004, 2005), based on a multidisciplinary analysis of the data available on the higher-level phylogeny, biogeographic distribution, physiology and ecology of catfishes, suggested that these fishes originated at a time when there were still some Pangean connections between Laurasia and Gondwana. That is, much before the age of the oldest catfish fossil discovered to date (ca. 72–75 Mya). A similar scenario was defended by Briggs' (2005) Fig. 6, where the origin of otophysan groups such as the cypriniforms and the siluriforms were proposed to be of Late Jurassic origin (about 150–160 Mya). The results obtained in the present work are somewhat similar to those proposed under Briggs' scenario, with the times of origin for the Cypriniformes being 183 Mya and for the Siluriformes being 173 Mya.

The substantial differences between the divergence times defended by papers such as Diogo (2004, 2005) and Briggs (2005) and by the results of the present work and those suggested by direct evidence from the fossil record lead us to an old and much discussed question: is the absence of evidence, evidence of absence? This subject has been widely discussed, and was the subject of Lundberg's (1998) overview on the temporal context of the diversification of Neotropical fishes. This author (1998: 64), referring precisely to the otophysan fishes, stated: "one possible answer, especially for the absence in the Early Cretaceous record of taxa like cichlids placed high up in the tree of acanthomorphs, is yes, they just had not evolved by then. The other possible answer perhaps more applicable to otophysans for which there is a basis for predicting a ghost-like existence, is no, some had originated and perhaps diversified but they escaped preservation or detection". One particularly elucidating empirical example given by Lundberg concerns a species from the order Siluriofrmes. *Corydoras revelatus*, a callichthyid record from the Late Palaeocene, ca. 58.5 Mya, was described more than three-quarters of a century ago by Cockerell (1925). However, despite the heavy bony armour in which callichthyid catfishes are encased, there are no subsequent fossil records of these fishes at least until 20 million years later. As stated by Lundberg (1998), one of the

problems related with paleobiogeographic hypotheses is effectively that, although palaeontologists do acknowledge, in theory, that absence of evidence does not correspond to evidence of absence, they often do refer, in practice, to the absence of evidence as evidence of absence.

Diogo (2004, 2005) has stated that the palaeontological data available for certain otocephalan groups does in fact provide indirect evidence supporting that these groups had a much older origin than what might be indicated by a direct, exclusive analysis of the oldest fossil record available for those groups. An example given by this author is that, although the oldest catfish fossil discovered to date is about 72–75 Mya, a broader analysis of the catfish fossil record shows that by the Late Cretaceous these fishes already had a worldwide distribution. This indicates, according to that author, that the origin of catfishes may have occurred much before 72–75 Mya. Moreover, the catfish fossils that were found on the various continents at that period are from phylogenetically derived groups within the order, such as the ariids, and already present a morphology similar to that of extant siluriforms. The paleobiogeographic data on other otocephalan groups does also provide interesting indirect evidence supporting that those groups might have an older origin than that indicated by a direct and exclusive analysis of their oldest fossil. For example, Poyato-Ariza (1996) described a chanid fossil, from the order Gonorynchiformes, of about 140 Mya. This is particularly remarkable, since this means that at about 140 Mya there were already fishes from an extant otocephalan family, i.e., the Chanidae. Therefore, if members of this extant gonorynchiform family were already there at about 140 Mya, this very likely means that the first Gonorynchiformes occurred well before. And, thus, that probably the very first otocephalans have appeared much before that, well before than the date (150 Mya) of the oldest otocephalan fossil discovered so far (see above). An illustrative example that the absence of palaeontological evidence does not mean evidence of absence concerns the otophysan fossil *Santanichthys diasii*. According to the recent paper by Filleul and Maisey (2004), *S. diasii* is a characiform from the Albian, being thus several million years older than the oldest characiform fossil described until the publication of their paper. According to Maisey (pers. comm.), this palaeontological discovery "make us seriously recalibrate the origin of the Otophysi and of the different otophysan orders, which might be significantly older than previously thought". This view is supported by the results obtained in the present work.

5. Conclusions

In general, the phylogenetic analysis of the present work resulted in a phylogenetic scenario that is highly congruent with that described in Stiassny's recent overview of teleost relationships (Stiassny et al., 2004). The only incongruent points are the clades Gonorynchiformes+Clupeomorpha and Gymnotiformes+Characifomes and the paraphyly of Characiformes. However, these points are in agreement with the results of other molecular phylogenetic analyses published in recent years. With respect to the divergence times obtained in the present study, they support the scenario defended in recent papers dealing with the origin and biogeography of otocephalan fishes: that the origin of the major otocephalan groups is probably much older than a direct, exclusive analysis of the oldest fossil of each of these groups might suggest. Of particular interest for biogeographical studies, the origin of all otophysan orders is estimated as having occurred before the Cretaceous (Fig. 2). As stressed by Diogo (2004, 2005) and Briggs (2005), it is likely that there were still some Pangean connections between Gondwana and Laurasia in the Late Jurassic, and perhaps even in the Early Cretaceous. If this is so, this would help to explain the Pangean distribution of cypriniforms, characiforms and siluriforms, groups of primary freshwater fishes with relatively few, and rather derived, marine members (note: although characiforms are today only found in Africa and South America, fossils of this order have been found in northern parts of the globe such as Europe—see, e.g., Diogo, 2005). It should, however, be kept in mind that hypotheses based on molecular estimates of the age of divergence between major lineages, such as those formulated in the present work, should be viewed with caution. They should be tested, and will hopefully stimulate further discussions. It is hoped that the present work will inform future phylogenetic, palaeontological, tectonic, biogeographical and molecular studies that might clarify the origin and distribution of otocephalans, and of teleostean fishes in general.

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