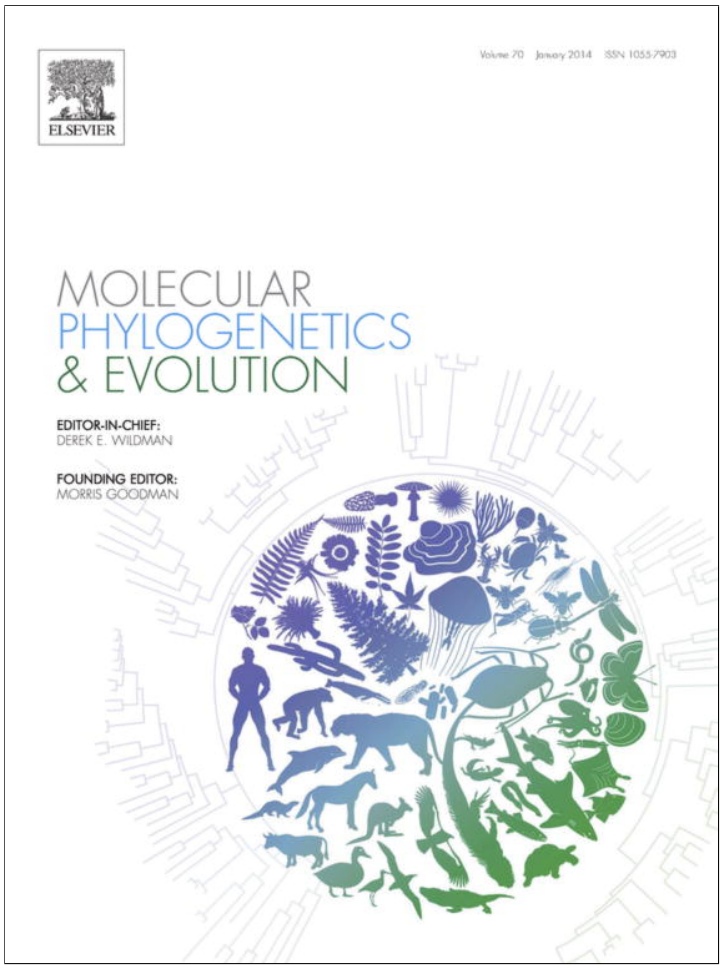

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From species divergence to population structure: A multimarker approach on the most basal lineage of Salamandridae, the spectacled salamanders (genus *Salamandrina*) from Italy

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ABSTRACT

The Apennine Peninsula is one of Europe's main glacial refugial areas and harbors a large number of lineages and species. Here, a pattern of higher genetic diversity in the south compared to that of the north is characteristic of most vertebrates; however, most studies that have produced these results have relied only on inferences based on mitochondrial DNA. The spectacled salamanders (genus *Salamandrina*) are endemic to the Apennine Peninsula and have diverged into two sibling species: *S. terdigitata* (in the south) and *S. perspicillata* (in the north), presumably in the late Miocene or early Pliocene. By sequencing one mitochondrial (*cytb*) and two nuclear genes (*RAG1* and *POMC*) and genotyping 10 microsatellite loci, we traced the evolution of these sibling species from their divergence to their contemporary population structure at a fine scale. Using a multilocus coalescent-based approach, we estimated the temporal divergence of both species at approximately 2.25 mya (million years ago), which, hence, is much younger than previous estimates. The classical pattern of *high genetic diversity in the south and lower diversity in the north* was confirmed only for some markers, and the demographic histories of the two species differed substantially. Whereas *S. perspicillata* (north) expanded from a single major refugium in the center of the Apennine Peninsula, populations of *S. terdigitata* (south) persisted through cooler periods in multiple refugia. Further, the fine-scale population genetic structure of 16 *S. perspicillata* populations revealed significant genetic differentiation, even across short geographic distances. The results of our study stress that for a better understanding of phylogeographic patterns and past demographic processes, both mitochondrial and multiple nuclear loci should be analyzed to avoid gene-specific, and possibly biased results.

1. Introduction

The integration of phylogeographical analyses of multiple taxa into a broader biogeographic context permits the identification of common patterns of spatial genetic diversity across large geographic scales (e.g., Bermingham and Moritz, 1998; Hewitt, 2004; Schmitt, 2007; Taberlet et al., 1998). Phylogeographic patterns of species occurring in temperate zones have been greatly influenced by climatic oscillations starting at the Pliocene–Pleistocene transition and lasting until the final glaciations at the end of the Pleistocene (Hewitt, 2000, 2001). Many temperate animal and plant species were unable to remain in the northern areas that were affected by ice and unfavorable climatic conditions and could only survive in the southern refugia. Glaciations have generated a well-documented pattern observed across many different taxa, showing high genetic diversity and degrees of endemism in the southern refugia, namely in the Iberian and Apennine Peninsulas (Gomez and Lunt, 2007), the Balkan and the Caspian/Caucasus regions (Hewitt, 2004). In contrast, comparably low genetic diversity was observed in rather recently, postglacially recolonized areas (Schmitt, 2007; Taberlet et al., 1998). Recent studies have also underlined the importance of extra-Mediterranean refugia in arctic/alpine species (e.g., Schmitt, 2009) and in continental species (e.g., Schmitt and Varga, 2012). Typically, the refugia acted as sources (sometimes repeatedly) to recolonize areas that became habitable after the ice retreated. Because most European lineages most likely diverged before the last glaciation cycles, in some cases

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even millions of years ago (Hewitt, 2000), it is commonly acknowledged that long separation times of lineages across ancestral geographic ranges, in combination with repeated colonization and extinction events, have shaped the current phylogeographic patterns of animal and plant species across Europe. Whereas initially higher genetic diversity in refugial areas was explained by prolonged demographic stability and larger population sizes, recent evidence has shown that the refugia did not necessarily contain large continuous populations, but frequently became fragmented, generating a pattern of 'refugia within refugia' (Gomez and Lunt, 2007). Isolation and secondary admixture of divergent populations from these microrefugia have played a major role in altering refugia into hotspots of genetic diversity (e.g., Canestrelli et al., 2012).

The Apennine Peninsula is unique among refugial areas in Europe because the mountain chains of the Alps prevented the (initial) postglacial expansion of lineages to the north (Bilton et al., 1998; Hewitt, 2011). It is inhabited by many distinct genetic lineages and endemic species (Buzan et al., 2008; Gippoliti and Amori, 2002; Ruedi et al., 2008), and harbors a significant phylogeographic structure in itself. Most vertebrate taxa from this region show a similar pattern of substantial genetic divergence between northern and southern lineages, with low diversity in the north and higher levels of differentiation in the south. For example, such pattern can be observed for numerous species, such as Mediterranean and Eurasian water shrews (*Neomys anomalus* and *Neomys fodiens*; Castiglia et al., 2007), the red squirrel (*Sciurus vulgaris*; Grill et al., 2009), the Italian mole (*Talpa romana*; Canestrelli et al., 2010), the bank vole (*Myodes glareolus*; Colangelo et al., 2012), the fat dormouse (*Glis glis*; Lo Brutto et al., 2011), the common dormouse (*Muscardinus avellanarius*; Mouton et al., 2012), the European pond turtle (*Emys orbicularis*; Fritz et al., 2005), the Asp viper (*Vipera aspis*; Barbanera et al., 2009), the Italian newt (*Lissotriton italicus*; Canestrelli et al., 2011) and the Italian crested newt (*Triturus carnifex*; Canestrelli et al., 2012). Most estimates of mitochondrial divergence time between southern and northern lineages are based on a single marker [cytochrome *b* (*cytb*)] and range from 0.313 million years ago (mya) (the Apennine fire belly toad, *Bombina pachypus*, Canestrelli et al., 2006) to 3.3 mya (the fat dormouse, Lo Brutto et al., 2011). However, two vertebrate taxa show a strikingly large mitochondrial divergence, indicating that northern and southern lineages on the Apennine chain represent even different species: based on 7% *cytb* sequence divergence, Aesculapian snakes were split into two distinct species (*Zamenis longissimus* and *Z. lineatus*; Lenk et al., 2001), and based on 17% *cytb* sequence divergence and substantial allozyme differentiation ($D_{Nei} = 0.47$) between northern and southern spectacled salamanders, they were considered separate species (*Salamandrina perspicillata* and *S. terdigitata*; Mattoccia et al., 2005; Nascetti et al., 2005). Notably, in both cases, the Volturno-Calore River drainage basin appears to have acted as a phylogeographic break between the southern and the northern species.

