Evolutionary Origin and Phylogeny of the Modern Holocephalans (Chondrichthyes: Chimaeriformes): A Mitogenomic Perspective

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Abstract

With our increasing ability for generating whole-genome sequences, comparative analysis of whole genomes has become a powerful tool for understanding the structure, function, and evolutionary history of human and other vertebrate genomes. By virtue of their position basal to bony vertebrates, cartilaginous fishes (class Chondrichthyes) are a valuable outgroup in comparative studies of vertebrates. Recently, a holocephalan cartilaginous fish, the elephant shark, *Callorhinchus milii* (Subclass Holocephali: Order Chimaeriformes), has been proposed as a model genome, and low-coverage sequence of its genome has been generated. Despite such an increasing interest, the evolutionary history of the modern holocephalans—a previously successful and diverse group but represented by only 39 extant species—and their relationship with elasmobranchs and other jawed vertebrates has been poorly documented largely owing to a lack of well-preserved fossil materials after the end-Permian about 250 Ma. In this study, we assembled the whole mitogenome sequences for eight representatives from all the three families of the modern holocephalans and investigated their phylogenetic relationships and evolutionary history. Unambiguously aligned sequences from these holocephalans together with 17 other vertebrates (9,409 nt positions excluding entire third codon positions) were subjected to partitioned maximum likelihood analysis. The resulting tree strongly supported a single origin of the modern holocephalans and their sister-group relationship with elasmobranchs. The mitogenomic tree recovered the most basal callorhinchids within the chimaeriforms, which is sister to a clade comprising the remaining two families (rhinochimaerids and chimaerids). The timetree derived from a relaxed molecular clock Bayesian method suggests that the holocephalans originated in the Silurian about 420 Ma, having survived from the end-Permian (250 Ma) mass extinction and undergoing familial diversifications during the late Jurassic to early Cretaceous (170–120 Ma). This postulated evolutionary scenario agrees well with that based on the paleontological observations.

Key words: model organism, divergence time, Holocephali, Elasmobranchii, mitochondrial genome.

Introduction

The cartilaginous fishes (Class Chondrichthyes) comprising chimaeras, sharks, skates, and rays are the oldest living group of jawed vertebrates that diverged from a common ancestor of bony vertebrates (Osteichthyes: ray-finned fishes, coelacanths, lungfishes, and tetrapods) in the early Silurian about 420 Ma (Benton et al. 2009). Owing to their phylogenetic position, chondrichthyans provide a critical reference for our understanding of vertebrate genome evolution. The extant cartilaginous fishes comprising approximately 970 species (Nelson 2006) are divided into two major groups: Subclasses Holocephali (chimaeras) and Elasmobranchii (sharks, skates, and rays). Recently, one of the holocephalans, elephant shark (*Callorhinchus milii*), has attracted much attention in comparative genomics because of its relatively small genome (910 Mb) (Venkatesh et al. 2007). A low-coverage (1.4×) sequencing of the elephant shark genome and characterization of some gene loci have revealed that elephant shark has retained a higher number of ancestral vertebrate genes than bony vertebrates (Venkatesh et al. 2007; Yu et al. 2008; Larsson et al. 2009; Ravi et al. 2009). Furthermore, elephant shark and mammals were found to contain several thousand conserved noncoding elements (putative *cis*-regulatory elements) that have diverged beyond recognition in teleost fishes (Venkatesh et al. 2006; Wang et al. 2009). These findings suggest that genomes of teleost fishes may be evolving at a faster rate compared with elephant shark and mammals and underscores the importance of an outgroup, such as the elephant shark for understanding the evolution of vertebrate genomes.

The modern holocephalans are marine fishes inhabiting all the world's oceans with the exception of Arctic and

Antarctic waters (Didier 2004), with adult sizes ranging from 60 to 200 cm (Helfman et al. 2009). Most holocephalans are deep-water dwellers of the shelf and slope off continental landmasses, oceanic islands, seamounts, and underwater ridges, generally occurring at depths of around 500 m and deeper (Didier 2004). A few species inhabit shallower coastal waters, most notably *Hydrolagus colliei* and all three species of *Callorhinchus* (Didier 2004). The Chimaeriformes is the only extant order of Subclass Holocephali and the closest living relatives of Subclass Elasmobranchii (Nelson 2006), comprising only 39 extant species (Last and Stevens 1994; Compagno 2005; Didier 2008; Didier et al. 2008; Last et al. 2008) placed in six genera and three families (Didier 2004). Didier (1995) made detailed anatomical observations on the extant chimaeriforms and interpreted the morphological data in a cladistic manner. Her cladogram shows that the Rhinochimaeridae and Chimaeridae form a monophyletic group to the exclusion of the Callorhinchidae, although she questioned monophyly of the rhinochimaerids. Subsequently, no phylogenetic hypothesis of the extant chimaeriforms has been proposed based on either morphology or molecule.

The extant holocephalan species represent a small fraction of a previously successful and diverse group (Helfman et al. 2009). Fossil record revealed that the holocephalans apparently achieved their greatest diversity during the Carboniferous (359–299 Ma), and most of the descendant forms appear to have become extinct by the end of the Permian (251 Ma) (Grogan and Lund 2004). Some of the modern holocephalans should have evolved from Paleozoic ancestors (Benton 2005). Indeed, holotomography scanning of the skull and brain of a 300-My-old fossil holocephalan, iniopterygian, has suggested that the key features of the modern day holocephalan skull were established at least 300 Ma (Pradel et al. 2009). Grogan and Lund (2004) have suggested that holocephalans might have survived the end-Permian mass extinction by having sought refuge in or having adopted a deeper water lifestyle.

