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Phylogeny and biogeography of the family Salamandridae (Amphibia: Caudata) inferred from complete mitochondrial genomes

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ABSTRACT

Phylogenetic relationships of members of the salamander family Salamandridae were examined using complete mitochondrial genomes collected from 42 species representing all 20 salamandrid genera and five outgroup taxa. Weighted maximum parsimony, partitioned maximum likelihood, and partitioned Bayesian approaches all produce an identical, well-resolved phylogeny; most branches are strongly supported with greater than 90% bootstrap values and 1.0 Bayesian posterior probabilities. Our results support recent taxonomic changes in finding the traditional genera *Mertensiella*, *Euproctus*, and *Triturus* to be non-monophyletic species assemblages. We successfully resolved the current polytomy at the base of the salamandrid tree: the Italian newt genus *Salamandrina* is sister to all remaining salamandrids. Beyond *Salamandrina*, a clade comprising all remaining newts is separated from a clade containing the true salamanders. Among these newts, the branching orders of well-supported clades are: primitive newts (*Echinotriton*, *Pleurodeles*, and *Tylototriton*), New World newts (*Notophthalmus-Taricha*), Corsica-Sardinia newts (*Euproctus*), and modern European newts (*Calotriton*, *Lissotriton*, *Mesotriton*, *Neurergus*, *Ommatotriton*, and *Triturus*) plus modern Asian newts (*Cynops*, *Pachytriton*, and *Paramesotriton*).Two alternative sets of calibration points and two Bayesian dating methods (BEAST and MultiDivTime) were used to estimate timescales for salamandrid evolution. The estimation difference by dating methods is slight and we propose two sets of timescales based on different calibration choices. The two timescales suggest that the initial diversification of extant salamandrids took place in Europe about 97 or 69 Ma. North American salamandrids were derived from their European ancestors by dispersal through North Atlantic Land Bridges in the Late Cretaceous (~69 Ma) or Middle Eocene (~43 Ma). Ancestors of Asian salamandrids most probably dispersed to the eastern Asia from Europe, after withdrawal of the Turgai Sea (~29 Ma).

1. Introduction

The family Salamandridae is one of the most diverse groups of extant salamanders, including 20 genera and about 77 recognized species (AmphibiaWeb, http://amphibiaweb.org). Traditionally, the salamandrids are informally divided into two major subgroups, the "true salamanders" (*Chioglossa*, *Lyciasalamandra*, *Mertensiella*, and *Salamandra*) and the newts (all remaining extant genera). The true salamanders are smooth-skinned, while the newts differ from all other salamanders in having rough skin. The salamandrids exhibit considerable diversity in feeding morphology (Wake and Özeti, 1969), courtship behavior (Salthe, 1967; Houck and Arnold, 2003), and reproductive mode (Sever, 1992), making them a suitable model to investigate the evolution of such biologically significant features. Moreover, the family Salamandridae is discontinuously distributed across Europe, North America and East Asia, providing an appropriate group for the investigation of historical biogeography of these three regions.

A robust phylogeny of salamandrids is a necessary framework for studying the evolutionary questions listed above. Early morphological studies failed to produce a convincing phylogenetic hypothesis for salamandrids because of limited morphological characters and extensive homoplasy of those characters (Wake and Özeti, 1969; Zhao et al, 1988). Using a combination of morphological and mitochondrial DNA characters, Titus and Larson (1995) provided the first complete study of salamandrid phylogeny. Their analysis gave strong support to the monophyly of the Salamandridae and to some intergeneric groupings, but was unable to resolve many basal relationships within the family, particularly the placement of the newt genus *Salamandrina*. Recent molecular

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phylogenetic results for 10 genera reported by Frost et al. (2006) revised and resolved some weak nodes of Titus and Larson's study, but suffered from incomplete taxon sampling. Most recently, using a comprehensive sampling strategy, two independent studies used different mitochondrial fragments (tRNALeu–COI, ~2700 bp, Weisrock et al., 2006; partial 12S, 16S, and cytb, ~1700 bp, Steinfartz et al., 2007) to produce largely congruent phylogenetic hypotheses. Both studies agreed in rejecting monophyly of the old genera *Mertensiella*, *Euproctus*, and *Triturus*, taxonomic puzzles that have perplexed the herpetological community for decades. The phylogenetic position of the spectacled newts of the Italian peninsula, *Salamandrina*, was treated differently in the two studies. Steinfartz et al. (2007) found *Salamandrina* to be the sister taxon to all other extant salamandrids but with Bayesian posterior probability below 0.95; Weisrock et al. (2006) found *Salamandrina* either to be a sister taxon of the true salamanders or the remaining newts, depending on analytical methods used, and suggested the possibility of a basal polytomy for the three main lineages of salamandrids.

Mitochondrial DNA (mtDNA) has proven to be 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 data for phylogenetic analyses. Compared to small gene fragments, which may show poor phylogenetic performance in relation to deeply diverged lineages, the complete mitochondrial genome is expected to give more reliable estimations of evolutionary relationships in phylogenetic analyses and estimates of the timing of cladogenetic events. Previous studies demonstrated that mitogenomic data recovered robust phylogenies (with high statistical support) for many taxa (Mueller et al., 2004; Zhang et al., 2005, 2006), and thus may resolve questions of salamandrid phylogeny. More importantly, the considerable amount of DNA data from complete mitochondrial genomes is likely to decrease the uncertainty in branch length estimation and thus may improve the accuracy of divergence time estimates. Currently, the most complete molecular dating analysis for extant salamandrids is based on a mitochondrial cytb fragment of about 800 bp (Steinfartz et al., 2007).

Here, we reinvestigate the phylogenetic relationships of the Salamandridae with complete mitochondrial genomes, based on a sampling scheme that includes all relevant taxa, in particular representatives of all recognized genera. In addition to conventional phylogenetic tree-building methods, we test the reliability of different phylogenetic hypotheses. Based on the resulting phylogenies, we calculate evolutionary timescales of salamandrids with newly developed relaxed-clock Bayesian dating approaches.

2. Materials and methods

2.1. Taxon sampling for mitochondrial genomes

The family Salamandridae currently is comprised of 77 extant species, grouped into 20 genera (http://amphibiaweb.org). Our goal is to obtain a fully resolved, robust tree and our sampling strategy included all genera. Where availability of tissues permitted, we also included more than one species per genus so as to truncate potential long branches. We sampled a total of 35 salamandrid species. These species, together with two additional species (*Lyciasalamandra atifi* and *Paramesotriton hongkongensis*), for which full mitochondrial genome sequences already exist, formed 37 ingroup taxa in this study. Complete mitochondrial genomes of four non-salamandrid salamanders and one frog were retrieved from GenBank to serve as outgroup taxa in phylogenetic analyses. All species used in this study are listed in Table 1.

Table 1
List of species used in this study, along with GenBank Accession Nos. and vouchers (if applicable)