To date, most divergence-time estimates that have been applied to taxa that are distributed across the Apennine Peninsula have relied on mitochondrial markers. Whereas most studies have employed phylogenetic methods, some have used coalescent approaches (Canestrelli et al., 2007, 2010; Colangelo et al., 2012). Inferences based on a single gene or several linked genes can also lead to false conclusions because of stochasticity in the coalescent process. In contrast to nuclear genes, mitochondrial genes have a higher probability of showing reciprocal monophyly among different species because of a higher substitution rate and smaller effective population size. Sánchez-Gracia and Castresana (2012) have shown that a combination of mitochondrial and nuclear genes is the most efficient option for recovering species trees and estimating speciation times, particularly in recently diverged species. Obtaining accurate estimates of the divergence time of a species pair is an important prerequisite to understanding the evolutionary history and biogeography of species and the consequences of speciation, such as the degree of ecological differentiation and reproductive isolation.

Spectacled salamanders (genus *Salamandrina*) are endemic to the Apennine Peninsula and represent the sister taxon to a clade comprising all other extant Salamandridae (Steinfartz et al., 2007; Zhang et al., 2008). Despite their current geographic restriction, spectacled salamanders were widespread during the Lower Miocene through the Lower Pliocene according to fossil records found in central Europe, Spain, Sardinia and Greece (reviewed in Mattoccia et al., 2011). They are strikingly different from any other salamandrid species in their external morphology because they are the only salamandrid with four digits on each front and hind limb. Because of their conserved external morphology, their real divergence on the Apennine Peninsula remained cryptic for a long time. Mitochondrial sequence data and allozyme analysis, however, rather recently revealed that the northern and the southern lineages of *Salamandrina* should be considered sister taxa (Mattoccia et al., 2005; Nascetti et al., 2005). Phylogenetic divergence methods based on mitochondrial sequence data and allozyme distance data suggest that the two lineages, *Salamandrina perspicillata* in the north (ranging today from Liguria to northern Campania) and *S. terdigitata* in the south (ranging today from Campania to Calabria), split sometime between 4.7–16.4 mya, depending on the marker and calibration scenario used (Mattoccia et al., 2005, 2011; Nascetti et al., 2005). *Salamandrina*, therefore, shows the deepest divergence between any northern and southern lineage of any Italian vertebrate taxon studied until now. Notably, and despite substantial isolation, a hybrid zone has been found along the Matese Massif of northern Campania (Hauswaldt et al., 2011; Mattoccia et al., 2011).

In this study, we use a multilocus coalescent species-tree approach that was implemented in *BEAST (Heled and Drummond, 2010) to estimate the divergence time of these species. We complemented the phylogeographic information that was recovered from one mitochondrial and two nuclear genes by adding within-species divergence patterns from 10 newly developed microsatellite loci (Hauswaldt et al., 2012). Our study design encompasses 900 individuals from 37 localities across both species' ranges and was used to examine different levels of divergence from species differentiation to demographic processes within species.

2. Material and methods

2.1. Sampling and localities

A total of 900 individual salamanders were sampled from 32 localities across the range of *Salamandrina perspicillata* and from 5 localities across the range of *S. terdigitata* (Table 1, Fig. 1) with the permission of the Ministero dell'Ambiente e della Tutela del Territorio (DCN/2D/2003/15281). Sixteen of the 32 collection sites of *S. perspicillata* represent locations in the Lepini Mountains (see inset of Fig. 1), an area approximately 700 km^2 located 50 km southeast of Rome. Within the Lepini Mountains, individuals were sampled from 5 locations in the eastern chain of the mountain range and from eleven sites in the western chain of the mountain range. Genomic DNA was obtained from tail tips of adult and larval individuals, and also from eggs. Following sampling, all individuals were immediately released at the site of capture.

2.2. DNA extraction, amplification and sequencing

We amplified and sequenced 6–24 individuals per population for one gene fragment of the mitochondrial DNA (mtDNA) genome

Table 1
Sampling sites of *Salamandrina perspicillata* and *S. terdigitata*, sample code, geographic region (N = north, C = central, L–E = eastern and L–W = western chains of the Lepini, S = south), and the number of individuals analysed for each marker (Msat = microsatellite analysis).

	Locality name (abbreviation)	Code	Region	*cytb* (682 bp)	*POMC* (*341 bp*)	*RAG1* (*570 bp*)	Msat
Salamandrina perspicillata							
1	Rio Riazzu	RR	N	18	17	16	20
2	Bologna	BC	N	15	9	9	12
3	Molino del Pallone	MP	N	8	11	11	20
4	Borro al Diavolo	BD	N	15	12	11	22
5	Montaione	MO	N	17	8	8	21
6	Monte Nerone	MN	N	15	10	11	23
7	Sefro	SO	N	16	8	16	17
8	Mattere	MT	C	18	9	10	24
9	Terni	ER	C	21	12	10	20
10	Torrente Rigo	TR	C	10	10	10	14
11	Sorgente Pero	SP	C	16	8	9	19
12	Monte Livata	ML	C	15	8	11	18
13	Belle Facce	BF	C	18	8	8	–
14	Donzella	DO	C	23	19	11	17
15	Lombetto	LO	L–E	15	12	9	18
16	Pisciarello	PI	L–E	15	9	10	22
17	Cima Gionara	CG	L–E	14	12	8	19
18	Acquaviva	AQ	L–W	19	15	11	13
19	Rapiglio	RA	L–W	15	11	11	24
20	Sant' Angelo	SA	L–W	19	8	9	35
21	Valle Ota	VO	L–W	16	13	10	21
22	Capoccigli	CP	L–W	21	8	8	17
23	Acqua della Chiesa	AC	L–W	17	8	10	16
24	Ciccopano Nuove	CN	L–W	21	10	10	20
25	Ciccopano	CI	L–W	22	9	10	13
26	Le Pretelle	LP	L–W	24	8	8	32
27	Castellone	CA	L–W	16	8	10	15
28	Sant Erasmo	SE	L–W	21	13	12	22
29	San Martino	SM	L–E	24	10	10	15
30	La Roleca	RL	L–E	19	9	8	24
31	Majella	MJ	S	12	14	14	16
32	Casina Mainarde	CM	S	16	17	19	24
Σ				551	343	338	613
S. terdigitata							
33	Torrente Cerasuolo	ST		24	14	14	19
34	Vallone Cupo	VC		18	18	15	24
35	Foresta	FO		21	9	14	24
36	Torrente Rosa	SR		18	9	6	–
37	Fiumara Samo	FS		19	20	11	20
Σ				100	70	60	87

(*cytb*) and for two nuclear gene fragments: the proopiomelanocortin A gene (POMC) and the recombination activating gene 1 (RAG1) (see Table 1 for a general overview and Supplementary Table S4 for a detailed overview). The details of DNA extraction, primers, PCR and sequencing protocols, as well as sequence editing, haplotype identification, haplotype-network reconstruction, and basic sequence-diversity analyses (haplotype and nucleotide diversity), are described elsewhere (Hauswaldt et al., 2011). The haplotypes of all the genes were deposited in GenBank (accession numbers KF430817–KF431826), and alignments of (phased) sequence sets in the Dryad repository.

