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Molecular Phylogenetics and Historical Biogeography among Salamandrids of the "True" Salamander Clade: Rapid Branching of Numerous Highly Divergent Lineages in *Mertensiella luschani* Associated with the Rise of Anatolia

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Phylogenetic relationships among salamandrids of the "true" salamander clade are investigated using 2019 aligned base positions (713 parsimony informative) of 20 mitochondrial DNA sequences from the genes encoding ND1 (subunit one of NADH dehydrogenase), $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, ND2, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, $tRNA^{Tyr}$, and COI (subunit I of cytochrome *c* oxidase), plus the origin for light-strand replication (O_L) between the $tRNA^{Asn}$ and the $tRNA^{Cys}$ genes. Parsimony analysis produces a robust phylogenetic estimate for the relationships of the major groups of "true" salamanders. Strong support is provided for the sister taxon relationship of *Chioglossa* and *Mertensiella caucasica* and for the placement of *Salamandra* and *Mertensiella luschani* as sister taxa. These relationships suggest two vicariant events between Europe and Anatolia caused by the formation of seaways in the Mediterranean Basin. Molecular divergence indicates an Early Miocene separation of *Chioglossa* and *M. caucasica* and a Late Miocene separation of *Salamandra* and *M. luschani.* The traditional phylogenetic hypothesis of a monophyletic *Mertensiella* is statistically rejected, indicating that southwestern and northeastern Anatolian populations have separate historical biogeographic origins. Therefore, we recommend placement of *M. luschani* in the genus *Salamandra.* Within *M. luschani,* six highly divergent lineages showing 7.6 to 10.1% pairwise sequence divergence are identified. Tests using four-taxon subsamples suggest that these lineages diverged nearly simultaneously in the Late Miocene, approximately 6 to 8 million years ago, when extensive uplifting of Anatolia occurred in response to the Arabian collision.

Key Words: **Amphibia; Caudata; Salamandridae; *Chioglossa; Mertensiella; Salamandra;* biogeography; mitochondrial DNA; phylogenetics.**

INTRODUCTION

The "true" salamander clade of the Salamandridae is distributed predominantly across Europe and Anatolia, providing an interesting group for the investigation of historical biogeography of these two regions. There are three genera currently recognized in the "true" salamanders. The monotypic genus *Chioglossa* is restricted to the Iberian Peninsula (Fig. 1). The genus *Salamandra* occurs across Europe with disjunct populations in North Africa and eastern Anatolia. The genus *Mertensiella* is recognized as two distinct species. *Mertensiella caucasica* occurs on the northern margins of the Anatolian Plateau in the lesser Caucasus of Georgia and Turkey. *Mertensiella luschani* is found along the southwestern Turkish coast on the southern margin of the Anatolian Plateau and adjacent Aegean Islands of Greece (Fig. 2).

Two main hypotheses have been proposed for the phylogenetic relationships among genera and species of "true" salamanders. The traditional hypothesis (Özeti, 1967; Wake and Özeti, 1969), which has a monophyletic *Mertensiella*, suggests that the Anatolian populations have a single historical biogeographic origin followed by a north–south vicariant event across the Anatolian Plateau. Recent molecular phylogenetic studies suggest an alternative phylogenetic hypothesis, which places *Chioglossa lusitanica* as the sister taxon to *M. caucasica* and the genus *Salamandra* as the sister taxon to *M. luschani* (Titus and Larson, 1995; Veith *et al.*, 1998). This second hypothesis suggests two separate vicariant events between Europe and Anatolia, indicating that taxa within Anatolia do not form a monophyletic group. The closing of the

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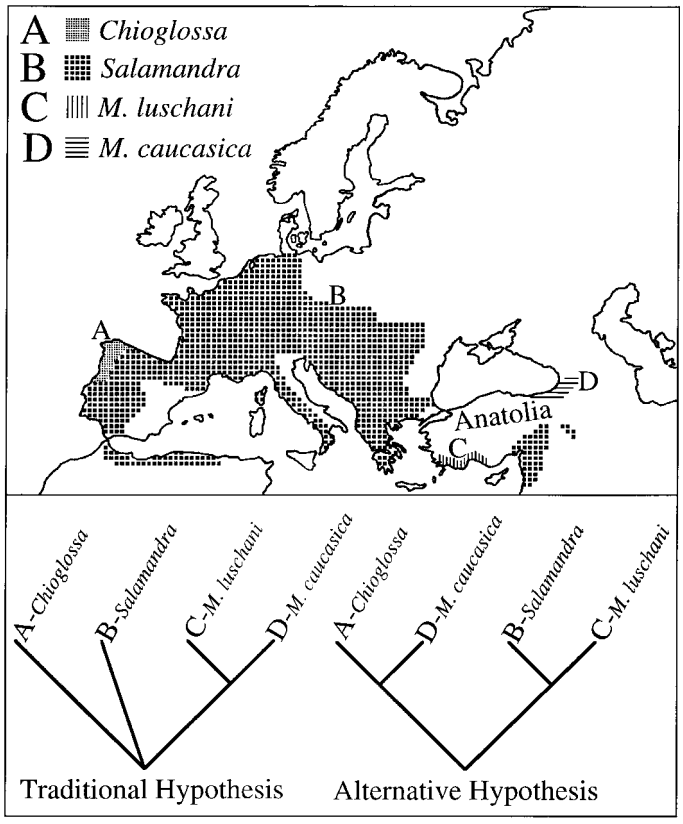


FIG. 1. Distribution of salamandrid taxa used in this study. *Chioglossa* (A) is isolated in the mountains of northern Iberia. The genus *Salamandra* (B) occurs across Europe with disjunct populations in North Africa and eastern Anatolia. *Mertensiella luschani* (C) is isolated along a narrow stretch of the southwestern Turkish coast and adjacent Aegean Islands of Greece. See Fig. 2 for more detail. *Mertensiella caucasica* (D) is restricted to the Lesser Caucasus of northeastern Turkey and Georgia. Shown below are the two main hypotheses for relationships among genera and species of "true" salamanders. The traditional hypothesis (Özeti, 1967; Wake and Özeti, 1969), which has a monophyletic *Mertensiella* (C and D), suggests a single historical biogeographic origin for Anatolian populations followed by a north–south vicariant event across the Anatolian Plateau. The alternative hypothesis derived from recent molecular phylogenetic studies (Titus and Larson, 1995; Veith *et al.*, 1998) places *Chioglossa* (A) as the sister taxon to *M. caucasica* (D) and the genus *Salamandra* (B) as the sister taxon to *M. luschani* (C). This second hypothesis suggests separate historical biogeographic origins for southwestern and northeastern Anatolian populations.

Tethys Sea by northward movement of the African Plate caused periodic isolation of Anatolia from Central Europe (Steininger and Rogl, 1984). Therefore, multiple connections between Anatolia and Europe followed by vicariant separations may be expected.

Both previous molecular phylogenetic studies addressing relationships among "true" salamanders use mitochondrial ribosomal RNA genes and resolve concordant phylogenetic topologies (Titus and Larson, 1995; Veith *et al.*, 1998). Both studies statistically reject monophyly of the genus *Mertensiella*, but neither study provided statistical support for all major groupings of "true" salamanders.

M. luschani is narrowly distributed along ~350 km of the southwestern Turkish coast and adjacent islands in the Aegean Sea. Within this geographically limited distribution, extensive color variation occurs and is used to diagnose nine subspecies (Plate 1). Similar diversity in color variation is found among subspecies of the western North American salamander, *Ensatina eschscholtzii*, but across a much larger geographic area (~1350 km; Stebbins, 1949). Because subspecies of *M. luschani* have small distributions restricted to isolated limestone outcrops (Baran and Üçüncü, 1994; Polymeni, 1994), considerable genetic divergence may occur among these populations.

New mitochondrial DNA data are used to estimate relationships among "true" salamanders using 2019 aligned base positions (713 parsimony informative). The new region sequenced extends from the protein-coding gene, ND1 (subunit one of NADH dehydrogenase), through the genes encoding $tRNA^{Ile}$, $tRNA^{Gln}$, $tRNA^{Met}$, ND2, $tRNA^{Trp}$, $tRNA^{Ala}$, $tRNA^{Asn}$, $tRNA^{Cys}$, and $tRNA^{Tyr}$, to the protein-coding gene COI (subunit I of cytochrome *c* oxidase), and includes the replication origin for the light strand (O_L) between the $tRNA^{Asn}$ and the $tRNA^{Cys}$ genes.