Species	Specimen voucher No.	GenBank Accession No.	References
Xenopus tropicalis	—	NC_006839	JGI direct submission
Ambystoma mexicanum	—	AJ584639	Arnason et al. (2004)
Andrias davidianus	—	AJ492192	Zhang et al. (2003a)
Rhyacotriton variegatus	—	AY728219	Mueller et al. (2004)
Ranodon sibiricus	—	AJ419960	Zhang et al. (2003b)
Calotriton asper	Vieites01	EU880307	This study
Chioglossa lusitanica	TP-MVZ01	EU880308	This study
Cynops cyanurus	TP-MVZ02	EU880309	This study
Cynops ensicauda	MVZ238530	EU880310	This study
Cynops orientalis	MVZ230345	EU880311	This study
Cynops orphicus	MVZ241428	EU880312	This study
Cynops pyrrhogaster	TP-MVZ03	EU880313	This study
Echinotriton andersoni	MVZ232187	EU880314	This study
Echinotriton chinhaiensis	TP26195	EU880315	This study
Euproctus montanus	TP-MVZ04	EU880316	This study
Euproctus platycephalus	DBW-MVZ01	EU880317	This study
Lyciasalamandra flavimembris	MVZ230148	EU880318	This study
Lyciasalamandra atifi	—	AF154053	Zardoya and Meyer (2001)
Lissotriton vulgaris	MVZ230731	EU880339	This study
Mertensiella caucasica	MVZ218721	EU880319	This study
Mesotriton alpestris	MVZ232177	EU880335	This study
Neurergus kaiseri	MVZ234201	EU880320	This study
Neurergus s. strauchii	MVZ236768	EU880321	This study
Notophthalmus meridionalis	MVZ250846	EU880322	This study
Notophthalmus viridescens	MVZ205720	EU880323	This study
Ommatotriton vittatus	MVZ230205	EU880338	This study
Pachytriton brevipes	MVZ231167	EU880324	This study
Pachytriton labiatus	MVZ230355	EU880325	This study
Paramesotriton caudopunctatus	MVZ236252	EU880326	This study
Paramesotriton deloustali	MVZ223628	EU880327	This study
Paramesotriton hongkongensis	—	AY458597	Zhang et al. (2005)
Paramesotriton laoensis	FMNH255452	EU880328	This study
Pleurodeles poireti	MVZ235673	EU880329	This study
Pleurodeles waltl	MVZ231894	EU880330	This study
Salamandra salamandra	MVZ236839	EU880331	This study
Salamandrina terdigitata	MVZ178848	EU880332	This study
Taricha granulosa	MVZ225502	EU880333	This study
Taricha rivularis	MVZ219804	EU880334	This study
Triturus cristatus	MVZ230726	EU880336	This study
Triturus marmoratus	MVZ148916	EU880337	This study
Tylototriton asperrimus	MVZ237103	EU880340	This study
Tylototriton wenxianensis	MVZ236638	EU880341	This study

2.2. Laboratory protocols

Total DNA was purified from frozen or ethanol-preserved tissues (liver or muscle) using the Qiagen (Valencia, CA) DNeasy Blood & Tissue Kit. A suite of 26 primers (Table 2) was used to amplify contiguous and overlapping fragments that covered the entire mt genome (Fig. 1). 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. PCR products were purified either directly via ExoSAP (USB) treatment or gel-cutting (1% TAE agarose) using the gel purification kit (Qiagen). Sequencing 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

Table 2
Primers used to amplify the complete salamandrid mito-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 16S2000H	AAACTGGGATTAGATACCCCACTAT GTGATTAYGCTACCTTTGCACGGT	1500	55
L2	LX12SN1 LX16S1R	TACACACCGCCCGTCA GACCTGGATTACTCCGGTCTGAACTC	1600	55
A	LX16S1 Met3850H	GGTTTACGACCTCGATGTTGGATCA GGTATGGGCCCAARAGCTT	1500	55
B	Ile3700L COI5350H	AGGRRYYACTTTGATARAGT AGGGTGCCRATRTCYTTRTGRTT	1600	50
C1	Ala5030L COI6600H	ACATCTTCTGAATGCAACCCA AAGTGYTGTGGRAARAATGT	1600	45
C2	COI5600L COII7000H	TTCCCTCGAATAAATAAYATAAG TGAAAGTGTAGTAGTTCTTCTAT	1400	45
E	Ser6800L Lys7700H	GAACCCCCITARRYTAATTTCAAGT CACCGRTCTWYAGCTTAAAAGGC	900	50
F	Lys7700L Arg9820H	AAGCAATAGCCTTTTAAGC AACCRAAATTTAYTRAGTCGAAAT	2100	50
G	Arg9820L Leu11720H	ATTTCGACTYAGTAAATTTYGGTT CATTACTTTTACTTGGRNTTGCACC	1900	50
H	SHis11540L SND512800H	TAGATTGTGATTCTAAAAAYGA ATTTTTCGAATGTCTTGTTC	1300	45
I	SND512660L SCB14280H	ATTGTAGCATTTTCAACATC GTGTCTGCTGTGTAGTGYAT	1600	45
M1	Glu14100L Pro15500H	GAAAAACCAAYGTTGTATTCAACTATAA AGAATTYTGGCTTTGGGTGCCA	1400–1600	50
M2	SThr15300L 12S600H	AAAACATCGGTCTTGTAAGCC TCGATTATAGAACAGGCTCCTCT	1400–1600	50

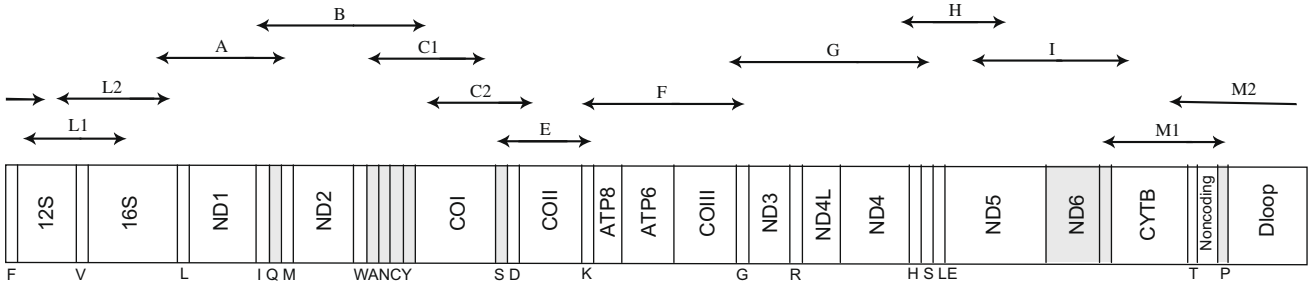

Fig. 1. Gene organization and sequencing strategy for mt genomes of salamandrids. Genes encoded by the L strand are shaded. Arrow headed segments denote the location of the fragments amplified by PCR with each pair of primers (see Table 2 for the primer DNA sequence associated with each fragment).

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, 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 sequences were aligned using Clustal X (Thompson et al., 1997) at default settings, respectively. 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. The parameter settings used in Gblock are: minimum number of sequences for a conserved position 22; minimum number of sequences for a flanking position 35; maximum number of contiguous nonconserved positions 8; minimum length of a block 5; no

gaps allowed. Finally, a DNA dataset combining all 16 Gblock-refined alignments was generated. Mueller et al. (2004) determined 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, was better than the other partition strategies. We thus 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). The mitogenomic alignment used in this study was deposited in TreeBASE under Accession No. SN3995.

2.4. Phylogenetic analyses

Maximum parsimony (MP) analyses were performed with PAUP* 4.0b8 (Swofford, 2001) using heuristic searches (TBR branch swapping; MULPARS option in effect) with 100 random-addition sequences. All sites were given an empirical weighting of a 2:1 transversion–transition rate. Support for internal branches in the parsimony analyses was assessed using 1000 bootstrap replicates, with 10 random-addition sequences performed in each replication. Partitioned maximum-likelihood (ML) phylogenetic analyses were performed on the DNA dataset by using RAxML 7.0.0 (Stamatakis, 2006) with independent GTR + I + Γ substitution models applied to 42 partitions. Robustness of the ML results was tested by bootstrapping analyses with 1000 replicates performed by RAxML. The partitioned Bayesian inference was done with MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001). The dataset was divided into 42 partitions as described above. The best-fitting nucleotide substitution models for each of the 42 partitions were selected using the hierarchical likelihood ratio test implemented in MRMODELTEST version 1.1b (http://www.ebc.uu.se/systzoo/staff/nylander.html). Metropolis-coupled Markov chain Monte Carlo (MCMC) analyses (with random starting trees) were run with one cold and three heated chains (temperature set to 0.1) for 20 million generations and sampled every 1000 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 3–10 million generations were discarded. To ensure that our analyses were not trapped in local optima, four independent MCMC runs were performed. Topologies and posterior clade probabilities from different runs were compared for congruence.