The whole mitogenome sequences have been shown to be useful not only for the phylogenetic analysis at higher taxonomic levels (Inoue et al. 2003; Miya et al. 2003; Zardoya et al. 2003) but also for the divergence time estimation dating back to more than 100 Ma (Pereira and Baker 2006; Kumazawa 2007; Azuma et al. 2008; Inoue et al. 2009). In this study, we assembled whole mitogenome sequences from eight holocephalans (including seven newly determined sequences) representing all the three families together with 17 other vertebrates. Unambiguously aligned mitogenomic sequences were subjected to phylogenetic analysis and divergence time estimation to reconstruct evolutionary history of the extant chimaeriforms that cannot be inferred from fossil record alone.

Materials and Methods

Taxon Sampling

We used eight species of chimaeriforms from all the three families, along with 17 other species chosen to represent major vertebrate lineages. Final rooting was done using two cyclostomes, *Petromyzon marinus* and *Lampetra fluviatilis*, based on the broadly accepted vertebrate phylogenies (Maisey 1986). The species used in this study are shown in table 1 with DNA Data Bank of Japan/European Molecular Biology Laboratory/GenBank accession numbers.

Table 1. List of the Species Used in This Study.

Classification[a]	Species	Accession Number
Agnatha		
Petromyzontidae	*Petromyzon marinus*	U11880
	Lampetra fluviatilis	Y18683
Actinopterygii		
Cyprinidae	*Cyprinus carpio*	X61010
Polymixiidae	*Polymixia japonica*	AB034826
Tetraodontidae	*Tetraodon nigroviridis*	DQ019313
Sarcopterygii		
Protopteridae	*Protopterus aethiopicus*	AB558409
Pipidae	*Xenopus tropicalis*	AY789013
Struthionidae	*Struthio camelus*	AF338715
Hominidae	*Homo sapiens*	J01415
Ornithorhynchidae	*Ornithorhynchus anatinus*	X83427
Elasmobranchii		
Heterodontidae	*Heterodontus francisci*	AJ310141
Scyliorhinidae	*Scyliorhinus canicula*	Y1606
Triakidae	*Mustelus manazo*	AB015962
Squalidae	*Squalus acanthias*	Y18134
Rajidae	*Okamejei kenojei*	AY525783
	Amblyraja radiata	AF106038
Plesiobatidae	*Plesiobatis daviesi*	AY597334
Holocephali		
Callorhinchidae	*Callorhinchus callorynchus*	HM147135
	C. capensis	HM147136
	Callorhinchus milii	HM147137
Rhinochimaeridae	*Rhinochimaera pacifica*	HM147141
	Harriotta raleighana	HM147140
Chimaeridae	*Hydrolagus lemures*	HM147139
	Chimaera monstrosa	AJ310140
	Chimaera fulva	HM147138

[a] Classifications follow Nelson (2006).

DNA Extraction, Polymerase Chain Reaction, and Sequencing

Fin clips of the specimens stored frozen or in 95% ethanol were used for DNA extraction. Total genomic DNA was extracted using the standard phenol–chloroform extraction method. In B.V.'s laboratory, the mitogenomes of 6 of the 7 holocephalan fishes (species whose mitogenome sequences have accession numbers that start with HM in table 1 excluding *Rhinochimaera pacifica*) were sequenced. We employed a modified version of the polymerase chain reaction (PCR)-based approach for sequencing the whole mitogenome sequence described in Miya and Nishida (1999). A pair of fish-versatile PCR primers, S-LA-16S-L (CGATTAAAGTCCTACGTGATCTGAGTTCAG) and H12293-Leu (TTGCACCAAGAGTTTTTGGTTCCTAAGACC), were used in a long PCR to amplify a large fragment of mitogenome (ca. 9 kb). The PCR product was gel purified and sequenced completely by the "shotgun" sequencing strategy. Then, specific primers were designed close to the ends of this sequence, and the remaining region of

the mitogenome was amplified by the long PCR. This PCR product was also completely sequenced by the shotgun sequencing strategy. The shotgun sequencing strategy comprised generation of random fragments of the PCR product by hydrodynamic shearing (Hydroshear, GeneMachines, San Carlos, CA). The ends of the fragments were filled by Klenow treatment. Fragments in the size range of 1–2 kb were gel purified and subcloned into the EcoRV site of pBluescript SK vector. Sequences of both ends of the plasmid inserts were determined using the BigDye Termination Cycle Sequencing Kit (Applied Biosystems) on an ABI 3730*xl* DNA analyzer. Sequences were processed and assembled using Phred–Phrap and Consed (www.phrap.org/phredphrapconsed.html).

Sequencing of the mitogenome of *R. pacifica* was done in M.M.'s laboratory. We followed the original version of PCR-based method developed by Miya and Nishida (1999) because all the fish-versatile PCR primers used in that study are available in M.M.'s laboratory.

Phylogenetic Analysis

The whole mitogenome sequences from the 23 gnathostomes plus two outgroups were arranged into typical gene order of vertebrates and manually aligned with the exception of the two rRNA genes. Amino acids were used for alignments of the protein-coding genes and secondary structure for alignment of tRNA genes. The two rRNA gene (12S and 16S) sequences were aligned using MAFFT (Katoh et al. 2005), and ambiguously aligned positions were manually excluded. All sequences from L-strand encoded genes (eight tRNA genes) were converted into complementary strand sequences. A total of 9,744, 1,120, and 1,793 nt positions were unambiguously aligned for the 12 protein-coding genes (except for ND6), 22 tRNA genes, and 2 rRNA genes, respectively (total 12,657 positions). The third codon positions of the protein-coding genes were excluded from the data set because of extremely high substitution rates (and the resulting multiple hits) and heterogeneous base composition as sources of systematic noise in the phylogenetic analysis at this taxonomic level (Miya and Nishida 2000; Broughton 2010). Consequently, 9,409 nt positions were available for phylogenetic analysis (hereafter called 12TR data set). To complement results based on the nucleotide sequences, 12 protein-coding genes were translated into amino acids, and they were concatenated to the aligned tRNA and rRNA gene sequences (total 6,161 positions; hereafter called ATR data set). For divergence time estimation using the latter data set, we had to exclude nucleotide sequences from the tRNA and rRNA genes because MCMCTREE (Yang 2007) does not accept a mixed data set (hereafter called AA data set). These three aligned sequences are available in supplementary file 1 (Supplementary Material online).

