

A Bayesian Approach on Molecules and Behavior: Reconsidering Phylogenetic and Evolutionary Patterns of the Salamandridae with Emphasis on *Triturus* Newts

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ABSTRACT The monophyly of European newts of the genus *Triturus* within the family Salamandridae has for decades rested on presumably homologous behavioral and morphological characters. Molecular data challenge this hypothesis, but the phylogenetic position of *Triturus* within the Salamandridae has not yet been convincingly resolved. We addressed this issue and the temporal divergence of *Triturus* within the Salamandridae with novel Bayesian approaches applied to DNA sequence data from three mitochondrial genes (12S, 16S and *cytb*). We included 38 salamandrid species comprising all 13 recognized species of *Triturus* and 16 out of 17 salamandrid genera. A clade comprising all the “Newts” can be separated from the “True Salamanders” and *Salamandrina* clades. Within the “Newts” well-supported clades are: *Tylotriton–Pleurodeles*, the “New World Newts” (*Notophthalmus–Taricha*), and the “Modern Eurasian Newts” (*Cynops*, *Pachytriton*, *Paramesotriton* = together the “Modern Asian Newts”, *Calotriton*, *Euproctus*, *Neurergus* and *Triturus* species). We found that *Triturus* is a non-monophyletic species assemblage, which includes four groups that are themselves monophyletic: (i) the “Large-Bodied *Triturus*” (six species), (ii) the “Small-Bodied *Triturus*” (five species), (iii) *T. alpestris* and (iv) *T. vittatus*. We estimated that the last common ancestor of *Triturus* existed around 64 million years ago (mya) while the root of the Salamandridae dates back to 95 mya. This was estimated using a fossil-based molecular dating approach and an explicit framework to select calibration points that least underestimated their corresponding nodes. Using the molecular phylogeny we mapped the evolution of life history and courtship traits in *Triturus* and found that several *Triturus*-specific courtship traits evolved independently. *J. Exp. Zool. (Mol. Dev. Evol.)* 308B:139–162, 2007. © 2006 Wiley-Liss, Inc.

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Salamanders and newts of the family Salamandridae are at present distributed over North America and Eurasia. Despite a profound diversity in life history traits, including courtship and reproductive modes, the monophyly of the Salamandridae is strongly supported (Weisrock et al., 2005). Among the 17 extant salamandrid genera, the genus *Triturus* has the widest distribution and the highest number of species, since it is found throughout Europe except for northern Scandina-

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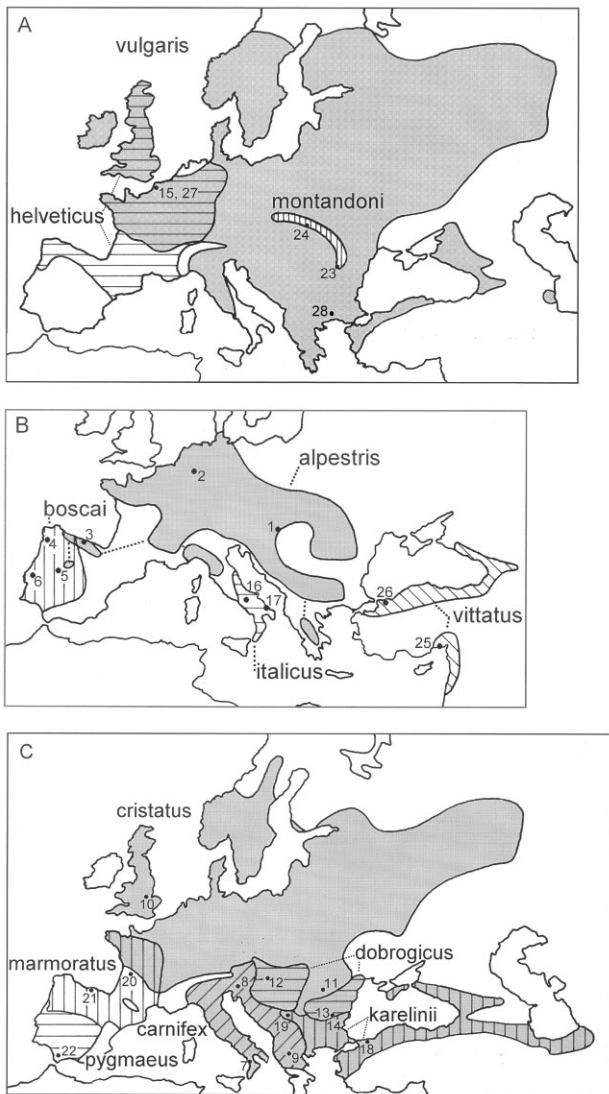


Fig. 1. Distribution of species currently included in the genus *Triturus*: (A) *T. helveticus* (horizontal hatching), *T. montandoni* (vertical hatching) and *T. vulgaris* (shaded); (B) *T. boscai* (vertical hatching), *T. italicus* (horizontal hatching), *T. vittatus* (diagonal hatching) and *T. alpestris* (shaded); (C) *T. marmoratus* (vertical hatching), *T. pygmaeus* (horizontal hatching), *T. carnifex* (diagonal hatching, shaded), *T. dobrogicus* (horizontal hatching, shaded), *T. karelinii* (vertical hatching, shaded) and *T. cristatus* (shaded, no hatching). Numbers refer to the sampling localities listed in Appendix A.

via and the Mediterranean islands (Fig. 1). The monophyly of *Triturus* was generally assumed on the basis of overall similarity in morphology and traits of courtship behavior. *Triturus* newts display a biphasic life style with aquatic reproduction, typically taking place in ponds and stagnant waters during spring and early summer, and a subsequent terrestrial phase until the next repro-

ductive event (Jehle and Faber, 2003). They are traditionally arranged into three groups according to body size: (1) a group of large-bodied newts (~16 cm total length) composed of four so-called crested species (*Triturus cristatus*, *T. carnifex*, *T. dobrogicus* and *T. karelinii*) and two so-called marbled species (*Triturus marmoratus* and *T. pygmaeus*, formerly *T. marmoratus pygmaeus*), (2) a group of intermediate-sized newts (~12 cm) composed of the alpine newt (*T. alpestris*) and the banded newt (*T. vittatus*), and (3) a group of small-bodied newts (~8 cm) composed of *T. boscai*, *T. helveticus*, *T. italicus*, *T. montandoni* and *T. vulgaris*.

The first systematic classification of *Triturus* and other salamandrid species dates back to Bolkay ('28). Based on cranial osteological characters, Bolkay proposed the monophyly of *Triturus* within the Salamandridae, although he also included in the genus the taxon now called *Neurergus crocatus*. The monophyly of *Triturus* was further supported by the first comprehensive salamandrid phylogeny based on morphological characteristics of the feeding apparatus (Wake and Özeti, '69). With the monophyly of *Triturus* more or less taken for granted, the design and goals of subsequent studies focused on the species relationships within *Triturus* rather than on the position of *Triturus* within the Salamandridae (e.g., Arntzen and Sparreboom, '87; Macgregor et al., '90). The most convincing phylogeny using this approach is the one from Arntzen and Sparreboom ('89). It combined allozymes and explicit behavioral data from the courtship for nine *Triturus* species to reconstruct an intrageneric phylogeny. This study supported the grouping of the large-bodied species (*T. cristatus*, *T. marmoratus* and allies) together with *T. alpestris* and *T. vittatus* as one clade, separate from the small-bodied species *T. boscai*, *T. helveticus*, *T. italicus*, *T. montandoni* and *T. vulgaris*. The monophyly of *Triturus* was challenged by the analysis of mtDNA sequence data (Caccone et al., '94, '97; Titus and Larson, '95) and other studies (Steinfartz et al., 2002; Garcia-Paris et al., 2004; Carranza and Amat, 2005; Litvinchuk et al., 2005). Recently, Garcia-Paris et al. (2004) and Litvinchuk et al. (2005) independently proposed a taxonomic revision of the genus *Triturus*. The first team proposed the erection of three genera (*Triturus*, including all the large-bodied species plus *T. vittatus*, *Lisso-triton*, comprising all small-bodied species, and *Mesotriton*, with only *T. alpestris*). The second team proposed the inclusion of *T. vittatus* in its

own genus, *Ommatotriton*, and the name *Lophinus*, for the small-bodied *Triturus* (SBT) species. In this paper, we refrained from applying either of these two revisions because we felt that both were based on incomplete taxon sampling and we adhere to the taxonomy of *Triturus* used by Frost et al. (2006).

Here, we investigate the phylogenetic position of *Triturus* with molecular data, based on a full sampling scheme of *Triturus* species and a comprehensive representation of other salamandrid species. We document a deep phylogenetic divergence of *Triturus* and provide a temporal calibration based upon fossil evidence that was then cross-validated with palaeogeographic data. Both, phylogenetic reconstruction and dating are accomplished using newly developed Bayesian approaches. We furthermore exploit the phylogenetic hypothesis to reconstruct the evolution of courtship traits within the Salamandridae in terms of the degree of body contact, with a focus on the primarily male-mediated *Triturus* courtship traits (Halliday, '77, '90). With *Triturus* being a model system in evolutionary ecology (Schmidt and van Buskirk, 2005), limb development and regeneration (Blanco and Alberch, '92; Rienesl and Wagner, '92; Wagner et al., '99; Endo et al., 2004; Wagner, this issue), population and conservation genetics (Jehle et al., 2005a,b), and ethology (Houck and Arnold, 2003), we believe that a robust phylogeny of *Triturus* is a valuable scientific contribution that will allow the evolution of observed complex traits to be placed within a sound phylogenetic context.

