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A molecular phylogeny of 'true' salamanders (family Salamandridae) and the evolution of terrestriality of reproductive modes

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Abstract

Key innovations enable species to conquer new habitats. Within the family Salamandridae, particular adaptations to terrestrial life, such as the anatomy and physiology of the feeding apparatus, courtship behaviour and in some cases viviparity, allowed the 'true' salamanders (genera *Chioglossa*, *Mertensiella*, *Salamandra*) to shift from a semi-aquatic to a more terrestrial life cycle. We sequenced 423 base pairs of the 16S RNA gene of the mitochondrial DNA for all species of the 'true' salamanders. Based on the resulting phylogeny we discuss the evolution of terrestrial reproductive modes within this species group. We especially tested two hypotheses of monophyletic origin of specific adaptations to terrestriality within the 'true' salamanders: *Mertensiella caucasica*/*Mertensiella luschani*, whose congeneric relationship has already been questioned on the basis of morphological, behavioural and molecular data, and *Salamandra atra*/*Salamandra lanzai*, the two species of Alpine salamanders, which are characterized by melanism and matrotrophic viviparity. We tested alternative tree topologies and included additional published and unpublished data on morphology, allozyme polymorphisms, and further mtDNA sequences. Maximum parsimony and maximum likelihood analyses always rejected the hypothesis of monophyly of the two *Mertensiella* species. Although data on courtship behaviour of 'true' salamanders indicate the loss of a symplesiomorphic tail projection in *Salamandra* and *Chioglossa*, the most parsimonious explanation may at present be a convergent evolution of the projection as indicated by recent histological studies. Although our DNA sequence and additional allozyme data suggest that *S. atra* and *S. lanzai* do not form a monophyletic group despite their geographic proximity and shared traits, we cannot reject their monophyly. Using the flooding of the Strait of Gibraltar five million years ago for the separation of African and European species, a molecular 16S RNA clock was calibrated with 0.7% total sequence divergence per million years. Estimated times of divergence for further evolutionary splits within 'true' salamanders coincide with paleogeographical data.

Key words: Salamandridae – 'true' salamanders – sequencing – 16S RNA – mtDNA – molecular phylogeny – terrestriality – paleobiogeography

Introduction

Molecular data suggest that the earliest phylogenetic split within the Salamandridae separated the 'true' salamanders (genera *Chioglossa*, *Mertensiella*, and *Salamandra*) from the newts (genera *Cynops*, *Echinotriton*, *Euproctus*, *Neurergus*, *Notophthalmus*, *Pachytriton*, *Paramesotriton*, *Pleurodeles*, *Salamandrina*, *Taricha*, *Triturus* and *Tylototriton*; Titus and Larson 1995). Morphological adaptations to terrestrial life are characteristic of the 'true' salamanders and are exemplified by the functional morphology of the feeding apparatus (Özeti and Wake 1969; Wake 1982; Miller and Larsen 1990), cloacal anatomy (Wahlert 1953; Sever 1992), reproductive biology (Tarkhnishvili 1994) and a specialized courtship behaviour (Salthe 1967).

Geographically, the three genera of 'true' salamanders are restricted to the region around the Mediterranean Sea. The monotypic genus *Chioglossa* occurs only on the Iberian Peninsula. Two species are described for the genus *Mertensiella*: *Mertensiella caucasica* from the Caucasus Mountains, and *Mertensiella luschani* from the coastal mountain ridges of Lycia in southern Asia Minor and some Greek islands (e.g. Karpathos). Six species are regarded as valid in the genus *Salamandra* (recently reviewed by Veith 1994): *Salamandra algira* (Northern Africa), *Salamandra corsica* (Corsica), *Salamandra infraimmaculata* (Asia Minor and Near East) and *Salamandra salamandra* (from the Iberian Peninsula to Greece, including large parts of central and north-eastern Europe) are regarded as the distinct species group of 'fire salamanders'. They are distinguished from the 'Alpine salamanders', *Salamandra atra* (almost the whole Alpine ridge including an isolated population in the Dinarian Alps of Serbia and Albania) and *Salamandra lanzai* (Monviso Massif, Italian Alps) by coloration and reproductive strategy. The Alpine salamanders are melanistic (with the exception of *Salamandra atra aurorae* from Bosco del Dosso, Alps, which shows differing degrees of yellowish pigmentation on the cover of head and trunk) and show matrotrophic viviparity (*sensu* Blackburn 1995). Substantial provision of extra-vitelline nutrients allow females to give birth to fully developed juveniles. In fire salamanders, which are black with yellow dots or stripes, females mainly give birth to larvae due to a provision of nutrients via the yolk (lecitotrophic viviparity).

Shared derived traits characterize taxa as members of monophyletic entities, originating from a common ancestor (Hennig 1950). This is the major principle of cladistic analysis. However, it is often difficult to decide whether a morphological character is synapomorphic. One criterion may be uniqueness among all members of a higher taxon (outgroup comparison; see Mayr and Ashlock 1991). This holds for a classical herpetological example of a synapomorphic trait that characterizes the two species of the genus *Mertensiella* (Salamandridae). Males of both species possess cutaneous papillae projecting dorsally over the base of the tail (henceforth 'tail projection') which they use for female stimulation and courtship synchronization (Schultschik 1994). No such organ is found in any other species of the Urodela and a similar projection is mentioned only for the plethodontid salamander *Eurycea multiplicata* (Noble 1931). Therefore, *Mertensiella* has been accepted as a monophyletic genus, although morphological similarities of both *Mertensiella* species to other species of the Salamandridae are evident (see below).

Based on 1010 molecular characters (DNA sequences of the mitochondrial genes 16S, 12S and Val-tRNA genes) Titus and Larson (1995) questioned the monophyly of *Mertensiella*. Their most parsimonious tree placed *M. luschani* as a sister taxon of the genus *Salamandra* (represented by *S. atra*), whereas *M. caucasica* was suggested to be the sister taxon of *Chioglossa*

lusitanica. However, they also obtained a tree that supported monophyly of the genus *Mertensiella*, which was not significantly longer than the most parsimonious one.

Within the genus *Salamandra*, a second case of monophyly is questioned. Two presumably independent characters, melanism and intra-uterine completion of larval development, are typical for *S. atra* and *S. lanzai*. Olivieri (1991) studied allozyme polymorphisms of numerous populations of *Salamandra*, including *S. atra* and *S. lanzai*. From her phenetic analysis she concluded that *S. lanzai* and *S. corsica* are sister taxa, thus contradicting the hypothesis of monophyly of the two Alpine species (see Grossenbacher 1994). However, Joger and Steinfartz (1994) performed a cladistic re-analysis of Olivieri's (1991) data and derived a tree with *S. atra* and *S. lanzai* forming a clade. Finally, Veith (1996) used a maximum parsimony approach to show that Olivieri's (1991) data are insufficient in deciding this controversy.