2.3. Divergence-time estimations between S. perspicillata and S. terdigitata

We used a coalescent-based approach to date the split between *S. perspicillata* and *S. terdigitata* using multilocus sequence data of nuclear and mitochondrial origin from multiple individuals sampled across the ranges of both species (Table 1). *BEAST (Heled and Drummond, 2010) is part of the BEAST v1.6.1 package (Drummond and Rambaut, 2007) and estimates a species tree from multiple gene trees along with the change in effective population size. According to simulations, the method outperforms a strict phylogenetic approach (Heled and Drummond, 2010). To calibrate the *BEAST inference, we estimated a substitution rate for the *cytb* gene, for which a large number of salamandrid species-specific haplotypes and various alternative calibration points are available. Accordingly, we included all (22) *cytb* haplotypes of *Salamandrina* and 34 additional species-specific salamandrid haplotypes (Table S1) to reconstruct a fossil-calibrated gene tree using BEAST. The sequences were aligned in MEGA5 (Tamura et al., 2011) using the MUSCLE algorithm (Edgar, 2004). Here and throughout the study, the best-fitting substitution model was estimated using the Akaike Information Criterion implemented in JMODELTEST v0.1.1 (Posada, 2008). Based on these results, we used the HKY85 (Hasegawa et al., 1985) model with invariant sites, a gamma heterogeneity distribution (HKY + G + I) and codon partitioning. To calibrate the *cytb* gene tree, we constrained four nodes using uniform priors from previous studies. Accordingly, we used the fossil calibration points C3 (23–50 mya) and C5 (15–30 mya) applied previously in the study of Steinfartz et al. (2007). The upper bounds for the fossil calibrations were defined according to the confidence interval of the corresponding nodes of the Salamandridae chronograms generated by Zhang et al. (2008). Additionally, the split between the Corsica-Sardinia *Euproctus* newts and the continental *Triturus* species complex was constrained to 20–30 mya (following Zhang et al., 2008), and the separation between *Euproctus platycephalus* on Sardinia and *E. montanus* on Corsica was constrained to 9–15 mya following Caccone et al. (1997). We used a log-normal relaxed clock and a Yule process model of speciation (Drummond

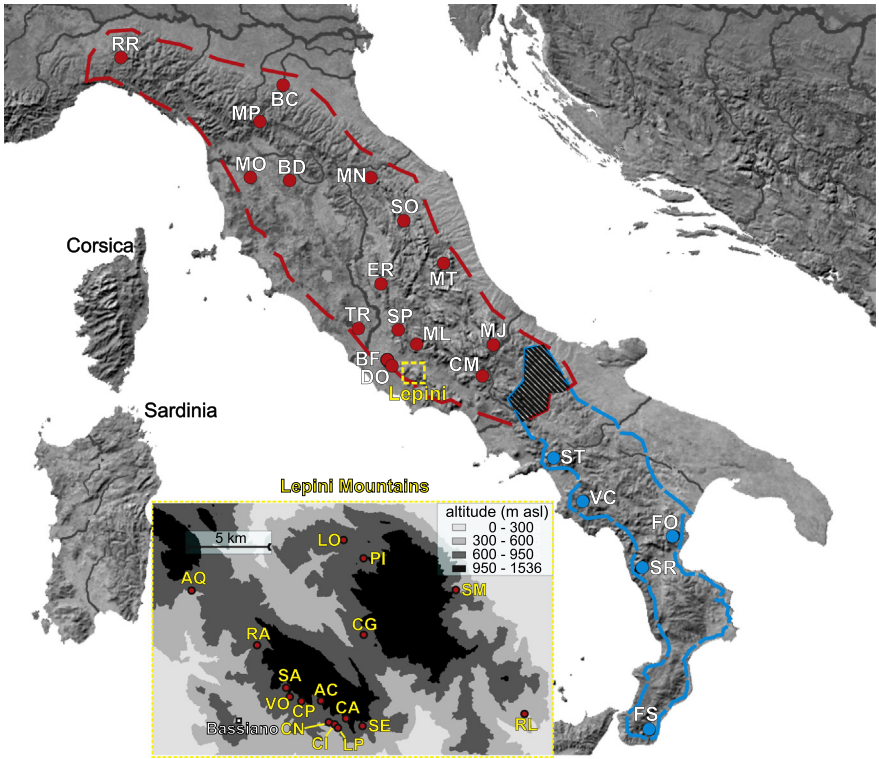

Fig. 1. Map showing geographic distribution and sampling locations for *Salamandrina perspicillata* (in red) and *S. terdigitata* (in blue), and hatched the contact zone. The insert shows the 16 sampling sites from the Lepini Mountains. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

et al., 2006; Yule, 1924). Five independent chains of 50×10^6 generations were run, sampled every 5,000 generations, discarding the initial 10% as burn-in. Convergence and mixing of runs were checked with TRACER v1.5 (Rambaut and Drummond, 2009). The five runs were combined, and the resulting median of the posterior distribution of the parameter *meanRate* was used as a *cytb* mutation rate in the following multi-locus divergence-time estimations.

Subsequent *BEAST species-tree analyses were performed using the *cytb*, *POMC* and *RAG1* datasets simultaneously. Because multiple individuals of each species are required to estimate all the species-tree parameters, we performed the analysis using only two species of *Salamandrina* because most other salamandrid species were represented in GenBank only by single individuals or not represented at all for *RAG1* and *POMC* genes. Before estimating the species-tree, however, we had to reduce the dataset to allow for realistic computation times using a randomization process (www.random.org). Individuals were randomly drawn from all five populations of *S. terdigitata* and from all 17 populations of *S. perspicillata* (all the populations outside the Lepini Mountains and a single randomly drawn population from the Lepini Mountains (CA); refer to Table S4). For each species, 100 sequences were randomly chosen from the *cytb* dataset and 60 from the *RAG1* dataset. Because of the limited variation in the *POMC* gene dataset, we used only 20 randomly chosen individuals (i.e., 40 phased sequences derived from 20 individuals) of *S. perspicillata* and 35 individuals of *S. terdigitata* (see Table S4). Whereas the HKY substitution model fit the *cytb* dataset the best, the Jukes-Cantor model was selected for the two nuclear genes. Before running the multilocus analysis, we ran each gene separately under a log-normal relaxed clock model and a species-tree prior. We performed these exploratory runs to optimize the parameters and operators, to check for independent convergence of each gene-tree, and to check whether a relaxed clock model fits the data. We rejected the relaxed clock model for all three genes, as the posterior distribution of the standard deviation of the log-normal relaxed clock (*stdv.ucld*) included zero. Therefore, the species-tree based on all three genes was inferred using a strict clock model. To calibrate the species-tree, we fixed the *cytb* substitution rate to the value derived from the calibrated gene-tree (see above). The mutation rates for *RAG1* and *POMC* were co-estimated using the same software. We ran five chains of 5×10^8 steps, sampling every 5×10^4. Mixing and convergence of the runs were checked with TRACER. The results of the five runs were combined, and the median and confidence intervals of the posterior distribution of the *speciestree.rootHeight* parameter was used as an estimate of divergence time between the two species.

2.4. Microsatellite loci genotyping and analyses

We genotyped between 13 and 35 individuals from 35 populations for 10 microsatellite loci (Tables 1 and S4). Individuals from BF (*S. perspicillata*) and SR (*S. terdigitata*) were not genotyped. Details on the primer combinations used, the amplification, and the genotyping protocols are described in detail in Hauswaldt et al. (2012). MICRO-CHECKER (Van Oosterhout et al., 2004) was used to test for the presence of null alleles, large allele dropouts and scoring errors because of stutter peaks. All the loci were tested for departure from linkage equilibrium using ARLEQUIN 3.5.1.2 (Excoffier and Lischer, 2010) with 10^4 permutations. Estimates of allelic diversity, i.e., expected (H_E) and observed (H_O) heterozygosities, were obtained using GENALEX (Peakall and Smouse, 2006). FSTAT (Goudet, 2001) was used to calculate allelic richness (A_R) based on a minimum of 11 individuals per population. With the identical software, we also tested for differences in genetic diversity, measured as allelic richness, and F_{ST}, H_O and H_E between the northern, central and Lepini populations of *S. perspicillata*. All tests

were conducted using 10^4 randomizations. GENEPOP (Raymond and Rousset, 1995) was used to assess the Hardy–Weinberg equilibrium of each locus and population using 10^4 dememorizations, 10^3 batches and 10^4 iterations per batch.

2.5. Analysis of large-scale population structure within species

We analyzed the large-scale structure of *S. perspicillata* and *S. terdigitata* populations based on sequence and microsatellite loci datasets. For the microsatellite loci, we used the multilocus Bayesian clustering of individual genotypes implemented in BAPS5 (Corander et al., 2008) to infer the population structure within both species separately. As a first step, a group-level mixture analysis was performed to define the number of clusters followed by an admixture analysis based on the mixture clustering of individuals. All the analyses were run for different values of assumed genetic clusters (K) with ten replicate runs for each value of K. The K with the highest posterior probability (across the 10 runs) was considered the most likely number of genetic clusters. We analyzed the population genetic structure within *S. perspicillata* for the complete set of 31 populations with K ranging from 2 to 31. For *S. terdigitata*, the population structure was analyzed for four populations with K ranging from 2 to 4.

