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Mitochondrial DNA sequence analysis of the spectacled salamander, *Salamandrina terdigitata* (Urodela: Salamandridae), supports the existence of two distinct species

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ABSTRACT

Salamandrina is a monotypic genus of the family Salamandridae endemic to Italy. Forty five individuals of the spectacled salamander, *Salamandrina terdigitata*, representing 11 populations throughout the whole distribution range were examined for sequence variation of three mitochondrial DNA genes encoding the 12S and 16S ribosomal RNA's and cytochrome *b* (1324 bp). The results indicate the existence of two genetically distinct and geographically non-overlapping mtDNA lineages. The first lineage includes the southern populations and the second one comprises the central-northern populations. The degree of genetic divergence between the two groups is high and comparable to distances calculated from homologous sequences available in GenBank between other Salamandrid species. As a result, the genus *Salamandrina* probably requires splitting into two species. We also compare substitution rates associated with the mitochondrial genes employed across all Salamandrids studied so far, and discuss two possible palaeogeographic scenarios which could have shaped the splitting of the two *Salamandrina* lineages. These findings have also important implications for management and conservations of the spectacled salamander, which is protected under several international and regional conventions and directives.

Key words: *Salamandrina*, phylogeny, mtDNA, 12S, 16S, cytochrome *b*, genetic divergence

INTRODUCTION

The genus *Salamandrina* Fitzinger, 1826 is the sole extant representative of an ancient lineage of the family Salamandridae (cf. Thorn, 1969), whose phylogenetic relationships are still questioned, even if it appears to be more related to the "newts" rather than to the "true

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salamanders" (Titus & Larson, 1995). *Salamandrina* has long been considered a monotypic genus, endemic to Italy where its sole representative, *S. terdigitata* (Fig.1), is discontinuously distributed from the northern Apennines (westernmost portion of Genoa province) to the tip of Calabria. The great majority of records are from the Tyrrhenian side, less records are from the Adriatic one (Zuffi, 1999) and the southern hilly side of the Po Valley, where it is restricted just to few sites (Barbieri *et al.*, 1989; Barbieri, 2001).

FIGURE 1. A female specimen of *Salamandrina terdigitata* from Lepini Mountains (Latium, Central Italy).

Total adult length is usually 8–9 cm (Vanni, 1980; Zuffi, 1999), with a maximum of 12.3 cm recorded in populations from the Lepini Mts. (Angelini *et al.*, 2001). This species is usually found between 200 m and 900 m although there are some records from the sea level and up to 1500 m (Barbieri & Pellegrini, in press). The breeding sites are represented by slow running streams, pools, ponds, sources, springs, fountain watering places for livestock and, sometimes, stony wells, tanks and natural and artificial caves (Corsetti, 1999; Zuffi, 1999; Angelini & Cari, 2002; Mattoccia & Romano, unpublished data).

The spectacled salamander is listed in Appendix II of the Bern Convention, included in DPR n.357/97 (Habitat Directive, Annex II and IV) and it is strictly protected by Regional Laws in several Italian Regions. Although considered at "lower risk" by a few authors (e.g. Scalera, 1998), others have recommend that *Salamandrina* deserves particular attention and should be considered an endangered species (Andreone & Luiselli, 2000). In spite of the remarkable value of this species (it is the icon of the Italian Zoology Society, U.Z.I.), there is a surprising lack of studies concerning its genetic structure and molecular geographical variation. This preliminary study attempts to fill in this gap.

MATERIALS AND METHODS

Sampling

Forty-five individuals of *Salamandrina terdigitata* were sampled from 11 populations covering the whole distribution range of the species from Liguria to Calabria (Fig. 2). Sampling localities and numbers of individuals collected are reported in Table 1. All samples were obtained from live animals by removing approximately 2 mm^3 from the tail tip (Arntzen *et al.* 1999; Rowe *et al.*, 1999; Taberlet & Luikart, 1999). Tissue samples were stored in 95% ethanol and the individuals were released.

FIGURE 2. Map of Italy showing the sampling sites of *Salamandrina terdigitata*.

Sampling was carried out under authorization of the "Ministero dell'Ambiente e della Tutela del Territorio" of Italy (permission DPN/2D/2004/17393).

TABLE 1. Details of the sampling sites and numbers of specimens analysed (N) of *Salamandrina terdigitata.*

REGION	Code	Localities	Province	N
Liguria	LIG	Rio Chiapeto, S. Martino	Genova	5
Umbria	UMB	Collescipoli	Terni	6
Latium	TAN	Osteria del Tancia	Rieti	5
Latium	TOR	Torrecchia Vecchia	Latina	7
Latium	GOR	Fosso delle Pietracquare	Rome	3
Latium	LOM	Lombetto	Rome	3
Latium	ERA	S. Erasmo	Latina	2
Latium	AUR	Valle Piana	Latina	5
Campania	PIC	Fiume Calore	Salerno	1
Basilicata	POL	Bosco Magnano	Potenza	5
Calabria	ASP	Fiumara La Verde	Reggio Calabria	3