To investigate phylogenetic relationships among the "true" salamanders and to test support for two vicariant events between Europe and Anatolia, *C. lusitanica* from the northern coast of Spain and *M. caucasica* from the Lesser Caucasus of Georgia are sampled. In the

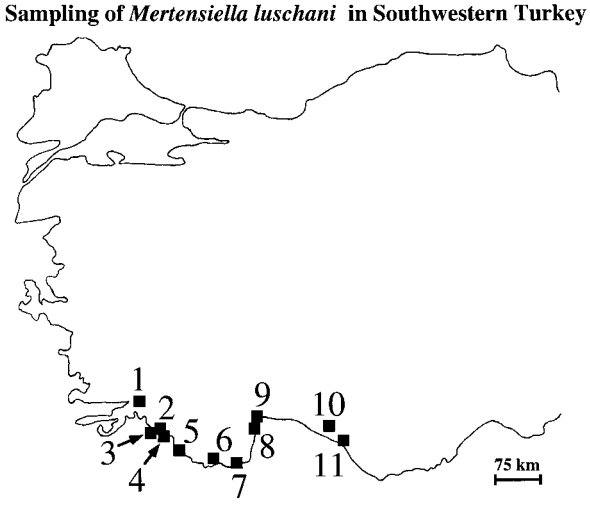


FIG. 2. Location of *Mertensiella luschani* populations used in this study, including all eight subspecies endemic to southwestern Anatolia. Numbers refer to populations listed under Materials and Methods: (1) *M. l. flavimembris*, (2) *M. l. fazilae* Gökceovacik Köyü, (3) *M. l. fazilae* Domuz Island, (4) *M. l. fazilae* Tersane Island, (5) *M. l. luschani*, (6) *M. l. basoglui*, (7) *M. l. finikensis*, (8) *M. l. billae*, (9) *M. l. antalyana*, (10) *M. l. atifi* Fersin Köyü, and (11) *M. l. atifi* Türbelinaz.

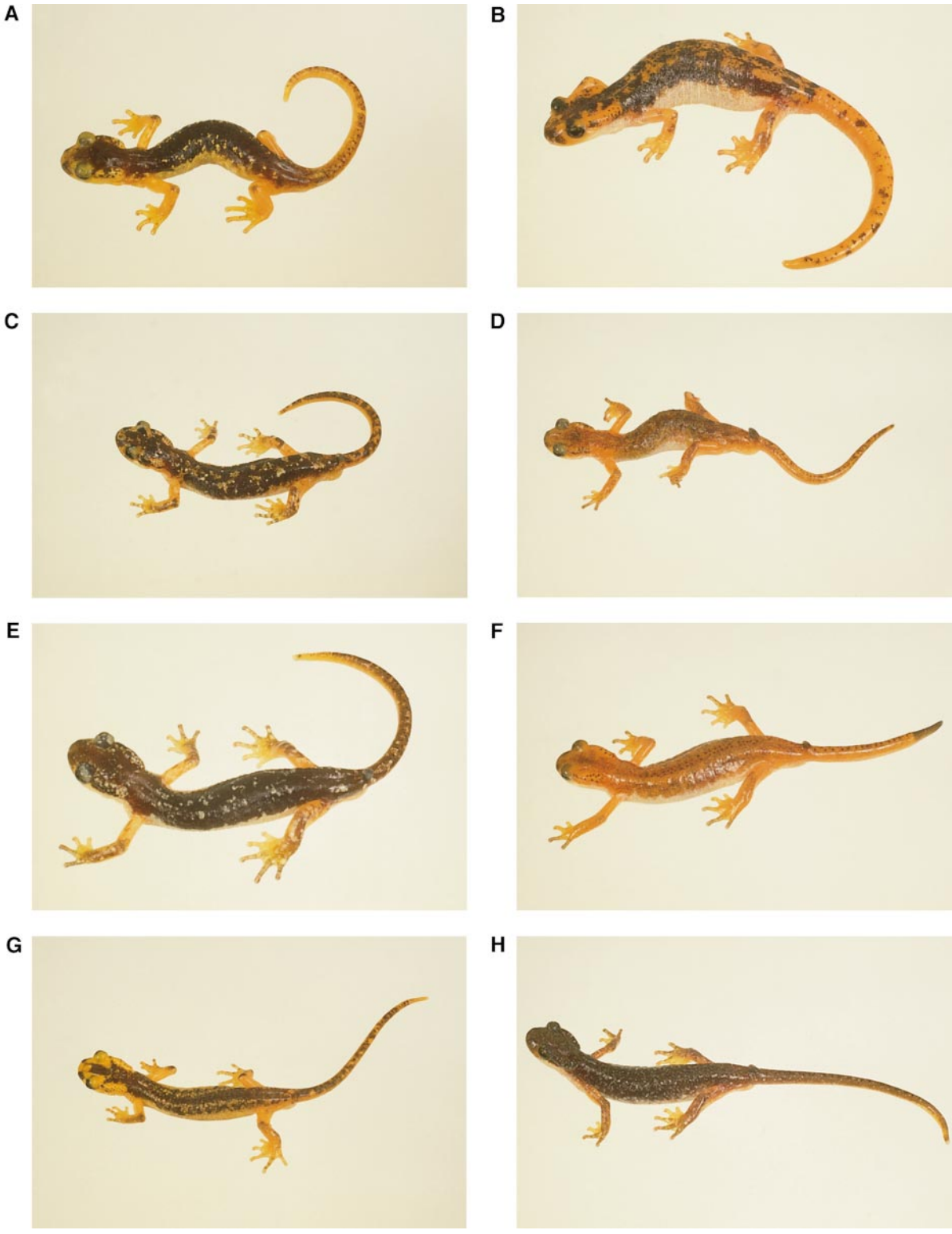


PLATE 1. Color patterns among the eight Anatolian subspecies of *Mertensiella luschani*. (A) *M. l. flavimembris* (MVZ 230148); (B) *M. l. fazilae* (MVZ230150); (C) *M. l. luschani* (MVZ230165); (D) *M. l. basoglui* (MVZ230171); (E) *M. l. finikensis* (MVZ230177); (F) *M. l. billae* (MVZ230184); (G) *M. l. antalyana* (MVZ230190); (H) *M. l. atifi* (MVZ230196). Taxa are arranged in order of their distribution from west to east (see Fig. 2).

genus *Salamandra, S. salamandra* is sampled from Spain and Ukraine to represent both ends of its European distribution. *Salamandra infraimmaculata,* isolated in eastern Turkey and adjacent countries, is also sampled. This sampling covers two of the six major clades identified in a previous molecular phylogenetic study of the genus *Salamandra* (Steinfartz *et al.*, 2000). In addition, 11 populations representing all eight of the Anatolian subspecies of *M. luschani* are included and used in biogeographic tests.

MATERIALS AND METHODS

Specimen Information

Museum numbers and localities for voucher specimens from which DNA was extracted and GenBank accession numbers are presented below. Acronyms are CAS for California Academy of Sciences, San Francisco, and MVZ for Museum of Vertebrate Zoology, University of California at Berkeley. Populations of *M. luschani* are numbered as in Fig. 2. *Notophthalmus viridescens:* MVZ230959, AF296616, 2.4 miles south of Weldon Springs at I-40 on Hwy 94, St. Charles Co., Missouri. *Pachytriton labiatus:* CAS194298, AF296618, 6.8 km south of Zhangcun (30° 58′ N 119° 22′ E), Jiaxing Prefecture, Zhejiang Province, China. *Triturus vulgaris:* CAS182922, AF296619, Adler City (42° 25′ N 39° 56′ E), Adler District of Sochi Municipality, Krasnodarsky Territory, Russia. *Tylototriton taliangensis:* CAS195126, AF296617, vicinity of elev. 2400 m, 9.5 km north of Tuowo (28° 49′ N 102° 17′ E) on the Hanyuan to Xichang Rd., then 1.4 km NNE on dirt Rd., Liangshan Yizu Autonomous Prefecture, Sichuan Province, China. *C. lusitanica:* MVZ230958, AF296620, San Martin de Luina, Asturias, Spain. *S. salamandra:* MVZ186046, AF296622, Cadiz, Andalusia, Spain. *S. salamandra:* MVZ230725, AF296623, Elev. 600 m, valley of Latorista River, Mukachevo District, Trans Karphatien Region, Ukraine. *S. infraimmaculata:* MVZ230199, AF296624, Harbiye (36° 09′ N 36° 09′ E), Hatay Province, Turkey. *M. caucasica:* MVZ218721, AF296621, approx. 10 km SSE of Borzhomi (41° 51′ N 43° 23′ E), Georgia. (1) *M. luschani flavimembris:* MVZ230148, AF296635, Cicekli Köyü (37° 04′ N 28° 30′ E), 7 km east by road of Ula, Mugla Province, Turkey. (2) *M. l. fazilae:* MVZ230150, AF296630, Gökceovacik Köyü (36° 47′ N 28° 59′ E), 10 km north by road of Göcek, Mugla Province, Turkey. (3) *M. l. fazilae:* MVZ230159, AF296627, Domuz Adasi (36° 39′ N 28° 54′ E) in Fethiye Bay, Mugla Province, Turkey. (4) *M. l. fazilae:* MVZ230153, AF296628, Tersane Adasi (36° 40′ N 28° 56′ E) in Fethiye Bay, Mugla Province, Turkey. (5) *M. l. luschani:* MVZ230165, AF296632, Dodurga Köyü, 9 km southwest by road of Esen (36° 27′ N 29° 16′ E), Mugla Province, Turkey. (6) *M. l. basoglui:* MVZ230171, AF296633, Nardarlar Köyü, 17 km ENE of Kas (36° 12′ N 29° 38′ E) via Finike Hwy, Antalya Province, Turkey. (7) *M. l. finikensis:* MVZ230177, AF296631, 3 km south by road of Finike (36° 18′ N 30° 09′ E), Antalya Province, Turkey. (8) *M. l. billae:* MVZ230184, AF296626, Limestone hills at Büyük Calticak Beach, 20 km SSW by Finike Hwy of Antalya (36° 53′ N 30° 42′ E), Antalya Province, Turkey. (9) *M. l. antalyana:* MVZ230190, AF296625, Hurma Köyü, 9 km SW of Antalya (36° 53′ N 30° 42′ E) along Altinyaka Rd., Antalya Province, Turkey. (10) *M. l. atifi:* MVZ230197, AF296629, Fersin Köyü (36° 49′ N 31° 47′ E), 32 km northeast by Akseki Rd. of Kizilot, Antalya Province, Turkey. (11) *M. l. atifi:* MVZ230196, AF296634, Türbelinaz, 23 km north by road of Alanya (36° 33′ N 32° 01′ E), Antalya Province, Turkey.