Alternative phylogenetic topologies were tested using the parsimony-based Templeton Test (Templeton, 1983), the likelihood-based Kishino–Hasegawa (KH) test (Kishino and Hasegawa, 1989), and the approximately unbiased (AU) test (Shimodaira, 2002). To perform the KH and AU tests, the first step was to reconstruct alternative tree topologies. PAUP heuristic searches under a GTR + I + Γ model and incorporating a topological constraint were conducted in order to identify the highest-likelihood topology that satisfied a given hypothesis. Second, PAUP was used to produce a log file for the site-wise log-likelihoods of alternative trees given the concatenated data set with a GTR + I + Γ model. The log file generated was then submitted to the CONSEL program (Shimodaira and Hasegawa, 2001) to calculate the *P*-value for each alternative topology by the AU test and the KH test. The Templeton test was done by using 1000 RELL bootstrap replicates, implemented in PAUP* v4.0. The search strategy for finding alternative phylogenetic hypotheses for use in Templeton tests followed a similar methodology.

2.5. Molecular dating

The tree shown in Fig. 2 was used as the reference topology to perform the molecular dating analyses. A frog sequence (*Xenopus tropicalis*) served as outgroup, allowing the tree relating the remaining 41 ingroup sequences to be rooted. We chose five nodes as our primary calibration points. The ingroup root of the tree, the split between Cryptobranchoidea and Salamandroidea, is constrained between 151 and 170 million years ago (Ma). This is based on the oldest known salamander fossil, the salamandroid-like *Iridotriton hechti*, dated to 151 Ma (Evans et al., 2005) and a proposed maximal bound for the origin of Caudata (170 Ma; Marjanović and Laurin, 2007). The lower limit for the split between Hynobiidae and Cryptobrachidae 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 145 Ma (as used by Roelants et al., 2007), which is a more conservative minimum age for this problematic fossil than the original assumption of a Middle Jurassic age by Gao and Shubin. The occurrence of the common ancestor of the Salamandridae is constrained between 55 and 151 a. This is based on the oldest known fossil of the family, the newt-like *Koalliella genzeli*, dated to 55–65 Ma (Estes, 1981), and the oldest known fossil of salamanders, the salamandroid-like *Iridotriton hechti* as mentioned above. The lower limit for the split between *Tylototriton* and *Pleurodeles* is set to 44 Ma, based on a *Tylototriton*-related salamandrid fossil, *Chelotriton weigelti* from the middle Eocene (Milner, 2000). The *Taricha-Notophthalmus* split is constrained to be greater than 23 Ma, based on a nearly complete fossil skeleton of *Taricha oligocenica* from the upper Oligocene (Estes, 1981). The *Cynops-Paramesotriton* split is constrained to be greater than 15 Ma, based on the nearly complete fossil of *Procynops miocenicus* from the upper Miocene, which is apparently closely related to *Cynops* (Estes, 1981). The three calibration points within the Salamandridae are identical to those used by Steinfartz et al. (2007), making the dating results of both studies comparable.

To provide an alternative to time estimates that were old (because of the absence of recent calibration points), we introduced an additional calibration point based on indirect biogeographic inference: the split between Corsica-Sardinia *Euproctus* and the continental *Triturus* complex (Node 5; Fig. 3). Previous studies (Caccone et al., 1994, 1997; Steinfartz et al., 2000) suggested that this split was likely caused by the disjunction of the Corsica-Sardinia microplate from the Iberian Peninsula. Because the date of this geological event is somewhat controversial, either in the Late Oligocene about 29 Ma (Alvarez, 1972; Boccaletti et al., 1990) or in the Early Miocene 24–20 Ma (Robertson and Grasso, 1995; Carmignani et al., 1995; Meulenkamp and Sissingh, 2003), we used a broad time range of 30–20 Ma for this event and constrained the split between Corsica-Sardinia *Euproctus* and the continental *Triturus* complex within this time range.

Bayesian inference under various relaxed-clock models, implemented by MultiDivTime (Thorne and Kishino, 2002) and BEAST version 1.4.7 (Drummond et al., 2006), was used to perform the molecular dating process. We did not use the penalized likelihood method implemented in R8S (Sanderson, 2003) because that method uses only phylogenetic topology and branch length information derived from third-party programs and is unable to perform a multiple-locus analysis. Our saturation analysis (results not shown) indicates that mitochondrial third codon positions exhibit high saturation level when analyzing both outgroup and ingroup sequences. Therefore, these sites were excluded from our molecular dating analysis. As a result, the DNA dataset used in molecular dating contains 29 partitions as follows: a separate partition for each of the two ribosomal RNAs, the concatenated tRNAs, and first and second codon positions for thirteen protein-coding genes.

In the MultiDivTime analyses, optimized branch lengths with their variance–covariance matrices for the DNA dataset were estimated for each partition with the program Estbranches_dna, using an F84 + G model with parameters estimated by PAML (Yang, 1997). The priors for the mean and standard deviation of the

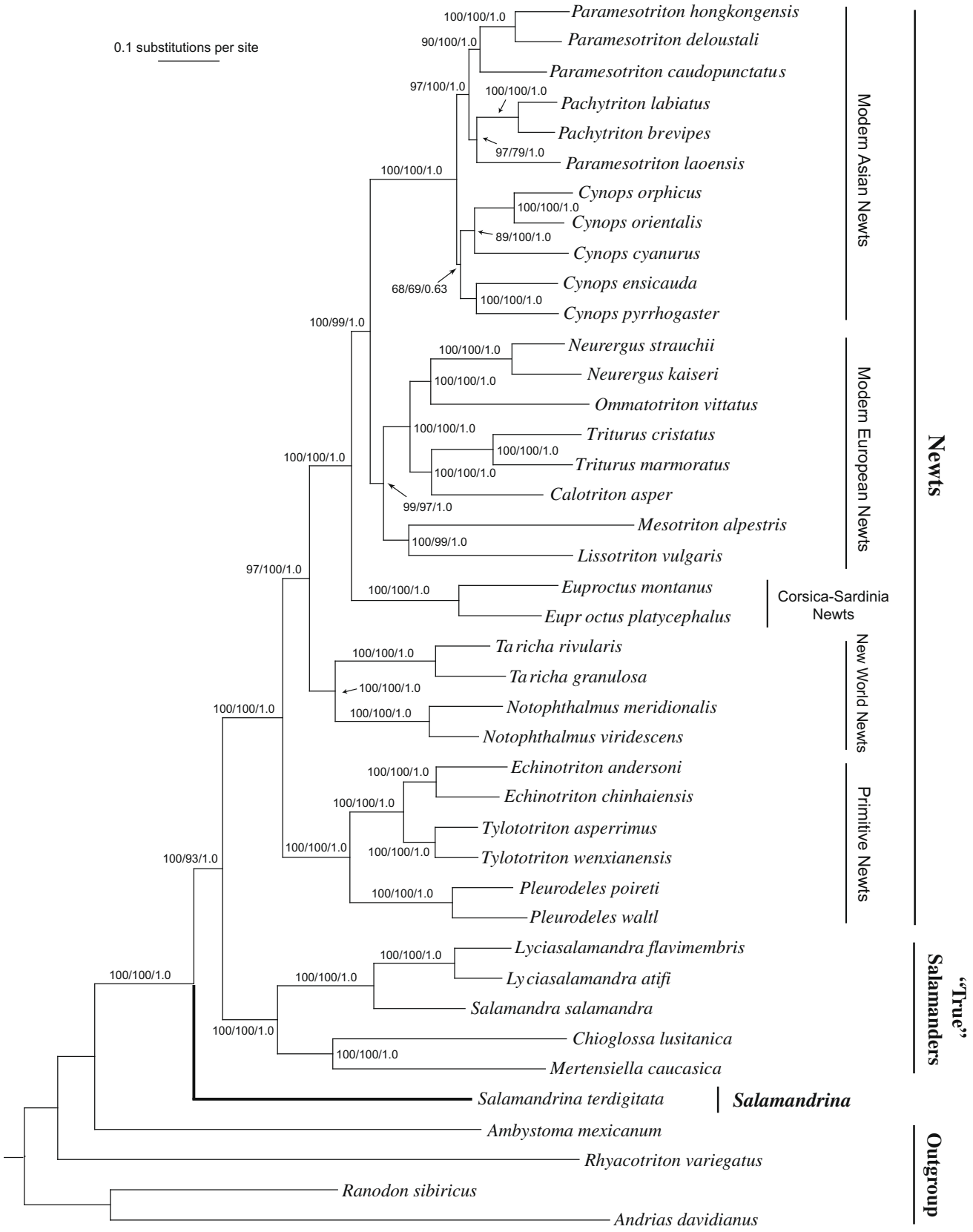

Fig. 2. Phylogenetic relationships of extant salamandrids inferred from mitochondrial genome sequences. The DNA dataset (14461 sites) was analyzed with weighted maximum parsimony, partitioned maximum likelihood, and partitioned Bayesian inference. All approaches produce identical topology, and their branch-support measures are mapped to the phylogram. Left numbers along branches represent weighted maximum parsimony bootstrap values, middle numbers represent partitioned maximum likelihood bootstrap values and right numbers represent partitioned Bayesian posterior probabilities. Branch lengths were estimated by partitioned maximum-likelihood analysis. Frog outgroup (*Xenopus*) is not shown.