MATERIAL AND METHODS

Molecular methods

Sampling

We analyzed DNA sequence variation in the mitochondrial cytochrome *b* gene (*cytb*) for 162 individuals representing 25 non-*Triturus* salamandrid species and all 13 species of *Triturus*. Our taxonomic sampling covers 16 out of 17 extant salamandrid genera, i.e., all except for *Echinotriton* (cf. Duellman and Trueb, '86; Frost et al., 2006). For each *Triturus* species, we sampled one to nine individuals in up to three populations. Sampling localities are shown in Figure 1. As outgroups we used five representative species from four urodelan families: *Ambystoma mexicanum*, Ambystomatidae; *Andrias davidianus*, Cryptobranchidae; *Ranodon sibiricus*, Hynobiidae; *Boli-*

toglossa zapoteca and *Ensatina escholtzii*, Plethodontidae. To increase the resolution of the deep nodes of the phylogeny, we added published sequences of the small and large ribosomal mtDNA genes (12S and 16S) for 21 species (Titus and Larson, '95). Appendix A provides the information on sampling localities, taxonomic status, number of individuals, *cytb* haplotypes detected per locality, voucher material and GenBank accession numbers for both published and newly obtained sequences.

DNA extraction, sequencing and alignment

Total genomic DNA was extracted from either frozen or ethanol-preserved muscle tissue using the EasyDNA kit (Invitrogen, Carlsbad, CA). Approximately 800 bp (base pairs) of *cytb* were amplified using the primer combination MVZ15-MVZ16 (Moritz et al., '92). For DNA sequencing, we used the MVZ15 and MVZ16 primers, the nested MVZ18 and MVZ25 primers (Moritz et al., '92) and the universal primers *cytb* B2 and *cytb* Glu (Kocher et al., '89). In some cases, it was necessary to sequence the 800 bp fragment in two overlapping fragments (each ca. 450 bp long) by using the *cytb* B2-*cytb* GLU and MVZ25-MVZ16 primer pairs. PCR cycling conditions of the different primer combinations followed published protocols (Moritz et al., '92; Caccone et al., '97). DNA sequences were obtained with an automated DNA sequencer (Applied Biosystems 377 or 3100), following the manufacturer protocols. To increase accuracy, both DNA strands were sequenced for each individual. DNA chromatographs were edited using Sequencer 4.1 (Gene Codes Corporation).

All distinct *cytb* haplotypes were aligned using Clustal X (Thompson et al., '97) and all subsequent phylogenetic analyses were based on haplotypes. Nucleotide base composition, the transition-transversion ratio and percentage sequence divergence were assessed with the program PAUP 4.10b (Swofford, 2003). Data for the 12S and 16S genes were from Titus and Larson ('95: Fig. 3). Their alignment and character selection were used.

Tree reconstruction: choice of the substitution model

Separate and combined Bayesian analyses were used for the tree reconstruction. For each of three mtDNA partitions (12S with 21 taxa, 16S with 21

taxa and *cytb* with 104 taxa), we tested five evolutionary models using a Likelihood Ratio Test (LRT) against the most complex GTR+G+I-model. The five chosen models represent all combinations between two possible transition matrices (GTR and HKY) and three ways to accommodate among-site variation: (1) identical rates for all sites, (2) rates variable but following a gamma distribution Γ and (3) rates variable but with a percentage of invariable sites (I). For the *cytb* partition, we also explored a site-specific (SS) model with different substitution rates for the first, second and third codon position (GTR+SS). We evaluated the performance of this model under the Akaike information criterion (Akaike, '83).

The full dataset consists of 104 haplotypes with complete *cytb* sequences, but with missing data in the 12S and 16S partition for many taxa. This dataset was first analyzed using a general mixed model, in which each partition is allowed to evolve under a different GTR+G+I model, but with a topology that is shared across partitions (a plausible assumption since mitochondrial recombination is rare). We then used a Bayesian approach to choose the other parameters than can be shared among data partitions (Suchard et al., 2001). We tested all possible sub-models that shared a specific set of parameters across partitions against the general mixed model. Each comparison produced a Bayes factor estimated using a Savage–Dickey ratio (Verdinelli and Wasserman, '95). The preferred sub-model was subsequently used in the phylogenetic reconstruction. We prefer the Bayesian approach to the LRT one because it does not require finding the maximum likelihood (ML) set of values for each tested sub-model. Furthermore, rather than conducting the comparison over a point estimation of likelihood, as in ML, the Bayesian approach performs it over a sample of the entire posterior distribution.

The Bayes factor statistics were evaluated using the table of Jeffreys in Kass and Raftery ('95). We transformed the values of the Bayes factor into a natural logarithm to illustrate the results of the test graphically: a value of 0 corresponds to a Bayes factor 1, $-\ln(10)$ and $\ln(10)$ are the thresholds for strong support for the general and for the sub-model, respectively. Analogously, $-\ln(3)$ and $\ln(3)$ are the thresholds for moderate support for the two competitive hypotheses. Altogether, we tested 11 parameters (five mutation-specific rates multipliers, four base frequencies, the α parameter of the gamma distribution, and the percen-

tage of invariant sites) to examine if they were shared between the three data partitions (*cytb*, 12S and 16S).

In addition, we conducted two tests to examine how branch-length should be shared across partitions. The Bayesian analysis implemented in the program MrBayes 3.0b4 deals with branch-length diversity across data partitions in three ways: (i) all partitions have the same branch length, (ii) branch-length differs between partitions by a fixed scaling factor, (iii) each partition has a separate set of branch-length parameters. To deal with branch-length diversity across data partitions, we propose two new test statistics that were tested with simulated data. We tested if the percent difference in branch-length proportion (Δ BPL) between pairs of partitions has an increased probability at value zero than its respective prior probability. Δ TL is simply the difference of the sum of all branch-length parameters between two partitions. Δ BPL, is defined as

$$blp = \frac{bl}{TL},$$

$$\Delta BLP = \frac{blp_{1i} - blp_{2i}}{[blp_{1i}, blp_{2i}]}, \quad i = 1 \dots m,$$

where *bl* is a branch-length parameter for a given branch in a given partition for a given topology, *TL* is the total tree length for that specific partition and topology, and *i* is the branch index going from 1 to *m*, the total number of branches in the topology. In contrast to existing test statistics, this is not performed on the entire topologies but as pair-wise comparisons on the same branch-length for the two partitions. To avoid under evaluation of differences between short branches, each difference was divided by the mean of the branch-length proportion for that branch in the two partitions. If both tests (Δ TL and Δ BLP), detect a Bayes factor greater than 1, the branch-length should be considered the same for all partitions, corresponding to case (i). If the scaled difference of Δ BLP has a Bayes factor <1 (irrespective of the results from the total branch-length test), a different set of branch-length for the two tested partitions should be used (case ii). If Δ BLP results in a Bayes factor >1 , but Δ TL is <1 , SS parameters and one set of branch-length parameters should be used (case iii).

In order to test whether the newly devised statistics can detect differences of branch-length across partitions, we simulated two datasets each

with two 800 bp partitions. Both datasets have different transition matrix values and base frequencies for each partition with values taken from the estimated model. One dataset uses branch-lengths from *cytb* for both simulated partitions, while the other uses different sets. The simulation of the dataset was performed with the sub-routine “Evolver” in the PAML package (Yang, '97). The Markov chains, used to estimate the posterior distribution of both the original and the simulated data under the general mixed model from which all test statistics were estimated, were run for 5,000,000 generations with burn-in of 1,000. This unusually high generation number was adopted to obtain a detailed description of the posterior distribution of the parameters.

Tree reconstruction: Bayesian phylogenetic inference

All Bayesian analyses were performed with MrBayes 3.0b4. Each of the four Bayesian analyses (the three data partitions *cytb*, 12S plus 16S and the full dataset) was run for 2,000,000 generations using six differentially heated chains, sampling every 1,000 generations, and discarding the first 500,000 generations as burn-in. We checked for algorithm convergence in the full dataset by running two separate analyses and by graphical analysis with a Python script that compares clades's posterior probabilities (PP) from two or more MrBayes analyses (available from SV). Topological PP were summarized using a majority consensus tree, as implemented in PAUP 4.10b. The strength of alternative topological hypotheses for each of the nodes supported with low PP was evaluated by calculating the frequency of the hypotheses in the posterior distribution, as described in Vicario et al. (2003). We tested 24 alternative hypotheses focusing mainly on the position of *Triturus*.

We evaluated the impact of missing data by comparing the phylogeny obtained from the full dataset of 104 haplotypes (missing data in the 12S and 16S partition), with the one obtained from the restricted set of 21 haplotypes (21 taxa) with no missing data in all three partitions (hereafter referred to as “backbone dataset” and “backbone phylogeny”). We used the same evolutionary model for the two datasets, re-estimating each time the parameters' values. Following Wiens (2003), we assume that the missing data do not affect the results if both datasets result in a congruent phylogeny.

Molecular dating

The Bayes factor analysis (see section above) indicated that branch-lengths across the three DNA partitions were not compatible. Since only a sub-set of samples was available for the 12S and 16S data partitions, we carried out time estimations only on the *cytb* dataset.

In order to estimate the timing of the radiation of Salamandridae and of the *Triturus* taxa in particular, we performed a molecular clock analysis that took the three recognized sources of error into account: the topological, the branch-length and the calibration error (Sanderson et al., 2004). In a Bayesian framework, this involves estimating a posterior distribution for divergence times that integrates across the three sources of uncertainty (Drummond et al., 2002). We approximated this posterior distribution with a series of point ML estimations based on a sample of 2,000 trees (topologies+branch-length) from the posterior distribution generated by the phylogenetic inference. Several calibration points were included in the ML estimations to deal with uncertainty in the calibration time. Because the LRT on *cytb* rejected the molecular clock model ($P < 0.01$), these estimations were performed assuming a relaxed molecular clock, as implemented in the r8s software (Sanderson, '97, 2002). The sampled topologies and branch-lengths were modified to match the requirements of r8s (see r8s manual, Sanderson, '97, 2002). That is, we pruned the outgroup taxa (see Appendix A) from the analysis, but kept the root position to avoid performing the time estimation on a basal polytomy. We also pruned all terminal taxa that were at the end of branches with < 1 expected mutations, reducing the dataset from 104 to 60 *cytb* haplotypes. The 2,000 resulting chronograms were translated into distance matrices with the program TreeEdit (Rambaut and Charleston, 2002) and yielded the approximate posterior distribution of divergence times. To reduce the effect of asymmetric tails, we reported the mode, rather than the mean as the summary statistic (Hedges and Shah, 2003), and used the 95% Highest Probability Density (95% HPD), rather than the central 95% probability region, as confidence interval. Both mode and 95% HPD were calculated using the R package (R_Development_Core_Team, 2004).