DNA sources, PCR amplification and sequencing

Genomic DNA was extracted using CTAB protocol (Doyle & Doyle, 1987). PCR amplifications of 416-bp of the 12S rRNA, 576-bp of 16S rRNA and 463-bp of cyt*b* genes were carried out using primers 12Sa and 12Sb (Kocher *et al.*, 1989), 16Sa and 16Sb (Simon *et al.*, 1994), and L14724 and H15149 (Caccone *et al.*, 1997), respectively. Double stranded amplifications were performed with a Perkin-Elmer-Cetus thermal cycler in 25 µl of a solution containing genomic DNA (10-100 ng), 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, each dNTP at 2.5 mM, each primer at 1 µM and 1 unit of Amplitaq (Perkin-Elmer). Thermocycling conditions were: 2 min. denaturation at 94° C, 35 cycles at 94° C for 1 min., 50° C for 1 min., 72° C for 1'30 sec., with final extension at 72° C for 6 min. for the primer pair 12Sa and 12Sb; 2 min. at 94° C, 30 cycles at 94° C for 30 sec., 50° C for 30 sec., 72° C for 45 sec., 72° C for 2 min. for 16Sa and 16Sb; 2 min. at 94° C, 40 cycles at 94° C for 1 min., 48° C for 1 min., 72° C for 1'30 sec., 72° C for 6 min. for the primer pair L14724 and H15149. PCR fragments were purified by using GFXTM DNA and Gel Band purification Kit from Amersham Pharmacia Biotech and sequenced in both directions for each individual using the BigDye terminator ready-reaction kit. Sequences were determined with an automated sequencer ABI 3100 (Perkin-Elmer Applied Biosystems). Sequences were aligned by using Clustal X (Thomson *et al.*, 1997)**.** Sequences have been submitted to GenBank, accession numbers AY898727 to AY898739.

Phylogenetic analyses

The smooth newt, *Triturus vulgaris*, (GenBank Accession numbers U55948 for cyt*b*, U04704 for 12S and U04705 for 16S) was used as the outgroup. The suitability of combin-

ing sequence data from the three genes was assessed by the Partition Homogeneity Test (Farris *et al.*, 1994). This test was performed with PAUP* 4.0β10 (Swofford, 2002). Aligned sequences were analysed by four different methods: maximum parsimony (MP; heuristic searches, 100 random stepwise additions, TBR branch-swapping algorithm (Farris, 1970), maximum likelihood (ML; heuristic searches, 100 random stepwise additions, TBR branch-swapping algorithm) (Felsenstein, 1981), neighbour-joining (NJ) (Saitou & Nei, 1987) and Bayesian analysis (Huelsenbeck, 2000; Larget & Simon, 1999; Mau & Newton, 1997; Mau *et al.*, 1999; Rannalla & Yang, 1996). MP, ML and NJ were carried out using PAUP* 4.0β10 and Bayesian analysis was performed using MrBayes (Huelsenbeck, 2000). MP searches were run giving equal weight to all substitutions and weighting transition three times transversions (TV3 × Ti) according to the observed transitions/transversions ratio(Ti/Tv=2.8). The MP analyses was repeated including (as a fifth character) and excluding gaps. ModelTest (Posada & Crandall, 1998) was employed to determine the best sequence evolution model for our data set. According to the results of this test, ML analysis was carried out using the HKY85+Γ model of character evolution (variable rates, shape parameter $\alpha = 0.1023$). NJ analyses were performed on HKY distances calculated with the same ModelTest parameters used for ML analyses. The same model of DNA substitution was employed for the Bayesian approach to estimate the posterior probabilities of the best set of trees. The MrBayes program was run for 2 million generations and a tree was sampled every 100 generations. From the 20,000 trees found, the first 10% were exuded in order to include only trees for which convergence of the Markov chains had been reached. The remaining trees were used to construct a 50% majority rule consensus tree using PAUP* 4.0β10.

Bootstrap supports (Felsenstein, 1985) for the resulting phylogenetic hypotheses were calculated using 1000 replicates for MP and NJ and 100 replicates for ML. Alternative phylogenetic hypotheses were tested using the Templeton test (1983), and Shimodaira-Hasegawa log-likelihood test (Shimodaira & Hasegawa, 1999) as implemented in PAUP* 4.0β10.

To test for rate constancy among lineages and molecular clock hypothesis, we used the log-likelihood ratio test (Goldman, 1993), which compares the log-likelihood of the ML trees with and without assuming a molecular clock.

Intraspecific gene genealogies were inferred using TCS program (Clement *et al.*, 2000**)** wich implements the Templeton *et al.* (1992) statistical parsimony method.

Results

Sequence variation

A total of 1455 bp of mtDNA were sequenced for each of the 45 specimens of *S. terdigitata* included in the study. The final length of our alignment (including the outgroup) was 1324 bp (a fragment of 373 bp of 12S gene, 525 bp of 16S gene and 426 bp of cyt*b*

gene). Most of mutational ingroup changes were base pair substitutions. Gaps occurred only in the 16S rDNA and consisted of single indels. The number of variable sites, informative sites and percentage of Adenine+Thymine (A+T) composition in the three gene fragments sequenced are shown in Table 2. The number of variable sites were similar between the two ribosomal genes and higher in the cyt*b* gene. The majority of the cyt*b* gene substitutions occurred in the third codon position (80.8%). Considering the ingroup data set, all the variable sites, but one in the 16S gene, resulted parsimony-informative.