We set four partitions for these unambiguously aligned nucleotide sequences (12TR data set), assuming that functional constraints on sequence evolution are more similar within codon positions (or types of molecule) across genes than across codon positions (or types of molecule) within genes, at least for a set of mitochondrial genes. Actually, this partitioning scheme (codon positions + type of molecule) resulted in the largest improvement of the likelihood scores compared with the gene-by-gene partitioning in the phylogenetic analysis of pufferfish mitochondrial genomes (Yamanoue Y, unpublished data) and that of ten nuclear protein-coding genes from various actinopterygians (Li et al. 2008). We set only three partitions for the ATR data set to avoid overparameterization through gene-by-gene partitioning for the 12 protein-coding genes.

The aligned sequences were subjected to maximum likelihood (ML) analysis using RAxML ver. 7.2.6 (Stamatakis 2006). Although Modeltest (Posada and Crandall 1998) selected general time reversible (GTR) + Γ + I (Yang 1994) as the optimum models of sequence evolution based on AIC criterion for all partitions from the 12TR data set, we did not use "I" (proportion of invariant sites) because the parameter is sensitive to the number and divergence of sequences included in the data set (Yang 2006). For amino-acid sequences in the ATR data set, we used MTREV replacement matrix (Adachi and Hasegawa 1996), with stationary amino acid frequencies estimated from the data set and four categories of gamma-distributed rates across sites.

We used the topological constraint tree option (–f g) enforcing monophyly of the bony vertebrates (Osteichthyes) because analyses without that constraint yielded unusual trees that are inconsistent with the conventionally accepted relationships among major vertebrate lineages (Maisey 1986; Kikugawa et al. 2004). We confirmed that the topological constraint did not affect ingroup relationships of the chimaeriforms as well as the relationship between chimaeriforms and elasmobranchs. We performed a rapid bootstrap (BS) analysis with 1,000 replications (–f a option). This option performs BS analysis using GTRCAT, which is GTR approximation with optimization of individual per site substitution rates and classification of those individual rates into certain number of rate categories. After implementing the BS analysis, the program uses every fifth BS tree as a starting point to search for the ML tree using a specified model of sequence evolution and saves the top 10 best-scoring ML trees (fast ML searches). Finally, RAxML calculates more accurate likelihood scores (slow ML searches) for those ten trees and puts BS probabilities on the best-scoring ML tree.

For the 12TR data set, probabilities of alternative phylogenetic hypotheses were calculated using the likelihood-based approximately unbiased (AU) test (Shimodaira 2002) as implemented in CONSEL v.0.1k (Shimodaira and Hasegawa 2001). No such test was available for the mixed data set (ATR data set).

Divergence Time Estimation

A relaxed molecular clock Bayesian method implemented in MCMCTREE program in PAML 4.4 (Yang 2007) was used for dating analysis. The constrained best-scoring ML tree from the 12TR data set was used for divergence time estimation, but two cyclostomes were excluded from the analysis because MCMCTREE does not require outgroups

Table 2. Maximum (U) and Minimum (L) Time Constrains (Ma) on Nodes Used for Dating in figure 2.

Number	Constraints	Divergence
1	U 463[a]	Osteichthyes/Chondrichthyes
	L 422[a]	
2	U 422[a]	Actinopterygii/Sarcopterygii
	L 416[a]	
3	L 150[a]	Cypriniformes/Tetraodontiformes
6	U 350[a]	Amphibia/Amniota
	L 330[a]	
7	U 330[a]	Aves/Mammalia
	L 312[a]	
8	U 191[a]	Prototheria/Theria
	L 163[a]	
9	L 410[b]	Elasmobranchii/Holocephali
10	L 190[c]	Batoidea/Selachii
11	L 176[c]	Plesiobatidae/Rajidae
14	L 176[c]	Heterodontidae/Triakidae
15	L 165[c]	Scyliorhinidae/Triakidae
16	L 161[d]	Callorhinchidae/Chimaeridae
19	L 84[e]	Rhinochimaeridae/Chimaeridae

[a] Benton et al. (2009).
[b] Coates and Sequeira (2001).
[c] Heinicke et al. (2009).
[d] Ward and Duffin (1989).
[e] Cappetta et al. (1993).

and works with sequence data from the ingroup species only. The ML estimates of branch lengths were obtained using BASEML and CODEML (in PAML) programs under the GTR + Γ and MTREVF + Γ substitution models for the 12TR and AA data sets, respectively, with the gamma priors set at 0.5. Two priors, the overall substitution rate (rgene gamma) and rate-drift parameter (sigma2 gamma), were set at G (1, 20) and G (1, 4.5) for the 12TR data set and G (1, 14.3) and G (1, 4.5) for the AA data set, respectively, using the strict molecular clock assumption with 443 Ma constraint (an average of the upper and lower constraints for the node; see table 2) to the divergence between Osteichthyes and Chondrichthyes. The independent rates (IRs) model (Rannala and Yang 2007) 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 correlated rates model in recent studies (Zhong et al. 2009). The parameters of the birth–death process for tree generation with species sampling (Yang and Rannala 1997) were fixed at $\lambda = \mu = 1$ and $\rho = 0$, so that the priors are similar to those used in previous mitogenomic studies (Azuma et al. 2008; Setiamarga et al. 2009) using MULTIDIVTIME (Thorne and Kishino 2002). A loose maximum bound for the root was set at <10.0 (= 1,000 Ma).