We used six different fossil datings that represent five calibration points (C1–C5, Fig. 3) for nodes with a $PP \geq 0.95$. C1 refers to the root of the tree, the common ancestor of the Salamandridae,

which is constrained between 55 and 160 million years ago (mya). This is based on the oldest known fossil of the family, the newt-like *Koalliella genzeli*, dated to 65–55 mya (Estes, '81), and *Chunerpeton tianyiensis* (Cryptobranchidae), the earliest known crown group of urodeles, dated to 160 mya (Gao and Shubin, 2003). C2 corresponds to the lower limit for the split between *Tylotriton* and *Pleurodeles*. This is based on *Chelotriton weigelti* (Herre, '35; Rocek, '96; Milner, 2000; but see Carranza and Arnold, 2004), a fossil from the middle Eocene in a stratum that corresponds to the Mammal Paleogene 13 (MP13; Haubold and Thomae, '90), dated at 44 mya (Mertz et al., 2000). The lower limit for our calibration point C3 is the nearly complete fossil skeleton of *Taricha oligocenica* from the upper Oligocene, which shows strong similarities with *Taricha* from the Holocene and is dated to 22 mya (Estes, '81). C4 corresponds to the crown of the large-bodied *Triturus* (LBT) (crested and marbled newts), which should be older than the Lower Miocene fossils of *Triturus*, cf. *T. marmoratus* (Estes, '81), dated to 24.2–23.8 mya (Böhme, 2003). For calibration point C5, we refer to the nearly complete fossil of *Procyonops miocenicus* (Estes, '81), which shows several similarities with *Cynops orientalis*. The Shanwang series from which the fossil originates was dated to 18–15 mya (Qiu and Qiu, '95), which sets the lower limit of the node of the Modern Asian Newts (*Cynops*, *Pachytriton* and *Paramesotriton*) at 15 mya.

Sanderson's method requires fixing at least one of the calibration points, while the others can be constrained to be smaller or larger than a given value. We performed the complete analysis (making point likelihood estimates for each of the 2,000 trees from the posterior distribution) four times, each time fixing one of the four internal points (C2–C5), while leaving the root bounded between 160 and 55 mya, and constraining the other calibrations to be older than the fossil date. Assuming that each fossil potentially underestimates the true age of a node, we identified which fossils are closest in age to the node that it calibrates. This was accomplished by finding which of the four analyses produced age estimates for the unfixed internal nodes, that were older than the age of the fossils at those nodes. To infer the times of divergence, we used an analysis in which all multiple fossil calibrations contributed to the estimation, although in different ways. The fossils that produced the least underestimation of

the age of the node were used as fixed calibration points, while the others were used as minimum age constraints.

Life history and behavioral character evolution

Using the molecular phylogeny, we traced back the evolution of two life history traits (reproduction and adult habitat use) and the degree of body contact during courtship for the "Newts" clade (see Fig. 3 for names of clades and included species). Additionally, we analyzed the evolution of nine *Triturus*-specific male-mediated courtship displays (see Appendix B for description of traits). Evolutionary reconstructions were performed on the Bayesian molecular tree using the program SIMMAP (Bollback, 2005).

Since the taxa for which both molecular and behavioral data were available overlapped only partially, the mapping was restricted to 28 taxa (Appendix A). Because a single species was represented by several mtDNA haplotypes, we assigned the behavioral characters of one species to all of its mitochondrial haplotypes.

The probability for a character change was assumed to be proportional to time and it was estimated from the branch-length from the *cytb* data using r8s (Sanderson, 2002). Of the 2,000 trees sampled from the posterior distribution, 142 contained internal polytomies and were discarded from the analysis. For each of the remaining chronograms we sampled 10 possible sets of rate multipliers from the default a priori distribution. From each of these sets we sampled 100 possible histories of the characters. The resulting 1,858,000 simulated character histories were summarized by collecting the frequencies of each character state. This was carried out for a sub-set of 10 internal nodes and four terminal taxa that were of special interest to evaluate character evolution within *Triturus* and its closely related taxa.

RESULTS

Molecular analysis

Cytb sequence variation

The *cytb* alignment was 839 bp long, with no inferred deletions or insertions. Using the general vertebrate mitochondrial code all *cytb* sequences could be translated into aminoacid sequences without stop codons, with the exception of *T. alpestris* (see next section). Sixty unique

haplotypes were observed for *Triturus* with no haplotype shared between species. Among salamandrids the maximum sequence divergence value for *cytb* was 29.1%, while for comparisons between salamandrids and non-salamandrids maximum divergence was 30.1%, indicating a saturation of *cytb* sequences at the more inclusive phylogenetic levels. Within the “True Salamanders” the deep intra-specific sequence divergence found between western and eastern populations of *Mertensiella caucasica* (range 11.3–14.5%) confirms previous work (Tarkhnishvili et al., 2000). Between small-bodied *Triturus* and LBT, maximum sequence divergence for the *cytb* fragment was 17.5% and 15.0%, respectively. Maximum *cytb* divergence between all species of *Triturus* was 19.8%.

The percentage *cytb* sequence divergence for the 819 bp fragment varied across *Triturus* species. Intra-specific sequence variation of the LBT species ranged from 0.1 to 0.6% (corresponding to 1–5 substitutions), while between-species comparisons ranged from 5.0 to 6.8%. For the SBT differentiation at the intra-specific level was 8.5% in *T. boscai*, 2.1% in *T. italicus*, 4.2% in *T. montandoni* and 4.5% in *T. vulgaris*. The intermediate-sized *T. vittatus* displayed a high level of intraspecific sequence divergence (up to 10.6% between populations). Since *T. alpestris* and *T. helveticus* have been sampled only from a few localities (see Appendix A), a pattern of intra-specific divergence for these species cannot be inferred.

Authenticity of *cytb* sequences

Analyses for the occurrence of numt genes (non-function nuclear gene copies of mtDNA origin) from base composition (Collura et al., '96) gave no positive clues (no stop codons, no abrupt change in amino acid sequences, no anomalous base composition) except for *T. alpestris*. Samples sequenced for this species had a 42 bp stretch (position 19–60) that could not be aligned and potentially included stop codons. Thus it was discarded from the analysis. The remaining 777 bp of the *cytb* fragment, however, aligned properly in the dataset alignment. We also suspect that the sequences obtained from the amplification of the 5' end of the *cytb* fragment in *T. italicus* is from a pseudogene or numt gene. We reached this conclusion on the basis of a phylogenetic criterion to check for spurious positions of putative pseudogene sequences in an otherwise well-established clade

(Bensasson et al., 2001). This is the only species for which we could not amplify the whole 800 bp in a single PCR reaction. When we included the whole *cytb* fragment from this species in any phylogenetic analysis, we obtained a placement of this small-bodied newt as sister taxon to the LBT species. A placement within the LBT species was obtained when the analysis was performed on only the 5' PCR fragment. However, when we carried out the phylogenetic analyses using only the 3' PCR fragment, amplified by the newt-specific primers MVZ25–MVZ16, *T. italicus* clustered with the other SBT species with high support. Multiple attempts to amplify the whole *cytb* fragment from *T. italicus* either with a variety of newly designed primers or by using long-range PCR methods failed. Interestingly, *T. italicus* has neither unpigmented eggs nor the so-called chromosome-1 syndrome (Rusconi, 1821; Horner and Macgregor, '85) characteristic of LBT species. We carried out all the remaining phylogenetic analyses using only the 3' PCR fragment for this species.

Tree reconstruction: choice of the substitution model

We chose to use the GTR+G+I for the three partitions since this model was the preferred model for the *cytb* ($P < 0.001$) and for both the rDNA's ($P \sim < 0.05$) data partitions. To define the combined mixed model, we used Bayes factor tests (BF) to decide how many parameters could be shared across partitions (the five mutation-specific rates multipliers, the four base frequencies, the α parameter of the Γ distribution, the percentage of invariant sites, the total length of the tree and the proportion of the branch-lengths). Figure 2 shows the results of the 39 BF. For each parameter, three tests (a, b and c; Fig. 2) were performed to examine if a given parameter could be shared in any two of the three partitions.