TABLE. 2. Percentage of variable sites, parsimony informative sites and A+T by genes and third codon position across all the populations of *Salamandrina terdigitata*

Region and codon position	% variable sites		% informative sites	Number sites	A+T
	All taxa	Ingroup			
12S + 16S + cyt*b*	16.8	6.2	6.10	1324	60.6
Cyt*b*	23.9	11	11	426	61.2
Cyt*b* III	57	26.8	26.8	142	65.7
12S	12.9	4	4	373	59.9
16S	13.7	3.8	3.6	525	60.5

The mean percentage of A+T was similar in the three sequences (61.2%; 59.9%; 60.5%) and was just a bit higher in the third codon position of the cyt*b* (58.1%, 59.8%, 65.7% first, second and third position respectively). The sequences of the three genes showed a deficit of guanine particularly in third codon positions of cyt*b* (only 5%), which is a typical characteristic of the vertebrate mitochondrial genes encoding for proteins. Most of the nucleotide substitutions were transitions. Transversions occurred in comparison between ASP, POL, PIC and the other populations (36% of the nucleotide substitutions) and between *Salamandrina* populations and outgroup (43%). To detect saturation of base pair substitutions we plotted transitions and transversions versus uncorrected p distances for gene 12S, 16S, and cytochrome *b* genes and for third position of cyt*b* only. The result displayed no evidence of saturation (data not shown).

Phylogenetic analyses

The results of the Partition Homogeneity test showed that the three genes sequenced were not phylogenetically incongruent and therefore it was decided to combine them for further phylogenetic analyses ($P = 0.7$). Table 3 reports the HKY85 distances calculated for the combined gene fragments. Sequence divergence estimates exhibited the highest differentiation value between the southern populations (ASP, POL, PIC) and the other northern and central populations. The average sequence divergence between these two

groups was 6.40% Within each group, the populations were genetically very similar, sequence divergence ranging from 0 to 0.23% among central and northern populations, and from 0 to 0.08% among southern populations.

TABLE. 3. HKY85 DNA distances calculated on the combined 12S, 16S and cyt*b* (below Diagonal), number of substitutions (above Bars) and number of transversions (below Bars) between all pairs mitochondrial DNA haplotypes.

Code of the sample	LIG	TAN	TOR	AUR1	AUR2	ERA	GOR	LOM4	LOM5	LOM6	UMB	ASP	POL	PIC	*T. vulgaris*
LIG	-	0/0	0/0	2/0	2/0	2/0	2/0	3/0	2/0	1/0	2/0	79/23	80/23	80/23	192/82
TAN	0.0000	-	0/0	2/0	2/0	2/0	2/0	3/0	2/0	1/0	2/0	79/23	80/23	80/23	192/82
TOR	0.0000	0.0000	-	2/0	2/0	2/0	2/0	3/0	2/0	1/0	2/0	79/23	80/23	80/23	192/82
AUR1	0.0015	0.0015	0.0015	-	2/0	0/0	0/0	1/0	0/0	1/0	2/0	79/23	80/23	80/23	191/82
AUR2	0.0015	0.0015	0.0015	0.0015	-	2/0	2/0	3/0	2/0	1/0	2/0	77/23	78/23	78/23	190/82
ERA	0.0015	0.0015	0.0015	0.0000	0.0015	-	0/0	1/0	0/0	1/0	2/0	79/23	80/23	80/23	191/82
GOR	0.0015	0.0015	0.0015	0.0000	0.0000	0.0000	-	1/0	0/0	1/0	2/0	79/23	80/23	80/23	191/82
LOM4	0.0023	0.0023	0.0023	0.0008	0.0023	0.0008	0.0008	-	1/0	2/0	3/0	80/23	81/23	81/23	192/82
LOM5	0.0015	0.0015	0.0015	0.0000	0.0015	0.0000	0.0000	0.0008	-	1/0	2/0	79/23	80/23	80/23	191/82
LOM6	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008	0.0008	0.0015	0.0008	-	1/0	78/23	79/23	79/23	191/82
UMB	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0015	0.0023	0.0015	0.0008	-	77/23	78/23	78/23	191/82
ASP	0.0631	0.0631	0.0631	0.0631	0.0614	0.0631	0.0631	0.0631	0.0631	0.0623	0.0614	-	1	1	183/82
POL	0.0640	0.0640	0.0640	0.0640	0.0623	0.0640	0.0640	0.0640	0.0640	0.0631	0.0623	0.0008	-	0	184/82
PIC	0.0639	0.0639	0.0640	0.0640	0.0623	0.0640	0.0640	0.0640	0.0640	0.0631	0.0623	0.0008	0.0000	-	184/82
T. vulgaris	0.1648	0.1648	0.1648	0.1628	0.1628	0.1638	0.1638	0.1638	0.1638	0.1638	0.1638	0.1559	0.1569	0.1569	-

Trees constructed using different methodologies showed similar topologies. Templeton (1983) and Shimodaira-Hasegawa (1999) tests supported that MP (unweighted and weighting Tv3xTi, with or without considering gaps), NJ, ML and Bayesian topologies were statistically undistinguishable (TEMP: P = 0.317; SH: P = 0.07-1). Figure 3 shows the ML tree and summarises the results of the other phylogenetic approaches employed in the study. The tree revealed a clear split of *S. terdigitata* haplotypes into two major clades: a southern clade (TER) including the haplotypes observed in the samples of Campania, Basilicata and Calabria, and a north-central clade (PER) comprising the haplotypes found in the samples from Liguria to Latium. The two clusters were strongly supported by bootstrap values of 100%. Within the two major lineages, some sub-clusters can be distinguished. The PER group included three branching: (i) *UMB* population (Umbria), (ii) *AUR*, *GOR*, *LOM* and *ERA* populations (Lepini Mts. and Aurunci Mts., Latium region), and (iii) *LIG* population (Liguria) together with *TOR* and *TAN* populations (Latium). The TER group included: (i) ASP population (Calabria) and (ii) POL and PIC populations (Basilicata and Campania respectively).