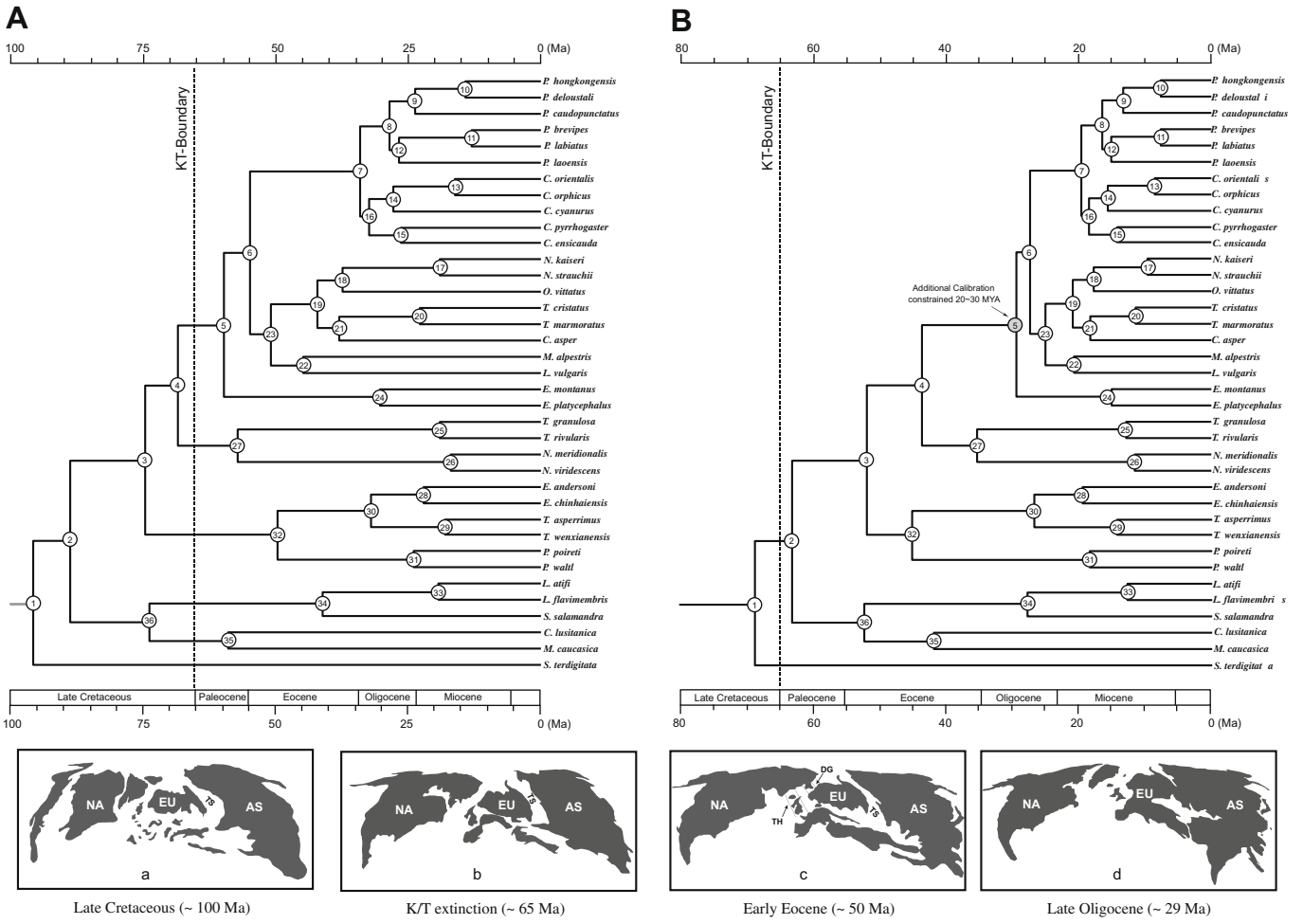

Fig. 3. Two proposed timescales for salamandrid evolution. The two timescales are according to the results estimated by BEAST based on primary calibration (A) or plus additional calibration (B). Non-salamandrid species are not shown in the timetrees. Detailed time estimates can be found in Table 4 to nodes with numbered circles above them. The additional calibration point is indicated as shaded circles with constraint information beside them. The paleomaps of the Northern Hemisphere during four important geologic periods are illustrated below the trees. *Abbreviations:* NA, North America; EU, Europe; AS, Asia; DG, "De Geer" North-Atlantic land bridge; TH, "Thulean" North-Atlantic land bridge; TS, Turgai Sea.

ingroup root age, rttm and rttmsd were set according to our ingroup constraints of 151–170 Ma (i.e., rttm = 1.60, rttmsd = 0.1), respectively. The prior mean and standard deviation for the Gamma distribution describing the rate at the root node (rtrate and rtratesd) were both set to 0.15. These values were based on the median of the substitution path lengths between the ingroup root and each terminal, divided by rttm. The prior mean and standard deviation for the Gamma distribution of the parameter controlling rate variation over time (i.e., brownmean and brownsd) were both set to 0.5. To allow the Markov chain to reach stationarity, the Markov chain Monte Carlo algorithm completed 200,000 initial cycles before the state of the Markov chain was sampled. Thereafter, the Markov chain was sampled every 100 cycles until a total of 10,000 samples was collected. To test whether or not the Markov chain was converging, three independent runs were performed. In the BEAST analyses, the uncorrelated lognormal model was used to describe the relaxed-clock, while GTR + I + Γ was used to describe the substitution model for 29 partitions of the dataset. The Yule process was used to describe speciation. Unlike MultiDivTime, BEAST does not require a fixed topology for time estimation; the tree shown in Fig. 2 was used only as the starting topology and has no influence on final results. A test MCMC run with 10 million generations was first performed to optimize the scale factors of the priori function. The final MCMC chain was run twice for 100 million generations sampled every 1000 generations. Burn-in and convergence of the chains were determined with Tracer 1.4. The measures of effective sample sizes (ESS) were used to determine the Bayesian statistical significance of each parameter.

3. Results

3.1. General features of salamandrid mtDNA

The complete nucleotide sequences of the L strands of the mt genomes of 35 salamandrid salamanders were determined. Total length ranged from 16,252 to 17,023 bp. As in most of the published higher vertebrate sequences, all 35 newly sequenced salamandrid mt genomes encode for two rRNAs, 22 tRNAs, and 13 protein-coding genes, with the exception of *Cynops pyrrhogaster*, whose tRNA-Ser (UCN) gene is unusually short and loses function. The long non-coding region between tRNA-Thr and tRNA-Pro genes, which is observed in all published salamander mtDNAs, is also present in all 35 new salamandrid sequences, ranging from 89 to 885 bp; this is the major cause of length variation among salamandrid mtDNAs.