The MCMCTREE program allows for minimum (lower) and maximum (upper) time constraints, and it has been argued that multiple calibration points would provide overall more realistic divergence time estimates (Benton and Donoghue 2006). We therefore sought to obtain an optimal phylogenetic coverage of calibration points across our tree, although we could set maximum constraints based on fossil records only for the five nodes (table 2). Other than those five well-established nodes, eight additional nodes were reasonably chosen to constraint their minimum ages only (total 18 time constraints for 13 nodes; table 2). 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 they are small but not zero for the maximum bounds (Yang and Rannala 2006). All time constraints are provided with a unit of 100 Ma (i.e., 1 = 100 Ma) because some of the model components in the Bayesian analysis are scale variant and the node ages should fall between 0.01 and 10 (Yang 2007). Those calibration nodes with minimal (lower) bound only was set as L (t_{min}) and with both minimal and maximal bounds set as B (t_{min}, t_{max}). The former setting (L) assumes a heavy-tailed density based on a truncated Cauchy distribution of $P = 0.1$ and $c = 1$ as the default (Yang 2007).

Markov chain Monte Carlo (MCMC) approximation with a burn-in period of 50,000 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, at least two replicate MCMC runs were performed with two different random seeds for each analysis. Also distributions of parameter values from MCMC samples were visualized using Tracer 1.5 (available from http://tree.bio.ed.ac.uk/software/tracer/) to check mixing, choose a suitable burn-in, and look for trends that might suggest problems with convergence. The number of samples (10,000) was large enough to reach effective sample sizes (>200) for all parameters estimated in this study.

Results and Discussion

Genome Organization

The contents of mitogenomes from the seven species of holocephalans sequenced in this study include 2 rRNA, 22 tRNA, and 13 protein-encoding genes, plus the putative control region, as found in other vertebrates. Their gene arrangements are identical to those of the typical vertebrates, with total lengths ranging from 16,758 bp (*Callorhinchus callorynchus*) to 24,889 bp (*R. pacifica*). Of the known 1,343 mitogenome sequences from gnathostomes (as of 21 April 2010), the latter represents the second longest mitogenome following that of prickly gecko *Heterotopias binge* (25,972 bp) (NCBI Organelle Genome Resources: http://www.ncbi.nlm.nih.gov/genomes/OrganelleResource.cgi?taxid=7776).

The long mitogenomes of the holocephalans are due to an extremely long noncoding region between the $tRNA^{Thr}$ and $tRNA^{Pro}$ genes that are located adjacent to each other in most vertebrates. Of the eight chimaeriforms, the five species from the two derived families (Rhinochimaeridae and Chimaeridae) share this long noncoding region (1,534–7,999 bp). Arnason et al. (2001) first found this intergenic spacer in *Chimaera monstrosa* (Chimaeridae), and the present finding suggests that it represents a molecular synapomorphy that was originated in a common ancestor of the two families (see below). A similar (but considerably

shorter) intergenic spacer between $tRNA^{Thr}$ and $tRNA^{Pro}$ genes (26–99 bp) was found in various vertebrates, such as the toad (Roe et al. 1985), ostrich (Harlid et al. 1997), and cods (Bakke et al. 1999), and much longer one (244–688 bp) has been reported in species of salamanders (McKnight and Shaffer 1997).

Arnason et al. (2001) also located the actual control region downstream of the $tRNA^{Pro}$ gene by detecting two copies of conserved sequence block 2 (CSB-2) and one of CSB-3. We confirmed their observation among mitogenomes from the rest of the four chimaerids and rhinochimaerids.

Phylogenetic Relationships

By assuming monophyly of the Osteichthyes (Actinopterygii + Sarcopterygii; an arrowhead in fig. 1), partitioned ML analysis using only nucleotide sequences (12TR data set) recovered a conventional tree topology of major vertebrate lineages (fig. 1) (Maisey 1986; Kikugawa et al. 2004). Three species of the actinopterygians and four species of the sarcopterygians form reciprocal monophyletic groups, and they are together sister to the Chondrichthyes (Holocephali + Elasmobranchii). Monophyly of the chondrichthyans as well as that of the two subgroups (holocephalans and elasmobranchs) are recovered with 100% bootstrap probabilities (BPs), consistent with the well-established morphology-based hypotheses (Maisey 1984, 1986; Grogan and Lund 2004). Molecular studies have not been designed to specifically address these relationships, although recent studies based on a broad taxon sampling have supported the monophyly of these groups (Arnason et al. 2001; Mallatt and Winchell 2007).

Partitioned ML analysis of the mixed data set (amino-acid sequences from the 12 protein-coding genes plus nucleotide sequences from the tRNA and rRNA genes; ATR data set) recovered an identical tree topology except for the placement of *Protopterus* (lungfish). Actually, the lungfish, one of the sarcopterygians, was placed at the most basal position in the actinopterygians with 66% BP (supplementary fig. S1, Supplementary Material online), and this phylogenetic position is inconsistent with the broadly accepted phylogeny of vertebrates (Maisey 1986; Kikugawa et al. 2004). Thus, our subsequent discussions will be focused on the results based on the nucleotide sequences (12TR data set).