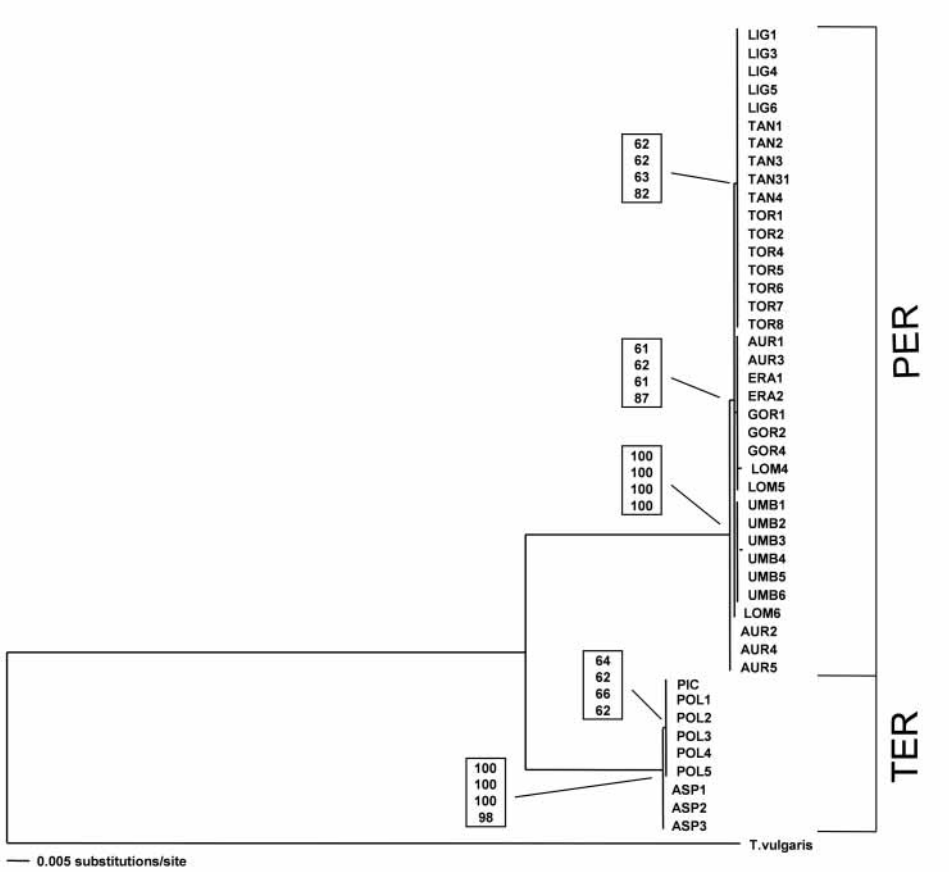

FIGURE 3. Maximum likelihood tree for *Salamandrina terdigitata* samples plus outgroup, based on combined 12S, 16S and cyt*b*. HKY85+Γ model (-ln = 2864.386; shape parameter α = 0.1203) was assumed. Numbers in boxes (from top to bottom) are bootstrap support values for ML (100 replicates), MP (1000 replicates) and NJ (1000 replicates) and posterior probability percentages in the Bayesian analysis (2 million generators). Specimens are identified by code of sampling locality (as in Tab.1) and by a progressive number.

Figure 4 shows the results of the network analysis based on statistical parsimony. Among the 8 mtDNA haplotypes we detected, 6 were grouped in a cluster including the central-northern populations and 2 in another one that groups the southern populations.

To estimate evolutionary times for the lineages split, we tested rate homogeneity for nucleotide substitutions between the two lineages of *S. terdigitata*. The likelihood ratio test revealed no significant differences between the ML tree, assuming a molecular clock (-ln L = 2872.261), and the tree without clock (ln L = -2864.386; d.f. = 44, P > 0.995). Thus, the sequences sample utilised meets the requirements of a constant substitution rate.

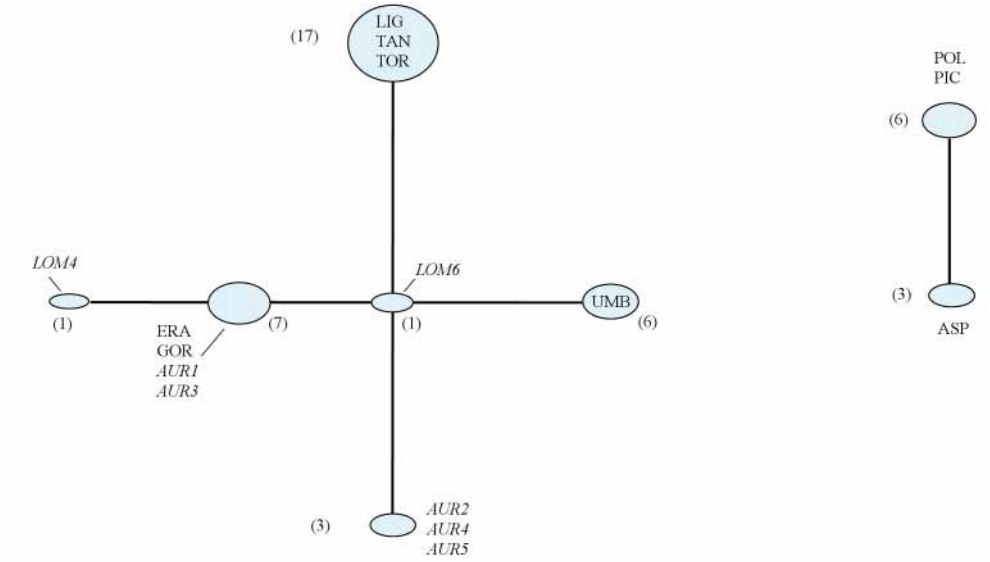

FIGURE 4. Haplotype network of *Salamandrina terdigitata*, reconstructed using parsimony probability as implemented in TCS. Each circle in the network corresponds to one observed haplotype. Size of circles is proportional to the number of individuals (in parentheses) carrying a given haplotype. The three letters codes of the populations are indicated; numbered codes in italic mean different individuals when more than one haplotype was found in a population.