Genomic DNA was extracted from liver or muscle using the Qiagen QIAamp tissue kit. Amplification of genomic DNA was conducted using denaturation at 94°C for 35 s, annealing at 50°C for 35 s, and extension at 70°C for 150 s with 4 s added to the extension per cycle for 30 cycles. Negative controls were run for all amplifications. Amplified products were purified on 2.5% Nusieve GTG agarose gels and reamplified under similar conditions. Reamplified double-stranded products were purified on 2.5% acrylamide gels (Maniatis *et al.*, 1982). Template DNA was eluted from acrylamide passively over 3 days with Maniatis elution buffer (Maniatis *et al.*, 1982). Cycle sequencing reactions were run using the Promega fmol DNA-sequencing system with a denaturation at 95°C for 35 s, annealing at 45–60°C for 35 s, and extension at 70°C for 1 min for 30 cycles. Sequencing reactions were run on Long Ranger sequencing gels for 5–12 h at 38–40°C.

Amplifications from genomic DNA were done for all taxa using two main primer combinations: L3002–H4419 and L4437–H5934 (Table 1). A third pair of primers was used to amplify a segment that bridged these two amplified regions. This additional primer pair varied across taxa. *C. lusitanica, M. caucasica, M. luschani atifi* Fersin Köyü, *M. luschani billae, M. luschani finikensis,* and all *Salamandra* were amplified with L4221–H4980 (Table 1). The outgroup taxon, *N. viridescens,* and all remaining *M. luschani* populations were amplified with L4160–H4980 (Table 1). Additional outgroup taxa were amplified with the primer pair L3838–H4980 (Table 1).

Procedure for Alignment of DNA Sequences

DNA sequences encoding part of ND1, all of ND2, and part of COI were aligned by amino acid using MacClade (Maddison and Maddison, 1992). Alignments of sequences encoding tRNAs were constructed manually based on secondary structural models (Kumazawa and Nishida, 1993; Macey and Verma, 1997). Secondary structures of tRNAs were inferred from primary structures of the corresponding tRNA genes using these models. Unalignable

TABLE 1

Primers Used in This Study

Human position	Gene	Sequence	Reference
L3002	16S	5′-TACGACCTCGATGTTGGATCAGG-3′	Macey *et al.* (1997a)
L3878	ND1	5′-GCCCCATTTGACCTCACAGAAGG-3′	Macey *et al.* (1998b)
L4160	ND1	5′-CGATTCCGATATGACCARCT-3′	Kumazawa and Nishida (1993)
L4221	tRNA*Ile*	5′-AAGGATTACTTTGATAGAGT-3′	Macey *et al.* (1997a)
H4419	$tRNA^{Met}$	5′-GGTATGGGCCCAAAAGCTT-3′	Macey *et al.* (1998b)
L4437	$tRNA^{Met}$	5′-AAGCTTTCGGGCCCATACC-3′	Macey *et al.* (1997a)
L4882a	ND2	5′-TGACAAAAACTAGCCCC-3′	Macey *et al.* (2000)
L4882b	ND2	5′-TGACAAAAAATTGCNCC-3′	Macey *et al.* (2000)
H4980	ND2	5′-ATTTTTCGTAGTTGGGTTTGRTT-3′	Macey *et al.* (1997a)
H5692	$tRNA^{Asn}$	5′-GCGTTTAGCTGTTAACTAAA-3′	This study
L5551	$tRNA^{Trp}$	5′-GACCAAAGGCCTTCAAAGCC-3′	Macey *et al.* (1997b)
H5934	COI	5′-AGRGTGCCAATGTCTTTGTGRTT-3′	Macey *et al.* (1997a)
H6159	COI	5′-GCTATGTCTGGGGCTCCAATTAT-3′	This study

Note. Primers are designated by their 3′ ends which correspond to the position in the human mitochondrial genome (Anderson *et al.*, 1981) by convention. H and L designate primers that extend the heavy and light strands, respectively. Positions with mixed bases are labeled with standard one-letter codes: R = G or A, and N = any base.

sequences from some length-variable intergenic regions between the $tRNA^{Ile}$ and the $tRNA^{Gln}$ genes (positions 452–455) and between the $tRNA^{Gln}$ and the $tRNA^{Met}$ genes (positions 526–528) were excluded from phylogenetic analyses. In addition, positions 1628–1643 encoding the C-terminal end of ND2, as well as portions of the origin for light-strand replication (positions 1869–1883), were not used because of considerable length variation (Fig. 3).

Phylogenetic Analysis

Phylogenetic trees were estimated using PAUP* beta version 4.0b2 (Swofford, 1999) with 100 heuristic searches using random addition of sequences. Bootstrap resampling was applied to assess support for individual nodes using 1000 bootstrap replicates with 100 random additions per replicate. Decay indices (= "branch support" of Bremer, 1994) were calculated for all internal branches of the tree. To calculate decay indices, a phylogenetic topology containing the single node in question was constructed using MacClade (Maddison and Maddison, 1992) and analyzed as a constraint in PAUP* beta version 4.0b2 (Swofford, 1999) with 100 heuristic searches featuring random addition of sequences. These searches retained trees that violated the imposed constraint. The decay index was then tabulated as the difference in length between the shortest tree violating the constraint and the overall shortest tree.

The Wilcoxon signed-ranks test (Felsenstein, 1985; Templeton, 1983) was used to examine the statistical significance of the overall shortest tree relative to alternative hypotheses. This test asks whether the most parsimonious tree is significantly shorter than an alternative or whether their difference in length can be attributed to chance alone (Larson, 1998). Wilcoxon signed-ranks tests were conducted as one- and two-tailed tests. Felsenstein (1985) showed that one-tailed probabilities are close to the exact probabilities for this test but are not always conservative, whereas the two-tailed test is always conservative. Tests were conducted using PAUP* beta version 4.0b2 (Swofford, 1999), which incorporates a correction for tied ranks.

Alternative phylogenetic hypotheses were tested using the most parsimonious phylogenetic topologies compatible with them. To find the most parsimonious tree(s) compatible with a particular phylogenetic hypothesis, phylogenetic topologies were constructed using MacClade (Maddison and Maddison, 1992) and analyzed as constraints using PAUP* beta version 4.0b2 (Swofford, 1999) with 100 heuristic searches with random addition of sequences.

To evaluate whether a hard polytomy (simultaneous or nearly simultaneous branching from a common ancestral lineage) or a soft polytomy (successive branching of lineages that have relatively short intervals of time between them) exists in areas of the tree that have weak branch support, the four-taxon test of Felsenstein (1985) was employed as implemented in Jackman *et al.* (1999). This test evaluates whether removing taxa that potentially divide long branches increases support for phylogenetic groupings of the remaining taxa that lacked strong support in the phy-

TABLE 2

Significant Decay Index Values for the Four-Taxon Test of Felsenstein (1985)

Number of parsimony-informative sites	Significant decay value
53–65	10
66–79	11
80–97	12

logenetic analysis of all taxa (see Figure 3 in Jackman *et al.*, 1999). Critical values of the decay index for four-taxon statements (Table 2) were derived from a computer program written by J. Felsenstein for use in Felsenstein (1985). This test was applied to lineages within *M. luschani* using *Salamandra* populations and species as outgroups.

RESULTS

Mitochondrial DNA sequences ranging in size from 2036 to 2048 bases for 20 salamandrids are presented in Fig. 3.

Authentic Mitochondrial DNA

Several observations suggest that the DNA sequences analyzed here are from the mitochondrial genome and are not nuclear-integrated copies of mitochondrial genes (see Zhang and Hewitt, 1996). Protein-coding genes do not have premature stop codons, suggesting that these sequences represent functional copies that encode a protein. Transfer RNA genes specify tRNAs with stable secondary structures, indicating functional genes. The presence of strand bias further supports our conclusion that the DNA sequences reported here are from the mitochondrial genome. The sequences reported here show strong strand bias against guanine on the light strand (G = 11.1–14.1%, A = 32–35.6%, T = 25.2–31%, and C = 23.3–26.6%), which is characteristic of the mitochondrial genome but not the nuclear genome.

Genic Variation

The majority of variable and informative sites are in protein-coding genes (81.8 and 86.4%, respectively; Table 3). Third codon positions contain approximately half of all phylogenetic information (52.6%), with first and second positions containing 23.4 and 10.4% of the phylogenetic information, respectively. All tRNA genes contain phylogenetic information in regions encoding stems and loops. However, only 6.4% of the total phylogenetic information is from tRNA stems, suggesting that compensatory changes within stem regions do not compromise the phylogenetic analysis.