Without the constraint of osteichthyan monophyly, "fishes" (chondrichthyans, actinopterygians, *Protopterus*) form a monophyletic group, which is sister to tetrapods (results not shown) as demonstrated in previous mitogenomic studies (Rasmussen and Arnason 1999; Arnason et al. 2001). It appears that the extremely long branch from the outgroups (two lampreys) attracts an incorrect internal branch of the gnathostome phylogenies because an unrooted tree without those two lamprey is congruent with the conventional vertebrate phylogenies. Despite seemingly incorrect rooting position in the unconstrained phylogenies, monophyly of the chondrichthyans as well as that of the subgroups (holocephalans and elasmobranchs) are confidently reproduced with 100% BPs. It is expected that bisection of the long internal branch from the outgroups by distantly related cyclostomes (hagfishes) would result in more reasonable vertebrate phylogenies outside the chondrichthyans.

Within the holocephalans, each of the three families (Callorhinchidae, Rhinochimaeridae, Chimaeridae) is recovered as monophyletic with 100% BP and the latter two families (rhinochimaerids and chimaerids) form a monophyletic group to the exclusion of the callorhinchids (fig. 1). All internal nodes are supported by 100% BPs except for a sister-group relationship between *Callorhinchus milli* and *Callorhinchus. capensis* (88%).

Didier (1995) made extensive anatomical observations on all extant chimaeriform genera and showed a cladogram depicting her cladistic interpretation of the morphological data. As in the mitogenomic tree in figure 1, callorhinchids were placed as the most basal position, although the three lineages comprising *Rhinochimaera*, *Neoharriotta* + *Harriotta* (Rhinochimaeridae), and *Chimaera* + *Hydrolagus* (Chimaeridae) formed a trichotomy in her cladogram because of the uncertain phylogenetic position of the two rhinochimaerids (*Neoharriotta* + *Harriotta*) with respect to the other genera. Although we were unable to collect *Neoharriotta* in this study, the mitogenomic tree strongly supports monophyly of the rhinochimaerids and its sister-group relationship with the chimaerids with 100% BP (fig. 1).

The interrelationships among the holocephalan families have not yet been addressed with molecular data. However, in their study of elasmobranchs based on the mitochondrial rRNA genes, Douady et al. (2003) sampled five species of the holocephalans as outgroups in one of the data sets and reported that a rhinochimaerid was embedded within chimaerids, rendering the latter family paraphyletic. Strangely, Blast searches of their downloaded sequence from *R. pacifica* (AF288203) show 99.4% matches against that of *C. monstrosa* (AJ310140), whereas the former matches only 93.7% against the same species used in this study. The tissue sample used in this study was taken from a neotype of the species designated by experts (Didier and Nakaya 1999) with a voucher specimen (CBM-ZF 6140). Therefore, authenticity of the sequence used in Douady et al. (2003) is dubious, and our phylogenies depicted in figure 1 should be more reasonable and reliable.

Extremely short terminal branches from the three species of *Callorhinchus* are notable because they exhibit disjunctive geographic distributions along coastal waters of the three continents in the southern hemisphere (*C. callorynchus* from southern South America, *C. capensis* from southern Africa, and *C. milli* from New Zealand and southern Australia) (Didier 2004). Actually, the K2P distances (Kimura 1980) calculated from "barcoding" sequences (partial sequences from the COI gene; 655 bp) for the three species range from 2.03% to 3.31%, falling between the reported mean distances of within-species (0.39%) and within-genera (9.93%) from Australian fishes (Ward et al. 2005). Such marginal genetic differentiations are reflected in their morphology, and Didier (2004) even stated that they are morphologically nearly indistinguishable.