The population structure was investigated further by F_{ST} (microsatellite loci) and Φ_{ST} (*cytb*: Kimura 2P model) analyses using ARLEQUIN. The significance was tested using 10^4 permutations. The partitioning of genetic variance was further examined by analysis of molecular variance (AMOVA; Excoffier et al., 1992) as implemented in ARLEQUIN. For a hierarchical AMOVA for the microsatellite loci and *cytb* sequence data within *S. perspicillata*, we grouped populations into the groups indicated by the Bayesian clustering analysis (see above) and obtained significance levels of the components of variance and the corresponding statistics by comparing the actual values with the distribution of 10,000 randomization values.

2.6. Test of population expansion

We tested different groupings of populations for signs of recent population expansion based on sequence data: (1) all 32 populations of *S. perspicillata*, (2) the 16 populations of *S. perspicillata* from the Lepini populations and (3) all 5 populations of *S. terdigitata*. We calculated Tajima's (1989) D statistics, Fu's (1997) Fs and Ramos-Onsins and Rozas's (2002) R_2 with DnaSP 5.10.01 (Librado and Rozas, 2009) for all three gene segments. Significantly negative values of Tajima's D reflect an excess of rare variation, indicating either population expansion or positive selection. Whereas this test is based on nucleotide diversity, Ramirez-Soriano et al. (2008) found that the most powerful tests for detecting sudden population expansion or bottlenecks are those based on haplotype frequencies such as the Fs statistic of Fu (1997) and the R_2 statistics of Ramos-Onsins and Rozas (2002), which compares the difference between the number of singleton mutations and the average number of nucleotide differences. For example, a population that has experienced a bottleneck will show large statistically significant positive values for Fs, indicating a deficit of rare haplotypes.

The significance of D, Fs and R_2 were determined using the coalescent algorithm implemented in DnaSP by comparing the observed value with a null distribution generated by 10^3 replicates, the empirical sample size and the observed number of segregating sites. For the genotype data, we used the Wilcoxon test as implemented in BOTTLENECK (Cornuet and Luikart, 1996) to test whether any population had experienced a recent bottleneck. This test compares whether the observed heterozygosity is higher than expected from the observed number of alleles under the assumption of mutation-drift equilibrium. We used the recommended settings for the two-phase model with 95% single-step, 5% multi-step mutations and a variance among multiple steps of 12 (Piry et al., 1999).

2.7. Analysis of fine-scale population structure within the Lepini area (S. perspicillata)

Because we were also interested in analyzing the current levels of gene flow within species, we studied the fine-scale population structure among sixteen populations in the Lepini Mountains based on the differentiation of microsatellite loci (Fig. 1). We used BAPS5 to estimate the number of genetic clusters with K ranging from 2 to 16 and 10 runs per K. Additionally, we tested for panmixia between every pair of sampled locations using the allele frequency heterogeneity contingency tests of Raymond and Rousset (1995). According to Waples and Gaggiotti (2006), this simple algorithm is powerful for detecting distinct population units, even if the number of migrants per generation is comparably large. To combine the P values for the different loci, we used chi-square tests implemented in TFPGA v1.3 (Miller, 1997), which have proven to be more powerful when the number of alleles is small (Ryman et al., 2006). In TFPGA, we used 2000 dememorization steps and 20 batches with 5000 permutations per batch. For these and all the other statistical tests, we used 5% as critical significance level, and if multiple tests were performed, we corrected the P-values according to a Bonferroni sequential adjustment (Rice, 1989).

3. Results

3.1. Mitochondrial and nuclear sequence variation

For 552 individuals of *S. perspicillata*, we obtained 682 bp (aligned positions) of the *cytb* gene and found 16 segregating sites, of which six represented non-synonymous substitutions, representing 17 distinct haplotypes. Four of these haplotypes ($Cytb_{I}$, $Cytb_{VII}$, $Cytb_{XX}$, $Cytb_{XXI}$) were shared among populations of *S. perspicillata* (Table 2 and Fig. 2). For the 100 *S. terdigitata* individuals that were analyzed, we found seven segregating sites, all representing synonymous substitutions, representing five distinct haplotypes. Here, only the common haplotype ($Cytb_{X}$) was shared by four populations, whereas population FO was fixed for haplotype $Cytb_{XIII}$, which differs from all the remaining haplotypes by at least four mutational steps. As a consequence, the average haplotype diversity was higher in *S. terdigitata* (0.4) than in *S. perspicillata* (0.26). None of the *cytb* haplotypes were shared between species, and haplotypes differed by at least 96 mutational steps. Whereas within-species uncorrected average p-distance was 0.00042 and 0.00208 for *S. perspicillata* and *S. terdigitata*, respectively, it was 0.145 between species.

Compared to the mitochondrial gene segment, the nucleotide variation and the number of haplotypes were low for the nuclear *POMC* (341 bp) and *RAG1* (570 bp) gene segments in both species (Table 2). The genetic diversity of *POMC* was much lower in *S. perspicillata* than in *S. terdigitata*, with a 30-fold lower haplotype and an 80-fold lower nucleotide diversity. Haplotype $POMC_{I}$ was shared among all the individuals of the northern species, and only five individuals were heterozygous with another allele ($H_O = 0.014$). Among the 70 individuals of *S. terdigitata*, seven segregating sites defined five haplotypes, and 13 individuals were heterozygous ($H_O = 0.18$; see Table 2). For this marker, we also found evidence of haplotype sharing between species: the dominant haplotype $POMC_{I}$ in *S. perspicillata* was also found in two individuals from population FS (one homozygous, the other heterozygous) and in one homozygous individual from population SR (see Fig. 2). The uncorrected average p-distance for *POMC* within *S.*

Table 2
Summary of regional and within-population diversity for *cytb*, *POMC*, and *RAG1* gene sequences; number of individuals sequenced per population (N); number of haplotypes (H); haplotype diversity (h); sequence diversity (k); nucleotide diversity $\times 10^2$ (π), observed heterozygosity (Ho). Summary and average values for the two species and the Lepini Mountains are in bold.