Phylogenetic Relationships

A single tree of 2654 steps is produced from parsimony analysis of the 2019 (713 informative) base positions from the 20 aligned DNA sequences (Fig. 4). Support for phylogenetic relationships among outgroup taxa is weak.

Monophyly of the "true" salamanders is well supported relative to the outgroup taxa sampled (bootstrap 99%, decay index 25). Within the "true" salamanders, the genus *Mertensiella* does not appear monophyletic. *M. caucasica* is well supported as the sister taxon to *C. lusitanica* (bootstrap 98%, decay index 20), whereas *M. luschani* is well supported as the sister taxon to *Salamandra* (bootstrap 100%, decay index 35). *Salamandra* appears monophyletic with strong support (bootstrap 100%, decay index 41). Within *Salamandra*, the samples from Spain and Ukraine referred to *S. salamandra* form a well-supported clade (bootstrap 100%, decay index 27) matching the results of Steinfartz *et al.* (2000).

Monophyly of *M. luschani* is well supported (bootstrap 100%, decay index 41). Within *M. luschani*, there is little support for relationships among the 11 populations sampled, with three exceptions. The 2 populations of *M. l. atifi* form a well-supported clade (bootstrap 100%, decay index 39) as do the 3 populations of *M. l. fazilae* (bootstrap 100%, decay index 57). In addition, *M. l. basoglui*, *M. l. finikensis*, and *M. l. luschani* form a well-supported monophyletic group (bootstrap 100%, decay index 15). Relationships between these three groups and *M. l. antalyana*, *M. l. billae*, and *M. l. flavimembris* are poorly supported with bootstrap values of 52% or less and decay indices of 2 to 4.

Four alternative phylogenetic hypotheses are statistically compared to the results of the phylogenetic analysis presented above:

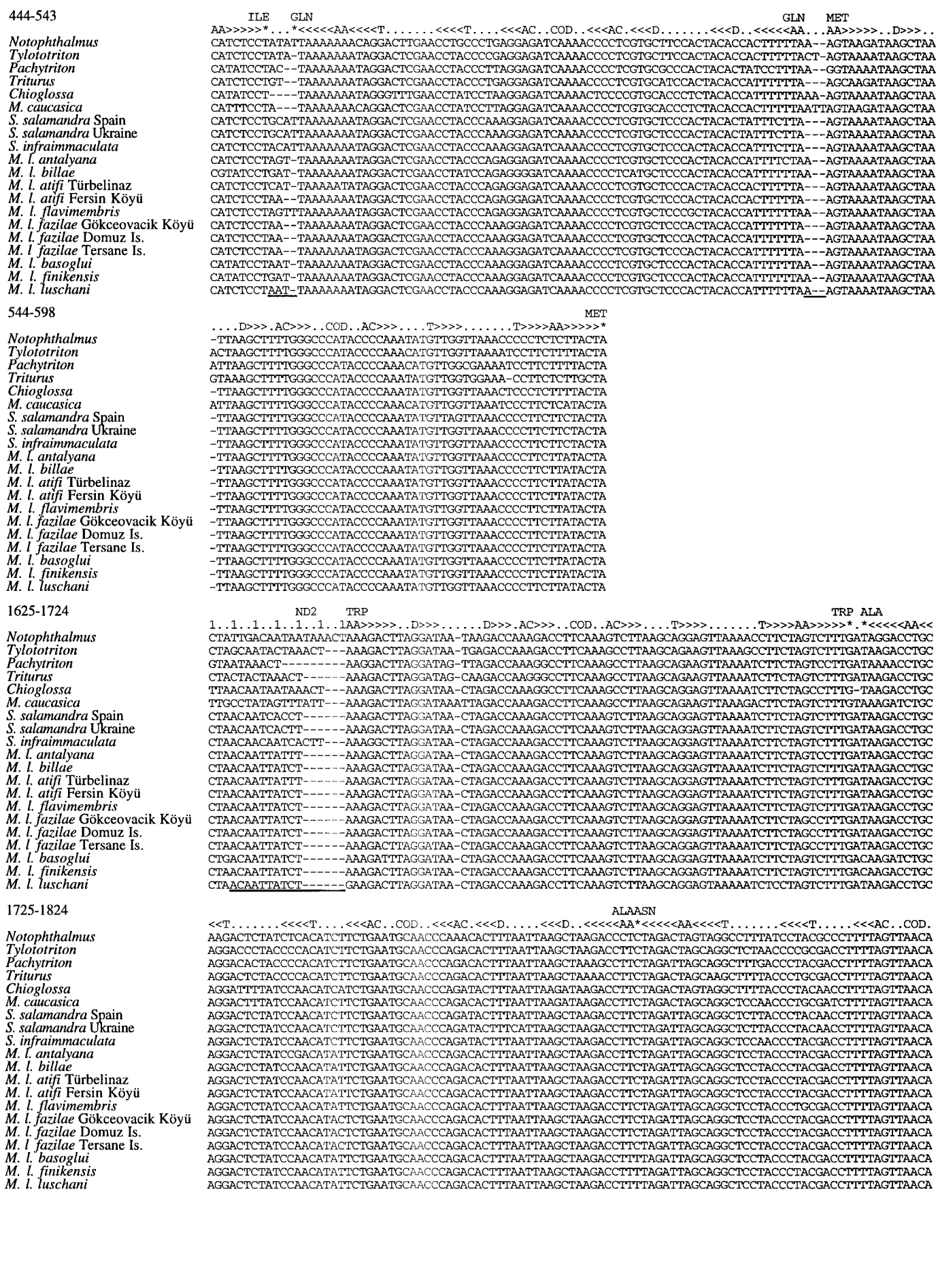
(1) The overall shortest tree (Fig. 4) depicts the "true" salamanders as monophyletic relative to the outgroup taxa sampled. When this tree is compared to the shortest alternative tree (A1 in Appendix) showing the "true" salamanders as a nonmonophyletic group, this alternative is rejected by the Wilcoxon signed-ranks test using the two-tailed test (Table 4).

(2) The genus *Mertensiella* is not resolved as a monophyletic group in the overall shortest tree (Fig. 4). When the overall shortest tree is compared to the shortest alternative trees (B1–16 in Appendix) depicting a monophyletic *Mertensiella*, the alternative trees are rejected by the Wilcoxon signed-ranks test using the two-tailed test.

(3) The overall shortest tree (Fig. 4) places *M. luschani* as the sister taxon to the genus *Salamandra*. When this tree is compared to the shortest alternative tree (C1 in Appendix) in which *M. luschani* and *Salamandra* do not form a monophyletic group, this alternative tree is rejected by the Wilcoxon signed-ranks test using the two-tailed test.

(4) The overall shortest tree (Fig. 4) places *M. caucasica* and *Chioglossa* as sister taxa. When this tree is compared to the shortest alternative trees (D1 and 2 in Appendix) in which these taxa do not form a monophyletic group, these alternatives are rejected by the Wilcoxon signed-ranks test using the one-tailed test.

Within the ingroup, all branches are well supported (a minimum bootstrap value of 97% and a decay index of 15) except for some branches within *M. luschani*. The phylogenetic analysis identifies three well-supported clades within *M. luschani*, but the relationships


444-543
544-598
1625-1724
1725-1824
Notophthalmus
Tylototriton
Pachytriton
Triturus
Chioglossa
M. caucasica
S. salamandra Spain
S. salamandra Ukraine
S. infraimmaculata
M. l. antalyana
M. l. billae
M. l. atifi Türbelinaz
M. l. atifi Fersin Köyü
M. l. flavimembris
M. l. fazilae Gökceovacik Köyü
M. l. fazilae Domuz Is.
M. l fazilae Tersane Is.
M. l. basoglui
M. l. finikensis
M. l. luschani

