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A mitogenomic perspective on the phylogeny and biogeography of living caecilians (Amphibia: Gymnophiona)

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ABSTRACT

The caecilians, members of the amphibian Order Gymnophiona, are the least known Order of tetrapods, and their intra-relationships, especially within its largest group, the Family Caeciliidae (57% of all caecilian species), remain controversial. We sequenced thirteen complete caecilian mitochondrial genomes, including twelve species of caeciliids, using a universal primer set strategy. These new sequences, together with eight published caecilian mitochondrial genomes, were analyzed by maximum parsimony, partitioned maximum-likelihood and partitioned Bayesian approaches at both nucleotide and amino acid levels, to study the intra-relationships of caecilians. An additional multiple gene dataset including most of the caecilian nucleotide sequences currently available in GenBank produced phylogenetic results that are fully compatible with those based on the mitogenomic data. Our phylogenetic results are summarized as follow. The caecilian family Rhinatrematidae is the sister taxon to all other caecilians. Beyond Rhinatrematidae, a clade comprising the Ichthyophlidae and Uraeotyphlidae is separated from a clade containing all remaining caecilians (Scolecomorphidae, Typhlonectidae and Caeciliidae). Within this large clade, Scolecomorphidae is the sister taxon of Typhlonectidae and Caeciliidae but this placement did not receive strong support in all analyses. Caeciliidae is paraphyletic with regard to Typhlonectidae, and can be divided into three well-supported groups: Caeciliidae group 1 contains the African caeciliids *Boulengerula* and *Herpele*; Caeciliidae group 2 contains *Caecilia* and *Oscaecilia* and it is the sister taxon of Typhlonectidae; Caeciliidae group 3 comprises the remaining species of caeciliids. The mitochondrial genome data were also used to calculate divergence times for caecilian evolution using the penalized likelihood method implemented in the program R8S. The newly obtained dating results are compatible with (but a little older than) previous time estimates mainly based on nuclear gene data. The mitogenomic time tree of caecilians suggests that the initial diversification of extant caecilians most probably took place in Late Triassic about 228 (195–260) Ma. Caeciliids currently distributed in India and the Seychelles diverged from their African and American relatives most probably in Late Jurassic about 138 (112–165) Ma, fairly close to the time (~130 Ma) when Madagascar–India–Seychelles separated from Africa and South America. The split between the Indian caeciliid *Gegeneophis* and Seychellean caeciliids occurred about 103 (78–125) Ma, predated the rifting of India and the Seychelles (~65 Ma).

1. Introduction

Caecilians (Gymnophiona), together with frogs and toads (Anura) and newts and salamanders (Caudata), constitute the three living orders of the Class Amphibia. They are readily distinguished from frogs and salamanders by their elongate, annulate and limbless body form. Caecilians are found in most of the tropical regions of South-East Asia, Africa, the Seychelles islands and Central and South America. They have a primarily terrestrial, surface-cryptic or burrowing lifestyle as adults, except for the Typhlonectidae, a South American group that is secondarily aquatic or semiaquatic. Because of their secretive habits, caecilians are usually not frequently observed in the wild and many aspects of their biology are poorly known.

There are currently 33 genera and 176 caecilian species recognized, grouped into six families: Caeciliidae, Ichthyophiidae, Rhinatrematidae, Scolecomorphidae, Typhlonectidae and Uraeotyphlidae (AmphibiaWeb, 2009). The broad outlines of caecilian phylogeny were established largely based on analyses of morphological and life-history data (Nussbaum, 1977, 1979; Duellman and Trueb, 1986; Nussbaum and Wilkinson, 1989; Wilkinson and

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Nussbaum, 1996, 2006) and molecular data (Hass et al., 1993; Hedges and Maxson, 1993; Hedges et al., 1993; Wilkinson et al., 2002, 2003; San Mauro et al., 2004; Frost et al., 2006; Roelants et al., 2007). In most of those analyses, Caeciliidae, Typhlonectidae and Scolecomorphidae were put into a group informally known as higher caecilians, formalized by Wilkinson and Nussbaum (2006) as the Teresomata, and Uraeotyphlidae, Ichthyophiidae (sometimes Uraeotyphlidae + Ichthyophiidae as a whole unit) and Rhinatrematidae were recovered as successively more distant outgroups to the higher caecilians. Wilkinson and Nussbaum (2006) designated the Ichthyophiidae + Uraeotyphlidae as the Diatriata, and the five non-rhinatrematid families as the Neocaecilia. However, major problems remain. For example, the largest family of caecilians, the Caeciliidae, which includes 101 of the 176 currently recognized species (AmphibiaWeb, 2009), is probably paraphyletic with respect to the Typhlonectidae and possibly the Scolecomorphidae and the interrelationships of its constituent genera are still under debate (Wilkinson et al., 2003; Wake et al., 2005; Frost et al., 2006; Roelants et al., 2007; Loader et al., 2007, and see below). More uncertain is the position of the Scolecomorphidae, which might be either the sister group of Caeciliidae plus Typhlonectidae (Wilkinson and Nussbaum, 1996; Roelants et al., 2007) or within Caeciliidae (Wilkinson et al., 2003; Frost et al., 2006).

The current distribution of extant caecilians is generally thought to reflect a Gondwanan origin of the order and consequent diversification with the breakup of Gondwana (Duellman and Trueb, 1986; Hedges et al., 1993; Wilkinson et al., 2002; San Mauro et al., 2005). Time tree analyses are useful to explain how the current distribution of living caecilians developed. However, the fossil record of caecilians is poor and mainly consists of fragments of vertebrae and jaws (Estes and Wake, 1972; Rage, 1986; Evans et al., 1996; Wake et al., 1999) and putative stem-group caecilians of uncertain affinities (Jenkins and Walsh, 1993; Carroll, 2000; Jenkins et al., 2007; Evans and Sigogneau-Russell, 2001). Time information extracted from molecular data is an alternative method to improve our knowledge of caecilian evolution when fossil records are insufficient. Wilkinson et al. (2002) used mitochondrial ribosomal RNA sequences and the average distance method (Kumar and Hedges, 1998) to generate the first molecular time scale for some caecilian divergences. Later studies (San Mauro et al., 2005; Roelants et al., 2007) used nuclear protein-coding gene sequences and relaxed clock methods (Bayesian, Thorne and Kishino, 2002; penalized likelihood, Sanderson, 2003) and provided largely compatible results but still with some differences. For example, the mean divergence times between the Diatriata and the Teresomata were estimated to be 178 Ma (Wilkinson et al., 2002), about 192 Ma (San Mauro et al., 2005), 188 or 196 Ma (Roelants et al., 2007), 200 Ma (this study), respectively. Compared to other vertebrate groups, studies of divergence times for caecilians are few and we believe that more efforts should be made to generate new data and analyses.

It has been shown that mitochondrial DNA (mtDNA) is a useful marker system in numerous phylogenetic analyses of vertebrate relationships because of its maternal mode of inheritance and relative lack of recombination (Saccone et al., 1999). Moreover, mtDNA is a moderate-scale genome suitable for complete sequencing and thus provides substantial amounts of DNA and amino acid data for phylogenetic analyses. Compared to small mitochondrial gene fragments used in some previous molecular studies (Hedges et al., 1993; Wilkinson et al., 2002, 2003) which cannot effectively resolve higher-level relationships of caecilians, the complete mitochondrial genome is expected to give more reliable results in phylogenetic analyses. More importantly, the considerable quantity of DNA data in complete mitochondrial genomes would decrease the uncertainty in branch length estimation and thus help to improve the accuracy of divergence time estimates. San Mauro et al. (2004) sequenced five caecilian mitochondrial genomes and presented the first mitogenomic phylogeny for living caecilians at the family level. However, the largest (57% of all species) caecilian family, Caeciliidae, is represented by only one sequence in their dataset. Therefore, it is necessary to increase the number of mitochondrial genome sequences of Caeciliidae and to construct a more comprehensive data set to further study a number of caecilian phylogenetic questions.

Here we report new complete mitochondrial genomes for thirteen caecilian species, including twelve species of caeciliids and one additional rhinatrematid. These new sequences are compared with the eight previously described caecilian mitochondrial genomes (Zardoya and Meyer, 2000; San Mauro et al., 2004, 2006; Zhang et al., 2005). In addition to conventional phylogenetic tree-building methods, we also use tree-based topology comparison to test the reliability of different phylogenetic hypotheses. Based on the resulting phylogenies, we calculate the evolutionary timescale of caecilian divergences with relaxed clock dating approaches (see Section 2.6).

2. Materials and methods

2.1. Taxon sampling for mitochondrial genomes

Our sampling included twelve species of the family Caeciliidae, representing a wide geographic distribution (Central America, South America, West Africa, East Africa and the Seychelles). We also included an additional species (*Epicrionops niger*) representing the family Rhinatrematidae. In addition to our new caecilian sequences, we downloaded eight published caecilian mitochondrial genomes from GenBank, including the caecilian families Ichthyophiidae, Rhinatrematidae, Scolecomorphidae, Typhlonectidae and Uraeotyphlidae, so our data set comprises 21 caecilian species and all currently recognized families. For outgroups, we selected two lobe-finned fishes (latimeria [*Latimeria chalumnae*] and lungfish [*Protopterus dolloi*]), two reptiles (alligator [*Alligator mississippiensis*] and chicken [*Gallus gallus*]), one mammal (human [*Homo sapiens*]), one frog (pipid [*Silurana tropicalis*]) and two salamanders (cryptobranchid [*Andrias davidianus*] and hynobiid [*Ranodon sibiricus*]). Moreover, we sequenced a South American pipid frog, *Pipa pipa*, which is used together with the African pipid frog *Silurana tropicalis* as an external calibration point, reflecting the biogeographic event of the final separation between Africa and South America (see Roelants et al., 2007 for discussion). Detailed information for all species used in this study is listed in Table 1.