In the present paper we provide new data that will contribute to both debates of monophyly within 'true' salamanders using 16S mtDNA sequences as well as additional published and unpublished morphological and molecular data. In contrast to previous authors we use a complete set of 'true' salamander species. Based on the systematic relationships of 'true' salamanders we investigate hypotheses about historic vicariance events and the evolution of adaptations of reproduction to terrestrial life.

Material and methods

Specimens examined

DNA sequences of the mitochondrial 16S RNA gene were obtained from samples of *Chioglossa lusitanica* (two specimens from Portugal), *Mertensiella caucasica* Waga, 1876 (two specimens, Caucasus Mountains, Georgia), *M. luschani* Steindachner, 1891 (two specimens, Antalya, Turkey), *Salamandra algira* Bedriaga, 1883 (one specimen, Algeria), *S. atra aurorae* Trevisan, Pederzoli-Trevisan and Callegarini, 1982 (one specimen, Bosco del Dosso, Italy), *S. atra atra* Laurenti, 1768 (one specimen, Austria), *S. corsica* Savi, 1838 (two specimens, Corsica, France), *S. infraimmaculata* Martens, 1885 (five specimens, Tel Dan and Mt. Meron, Israel), *S. lanzai* Nascetti, Andreone, Capula and Bullini, 1988 (one specimen, Monviso Massif, Italy) and *S. salamandra* Linnaeus, 1758 (five specimens, Paikon Mts., Greece). The salamandrid species *Salamandrina terdigitata* Lacépède, 1788 (one specimen, Italy), *Cynops orientalis* David, 1871 (one specimen, China) and *Neurergus strauchii* Steindachner, 1887 (one specimen, Turkey) were included for outgroup comparison.

Amplification and sequencing

DNA was extracted either from blood using the Qiagen extraction kit, or from liver or muscle using standard phenol extraction techniques. Using the versatile primers 16SA (light chain) and 16SB (heavy chain) (Palumbi et al. 1991), we amplified a piece of about 630 base pairs (bp) via the polymerase chain reaction (PCR). The PCR technique of Gyllensten and Ehrlich (1988) was used to produce single-stranded DNA (asymmetric PCR with 16SB as the limiting primer). Single-stranded PCR products were purified in Millipore 30 000 MW spin columns. We sequenced up to 423 bp per specimen according to the sequencing protocol of Kocher et al. (1989). This segment is homologous to bp positions 4106–4538 of the *Xenopus* mitochondrial genome (Roe et al. 1985). Sequences will be available in GenBank.

Sequence analysis

Sequences were aligned using CLUSTAL W (Higgins and Sharp 1993). Alignments were subsequently adjusted manually by taking secondary structural models (Orti et al. 1996) into consideration.

Maximum parsimony (MP) trees were calculated under three models of character substitution: Jukes and Cantor's (1969) one parameter model, Kimura's (1980) two-parameter model, and a specific TS : TV weighting model for conserved (1.7 : 1) and variable (1.3 : 1) regions according to their intrinsic, overall pairwise TS : TV ratios (data not shown). We regarded those parts of the gene as conserved that showed no or only minor sequence difference when aligned to the *Xenopus* 16S sequence (Roe et al. 1985), thus allowing us to detect regions that are evolutionarily constrained. Gaps were treated as providing phylogenetic signal; they were rare among ingroup taxa and never exceeded two bp in length. For confirmation of tree topologies, the distance-based neighbour-joining method (NJ; Saitou and Nei 1987) was performed using the Kimura distance (Kimura 1980) under the two-parameter substitution model. This combination proved to be consistent under a wide variety of conditions in simulation studies (Huelsenbeck and Hillis 1993). One hundred bootstrap replicates (Felsenstein 1985) were run for all MP and NJ analyses. The phylogenetic programs PAUP (Swofford 1993; Version 3.1.1) and PHYLIP (Felsenstein 1993; Version 3.5c) were used for the analyses.

Trees representing alternative hypotheses of clade formation within the 'true' salamanders were tested for significant length (least number of steps required to explain the observed data set) or log likelihood differences. For the first approach we used Felsenstein's (1993) modification of Templeton's (1983) test for comparing trees under the maximum parsimony criterion as implemented in PHYLIP. It uses the mean and variance of step differences between trees, taken across sites. It is similar to the test for log likelihood differences of trees constructed under the maximum likelihood criterion (Kishino and Hasegawa 1989). We performed the latter using the program MOLPHY (version 2.2) of Adachi and Hasegawa (1992). These tests produced slightly different results, therefore test statistics are given for both.

Available data sets

In addition to our own 16S sequence data (OWN16S), we included the 12S and 16S sequence data of Titus and Larson (1995; TL95SEQ) that did not overlap with our sequence data. They are homologous to bp positions 2319–3334 of the *Xenopus* mitochondrial genome (Roe et al. 1985). Morphological data as coded by Titus and Larson (1995; TL95MORPH) and two sets of allozyme data (VS95, Veith and Steinfartz unpublished; OLI91, Olivieri 1991) were also analysed. Availability of different character sets for each species is indicated in Table 1. Allozyme data were binary coded. We manually realigned the Titus and Larson (1995) DNA sequences with special emphasis on the ingroup taxa. This allowed us to include those portions of the sequences that were excluded as bad alignments by Titus and Larson (1995) since they analysed a phylogenetically more widespread set of taxa of the family Salamandridae.

None of the additional data sets covered all species of our sequence analysis (Table 1). Therefore, depending on the tested hypothesis, only subsets of the additional data were analysed in combination with our 16S sequences. Additional data for *S. atra* and *S. salamandra* were analysed in combination with our *S. a. atra* and *S. salamandra*-B sequences.

Results

Sequence divergence

The sequence covered two large loops of the 16S RNA gene, one of which was extremely variable. Altogether, three variable (23–76, 110–182, 220–288) and four constant regions (1–22, 77–109, 183–219, 289–423) were designated.

For our *S. salamandra* and *S. infraimmaculata* samples, two different haplotypes were found with a sequence divergence of 0.5% and 1.0%, respectively (Table 2). The two subspecies of *S. atra* did not show any sequence difference. However, there were minor differences at positions 424–500 (they are not included in the analyses because we could sequence them only in a few specimens).