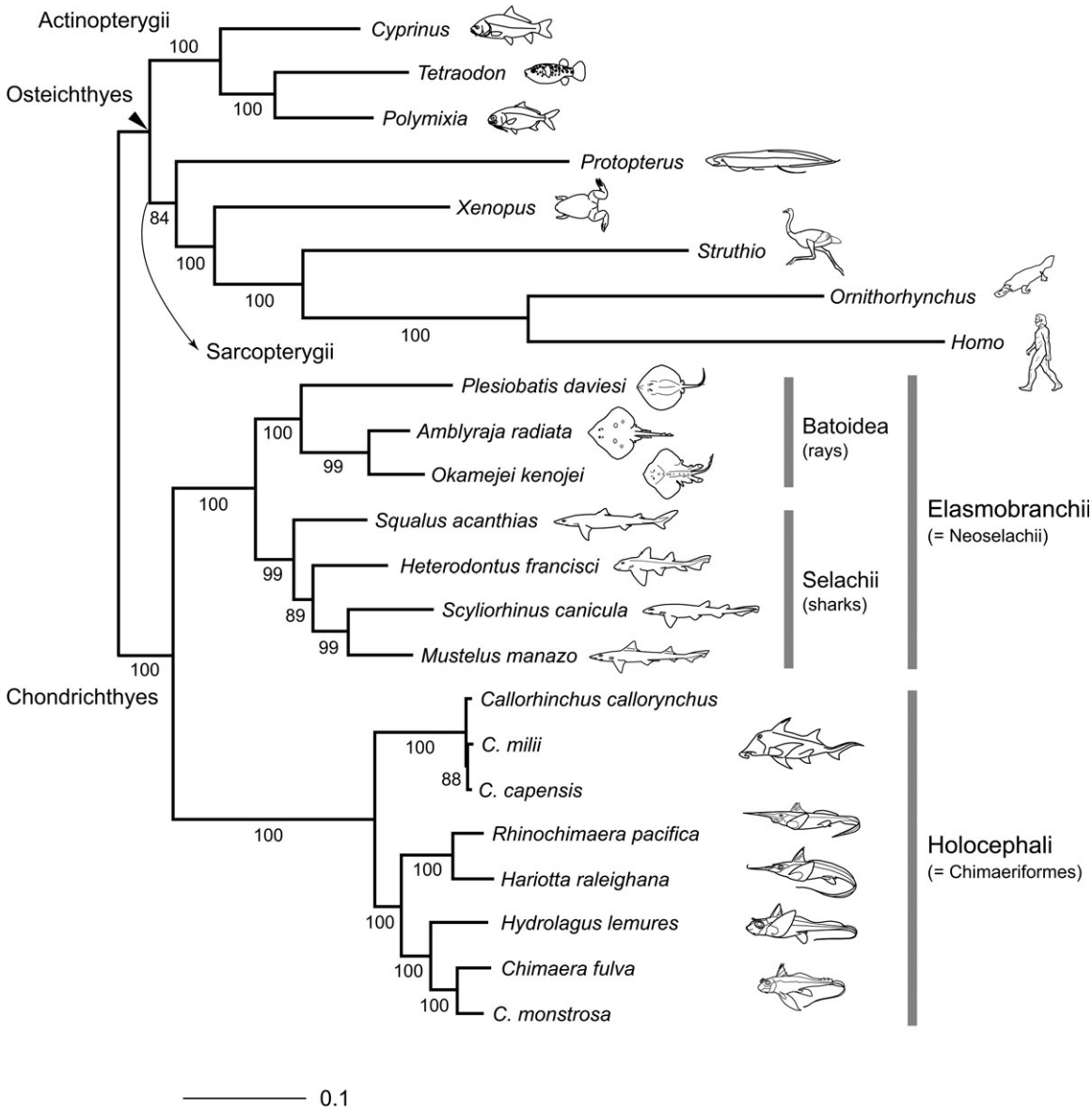

Fig. 1. The best-scoring ML tree derived from the 12TR data set from 25 vertebrates (total 9,409 positions excluding entire third codon positions). An arrowhead denotes a node enforcing monophyly of the bony vertebrates (Osteichthyes) using a −f g option in RAxML. Numeral beside internal branches indicate BPs based on 1,000 replicates. Scale indicates expected number of substitution per site. Extremely long branches from the two outgroups (lampreys) are deleted.

It should be noted that the mitogenomic tree strongly supports a basal divergence between sharks and rays with 100% BP (fig. 1). This is consistent with the traditional view based on morphology (Bigelow and Schroeder 1948) as well as the results from recent molecular phylogenetic studies based on comprehensive character and taxon sampling (Douady et al. 2003; Maisey et al. 2004; Winchell et al. 2004; Mallatt and Winchell 2007) but inconsistent with the recent morphological studies that support a position for rays deeply nested within nongaleomorph sharks called "Squalea" (Shirai 1992, 1996; de Carvalho 1996). When the latter hypothesis (monophyly of *Squalus* + rays) is constrained in tree searches, a likelihood difference between the unconstrained (fig. 1) and constrained best-scoring ML tree is large enough (73.912) to statistically reject the morphology-based hypothesis (AU test, $P < 0.000$). Interestingly, a recent molecular phylogenetic study on trypanorhynch tapeworms—abundant groups of metazoan parasites of elasmobranchs—also supports independent lineages of sharks and rays (Olson et al. 2010). In their study of the early diversification of neoselachians (modern sharks and batoids) based on the fossil record, Kriwet et al. (2009) stated that the molecular phylogenies (basal divergence of sharks and rays) are more congruent with the fossil record, whereas the phylogenetic hypotheses based on morphological characters (de Carvalho 1996; Shirai 1996) require long ghost lineages to be congruent with the fossil record. Thus, independent lines of molecular evidence from elasmobranchs and their parasites plus the fossil record together provide additional support for the basal

divergence between sharks and rays, a more traditional view in elasmobranch systematics before the emergence of cladistic methodology (Bigelow and Schroeder 1948).

Substitution Rate Difference

As clearly seen in figure 1, ML estimates of the branch lengths from the root to tips among chondrichthyans are similar and they are remarkably shorter than those of the sarcopterygians (particularly those of mammals). Actually, the mean branch length of chondrichthyans (0.28 ± 0.032 substitution per site) is significantly shorter ($P = 0.0069$) than that of sarcopterygians (0.65 ± 0.17). A previous estimate of substitution rates in mitogenomes of cartilaginous fishes and mammals using two mitochondrial protein-coding gene sequences (cytochrome *b* and COI) has shown that the nucleotide substitution rate in sharks is seven- to eight-fold slower than in mammals (Martin et al. 1992). Likewise, analysis of amino acid sequences of the cytochrome *b* and ND2 genes from cartilaginous fishes and mammals has shown that the evolutionary rate of amino acid sequences in cartilaginous fishes is much slower than in mammals (Kumazawa et al. 1999). Our analysis of whole mitogenomes provides further support that tetrapods in general have a higher substitution rate than cartilaginous fishes.

Divergence Time Estimation

Overall MCMCTREE analysis of the divergence times based on the 12TR (nucleotides only) and AA (amino acids only) data sets provides similar results, with an average of 11.5 My younger ages in the former estimation. Considering the long evolutionary history of vertebrates (>400 My), these differences are minor, and we consider that our estimations of the node ages are relatively robust against the data treatment. For simplicity and comparisons with other studies, our subsequent discussions will be focused on the results based on the nucleotide sequences (12TR data set). Estimated node ages based on AA data set can be found in supplementary table S1 (Supplementary Material online).

MCMCTREE analysis of the divergence times with the assumption of IRs across nodes indicates that an ancestral lineage of the modern holocephalans diverged from a common ancestor of the elasmobranchs during the late Silurian about 421 Ma (fig. 2) with a 95% credible interval of 410–447 Ma (table 3). Coates and Sequeira (2001) estimated that holocephalans and elasmobranchs have diverged by 410 Ma on the basis of the earliest fossil assignable to the Chondrichthyes from the Silurian (444–416 Ma) and subsequent diversification of many representatives of extinct groups during the Devonian (416–359 Ma). Our molecular estimate agrees well with that based on the fossil records despite the poorly fossilized cartilaginous skeleton of chondrichthyans (Maisey et al. 2004).