3.2. Phylogenetic analysis

The DNA data set combining two rRNAs, the concatenated tRNAs, and 13 protein-coding gene alignments contains 14461 characters (6006 constant, 1321 highly variable, and 7134 parsimony-informative). Weighted MP, partitioned ML and partitioned Bayesian analyses all produce identical topologies. The resulting phylogeny is well resolved; most branches are strongly supported with greater than 90% bootstrap values (BS) and 1.0 Bayesian posterior probabilities (PP). To test the consistency of our results, we also used equal-weighting MP, unpartitioned ML, and unpartitioned Bayesian methods that are more assumption-independent to repeat our phylogenetic analyses, and obtained identical topologies and similar statistical support (results not shown). Fig. 2 shows the ML tree obtained from the complete mitochondrial genome using independent GTR + I + Γ models applied to 42 data partitions and summarizes the statistical results of the other phylogenetic methods employed in the study.

Our phylogeny is well resolved and contains several distinct clades (Fig. 2). The endemic Italian genus *Salamandrina* is recovered as sister to all other salamandrids. A clade of "true salamanders" (*Chioglossa*, *Lyciasalamandra*, *Mertensiella*, and *Salamandra*) is strongly supported, and is sister to another well-supported clade that includes all newts except *Salamandrina*. The first branch within the newt clade is the "primitive newts" (*Echinotriton*, *Pleurodeles*, and *Tylototriton*). Species of these genera share features of cranial anatomy long considered ancestral and the clade has a long fossil record (Estes, 1981). The second branch within the newt clade, "New World newts", includes both North American genera, *Notophthalmus* and *Taricha*. The two *Euproctus* species of Corsica-Sardinia are sister to all remaining Eurasian newts and we therefore give the informal name "Corsica-Sardinia newts" to this clade. A clade comprising the Asian genera *Cynops*, *Pachytriton*, and *Paramesotriton* (informally called "modern Asian newts") is strongly supported. European continental genera (*Calotriton*, *Lissotriton*, *Mesotriton*, *Neurergus*, *Ommatotriton*, and *Triturus*) form a clade and we term "modern European newts". Subsequent description and discussion of results will use the clade names listed in Fig. 2.

The only ambiguous parts of our mitogenomic trees are the placement of the recently described species *Paramesotriton laoensis* and the monophyly of the genus *Cynops*. Both MP and Bayesian analyses strongly support a clade of *Paramesotriton laoensis* + *Pachytriton* (MPBS = 97%, PP = 1.0). Bootstrap support of the partitioned ML analysis for this grouping is 79%. Likelihood-based topological tests found that an hypothesis of a *Paramesotriton laoensis* + *Pachytriton* clade is not significantly better than two alternative hypotheses: (1) monophyly of *Paramesotriton* and (2) a clade with the following topology (*Paramesotriton laoensis*, (remaining *Paramesotriton*, *Pachytriton*)) ($P > 0.05$; Table 3). The monophyly of *Cynops* is only moderately supported in our mitogenomic tree (MPBS = 68%, MLBS = 69%, PP = 0.63). Topological tests based on both parsimony and likelihood were unable to reject paraphyly of *Cynops* ($P > 0.05$; Table 3).

3.3. Divergence times

The final sequence length for the molecular dating analysis is 10,755 nt after excluding all third codon positions. The estimated divergence times for the nodes of the phylogeny are summarized in Table 4. Generally, mean age estimates averaged 10–15% higher in MultiDivTime analyses than in BEAST analyses, but 95% confidence intervals (CI) of the BEAST analyses normally overlapped with the MultiDivTime's 95% CI, suggesting that the time estimates from the two programs are congruent. When using only primary calibration points, both MultiDivTime and BEAST gave a Late Cretaceous (90–100 Ma) origin for extant salamandrids (Node 1; Table 4), and the divergences of the major clades took place before Eocene (>55 Ma; Table 4). On the other hand, when using the additional Mediterranean biogeographic calibration point (see Section 2 for details), the root of extant salamandrids was estimated about Near Paleocene (65–80 Ma, Late Cretaceous to Early Paleocene; Node 1; Table 4) and the divergences of the major clades occurred during the Eocene-Oligocene period (30–55 Ma; Table 4). These dating differences are discussed below.

4. Discussion

4.1. Phylogeny and systematics of salamandrids

Using a mitochondrial fragment of ~2700 bp and nearly complete taxon sampling, Weisrock et al. (2006) presented a comprehensive view of salamandrid phylogeny. Their results are largely in agreement with Steinfartz et al.'s (2007) molecular study based on different mitochondrial fragments of about 1700 bp. However, in both studies, some uncertainties remained and many clades were strongly supported only in their Bayesian analyses. By increasing the amount of mitochondrial sequence, we have generated a more robust salamandrid phylogeny. We used various phylogenetic analytical methods, such as bootstrapping MP, bootstrapping partitioned ML and partitioned Bayesian inference, to reconstruct and evaluate our trees. In general, our mitogenomic trees are topologically similar to the previous results (Weisrock et al., 2006; Steinfartz et al., 2007) but most nodes that were pre-

Table 3
Statistical comparisons among alternative hypotheses of salamandrid relationships using AU test, KH test, and Templeton test

Alternative topology tested	Likelihood-based			Parsimony-based
	$\Delta\ln L$[a]	AU test	KH test	Templeton test Δsteps[b] (P value)
Best tree	—	—	—	—
Salamandrina sister to all remaining Newts	29.5	$P = 0.029^*$	$P = 0.037^*$	68 ($P = 0.0001^*$)
Salamandrina sister to "true" salamanders	25.6	$P = 0.013^*$	$P = 0.016^*$	66 ($P = 0.0002^*$)
Old *Mertensiella* monophyly	408.5	$P = 0^*$	$P = 0^*$	352 ($P < 0.0001^*$)
Old *Triturus* monophyly	277.8	$P = 0^*$	$P = 0^*$	200 ($P < 0.0001^*$)
Old *Euproctus* monophyly	322.6	$P = 0^*$	$P = 0^*$	226 ($P < 0.0001^*$)
Cynops non-monophyly	1.7	$P = 0.604$	$P = 0.414$	13 ($P = 0.1730$)
Paramesotriton monophyly	10.7	$P = 0.214$	$P = 0.189$	40 ($P = 0.0006^*$)
P. laoensis sister to *Paramesotriton*+*Pachytriton*	12.0	$P = 0.187$	$P = 0.158$	39 ($P = 0.0009^*$)

[a] Log likelihood difference for the trees being tested.
[b] Difference in minimum numbers of mutational steps for the paired trees being tested.
* $P < 0.05$, significant difference.

Table 4
Divergence time means and 95% confidence intervals calculated by MultiDivTime and BEAST