The average sequence divergence between species of the genus *Salamandra* was 4.5%, whereas between members of the three

Table 1. Data sets used in the analyses

Data set *n* characters data type data coding	OWN16S 423 DNA multistate	TL95SEQ 1012 DNA multistate	TL95MORPH 48 morphology multistate	VS95 75 allozymes bin.state	OLI91 133 allozymes bin.state
Ingroup taxa					
S. algira	X	–	–	–	X
S. a. atra	X	X	X	–	X
S. a. aurorae	X	–	–	–	X
S. corsica	X	–	–	–	X
S. infraimmaculata	X	–	–	–	X
S. lanzai	X	–	–	–	X
S. salamandra	X	X	X	X	X
M. luschani	X	X	X	X	–
M. caucasica	X	X	X	X	–
C. lusitanica	X	X	X	X	–
Outgroup taxa					
Salamandrina	X	X	X	X	–
Neurergus	X	X	X	X	–
Cynops	X	X	X	X	–

OWN16S, own 16S sequence data (423 bp); TL95SEQ, 16S and 12S sequence data of Titus and Larson (1995); TL95MORPH, morphological data of Titus and Larson (1995); VS95, unbublished allozyme data of Veith and Steinfartz; OLI95, allozyme data of Olivieri (1991).

Table 2. Number of base substitutions (below diagonal) and percentage of sequence divergence (above diagonal) for 423 bp of the 16S RNA gene

	Taxon	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
(1)	*S. algira*		4.5	4.5	4.7	5.5	5.0	3.5	4.5	3.5	8.7	11.3	11.8	11.3	13.9	17.5
(2)	*S. a.atra*	19		0.0	6.1	4.5	4.0	3.3	4.7	4.3	9.5	11.8	12.5	12.3	15.1	16.5
(3)	*S. a.aurorae*	19	0		6.1	4.5	4.0	3.3	4.7	4.3	9.5	11.8	12.5	12.3	15.1	16.5
(4)	*S. corscia*	20	26	26		6.7	6.6	5.2	2.9	4.5	11.3	12.8	13.7	13.7	15.8	18.0
(5)	*S. infraimmaculata*	23	19	19	28		1.0	5.0	5.3	5.0	9.3	11.4	12.1	12.4	15.2	18.5
(6)	*S. infraimmaculata* B	21	17	17	28	4		4.5	5.5	4.5	8.7	11.6	12.1	12.3	15.1	18.3
(7)	*S. lanzai*	15	14	14	22	21	19		3.9	3.1	9.0	10.9	11.6	11.1	14.7	17.0
(8)	*S. salamandra* A	17	18	18	11	20	21	15		0.5	11.6	10.5	12.1	12.4	14.5	16.8
(9)	*S. salamandra* B	15	18	18	19	21	19	13	2		9.9	10.4	11.3	12.3	14.2	17.3
(10)	*M. luschani*	37	40	40	48	39	37	38	44	42		12.3	14.2	13.0	15.8	17.8
(11)	*M. caucasica*	48	50	50	54	48	49	46	40	44	52		10.6	13.9	16.3	18.3
(12)	*C. lusitanica*	50	53	53	58	51	51	49	46	48	60	45		15.4	18.2	19.5
(13)	*Cynops*	48	52	52	58	52	52	47	47	52	55	59	65		12.1	17.0
(14)	*Neurergus*	59	64	64	67	64	64	62	55	60	67	69	77	51		19.5
(15)	*Salamandrina*	70	66	66	72	74	73	68	64	69	71	73	78	68	78	

genera an average of 11.2% was found (Table 3). Between 'true' salamanders and newts the average sequence divergence was 16.6%.

Table 3. Percentage of sequence divergence at 423 sites of the mitochondrial 16S RNA gene between taxa of 'true' salamanders on different taxonomical levels; only one haplotype per polymorphic species was used (*S. a. aurorae*, *S. infraimmaculata* B, *S. salamandra* B) to avoid biased averages; *n* = number of pairwise comparisons

Taxonomical level	n	min	mean	max	SD
Within *Salamandra*	15	3.1	4.5	6.7	1.0
Within 'true' salamanders	21	10.0	11.2	14.2	1.5
Between 'true' salamders and newts	18	11.1	16.6	18.5	1.6

Phylogeny within 'true' salamanders

Concerning the relative positions of the two *Mertensiella* species, *C. lusitanica* and the genus *Salamandra*, the tree topologies calculated under different algorithms and TS:TV weighting regimes were concordant. *Chioglossa lusitanica* and *M. caucasica* always appeared to be sister taxa with bootstrap values between 93 and 98% (Fig. 1). Depending on which *Salamandra* species was included in a four-taxon analysis of monophyly of *Mertensiella* (*M. caucasica*, *M. luschani*, *C. lusitanica* and *Salamandra*), the bootstrap support for polyphyly/paraphyly of *Mertensiella* was between 93 and 98% (average: 95.5%). The hypothesis that *M. caucasica* and *M. luschani* are sister-taxa was supported by bootstrap values between 1 and 3% with an average of 2% (hypothesis #1 in Table 4). However, whether *Salamandra*, *M. luschani* or a clade formed by both taxa are the sister taxon/clade of *M. caucasica* and *C. lusitanica* still remains