2.2. Laboratory protocols

Total DNA was purified from frozen or ethanol-preserved tissues (liver or muscle) using the Qiagen (Valencia, CA) DNeasy Blood and Tissue Kit. A suite of 26 primers (Table 2) was used to amplify contiguous and overlapping fragments that covered the entire caecilian mt genome (Fig. 1). The frog mt genome (*Pipa pipa*) was amplified by a different suite of primers which will be published elsewhere (Zhang et al., unpublished data). PCR reactions were performed with AccuTaq LA DNA Polymerase (SIGMA) in total volumes of 25 μl, using the following cycling conditions: an initial denaturing step at 96 °C for 2 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 45–55 °C (see Table 2) for 60 s, and extending at 72 °C for 5 min; and a final extending step of 72 °C for 10 min. For a few fragments we could not amplify with universal primers, we designed new primers according to sequences of their adjacent fragments to cover them. PCR products were purified either directly via ExoSAP (USB) treatment or gel-cutting (1% TAE agarose) using the gel purification kit (Qiagen). Sequencing

Table 1
List of all outgroup and ingroup species used in this study; species names are shaded for new mitochondrial genome sequences.

Species	Taxonomy	Voucher No.	GenBank Accession No.	Rough collection locality
Latimeria chalumnae	Coelacanthiformes	–	NC_001804	–
Protopterus dolloi	Dipnoi	–	NC_001708	–
Alligator mississippiensis	Crocodylidae	–	NC_001922	–
Gallus gallus	Aves	–	NC_001323	–
Homo sapiens	Mammalia	–	NC_001807	–
Silurana tropicalis	Anura: Pipidae	–	NC_006839	–
Andrias davidianus	Caudata: Cryptobranchidae	–	AJ492192	–
Ranodon sibiricus	Caudata: Hynobiidae	–	AJ419960	–
Gegeneophis ramaswamii	Gymnophiona: Caeciliidae	MW 331	AY456250	Thenmalai, India
Siphonops annulatus	Gymnophiona: Caeciliidae	BMNH 2005.9	AY954506	Dominguez Martins, Brazil
Ichthyophis glutinosus	Gymnophiona: Ichthyophiidae	MW 1733	AY456251	Peradeniya, Sri Lanka
Ichthyophis bannanicus	Gymnophiona: Ichthyophiidae	Personal collection	AY458594	Beiliu, GX, China
Rhinatrema bivittatum	Gymnophiona: Rhinatrematidae	BMNH 2002.6	AY456252	Kaw, French Guyana
Scolecomorphus vittatus	Gymnophiona: Scolecomorphidae	BMNH 2002.100	AY456253	Amani, Tanzania
Uraeotyphlus cf. oxyurus	Gymnophiona: Uraeotyphlidae	MW 212	AY456254	Payyanur, India
Typhlonectes natans	Gymnophiona: Typhlonectidae	BMNH 2000.218	AF154051	Potrerito, Venezuela
Boulengerula boulengeri	Gymnophiona: Caeciliidae	CAS168822	GQ244464	Lushoto Dist.,Tanzania
Boulengerula taitana	Gymnophiona: Caeciliidae	MVZ179505	GQ244465	Taita Hills, Kenya
Caecilia volcani	Gymnophiona: Caeciliidae	MVZ231242	GQ244466	Fortuna, Panama
Dermophis mexicanus	Gymnophiona: Caeciliidae	MVZ179061	GQ244467	Finca Santa Julia, Guatemala
Geotrypetes seraphini	Gymnophiona: Caeciliidae	MVZ252475	GQ244469	Ajenjua Bepo F.R., Ghana
Grandisonia alternans	Gymnophiona: Caeciliidae	MVZ258026	GQ244470	La Digue, Seychelles
Gymnopis multiplicata	Gymnophiona: Caeciliidae	MVZ171331	GQ244471	Tortuguero, Costa Rica
Hypogeophis rostratus	Gymnophiona: Caeciliidae	MVZ258025	GQ244472	La Digue, Seychelles
Microcaecilia sp.	Gymnophiona: Caeciliidae	IWK0128	GQ244473	Guyana
Oscaecilia ochrocephala	Gymnophiona: Caeciliidae	MVZ222472	GQ244474	Santa Clara de Arajan, Panama
Praslinia cooperi	Gymnophiona: Caeciliidae	UMMZ192933	GQ244475	Silhouette, Seychelles
Schistometopum thomense	Gymnophiona: Caeciliidae	CAS219292	GQ244476	Sao Tome
Epicrionops niger	Gymnophiona: Rhinatrematidae	CPI103W8	GQ244468	Guyana
Pipa pipa	Anura: Pipidae	MVZ247508	GQ244477	Berceba, Suriname

was performed directly with the corresponding PCR primers using the BigDye Deoxy Terminator cycle-sequencing kit v3.1 (Applied Biosystems) in an automated DNA sequencer (ABI PRISM 3730) following manufacturer's instructions. For some large PCR fragments, specific primers were designed according to newly obtained sequences to fulfill primer walking. To make sure we did not amplify nuclear copies of mitochondrial fragments, we carefully examined our contig assemblies and found no incongruence in any overlapping regions, and no stop codons in protein-coding genes, which supports the reliability of our sequences.

2.3. Mitogenomic alignment preparation

All sequences from the L-strand-encoded genes (ND6 and eight tRNA genes) were converted into complementary strand sequences. Thirteen protein-coding, 22 tRNA and two rRNA gene se-

Table 2
Primers used to amplify the complete caecilian mitochondrial genomes (see Fig. 1 to trace fragments along the genome).

Fragment name	Primer name	Sequence (5′–3′)	Approximate product length (bp)	Annealing temperature (°C) used in the PCR
L1	12SAL[a]	AAACTGGGATTAGATACCCCACTAT	1500	55
	16S2000H[a]	GTGATTAYGCTACCTTTGCACGGT		
L2	LX12SN1[a]	TACACACCGCCCGTCA	1600	55
	LX16S1R[a]	GACCTGGATTACTCCGGTCTGAACTC		
C1	LX16S1[a]	GGTTTACGACCTCGATGTTGGATCA	1500	55
	Met3850H[a]	GGTATGGGCCCAARAGCTT		
C2	CP2F	TTAAGGAYCAYTTTGATAGA	2400	50
	CP2R	ACYTCTGGRTGDCCAAARAATCA		
C3	Amp-P3F[b]	CAATACCAAACCCCCTTRTTYGTWTGATC	900	45
	Amp-P3R[b]	GCTTCTCAGATAATRAAYATYATTA		
C4	Amp-P4F[b]	GGMTTTATYCACTGRTTYCC	1400	50
	Amp-P4R[b]	AAATTGGTCAAAKAARCTTAGKRTCATGG		
C5	8.2 L8331[b]	AAAGCRTYRGCCTTTTAAGC	1550	50
	MNCN-COIIIR[b]	ACRTCTACRAAGTGTCARTATCA		
C6	CP6F	TTTAYGGMTCHACATTYTTTGT	1600	50
	CP6R	GCTTCTACRTGDGCTTTWGG		
C7	CP7F	GAACGHTTAAAYGCHGGHACATA	1000	50
	CP7R	AAGAGANTTRNGGARTTTAACC		
C8	CP8F	ATAGTTTAATAAAAAYAYTARATTGTG	1300	45
	Lati-ND5 R1[b]	CCYATYTTTCKGATRTCYTGYTC		
C9	CP9F	AGYCAACTHGGMYTAATRATAGT	1600	50
	CP9R	TCDGCTGTATARTGTATDGCTA		
C10	CP10F	TCTGAAAAACCAYCGTTGTWMTTCAAC	1150	50
	CP10R	TTCAGYTTACAAGRCYGRYGYTTT		
C11	CP11F	TGAATYGGMGGHCAACCMGTAGAA	1400–1600	50
	12S600H[a]	TTATCGATTATAGAACAGGCTCCTCT		

[a] Zhang et al. (2008).
[b] San Mauro et al. (2004).

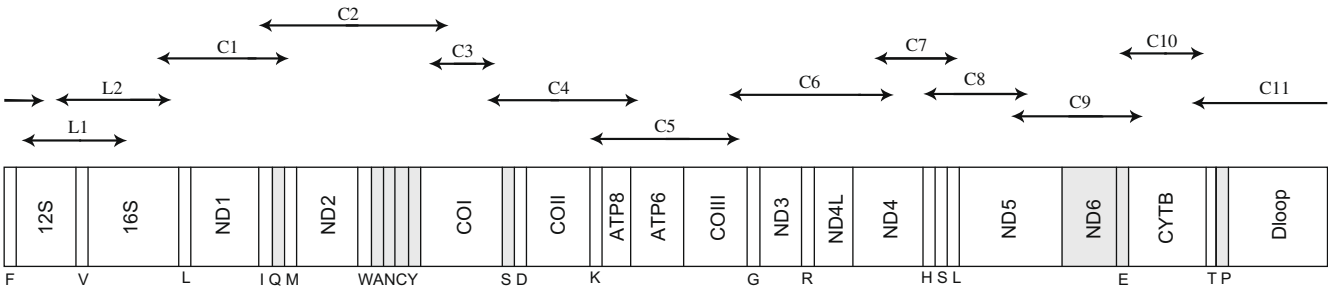


Fig. 1. Gene organization and sequencing strategy for mitochondrial genomes of caecilians. Genes encoded by the L strand are shaded. Arrow-headed segments denote the location of the fragments amplified by PCR with each pair of primers (see Table 2 for the primer DNA sequence associated with each fragment).

quences were aligned using Clustal W (Thompson et al., 1997) at default settings. All 22 tRNA alignments were then combined to generate a concatenated alignment. To avoid artificial bias in refining alignments, we used Gblocks (Castresana, 2000) to extract regions of defined sequence conservation from the two rRNAs, concatenated tRNAs, and 13 protein-coding gene alignments at default settings. Finally, a DNA dataset combining all 16 Gblock-refined alignments was generated. Mueller et al. (2004) pointed out that a partition strategy for mitogenome data that defined a separate partition for each ribosomal RNA, the concatenated tRNAs, and each codon position in each protein-coding gene is better than other partition strategies. We therefore followed their suggestion and divided our DNA dataset into 42 partitions according to genes and codon positions (tRNAs, 2 rRNAs, every codon position for 13 protein genes). Model selection for each partition was done according to the Akaike information criterion (AIC) as implemented in MrModelTest 2.2 (http://www.ebc.uu.se/systzoo/staff/nylander.html). The best fitting model for each partition was used in subsequent Bayesian phylogenetic analyses. In addition to the DNA alignment, we made an amino acid alignment of the deduced amino acid sequences of all 13 mt protein-coding genes using a similar methodology.