Nodes	MultiDivTime		BEAST	
	Primary calibration	Plus additional calibration	Primary calibration	Plus additional calibration
1	101.3 (91.2,111.4)	79.4 (72.8,86.1)	96.7 (79.9,113.1)	68.8 (57.7,83.0)
2	92.9 (83.6,102.3)	69.7 (64.7,74.9)	89.6 (74.2,104.7)	63.2 (53.8,75.9)
3	74.2 (66.4,82.5)	52.6 (49.9,55.4)	75.3 (63.0,88.6)	51.9 (47.5,57.7)
4	69.6 (61.9,77.6)	43.7 (41.2,46.2)	69.1 (57.6,81.5)	43.5 (34.2,51.1)
5	62.7 (55.4,70.3)	29.9 (29.5,30.0)	60.3 (49.8,71.4)	29.3 (27.8,30.0)
6	58.8 (51.8,65.9)	29.1 (27.9,29.9)	55.3 (45.1,65.3)	27.2 (25.6,28.7)
7	34.3 (29.7,39.2)	17.9 (16.1,19.6)	34.3 (27.6,40.9)	19.5 (16.9,21.5)
8	29.5 (25.4,34.0)	15.6 (13.9,17.3)	28.7 (22.4,35.0)	16.3 (13.1,18.8)
9	23.7 (20.0,27.6)	12.5 (10.9,14.3)	23.8 (18.0,29.9)	13.2 (9.9,16.5)
10	15.0 (12.3,18.2)	8.0 (6.7,9.6)	14.2 (9.2,19.4)	7.5 (4.4,11.8)
11	13.0 (10.4,15.9)	6.9 (5.6,8.2)	13.0, (7.8,18.2)	7.5 (3.9,11.2)
12	27.4 (23.3,31.8)	14.5 (12.8,16.3)	26.9 (20.4,33.5)	15.0 (11.5,18.3)
13	16.1 (13.2,19.3)	8.6 (7.2,10.1)	16.2 (10.4,22.5)	8.4 (3.7,12.7)
14	29.0 (24.7,33.5)	15.4 (13.5,17.3)	28.0 (21.6,34.7)	15.5 (12.1,18.8)
15	26.2 (22.2,30.6)	13.8 (12.0,15.7)	26.5 (19.5,33.3)	14.1 (9.9,17.9)
16	32.5 (28.0,37.2)	17.0 (15.2,18.8)	32.6 (26.0,39.2)	18.3 (15.7,20.6)
17	21.1 (17.4,25.2)	11.1 (9.3,12.9)	19.1 (12.1,26.4)	9.5 (5.4,13.8)
18	42.6 (36.5,49.1)	22.1 (19.8,24.4)	37.7 (28.8,46.7)	17.6 (12.2,21.7)
19	46.8 (40.7,53.3)	24.0 (22.0,26.0)	42.5 (33.5,51.4)	20.8 (17.4,23.9)
20	26.6 (22.3,31.3)	14.0 (12.1,16.0)	22.9 (14.6,30.7)	11.3 (7.4,15.4)
21	41.8 (36.1,47.9)	21.7 (19.5,23.8)	38.3 (29.7,47.3)	18.1 (14.0,21.4)
22	50.2 (43.2,57.7)	25.7 (23.1,28.2)	45.2 (36.4,54.7)	20.6 (17.1,24.3)
23	55.8 (49.1,63.0)	28.1 (26.4,29.5)	51.3 (41.5,60.8)	24.9 (22.3,27.0)
24	31.5 (26.3,37.0)	15.6 (13.5,17.9)	30.5 (18.4,42.4)	14.9 (8.5,21.1)
25	21.5 (17.7,25.5)	14.7 (12.4,17.3)	19.1 (11.1,28.1)	12.7 (4.7,19.8)
26	19.6 (15.8,23.9)	13.2 (10.8,15.8)	17.0 (10.5,24.6)	11.4 (5.5,18.6)
27	55.4 (48.5,62.7)	36.4 (33.3,39.6)	57.7 (46.2,69.4)	35.2 (26.7,44.6)
28	26.3 (21.8,31.2)	21.8 (18.8,24.9)	22.2 (15.1,29.8)	19.4 (11.2,28.3)
29	19.4 (16.0,23.2)	16.3 (13.9,18.8)	18.1 (11.1,25.3)	14.2 (5.7,22.2)
30	33.2 (28.3,38.7)	28.1 (25.1,30.7)	32.2 (24.3,41.0)	26.7 (18.9,35.6)
31	24.5 (20.4,29.0)	20.6 (17.9,23.5)	24.1 (15.9,32.0)	18.3 (7.9,26.7)
32	51.8 (45.6,58.8)	44.3 (44.0,45.2)	50.1 (44.0,58.9)	45.1 (44.0,47.3)
33	20.9 (17.1,25.2)	16.1 (13.1,19.4)	19.4 (11.4,28.1)	12.6 (5.3,20.8)
34	43.4 (37.2,50.2)	33.5 (28.9,38.5)	41.5 (28.7,55.0)	27.7 (16.1,39.9)
35	71.2 (62.0,81.0)	53.4 (46.9,60.1)	59.5 (43.4,75.3)	41.8 (25.8,55.4)
36	81.2 (72.0,90.7)	61.2 (55.4,67.4)	74.5 (59.0,90.1)	52.3 (41.2,63.5)

Letters for nodes are corresponding to Fig. 3. See Section 2 for calibration choices.

viously not resolved or weakly supported received strong support in the current study. To reduce redundancy, we discuss only those nodes previously weakly supported or unreported.

One of the major contributions of this study is robust determination of the placement of the genus *Salamandrina*. A basal polytomy is found consistently in previous higher-level studies of salamandrid phylogeny (Titus and Larson, 1995; Steinfartz et al., 2007; Weisrock et al., 2006) among three major salamandrid lineages: (1) the endemic Italian *Salamandrina*, (2) a lineage ancestral to the mostly European true salamanders, and (3) and a lineage ancestral to all newts excluding *Salamandrina*. Weisrock et al. (2006) proposed two hypotheses: either *Salamandrina* is the sister lineage to the true salamanders or it is sister to a clade containing all remaining newts. Steinfartz et al. (2007) found *Salamandrina* to be the sister taxon to all other extant salamandrids but without significant support (Bayesian posterior probability < 0.95). Our mitogenomic analyses favored the result of Steinfartz et al. (2007) but with strong statistical support. *Salamandrina* is collectively resolved as sister to all other salamandrids by all methods (BS > 90% and PP = 1.0, Fig. 2) and both hypotheses suggested by Weisrock et al. (2006) are rejected by our topological tests ($P < 0.05$, Table 3).The family Salamandridae has long been informally divided into two major subgroups, the true salamanders (*Chioglossa*, *Lyciasalamandra*, *Mertensiella* and *Salamandra*) and the newts (all remaining extant genera). Our finding indicates that the commonly used term "newts" does not refer to a monophyletic group. *Salamandrina* has particular features of its biology that differentiate it from all other newts, such as courting in mainly terrestrial settings (different from all modern newts) and lacking ventral amplexus (different from all primitive newts) (Houck and Arnold, 2003). Therefore, it is best considered as a unique clade, neither salamander nor newt in the conventional sense of these vernacular terms.

Our results agree with previous studies of salamandrid phylogeny (Weisrock et al., 2006; Steinfartz et al., 2007) in placing *Calotriton*, *Cynops*, *Euproctus*, *Lissotriton*, *Mesotriton*, *Neurergus*, *Ommatotriton*, *Pachytriton*, *Paramesotriton*, and *Triturus* into a large clade (Fig. 2). However, our trees firmly place Corsica-Sardinia *Euproctus* as the sister taxon to the remaining genera (Fig. 2; MPBS 100% and MLBS 99%). Weisrock et al. (2006) reported only 63% MPBS for this node and Steinfartz et al. (2007) found *Euproctus* to be nested within the larger clade (PP < 0.90). Furthermore, our phylogenetic analyses group all other European genera of this clade (except *Euproctus*; that is, *Calotriton*, *Lissotriton*, *Mesotriton*, *Neurergus*, *Ommatotriton*, and *Triturus*) into a newly recognized, fully resolved clade (modern European newts; Fig. 2), whose sister taxon is a clade that comprises the modern Asian genera *Cynops*, *Pachytriton* and *Paramesotriton*. This well-supported grouping (BS > 95% and PP = 1.0) has not been found in other studies. *Calotriton* is sister to *Triturus*. *Ommatotriton* is the closest relative of *Neurergus*. And *Lissotriton* and *Mesotriton* form a strongly supported clade, sister to the clade comprising *Calotriton*, *Triturus*, *Ommatotriton*, and *Neurergus*. These relationships were partly reported in previous studies (Busack et al., 1988; Giacoma and Balletto, 1988; Halliday and Arano, 1991; Macgregor et al., 1990; Titus and Larson, 1995; Zajc and Arntzen, 1999; Steinfartz et al., 2007; Weisrock et al., 2006) but never so strongly supported by all analytical methods.