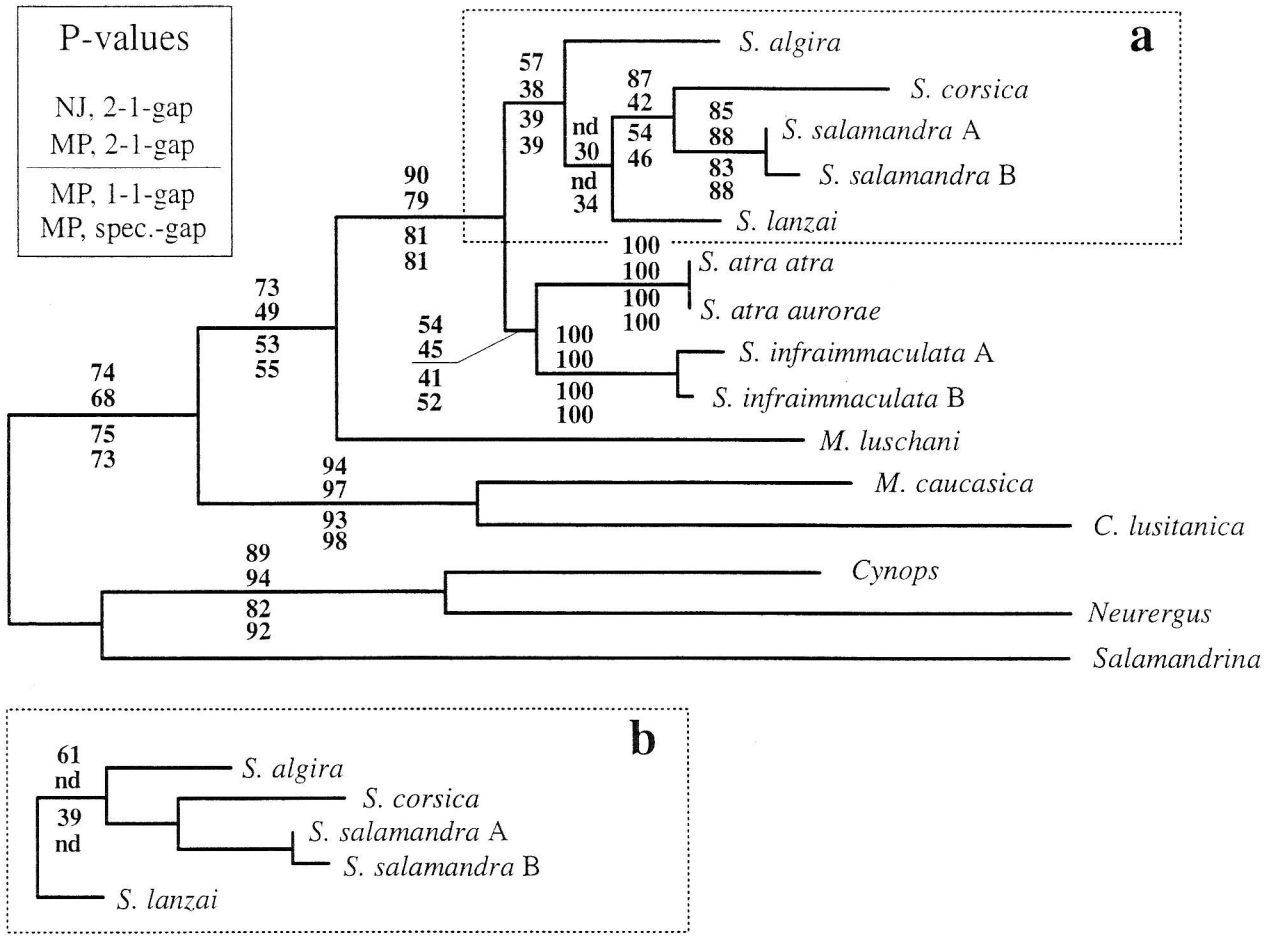

Fig. 1. Hypotheses of 'true' salamander phylogeny using 423 bp of the 16S mtDNA; bootstrap-values are given for different tree algorithms (neighbour joining = NJ, maximum parsimony = MP) and for different weightings of transitions and transversions under MP (2–1-gap, 1–1-gap, specific weighting); an alternative tree topology within *Salamandra* is given in the insert

Table 4. Bootstrap support for different hypotheses of monophyly within 'true' salamanders. 100 bootstrap replicates were run in each MP analysis. A: Data set OWN16S, using different *Salamandra* species as ingroup; B: different data sets and their combinations. TS : TV ratio for sequence data = 2 : 1, character states of other data unordered; *M. luschani* (*Ml*), *M. caucasica* (*Mc*), *C. lusitanica* (*Cl*) and one *Salamandra* (*S*) species each were tested; bootstrap values are given for different clades of species; outgroups: *Salamandrina* in A, *Cynops*, *Salamandrina* and *Neurergus* in B

	Hypothesis									
	1 *Ml/Mc*	2 *S/Cl*	3 *S/Ml*	4 *Mc/Cl*	5 *S/Mc*	6 *Cl/Ml*	7 *S/Ml/Mc*	8 *Ml/Mc/Cl*	9 *S/Ml/Cl*	10 *S/Mc/Cl*
A										
algira	1	1	76	98	0	0	1	10	0	13
atra	3	1	35	95	0	0	0	50	2	16
corsica	2	0	39	97	<1	1	2	33	0	25
infraimmaculata (B)	2	2	74	95	0	0	2	10	2	15
lanzai	2	2	44	95	1	<1	1	18	1	37
salamandra (B)	2	4	24	93	1	2	2	12	0	63
Mean	2.0	1.7	48.7	95.5	0.4	0.6	1.3	22.2	0.8	28.2
SD	0.6	1.2	19.6	1.6	0.4	0.7	0.7	14.8	0.9	17.6
B										
OWN16S	2	4	24	93	1	2	2	12	0	63
TL95SEQ	0	0	80	91	0	0	2	20	0	63
OWN16S + TL95SEQ	0	0	66	97	0	0	1	0	2	33
OWN16S + TL95SEQ + TL95MORPH	0	0	71	95	0	0	5	0	1	30
OWN16S + TL95SEQ + TL95MORPH + VS95	0	0	70	93	0	0	8	0	1	31