2.4. Multiple gene alignments with a denser taxon sampling

Although the goal of this study is to show what whole mitochrondrial genome data contribute to analysis of the relationships of the caecilians, we also want to see whether the result from mitogenomes is still supported by a multiple gene data with a denser taxon sampling. To this end, we downloaded all available caecilian nucleotide sequences from GenBank and compiled a multiple gene data set combining three mitochondrial gene fragments (12S, 16S and CytB) and four nuclear genes (RAG1, NCX1, SLC8A3 and CXCR4). A frog (*Pipa pipa*) and a salamander (*Andrias davidianus*) were used as outgroup in this dataset. Compared with the mitogenome data set, the caecilian species included in this multiple gene data set increased from 21 to 41. Detailed information (species names, accession numbers, etc.) about this multiple gene data set can be found in the online Supplementary material.

2.5. Phylogenetic analyses

Maximum parsimony (MP) analyses were performed using heuristic searches (TBR branch swapping; MULPARS option in effect) with 100 random-addition sequences by PAUP* 4.0b10 (Swofford, 2001). All sites were given equal weight in the parsimony analysis. ML analyses were applied to the DNA data under a partitioned scheme, using RAxML 7.0.3 (Stamatakis, 2006) with independent GTR+I+Γ substitution models defined to each partition. For the amino acid data, the mtREV24 model (Adachi and Hasegawa, 1996) was used. The Bayesian inferences were made using MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001) with one cold and three heated chains (temperature set to 0.1) for 20 million generations and sampled every 1000 generations. Due to computation cost, the BIs for the amino acid data were run for one million generations and sampled every 100 generations. The burn-in parameter was empirically estimated by plotting $-\ln L$ against the generation number by using Tracer version 1.4 (http://evolve.zoo.ox.ac.uk/beast/help/Tracer), and the trees corresponding to the first 15–50% generations were discarded. To ensure that our analyses were not trapped in local optima, four independent MrBayes runs were performed. Topologies and posterior clade probabilities from different runs were compared for congruence.

Branch support was evaluated with non-parametric bootstrap proportions (1000 pseudoreplicates) and Bayesian posterior probabilities. Approximately unbiased (AU) (Shimodaira, 2002) and Shimodaira–Hasegawa (SH) (Shimodaira and Hasegawa, 1999) tests were used to evaluate alternative caecilian phylogeny hypotheses. The SH test is a well known method for testing posterior hypotheses emerging from the analysis of the data. Compared to the SH test, the AU test aims to provide better control of type-1 errors (the rejection of potentially valid hypotheses) by simultaneous comparison of multiple hypotheses (Shimodaira, 2002). The tests were carried out using CONSEL version 0.1f (Shimodaira and Hasegawa, 2001) with per-site log likelihoods calculated by RAxML 7.0.3 (Stamatakis, 2006) through the option "-f g".

2.6. Molecular dating

For external calibration points outside the amphibian lineages, we used the lungfish-tetrapod split (419–408 Ma; Müller and Reisz, 2005), the Amphibia–Amniota split (330–360 Ma; derived from Benton and Donoghue, 2007; Marjanović and Laurin, 2007), the mammal–bird split (>312 Ma; Benton and Donoghue, 2007), and the bird–crocodile split (235–251 MYA; Müller and Reisz, 2005; Benton and Donoghue, 2007). Within the amphibians, the frog–salamander split was constrained to be greater than 250 Ma (*Triadobatrachus massinoti*, Rage and Rocek, 1989; *Czatkobatrachus polonicus*, Evans and Borsuk-Bialynicka, 1998). Recently, a stem batrachian, *Gerobatrachus hottoni,* was found in Early Permian, Leonardian stratum (280–270 Ma) and then suggested as a lower limit on the divergence between frogs and salamanders (Anderson et al., 2008). We therefore used a conservative value (280 Ma) as the maximum bound for the frog–salamander split. The lower limit for the split between *Ranodon* and *Cryptobranchus* is based on the Mid-Jurassic–Early Cretaceous fossil salamander *Chunerpeton tianyiense* (Gao and Shubin, 2003). Because the dating of *Chunerpeton tianyiense* is still controversial, we used the Jurassic–Cretaceous boundary, at 145 Ma, as its age. The minimum of 86 Ma for the split between the South American pipid frog *Pipa* and the African pipid *Silurana tropicalis* corresponds to the youngest estimated age for the final separation between Africa and South America (see Roelants et al., 2007 for discussion). The split between the Indian caeciliid *Gegeneophis* and the Seychelles caeciliid *Praslinia* was con-

strained to be greater than the final separation between India and Seychelles at ~65 Ma (Briggs, 2003). This paleogeographic event is unlikely to provide an overestimation of the divergence between Indian and Seychelles caeciliids; all relevant published caecilian molecular phylogenetic studies, as well as this study, have indicated a sister-clade relationship between the Indian *Gegeneophis* and the Seychelles genera *Praslinia*, *Hypogeophis* and *Grandisonia,* which suggests that a clade containing all the Seychelles caeciliid genera had already split from the Indian caeciliids before the breakup of India–Seychelles. These constraints are illustrated in the relevant figures.

We used the program R8S 1.71 (Sanderson, 2003) rather than the program MultiDivTime (Thorne and Kishino, 2002) to perform our relaxed clock dating analyses for two reasons: (i) R8S can use any third party programs to estimate branch lengths thus can use more sophisticated model such GTR+Γ while the MultiDivTime can only use F81+Γ model. (ii) Although R8S treats all data as a single partition, but if we estimate branch lengths using a partitioned scheme in a third party program, this shortcoming can be partly avoided. Therefore, we used MrBayes to generate tree samples with branch lengths under a partitioned scheme (29 partitions as in phylogenetic analyses). These trees were used as the input data for the program R8S. The *Latimeria chalumnae* sequence served as outgroup, allowing the tree relating the remaining 29 ingroup sequences to be rooted. Analyses were performed with a truncated-Newton (TN) optimization algorithm and a log penalty function as suggested by the program manual. The optimal smoothing parameter was determined by the cross-validation method implemented in R8S. Credibility intervals for the PL age estimates were obtained by replicate PL analyses of 1000 trees, randomly sampled from the posterior tree set produced by MrBayes. Because these trees approximate the posterior distribution of both phylogenetic relationships and branch lengths, so will the derived 95% CIs.

3. Results

3.1. General features of caecilian mtDNA

The complete nucleotide sequences of the L strands of the mitochondrial genomes of 13 caecilian species were determined. Total length ranges from 15,973 to 16,315 bp. As in most of the published higher vertebrate sequences, all 13 newly sequenced caecilian mitochondrial genomes encode for two rRNAs, 22 tRNAs, and 13 protein-coding genes, with the exception of *Gymnopis multiplicata,* whose tRNA-Val gene has a 2-bp deletion in its anticodon loop and thus loses primary function.

The mitochondrial genomes of *Dermophis mexicanus* and *Gymnopis multiplicata* have long noncoding regions between tRNA-Phe and 12S rRNA genes of 153 and 161 bp, respectively, which is a new mitochondrial genome feature in vertebrates. No secondary structures, tandem repeats, or functional ORFs are found in these intergenic regions, and BLAST searches produce no informative matches. Further analyses of the intergenic region of *D. mexicanus* indicate that its 3′ end contains a tRNA-Phe pseudogene (75% similarity to the normal one). Although the anticodon sequences of this pseudogene are conserved, it has two mismatch mutations on the right arm of its anticodon stem, indicating loss of primary function (Fig. 2). The tRNA-Phe pseudogene in *G. multiplicata* is shortened to only 61 nt relative to a normal size of 71 nt. The upstream portion beside the tRNA-Phe pseudogene of *D. mexicanus* and *G. multiplicata* is highly AT-rich (>80%), somewhat like the compositional characteristics of caecilian mitochondrial D-Loop regions. Therefore, we postulate a possible pathway for the formation of this region: tandem duplication presumably occurred from the D-Loop (partial 3′ end) to the tRNA-Phe gene but deletions of redundant genes did not take place; the extra D-Loop region and the tRNA-Phe gene likely underwent a random mutation process, resulting in the unusual noncoding region in *Dermophis* and *Gymnopis* mtDNA (Fig. 2). This unique mitogenomic feature shared by *Dermophis* and *Gymnopis* appears to be strong evidence that *Dermophis* and *Gymnopis* are monophyletic.