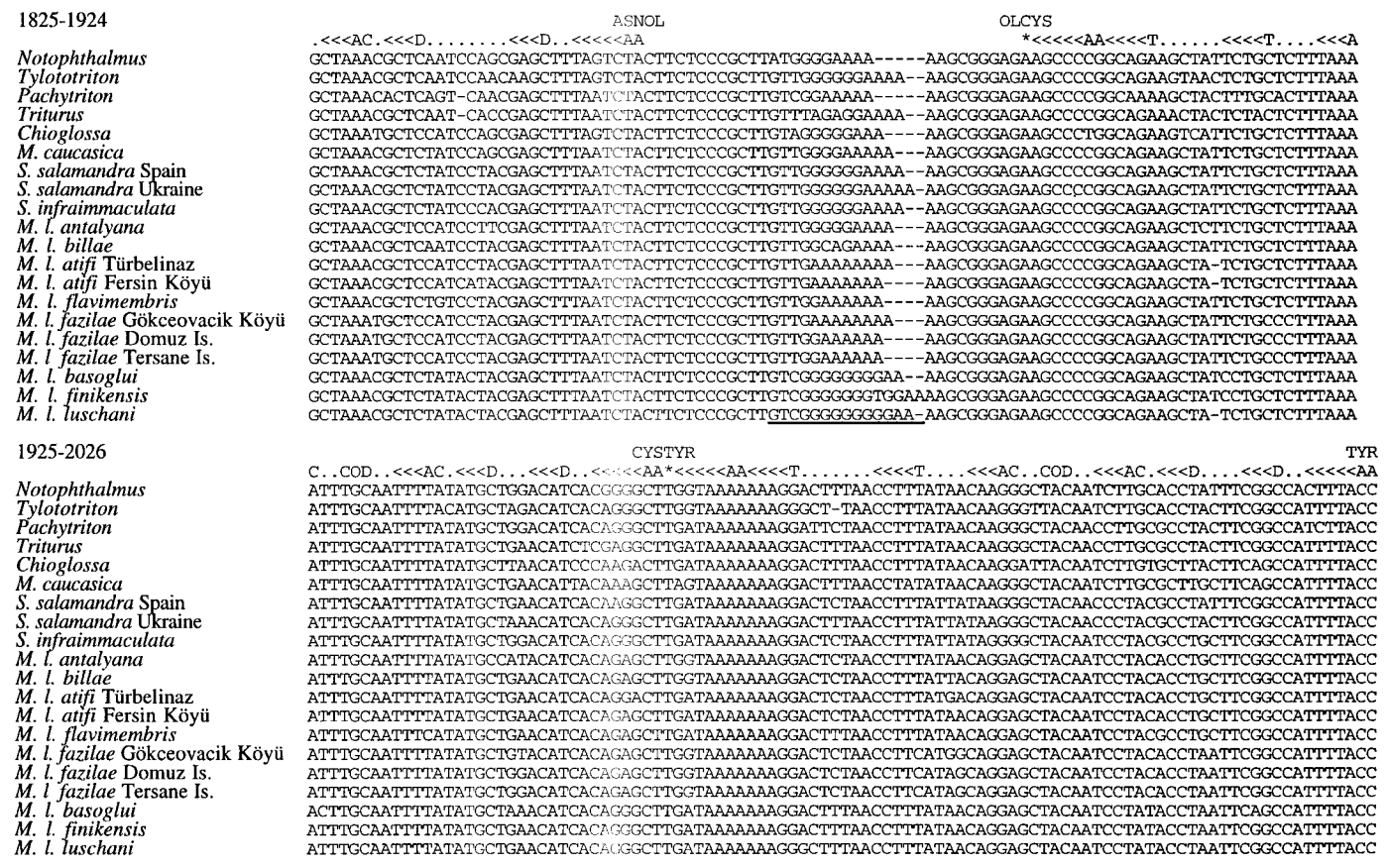


FIG. 3—*Continued*

among these three clades and three additional *M. luschani* lineages are recovered with only weak support. Branch lengths between these six lineages are relatively short (Fig. 5). To test whether the six lineages of *M. luschani* whose relationships are not well resolved represent a hard polytomy, the four-taxon test of Felsenstein (1985) as implemented by Jackman *et al.* (1999) is employed. Between the six *M. luschani* lineages and the *Salamandra* lineage, there are 318 quartets of populations. Of these quartets, only 17 have significant decay index values (5.3%). Application of the 95% significance criterion therefore fails to reject the hypothesis that the poorly supported region between the six *M. luschani* lineages represents a hard polytomy.

DISCUSSION

Historical Biogeography among Major Lineages of "True" Salamanders

The phylogenetic analysis conducted here provides further support for the hypothesis that *C. lusitanica* is the sister taxon to *M. caucasica* and that the genus *Salamandra* is the sister taxon to *M. luschani* (Titus and Larson, 1995; Veith *et al.*, 1998). This hypothesis

FIG. 3. Length-variable regions among the 20 aligned mitochondrial DNA sequences as used in the phylogenetic analysis. Four regions totaling 38 positions were excluded from the analysis and are indicated with underlines. Positions 1–443 from the ND1 and $tRNA^{Ile}$ gene and positions 2026–2057 from the COI gene are not shown because they have no length variation. Also not shown are positions 599–1624 in the ND2 gene. Three amino acid positions have length variation in the ND2 gene. The four outgroup taxa have an extra amino acid at the 96th codon position and three gaps are placed in members of the "true" salamanders at alignment positions 884–886. *Notophthalmus* has an amino acid deleted at codon position 321 and gaps are placed at alignment positions 1559–1561. *Chioglossa* has an amino acid deleted at codon position 331 and gaps are placed at alignment positions 1589–1591. Sequences are presented as light-strand sequence and tRNA secondary structure is designated above the sequence. Stems are indicated by arrows in the direction encoded: AA, amino acid-acceptor stem; D, dihydrouridine stem; AC, anticodon stem; and T, TΨC stem. The tRNA anticodons are designated COD. Asterisks indicate the unpaired 3′ tRNA position 73. Periods indicate bases located outside stem regions; 1 depicts the first positions of codons in protein-coding sequences. O_L represents the origin for light strand replication.

TABLE 3

Distribution of Phylogenetically Informative and Variable Positions

Protein-coding genes	ND1			ND2			COI		
Codon position	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Variable	53	18	118	172	90	320	1	2	6
Informative	40	11	97	126	63	273	1	0	5

tRNA genes	$tRNA^{Ile}$		$tRNA^{Gln}$		$tRNA^{Met}$		$tRNA^{Trp}$	
Secondary structure	Stem	Loop	Stem	Loop	Stem	Loop	Stem	Loop
Variable	15	8	13	9	8	9	11	6
Informative	9	5	4	7	4	4	5	5

tRNA genes	$tRNA^{Ala}$		$tRNA^{Asn}$		$tRNA^{Cys}$		$tRNA^{Tyr}$	
Secondary structure	Stem	Loop	Stem	Loop	Stem	Loop	Stem	Loop
Variable	11	10	8	16	10	13	15	11
Informative	4	6	4	11	5	5	11	8

	Protein-coding genes			tRNA genes		
Position/structure	1st	2nd	3rd	Stem	Loop	Total aligned sequence
Variable	226	110	444	91	82	953
Informative	167	74	375	46	51	713

Note. No variation is observed in the unambiguously aligned portion of the replication origin for light-strand synthesis between the genes encoding $tRNA^{Asn}$ and $tRNA^{Cys}$.

suggests two separate vicariant events between Europe and Anatolia, indicating that taxa on the northern and southern margins of the Anatolian Plateau do not form a monophyletic group (Fig. 1).

The region of the mitochondrial genome sequenced has been found to evolve in a clock-like manner among a wide range of vertebrates with a consistent rate of change per lineage per million years [fish 0.65% (Bermingham *et al.,* 1997); hynobiid salamanders 0.64% (unpublished data of the authors); frogs of the genus *Bufo* 0.69% (Macey *et al.,* 1998b); lizards of the genus *Laudakia* 0.65% (Macey *et al.,* 1998a); lizards of the genus *Teratoscincus* 0.57% (Macey *et al.,* 1999b)].

Pairwise sequence divergence between *Chioglossa* and *M. caucasica* is 18.8%. Applying the pairwise rate of 1.28% (0.64% per lineage) change per million years derived from four geologic events in hynobiid salamanders and assuming a linear rate of change, *Chioglossa* and *M. caucasica* are calculated to have diverged approximately 15 MYBP. Mitochondrial DNA is not expected to show linear divergence beyond 10 million years (Moritz *et al.,* 1987) and therefore this divergence is probably an underestimate. The most recent continuous land connection between the Lesser Caucasus of northeastern Anatolia through central Europe to the Iberian Peninsula beyond 15 million years ago is in the Oligocene between 23 and 25 MYBP; Europe and Anatolia rapidly separated in the Early Miocene 20 MYBP (Fig. 6). At this same time (between 22 and 17 MYBP) climatic changes coupled with the rise of the central European mountain systems occurred (Vennemann and Hegner, 1998). Hence, the formation of a seaway, the rise of the Alps, and a shift in climate disrupted favorable habitat causing vicariant separation of *Chioglossa* and *M. caucasica.*

Pairwise sequence divergence between *Salamandra* and *M. luschani* is 14.1%, which corresponds to a divergence date of 11 MYBP. Between 14.5 and 12 MYBP a continuous land connection existed between Europe and Anatolia, which subsequently separated 12 MYBP (Fig. 6). The molecular calibration of 11 MYBP for the divergence of these taxa is expected to be a slight underestimate because of the nonlinear evolution of mitochondrial DNA beyond 10 million years, which is consistent with the geologic divergence of 12 MYBP.

Recurring vicariant separation of Anatolian amphibians and reptiles from populations in Iberia and central Europe may be a major theme in the biogeography of the Mediterranean Basin. The distributional pattern of northern Iberia and northeastern Anatolia, observed for *Chioglossa* and *M. caucasica,* is matched in the frog family Pelodytidae. In addition, the amphisbaenian genus *Blanus* occurs in Iberia and southern Anatolia, areas occupied by *Salamandra* and *M. luschani,* respectively.