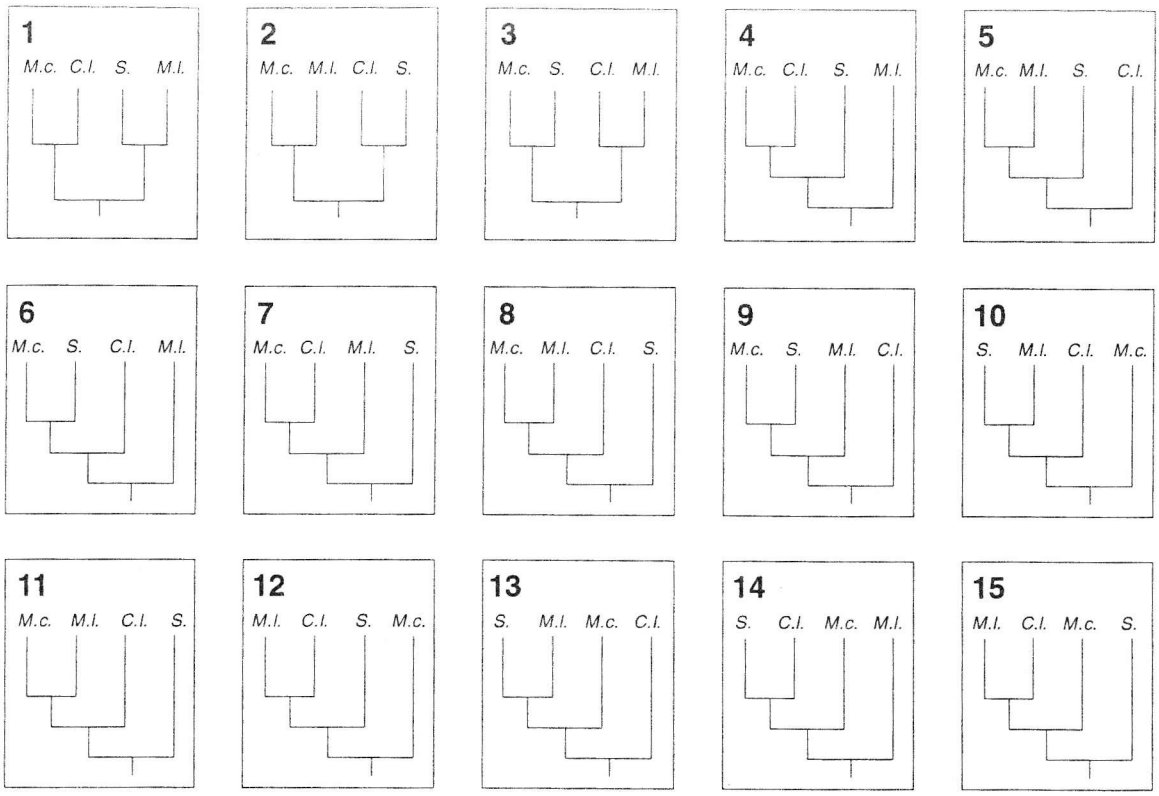

Fig. 2. Possible tree topologies of within-'true'-salamander evolution when applying the four-taxon case (*C. lusitanica* [*C.l.*], *M. caucasica* [*M.c.*], *M. luschani* [*M.l.*], genus *Salamandra* [*S.*])

unclear (average bootstrap support for these hypotheses: 28.2, 22.2 and 48.7%, respectively).

Inclusion of additional data sets into the analyses confirmed the strong support for the sister relationship of *M. caucasica* and *C. lusitanica* and weakened the support for monophyly of the genus *Mertensiella* (Table 4). The realignment of the Titus and Larson (1995) data increased their statistical significance for the lack of a sister relationship between the two *Mertensiella* species in comparison to their analysis.

If we consider the question of monophyly of *Mertensiella* species to be a four taxon case (*Salamandra*, *Chioglossa*, *M. luschani* and *M. caucasica* with *Cynops*, *Salamandrina* and *Neurergus* as outgroups), there are 15 possible tree topologies (Fig. 2). Tree #1 was the best under both the maximum parsimony and the maximum likelihood criteria. Only trees #4 and #7 were not significantly different in length and likelihood from tree #1(Table 5). All other trees were significantly less parsimonious or likely (henceforth 'worse') than tree #1. Hypotheses #4 and #7 show *M. caucasica* and *C. lusitanica* as sister-taxa as for hypothesis #1. Stepwise addition of further data sets (TL95SEQ, TL95MORPH and VS95) almost always showed tree #4 to be equally good as tree £1. In addition, tree £13 where *Salamandra* and *M. luschani* are sister taxa was not significantly worse than tree #1 under the maximum parsimony approach and, when including all available data, also under the maximum likelihood approach (Table 6).

Phylogeny within *Salamandra*

Monophyly of the *Salamandra* clade was supported by different analyses with bootstrap values in the range of 79–90% (Fig. 1). Two major clades consistently formed within the genus in all analyses: (*S. atra* + *S. infraimmaculata*) and (*S. algira* + *S. corsica* + *S. lanzai* + *S. salamandra*). The topology within the latter clade varied, depending on which phylogenetic method was used (Fig. 1). The highest bootstrap values were achieved when using the neighbour-joining algorithm, supporting a first split separating *S. atra* and *S. infraimmaculata* from the other species. Subsequently, *S. lanzai* and then *S. algira* separated from the remaining two, *S. corsica* and *S. salamandra*.

A comparison of seven different tree topologies within the genus *Salamandra*, representing the two alternative hypotheses in combination with biogeographically plausible scenarios of speciation, showed that none of the alternative topologies is significantly worse than the best tree under the maximum parsimony (tree #1) and the maximum likelihood (tree #7) approach, respectively (Table 7). The same holds when allozyme data (OLI95) are included. Therefore, none of these alternatives can be ruled out with currently available data.

Discussion

Nonmonophyly of *Mertensiella*

Using our 16S sequence data alone and in combination with additional data sets of mtDNA sequences, allozyme polymorphisms and morphological characters result in a tree that unambiguously contradicts the hypothesis of monophyly of the genus *Mertensiella*. Alternative tree topologies are significantly worse with the exception of those that show a relationship between *M. caucasica* and *C. lusitanica* as sister taxa. Therefore, *Mertensiella* may be either polyphyletic or paraphyletic.

This result supports previous hypotheses based on morphological, ecological, biochemical and molecular characters

Table 5. Comparison of all possible tree topologies within 'true' salamanders. *C. lusitanica*, *M. luschani*, *M. caucasica* and *Salamandra* using 423 bp of 16S RNA mtDNA sequences; tree numbers correspond to those in Fig. 2; topology within *Salamandra* and outgroups according to Fig. 1; tree #1 was used as null-hypothesis since it was the best tree under both the maximum parsimony (TS : TV = 1 : 1, gaps included) and the maximum likelihood criterion; tree topologies were tested for different step length using Felsenstein's (1993) modification of Templeton's (1983) and for log likelihood differences using Kishino and Hasegawa's (1989) tests; trees under the maximum parsimony approach were treated as different in length if their mean difference was more than 1.96 times their SD (standard deviation) different from the best tree (Felsenstein 1993); SE = standard error

	Maximum parsimony analysis				Maximum likelihood analysis			
tree #	steps	Δ steps	SD	sign. worse?	ln L	Δ ln L	SE	sign. worse?
1	286 (most parsimonious tree)				−2766.0 (best tree)			
2	298	12	4.70	Yes	−2791.5	−25.6	13.0	Yes
3	299	13	4.59	Yes	−2796.0	−30.0	11.7	Yes
4	288	2	2.45	No	−2768.5	−2.6	5.5	No
5	296	10	4.70	Yes	−2791.7	−25.7	12.3	Yes
6	299	13	4.36	Yes	−2795.0	−29.1	11.8	Yes
7	289	3	2.24	No	−2768.6	−2.6	5.4	No
8	299	13	4.59	Yes	−2792.3	−26.4	12.6	Yes
9	298	12	4.47	Yes	−2796.1	−30.2	11.1	Yes
10	294	8	3.47	Yes	−2782.8	−16.9	8.9	Yes
11	297	11	4.80	Yes	−2789.9	−24.0	12.8	Yes
12	298	12	4.70	Yes	−2793.2	−27.3	11.8	Yes
13	294	8	3.47	Yes	−2784.1	−18.2	8.2	Yes
14	296	10	4.70	Yes	−2790.9	−25.0	13.1	Yes
15	299	13	4.59	Yes	−2793.4	−27.5	12.1	Yes