Site	*cytb*						*POMC*							*RAG1*				
	N	H	Ht	h	k	π	N	H	Ht	h	k	π	Ho	N	H	Ht	h	k	π	Ho
RR	18	1	I	–	–	–	17	1	I	–	–	–	–	16	5	I, II, III IV, V	0.615	3.23	0.567	0.438
BC	15	2	I, II	0.133	0.133	0.02	9	1	I	–	–	–	–	9	3	I, IV, V	0.621	3.954	0.694	0.778
MP	8	1	I	–	–	–	11	1	I	–	–	–	–	11	3	I, IV, V	0.680	3.961	0.695	0.636
BD	15	1	I	–	–	–	12	1	I	–	–	–	–	11	3	I, IV, V	0.558	3.338	0.586	0.636
MO	17	2	I, XIV	0.382	0.382	0.056	8	1	I	–	–	–	–	8	3	I, IV, V	0.708	4.250	0.476	0.625
MN	15	2	I, XV	0.284	0.284	0.036	10	1	I	–	–	–	–	11	3	I, IV, V	0.662	3.779	0.663	0.545
SO	16	1	I	–	–	–	8	1	I	–	–	–	–	16	2	I, IV	0.063	0.313	0.055	0.063
MT	18	1	I	–	–	–	9	1	I	–	–	–	–	10	3	I, IV, V	0.589	3.537	0.620	0.600
ER	21	4	I, VI, VII, VIII	0.557	0.6570	0.096	12	1	I	–	–	–	–	10	3	I, IV, V	0.637	4.032	0.707	0.600
TR	10	2	I, VII	0.356	0.356	0.052	10	1	I	–	–	–	–	10	2	IV, V	0.479	2.874	0.504	0.700
SP	16	4	I, XVIII, IX, XX	0.650	0.775	0.114	8	1	I	–	–	–	–	9	4	I, IV, V, XI	0.739	4.131	0.725	0.333
ML	15	2	XVI, XVII	0.133	0.267	0.039	8	1	I	–	–	–	–	11	2	I, V	0.247	1.727	0.303	0.273
BF	18	2	I, XXI	0.523	0.523	0.077	8	1	I	–	–	–	–	8	3	I, IV, V	0.575	3.05	0.535	0.500
DO	23	1	I	–	–	–	19	1	I	–	–	–	–	11	4	I, IV, V, X	0.636	3.779	0.663	0.818
LO	15	1	I	–	–	–	12	2	I, VIII	0.083	0.083	0.024	0.083	9	3	I, IV, V	0.66	4.02	0.705	0.778
PI	15	1	I	–	–	–	9	1	I	–	–	–	–	10	3	I, IV, V	0.674	4.211	0.739	0.700
CG	14	2	I, XX	0.143	0.143	0.021	12	1	I	–	–	–	–	8	3	I, IV, V	0.708	4.208	0.738	0.875
AQ	19	2	I, XXI	0.105	0.105	0.015	15	2	I, II	0.067	0.067	0.02	0.067	11	3	I, IV, V	0.628	4.004	0.703	0.727
RA	16	1	I	–	–	–	11	1	I	–			–	11	3	I, IV, V	0.658	4.121	0.723	0.700
SA	19	1	I	–	–	–	8	1	I	–			–	9	3	I, IV, V	0.68	4.183	0.734	0.667
VO	16	1	I	–	–	–	13	2	I, II	0.077	0.077	0.023	0.077	10	3	I, IV, V	0.695	4.168	0.731	0.700
CP	21	2	I, XXII	0.257	0.257	0.038	8	1	I	–	–	–	–	8	3	I, IV, V	0.508	2.958	0.519	0.500
AC	17	1	I				8	2	I, II	0.125	0.125	0.037	0.125	10	4	I, IV, V, X	0.689	4.142	0.727	0.500
CN	21	2	I, XX	0.495	0.495	0.073	10	1	I	–	–	–	–	10	3	I, IV, V	0.584	3.632	0.637	0.500
CI	22	1	I				9	1	I	–	–	–	–	10	3	I, IV, V	0.542	3.321	0.583	0.700
LP	24	2	I, XXIII	0.083	0.083	0.012	8	1	I	–	–	–	–	8	2	I, V	0.233	1.633	0.287	0.250
CA	16	2	I, XXIV	0.458	0.458	0.067	8	1	I	–	–	–	–	10	3	I, IV, V	0.674	4.211	0.739	0.700
SE	21	1	I	–	–	–	13	2	I, II	0.077	0.077	0.023	0.077	12	3	I, IV, V	0.62	3.804	0.667	0.583
SM	24	1	I	–	–	–	10	1	I	–	–	–	–	10	3	I, IV, V	0.532	3.395	0.596	0.600
RL	19	1	I	–	–	–	9	1	I	–	–	–	–	8	3	I, IV, V, IX	0.742	4.417	0.775	0.625
Lepini	**298**	**6**	**I, XX–XXIV**	**0.123**	**0.125**	**0.018**	**163**	**3**	**I, II, VIII**	**0.030**	**0.030**	**0.009**	**0.031**	**154**	**5**	**I, IV, V, IX, X**	**0.635**	**3.90**	**0.684**	**0.545**
MJ	12	1	I	–	–	–	14	1	I	–	–	–	–	14	2	I, V	0.349	2.444	0.429	0.286
CM	16	2	I, III	0.400	0.400	0.059	17	1	I	–	–	–	–	19	3	I, IV, V	0.582	3.760	0.660	0.526
S. per.	**551**	**17**	**I–VI, VII–VIII, XIV–XXIV**	**0.264**	**0.286**	**0.042**	**343**	**3**	**I, II, VIII**	**0.015**	**0.015**	**0.004**	**0.014**	**338**	**8**	**I, II, III, IV, V, IX, X XI**	**0.645**	**3.877**	**0.680**	**0.559**
ST	24	3	IX, X, XI	0.236	0.243	0.036	14	1	VII	–	–	–	–	14	3	VI, VII, VIII	0.455	0.772	0.136	0.286
VC	18	2	X, XII	0.111	0.111	0.016	18	2	IV, VII	0.322	0.644	0.189	0.278	15	2	VI, VII	0.186	0.372	0.065	0.200
FO	21	1	XIII	–	–	–	9	1	IV	–	–	–	–	14	3	VI, VII, VIII	0.140	0.209	0.037	0.071
SR	18	1	X	–	–	–	9	3	I, IV, VII	0.523	1.359	0.399	0.444	6	2	VI, VII	0.303	0.606	0.106	–
FS	19	1	X	–	–	–	20	5	I, III, IV, VI, VII	0.277	1.000	0.293	0.20	11	2	VI, VII	0.519	1.039	0.182	0.545
S. ter.	**100**	**5**	**IX – XIII**	**0.397**	**1.42**	**0.208**	**70**	**5**	**I, III, IV, VI, VII**	**0.468**	**1.116**	**0.327**	**0.186**	**54**	**3**	**VI, VII, VIII**	**0.415**	**0.780**	**0.137**	**0.217**

perspicillata was 0.00004 and within *S. terdigitata* was 0.00327, whereas it was 0.012 between species.

In contrast to *POMC*, the genetic diversity of *RAG1* was higher in *S. perspicillata* (eleven segregating sites producing eight haplotypes, three of them shared among all the populations) than in *S. terdigitata* (two segregating sites producing three haplotypes were found for the 60 individuals analyzed). Every population of *S. perspicillata* contained at least two haplotypes, and the highest number of haplotypes ($n = 5$) was found in the northernmost population, RR (Table 2 and Fig. 2). The average observed heterozygosity that was found for *S. perspicillata* ($H_O = 0.56$) was more than twice as high as that for *S. terdigitata* ($H_O = 0.22$). The uncorrected average *p*-distance within each species was 0.006 and 0.001, respectively, and it was 0.007 between the two species.

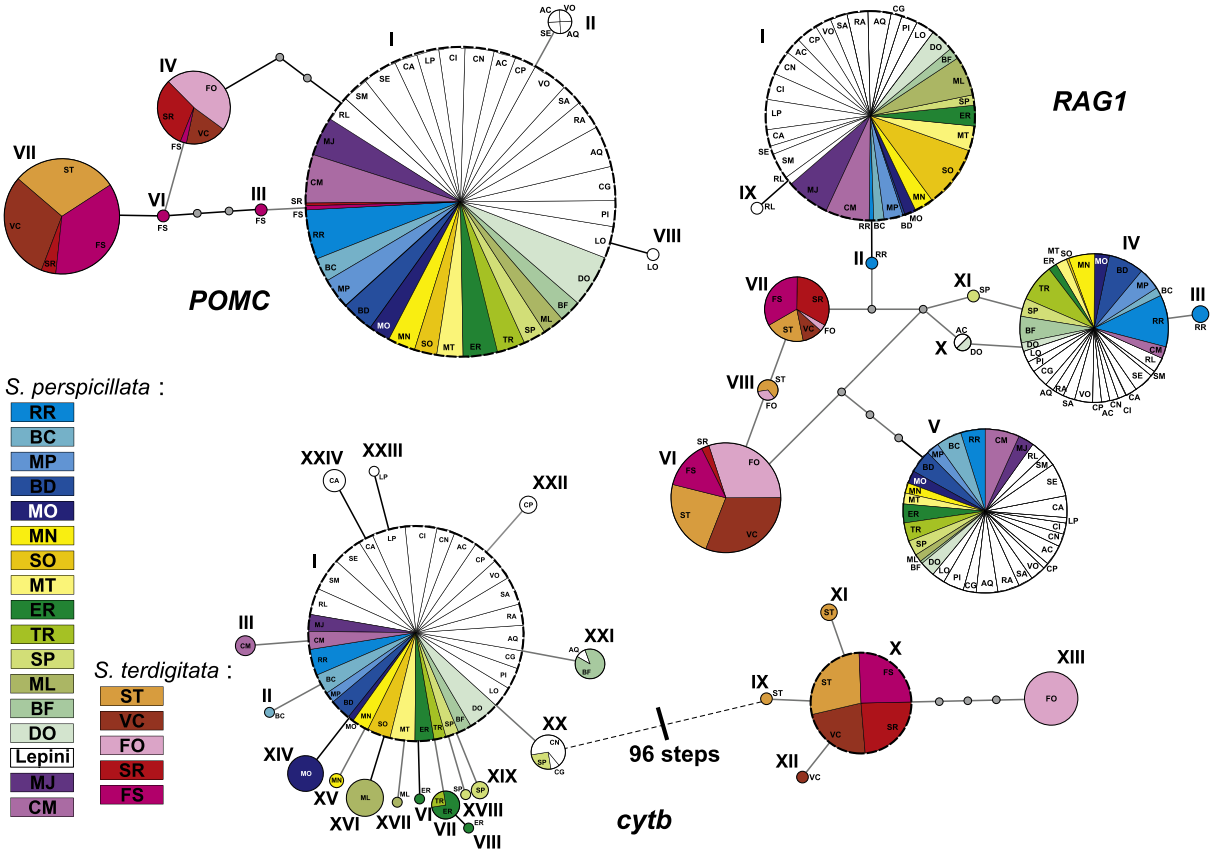

Fig. 2. Parsimony networks for the cyt*b*, *RAG1* and *POMC* genes for *Salamandrina perspicillata* and *S. terdigitata*. Connections between haplotypes equal one mutational step unless indicated otherwise. Small grey circles indicate unsampled or missing haplotypes. Gray connections indicate synonymous and black connections nonsynonymous mutations. Circle sizes represent number of copies found of each haplotype, and pie slices indicate the frequency of a haplotype in a given population. E.g. $Cytb_{XIII}$ was found in 12 individuals in population FO; $POMC_{IV}$ was found 6 times in VC. Note that per nuclear gene two copies of homologous DNA are sampled per individual. A dotted line around a haplotype indicates that it is inferred to be the ancestral haplotype for the network.