The two vicariant events between Anatolia and Europe separating *Chioglossa* from *M. caucasica* and *Salamandra* from *M. luschani* predate the rise of the

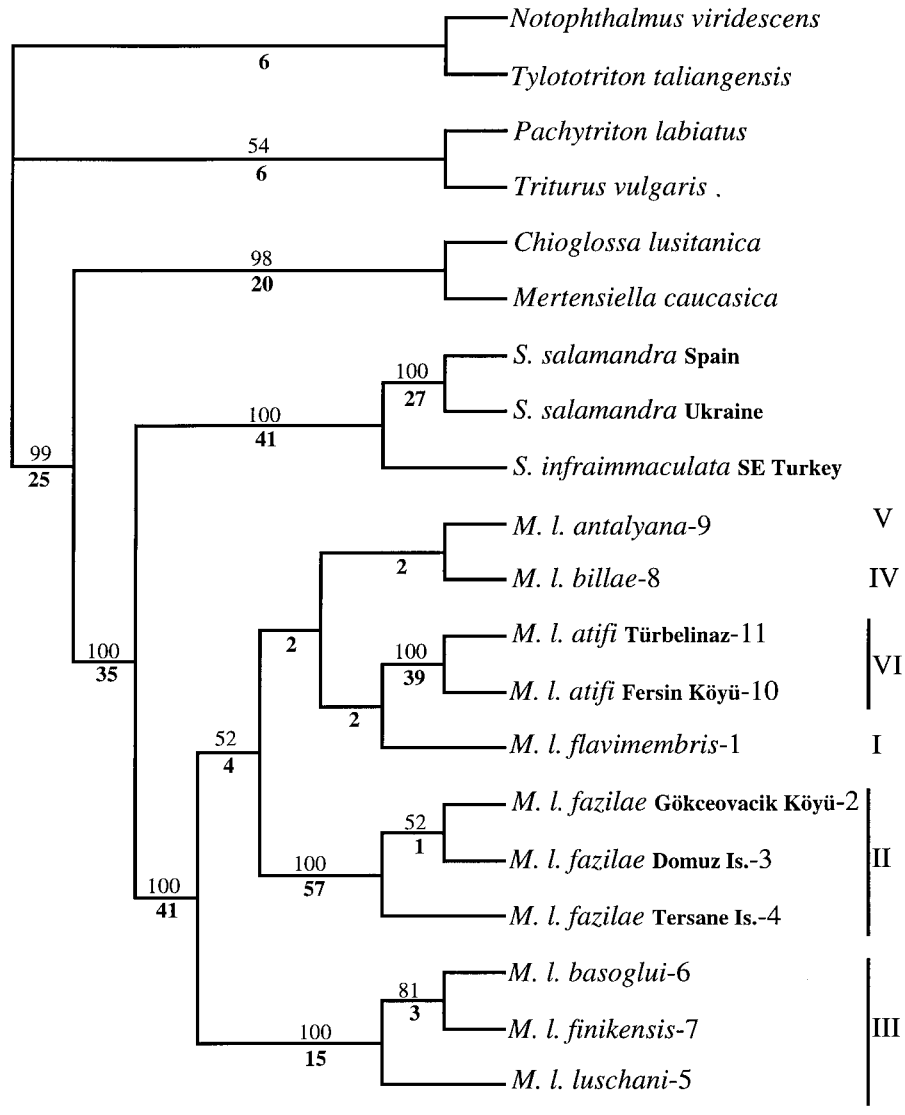


FIG. 4. Single most parsimonious tree generated from the 2019 aligned positions (713 phylogenetically informative) used in the phylogenetic analysis. The tree has a length of 2654 steps and a consistency index of 0.53. Bootstrap values are presented above branches and decay indices are shown in boldface below branches. *Mertensiella luschani* populations are numbered as in Fig. 2 and under Materials and Methods. The six lineages of *M. luschani* are depicted with bars and Roman numerals (see Discussion and Fig. 7).

Plateau of Anatolia. The Anatolian Plateau uplifted 5 to 10 MYBP due to acceleration of northward movement of the Arabian plate (Quennell, 1984; Steininger and Rögl, 1984). Divergences within *Salamandra* and *M. luschani* appear to be concordant with the rise of the Anatolian Plateau. Whereas European *Salamandra* populations show only 3.7% sequence divergence (3 MYBP) from Spain to Ukraine, the sequence divergence between these populations and the sample from southeastern Turkey is 7.45%, which suggests a divergence date of 5.8 MYBP. At this time, a continuous land connection existed between central Europe and Anatolia (Steininger and Rögl, 1984), but intense folding of the plateau (Quennell, 1984) isolated these populations from each other.

Six major lineages are identified within *M. luschani*. Subsampling using four-taxon combinations suggests that these six lineages diverged relatively rapidly in time. Pairwise sequence divergence between any of these six lineages ranges from 7.6 to 10.1% (Table 5, Fig. 7), suggesting divergence times between 5.9 and 7.9 MYBP. These estimates are consistent with Late Miocene vicariance caused by the rise of Anatolia due to acceleration of the Gondwanan fragment of Arabia (Quennell, 1984).

Morphological Evolution and Taxonomic Implications

Phylogenetic results of this study and of previous molecular studies (Titus and Larson, 1995; Veith *et al.*, 1998) do not support monophyly of the genus *Merten-*

TABLE 4

Results of Wilcoxon Signed-Ranks Tests

Hypothesis tested	Alternative tree	*N*	*Z*	*P*
1. "True" salamanders do not form a clade	A1	47	3.6466	<0.0005**
2. Monophyly of *Mertensiella*	B1	174	4.1111	<0.0001**
	B2	189	4.6535	<0.0001**
	B3	219	4.1564	<0.0001**
	B4	174	4.8857	<0.0001**
	B5	193	4.5370	<0.0001**
	B6	197	4.5616	<0.0001**
	B7	193	4.5348	<0.0001**
	B8	202	4.4125	<0.0001**
	B9	199	4.5045	<0.0001**
	B10	198	4.4480	<0.0001**
	B11	202	4.4728	<0.0001**
	B12	210	4.3918	<0.0001**
	B13	218	4.1111	<0.0001**
	B14	215	4.2209	<0.0001**
	B15	204	4.4189	<0.0001**
	B16	215	4.3120	<0.0001**
3. *M. luschani* and *Salamandra* do not form a clade	C1	61	4.4813	<0.0001**
4. *M. caucasica* and *Chioglossa* do not form a clade	D1	70	2.3905	0.0168**
	D2	110	1.9069	0.0282*

Note. A significant result denotes rejection of the hypothesis as stated. One-tailed probabilities are listed with a single asterisk denoting significance using the one-tailed test, and two asterisks denote significance using the two-tailed test.

siella, requiring independent origins of the dorsal tail tubercle observed in males of the two species. Histological studies reveal differences in morphologies of the tubercle glands in the two species of *Mertensiella* (Sever *et al.*, 1997), suggesting that these tubercules are not homologous across these species. Tubercular structures therefore appear to have originated independently in the two species of *Mertensiella.*

Strong support for nonmonophyly of the genus *Mertensiella* poses a taxonomic problem. *M. caucasica* could be placed in the genus *Chioglossa* or *M. luschani* could be placed in the genus *Salamandra*. *M. caucasica* is the type species of the genus *Mertensiella* (Wolterstorff, 1925). If this genus is retained in a monophyletic taxonomy, it must refer only to *M. caucasica.* Therefore, without creating a new taxon, *M. luschani* should

TABLE 5

Pairwise Comparisons of DNA Sequences among Salamandrids Used in This Study

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. *Notophthalmus viridescens*	—	18.6	19.2	18.2	19.7	21.1	19.9	20.3	20.3	21.4	20.4	21.4	21.4	20.8	21.0	21.0	20.8	20.6	20.1	21.2
2. *Tylototriton taliangensis*	374	—	20.2	19.9	21.5	20.7	20.8	20.7	20.5	20.2	20.3	20.6	20.3	20.0	21.6	21.3	21.2	20.2	20.5	20.7
3. *Pachytriton labiatus*	387	407	—	19.1	22.9	23.4	21.0	20.6	21.4	21.5	21.4	21.9	21.5	21.4	21.6	21.7	21.6	21.4	21.1	22.4
4. *Triturus vulgaris*	367	402	385	—	21.3	21.9	21.8	21.3	21.5	22.3	21.7	22.6	22.6	21.6	22.2	22.1	22.0	21.4	21.2	21.5
5. *Chioglossa lusitanica*	396	432	460	428	—	18.8	19.2	19.1	19.8	21.3	20.6	21.3	20.9	20.9	20.9	21.1	20.8	20.6	20.3	22.1
6. *Mertensiella caucasica*	424	418	471	441	378	—	20.2	20.1	19.8	21.0	20.5	20.1	19.9	20.2	20.9	20.9	20.8	19.6	20.5	21.0
7. *S. salamandra* Spain	401	418	423	439	385	407	—	3.7	7.4	15.0	13.6	14.3	14.0	14.1	14.6	14.4	14.3	14.2	14.1	15.0
8. *S. salamandra* Ukraine	408	417	415	428	384	405	75	—	7.5	14.6	13.5	14.2	14.0	14.1	14.2	14.2	14.2	13.5	13.9	14.5
9. *S. infraimmaculata*	408	413	430	433	398	399	150	152	—	14.1	13.5	14.0	13.2	14.1	14.4	14.3	14.3	13.7	13.6	14.6
10. *M. l. antalyana*	430	407	433	449	429	422	303	295	284	—	7.8	9.8	9.0	8.9	9.2	9.1	9.2	9.6	9.5	10.0
11. *M. l. billae*	411	408	430	437	414	412	274	271	271	157	—	8.2	7.6	7.9	8.0	7.9	7.8	8.4	7.6	8.7
12. *M. l. atifi* Türbelinaz	431	415	441	454	427	404	287	285	281	197	166	—	3.1	9.6	10.0	10.1	10.0	9.3	8.9	9.5
13. *M. l. atifi* Fersin Köyü	430	409	433	454	420	401	282	281	266	182	153	63	—	8.8	9.3	9.4	9.3	9.0	8.5	9.2
14. *M. l. flavimembris*	419	402	431	434	420	406	284	284	285	180	160	193	178	—	9.7	9.5	9.5	9.1	8.4	9.9
15. *M. l. fazilae* Gökceovacik	423	434	435	447	420	420	294	286	290	186	161	202	188	195	—	1.0	1.2	9.4	8.9	9.9
16. *M. l. fazilae* Domuz Is.	422	429	437	445	424	421	290	286	289	184	159	203	189	191	20	—	0.7	9.0	8.6	9.5
17. *M. l. fazilae* Tersane Is.	418	427	434	442	419	418	288	286	288	186	157	202	188	192	24	14	—	8.9	8.5	9.6
18. *M. l. basoglui*	414	407	431	430	414	394	285	272	277	193	170	188	182	184	189	181	180	—	4.2	6.2
19. *M. l. finikensis*	405	412	424	427	408	412	283	279	274	191	153	179	172	169	179	173	171	84	—	5.6
20. *M. l. luschani*	426	416	450	433	445	423	302	291	294	201	175	192	185	200	200	192	193	125	113	—