Recently, Heinicke et al. (2009) assembled published nucleotide sequences of the nuclear RAG1 and mitochondrial 12S/16S rRNA genes from 53 of 55 families of the chondrichthyans and concatenated these sequences in a single data set to perform divergence time estimation using MULTIDIVTIME (Thorne and Kishino 2002). Note that although 53 of 55 families are represented in their study, relatively few families are represented by all three genes as a consequence of concatenating the data from different studies with few overlapping taxa. Unlike our molecular estimation, their study provided much older age for the divergence between holocephalans and elasmobranchs (471 Ma with a 95% credible interval of 434–494 Ma). This difference may be due partly to a lack of time constraints for the divergence between Osteichthyes and Chondrichthyes in Heinicke et al. (2009). Indeed, the estimated node age for the divergence between holocephalans and elasmobranchs without the upper and lower time constraints became ~30 My older than that with the time constraints (results not shown). Following Benton et al. (2009), we set 422 Ma for minimum and 463 Ma for maximum ages for this divergence (table 2), and these two constraints are likely to stabilize the adjacent younger node in an effective manner (node number 9 in fig. 2).

To investigate the effects of rate heterogeneity on divergence time estimation (most notably 2–3 times higher substitution rates in bird and mammals compared with fishes; fig. 1), we also performed MCMCTREE analysis without sequences from these three amniotes (*Struthio*, *Ornithorhynchus*, and *Homo*). The resulting estimated node ages were generally younger than those of the above estimation, although the differences were relatively small, with an average of −4.9 My. Thus, we consider that the effects of remarkable rate heterogeneity on the divergence time estimation are minimal, at least with the present data set, priors, and analytical method.

Based on the fossil record, Grogan and Lund (2004) suggested that the holocephalans apparently achieved its greatest diversity during the Carboniferous (360–300 Ma), and most of the descendant forms appear to have become extinct by the end of the Permian about 250 Ma. Grogan and Lund (2004) also stated that all extant forms can be traced to *Eomanodon* from the mid-Jurassic deposit about 161 Ma (Ward and Duffin 1989). Therefore, there are no fossil records referable to the modern holocephalans for nearly a 100 My from the end-Permian to the Jurassic. Stahl (1999) even argued that the modern chimaeroids are not likely to share a direct ancestry with the Paleozoic forms because it is unlikely for lineages to persist for such an extended period of time. Grogan and Lund (2004) disagreed with her idea and argued that a lack of evidence does not equate as evidence of extinction or loss. Instead, they speculated that some holocephalans might have survived by having sought refuge in or having adopted a deeper water lifestyle. According to their theory, any remains of these forms would, necessarily, have a very low probability of preservation and recovery due to inaccessibility, lower potential of fossilization, and loss due to subductive forces acting on the ocean floor (Grogan and Lund 2004).

Our timetree agrees well with the hypothesis of Grogan and Lund (2004). Actually, a common ancestral lineage of the modern holocephalans originated in the late Silurian about 420 Ma, having survived from the end-Permian