3.2. Divergence time

The substitution rate per lineage of the analyzed *cytb* gene segment for the Salamandridae was 1.02%/site/my (95% HPD: 0.86–1.22%) based on four fossil/geological calibration points using the conventional phylogenetic approach of BEAST. By fixing the substitution rate for the *cytb* gene while co-estimating the mutation rates of both nuclear genes (*POMC* and *RAG1*), the multilocus species-tree approach with *BEAST estimated that the divergence between the two species of *Salamandrina* occurred approximately 2.25 mya (95%HPD: 582,000 years – 6.4 mya). We observed convergence of the MCMC runs, with similar results found across five independent runs for the mutation rate estimation and species-tree inference (*BEAST runs), and all the parameters had effective samples sizes (ESS) larger than 200.

3.3. Microsatellite variation

No evidence for linkage disequilibrium was found for any pair of loci. MICRO-CHECKER showed no evidence of scoring errors because of stuttering or large allele dropout. Whereas some loci differed significantly from Hardy–Weinberg expectations in single populations, which might be attributable to the presence of null alleles (e.g., NG4 and B8), this pattern was not consistent across populations, and therefore, we included all loci in further analyses (Table 3). Whereas in *S. perspicillata* all the loci were polymorphic, locus D4 was fixed in *S. terdigitata*. Genetic diversity, as assessed by A_R, H_O and H_E, was higher in *S. perspicillata* than in *S. terdigitata* (Table 3). In *S. perspicillata*, genetic diversity was significantly lower in the six northern populations when compared to the central populations: $A_R = 3.52$ vs. 4.76 ($P < 0.001$), H_O=0.51 vs. 0.66 ($P = 0.013$) and $H_E = 0.49$ vs. 0.67 ($P < 0.001$). Among the 16 populations from the Lepini Mountains, the levels of genetic diversity were on average slightly lower than those of the central populations (Table 3). In *S. terdigitata*, FO showed the highest genetic diversity, and one private allele (241 at locus NC7) was found at a high frequency (>30%). Across all the loci and individuals analyzed, a total of 114 alleles were found, with 49 alleles that were diagnostic for *S. perspicillata* and eight for *S. terdigitata*.

3.4. Population genetic structure and evidence of demographic changes

The AMOVA revealed significant population structure within *S. perspicillata* ($cytb\Phi_{ST} = 0.43$, microsatellite loci $F_{ST} = 0.15$, both $P < 0.0001$; Table 4). On a large scale (i.e., excluding the area of the Lepini Mountains), all the pairwise population F_{ST} comparisons for *S. perspicillata* that were based on the microsatellite loci showed significant differentiation with the exception of three comparisons (MN × BC, MN × MP, and SO × MO; Table S4). Among the four populations of *S. terdigitata*, the AMOVA results indicated even stronger population structure compared with *S. perspicillata* ($cytb\Phi_{ST} = 0.95$, microsatellite loci $F_{ST} = 0.19$, both $P < 0.0001$), with all the pairwise comparisons significantly different from zero (Tables 4 and S2).

Table 3
Summary of the analyses of ten microsatellite loci of *Salamandrina perspicillata* and *S. terdigitata*. *N*, number of individuals analysed; AR, mean allelic richness (±SD) based on at least 11 individuals; Ho, observed heterozygosity (±SD); He, expected heterozygosity (±SD); HWE, loci out of Hardy–Weinberg equilibrium (HWE) after Bonferroni corrections; A_N, loci that show potential problems with null alleles; AP, number of private alleles.

	N	A_R	H_o	H_e	HWE	A_N	A_P
RR	20	3.85 ± 1.73	0.39 ± 0.26	0.45 ± 0.3	NG4	NG4	–
BC	12	3.25 ± 1.44	0.55 ± 0.29	0.50 ± 0.16	NG4	10H	–
MP	20	3.91 ± 2.1	0.61 ± 0.22	0.55 ± 0.19	–	–	1
BD	22	3.03 ± 1.26	0.52 ± 0.29	0.45 ± 0.2	NG4	–	–
MO	21	2.91 ± 1.07	0.43 ± 0.28	0.39 ± 0.25	–	–	–
MN	23	4.17 ± 1.54	0.52 ± 0.2	0.56 ± 0.19	10H, B8	10H; B8	–
SO	17	3.51 ± 1.21	0.55 ± 0.2	0.54 ± 0.15	–	–	–
Mean North		**3.52 ± 1.51**	**0.51 ± 0.07**	**0.49 ± 0.06**	**–**	**–**	
MT	24	4.66 ± 1.49	0.65 ± 0.17	0.68 ± 0.09	10H, NG4	NG4	–
ER	20	5.13 ± 2.52	0.71 ± 0.16	0.67 ± 0.17	A3X	–	1
TR	14	3.90 ± 1.61	0.53 ± 0.22	0.58 ± 0.16	NG4	D4	1
SP	19	5.30 ± 1.89	0.64 ± 0.16	0.70 ± 0.11	–	B8; D4	1
ML	18	5.45 ± 1.64	0.72 ± 0.11	0.73 ± 0.09	–	–	–
DO	17	4.14 ± 1.29	0.71 ± 0.16	0.63 ± 0.12	–	–	1
Mean Central		**4.76 ± 1.80**	**0.66 ± 0.07**	**0.67 ± 0.05**	**–**	**–**	
LO	18	4.76 ± 1.76	0.64 ± 0.19	0.65 ± 0.14	–	H2	–
PI	22	5.06 ± 2.04	0.67 ± 0.18	0.66 ± 0.14	10H	D4	1
CG	19	4.84 ± 1.9	0.58 ± 0.25	0.63 ± 0.19	–	NG4	1
AQ	13	5.33 ± 1.53	0.62 ± 0.15	0.66 ± 0.09	NG4	B8; NG4	–
RA	24	4.38 ± 1.42	0.58 ± 0.2	0.59 ± 0.18	–	D4	1
SA	35	4.93 ± 1.72	0.69 ± 0.14	0.69 ± 0.1	NG4	D4	2
VO	21	4.72 ± 1.7	0.64 ± 0.12	0.66 ± 0.09	–	–	–
CP	17	4.76 ± 1.43	0.69 ± 0.16	0.69 ± 0.09	NG4	NG4	–
AC	16	4.87 ± 1.88	0.67 ± 0.21	0.65 ± 0.14	3F	–	–
CN	20	4.58 ± 1.29	0.70 ± 0.12	0.66 ± 0.08	–	–	–
CI	13	4.17 ± 1.19	0.66 ± 0.12	0.59 ± 0.12	–	–	–
LP	32	3.88 ± 1.06	0.59 ± 0.2	0.58 ± 0.15	–	D4	–
CA	15	4.98 ± 1.17	0.67 ± 0.18	0.66 ± 0.1	–	NG4	–
SE	22	4.34 ± 1.53	0.55 ± 0.2	0.61 ± 0.18	B8	A11; B8; NG4	–
SM	15	4.83 ± 1.96	0.70 ± 0.15	0.66 ± 0.12	–	–	–
RL	24	4.70 ± 1.66	0.64 ± 0.22	0.61 ± 0.19	–	B8	2
Mean Lepini		**4.69 ± 1.57**	**0.64 ± 0.05**	**0.64 ± 0.03**	**–**	**–**	
MJ	16	3.98 ± 1.26	0.54 ± 0.16	0.54 ± 0.18	–	–	–
CM	24	4.08 ± 1.79	0.48 ± 0.24	0.51 ± 0.23	D9X, NG4	NG4	1
Mean *S. perspicillata*		**4.40 ± 1.67**	**0.61 ± 0.21**	**0.60 ± 0.17**	**–**	**–**	
ST	19	2.98 ± 1.76	0.35 ± 0.35	0.38 ± 0.31	D9X	D9X	–
VC	24	3.23 ± 2.14	0.32 ± 0.32	0.33 ± 0.31	–	D9X; NG4	2
FO	24	4.27 ± 3.08	0.48 ± 0.31	0.53 ± 0.32	A3X, D9X, NG4	NG4	1
FS	20	3.12 ± 1.83	0.36 ± 0.31	0.38 ± 0.26	A11	A11; D9X	–
Mean *S. terdigitata*		**3.40 ± 2.24**	**0.38 ± 0.31**	**0.41 ± 0.30**	**–**	**–**	