Note. Percentage sequence divergence is shown above the diagonal and number of base substitutions between sequences (2019 aligned base positions) is shown below the diagonal. Taxa are abbreviated with *S.* representing *Salamandra* and *M. l.* representing *Mertensiella luschani.*

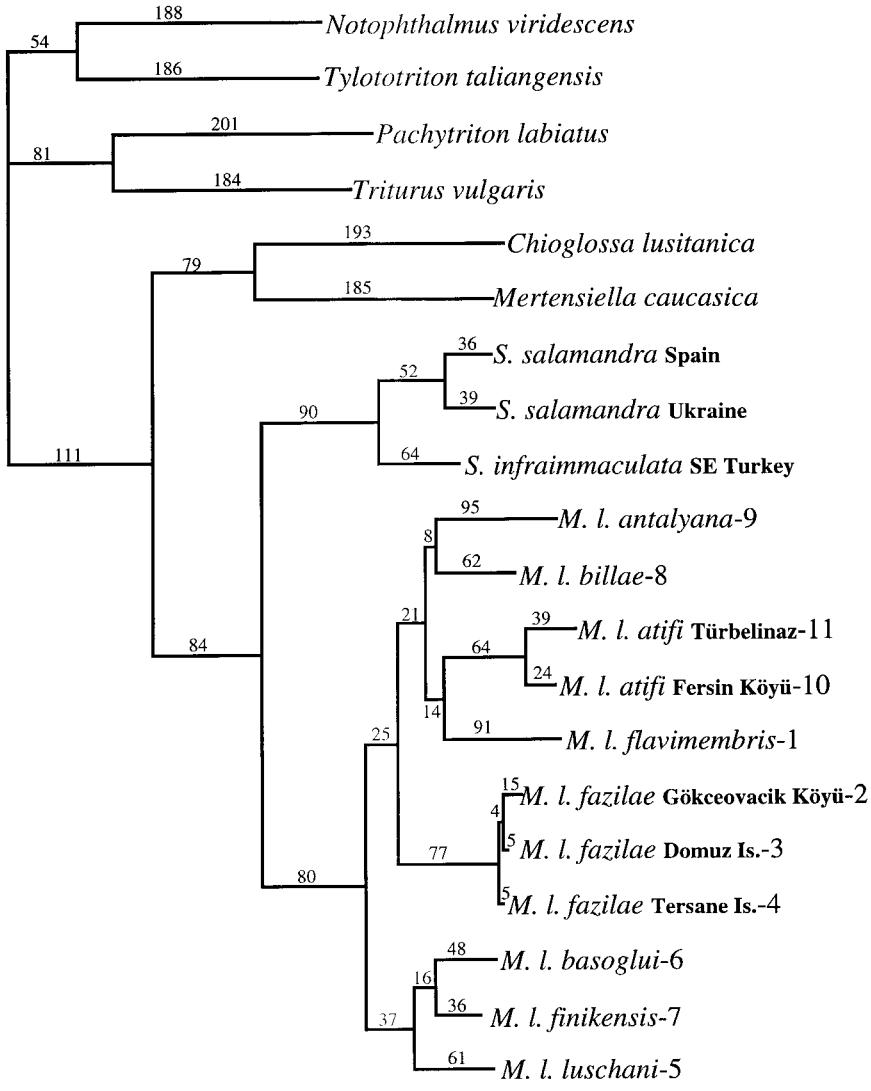


FIG. 5. Phylogram of the single most parsimonious tree showing branch lengths optimized with accelerated transformations. Relatively even branches are observed among "true" salamanders, implying even rates of change through time among lineages. Short internal branch lengths (8–37 steps) separate the six lineages of *Mertensiella luschani. M. luschani* populations are numbered as in Fig. 2 and under Materials and Methods.

be placed in the genus *Salamandra.* In addition, the morphologies of *M. luschani* and *Salamandra* are quite similar, in contrast with the morphologies of *M. caucasica* and *Chioglossa,* which are more different (Özeti, 1967; Wake and Özeti, 1969).

Color variation in different populations of *M. luschani* (Plate 1) has attracted considerable attention in recent years and is used as a major diagnostic character for the recognition of distinct taxonomic units (Baran and Ücüncü, 1994; Mutz and Steinfartz, 1995; Polymeni, 1994). Nine subspecies of *M. luschani* are currently recognized. Our sampling of 11 populations belonging to eight of the nine subspecies identifies six major groups. Pairwise sequence divergence between mitochondrial DNA haplotypes from any two of these groups is exceptionally high, greatly exceeding the amount of divergence expected to occur within a single species (Table 6). Comparisons of this same segment of mitochondrial DNA in sister taxa recognized as species in other amphibians and reptiles are lower (4.2–6.9%; Macey *et al.,* 1998a,b, 1999a,b, 2001) than any pairwise comparison between the six lineages of *M. luschani* (7.6–10.1%). *M. luschani* populations are isolated from each other on limestone outcrops, and movement between populations is highly unlikely.

The six lineages of *M. luschani* are diagnosable via color patterns (Plate 1) and probably represent monophyletic groups (Baran and Ücüncü, 1994). Therefore, applying the phylogenetic species concept (Cracraft, 1989), it is likely that six species could be recognized among Anatolian populations. These six taxa, if placed in the genus *Salamandra,* would include (I) *Salaman-*

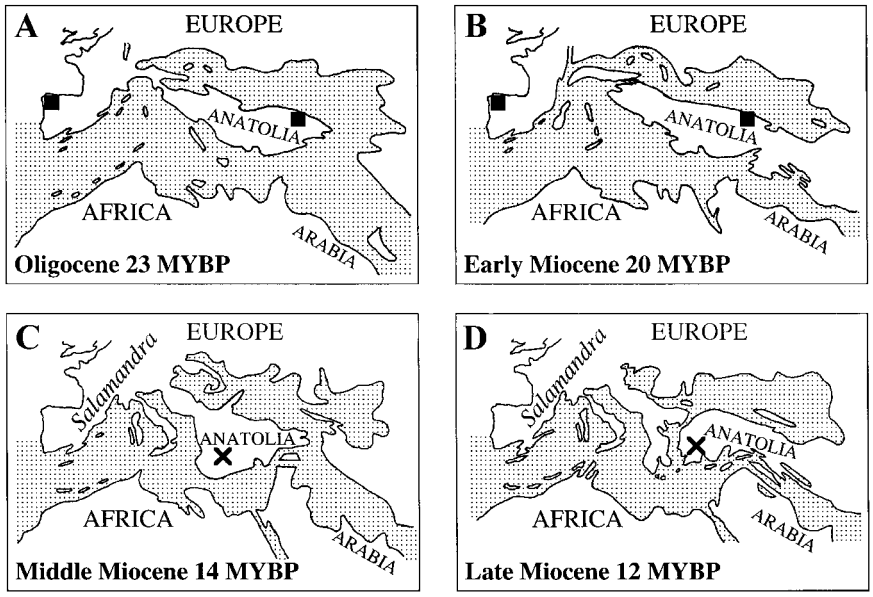


FIG. 6. Paleoreconstruction of the Mediterranean Basin illustrating the closing of the Tethys Sea (stippling) and suggested vicariant events fragmenting major lineages of "true" salamanders (maps after Steininger and Rogl, 1984). (A) In the Oligocene (23 MYBP) central Europe and Anatolia are connected by continuous land. Squares show the current distribution of *Chioglossa* in the Iberian Peninsula and *M. caucasica* in northwestern Anatolia. (B) During the Early Miocene (20 MYBP) central Europe is separated from Anatolia by a seaway, which may be responsible for the divergence between *Chioglossa* and *M. caucasica*. (C) In the Middle Miocene (14 MYBP) central Europe and Anatolia are connected by continuous land. *Salamandra* is currently distributed from the Iberian Peninsula through central and southern Europe. "X" denotes the current distribution of *M. luschani* in southwestern Anatolia. (D) During the Late Miocene (12 MYBP) central Europe is separated from Anatolia by a seaway and this separation may be responsible for the divergence between *Salamandra* and *M. luschani*.

dra flavimembris, (II) *S. fazilae*, (III) *S. luschani*, (IV) *S. billae*, (V) *S. antalyana*, and (VI) *S. atifi* (Fig. 7). However, further evidence of differentiation using additional molecular markers is needed before concluding that these six taxa represent distinct phylogenetic species.