3.2. Phylogenetic analyses of the mitogenomic data set

The mitogenomic DNA data set combining two rRNAs, the concatenated tRNAs, and 13 protein-coding gene alignments contains 13201 characters (4420 constant, 1264 parsimony-uninformative, and 7517 parsimony-informative). Within caecilians, the number of parsimony-informative characters is 6400. When all 3rd codon positions are excluded, the DNA data set contains 9768 characters (4391 constant, 1180 parsimony-uninformative, and 4197 parsimony-informative). Within caecilians, the number of parsimony-informative sites is 3244. The protein data set derived from the deduced amino acid sequences of all 13 mitochondrial protein-coding genes contains 3434 characters. Of these, 1321 are constant, 508 are parsimony-uninformative, and 1605 are parsimony-informative. Within caecilians, the number of parsimony-informative characters is 1248.

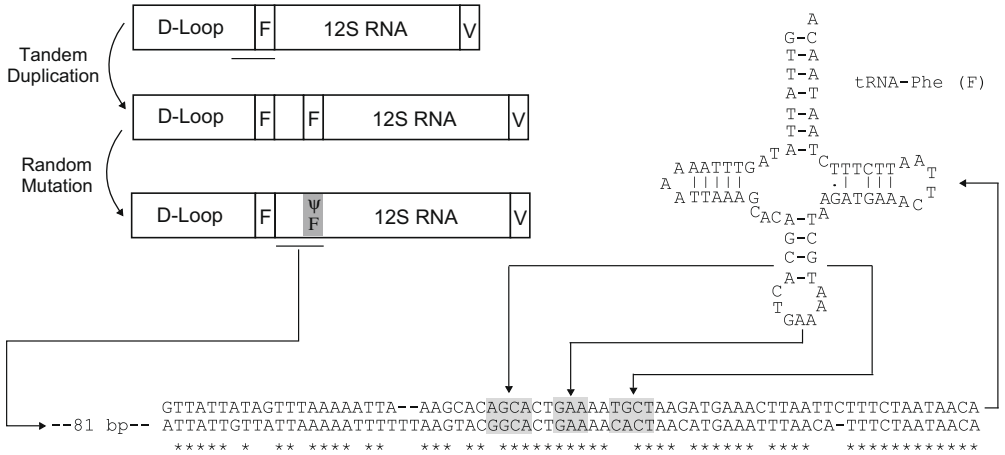
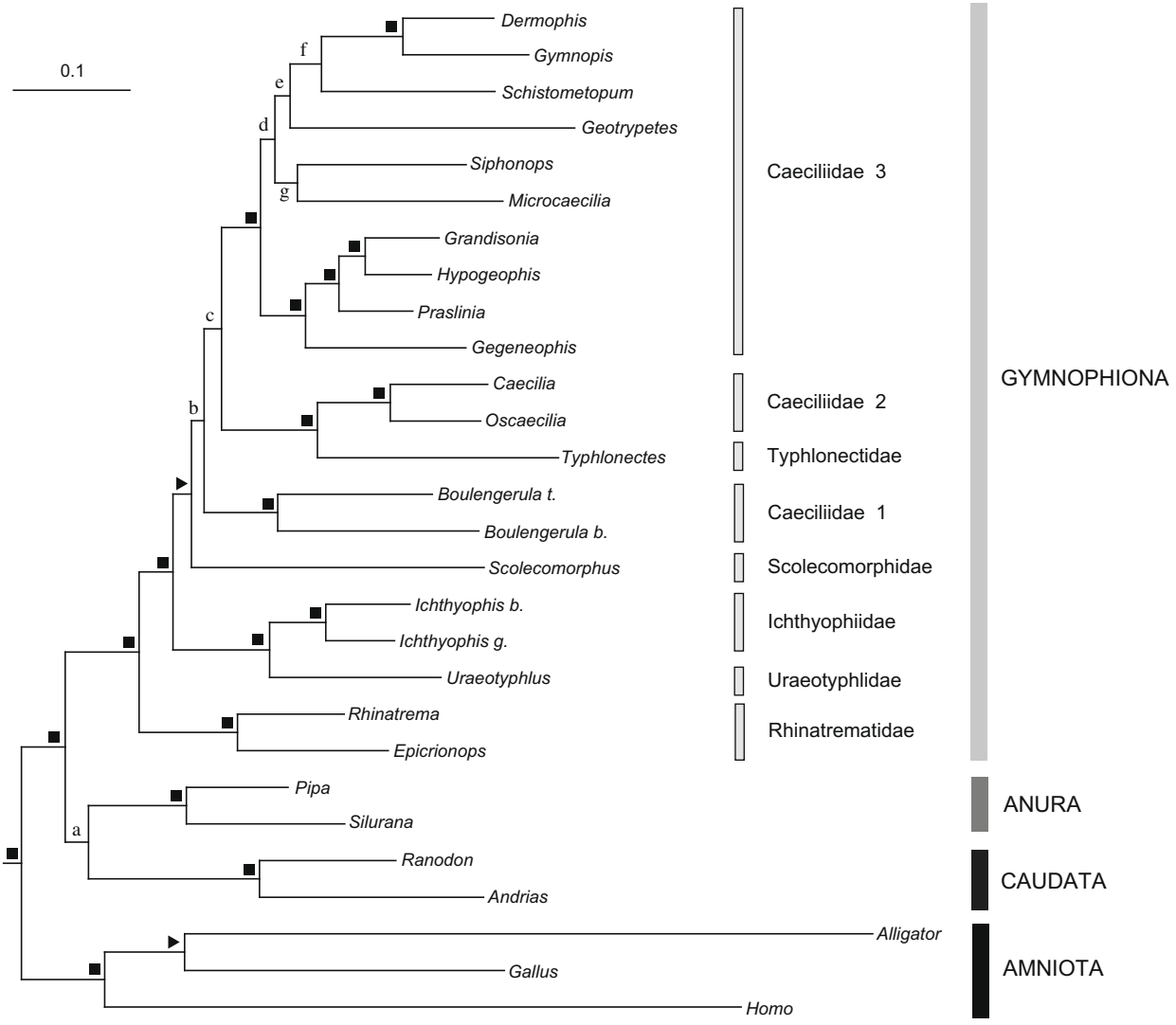


Fig. 2. Proposed mechanism of the formation of the unusual noncoding region between tRNA-Phe and 12S RNA genes in the mitochondrial genomes of *Dermophis* and *Gymnopis*. Tandem duplication presumably occurred from the D-Loop (partial 3′ end) to the tRNA-Phe gene, but deletions of redundant genes did not occur. The extra D-Loop region and the tRNA-Phe gene likely underwent a random mutation process, resulting in the unusual noncoding region in *Dermophis* and *Gymnopis* mtDNA. A sequential analysis of the noncoding region in *Dermophis mexicanus* is also presented. Although the anticodon sequences are conserved, the *Dermophis* tRNA-Phe pseudogene has lost the potential to fold into a stable anticodon stem, indicating loss of primary function.

Maximum parsimony analyses on both the DNA data set (with or without 3rd codon positions) and the protein data set produced somewhat different trees, but the conflicting parts always received low bootstrap support (<60%). Therefore, we do not show the MP trees separately but present bootstrap support for those branches congruent both in the MP analyses and other tree-building methods (Fig. 3). Partitioned ML and Bayesian analyses of the DNA data set (without 3rd codon positions) and protein data set all produce identical topologies. The DNA data set (includes all sites) produced similar trees that differed only in two nodes: Bayesian analysis of the mt DNA data (includes all sites) suggests a close relationship between Caudata and Gymnophiona rather than an Anura–Caudata clade recovered by the other two data sets (Node a, Fig. 3); the mt DNA data (includes all sites) differed from the other two data sets in placing Scolecomorphidae as sister group only to *Boulengerula* rather than to the entire Caeciliidae + Typhlonectidae clade



	mitochondrial DNA all sites			mitochondrial DNA 3rd codon excluded			mitochondrial protein		
Nodes	MP-BP	ML-BP	Bayesian-PP	MP-BP	ML-BP	Bayesian-PP	MP-BP	ML-BP	Bayesian-PP
a	--	58	----	78	87	1.00	91	91	1.00
b	--	--	----	--	43	0.96	43	60	0.99
c	--	58	0.99	--	77	1.00	42	85	1.00
d	60	66	1.00	62	71	1.00	--	72	1.00
e	80	95	1.00	70	86	1.00	--	55	0.99
f	--	100	1.00	98	99	1.00	78	58	0.90
g	68	73	1.00	73	88	1.00	--	54	1.00

Fig. 3. Phylogenetic relationships of amniotes, frogs, salamanders, and caecilians inferred from analyses of mitochondrial genome data (DNA level and protein level). Branches with letters have branch support values given below the tree for maximum parsimony bootstrapping (MP-BP), maximum-likelihood bootstrapping (ML-BP) and Bayesian posterior probabilities (Bayesian-PP). Branches with bootstrap support >90% and Bayesian posterior probability >0.99 are indicated as filled squares; branches with bootstrap support 80–90% and Bayesian posterior probability 0.95–0.99 are indicated as right-pointing filled triangles. Hyphens indicate nodes that are not supported in the corresponding analyses. Branch lengths were estimated by partitioned maximum-likelihood analysis on mitochondrial DNA data without 3rd codon. Lobe-finned fish outgroup is not shown.