The Bayesian clustering analysis (BAPS) of 613 individuals from 31 populations of *S. perspicillata* that were genotyped for ten microsatellite loci yielded an optimal partition into 13 genetic clusters (Fig. 3a). In the north, populations RR, BD and MO were recovered as single clusters, whereas all the individuals from BC, MP, MN and SO formed one genetic unit. Similarly, the central populations MT, ER and SP shared the same cluster, whereas TR, ML and DO each could be distinguished as separate genetic entities. When analyzed at a large-scale, three distinct genetic clusters could be found for the Lepini Mountains (Fig. 3a). Individuals of population LP and individuals of SE together with those from RL formed separate clusters. The third cluster was represented by individuals from the remaining populations. Finally, individuals from the two southernmost populations of *S. perspicillata*, MJ and CM, each were assigned to separate genetic clusters. Using these 13 groups, the AMOVA partitioned a significant proportion of the genetic variance among groups (microsatellite loci: 12.6%, *cytb*: 31.4%; Table 4). In *S. terdigitata,* the BAPS analysis assigned each individual unambiguously to its population of sampling, and each population itself formed a separate genetic entity (Fig. 3c).

Evidence for population expansion based on the DNA sequence data was found for *S. perspicillata* but not for *S. terdigitata* (Table S3). For *S. perspicillata*, the combined *cytb* dataset of 31 populations and the subset of Lepini populations provided a strong indication of past population expansion for all three neutrality tests applied. For the *POMC* gene segment, both Tajima's *D* and

Table 4
Overall F_{ST} for each species and hierarchical AMOVA results based on 10 microsatellite loci for *Salamandrina perspicillata* and 9 loci for *S. terdigitata*, as well as *cytb*. For *S. perspicillata*, we partitioned the dataset into the 13 groups defined by BAPS. For the separate analysis of the Lepini populations, we used the four groups defined by BAPS.

Species	Source of variation	Microsatellite			*cytb*		
		Percentage of variation	*F* statistic	*P*	Percentage of variation	*F* statistic	*P*
S. perspicillata	Among populations	15.71	$F_{ST} = 0.15$	<0.0001	42.52	$\Phi_{ST} = 0.42$	<0.0001
13 Groups	Among groups	12.61	$F_{CT} = 0.12$	<0.0001	31.35	$\Phi_{CT} = 0.31$	<0.05
	Within groups	4.97	$F_{SC} = 0.05$	<0.0001	15.08	$\Phi_{SC} = 0.22$	<0.0001
Lepini (4 groups)	Among groups	4.43	$F_{CT} = 0.04$	<0.0001	−8.98	$\Phi_{CT} = -0.09$	n.s.
	Within groups	4.33	$F_{SC} = 0.04$	<0.0001	27.17	$\Phi_{SC} = 0.21$	<0.0001
S. terdigitata	Among populations	19.82	$F_{ST} = 0.19$	<0.0001	95.51	$\Phi_{ST} = 0.95$	<0.0001

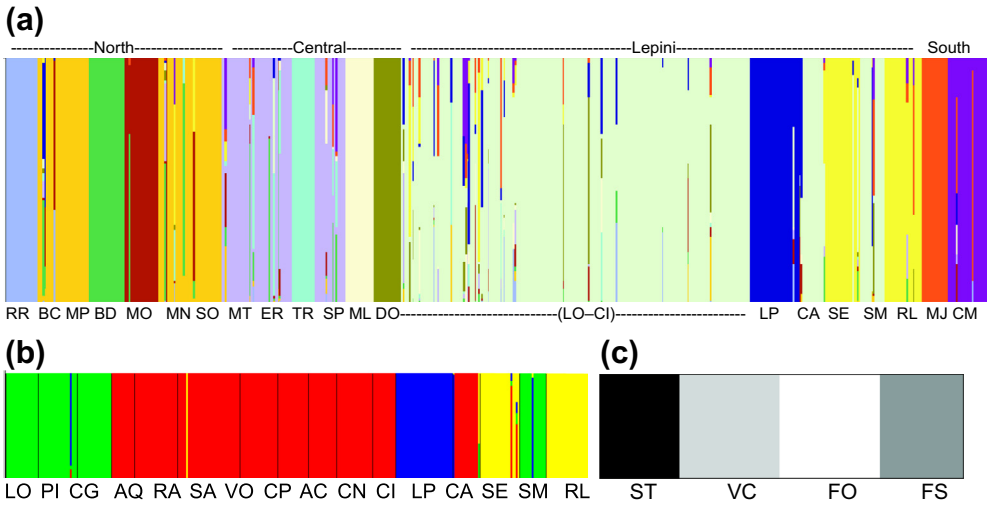

Fig. 3. Population admixture plots obtained with BAPS. Each vertical bar represents an individual and the height of each colored segment of a bar the probability of that individual assigned to each cluster. Populations are in the same order as in Table 1. (a) 31 Populations of *Salamandrina perspicillata* ($K = 13$), (b) 16 *S. perspicillata* populations from the Lepini ($K = 4$) and (c) 4 populations of *S. terdigitata* ($K = 4$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fu's *Fs* were also negative, although not significantly, whereas the *RAG1* segment yielded no support for population expansion. For *S. terdigitata*, all the estimators of the three genes supported a stable population size (Table S3). Based on the microsatellite loci data, we did not find any support for a recent genetic bottleneck for any of the 35 populations that were tested.

3.5. Fine-scale population structure

The AMOVA analyses revealed significant population structure among the 16 populations from the Lepini Mountains (*cytb* $\Phi_{ST} = 0.23$; microsatellite $F_{ST} = 0.074$, both $P < 0.0001$). Of the 120 pairwise F_{ST} comparisons, 15 were not significantly different from zero after sequential Bonferroni correction, and eight of these pairs, all of them found in the western chain, showed no significant heterogeneity of allele frequencies when analyzed with contingency table tests; five of these eight pairs involved populations AQ and AC (see Table S2).

When analyzing individuals from the 16 sampling locations in the Lepini Mountains with BAPS, four genetic clusters were detected (Fig. 3b). Thus, compared to the Bayesian clustering analysis of all the populations of *S. perspicillata* combined, one additional cluster was detected, comprising four populations from the eastern chain (LO, PI, CG, and SM) that now were delineated from the western chain. No difference in genetic diversity was found between populations from the western and eastern chains. When using the suggested four groups of the Bayesian cluster analysis, an AMOVA could explain a small, but significant, proportion of the genetic variance ($F_{CT} = 0.04$; $P < 0.0001$) for the microsatellite loci but not for the mitochondrial dataset (Table 4).