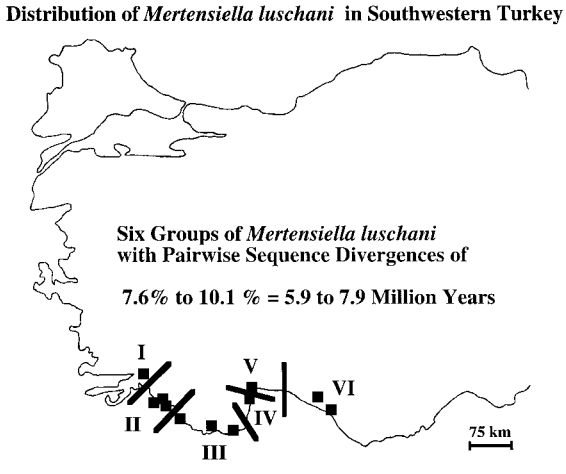


FIG. 7. Distribution of the six mitochondrial lineages of *Mertensiella luschani* identified in this study. Sequence divergences between pairs of populations belonging to different lineages range from 7.6 to 10.1%. Bars delineate the five breaks between the six lineages: (I) *M. l. flavimembris;* (II) *M. l. fazilae;* (III) *M. l. luschani, M. l. basoglui,* and *M. l. finikiensis;* (IV) *M. l. billae;* (V) *M. l. antalyana;* and (VI) *M. l. atifi*. Using the pairwise rate of 1.28% divergence between lineages per million years, these lineages are estimated to have diverged between 5.9 and 7.9 MYBP.

Genetic Diversity in Tectonic Collision Zones

The southern margin of Anatolia represents a collision zone between ancient Gondwanan tectonic fragments that are sandwiched between Laurasian plates to the north and the Gondwanan fragment of Arabia to the south. The intense Miocene mountain building in Anatolia results from the Arabian collision. The high levels of genetic diversity detected in *M. luschani* across a narrow region (~350 km) of this tectonic collision zone may represent a common pattern in geologically active areas. Conservation efforts should be focused in collision zones for two reasons: (1) tectonic plates introduce new faunal elements (Macey *et al.*, 2000), potentially increasing diversity, and (2) mountain building in suture zones is important in causing high levels of genetic diversity among faunal elements. Future studies assessing genetic variation in taxa that occur in geologically active areas will be important in testing this hypothesis.

APPENDIX

Alternative hypotheses used in Wilcoxon signed-ranks tests (Felsenstein, 1985; Templeton, 1983).

TABLE 6

Comparative Pairwise Sequence Divergences between Species of Amphibians and Reptiles

Taxa	Family	Taxa compared	Pairwise sequence divergences (%)	Reference
Bufo	Bufonidae	*B. andrewsi* and *B. gargarizans*	6.0–6.9	Macey *et al.* (1998b)
Rana	Ranidae	*R. aurora, R. cascadae,* and *R. muscosa*	7.0–8.4	Macey *et al.* (2001)
Laudakia	Agamidae	*L. caucasia* and *L. erythrogastra*	4.2–5.3	Macey *et al.* (1998a)
Teratoscincus	Gekkonidae	*T. przewalskii* and *T. roborowskii*	6.5	Macey *et al.* (1999b)
Elgaria	Anguidae	*E. kingii* to *E. multicarcinata/E. panamintina/E. paucicarinata*	4.8–5.9	Macey *et al.* (1999a)

Note. Sequence divergences are calculated for the same segment of mitochondrial DNA spanning from the ND1 gene to the COI gene. Bufonid frogs include only the first half of this segment, spanning from the ND1 gene to the ND2 gene.

Lengths of trees are given in parentheses. Numbers refer to the following taxa: (1) *Notophthalmus viridescens,* (2) *Tylototriton taliangensis,* (3) *Pachytriton labiatus,* (4) *Triturus vulgaris,* (5) *Chioglossa lusitanica,* (6) *Mertensiella caucasica,* (7) *Salamandra salamandra* Spain, (8) *Salamandra salamandra* Ukraine, (9) *Salamandra infraimmaculata* Turkey, (10) *Mertensiella luschani antalyana,* (11) *M. l. billae,* (12) *M. l. atifi* Türbelinaz, (13) *M. l. atifi* Fersin Köyü, (14) *M. l. flavimembris,* (15) *M. l. fazilae* Gökceovacik Köyü, (16) *M. l. fazilae* Domuz Is., (17) *M. l. fazilae* Tersane Is., (18) *M. l. basoglui,* (19) *M. l. finikensis,* and (20) *M. l. luschani.*

The most parsimonious tree derived by constraining the "true" salamanders not to form a monophyletic group (length of 2679): A1. (1, (2, (((3, 4), (5, 6)), (((7, 9), 8), ((((10, 11), ((12, 13), 14)), ((15, 16), 17)), ((18, 19), 20)))))).

The 16 equally most parsimonious trees derived by constraining the genus *Mertensiella* to form a monophyletic group (length of 2721): B1. (1, ((2, (((5, (((((10, 18), (19, 20)), 17), (14, (15, 16))), ((11, 13), 12))), ((7, 9), 8)), 6)), (3, 4))). B2. (1, ((2, (((5, ((((10, (19, 20)), (17, 18)), (14, (15, 16))), ((11, 13), 12))), ((7, 9), 8)), 6)), (3, 4))). B3. (1, ((2, (((5, (((((10, 18), (19, 20)), 17), (14, (15, 16))), (11, (12, 13)))), ((7, 9), 8)), 6)), (3, 4))). B4. (1, ((2, (((5, ((((10, (19, 20)), (17, 18)), ((11, 13), 12)), (14, (15, 16)))), ((7, 9), 8)), 6)), (3, 4))). B5. (1, ((2, (((5, ((((10, (14, (15, 16))), (19, 20)), (17, 18)), ((11, 13), 12))), ((7, 9), 8)), 6)), (3, 4))). B6. (1, ((2, (((5, (10, (((((11, 13), 12), (14, (15, 16))), (17, 18)), (19, 20)))), ((7, 9), 8)), 6)), (3, 4))). B7. (1, ((2, (((5, ((((10, (19, 20)), (17, 18)), (14, (15, 16))), (11, (12, 13)))), ((7, 9), 8)), 6)), (3, 4))). B8. (1, ((2, (((5, (10, (((((11, 13), 12), (17, 18)), (19, 20)), (14, (15, 16))))), ((7, 9), 8)), 6)), (3, 4))). B9. (1, ((2, (((5, (((((10, (14, (15, 16))), (19, 20)), 17), 18), ((11, 13), 12))), ((7, 9), 8)), 6)), (3, 4))). B10. (1, ((2, (((5, ((((10, (14, (15, 16))), (19, 20)), (17, 18)), (11, (12, 13)))), ((7, 9), 8)), 6)), (3, 4))). B11. (1, ((2, (((5, (10, ((((11, (12, 13)), (14, (15, 16))), (17, 18)), (19, 20)))), ((7, 9), 8)), 6)), (3, 4))). B12. (1, ((2, (((5, (10, ((((11, 13), 12), (17, (18, (19, 20)))), (14, (15, 16))))), ((7, 9), 8)), 6)), (3, 4))). B13. (1, ((2, (((5, (10, (((((11, (12, 13)), 18), 17), (19, 20)), (14, (15, 16))))), ((7, 9), 8)), 6)), (3, 4))). B14. (1, ((2, (((5, (10, ((((((11, 13), 12), 17), 18), (19, 20)), (14, (15, 16))))), ((7, 9), 8)), 6)), (3, 4))). B15. (1, ((2, (((5, (((((10, (14, (15, 16))), (19, 20)), 17), 18), (11, (12, 13)))), ((7, 9), 8)), 6)), (3, 4))). B16. (1, ((2, (((5, (10, (((11, (12, 13)), (17, (18, (19, 20)))), (14, (15, 16))))), ((7, 9), 8)), 6)), (3, 4))).

The most parsimonious tree derived by constraining *Mertensiella luschani* and *Salamandra* not to form a monophyletic group (length of 2689): C1. (1, (2, ((3, 4), (((5, 6), ((7, 9), 8)), ((((10, 11), ((12, 13), 14)), ((15, 16), 17)), ((18, 19), 20)))))).

The two most parsimonious trees derived by constraining *Mertensiella caucasica* and *Chioglossa* not to form a monophyletic group (length of 2674): D1. (1, (2, ((3, 4), ((5, (((7, 9), 8), ((((10, 11), ((12, 13), 14)), ((15, 16), 17)), ((18, 19), 20)))), 6)))). D2. (1, ((2, (3, 4)), ((5, (((7, 9), 8), ((((10, 11), ((12, 13), 14)), ((15, 16), 17)), ((18, 19), 20)))), 6))).

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