(Node b, Fig. 3). Notably, the two different results for nodes a and b derived from the mt DNA data (includes all sites) did not receive strong support (bootstrap <50% and Bayesian PP < 0.90). Considering that an Anura–Caudata clade was consistently supported in recent studies (Zardoya and Meyer, 2001; San Mauro et al., 2004, 2005; Zhang et al., 2005; Frost et al., 2006; Roelants et al., 2007) and mitochondrial 3rd codon positions tend to be fast evolving and often show poor performance in resolving ancient divergence events (Zardoya and Meyer, 1996), we suggest that the phylogenetic results derived from the mt DNA data (without 3rd codons) and protein data are more reliable. Fig. 3 shows the ML tree obtained from the mt DNA data without 3rd codons using independent GTR+I+Γ models applied to 29 data partitions; it summarizes the statistical results of the other data sets and phylogenetic methods employed in the study.

The multiple gene dataset combining three mitochondrial gene fragments (12S, 16S and CytB) and four nuclear genes (RAG1, NCX1, SLC8A3 and CXCR4) contains 5993 characters and 47% missing data. Of the 5993 sites, 4023 are constant, 604 are parsimony-uninformative, and 1366 are parsimony-informative. Equally weighted maximum parsimony and partitioned (partitioned by genes) maximum-likelihood analyses produced nearly identical tree topologies (Fig. 4). Although the multiple gene dataset uses different genetic loci and caecilian species sampling, its resulting

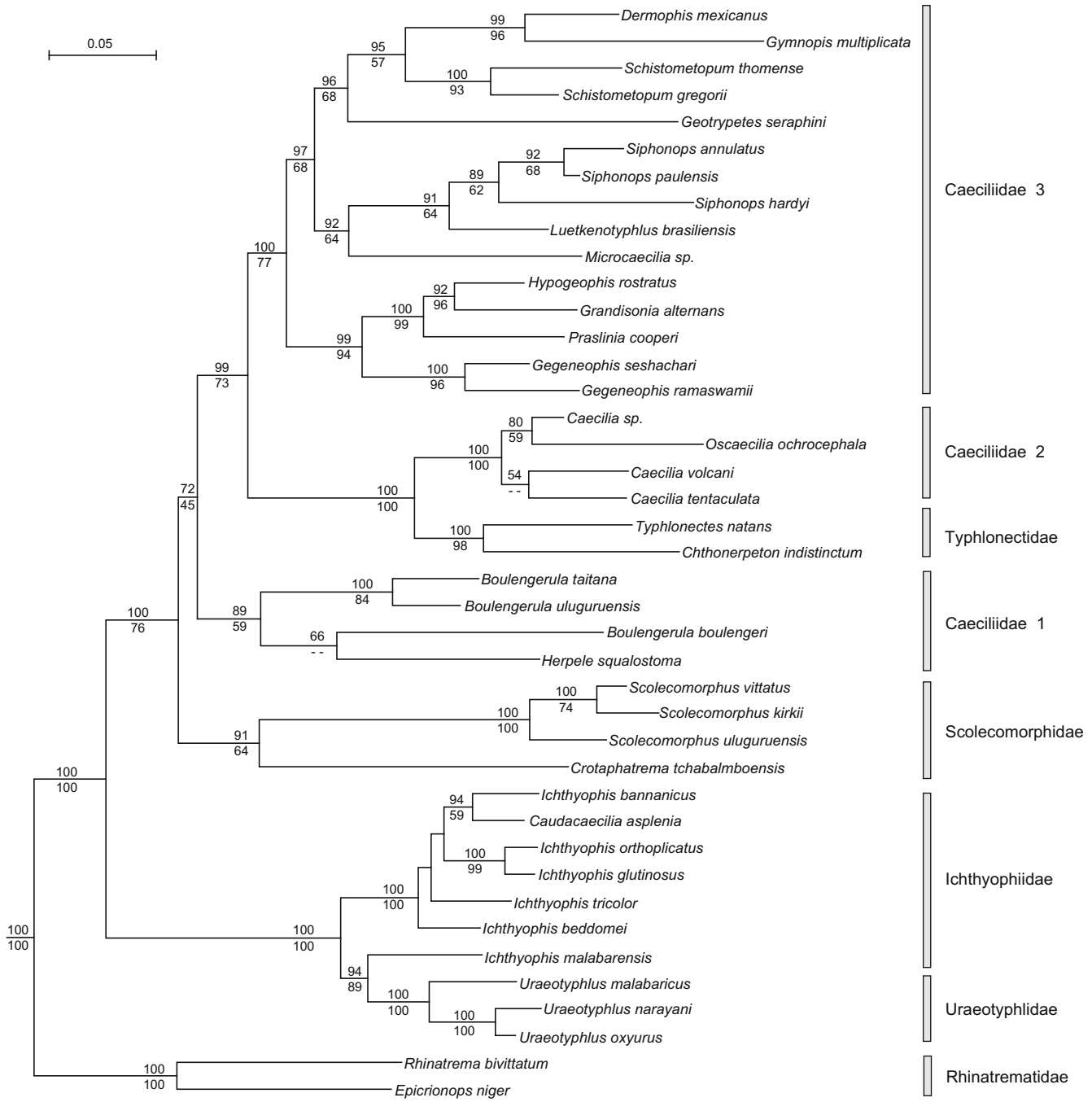


Fig. 4. Phylogenetic relationships (ML phylogram) of caecilians inferred from a multiple gene data set combining three mitochondrial gene fragments (12S, 16S and CytB) and four nuclear genes (RAG1, NCX1, SLC8A3 and CXCR4). Numbers above branches represent bootstrap support for ML (7 GTR+Γ+I models for 7 gene partitions) and number below branches represent bootstrap support for MP (equally weighting). Hyphens indicate nodes that are not supported in the corresponding analyses. Outgroup taxa (a frog, *Pipa pipa,* and a salamander, *Andrias davidianus*) are not shown.

tree topology is completely in congruence with the caecilian relationships inferred from whole mitochondrial genomes (Figs. 3 and 4). Because the goal of our paper is to show what whole mitochrondrial genome data contribute to analysis of the relationships of the caecilians and the multiple gene data produced similar result to the mitogenomic data, we will mainly focus on interpreting our mitogenomic results.

As did previous studies (Nussbaum and Wilkinson, 1989; Wilkinson et al., 2002, 2003; Wake et al., 2005; San Mauro et al., 2004; Frost et al., 2006; Roelants et al., 2007), we find that Rhinatrematidae is the monophyletic sister group of the remaining caecilians (Figs. 3 and 4). The monophyly of Diatriata (Ichthyophiidae and Uraeotyphlidae; Wilkinson and Nussbaum, 2006) is also well supported (Figs. 3 and 4). Within higher caecilians, Scolecomorphidae is recovered as the sister group of Caeciliidae and Typhlonectidae but this placement did not received strong support both in the analyses of mitogenomic data or multiple gene data (Node b, Figs. 3 and 4). As expected from previously published molecular studies, the commonly recognized family Caeciliidae is paraphyletic. In accordance with the Frost et al.'s (2006) results, we find that traditional Caeciliidae can be divided into three well-supported groups: Caeciliidae group 1 contains the African caeciliids *Boulengerula* and *Herpele*; Caeciliidae group 2 contains *Caecilia* and *Oscaecilia* and is the sister group of Typhlonectidae; Caeciliidae group 3 comprises the remaining caeciliid species in our sample (Fig. 3). We adopt the informal names for these clades used by Frost et al. (2006). Within Caeciliidae group 3, caeciliids from India and Seychelles (*Gegeneophis*, *Grandisonia*, *Hypogeophis* and *Praslinia*) formed a well-supported clade with respect to other African and American caeciliids (*Geotrypetes*, *Schistometopum*, *Gymnopis*, *Microcaecilia*, *Dermophis* and *Siphonops*), which probably reflects the breakup between India–Madagascar–Seychelles and Africa–South America in Late Jurassic (~130 Ma).

Results of AU and SH tests of alternative tree topologies regarding the placement of Scolecomorphidae, based on the four datasets used in the phylogenetic reconstruction, are summarized in Fig. 5. Four possible placements of Scolecomorphidae were tested: (a) Scolecomorphidae is the sister group of both Caeciliidae and Typhlonectidae (this study; Roelants et al., 2007); (b) Scolecomorphidae is the sister group of Caeciliidae group 1 (weakly supported in this study by the mt DNA data including all sites); (c) Scolecomorphidae is the sister group of Caeciliidae group 2 and 3 plus Typhlonectidae (Wilkinson et al., 2003); (d) Scolecomorphidae is the sister group of Caeciliidae group 3 (Frost et al., 2006). In summary, Hypothesis D can be rejected by most datasets and tests ($P < 0.05$; Fig. 5). The difference among Hypotheses A, B, C remains ambiguous, and only the AU test of the multiple gene dataset can reject Hypothesis C. None of the tests allow us to reject Hypotheses A and B, although we note that Hypothesis A always receives the highest P values in all tests.