All tests for the mutation-specific rate multipliers (Fig. 2—Transition Matrix tests) are negative, indicating that the rate multipliers should be different across each data partition. All tests involving the α parameter of the Γ distribution (Fig. 2—Site Variability, alpha) resulted in the natural logarithm of the Bayes factor values being $> \ln(10)$, thus strongly supporting the use of a single parameter across the three partitions. All tests involving the percentage of invariable sites (Fig. 2—Site Variability, pinv) resulted in positive values for the log-transformed Bayes factors,

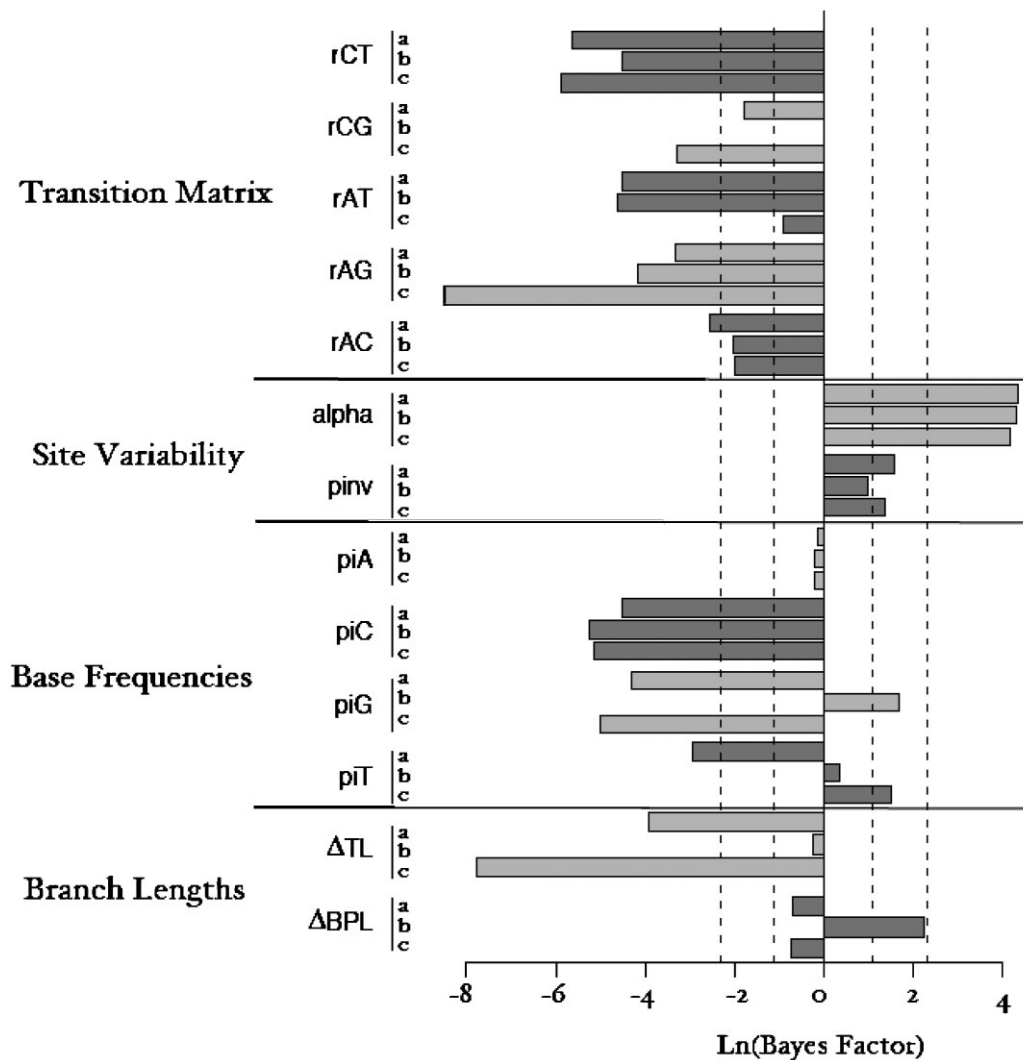


Fig. 2. Bar plot of the Bayes factor tests carried out to evaluate which parameters can be shared across each data partition. The natural log of the Bayes factor (horizontal axis) is shown for 39 tests. Negative and positive values indicate that parameters should either be kept separate or should be shared, respectively. The dashed vertical lines mark the boundaries of moderate ($>\ln(3)$ and $<\ln(-3)$) and strong ($>\ln(10)$ and $<\ln(-10)$) support for either sharing or not sharing parameters across partitions. Each test has been carried out on three pair-wise comparisons among mtDNA partitions (a = cyt *b*-12S, b = 12S-16S and c = cyt*b*-16S). The bars showing the result for each set of tests (a, b and c) are alternatively shaded in dark or light gray to separate consecutive sets of tests. Parameters were tested for the Transition Matrix (rAC, rAG, rAT, rCG, rCT for the five mutation-specific rate multipliers of the GTR transition matrix), for Site Variability (alpha for the shape parameters of the gamma distribution and pinv for proportion of invariable sites), for Base Frequencies (piA, piC, piG, piT) and for Branch Lengths (Δ TL and Δ BLP).

suggesting that parameters could be shared across partitions.

The base frequency tests gave heterogeneous results (Fig. 2—Base Frequencies). None of these had lnBayes factor values $>\ln(10)$, while several tests had values $<-\ln(10)$, thus strongly suggesting that base frequencies should be modeled separately across partitions.

The branch-length tests (Fig. 2 Branch Length) gave different results across partitions. The tests

supported strongly that the total tree length (TL) of the cyt*b* and rDNA genes are different, while there was weak support for a difference in branch proportion between cyt*b* and the ribosomal genes. We decided therefore not to share any branch parameters between these two partitions, thus adopting the case (iii) for this part of the model. Tests indicate that branch proportions are not different between the two rDNA genes (BF = 8.78, with BF = 10 being the threshold for strong

support), while there is a weak support for different TLs. Thus, for these partitions we adopted the case (ii) scenario (i.e., shared branch-length parameters across the ribosomal partitions but with SS rates to account for the TLs difference).

The resulting model can be summarized as 3(GTR+Pi)+2(Br+SS)+I+G, given that we used three transition matrices with the GTR model, different base frequencies for each of the partitions, two sets of branch-lengths (one for *cytb* and one for 12S and 16S), two SS parameters for partitions 12S and 16S, and one parameter for all data for both the Γ distribution and the percentage of invariant sites.

Tree reconstruction: molecular phylogenetic inference

When we used the GTR+G+I model for the *cytb* data partition, the root of the Salamandridae is not resolved. *Chioglossa lusitanica* is not monophyletic with the remaining salamandrid species and the “Newts” clade is only weakly supported (PP = 0.72). Nodes within the “Newts” are overall well supported and the topology is very similar to the one obtained for the full dataset analysis (see below). The Bayesian phylogenetic reconstruction based on the 12S partition clearly resolves the outgroup from the ingroup taxa but has few other clades supported with more than 0.95 PP. Both the “Newts” and *Triturus* (only represented by *T. alpestris* and *T. karelinii*) are not monophyletic. The phylogenetic reconstruction based on the 16S partition is less resolved than the one based on the 12S data partition, with few sister taxon relationships resolved, including the outgroup vs. ingroup one (results not shown, but available from the corresponding author).

Figure 3 shows the Bayesian tree obtained from the full dataset analysis of the 12S, 16S and *cytb* partitions using the 3(GTR+Pi)+2(Br+SS)+I+G model. Subsequent description and discussion of the result will use the clade name defined in Figure 3. Table 1 shows the support of the hypotheses that make up the posterior distribution for the four unresolved nodes at the base of the radiation of the “Modern Eurasian Newts”. None of these hypotheses support monophyly of the genus *Triturus*, even if *Calotriton asper* and *Neurergus strauchii* are included in *Triturus* (Table 1, hypotheses H1–H8). For the initial split within the “Modern Eurasian Newts” three hypotheses make up 97% of the PP (Table 1—H9,

H14 and H11): H9 corresponds to the topology shown in Figure 3 (PP = 0.66); H14, the “Modern Asian Newts” and *T. alpestris* together in one clade, with the remaining taxa being their sister clade (PP = 0.16); H11, the SBT is the sister taxon to a clade of the remaining taxa (PP = 0.15). The phylogenetic position of *Neurergus strauchii* is described by two major hypotheses covering up to 98% of the posterior distribution (Table 1 H18 and H19). The preferred hypothesis H18 (PP = 0.84; Fig. 3) groups *N. strauchii* as the sister species to *T. vittatus*. H19 places *N. strauchii* as the sister taxon of the LBT+*Calotriton asper*, although with low support (PP = 0.15).