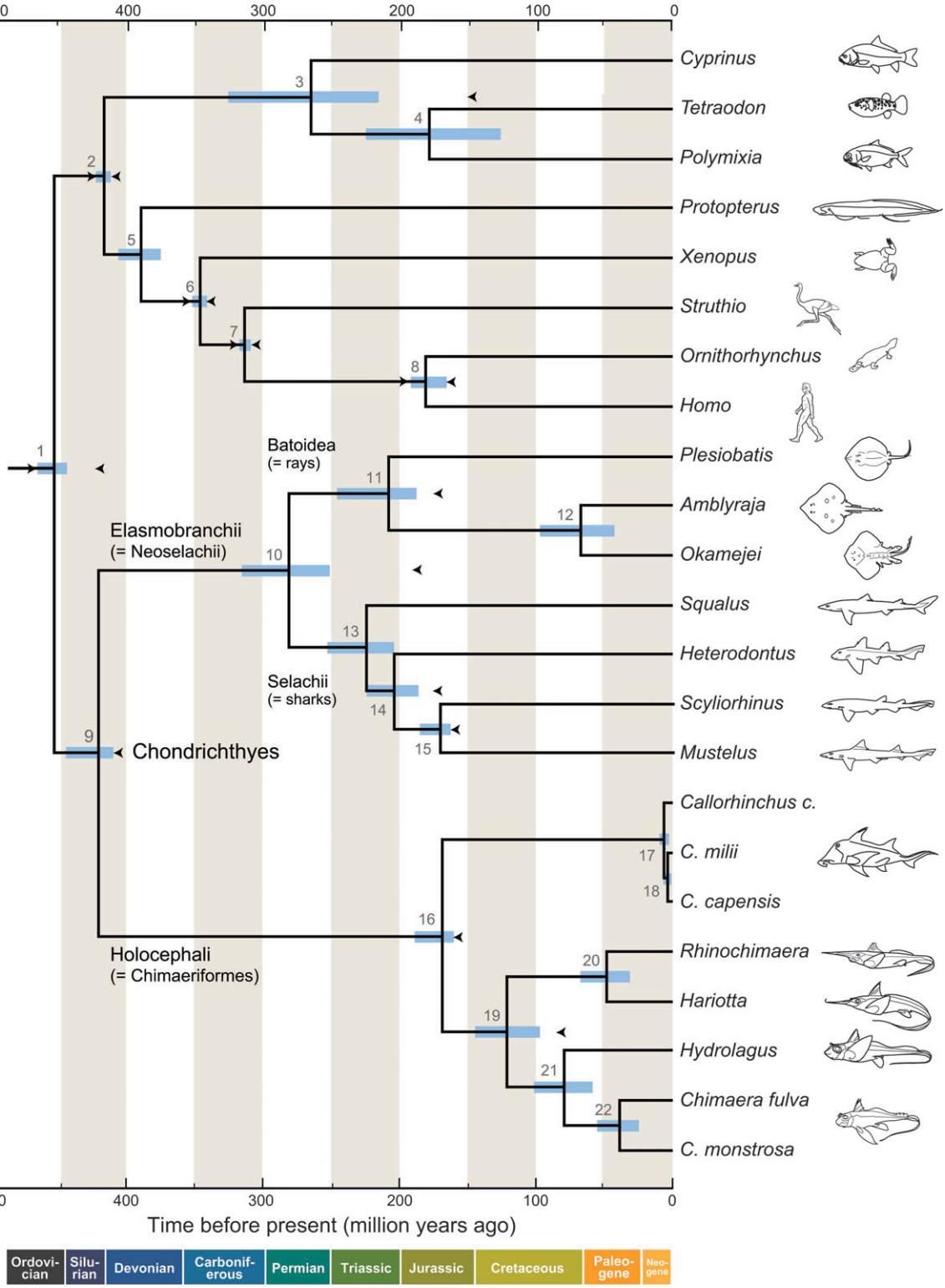

FIG. 2. Timetree derived from the relaxed molecular clock method implemented in MCMCTREE in PAML 4.4 (Yang 2007). A total of 13 nodes used for the time constraints are indicated by arrowheads (see table 2). Horizontal bars indicate 95% credible intervals of the divergence time estimates. All estimated ages and their 95% credible intervals are listed in table 3 with node numbers.

(250 Ma) and end-Triassic (200 Ma) mass extinction events without leaving any extant lineages until the first divergence 167 Ma (fig. 2). This is also consistent with the suggestion of Pradel et al. (2009) who observed that the skull and brain of fossil chimaeroid (iniopterygian) shares many unique features with living holocephalans and argued that the holocephalan specializations may be traced back to as early as Devonians (416–360 Ma).

Survival of the common ancestor of the modern holocephalans through the consecutive mass extinction events

Table 3. Comparisons of Divergence Time Estimates (posterior mean and 95% credible interval in Ma).

Node	This Study	Heinicke et al. (2009)
1	457 (443–464)	—
2	419 (416–422)	—
3	264 (217–325)	—
4	177 (128–226)	—
5	392 (375–408)	—
6	349 (341–351)	—
7	314 (312–323)	—
8	184 (166–192)	—
9	421 (410–447)	471 (434–494)
10	281 (251–318)	393 (354–431)
11	209 (178–247)	124 (98–159)
12	66 (43–97)	—
13	225 (204–253)	350 (309–392)
14	203 (188–228)	318 (279–359)
15	169 (165–184)	227 (195–261)
16	167 (161–190)	220 (125–320)
17	6.0 (3.7–9.2)	—
18	3.7 (1.9–6.2)	—
19	122 (98–146)	107 (51–182)
20	47 (31–68)	—
21	79 (59–102)	—
22	39 (26–56)	—

leads to the subsequent familial diversification during the Mesozoic. It is estimated to have diverged into the callorhinchids and the remaining two families in the late Jurassic 167 Ma (161–190 Ma), followed by the divergence into rhinochimaerids and chimaerids 122 Ma (98–146 Ma). These estimates are roughly concordant with those of the recent molecular study (Heinicke et al. 2009). Posterior distributions of their estimated ages, however, involved large credible intervals, owing apparently to short sequences and numerous missing positions resulting from the data concatenation from different studies. In their study, the above two familial divergences of holocephalans were estimated to be 220 Ma (125–320 Ma) and 107 Ma (51–182 Ma), respectively (table 3).

Finally, estimated divergence times of the elasmobranchs should be mentioned, although this study is not designed to address this issue. All neoselachians (modern sharks and batoids) before the Jurassic had been known from isolated teeth (Underwood 2006), and the first definite neoselachians was known from the Middle Triassic (Anisian) ~240 Ma, being represented by teeth of a synechodontiform *Mucrovenator* (Cuny et al. 2008). Kriwet et al. (2009) argued that no lineages of "living" neoselachian were present in the Late Triassic and that they originated in the Early Jurassic around 195 Ma (Maisey et al. 2004; Kriwet and Klug 2008). Recently, however, Klug (2010) performed cladistic analysis of the extinct †Synechodontiformes plus neoselachians and found that the former is sister to all living sharks. Consequently, the concept of neoselachian systematics needs to be enlarged to include this completely extinct group, which is considered to represent stem-group neoselachians. Thus, origin of modern sharks can be traced back into the Late Permian (250 Ma) based on the fossil record of †Synechodontiformes (Klug 2010).

In our timetree, the basal divergence between sharks and rays is estimated to have occurred during the Permian 281 Ma (251–318 Ma; fig. 2), ~90 My earlier than the age of unambiguous fossil record referable to the modern order (195 Ma) but more concordant with the age of stem-group neoselachians (250 Ma) represented by †Synechodontiformes (Klug 2010). It is argued that the most probable age of the divergence should be always older than the fossil minimum because the acquisition of the apomorphies will postdate the actual divergence (Steiper et al. 2008). It should be noted, however, our estimate of the basal divergence between sharks and rays (281 Ma) is >100 My younger than that of the independent molecular estimate (393 Ma) by Heinicke et al. (2009). Thus, our significantly younger estimate requires a shorter ghost range in the fossil record for early divergences within neoselachians.

Supplementary Material

Supplementary file 1, table S1, and figure S1 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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