Discussion

Systematic issues

The results clearly show that there are two genetically distinct and geographically vicariant mtDNA lineages of *Salamandrina terdigitata* in Italy. Although distances between populations range from 170 to 330 Km within the TER group, and from 2 to 510 Km within the PER group, we found very little genetic differentiation within each of the two lineages. The extent of the genetic divergence between the two groups is striking, especially in the face of the lack of noticeable morphological or ecological discriminating traits.

To weigh up the significance of this result, we compared the genetic distances found within the spectacled salamander with other Salamandridae genera from data available in GenBank. Namely, we used partial sequences of cyt*b* gene and 12S ribosomal gene for 27 and 18 species of 9 and 5 genera respectively (for details see Appendix I). Tamura-Nei distances for all congeneric species pairs were calculated on the homologous gene regions for cyt*b* and 12S separately, based on the results of ModelTest (Tamura & Nei 1993; Posada & Crandall, 1998). Actually, the 62% (number of comparisons N=34; cyt*b*) and 42% (N=26; 12S) of pairwise distances between congeneric species showed values lower or equal to those exhibited by *Salamandrina*.

The aforesaid percentages increased to 79% (N=26) and 55% (N=20) for cyt*b* and 12S respectively, by excluding the comparisons between non-monophyletic species (i.e. *Euproctus asper* and some species of *Triturus*; see Caccone, 1997; Steinfartz, 2002; Macgregor *et al.*, 1990). This procedure was employed because the deep sequence divergence between some species was inevitably expected by virtue of their non-monophyletic relationships. These data indicate that the average level of the mtDNA sequence divergence between the TER and PER *Salamandrina* assemblages is clearly of the same extent as those found between other salamandrid species. Moreover, the finding of a phylogeographic break between lineages, instead of any shape of gradual variation, suggests the past occurrence of long-term, extrinsic barriers to gene flow between two groups of populations. These facts strongly suggest the existence of two distinct species in the genus *Salamandrina*.

The value of mitochondrial divergences as indicators for taxonomic distinctness can be questionable. Our hypothesis of two distinct species of *Salamandrina* should be tested data from nuclear markers. Available morphological data are still insufficient to highlight discriminant characters between the two clades. Vanni (1980) asserts that *Salamandrina* shows insignificant morphological variation across its range and consequently no splitting into different subspecies (or species) has been proposed so far. However, among 46 specimens examined by this author, only one came from southern Italy. Therefore a more detailed study based on population samples from the whole range is badly needed.

If future taxonomic studies will corroborate the taxonomic distinction of the PER and TER lineages at the species level, two names are apparently available. *Salamandrina perspicillata* (Savi, 1821), a name widely employed until the beginning of the 20th century, is regarded as a junior synonym of *Salamandrina terdigitata* (Lacépède, 1788). Savi (1821) established the type locality of "*Salamandra perspicillata*" in the Mugello (Tuscany Apennines, Central Italy) while Lacépède (1788) indicated the type locality of "*Salamandra ter-digitata*" in the volcano Vesuvius, near Naples (Southern Italy). Considering the type localities of the two synonyms only, the central-northern clade PER should correspond to *Salamandrina perspicillata* (Savi, 1821) while *Salamandrina terdigitata* (Lacépède, 1788) should refer to the southern clade TER.

Molecular clocks and evolutionary scenarios

As an attempt to date the separation of the two lineages we first analysed evolutionary rates deriving from calibrations made for other salamandrids. Most of the available substitution rates showed concordant values ranging from 0.8% to 1%, per site per million years, for cyt*b*, and 0.38% for 12S and 16S ribosomal genes. These calibrations had been obtained using either fossil records, or dated palaeogeographic scenarios: *Taricha* and *Notophthalmus* (Tan & Wake, 1995), *Euproctus* (Caccone *et al.*, 1994, 1997) and *Ambystoma* (Spolsky *et al.*, 1992). Instead, Carranza and Arnold (2004), obtained evolutionary rates faster than those above reported, 1,95% for cyt*b* and 0,95% for 12S ribosomal

gene, calibrating the split of *Pleurodeles poireti* from *P. waltl* against the time of the opening of the Gibraltar Strait. These authors also suggest that these differences could be due either to unrelatedness of the Salamandrid taxa analysed, or to bias in the calibration procedures considered. In the *Taricha-Notophthalmus* calibration, the attribution of fossil records to *Notophthalmus* could be uncertain, and, the non-monophyly of the genus *Euproctus* (Caccone *et al.*, 1997; Steinfartz *et al.* 2002), would weaken the calibration based on the vicariance hypothesis.