Table 6. Comparison of all possible tree topologies within the 'true' salamanders, *M. luschani*, *M. caucasica*, *C. lusitanica* and *Salamandra* (*S. salamandra* B) using our own 16S data (1 = OWN16S) and additional data sets (2 = TL95SEQ, 3 = VS95, 4 = TL95MOPRPH); *Cynops*, *Neurergus* and *Salamandrina* were used as outgroups; test algorithms for maximum parsimony (MP) and maximum likelihood (ML) analyses according to Table 5; it is indicated whether the alternative tree topologies are (yes) or are not (no) significantly worse than the best tree (bt)

	[1]		[1, 2]		[1, 2, 3]		[1,2,3,4]	
Tree #	MP	ML	MP	ML	MP	ML	MP	ML
1	bt	bt	bt	bt	bt	bt	bt	bt
2	yes	yes	yes	yes	yes	yes	yes	yes
3	yes	yes	yes	yes	yes	yes	yes	yes
4	no	no	no	no	no	no	no	yes
5	yes	yes	yes	yes	yes	yes	yes	yes
6	yes	yes	yes	yes	yes	yes	yes	yes
7	no	no	yes	yes	yes	yes	yes	yes
8	yes	yes	yes	yes	yes	yes	yes	yes
9	yes	yes	yes	yes	yes	yes	yes	yes
10	yes	yes	yes	yes	yes	yes	yes	yes
11	yes	yes	yes	yes	yes	yes	yes	yes
12	yes	yes	yes	yes	yes	yes	yes	yes
13	yes	yes	no	yes	no	yes	no	no
14	yes	yes	yes	yes	yes	yes	yes	yes
15	yes	yes	yes	yes	yes	yes	yes	yes

(e.g. Bolkay 1928; Özeti and Wake 1969; Wake and Özeti 1969; Tarkhnishvili 1994; Titus and Larson 1995). Since the included characters are of different kinds (mitochondrial and nuclear genes, morphological data) and their number is large, we conclude that the hypothesis of monophyly of the genus *Mertensiella* can be rejected unambiguously.

Nonmonophyly of *S. atra* and *S. lanzai*

All tree topologies derived from different analyses of our 16S data suggest that the Alpine salamanders, *S. atra* and *S. lanzai*, do not form a monophyletic group. This result is in concordance with Olivieri's (1991) allozyme data (see also Veith 1996). However, bootstrap support for dichotomies within *Salamandra* were always rather weak (Fig. 1) due to short internal branches. This result hints at a rapid radiation of the genus (see also Veith 1996). Consequently, alternative hypotheses of intra-generic evolution of *Salamandra* cannot be ruled out statistically since none of them were significantly less parsimonious or less likely than the best trees in the maximum parsimony and the maximum likelihood analyses, respectively (Table 7).

Hypotheses on the evolution of terrestriality of reproductive modes in 'true' salamanders

Evolutionary radiations of Salamandridae were dominated by selective regimes according to major kinds of occupied habitats (Özeti and Wake 1969). Ancestral salamandrids were probably restricted to montane habitats and their reproduction was adapted to running water (e.g. a mating pattern that involved a capture of the female by the male; Salthe 1967). Salthe (1967) hypothesized that most probably the conquest of lowland habitats by ancestral newts caused the basal splitting within the family. Molecular data (Titus and Larson 1995) and data on functional morphology (Özeti and Wake 1969; Wake and Özeti 1969; Miller and Larsen 1990), however, indicate that adaptations of 'true' salamanders for terrestriality in montane habitats caused them to evolve characteristics that distinguish them from their more aquatic sister taxon.

The more terrestrial life-cycle of 'true' salamanders requires a further specialization of the mating behaviour. Consequently, new mechanisms of courtship synchronization evolved that increase successful spermatophore transfer from males to

Table 7. Comparison of some tree topologies within the *Salamandra* clade: tree topology of outgroups according to Fig. 1; trees were tested for significant difference against the most parsimonious tree (#1) and the best tree under the maximum likelihood approach (#7) (for details see Table 5); *Sat* = *S. a. atra*, *Sau* = *S. a. aurorae*, *Sl* = *S. lanzai*, *Si* = *S. infraimmaculata*, *Ss* = *S. salamandra*, *Sc* = *S. corsica*, *Sal* = *S. algira*; SD, standard deviation; SE, standard error

		Maximum parsimony analysis				Maximum likelihood analysis			
	Tree # (topology)	steps	Δ steps	SD	sign. worse?	ln L	Δ ln L	SE	sign. worse?
1	((((*Sat*,*Sau*),*Sl*),(*Si*A,*Si*B)),(((*Ss*A,*Ss*B),*Sc*),*Sal*))	286 (most parsimonious tree)				−2768.3	−2.3	9.6	No
2	((((*Sat*,*Sau*),(*Si*A,*Si*B)),(*Ss*A,*Ss*B)),((*Sl*,*Sc*),*Sal*))	290	4	2.83	No	−2773.5	−7.5	8.1	No
3	(((((*Sat*,*Sau*),*Sl*),(*Si*A,*Si*B)),(*Ss*A,*Ss*B)),(*Sc*,*Sal*))	290	4	2.45	No	−2770.2	−4.3	12.3	No
4	(((*Sat*,*Sau*),*Sl*),((*Si*A,*Si*B),((*Ss*A,*Ss*B),(*Sc*,*Sal*))))	289	3	1.73	No	−2770.0	−4.0	10.6	No
5	(((*Sat*,*Sau*),*Sl*),((*Si*A,*Si*B),(*Sal*,((*Ss*A,*Ss*B),*Sc*))))	288	2	1.42	No	−2769.2	−3.2	9.2	No
6	((((*Sat*,*Sau*),*Sl*),(*Si*A,*Si*B)),(*Sal*,((*Ss*A,*Ss*B),Sc)))	286	0	0.00	No	−2768.3	−2.3	9.6	No
7	(((((*Sat*,*Sau*),*Sl*),*Sc*),(*Ss*A,*Ss*B)),*Sal*),(*Si*A,*Si*B))	291	5	2.65	No	−2766.0 (best tree)			

females. The males of *M. caucasica* and *M. luschani* use their tail projection to stimulate the females and to synchronize courtship (Rehberg 1981; Mudrack 1984; Klewen 1991; Schultschik 1994). In addition, it helps the male to place the female above the spermatophore and consequently makes spermatophore transfer more successful (Arnold 1987; Sever et al. 1997).