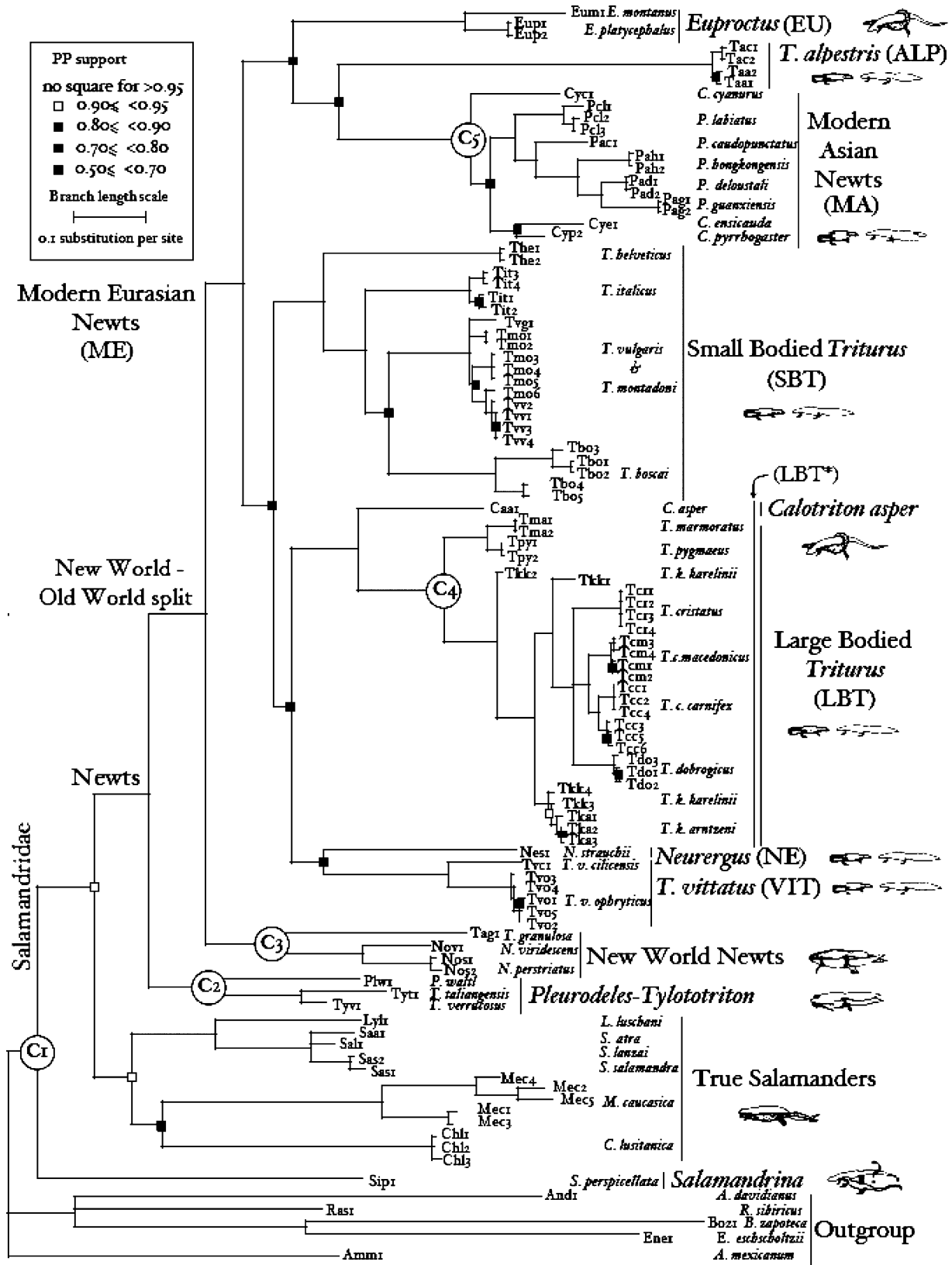
At the species level our phylogenetic analysis fully supports the reciprocal monophyly of the two sub-species of *T. carnifex* as well as the monophyly of most *Triturus* species haplotype groups. Two notable exceptions are the paraphyly of *T. k. karelinii* and the intermixing of *T. montandoni* and *T. vulgaris* haplotypes. Multiple and quite diverse *T. k. karelinii* haplotypes are grouped with a clade that includes the well-defined monophyletic *T. cristatus*, *T. carnifex* and *T. dobrogicus* haplotype assemblages. Although several well-resolved clades occur within the *T. montandoni*–*T. vulgaris* group, our analysis shows an intermixing of haplotypes for these two species, a finding reported previously (Babik et al., 2005).

Controlling for the effect of missing data: the backbone phylogeny

The analysis performed on the sub-set of 21 taxa without missing data for the three gene partitions (the backbone-dataset) resulted in a very similar topology (results available from the corresponding author) as the full dataset analysis (Fig. 3). All nodes with PP>0.95 in the backbone phylogeny are equally well supported in the full dataset analysis. Only three nodes are not well supported and just one of them is in disagreement with the full dataset analysis: the clade of *S. perspicillata*+ “True Salamanders” (PP = 0.76). In the full dataset analysis, the “True Salamanders” and “Newts” form a group (PP = 0.93) to the exclusion of *S. perspicillata*.

Molecular dating

When testing for the saturation behavior of the *cytb* gene for the included salamandrid taxa, we did not find evidence for strong saturation. Pairwise comparisons involving all three codon



positions are in the area between the maximum number of possible substitutions with a slope of 1 (equaling $Ti = Ti + Tv$) and a slope of 0.5 (equaling $Ti = Tv$). Only for a few comparisons we found signs of high saturation (data available from the corresponding author).

We associated five fossil data points with highly supported nodes in the phylogeny and evaluated which of the four internal nodes (the root was bounded) was best suited for the application of a fixed age calibration and a minimum age

constraint (see Fig. 3 and Material and Methods for details). The only calibration point that did not result in internal inconsistencies (i.e., pushing the age of the other three internal points beyond the established geological date of the reference fossil) was point C4, the crown of the LBT, dated at 24 mya. Fixing C2 pushed 92% of the distribution of estimate for the age of the LBT crown to the minimum threshold of 24 mya, while the time estimations for the other calibration points were all exceeding their minimum threshold. Conver-

TABLE 1. Topological hypotheses derived from the mtDNA sequence data and their respective posterior probabilities (PP) values for clades within the Modern Eurasian Newts (ME, Fig. 3)

Hypothesis	pp
Monophyly of <i>Triturus</i>	
H1 (LBT*+SBT+VIT+ALP)	0.0000
H2 (LBT*+SBT+VIT+ALP+NE)	0.0075
H3 (LBT*+SBT+ALP)	0.0035
H4 (LBT*+VIT+NE+ALP)	0.0030
H5 (SBT+MA+ALP)	0.0000
H6 (VIT+NE+MA+ALP)	0.0000
H7 (VIT+MA+ALP)	0.0015
H8 (LBT+MA+ALP)	0.0000
Basal nodes	
H9 ((LBT*+SBT+VIT+NE)+(MA+ALP+EU))	0.6617
H10 (((LBT*+(VIT+NE))+SBT)+(MA+ALP+EU))	0.3278
H11 (SBT+(LBT*+VIT+NE+MA+EU+ALP))	0.1499
H12 (SBT+((LBT*+VIT+NE)+(MA+EU+ALP)))	0.1359
H13 (LBT*+VIT+NE)+(ALP+MA)	0.0000
H14 ((ALP+MA)+(EU+VIT+LBT*+SBT+NE))	0.1624
H15 ((ALP+MA)+(EU+(VIT+LBT*+SBT+NE)))	0.1624
Paraphyly of <i>Euproctus</i>+<i>Calotriton</i>	
H16 (LBT*+EU)	0.0050
Position of <i>Neurergus</i>	
H18 (NE+VIT)	0.8355
H19 (NE+LBT*)	0.1510
H20 (NE+(LBT*+SBT+VIT))	0.0035
H21 (NE+TS)	0.0025
Sister taxa of <i>T. alpestris</i>	
H22 (EU+ALP)	0.0690
H23 (MA+ALP)	0.7500
H24 (ALP+(EU+MA))	0.0010

Bold highlights five groups of hypotheses. Each of the 24 hypotheses (H1–H24) is described in parenthetical notations, using the same clade abbreviations as in Figure 3. Indented hypotheses are nested in the previous ones.

Fig. 3. Majority rule consensus tree of the Bayesian phylogenetic inference for the Salamandridae dataset using the 3(GTR+Pi)+2Br+G+I model (104 haplotypes, 12S, 16S, *cytb* with missing data = “full dataset”). Nodes with PP values above 0.95 are unmarked; squares of increasingly darker colors identify nodes with decreasing support values (see inserted box). Haplotype labels are as in Appendix A. Groups of haplotypes are taxonomically classified by species and sub-species names. Vertical lines mark clades of haplotypes that are discussed in the text (clade name and acronym listed next to each line). A schematic drawing of the amplexus types is shown next to the clades (male in gray, females in white). Note that the degree of amplexus during courtship for *Salamandrina* is not verified and therefore marked with a question mark (see text). The fossil dated calibration points (white circles, C1–C5) used to estimate times of divergence are indicated on the corresponding node (age of the nodes listed in Table 2).

TABLE 2. Summary of the empirical posterior distribution of divergence times (mya) across Salamandridae

	Lower bound	Mode	Upper bound	Calibration
Root of Salamandridae	79.74	94.74	113.92	160 > Cr > 55
<i>Salamandrina</i> stem	72.36	94.97	117.56	
True Salamander—Newts	76.60	94.77	113.99	
True Salamander	61.99	74.92	91.63	
<i>C. lusitanica</i> —(<i>Lyciasalamandra</i> — <i>Salamandra</i>)	59.78	74.42	90.21	
<i>M. caucasica</i> —(<i>Lyciasalamandra</i> — <i>Salamandra</i>)	59.67	74.31	89.59	
Newts	63.36	73.54	87.25	
<i>C. lusitanica</i> — <i>M. caucasica</i>	56.46	70.03	89.59	
New World—Old World split	57.17	67.49	79.24	
<i>Triturus</i> MRCA	52.84	64.13	73.48	
Modern Eurasian	52.84	64.13	73.52	
<i>Notophthalmus</i> — <i>Taricha</i>	34.00	47.45	62.15	C3 > 22
<i>Tylotriton</i> — <i>Pleurodeles</i>	44	44	44	C2 = 44
Small-bodied <i>Triturus</i>	35.70	43.81	54.84	
Large-bodied <i>Triturus</i>	24	24	24	C4 = 24
Large-bodied <i>Triturus</i> + <i>C. asper</i>	33.54	38.76	47.31	
Modern Asian	19.85	23.91	31.65	C5 > 15
<i>Notophthalmus</i>	13.63	18.24	27.66	
<i>T. vittatus</i>	13.46	17.95	23.99	
<i>Euproctus</i>	12.15	16.57	22.74	
<i>Paramesotriton</i>	7.97	10.94	14.54	
<i>Tylotriton</i>	10.12	14.06	19.33	
<i>T. karelinii</i>	11.15	13.73	16.90	
<i>T. boscai</i>	7.98	11.41	14.61	
<i>T. marmoratus</i> — <i>pygmaeus</i>	4.29	06.82	9.20	
<i>T. italicus</i>	1.41	3.28	6.02	
<i>T. carnifex</i>	1.72	2.59	3.78	
<i>T. carnifex carnifex</i>	3.17	4.31	5.80	
<i>T. alpestris</i>	1.04	1.73	2.88	
<i>Notophthalmus perstriatus</i>	0.69	1.60	3.13	

The first column identifies the node (clades in Fig. 3). The third column lists the mode of the estimated age. Columns two and four provide the central 95% Confidence Intervals (2.5% and 97.5%). The last column lists the calibration points and their respective ages. If a clade is polyphyletic, the estimation is performed on the node of its most recent common ancestor (MRCA) and the genus name of such a node is followed by MRCA. For *Salamandrina* the estimation was performed on the stem node of the clade (*Salamandrina* stem).

sely, fixing either C3 or C5 pushed the remaining three calibration points towards their lower threshold (corresponding to the age of the fossil marking each point), which made them behave as if they were fixed for the totality of the trees sampled from the posterior distribution. This suggests that the fossil-based age of C3 and C5 strongly underestimates the age of their respective nodes, whereas C2 and C4 do not. Thus, we decided to use both C2 and C4 as fixed calibrations and the other two fossils as minimal age constraint for their nodes. Table 2 shows the estimated times of divergence for selected clades in the phylogeny, as well as 95% HPD confidence intervals (CI). The age of the root of the Salamandridae is estimated at 95 mya (CI 80–114). The stem node of the *Salamandrina* lineage is estimated at 95 mya (CI

72–117), the split between the “True Salamanders” and the “Newts” is estimated at 95 mya (CI 77–114), and the radiation of the Newts at roughly 74 mya (CI 63–87). The “New–Old World Split” clades dated at 67 mya (CI 57–69). The diversification of the Modern Eurasian Newts including the most recent common ancestor (MRCA) of the polyphyletic *Triturus* started at the same time (64 mya, CI 53–74). The diversification of the SBT at 44 mya (CI 36–55 mya) pre-dates that of the LBT at 24 mya (calibration point C4).