Considering the place of *Salamandrina* in the phylogenetic tree of Salamandridae (Titus & Larson, 1995), and the possibility of different evolutionary rates in unrelated genera, we attempted to recalculate evolutionary rates both in *Euproctus* and *Pleurodeles.* Caccone *et al.*'s (1997) calibration for *Euproctus* was based on two independent biogeographic events: the split of Corsica-Sardinia from the Pyrenees, and the disjunction of Sardinia from Corsica. While the first supposed cladogenetic event could be flawed by the uneven phylogenetic position of the Pyrenean taxon *Euproctus asper*, behaving as the sister taxon of *Neurergus* (Steinfartz *et al.* 2002), the second one appears perfectly sound for the calibration purpose. In fact, the disjunction between Corsica and Sardinia would have caused the separation of the ancestral populations of *E. montanus* and *E. platycephalus*, which clearly constitute a clade. Hence, substitution rates to be estimated for these two island species remain applicable. These rates differ from rates calculated by Carranza and Arnold (2004) for the separation of *P. poireti* from *P. walt*, although Veith *et al.* (2004) hypothesize an alternative scenario to the opening of the Strait of Gibraltar, maintaining the same rates calculated by Caccone *et al.* (1997).

To infer divergence time between the two lineages of *Salamandrina* we used two sources of calibration: the first one based on the split of *Euproctus montanus* from *E. platycephalus* (data set 1), and the second based on the split of *Pleurodeles poireti* and *P. waltl* (data set 2). Sequence data obtained from GenBank were representative of the two main clades within each of *Pleurodeles* species (see Appendix 1).

Table 4 reports for each of the two data sets genetic distances for different genes and classes of substitutions, calculated on the homologous gene regions (12S+16S+cyt*b* = 1340 bp for the data set 1 and 12S+cyt*b* = 767 bp for the data set 2) and corrected according to the Tamura-Nei substitution model chosen by Modeltest (Tamura & Nei, 1993; Posada & Crandall, 1998). The substitution rates estimated for *Euproctus* (calibration I and II) and *Pleurodeles* (calibration III), and the corresponding time dating for the split between *Salamandrina* lineages, are also showed. Disjunction of Sardinia from Corsica has been hypothesized to start 20-15 MYA and was completed 9 MYA (Alvarez, 1974; Bonin *et al.*, 1979; Boccaletti, 1990). We considered at least two possible time calibrations for *Euproctus*: 15 MYA (Caccone *et al.*, 1994, 1997), and 9 MYA.

Figures resulting from these attempts vary to a large extent. First, rates of cyt*b* and ribosomal genes of *Salamandrina* showed different ratios compared to both *Euproctus* and *Pleurodeles*. In fact, time estimates based on ribosomal genes were always lower than

those based on cyt*b*. This is due to cyt*b* gene evolving more than three times as fast as the ribosomal genes in spectacled salamander, while this ratio was 1.4 and 2.5 for *Euproctus* and *Pleurodeles* respectively. The splitting time of *Salamandrina* lineages obtained from *Pleurodeles* calibration, considering all substitutions, was among the lowest values, ranging from 4.9 MYA to 6.7 MYA. The splitting time based on *Euproctus* ranged from 4.7 MYA to 9.9 MYA in the calibration II, and from 7.9 MYA to16.4 MYA for calibration I. The estimates for 12S ribosomal gene were quite similar in calibrations II and III. Interestingly enough, from these last calibrations, all the time estimates based only on transversion data, were very similar (8.5-9.3 MYA).

TABLE 4. Tamura & Nei (TrN) distances, evolutionary rates (in italic) and different time estimates of splitting (in bold) between *Salamandrina* lineages, based on three different calibrations (see text for details).

		12S 16S	**12S 16S** Tv only	**12S**	**12S** Tv only	**Cytb**	**Cytb** Tv only
DATA SET 1	Calibration						
TrN distance between PER clade and TER clade of *Salamandrina*		0.037	0.012	0.040	0.011	0.122	0.029
TrN-distance between *Euproctus montanus* and *E. platycephalus*		0.062	0.012	0.076	0.022	0.111	0.029
Rate for *Euproctus* split at 15 MYA	**I**	*0.004*	*0.001*	*0.005*	*0.001*	*0.007*	*0.002*
Rate for *Euproctus* split at 9 MYA	**II**	*0.007*	*0.001*	*0.008*	*0.002*	*0.012*	*0.003*
Time (MYA) of *Salamandrina* split from cal. I		**9.06**	**15.00**	**7.88**	**7.43**	**16.43**	**15.00**
Time (MYA) of *Salamandrina* split from cal. II		**5.44**	**9.00**	**4.73**	**4.46**	**9.86**	**9.00**
DATA SET 2							
TrN distance between PER clade and TER clade of *Salamandrina*				0.039	0.011	0.133	0.031
TrN-distance between *Pleurodeles poireti* and *P. waltl*				0.042	0.007	0.105	0.019
Rate for *Pleurodeles* split at 5.3 MYA	**III**			*0.008*	*0.001*	*0.020*	*0.004*
Time (MYA) of *Salamandrina* split from cal. III				**4.91**	**8.50**	**6.68**	**8.60**

Different time estimates of splitting would obviously involve alternative palaeogeographic scenarios. The most recent time estimates inferred from calibrations II and III for

12S gene (4.7 and 4.9 MYA) date the split of the two *Salamandrina* lineages to the Lower Pliocene, a period characterized by a long-standing marine transgression. The main palaeogeographic outcome was a sound connecting the Adriatic to the Tyrrhenian Sea, separating the Apennine chain approximately at the border between Latium and Campania. Therefore, the Italian peninsula was separated in a central-northern part and in a southern fragmented part (La Greca, 1984; Pinna, 1989). Hence, *Salamandrina* ancestral populations would have remained isolated. Under this scenario (scenario A, Fig. 5) the palaeogeographic event would have determined both the phylogenetic split between the two *Salamandrina* lineages and their present distribution.