In the light of nonmonophyly of *Mertensiella*, two hypotheses may explain the absence of the tail projection in *Salamandra* and *Chioglossa*. (i) The tail projection is an ancestral character that was gained earlier in the evolution of 'true' salamanders and secondarily lost by *Chioglossa* (tree #7 in Fig. 2;) or *Chioglossa* and *Salamandra* (trees #1 and #4 in Fig. 2). (ii) It evolved twice in 'true' salamanders (homoplastic character; trees #1, 4 and 7 in Fig. 2). Trees #1 and #4 require three independent events: one gain and two separate losses. All the other trees under the two options require two independent evolutionary events. If we consider losses of a complex structure like the tail projection less costly than gains we can regard tree #7 under option (i) the most parsimonious one.

There is evidence from courtship behaviour that supports option (i). A common ancestor of 'true' salamanders may have possessed a tail projection, since similar elements of the *Mertensiella* courtship behaviour also occur in *Salamandra* and *C. lusitanica*, but are performed without a tail projection. These are head butting, body shifting (caudal rubbing of the female's cloaca) and positioning of the female above the spermatophore (Arnold 1987). This common ancestor of 'true' salamanders may have mated at least partially on land. In contrast to *M. luschani* and *Salamandra*, the *M. caucasica* male begins courtship in the water, while subsequent parts of the courtship, including spermatophore transfer, occur on land. Therefore, Schultschik (1994) argued that the species originally mated in water. This supposedly represents the ancestral type of mating behaviour, as indicated by data on courtship behaviour of other Salamandridae species (Salthe 1967). However, any of the above mentioned elements of 'true' salamander courtship behaviour may also have evolved as a symplesiomorphic character without the tail projection, followed by a convergent (or even parallel?) evolution of the tail projection in both *Mertensiella* species. Recent histological studies could show structural differences between the two species in gland distribution and gland size within the tail projections (Sever et al. 1997). To conclude from these differences, however, that projections may have evolved convergently seems too early since their temporal (age, season) and geographical variation are not yet studied.

Within 'true' salamanders, *M. caucasica* and *C. lusitanica* are oviparous, whereas *M. luschani* is matrotrophic viviparous and *Salamandra* species are either matrotrophic or lecitotrophic viviparous. In general, oviparity is considered the ancestral state (Wake and Özeti 1969). Thus, tree #1 in Fig. 2 reflects the most parsimonious explanation for the evolution of the reproductive mode in 'true' salamanders. A semi-aquatic ancestor of 'true' salamanders may have reproduced in fast-running mountain brooks prior to the evolution of a fully terrestrial mode in one descendant lineage. This hypothesis is also supported by comparative ecophysiological data on larvae of 'true' salamanders (Thiesmeier 1994).

However, the interpretation of the evolution of a complex life-history trait such as viviparity as an adaptation to terrestrial life, characteristic for *M. luschani* and *Salamandra*, is weakened by the fact that this feature is supposed to evolve surprisingly fast. This is indicated by studies on killifishes (Meyer and Lydeard 1993), for example and reptiles. In the latter, viviparity is thought to have evolved separately at least 98 times (Blackburn 1992). Even within *Salamandra*, several morphological and functional aspects of viviparity differ between species (e.g. mechanism of internal fertilization, intra-uterine feeding of larvae; see Häfeli 1971; Greven and Guex 1994; Guex and Greven 1994; Joly et al. 1994). Thus, matrotrophic viviparity which includes complete intrauterine development of larvae may have evolved several times even within *Salamandra* (see also Alcobendas et al. 1996). Nevertheless, lecitotrophic viviparity, as realised in most *S. salamandra* subspecies, may be considered the key innovation that enabled some populations to reproduce outside water and thus allowed them to live in high mountain (*S. atra*, *S. lanzai*, *S. salamandra bernardezi*, *S. salamandra fastuosa*) or extremely dry (*M. luschani*) habitats without open water.

Paleobiogeography and evolution of 'true' salamanders

Based on the flooding of the Strait of Gibraltar about five million years ago (Maldonado 1985), we estimated a rate of sequence divergence between *S. algira* from Africa and *S. salamandra* from Europe of 0.7% per million years when counting transitions and transversions. We use this calibration of the 16S molecular clock to discuss further splits within 'true' salamanders. We also consider fossil records. However, it must be emphasized that the fossil documentation from Western Europe is much better than that from Western Asia.

Morphologically 'true' salamanders are characterized by

derived structures adapted for terrestrial life, such as terrestrial feeding (Estes 1981; but see Wake and Özeti 1969 for a contrasting view). *Salamandra*-like urodelans are already known from the Upper Paleocene and the Lower Eocene of France and Belgium (Estes 1981; Rocek 1994). Together with the newt-like *Koalliella genzeli* Herre 1950 they are the oldest fossil records of salamandrids (Estes 1981). Consequently, the basal splitting within Salamandridae must have taken place more than 65 million years before present (MYBP).

The upper Eocene and Oligocene *Megalotriton filholi* Zittel 1890 may be the possible ancestor of the Oligocene and Miocene *Salamandra sansaniensis* Laret 1851 (Estes 1981). They must have been common throughout Central Europe since their fossil remains are known from many sites of France, Germany, former Czechoslovakia and Switzerland.

No fossil 'true' salamanders are recorded from Spain prior to the Lower Miocene period (Sanchiz 1977). This explains well the absence of 'true' salamanders on Sardinia, whereas species of the genus *Euproctus* that breed in virtually the same habitat (fast-running mountain brooks) live on Corsica and Sardinia. The latter already inhabited Iberia before the Corsica-Sardinia microplate separated from the Iberian mainland during the Oligocene period about 29 MYBP (Caccone et al. 1994).

In Central Europe *S. sansaniensis* must already have coexisted with the early and comparatively large *Chioglossa meini*, Estes and Hoffstetter 1976, by the Upper Oligocene and Lower Miocene periods 25 MYBP (Estes 1981). Thus, the first split within 'true' salamanders that presumably resulted in an oviparous and a viviparous lineage must have occurred at least during the Early Oligocene period. Viviparity as an adaptation to unfavourable environments with increasingly shorter periods for extra-uterine larval development must have evolved at that time in the ancient *Salamandra* lineage. Strong selective pressure may induce intra-uterine (better: intra-oviductal) development of embryos even in egg-laying forms (Wahlert 1953). This scenario, however, is only partially corroborated by our sequence data. The average sequence divergence between *C. lusitanica* and *M. luschani* (Table 2) allows for an estimation of a first splitting event within this species group about 20 MYBP.