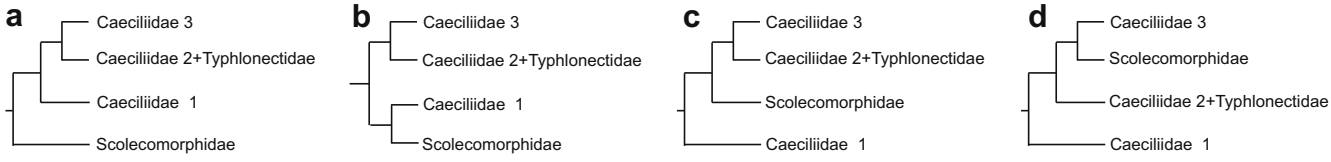
3.3. Divergence times

We provide four sets of time estimates for caecilian evolution using the mitogenome DNA data excluding 3rd codon and mitochondrial protein data under two calibration choices. The divergence times for the nodes of the phylogeny presented in Fig. 6 are summarized in Table 3. In general, when a maximal bound (280 Ma) for the origin of the Batrachia was used, penalized likelihood analyses provided mean time estimates 12.3 Ma (with DNA data) or 4.5 Ma (with protein data) younger on average than when the maximal bound was not applied. On the other hand, the average mean time difference between the DNA and protein analyses is 12.5 Ma when the Batrachia maximal bound was not enforced, while this average difference decreased to about 5.3 Ma when using the Batrachia maximal bound. According to our time estimates (Table 3), the initial split within living caecilians most probably occurred from Early to Mid-Triassic (228–252 Ma; Node 9, Table 3), but 95% confidence intervals for these estimates are wide, from Mid-Permian to Early Jurassic. The initial divergence within the higher caecilians (comprising scolecomorphids, caeciliids, and typhlonectids) most likely took place between very Late Triassic and Early Jurassic (184–206 Ma; Node 14, Table 3), although the wide 95% confidence intervals suggest that the divergence could have occurred during Late Triassic to Late Jurassic.

4. Discussion

4.1. Phylogeny and systematics of caecilians

San Mauro et al. (2004) used complete mitochondrial genomes to study the family-level relationships of living caecilians. However, their mtDNA data included only three species of Teresomata (one scolecomorphid, one typhlonectid and one caeciliid), so the largest component of caecilian phylogeny (the intra-relationships of Caeciliidae) was not considered in their study. By sampling an



	mitogenome all sites			mitogenome 3rd codon excluded			mitochondrial proteins			Multiple genes		
Hypotheses	ΔlnL	AU	SH	ΔlnL	AU	SH	ΔlnL	AU	SH	ΔlnL	AU	SH
a	----	0.670	0.804	----	0.728	0.844	----	0.796	0.878	----	0.838	0.933
b	3.1	0.413	0.611	4.4	0.391	0.553	5.9	0.309	0.465	5.2	0.240	0.474
c	3.9	0.495	0.670	6.0	0.346	0.539	8.4	0.208	0.345	7.2	0.022*	0.369
d	12.3	0.109	0.248	18.0	0.049*	0.117	26.1	0.006*	0.026*	54.9	0.002*	0.005*

Fig. 5. Alternative hypotheses of possible phylogenetic position of Scolecomorphidae. (a) Scolecomorphidae is the sister taxon of the clade comprising Caeciliidae and Typhlonectidae (this study; Roelants et al., 2007); (b) Scolecomorphidae is the sister taxon of Caeciliidae group 1 (weakly supported in this study); (c) Scolecomorphidae is the sister taxon of Caeciliidae group 2 and 3 plus Typhlonectidae (Wilkinson et al., 2003); (d) Scolecomorphidae is the sister taxon of Caeciliidae group 3 (Frost et al., 2006). Statistical confidence (P-values) for alternative hypotheses using AU and SH tests are given below the topologies. Asterisks indicate that the hypothesis received a P value <0.05 and can be rejected.

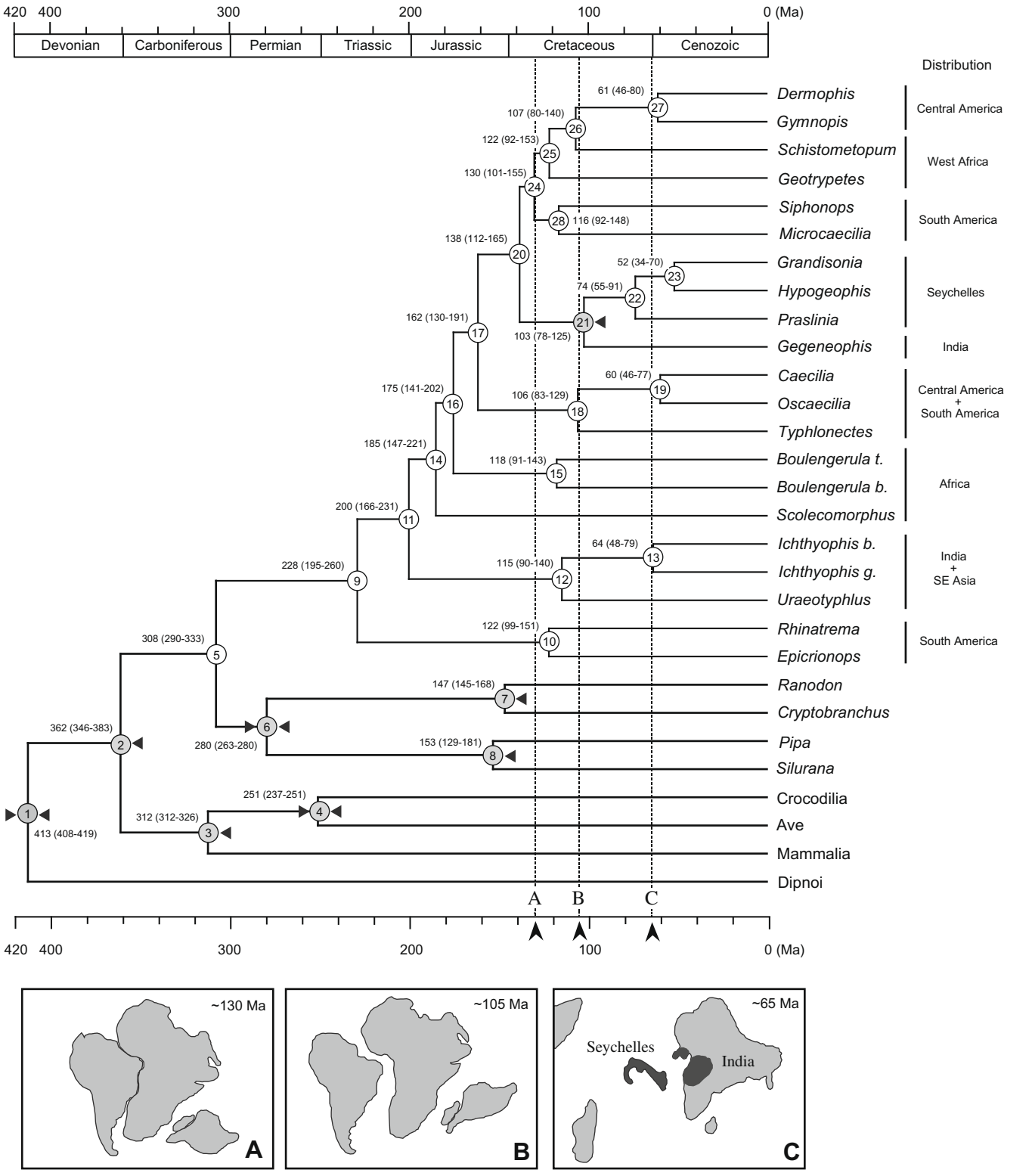


Fig. 6. Evolutionary timetree of extant caecilians based on the penalized likelihood method implemented in R8S, and 11 time constraints derived from fossil and paleogeographic evidence (see Section 2). The calibration points are indicated as shaded circles with left/right (minimum bound/maximum bound) pointing triangles beside them. Numbers and numbers in parentheses beside the nodes represent divergence time mean and 95% credibility intervals, averaging from the mitogenomic DNA and protein dating results with the Batrachia maximum time constraint. More detailed time estimates are given in Table 3; node numbers in the table correlate with circled node numbers in the figure. Plate-tectonic reconstruction of continents: (A) the Madagascar–Seychelles–India block separated from Africa while South America was still connected to Africa in Late Jurassic (~130 Ma); (B) the final separation of Africa and South America in Middle Cretaceous (~105 Ma); (C) the separation of India and the Seychelles at the K–T boundary (~65 Ma; the dark area denotes land currently covered by volcanic basalts).

additional thirteen caecilian species (including twelve caeciliid species), we have generated a more comprehensive caecilian phylogeny based on complete mitochondrial genomes. Our data add information regarding the relationships of the *Boulengerula* (see also Wilkinson et al., 2003 and Loader et al., 2007), and *Dermophis*, *Gymnopis*, *Caecilia* and *Oscaecilia* (see also Nussbaum and Wilkin-

Table 3
Divergence time means and 95% confidence intervals calculated by penalized likelihood method implemented in R8S. Letters for nodes correspond to those in Fig. 6. Dating analyses were performed for both mitogenomic DNA and protein data with/without a maximum bound (280 Ma) for the frog–salamander split (Batrachia).