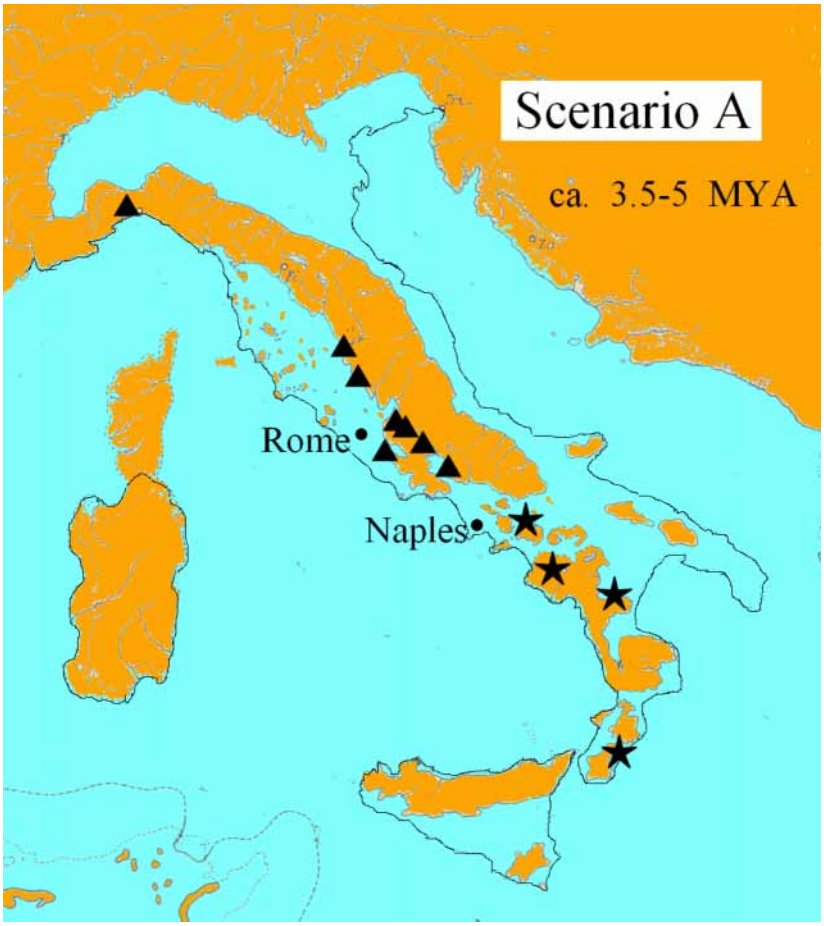

FIGURE 5. Palaeogeographic scenario (Scenario A). The Italian Peninsula in the Pliocene (modified from Pinna, 1989). Black triangles: sampling sites of *Salamandrina* populations included in the PER clade. Black stars: sampling sites of *Salamandrina* populations included in the TER clade. See text for details and confront to the Fig. 1.