Our results confirm previous molecular studies in grouping *Cynops*, *Pachytriton*, and *Paramesotriton* into a monophyletic group (Chan et al., 2001; Hayashi and Matsui, 1988; Titus and Larson, 1995; Steinfartz et al., 2007; Weisrock et al., 2006). Relationships within this clade remain difficult to resolve even with complete mitochondrial genome data. As in the previous results (Steinfartz et al., 2007; Weisrock et al., 2006), the monophyly of *Cynops* is not well-supported by Bayesian, likelihood or parsimony analyses (BS < 70% and PP = 0.63; Fig. 2) and a paraphyly hypothesis of *Cynops* cannot be rejected ($P > 0.05$; Table 3). The recently described salamandrid species from Laos, *Paramesotriton laoensis* (Stuart and Papenfuss, 2002), is recovered as sister to *Pachytriton* in our mitogenomic tree (Fig. 2), in contrast to previous results that place the species sister to a well-supported clade containing the genus *Pachytriton* and all remaining species of *Paramesotriton* (Weisrock et al., 2006). However, our result receives strong support only in parsimony analyses (MPBS 97%; alternative hypotheses $P < 0.05$, Table 3) but not in likelihood analyses (MLBS 79%; alternative hypotheses $P > 0.05$, Table 3), suggesting a soft polytomy problem here. The ML-corrected sequence divergence matrix (Supplementary Material, Table 1) also indicates that *P. laoensis* is closer to *Pachytriton* (avg. = 0.163) than to *Paramesotriton* (avg. = 0.176). Although *P. laoensis* is morphologically similar to other species of *Paramesotriton* in its skull morphology and vertebral number (12), it has a reduced tongue pad and probably fully aquatic lifestyle similar to that of *Pachytriton* (Stuart and Papenfuss, 2002). If our phylogenetic placement of *P. laoensis* is correct, this species likely represents a transitional form when the increasingly specialized *Pachytriton* stock evolved from *Paramesotriton* to become aquatic. Our results suggest that denser taxon sampling will be required to obtain a robust phylogenetic hypothesis for the modern Asian newts. This matter is treated in detail elsewhere (Stuart et al., in prep.).

Our phylogenetic results are useful in evaluating some recent taxonomic changes. Species formerly placed in *Triturus* have been sorted into *Lissotriton*, *Mesotriton* (Montori and Herrero, 2004), *Ommatotriton* (Litvinchuk et al., 2005), and *Triturus*, species of *Mertensiella* into *Lyciasalamandra* and *Mertensiella* (Veith and Steinfartz, 2004), and species of *Euproctus* into *Calotriton* and *Euproctus* (Montori and Herrero, 2004). These changes are rational according to our results because traditional versions of *Euproctus*, *Mertensiella*, and *Triturus* were polyphyletic (Fig. 2) and monophyly of these genera is consistently rejected (Table 3). Our ML-corrected sequence divergence matrix (Supplementary Material, Table 1) indicates that divergence within the currently recognized genera (all of which are monophyletic) ranges from 0.072 to 0.18. The pairwise divergence between *Lissotriton*, *Mesotriton*, *Ommatotriton*, and *Triturus*, between *Calotriton* and *Euproctus*, and between *Mertensiella* and *Lyciasalamandra* is greater than 0.3, a substantial divergence that could be interpreted as supporting recognition of these morphologically differentiated groups.

4.2. Salamandrid evolution timescale

In this study, we presented four sets of divergence time estimates (Table 4) inferred from different dating methods and calibration choices. Our results suggest that the time estimation difference between dating methods is slight, with 10–15% mean difference and largely overlapping confident intervals. We further calculated the rate covariance among adjacent branches of our data under different calibration choices. The results showed that rate covariance among adjacent branches is 0.0125 (primary calibration) or 0.0284 (plus additional calibration), very close to 0, which indicates that there is no strong evidence supporting rate-autocorrelation among adjacent branches in our data (Drummond et al., 2006). This result suggests that the program BEAST (without rate-autocorrelation assumption) may be more suitable to our data and calibration choices than the program MultiDivTime (based on rate-autocorrelation assumption). Therefore, to facilitate our interpretations regarding divergence times, we used time estimates from the BEAST analyses as our primary dating results.

Before this study, there were two studies that provided several time estimates for the major splits within Salamandridae (Larson et al., 2003; Steinfartz et al., 2007). Larson et al.'s time estimates (2003) were based on a global clock assumption and published vertebrate mtDNA substitution rates, and this approach was questioned by Steinfartz et al. (2007), who considered the timing estimates to be too young. Using very similar calibration choices (primary calibration), our time estimates (based on mitochondrial genomes, >10000 bp) are largely congruent with those estimated by Steinfartz et al. (2007) based on partial cytb fragments (~700 bp). However, this does not mean that using longer sequences to perform molecular dating is unnecessary. For example, in Steinfartz et al.'s (2007) study, the split of True Salamanders-Newts was estimated to be 94.77 Ma (CI 76.6–114.0), nearly identical to the estimates of the root of Salamandridae 94.74 Ma (CI 79.7–114.0); while in our study, the two splits were estimated to be 89.6 Ma (CI 74.2–104.7) and 96.7 Ma (CI 79.9–113.1), respectively. This apparently is because the branch length errors estimated by small datasets are higher than those by larger datasets.

Although when using the primary calibration choices our time estimates for Salamandridae evolution are largely in agreement with Steinfartz et al.'s (2007) results, this does not mean these estimates are reliable because both studies used very similar calibration choices. The primary calibration strategy that we used was to apply most fossil constraints as minima, with a maximum root constraint. We think this is logical because fossils often provide good minimal bounds, but not maximal bounds. However, this strategy leads to a consistent bias toward inflated dating estimates, as indicated by Yang and Rannala (2006). In such analyses, a single calibration point with a maximal bound can largely scale the tree (and all other calibrations will have little effect), and model-fitting artifacts can further increase basal branch lengths until the maximum age constraint is reached, which makes the date for each node a maximum possible age (Hugall et al., 2007). Furthermore, previous computer simulation analyses pointed out that when the rate variation across branches (σ) is too great (e.g., $\sigma > 0.25$), relaxed-clock methods will not give consistently correct time estimation using only sequence data without accurate fossil constraints (Rannala and Yang, 2007). In our case, the rate variation across branches is estimated to be about 0.29−0.43, implying that longer sequences cannot guarantee accurate time estimates and more fossil constraints are needed. Because of this concern, we included an additional calibration point with a relatively strict constraint (*Euproctus-Triturus* split) to check the effect of different calibrations.

The time estimation difference under different calibration choices is obvious: when applying the additional biogeographic calibration point, the average time estimates are 15–30 Ma younger than those from primary calibration (Table 4). Steinfartz et al. (2007) suggested that comparing the time with independently derived time estimates, such as those based on biogeographic events, is a way to test the reliability of one's molecular dating results. Therefore, we applied two nodes with biogeographic inferences used by Steinfartz et al. to evaluate our salamandrid timescales based on different calibration choices. The split between *Euproctus montanus* (Corsica) and *Euproctus platycephalus* (Sardinia) is likely to have occurred between 29 and 13–15 Ma (Caccone et al., 1994, 1997). The time estimations for this node are 30.5 Ma (CI 18–42) (primary calibration) and 14.9 Ma (CI 8.5–21.1) (plus additional calibration), all compatible with this dating but the fit of the former result could be due to the large confidence interval. Veith et al. (2004) proposed three possible biogeographic scenarios that could have led to the current phylogeographic patterns in *Pleurodeles*: (I) the split between *Pleurodeles waltl* and *P. poireti* was caused by the Messinian salinity crisis (ca. 5.33 Ma); (II) the Betic crisis caused the split, moving the cladogenetic event back to ca. 14 Ma; (III) the Betic crisis caused the split between the north-western and south-eastern populations of *P. waltl*, rather than between the two *Pleurodeles* species, moving the split between the two species back to ca. 35 Ma. Based on current salamandrid fossil records and molecular inference, they further suggested that hypothesis II is more plausible than the other two. The time estimations for this split in our analyses are 24.1 Ma (CI 16–32) (primary calibration) and 18.3 Ma (CI 8–27) (plus additional calibration). The young estimate overlaps hypothesis II but the old one is incongruent. In general, the younger timescale presented in this study is more compatible with independent biogeographic inferences.