Nodes	Without Batrachia Max limit		With Batrachia Max limit	
	DNA	Protein	DNA	Protein
1: Lungfish–Tetrapod split (ingroup root)	412 (408–419)	416 (408–419)	410 (408–419)	416 (408–419)
2: Origin of Tetrapods	368 (349–389)	363 (344–383)	362 (347–384)	361 (344-–382)
3: Bird–Mammal split	312 (312–326)	313 (312–327)	312 (312–326)	312 (312–325)
4: Bird–Crocodile split	251 (245–251)	251 (235–251)	251 (239–251)	251 (235–251)
5: Origin of living amphibians	327 (303–350)	316 (286–344)	307 (293–335)	309 (288–331)
6: Anura–Caudata split (Batrachia)	303 (278–332)	288 (255–320)	280 (265–280)	279 (259–280)
7: Cryptobrachidae–Hynobiidae split	150 (145–172)	152 (145–178)	145 (145–170)	148 (145–166)
8: South American–African pipid split	175 (147–205)	155 (117–190)	158 (138–188)	148 (120–173)
9: Origin of living caecilians	252 (227–277)	237 (199–270)	229 (199–261)	228 (191–259)
10: *Epicrionops–Rhinatrema* split	143 (119–165)	120 (95–150)	129 (105–152)	115 (93–150)
11: Ichthyophiidae–Scolecomorphidae split	221 (193–249)	208 (171–247)	200 (165–230)	200 (167–231)
12: Ichthyophiidae–Uraeotyphlidae split	134 (117–153)	113 (88–144)	121 (92–148)	108 (88–131)
13: Sri Lanka–Chinese Ichthyophiid split	77 (63–94)	60 (44–78)	70 (52–85)	57 (43–73)
14: Scolecomorphidae–Caeciliidae split	206 (173–234)	191 (154–232)	186 (143–227)	184 (150–214)
15: *Boulengerula boulengeri–taitana* split	139 (118–160)	114 (86–142)	125 (99–153)	110 (83–133)
16: *Boulengerula–Typhlonectes* split	197 (170–222)	180 (143–213)	177 (141–201)	173 (141–203)
17: *Typhlonectes–Gegeneophis* split	184 (160–209)	163 (124–195)	166 (132–192)	157 (127–189)
18: *Typhlonectes–Caecilia* split	123 (101–142)	105 (77–130)	110 (86–133)	101 (80–124)
19: *Caecilia–Oscaecilia* split	69 (54–86)	60 (41–77)	62 (48–81)	57 (43–72)
20: *Gegeneophis–Microcaecilia* split	156 (133–175)	141 (105–174)	140 (112–165)	136 (111–165)
21: *Gegeneophis–Praslinia* split	118 (99–139)	102 (67–131)	107 (81–129)	98 (75–121)
22: *Praslinia–Hypogeophis* split	85 (70–99)	73 (50–95)	76 (55–92)	71 (54–89)
23: *Hypogeophis–Grandisonia* split	61 (44–80)	50 (34–68)	55 (35–75)	48 (33–64)
24: *Geotrypetes–Microcaecilia* split	147 (124–165)	133 (100–164)	132 (103–154)	128 (99–156)
25: *Geotrypetes–Dermophis* split	137 (118–159)	124 (91–155)	124 (92–157)	119 (91–149)
26: *Schistometopum–Dermophis* split	118 (100–138)	112 (81–150)	106 (78–138)	108 (81–141)
27: *Gymnopis–Dermophis* split	67 (55–79)	64 (46–87)	60 (44–77)	62 (47–82)
28: *Microcaecilia–Siphonops* split	130 (109–149)	120 (89–149)	117 (93–149)	115 (91–146)

son, 1989, and Wake et al., 2005). Although the caecilian mitochondrial genomes used in this study are still limited, the current phylogenetic results based on mitochondrial genomes (Fig. 3) are fully compatible with those from the multiple gene dataset that contains fewer characters, more missing data, but more caecilian species (Fig. 4). Consistency between these two different datasets suggests that the relationships of caecilians inferred from mitogenomes is reliable and may be unlikely to be affected by insufficient caecilian taxon sampling.

In agreement with both previous morphological (e.g. Duellman and Trueb, 1986; Nussbaum and Wilkinson, 1989; Wilkinson and Nussbaum, 2006) and molecular (e.g. Wilkinson et al., 2003; San Mauro et al., 2004; Frost et al., 2006; Roelants et al., 2007) results, Rhinatrematidae is strongly supported as a monophyletic group and is the sister taxon of the remaining caecilians. A sister group relationship of Ichthyophiidae and Uraeotyphlidae (= Diatriata, Wilkinson and Nussbaum, 2006), which has been recovered as the sister group to higher caecilians (= Teresomata, Wilkinson and Nussbaum, 2006) in nearly all recent molecular studies of caecilian relationships (e.g. Wilkinson et al., 2002, 2003; San Mauro et al., 2004; Frost et al., 2006; Roelants et al., 2007), was also highly corroborated by our molecular data. Recently, Frost et al. (2006) synonymized Uraeotyphlidae with Ichthyophiidae based on the apparent paraphyly of *Ichthyophis* with regard to *Uraeotyphlus* (Gower et al., 2002; Frost et al., 2006). Because of the limited sampling of *Ichthyophis* species in our mitogenome dataset, our mitogenomic caecilian tree (Fig. 3) does not provide evidence to support or reject this merger. However, our multiple gene dataset, using DNA sequences of the key species *Ichthyophis malabarensis* that was the sister taxon to the Uraeotyphlidae in Gower et al.'s (2002) study shows that *Ichthyophis* is indeed paraphyletic with respect to *Uraeotyphlus* (Fig. 4).

The monophyly of higher caecilians (Scolecomorphidae, Typhlonectidae and Caeciliidae) with respect to other caecilians (Rhinatrematidae, Ichthyophiidae and Uraeotyphlidae) is well corroborated in all analyses (ML bootstrap >90%; MrBayes PP = 1.0; Figs. 3 and 4). As to the uncertain of the position of the Scolecomorphidae, which might be either the sister group of Caeciliidae plus Typhlonectidae (Roelants et al., 2007) or within Caeciliidae (Wake, 1993; Wilkinson et al., 2003; Frost et al., 2006), our phylogenetic results support the former hypothesis. Although this hypothesis did not receive strong branch support by the two datasets (Node b, Figs. 3 and 4) and most alternative hypotheses cannot be rejected in the two topological tests used here (Fig. 5), it was repeatedly favored by two kinds of molecular data (mitogenome and multiple genes) and thus might be closer to the real cladogenetic history. Because the Scolecomorphidae is most likely the sister group of the Typhlonectidae plus Caeciliidae and they possess many distinctive characters compared to Typhlonectidae and Caeciliidae (e.g. separate premaxillae and nasals, septomaxillae and prefrontals present, and stapes absent), we believe its family status should be maintained as recommended by Wilkinson and Nussbaum (2006).

The paraphyly of Caeciliidae with regard to Typhlonectidae has long been recognized (e.g. Nussbaum, 1979; Nussbaum and Wilkinson, 1989; Hedges et al., 1993;Wilkinson et al., 2002, 2003; Frost et al., 2006; Wilkinson and Nussbaum, 2006; Roelants et al., 2007). Our mitogenomic caecilian tree, together with the result of the multiple gene data, confirmed this result again by recovering a well-supported clade of *Typhlonectes* plus *Caecilia–Oscaecilia* deeply imbedded within Caeciliidae (Figs. 3 and 4). Hedges et al. (1993) and Frost et al. (2006) regarded Typhlonectidae as a subsidiary taxon (the Typhlonectinae). We think that taxonomy should not only be a way to classify organisms, but also a way to represent evolutionary history. Considering that many of the distinctions between Typhlonectidae and Caeciliidae are non-informative autapomorphic traits, and Typhlonectidae is always found imbedded within Caeciliidae in all relevant molecular studies, we tentatively agree with merging Typhlonectidae into Caeciliidae to make Caeciliidae a monophyletic group.

Since Nussbaum (1979) presented the first numerical analysis of caecilian relationships, many studies have addressed this issue

focusing on either larger scale relationships (e.g. Hedges et al., 1993; Hedges and Maxson, 1993; Hay et al., 1995; Frost et al., 2006; Roelants et al., 2007) or certain geographic areas (e.g. Gower et al., 2002; Wilkinson et al., 2002, 2003; Wake et al., 2005). Many of these studies are interested in the relationships within the Caeciliidae, which is the largest and most diverse group in terms of ecology, morphology and life-history (Wilkinson and Nussbaum, 2006). The two most recent molecular studies (Frost et al., 2006; Roelants et al. 2007) showed that a clade comprising the African *Boulengerula* and *Herpele* is the sister group of other caeciliids. Although our mitogenomic data did not include *Herpele*, our multiple gene dataset once again indicates a close relationship between *Boulengerula* and *Herpele* (Fig. 4). Therefore, the divergence of *Boulengerula* within Caeciliidae in our mitogenomic tree (Fig. 3) is also in agreement with previous findings. The monophyly of the former "Caeciliinae" (*Caecilia*, *Microcaecilia*, *Oscaecilia* and *Parvicaecilia*; Wake and Campbell, 1983; Duellman and Trueb, 1986; Hedges et al., 1993; but see Nussbaum and Wilkinson, 1989) is not supported because *Microcaecilia* is in Caeciliidae group 3 (Figs. 3 and 4). As expected based on the study by Wilkinson et al. (2003), we found the Seychellean caeciliids (*Grandisonia*, *Hypogeophis* and *Praslinia*) to be a clade, related to the Indian caeciliid *Gegeneophis* (Figs. 3 and 4). The position of the South American caeciliid *Siphonops* is variable in several analyses (e.g. Wilkinson et al., 2003; Frost et al., 2006; Roelants et al., 2007). Our results show *Siphonops* to be embedded within African and Central-South American caeciliids, the sister group of *Microcaecilia* and *Luetkenotyphlus* (Roelants et al., 2007), rather than embedded within India–Seychellean caeciliids (Frost et al., 2006) or diverging earlier in the tree (Wilkinson et al., 2003).