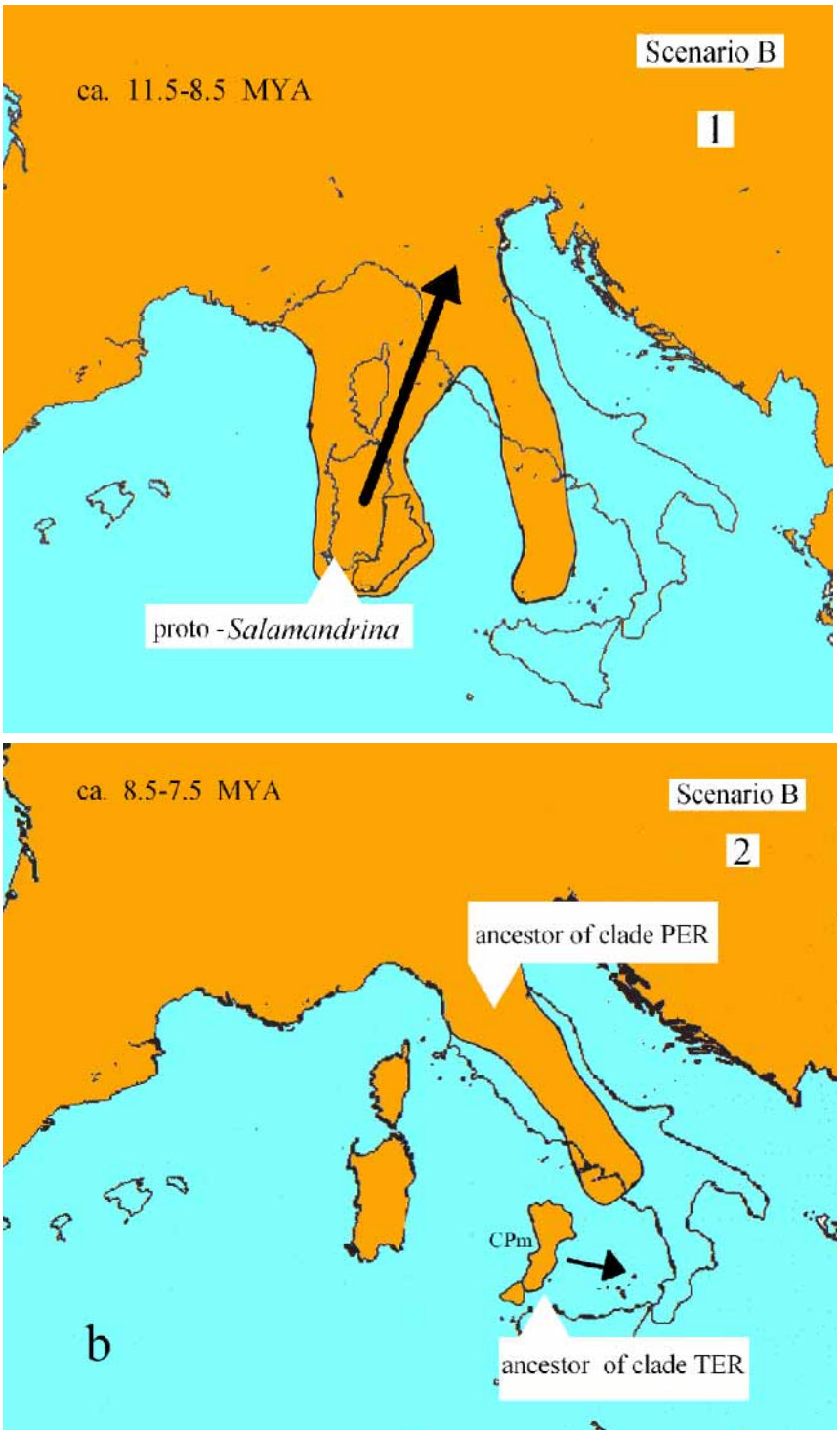

FIGURE 6. Palaeogeographic scenario (Scenario B). (1) The Tyrrhenian basin in the Tortonian time (modified from Orszag-Sperber *et al.*, 1993) with a land bridge connecting Sardinia with the peninsula. (2) Separation of the Calabro-Peloritan massif (CPm) from Sardinia (modified from Duermeijer *et al.*, 1998). Arrows hypothesize dispersal routes. See the text for comments.

All the other estimates would entail more ancient cladogenetic events. Excluding a few oldest values obtained from calibration I, all the remaining time splits range from 7 to 10 MYA, and from 8.5 to 9 MYA considering transversions only. These estimates bring back us to the Tortonian. At this time, some important events deeply affected the geography of the Tyrrhenian basin. The three main palaeogeographic events were: (i) Corsica and Sardinia were connected via land bridge to the exposed lands that will form the northern, central and part of southern Italian territories (Orszag-Sperber *et al.*, 1993); (ii) the separation between Sardinia and Corsica was completed ca. 9 MYA (Alvarez, 1974; Bonin *et al.*, 1979; Boccaletti, 1990); (iii) the separation of the Calabro-Peloritan massif (presently Sicily and southernmost portion of southwestern Italy) from Sardinia (8.6 and 7.6 MYA) and its drift to the present day position (Duermeijer *et al.*, 1998). Trying to imagine how these events could have affected genetic differentiation between two *Salamandrina* lineages, it is worth considering that the past presence of this genus in Sardinia is demonstrated by fossil records in the Lower Miocene (23-15 MYA) and Pliocene (5.3-1.8 MYA) of Oschiri, Sardinia (Estes, 1981; Sanchiz, 1977, 1983; Sanchiz & Mlynarski, 1979). To relate the palaeogeographic events and fossil data with *Salamandrina* evolutionary scenario, one should assume that an exchange of fauna, through the land bridge, allowed dispersal of the *Salamandrina* ancestor across Sardinia and the mainland (scenario B1, Fig. 6). The two lineages of *Salamandrina* could have begun to diverge either when the land bridge disappeared or, later, at the separation of the Calabro-Peloritan massif from Sardinia (scenario B2, Fig. 6). In any case, this last event was the only that could have produced the present distribution, carrying the southern *Salamandrina* lineage up to join to the rest of Italy. The Tortonian land bridge and the separation of the Calabro-peloritan massif from Sardinia were already invoked as probably routes of dispersion in other Amphibians (e.g. Fromhage *et al.*, 2004).

While each of these hypothetical scenarios is supported by a particular calibration, the first scenario appears to be better supported both by a more parsimonious evolutionary trajectory and because it is more robustly supported by evolutionary rates established on 12S ribosomal genes, which show a more linear clockwise behaviour.

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APPENDIX I. GenBank accession numbers for the species used in pairwaise comparisons in the text and in calibrations of Table 5.

Genus	Species	Genbank Accession Number cyt*b*/12S/16S
Euproctus	*asper, montanus, platycephalus*	U55945/U04694/U04695, U55946/U04696/U04697, U55947/U04698/U04699
Paramesotriton	*deloustali, guanxiensis, chinensis, fuzhongensis, caudopunctatus*	AY079480/AY079462, AY079488/AY079470, AY079478/AY079460, AY079484/AY079466, AY079475/AY079457
Tylototriton	*taliangensis, verrucosus*	AF295684/, AY336660/
Cynops	*cyanurus, pyrrogaster*	AF295681/, AF295682/
Pleurodeles	*waltl, poireti*	AY222515/AY222471, AY222513/AY222469, AY222507/AY222463, AY222511/AY222467
Triturus	*carnifex, marmoratus, vittatus, pygmaeus, vulgaris, alpestris*	U55949/U04702, AY046081/AY147252, AY336659/, AY046082/, U55948/U04704, /AY147256
Salamandra	*salamandra, atra, lanzai, algira*	AY222503/, AY042786/, AF356699/, AY247735/
Notophthalmus	*viridescens, perstriatus*	AF380361/, AF380363/
Taricha	*granulosa, rivularis*	AY627912/, L22880/
Neurergus	*kaiseri, microspilotus, strauchii, crocatus*	/AY147250, /AY147248, /AY147242, /AY147246