Life history and courtship evolution

Table 3 shows the results of the evolutionary reconstruction of reproduction and adult habitat use (Table 3a) and the evolution of nine male-mediated courtship traits (Table 3b) within the

TABLE 3. Ancestral state reconstruction of behavioral and life history characters within the Salamandridae with special emphasis on Triturus

Character	State	Internal nodes										Terminal nodes					
		Newts	<i>Pleurodeles-</i> <i>Tylostotriton</i>	World- New World split	New world Newts	Modern European Newts	<i>Euproctus</i>	Modern Asian Newts	<i>Pleurodeles</i>	LBT*	LBT	<i>T. alpestris</i>	<i>T. vittatus</i>	<i>T. vulgaris</i>	<i>T. N. strauchii</i>		
(a) Clasp of female by male	Absent	0.06	0.00	0.93	0.01	1.00	0.00	1.00	0.00	1.00	0.99	1.00	1	1	1	1	
	Ventral	0.94	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	
	Dorsal	0.00	0.00	0.07	0.99	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	0
	Caudal	0.00	0.00	0.00	0.00	0.00	1.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	0
	Terrestrial	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	0
Adult habitat	Semi-terrestrial	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	0.99	1.00	1	1	1	1	
	Aquatic	0.00	0.00	0.00	0.00	0.00	0.00	0.00	1.00	1.00	0.01	0.00	0	0	0	0	
Reproduction habitat	Terrestrial	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	
	Stream Pond	0.00	0.00	0.00	0.00	0.00	1.00	0.00	1.00	0.22	0.00	0.79	0	0	0	0	
(b) Break	Absent	0.99	0.99	0.95	1.00	0.44	1.00	0.00	1.00	0.02	0.00	0	0	0	0	0	
	Present	0.01	0.01	0.05	0.00	0.56	0.00	0.00	0.00	0.98	1.00	1	1	1	1	1	
	Absent	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.29	0.00	0	0	0	0	0	
	Present	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.71	1.00	1	1	1	0	0	
	Absent	1.00	1.00	1.00	1.00	1.00	0.00	1.00	0.00	0.29	0.00	0	0	0	0	0	
Waving	Present	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1	1	1	1	1	
	Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	0	
Fanning	Present	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.71	1.00	1	1	1	1	1	
	Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.29	0.00	1	0	0	0	0	
Rocking	Present	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.89	0.00	1	1	1	1	1	
	Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	1.00	0	0	0	0	0	
Wiggle tail	Present	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1	1	1	1	1	
	Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	0	
Retreat	Present	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	0.00	1	1	1	1	1	
	Absent	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0	0	0	0	0	
Push back	Present	1.00	0.99	1.00	1.00	1.00	1.00	1.00	1.00	0.97	0.90	0	0	0	0	0	
	Absent	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.03	0.10	1	1	1	1	1	

(a): Evolution of characters involved in the amplexus and of adult and reproductive habitat types for clades within the Newts. (b): The first two columns list the characters and their state (see Appendix B for character descriptions). The following 10 columns report the probabilities of each state for 11 fully supported nodes (names in Fig. 3). The last four columns show the observed state for four terminal taxa to allow immediate comparison with the state in the corresponding internal nodes.

“Newts” clade. Breeding in ponds is the ancestral state for this clade, whereas stream breeding has evolved at least three times independently (in *Euproctus*, *Calotriton* and in the “Modern Asian Newts” lineages). A semi-terrestrial life cycle is the ancestral condition for the “Newts”, whereas a switch to a fully aquatic life style has occurred at the base of the “Modern Asian Newts” lineage (Table 3a). Ventral contact is the ancestral state for the “Newts” clade, whereas a lack of body contact is the ancestral condition for the “Modern Eurasian Newts”, including *Triturus* (see also Fig. 3). Within this clade two distinct lineages *Euproctus* and *Calotriton* evolved independently a new form of body contact (Table 3a—caudal capture).

Our results suggest also that several male-mediated courtship behaviors have evolved independently in the Modern European Newts (Table 3b). The “wobble tail bent” trait evolved at least twice. The “retreat” and “waving” behaviors evolved only in some species of the SBT and the “rocking” display is found only in the LBT. The ancestral state for two of these traits, the “brake” trait ($PP_{\text{Absent}} = 0.44$, $PP_{\text{Present}} = 0.56$) at the crown of the Modern Eurasian Newts clade (Fig. 3), and the “fanning” trait ($PP_{\text{Absent}} = 0.71$, $PP_{\text{Present}} = 0.29$; Table 3b) at the LBT* clade, could not be resolved.

DISCUSSION

The phylogenetic divergence of Triturus within the Salamandridae

Based on a complete taxon sampling of all *Triturus* species, our mtDNA-based Bayesian analysis clearly supports that this genus is not monophyletic. Compared with the phylogeny of Titus and Larson ('95) based on the ribosomal 12S and 16S genes, our phylogeny provides new insights into the phylogenetic relations of the Salamandridae (Fig. 3). All newt-like salamandrid species are separated from the semi- or fully terrestrial salamanders, i.e., the “True Salamanders” and *Salamandrina*, in a distinct clade (“Newts”; Fig. 3). New phylogenetic aspects within the “Newts” are the clear separation of the *Taricha–Notophthalmus* clade (the “New World Newts”) from the “Modern Eurasian Newts” species. Moreover, the *Tylotriton–Pleurodeles* clade can be distinguished from either of these groups. Within this group all of the East Asian

taxa (*Cynops*, *Pachytriton* and *Paramesotriton* = the “Modern Asian Newts”) can be separated from all the other taxa (*Calotriton*, *Euproctus*, *Neurergus* and species of *Triturus*) distributed in Europe and the Near East.

While clearly *Triturus* is not a monophyletic assemblage of species ($PP = 0$; Fig. 3 and Table 1), the monophyly of the SBT (*T. boscai*, *T. helveticus*, *T. italicus*, *T. montandoni* and *T. vulgaris*) and that of the LBT (*T. cristatus*, *T. carnifex*, *T. dobrogicus*, *T. karelinii*, *T. marmoratus* and *T. pygmaeus*) is strongly supported and in line with the traditional view. The remaining *Triturus* species, *T. alpestris* and *T. vittatus*, are clearly outside these clades. Although we did not obtain high support for any phylogenetic grouping of *T. alpestris*, the combined probability that *T. alpestris* clusters with either the “Modern Asian Newts” or with *Euproctus* is relatively high ($PP = 0.82$). Furthermore, our backbone phylogeny strongly supports the close relationship of *T. alpestris* and the “Modern Asian Newts” ($PP = 0.98$) in line with Titus and Larson ('95). Thus, from a mitochondrial perspective we argue that *T. alpestris* should be considered distinct from the other *Triturus* species as the “Modern Asian Newts”. For *T. vittatus* we obtained moderate support ($PP = 0.84$) for a close relationship with *Neurergus strauchii*. This hypothesis together with a sister-clade relationship between *Neurergus* and the LBT* (LBT plus *Calotriton asper*) clade constitute the vast majority of the posterior distribution ($PP = 0.99$). Although we took into consideration only one *Neurergus* species, we expect this phylogenetic relationship to hold, as the monophyly of *Neurergus* within the Salamandridae appears to be well established (Steinfartz et al., 2002).

For most *Triturus* species, the observed haplotypes form reciprocally monophyletic groups characterized by high sequence divergence. This is particularly true for *T. boscai* in which we observed a maximum sequence divergence of 8.5%, in line with a deep differentiation reported by Martínez-Solano et al. (2004) for this species. The sharing of mitochondrial haplotypes by *T. montandoni* and *T. vulgaris* could possibly reflect past or ongoing hybridization or multiple introgression of mtDNA from *T. vulgaris* into *T. montandoni*, as previously suggested by Babik et al. (2005). The paraphyletic pattern of haplotypes in *T. karelinii* from Anatolia (Fig. 3) may reflect incomplete lineage sorting or the existence of a cryptic species (Wallis and Arntzen, '89).

In conclusion, the current data strongly support the non-monophyly of *Triturus*, but it does not allow discriminating between polyphyly and paraphyly, since to do so one would need to clearly identify a suite of characters that define the taxon name. To date, no consensus is available in the literature on this point.