4.2. Times of divergence and biogeography

The major difference among the four sets of time estimates in this study (Table 3) is whether the fossil batrachian *Gerobatrachus hottoni* (Anderson et al., 2008) is used as maximal constraint for the frog–salamander split. For example, when the Batrachian maximal bound was not applied, the origin of living amphibians is estimated to be 327 (303–350) Ma (based on mitogenomic DNA data), close to our previous mitogenomic estimate of 337 Ma (Zhang et al., 2005). This estimate decreases to 307 (293–335) Ma when the Batrachian maximal bound was used, resulting in an apparently much more recent origin. We are very cautious in using fossils as maximal constraints because fossils often provide good minimal bounds, but not maximal bounds. However, as pointed out by Anderson et al. (2008), *Gerobatrachus* is most plausibly not a relict form but a stem batrachian, so it can be used as a maximal limit for the Batrachian origin. Moreover, the time of this fossil (280–270 Ma) is highly congruent with the previously recommended maximal limit for the Batrachian origin based on biostratigraphy (275 Ma; Marjanović and Laurin, 2007). Therefore, the conservative usage of *Gerobatrachus hottoni* in our molecular dating analyses (280 Ma as its oldest age) is unlikely to provide too great an underestimate for the maximal limit of the Batrachian origin. In theory, using the same data, the time estimation difference between different analytical levels (mitochondrial DNA and mitochondrial protein in this case) should be minimal if branch length estimation is without errors. However, no currently available evolutionary model can unerringly describe substitution processes of molecular data, so branch length estimation is always somewhat approximate. Therefore, we averaged the mean time estimates from both DNA and protein analyses (with the Batrachia maximal constraint) to produce our primary dating result. We illustrate these average times in Fig. 6.

Previously, several studies provided some time estimates for major splits within caecilians (Wilkinson et al., 2002; San Mauro et al., 2005; Loader et al., 2007; Roelants et al., 2007). Wilkinson et al. (2002) used ~1000 bp mitochondrial rRNA sequences, the average distance method (assuming a global clock), and divergence rates inferred from biogeographic assumptions, to calculate the caecilian time scale. But their time scale gave only five time estimates, not including the root of the caecilian tree, and therefore was rather incomplete. Loader et al.'s results (2007) are also based on mitochondrial rRNA sequences but are relative time scales so they are difficult to compare and interpret. Using nuclear data and relaxed clock dating methods, San Mauro et al. (2005) and Roelants et al. (2007) generated similar time estimates for caecilian evolution except that Roelants et al.'s study used a larger data set and denser taxon sampling. In general, for deep nodes, our mitogenomic time estimates for caecilian evolution are compatible with those from nuclear data (San Mauro et al., 2005; Roelants et al., 2007), but for younger nodes, our estimates are closer to those from mitochondrial rRNA data (Wilkinson et al., 2002). For example, the time of the initial split within the modern caecilians (deep node) is 228 (195–260) Ma in our study, 214 (177–256) Ma in San Mauro et al.'s analysis (2005), 218 (192–242) Ma in Roelants et al.'s penalized likelihood analyses (2007); the *Gegeneophis–Praslinia* split (younger node), is estimated to be 103 (78–125) Ma (this study), 101 (79–128) Ma (Wilkinson et al., 2002) but 83 (66–105) Ma in Roelants et al.'s (2007) penalized likelihood analyses. Although the mean time estimation discrepancies among these time studies are apparent, confidence intervals for certain nodes are wide and largely overlapping, which makes it difficult to judge which studies' time estimates are more precise. Wide confidence intervals seem to be a general phenomenon for all current caecilian time studies, perhaps because few reliable constraints within caecilian lineages are available currently.

The fossil record of caecilians is poor and mainly consists of isolated vertebrae of uncertain affinities with modern caecilians (e.g. Estes and Wake, 1972; Rage, 1986; Evans et al., 1996; Hecht and LaDuke, 1997; Wake et al., 1999). In addition, two putative stem-group caecilians have been found: the older, *Eocaecilia micropodia* from the Lower Jurassic of Arizona, USA, possessed reduced limbs and a relatively long tail (Jenkins and Walsh, 1993; Carroll, 2000; Jenkins et al., 2007); the younger, *Rubricacaecilia monbaroni* from the Lower Cretaceous of Morocco, might also have had limbs (Evans and Sigogneau-Russell, 2001). The origin time of Gymnophiona based on fossil evidence is estimated to be the Early Jurassic (~190 Ma; Marjanović and Laurin, 2007). In all relevant time studies (this study; San Mauro et al., 2005; Roelants et al., 2007), the lower margin of the 95% confidence intervals for the origin of caecilians all overlap the Early Jurassic period, suggesting that the molecular dating results are also somewhat in congruence with the fossil estimation. However, considering the mean time estimates of all three studies (214, 218, or 228 Ma) always fall into the Late Triassic period, it's still possible that older caecilian fossil records can be discovered in the Late Triassic.

The distribution of the family Caeciliidae is almost entirely Gondwanan, suggesting that the main divergences of this group took place before the initial breakup of Gondwana in Late Jurassic (~150 Ma). Our time estimate for the origin of "Caeciliidae" (Node 16, Fig. 6) is about 175 (141–202) Ma, congruent with the biogeographic inferences. In all relevant caecilian phylogenetic studies, the Seychellean and Indian caeciliids (*Gegeneophis*, *Grandisonia*, *Hypogeophis* and *Praslinia*) are more closely related to each other than to any of the South American and African caeciliids, which is concordant with the sequence of the breakup of Gondwana. Based on this point, Wilkinson et al. (2002) assumed the split between the Seychellean and Indian caeciliids and other South American and African caeciliids to be at least as old as the separation between Madagascar–India–Seychelles and Afro-American

Gondwanaland (Fig. 6, illustration A), and fixed this node at geological estimate of 130 Ma (Smith et al., 1994). Our time estimate for this node (Node 20, Fig. 6) is 138 (112–165) Ma, fairly close to the geological estimate. Moreover, the estimate based on nuclear data (124 Ma; Roelants et al., 2007) is also largely congruent with this biogeographic assumption. Therefore, we agree with Wilkinson et al. in using this biogeographic time constraint as one of the possible calibration choices for future caecilian time tree studies. However, because the geological time is approximate and we cannot reject that the split occurred before the breakup, we suggest using this biogeographic constraint as a minimum bound, not a fixed one.

Considering that caecilians are not likely to be capable of long-distance transoceanic dispersal, one biogeographically surprising result in recent caecilian phylogenetic studies (this study; Wilkinson et al., 2003; Frost et al., 2006; Roelants et al., 2007) is that the caeciliid *Schistometopum,* which occurs on the West African island of Sao Tome (*S. thomense*) and in East Africa (*S. gregorii*), is consistently found to be closer to the Central American caeciliids *Dermophis* + *Gymnopis* rather than its potentially sympatric relatives – the West African *Geotrypetes* and *Boulengerula.* Because taxon sampling in all current studies is still incomplete, especially lacking many South American lineages, the strong grouping between the African *Schistometopum* and the Central American *Dermophis* + *Gymnopis* may be an analytical artifact caused by insufficient taxon sampling. However, this grouping could also be explained if the split between *Schistometopum* and *Dermophis* + *Gymnopis* was caused by (or predated) the breakup of Afro-American Gondwanaland. Our divergence time estimate for this split (Node 26, Fig. 6) is 107 (80–140) Ma, matching the final separation between Africa and South America in late Early Cretaceous (~105 Ma; illustration B, Fig. 6), which suggests the latter hypothesis (split predating separation of the continents) is more likely. Therefore, we postulate an evolutionary scenario (based on our samples) as followed. *Geotrypetes* separated from the ancestor of *Schistometopum* + *Dermophis* + *Gymnopis* before the breakup of Afro-American Gondwanaland. The ancestor of *Schistometopum* + *Dermophis* + *Gymnopis* was widely spread across Afro-American Gondwanaland in Early Cretaceous (~120 Ma) and was split into two groups restricted to Africa (giving rise to *Schistometopum*) and South America (giving rise to *Dermophis* + *Gymnopis*) respectively, when the South Atlantic Ocean finally formed (~105 Ma; see http://jan.ucc.nau.edu/~rcb7/globaltext2.html). The South American group further dispersed into Central America when the two landmasses were connected in the Cenozoic and subsequently became extinct from all southern areas. Because Sao Tome is a young volcanic island formed no earlier than the Oligocene (Dèruelle et al., 1991), the Sao Tome *Schistometopum thomense* probably colonized the island by a later transoceanic dispersal from the African mainland.

In many molecular studies (this study; Wilkinson et al., 2002, 2003; Roelants et al., 2007), the Seychellean caeciliid genera *Grandisonia*, *Hypogeophis* and *Praslinia* form a well-supported clade and its sister group is the Indian caeciliid genus *Gegeneophis*. It seems reasonable that such a grouping is caused by the vicariant event of the Seychelles separating from India. The rifting of India and the Seychelles took place 62.0–68.7 Ma (Collier et al., 2008), near the K–T boundary, when the Deccan Traps volcanism was active (illustration C; Fig. 6). However, our time estimate for the *Gegeneophis–Praslinia* split is about 103 (78–125) Ma, and even the divergence within the Seychellean caeciliids (~74 Ma; Node 22, Fig. 6) is older than the geological event. The time discrepancy suggests that the divergence between Seychellean and Indian caeciliids was not a consequence of the separation of the two areas, and some lineages of caeciliids on the Seychelles survived the Deccan Traps volcanism.

5. Conclusion

In this study, we have used complete mitochondrial genome sequences to address phylogenetic relationships and divergence times of caecilians. An additional multiple gene data with denser caecilian taxon sampling but more missing data produced fully compatible results compared to the mitogenomic caecilian tree, suggesting our mitogenomic analyses on caecilians are unlikely affected by insufficient taxon sampling. Our phylogenetic results and divergence estimates based on mitochondrial genomes are in good agreement with previous studies based on nuclear gene sequences, which suggests that the mitochondrial genome is effective in resolving deep phylogeny of organisms with ancient history such as caecilians. However, due to limited caecilian samples used in this study, some uncertainty of caecilian relationships, such as the validity of Typhlonectidae and the position of African *Schistometopum*, still remains. More complete taxon sampling that allows more extensive analysis of whole mitochondrial genomes of caecilians will doubtless alter and clarify concepts of the origins and relationships of lineages of caecilian amphibians.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2009.06.018.

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