After the Late Oligocene-Early Miocene period, 25–20 MYBP, formation of a continuous landmass, roughly corresponding to the Balkans and Turkey and including Iberia and Italy, separated Tethys and Paratethys and allowed for a distribution throughout the Mediterranean, from East to West and vice versa (Oosterbroek and Arntzen 1992). During this period, the probably montane, brook-dwelling, and oviparous *Chioglossa meini* and the viviparous *Salamandra sansaniensis* could spread from their Western origins towards the eastern Mediterranean as well as to the Iberian Peninsula. Comparable patterns of exchange of mammals between Asia and Africa support this hypothesis (see e.g. Steininger et al. 1985).

The vicariance of Iberia and Italy and the eastern Mediterranean areas (Oosterbroeck and Arntzen 1992) 20–15 MYBP, and the restoration of marine conditions between the Tethys and Paratethys 13–17 MYBP (Rögl and Steininger 1983) may have been responsible for the separation of *C. lusitanica* from *M. caucasica* and of *Salamandra* from *M. luschani*, respectively. Our sequence data support this scenario. The time of divergence between both pairs of taxa can be estimated to be about 14–15 million years ago. The congeneric frog species *Pelodytes punctatus* from Iberia and *Pelodytes caucasicus* show basically the same distributional pattern as *C. lusitanica* and *M. caucasica* and thus support this hypothesis, which is only contradicted by some fossil vertebrae from the Lower Miocene of Germany that may be attributed to a *Mertensiella*-like urodelan (Estes 1981).

Asian Minor-Transmediterranean (ATM) distribution again became possible 13–15 MYBP. This may have enabled western and eastern populations of 'true' salamanders to invade circum-Mediterranean areas. At that time, species like *C. lusitanica* and *M. caucasica* must have been more widely distributed than indicated by their present range (see Estes 1981). In addition, fossil records clearly indicate that even in the Lower Pliocene period a *M. caucasica*-like salamander lived in Central Europe (Sanchiz and Mlynarski 1979). Since *Salamandra* is by far the most widely distributed taxon of 'true' salamanders and since today the other taxa are endemic to rather restricted areas, it is obvious that plasticity related to the reproductive mode may have enabled it to spread much further than any of the other genera and more easily to survive periods of unfavourable climatic conditions.

Using our calibration of the 16S molecular clock we can also infer some preliminary hypotheses on a paleobiogeographic scenario of species evolution within *Salamandra*. The estimated time of divergence between *S. corsica* and *S. salamandra* is about six million years. This corresponds to the Messinian salinity crisis in the late Miocene period when major parts of the Mediterranean basin were dry and colonization of formerly unsettled areas was possible (see also Lanza 1988). The subsequent flooding of the basin isolated Corsica from the European mainland and caused a divergent evolution of Corsican and mainland populations.

Degrees of nucleotide divergence between *Salamandra* species range from only 3.1 (*S. lanzai* – *S. salamandra*) to 6.7% (*S. infraimmaculata* – *S. corsica*). This indicates that the major divergence within the genus took place between five and 10 MYBP. Therefore, it may be hypothesized that the genus' radiation was strongly correlated with the opportunity of an Asia Minor–Transmediterranean distribution about 13–15 MYBP during the early Serravallian period. A newly created mountain chain stretched from the Alps through the Dinarides and the Hellenides to Anatolia (Maldonado 1985). Ancestors of the present species could have settled large parts of Europe using this mountain bridge between Europe and Asia, where the genus probably originated. A first split within the genus could have separated the species' distribution more or less simultaneously into Iberian, Central and Eastern Mediterranean populations about 10 MYBP, during the final structuring of the Alps and the Neo-Pyrenees (Decourt et al. 1986).

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Zusammenfassung

Eine molekulare Phylogenie 'Echter' Salamander und die Evolution von Terrestrialität des Paarungsverhaltens

Innerhalb der Familie der Salamandridae sind die 'Echten' Salamander (Gattungen *Chioglossa*, *Mertensiella* und *Salamandra*) durch spezifische Anpassungen an eine terrestrische Lebensweise gekennzeichnet (Anatomy und Physiologie des Freßapparates: Paarungsverhalten, Viviparie, etc.), die es ihnen erlaubten, von einer eher semi-aquatischen zu einer mehr terrestrischen Lebensweise überzugehen.

Wir sequenzierten 423 Basenpaare des mitochondrialen 16S RNA-Gens aller Arten 'Echter' Salamander. Basierend auf den resultierenden

phylogenetischen Hypothesen diskutieren wir die Evolution terrestrischer Reproduktion innerhalb dieser Artengruppe. Insbesondere testeten wir zwei Hypothesen monophyletischen Ursprungs solcher Anpassungen: Monophylie von *Mertensiella caucasica* und *M. luschani*, deren Zuordnung zu einer gemeinsamen Gattung, vorgenommen aufgrund eines die Männchen beider Arten kennzeichnenden Schwanzwurzelhöckers, bereits anhand morphologischer, ethologischer und molekularer Daten angezweifelt wurde, sowie Monophylie der beiden Arten von Alpensalamandern, *Salamandra atra* und *S. lanzai*, die sich durch Melanismus und matrotrophe Viviparie von den übrigen *Salamandra*-Arten unterscheiden.

Wir testeten alternative Stammbäume statistisch und zogen weitere publizierte und unpublizierte morphologische, biochemische (Allozyme) und molekulare (mtDNA-Sequenzen) Daten hinzu. Alle Analysen widersprachen einem monophyletischen Ursprung der beiden *Mertensiella*-Arten. Daten zum Paarungsverhalten 'Echter' Salamander weisen darauf hin, daß der Schwanzwurzelhöcker der *Mertensiella*-Männchen entweder ein symplesiomorphes Merkmal aller oder zumindest der meisten 'Echten' Salamander ist, das bei *Salamandra* oder *Chioglossa* sekundär verloren ging, oder daß es ein konvergentes Merkmal ist. Obwohl die zur Zeit vorliegenden Daten andeuten, daß *S. atra* und *S. lanzai* keine Geschwistertaxa sind, kann dies nicht zweifelsfrei ausgeschlossen werden.

Wir nutzten die Öffnung der Straße von Gibraltar vor *c.* 5 Millionen Jahren, in deren Zuge die europäischen von den afrikanischen Populationen getrennt wurden, um eine molekulare 16S RNA-Uhr zu eichen (gesamte Sequenz-Divergenz: 0.7% pro 1 Million Jahre). Die hieraus ableitbaren Divergenzzeiten innerhalb der 'Echten' Salamander stimmen gut mit paläogeographischen Daten überein.

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