Life history and behavioral evolution

The Salamandridae show large differences in habitat selection, spanning the range from fully terrestrial in some of the “True Salamanders” (e.g., *Lyciasalamandra*) to entirely aquatic, such as the stream-adapted *Calotriton*, *Euproctus* and *Pachytriton* species. The courtship displays, which aim to transfer pheromones from the male to the female, have been interpreted as an evolutionary response to these differing environmental conditions. According to Houck and Arnold (2003), courtship behavior in the Salamandridae can be classified by the degree of physical contact between the male and the female into four broad categories (see also Fig. 3). (1) *Ventral amplexus*—the male holds the female on his back by clasping both of his forelimbs around hers—found in *Pleurodeles*, *Tylototriton* and all “True Salamanders”. (2) *Dorsal amplexus*—the male is positioned on the female’s back grasping her pectoral region either with the forelimbs (*Taricha*) or the hind limbs (*Notophthalmus*). (3) *Tail use*—the male restrains with his tail the female movements—found in *Calotriton* and *Euproctus*. (4) *Absence of physical contact*—no amplexus or other physical contact between the sexes (*Cynops*, *Neurergus*, *Paramesotriton*, *Pachytriton* and *Triturus*).

The spermatophore transfer takes place at varying times after the ventral or dorsal amplexus, but in *Euproctus* and *Calotriton* it occurs during the tail amplexus. *Notophthalmus* species can shortcut the clasping phase and proceed to sperm transfer, thus displaying no amplexus (Verrell, ’82). In *Salamandrina* a head-to-head courtship posture (without interlocking of the forelimbs) has been described (Strötgen, ’27; Houck and Arnold, 2003), but so far the observation was not validated under natural conditions (Utzeri et al., 2005; C. Angelini, pers. communication). Thus, we consider it unresolved whether or not physical contact is involved in their courtship. This ambiguity results in an unknown state for the male contact traits at the root of the Salamandridae. We used a Bayesian approach to reconstruct the ancestral state of various courtship

traits (Table 3). Each of the basal salamandrid clades (the “True Salamanders”, the *Pleurodeles*–*Tylototriton* clade and the “New World Newts”) shows body contact during courtship. Loss of body contact occurred in “Modern Eurasian Newts” clade. Within this group the species *Calotriton* and *Euproctus* display a caudal capture, a trait that has been interpreted as an adaptation to the stream environment (Salthe, ’67). As pond breeding is the ancestral condition for “Modern Eurasian Newts” clade (PP = 1.0; Table 3), the most likely explanation is that the transition to stream reproduction and the evolution of caudal capture has occurred independently for *Calotriton* and *Euproctus*. Some observed differences in caudal capture between the two taxa (Thiesmeier and Hornberg, ’90) would support a scenario of parallel evolution.

Male-mediated courtship traits have been best studied for *Triturus* species. On the basis of the molecular phylogeny (Fig. 3), we inferred the ancestral state for nine male-mediated *Triturus* courtship displays within the clade of the Modern Eurasian Newts (see Table 3 and Appendix B). With the exception of the synapomorphic “brake” trait displayed by all *Triturus* species and *Neurergus* (Sparreboom et al., 2000) and the “fanning” trait, all male-mediated displays were absent at the stem node of this clade. Our results suggest that several male-mediated courtship behaviors evolved independently in *Triturus* (see results for minimum number of independent gains). Thus, to explain the behavioral complexity found in *Triturus* we need to assume a high level of parallel evolution. New conceptual and experimental approaches are required to analyze and explain how complex courtship traits, long seen as homologous, could have evolved independently.

Dating the divergence of Triturus

The broad spectrum of morphological and behavioral diversity within the family Salamandridae renders it particularly worthwhile to estimate the timeframe associated with their evolutionary change. For the Salamandridae, molecular rates have been calibrated for single or combined mitochondrial genes by either using fossil records (e.g., *Taricha*, Tan and Wake, ’95) or paleogeographic events (e.g., in *Euproctus*: Caccone et al., ’94, ’97; Carranza and Arnold, 2004, radiation of the “True Salamanders”: Veith et al., ’98 and *Pleurodeles*: Veith et al., 2004). In their review on the systematics and phylogeny of

salamanders, Larson et al., (2003) provide several time estimates for the major splits within Salamandridae. These estimates were based on published vertebrate mtDNA substitution rates. They date the earliest divergence among extant salamandrid lineages at 48 mya. This estimate is at least 32 mya younger than ours (95 mya CI 80–114 mya), when using what we think are the most reliable calibration points (C2–C4). Even if the estimate of 95 mya is too old, the earliest divergence among extant salamandrid species must have pre-dated 48 mya, since a newt-like fossil, *Koalliella genzeli*, already existed in Europe in the Upper Paleocene (55 mya, Estes, '81). According to our scenario, the most recent ancestor of the *Triturus* species is dated at 64 mya (CI 53–73). The ages of *Triturus* clades are 44 mya (CI 36–55) for the SBT, 18 mya (CI 13–24) for *T. vittatus*, and 24 mya (fixed, C4 node) for the LBT. All these datings are considerably older than previous estimates (e.g., Oosterbroek and Arntzen, '92), with the exception of the work of Zhang et al. (2005, see below).

Given that our proposed time estimates differ from previous works, we need to evaluate the uncertainties associated with our dating approach in relation to other methods, based either on fossil or biogeographic evidences. For fossil-based calibrations, there are two main sources of error: the correct phylogenetic placement of the fossil and the degree of underestimation of the actual age of the node (i.e., how close is a given fossil to the actual node that it calibrates or temporal accuracy) which is also a function of the degree to which the fossil record is incomplete.

To minimize the first type of error we chose multiple calibration points, linked to well-supported nodes (Fig. 3) and associated to fossils that have been either well studied (e.g., *Chelotriton weigelti* for C2; *Triturus* cf. *T. marmoratus* for C4) or were available as more or less complete skeletons (e.g., *Taricha oligocenica* for C3, *Procyonops miocenicus* for C5). In this regard, we point out that we used *Chelotriton weigelti* for the calibration, even though Carranza and Arnold (2004) explicitly discard it to calibrate the split between *Tylostotriton* and *Pleurodeles* (under the rationale that the fossil has changed its classification from *Tylostotriton weigelti* to *Chelotriton weigelti*). We used this fossil because both the authors involved (Estes, '81; Rocek, '96) still consider *Tylostotriton* and *Chelotriton* more closely related to each other than to *Pleurodeles*.

To reduce the impact of the second source of error (temporal inaccuracy), we developed a method to evaluate the degree of underestimation associated with each calibration point and test for the relative compatibility of the time estimates of the different calibration points. Of the six fossil records tested, we found that only the fossils defining nodes C2 and C4 (Fig. 3) produced time estimates for all other nodes that were compatible (i.e., older) with the age of the fossils associated with them.

Our dating for the root of the Salamandridae (94 mya, CI 80–114) further highlights the sources of error in these types of estimates associated with the incompleteness of the fossil record. Is our estimate for this node unrealistically old given that there are no putative salamandrid fossil records older than 70 mya (Milner, 2000)? Given that fossils provide only minimal dates, the fact that currently no fossils older than 70 mya were ever found, does not mean that they could not exist. A recent finding of a new fossil of Cryptobranchids in China (Gao and Shubin, 2003) provides an example of the possible consequences of incomplete sampling. Here, the oldest record for this taxon was moved from the Paleocene (56 mya) to the middle Jurassic (160 mya, Gao and Shubin, 2003).

On the other hand one could argue that we have placed too much faith in the taxonomic identification of the fossils used for the C2 and C4 calibrations and that we associate them a priori with extant lineages. Thus it is interesting to explore what type of datings would be obtained if other combinations of calibration points for our time estimates were used. Calibrations C3 and C5 are compatible with each other, as are C2 and C4. If we assume that C3 and C5 are fixed calibration points rather than C2 and C4, and continue to use the same time range (160–55 mya) as constraint for the age of the root of the Salamandridae, we obviously obtain younger datings than the ones based on C2–C4. The root of Salamandridae becomes 55 mya old (CI 55–68), where it was 95 mya (CI 80–114) for the C2–C4 calibration. The most recent ancestor of the *Triturus* species becomes 38 mya old (CI 34–45), while it was dated at 64 mya (CI 53–73) under the C2–C4 setting. Although the C3–C5-based datings cannot be falsified given the uncertain taxonomy of the salamandrid fossil record, we propose that the datings based on the C2–C4 calibration points are probably closer to the actual divergence times of the clades. These two calibration points are

likely to be better estimators of the age of their nodes given that they produced older age estimates than the ones produced by the C3 and C5 calibration points (see methods and results sections). Interestingly, a dating for the Salamandridae root as old as ours C2–C4-based one has already been reported by Zhang et al. (2005). Using fossil calibration points at the base of Tetrapoda, they proposed a divergence for Salamandridae of 139 mya (CI 119–160) on the basis of full mtDNA data for multiple amphibian species, but including only two Salamandridae. This is a largely independent time estimate for the Salamandridae node, since it uses time calibrations based on different fossils than ours and a different methodological approach.

Another way to test the relative efficiency of the C2–C4 vs. C3–C5 calibration points is to compare the time of divergence obtained using these two sets of fossil-based calibrations with independently derived time estimates, such as the ones based on biogeographic events. For these calibrations, one assumes that a well-dated geological event is the causal force driving the cladogenetic events in the lineages of interest. As with fossil-based datings, calibrations based upon biogeographic events are minimum estimates. Unlike fossil-based estimates, however, the finding of the same vicariant distribution in a diverse array of organisms, whose taxa have been similarly affected by a geological event can be seen as corroborating the robustness of biogeographic-based datings.

The nodes leading to the two *Euproctus* species, *E. montanus* and *E. platycephalus*, provide one of these tests. Based on known and well-dated tectonic events in the Mediterranean, the separation of these two species is likely to have occurred between 29 and 13–15 mya (Caccone et al., '94, '97). Our fossil-based estimation (C2–C4 based) is compatible with this dating, since it suggests that *E. montanus* and *E. platycephalus* diverged 19 mya (CI 14–26; Table 2). On the other hand, the dating based on the C3–C5 calibration (10 mya CI 7–14) is younger and barely overlapping with the times proposed on biogeographic evidence.