4.3. Historical biogeography of salamandrids

Because the preference of the two salamandrid timescales generated in this study is not definitive, we discuss some major biogeographic events of salamandrid evolution under both timescales. The first biogeographic question is when and where the common ancestors of salamandrids occurred. Because most extant salamandrid genera occur in Europe and the oldest salamandrid fossil (*Koalliella genzeli)* was also found in Europe, Milner (1983) suggested that salamandrids originated in Europe. Our phylogenetic analyses support this hypothesis because the *Salamandrina* is endemic to Italy and the split between the true salamanders, with European and Near East distributions, and the newts, many of which are European, place basal members of all three clades in Europe. According to our timescales, extant salamandrids probably originated either in Late Cretaceous (~96.7 Ma; Fig. 3A) or in Near

Paleocene (~68.8 Ma; Fig. 3B). In Late Cretaceous, Europe was comprised of only many isolated land pieces (Fig. 3a); early salamandrid ancestors might have begun their diversification by colonizing different landmasses. If salamandrids originated later in Near Paleocene, close to the K/T boundary (~65.5 Ma), when a mass extinction event occurred, the common ancestors of salamandrids probably survived after the considerable destruction of biota during the K/T extinction but were divided into many isolated groups, causing their initial diversification. Such diversification after the K/T extinction was also proposed for other families of salamanders, such as Hynobiidae (Zhang et al., 2006) and Plethodontidae (Vieites et al., 2007). Currently, there are no putative salamandrid fossil records older than 70 Ma (Milner, 2000; Marjanović and Laurin, 2007), making the Near Paleocene origin more compatible. However, as pointed out by Steinfartz et al. (2007) "the fact that currently no fossils older than 70 Ma were ever found does not mean that they could not exist", so we prefer to leave the origin time for salamandrids an open question.

Another biogeographic issue is when and how the European salamandrids colonized North America. Milner (1983) suggested that salamandrids dispersed from Europe to North America in the early Cenozoic, probably via the North Atlantic land bridge (NALB); however, some authors argued that the North American salamandrids invaded from Asia by way of Beringia (Duellman and Trueb, 1986). According to our phylogenetic results (Fig. 2), the North American salamandrids are without doubt a clade but they show no direct affinity with the Modern Asian salamandrids, which makes the Beringia hypothesis questionable. Our phylogenetic results are compatible with the NALB hypothesis because the New World newts are nested within the large newt clade and their close relatives are modern Eurasian newts (Fig. 2). Our dating analyses estimated the split of New World newts from their Old World relatives either in Near Paleocene (~69.1 Ma, CI 58–82) or in Mid-Late Eocene (~43.5 Ma, CI 34–51). These results further question the "Beringia Hypothesis" because Beringia was not directly reachable from Europe until the closing of the Turgai Strait in the Late Oligocene (~29 Ma; Briggs, 1995).

The opening of the North Atlantic began in the Late Cretaceous (90 Ma) but terrestrial connections between Europe and North America persisted along various North Atlantic land bridges until at least the Late Eocene. Two major North Atlantic land bridges have been postulated (McKenna, 1983; Tiffney, 1985). During the Early Tertiary, the Thulean Bridge is supposed to have connected southern Europe to eastern North America through the British Isles, Greenland, and the Queen Elizabeth Islands (Fig. 3c); this land bridge is considered to be the most important route for exchange of the Europe-North America biota in the Early Tertiary; it closed in the Early Eocene (50 Ma). A more northern trans-Atlantic connection, the De Geer Bridge, persisted until the Late Eocene (39 Myr); it connected Scandinavia (Fennoscandia) to eastern North America through northern Greenland and the Canadian Arctic Archipelago (Fig. 3c). This route is considered far less important for biotic exchange than the Thulean Bridge because of its northern position. Our older divergence date for the split of the New World newts from the modern Eurasian newts is in Near Paleocene (~69.1 Ma, CI 58–82; Node 4, Table 4). This result is compatible with the fact that terrestrial connections between Europe and North America persisted from Late Cretaceous to Late Eocene but barely overlapped with the time duration of the two proposed land bridges. If this time is correct, we anticipate that Paleocene salamandrid fossils may be found in the present-day Greenland and North America. Currently, the oldest salamandrid fossil in North America is from Oligocene (Holman, 2006). On the other hand, our younger time estimate for the split of New World newts from their Old World relatives is about Mid-Late Eocene (~43.5 Ma, CI 34–51; Node 4, Table 4). This time is similar to an independent study of plethodontid salamanders based on nuclear genes, which estimated divergence time between North American *Hydromantes* and European *Speleomantes* + *Atylodes* at about 41 Ma (Vieites et al., 2007). The timing match between these studies implies that the separation between North American and European salamanders may have been the result of the same geological processes. The younger time (~43.5 Ma, CI 34–51) barely overlaps the duration of the Thulean Bridge but is compatible with the duration of the De Geer Bridge. If the younger estimate is correct, salamandrids might have dispersed from Europe to North America through the northern De Geer Bridge rather than the southern Thulean Bridge. As pointed out by Vieites et al. (2007), in the two global warming periods, Late Cretaceous and the Paleocene–Eocene boundary, climate was generally much warmer, nearly all the way to the pole, thus using a northern pathway for cold-adapted salamandrids is reasonable. Moreover, straight-line distances become increasingly shorter in high latitude areas, which may also facilitate the dispersal process.

Our salamandrid phylogeny strongly supports modern Asian newts (*Cynops*, *Pachytriton*, and *Paramesotriton*) as sister to the modern European newts (*Calotriton*, *Lissotriton*, *Mesotriton*, *Neurergus*, *Ommatotriton*, and *Triturus*) (Fig. 2). This relationship implies that the Asian newts probably diverged from their European relatives because of a vicariance or dispersal event between Europe and Asia. According to the older timescale, the split between modern European newts and modern Asian newts is in Early Eocene (~55.3 Ma, CI 45–65; Node 6, Table 4). During this time, Europe was mostly separated from Asia by the Turgai Sea and the possible dispersal route for salamandrids is some weak connection at the southern edge of the two continents (Fig. 3c). Our younger timescale shows that this split occurred in Late Oligocene (~27.2 Ma, CI 26–29; Node 6, Table 4), which coincides with the closing of the Turgai Strait (~29 Ma; Fig. 3d). Combining the cladogenetic and dating information, the regression of the Turgai Sea would have offered a dispersal opportunity into a new region with the availability of new niches for European salamandrids, which may have given rise to modern Asian newts in eastern Asia. On the other hand, the "modern Asian newts" are not the only salamandrids distributed in eastern Asia; some "Primitive newts" (*Tylototriton* and *Echinotriton*) now also live in eastern Asia and their close relatives are again European *Pleurodeles* (Fig. 2). There is fossil evidence of close relatives of *Tylototriton* in Europe in the Eocene, prior to disappearance of the Turgai Strait (Estes, 1981; Milner, 2000). To interpret the current distribution of primitive salamandrids, Estes (1981) assumed that *Tylototriton* dispersed to eastern Asia from Europe, after withdrawal of the Turgai Sea and with the loss of tropicality in Europe; the populations remaining in Europe evolved into *Tylototriton*-related groups such as *Chelotriton* and *Brachycormus* (extinct salamandrids). If his hypothesis is correct, one would expect the split between current Asian *Tylototriton* and their closest Asian relatives (if they exist) to have occurred after the closing of the Turgai Strait. Our younger and older dating results for the split between *Tylototriton* and their Asia-endemic relatives *Echinotriton* (Node 30; Table 4) are about 26.7 Ma (CI 19–36) and 32.2 Ma (CI 24–41), respectively. Both estimates span the time of the closing of Turgai Strait (~29 Ma), which is compatible with Estes' hypothesis.

In summary, our timescales suggest that extant salamandrids probably originated in Europe from Late Cretaceous (~97 Ma) to Early Paleocene (~69 Ma), dispersed into North America through NALB in Paleocene (~69 Ma) or Late Eocene (~44 Ma), and further dispersed into Asia in Early Eocene (~55 Ma) or Late Oligocene (~27 Ma) after the withdrawal of the Turgai Sea. Although both the older scenario and the younger scenario are congruent with paleogeological evidence, the younger one is more plausible according to current knowledge of the fossil record.

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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.2008.08.020.

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