Another node for which biogeographic-based estimates are available is the one marking the *Pleurodeles*–*Tylotriton* divergence. Veith et al. (2004) identified three possible biogeographic scenarios that could have led to the observed phylogeographic patterns in *Pleurodeles* in the western Mediterranean region. These scenarios can be dated using two vicariance events involving

the Iberian-African area; the disconnection of the Betic Arch, dated at ca. 14 mya, and the end of the Messinian salinity crisis, dated at ca. 5.33 mya. They proposed three different temporal hypotheses (calibrations I–III) to date the split between two species, *Pleurodeles waltl* and *P. poireti*. Calibration I (the youngest one): the split of these two species was determined by the Messinian salinity crisis (ca. 5.33 mya). Calibration II: the Betic crisis caused the split, moving the same cladogenetic event back to ca. 14 mya. Calibration III (the oldest one): the Betic crisis caused the split between the north-western and south-eastern populations of *P. waltl*, rather than between the two *Pleurodeles* species. Using a C2-only calibration, our divergence estimates for the *Pleurodeles*–*Tylotriton* split (54 mya, CI 44–72) overlaps only with their oldest time estimates for the same event (Calibration III: 59.9–77.6 mya). Using the C2–C4 calibration the age is fixed at 44 mya because C4 is a calibration point. Interestingly, Calibration III is also the only one of the three proposed by Veith et al. (2004) that is consistent with the current fossil record for *Pleurodeles* and *Tylotriton* (*Chelotriton weigelti*, 44 mya). On the other hand, our C3–C5-based estimate is 30 mya (CI 23–38). This estimate is still compatible with one of Veith et al.'s (2004) calibrations (Calibration II: 23–31 mya), but it is not compatible with known fossil evidence.

An additional node for which biogeographic-based estimates are available is the one marking the split of the “New World Newts” (*Notophthalmus* and *Taricha*) from the “Modern Eurasian Newts”. It is thought that newts colonized North America through either the North Atlantic or the Beringian land bridge. The North Atlantic land bridge closed around 56–53 mya, just after the Wasatchian–Sparnacian dispersal event (Eberle and Mckenna, 2002). Although the Beringian land bridge was practically open all over the Tertiary, it was not reachable from Europe until the closing of the Turgai Straits in the Early Oligocene (30 mya; Mckenna, '75; Sanmartin et al., 2001). Our C2–C4 divergence estimates for the split of “New World Newts” split from the “Modern Eurasian Newts” is 67 mya (CI 57–79). This is compatible with a North Atlantic land bridge and it overlaps with the Wasatchian–Sparnacian dispersal event (57–55 mya; Beard and Dawson, '99). Interestingly, our C2–C4 dating for the radiation of the “Modern Asian Newts” (24 mya, CI 20–32 mya) coincides with the closing of the Turgai Straits. This would have offered an

opportunity for dispersal into a new region with the availability of new niches, which may have favored speciation. The importance of the North Atlantic land bridge to dispersal between Europe and North America is also supported by a large comparative study of Sanmartin et al. (2001). This study, which comprises 57 molecular phylogenies from non-marine animal groups occurring mainly or exclusively in the Holarctic, found that the vast majority of the vicariance events between Europe and North America were compatible with time divergences based on the formation of this land bridge.

Our alternative C3–C5-based calibration estimates the divergence of the “New World Newts” to 41 mya (CI 35–48). This would roughly be compatible with a dispersal over the Beringian bridge after the closing of the Turgai Straits. However, it is implausible that this biogeographic event caused the “Old World Newts”–“New World Newts” split, because the cladogenetic event would be older than the closing of the Turgai Straits (30 mya). If we use the C3–C5-based calibration, three different scenarios for the MRCA of the Modern Asian Newts are possible, although admittedly speculative: (1) it dispersed into Asia long after the first opportunity for dispersal arose (closing of the Turgai straits), (2) its dispersal roughly coincided with the closing of the Turgai Straits, but cladogenesis followed at least 15 mya later; (3) dispersal and speciation followed the closing of the Turgai Straits almost immediately, but extinctions of ancestral “Modern Asian Newts” lineages would result in a younger age of the MRCA. Thus, if we use these younger calibration points, it also means that the radiation of the “Modern Asian Newts” clade would not be connected to any acknowledged biogeographic event, while with the C2–C4 calibration this clade diversification could be associated with the closing of the Turgai Straits.

In conclusion, we argue that the C2–C4 fossil-based dating provides time estimates that are consistent with datings from other fossil records as well as biogeographic events, although—undeniably—the fit could also be due to the large confidence intervals. Although we are aware that these datings are subject to a series of assumptions, first and foremost that the phylogenetic positions of the fossils used for the calibration of C2 and C4 are accurate, we think that our work will be valuable at two different levels. By highlighting the importance of having a correct phylogenetic position for the fossils used for our

calibrations, we hope that this will spur renewed interest in the systematics of salamandrid fossils. By using an explicit approach in deriving our time estimates, we hope to provide a general methodological framework for dating molecular phylogenies and for dealing with their inherent fallacies.

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APPENDIX: A

Appendix A gives a list of samples analyzed for the *cytb* gene. The first column identifies species and subspecies rank, the population and the museum location of the samples (ZMA = Zoological Museum Amsterdam, The Netherlands; RMNH = National Museum of Natural History (Naturalis), Leiden, The Netherlands; MNCN = Museo Nacional de Ciencias Naturales, Madrid, Spain). ZMA numbers refer to jars with multiple specimens rather than single individuals. The superior R next to a taxon name identifies the taxa for which 12S and 16S rDNA were available. The second column reports the individual field numbers for samples sequenced in this study. The next three columns list the number of samples and number of *cytb* haplotypes for each species and population, the acronyms used for the haplotypes, and their GenBank Accession numbers. (In parentheses are positions used in this study when the complete mtDNA genome sequence is known for this species).

Species/subspecies	Locality number and name, Museum registration number of voucher material	N	No. Hapl.	Haplotypes	GeneBank accession numbers
<i>TRITURUS</i> SAMPLES					
<i>Triturus alpestris alpestris</i> (Laurenti 1768) ^R					
1. Ajka, Hungary, ZMA8379	H2, H3, H4	6	2	Taa1	DQ821212
2. Bonn, Germany, MNCN17397-17399	D1, D2, D3	3	1	Taa2	DQ821209, DQ821210, DQ821211
<i>Triturus alpestris cyreni</i> Wolterstorff 1932					
3. Santillana-del-Mar, Spain, ZMA8378	Sa2, Sa3	2	2	Tac1, Tac2	DQ821213, DQ821214
<i>Triturus boscai</i> (Lataste 1879)					
4. Mirador de la Currota, Galicia, Spain no voucher specimens	124-1, 124-2	6	5	Tbo1, Tbo2	DQ821217, DQ821218
5. Pelahustán, Toledo, Castilla-La Mancha, Spain, no voucher specimens	Y8A, Y9A	2	1	Tbo3	DQ821219
6. San Bartolomeu dos Galegos, Portugal, no voucher specimens	118-1, 118-2	2	2	Tbo4, Tbo5	DQ821215, DQ821216
<i>Triturus carnifex carnifex</i> (Laurenti 1768)					
7. Fuscaldo, Italy, ZMA9108	405, 406, 408, 409	6	6	Tcc1, Tcc2, Tcc3, Tcc4	DQ821221, DQ821222, DQ821223, DQ821224
8. Kramplje, Slovenia, ZMA9132	313, 619	2	2	Tcc5, Tcc6	DQ821220, DQ821225
<i>Triturus carnifex macedonicus</i> (Karaman 1922)					
9. Ano Kaliniki, Greece, ZMA9085	602-605, 658, 660, 661, 674, 676	9	4	Tcm1, Tcm2, Tcm3, Tcm4	DQ821226, DQ821227, DQ821228, DQ821229
<i>Triturus cristatus</i> (Laurenti 1768)					
10. Peterborough, UK, ZMA9144 and 9199	583, 968, 972, 973	7	4	Tcr1, Tcr2, Tcr3	DQ821230, DQ821232, DQ821233
11. Sinaia, Romania, ZMA9156	700-702	4	3	Tcr1, Tcr4	DQ821231
<i>Triturus dobrogicus</i> (Kiritzescu 1903)					
12. Alap, Hungary ZMA9083	513, 514, 744	3	2	Tbo1, Tbo2	DQ821235, DQ821236
13. Svistov, Bulgaria, ZMA9160	748	1	1	Tbo2	DQ821237
14. Zimnicea, Romania, ZMA9171	705, Y15A	2	2	Tbo1, Tbo3	DQ821234

APPENDIX: B. TAXONOMIC IMPLICATIONS FOR *TRITURUS*

Our comprehensive mitochondrial phylogeny of *Triturus* within the Salamandridae, basically supports the taxonomic revisions found in both Garcia-Paris et al. (2004) and Litvinchuk et al. (2005), although some results agree with the proposal from one team, and others with the suggestions from the other team. Based on our results, we support the following taxonomic revisions with the intent to perturb as little as possible the previously proposed revisions. Since *Triturus cristatus* is the type species of the genus,

all members of the clade of LBT (*T. cristatus*, *T. carnifex*, *T. dobrogicus*, *T. karelinii*, *T. marmoratus* and *T. pygmaeus*) should maintain the genus name *Triturus*. *Triturus alpestris* and *Triturus vittatus* could be included in two monotypic genera, that is *Mesotriton* for *T. alpestris* (Garcia-Paris et al., 2004) and *Ommatotriton* for *T. vittatus* (Litvinchuk et al., 2005). Our data also support the placement of all SBT species (*T. boscai*, *T. helveticus*, *T. italicus*, *T. montandoni* and *T. vulgaris*) in their own genus. Proposed names for such a clade are *Lissotriton* (Garcia-Paris et al., 2004) and *Lophinus* (Litvinchuk et al., 2005).