4. Discussion

4.1. Estimating the divergence time of Salamandrina on the Apennine Peninsula

Based on our multilocus approach, the estimated median time of divergence between the two species of *Salamandrina* was 2.25 mya (HPD95 0.582–6.4 mya) and occurred during the early Pleistocene. This median estimate, therefore, is more than two million years younger than the previous estimates (Mattoccia et al., 2005, 2011; Nascetti et al., 2005), which had fallen either in the Lower Pliocene (4.7–4.9 mya) or into the Tortonian stage of the Miocene (7.3–11.6 mya). For these estimates two speciation scenarios have been invoked (Mattoccia et al., 2005, 2011): (1) The more recent estimate coincides with a period of marine transgression with a graben separating Latium from Campania; thus, separating the species into their current distributions. (2) The older estimate would fit a past distribution of *Salamandrina* across the Tortonian Land Bridge, with subsequent separation of the Calabro-Peloritan Massif, containing only the ancestor of the southern species, which subsequently travelled to its present position in southern Italy. According to our estimate, a third scenario can be invoked to explain the early evolutionary history of this genus. Approximately 2.6 mya, well-expressed glacial/interglacial cycles began in the Northern Hemisphere (Bertini, 2010) leading to drastic changes in climate with increased cooling, aridity and seasonality (Bertoldi et al., 1989) that were responsible for major vegetation changes, fragmentations of geographical distributions and even extinction of taxa (e.g. Bennett, 1997; Manzi et al., 2011). Whereas spectacled salamanders were widespread in Europe during the Miocene and Early Pliocene, climatic oscillations and biomic changes may have generated unsuitable habitat conditions for *Salamandrina*, leading to the extinction of these salamanders in northern Europe. The Apennines, which started to emerge during the Late Miocene following a West-East direction (Santangelo et al., 2012), could have served as a refugium during that period. However, this mountain chain has not been as continuous through time as tectonic faults have produced numerous subdivisions. Therefore, during certain periods, the area from the central to the northern Apennine was more like a narrow peninsula with disjunct islands farther in the south. With the lower bound of our estimate of the split between *S. perspicillata* and *S. terdigitata* coinciding with a period before the Messinian salinity crisis and the upper bound with the Gunz Glaciation (0.65–0.5 mya), most of the range of our divergence estimate coincides with a period of recurrent marine submersion of the Volturno-Calore River drainage basin, which lasted into the Early Pleistocene (Canestrelli et al., 2011; Romano et al., 1994). This geologic scenario, coupled with the climatic changes that occurred during this time, namely those during the overall cooling trend, in which one of the last periods of warm climate was recorded approximately 2.2 mya,

appears to be the most suitable explanation for the divergence of the two species. Additionally, the fine-scale analysis performed in the Lepini Mountains shows that valleys may act as dispersal barriers for these salamanders. Therefore, as the Volturno-Calore drainage basin is in a valley that represents a discontinuity in the Apennine chain, it could potentially act as a barrier even without complete flooding of the area. In Aesculapian snakes (*Zamenis longissimus* and *Z. lineatus*), the deepest lineage diversification has also been attributed to the submersion of the Volturno-Calore River drainage basin (Lenk et al., 2001). The same region also serves as a phylogeographic break in many other taxa including *Rana italica* (Canestrelli et al., 2008), *Bombina pachypus* (Canestrelli et al., 2006), *Triturus carnifex* (Canestrelli et al., 2012), and *Vipera aspis* (Barbanera et al., 2009). We acknowledge, however, that although our multilocus coalescence-based approach is currently the most comprehensive attempt to date the divergence of spectacled salamanders, the confidence interval, spanning from the Pliocene into the Middle Pleistocene, is large. This uncertainty might be attributed to the severe discordance regarding the genetic divergence of the mitochondrial and the nuclear gene segments that were analyzed. Evidence from the fine-scale analysis also supports this hypothesis (see discussion below) and helps explain why divergence estimates of *Salamandrina* that are based only on mtDNA are much older than the estimate presented here. Increasing the number of unlinked loci is necessary to narrow the confidence interval and provide an even more reliable timeframe for the divergence between these two species (Arbogast et al., 2002; Heled and Drummond, 2010).

4.2. General patterns of genetic diversity

High genetic diversity in the south – low diversity in the north is one of the key phylogeographic patterns found in many taxa across the Apennine Peninsula. In the genus *Salamandrina*, we found evidence for this pattern with lower haplotype and nucleotide diversity and heterozygosity in *cytb* and *POMC* in *S. perspicillata* compared with *S. terdigitata*. Yet for *RAG1* we found a different pattern, with lower genetic diversity in the southern species. Mattoccia et al. (2011), however, found lower mitochondrial diversity in the southern species. Possible reasons for this discrepancy could be that different parts of the *cytb* gene were analyzed and/or that the number of analyzed individuals differed. The segment that we analyzed is 350 bp longer, and per location, we sequenced on average 13 (6–24) versus an average of 4 individuals (1–8). Unfortunately, Mattoccia et al. (2011) did not make their *RAG1* sequences available (e.g., via GenBank), and therefore, we were unable to compare our results for this gene. Although the genetic diversity of the *POMC* gene in terms of haplotype and nucleotide diversity was much higher in the southern than in the northern species, the overall diversity of this marker was low. In this marker, we encountered haplotype sharing between the species: $POMC_{I}$, which was almost fixed in *S. perspicillata*, also occurred in two of the five populations of *S. terdigitata* (SR and FS). Whereas this finding could be considered as evidence for introgression, it seems more likely a consequence of incomplete lineage sorting and the retention of ancient polymorphism in *S. terdigitata*. First, SR and FS are in Calabria and at least 160 km southwards from the southern edge of the region containing heterospecific *RAG1* haplotypes. The two *S. terdigitata* populations that were sampled closest to the northern species (VC and ST) contained *S. terdigitata*-specific haplotypes only. Second, detecting the $POMC_{III}$ and $POMC_{IV}$ haplotypes that link the *S. perspicillata* and *S. terdigitata* haplotypes in the network in individuals from FS, which is the population at the tip of Calabria and the one most distant from the contact zone, can be taken as additional support for a shared ancestral polymorphism of *POMC* that is retained in the southern lineage.

4.3. Recent expansion in the north – stable population sizes in the south

Patterns of genetic diversity are also shaped by underlying demographic processes in a species' recent past population history. According to our dating, species of *Salamandrina* followed their independent evolutionary trajectories during the last two million years, and we were able to detect differences in demography and their possible consequences on the observed population structure.

The star-like arrangement of the *cytb* haplotype network of *S. perspicillata* (entire clade and subset from the Lepini Mountains), and the results of the three neutrality tests for *cytb* and *POMC* indicate shallow coalescence and recent population expansion. Mattoccia et al. (2011), however, discovered three mitochondrial haplogroups that coincided with geographic regions in this species. They found the highest genetic diversity in the populations from the central part of the Apennine, the Volsci Mountain Chain and concluded that this area most likely coincided with the single glacial refugium for the northern species. Within *S. perspicillata*, the overall genetic diversity decreased towards the northern part of the range, which is expected if the species expanded from a glacial refugium in central Italy, and we were able to confirm this pattern with the sequence data as well as with the microsatellite data. However, we found that mitochondrial and microsatellite diversities were not highest in the Lepini part of the Volsci Chain, but approximately 80 km northwest of there, in the Sabina. This area of SP was at a low elevation, near sea level, until approximately 1.5 mya, and ER is in an intermountain basin, the Tiber Valley. It is likely that the climate was milder in this region than in the more northern part of the Apennines. Therefore, the genetic diversity in *S. perspicillata* is best explained by ancestral polymorphism maintained by one comparatively large refugium from which the species has expanded recently.

In the southern species, we encountered a different pattern from that in the northern species, but also different from previous findings by Mattoccia et al. (2011). They reported four *cytb* haplotypes in *S. terdigitata* only, all of which were separated by single mutation steps, and they explained the lower genetic diversity (compared to *S. perspicillata*) by a more recent expansion of the southern clade from a single refugium. We found in population FO a *cytb* haplotype ($Cytb_{XIII}$) that differed by at least three mutational steps from the others and influenced our analyses regarding diversity and demography. However, with the other markers, we also found no evidence of expansion in the south. Furthermore, in *POMC*, we encountered more genetic differentiation in *S. terdigitata* compared to the northern species. In *S. terdigitata*, populations were much more differentiated genetically. This was particularly evident when considering the microsatellite data. Whereas we detected different degrees of admixture between populations in *S. perspicillata* (Fig. 3a), we found no sign of admixture between populations of *S. terdigitata* (Fig. 3c). Therefore, we suggest that in contrast to the northern species, the spectacled salamanders in the south survived in several smaller refugial areas.

4.4. Fine-scale analysis of the populations from the Lepini Mountains

Salamander populations from the Lepini Mountains showed significant genetic structure at the smallest geographical scale. Here, the strongest and geographically most obvious break was the Carpineto-Montelanico Valley, shaped by a fault of Plio-Pleistocene origin that separates the eastern and western slopes of the Lepini Mountains. Whereas the climate on the western slope is affected by the proximity of the sea, the eastern slope has a more continental climate. Nevertheless, individuals from the south-easternmost population of the western chain (SE) formed a distinct cluster together with a population of the eastern chain (RL) (Fig. 1). On the

western slope, the population farthest away (10 km on average) from all the others (AQ) showed the least genetic identity according to the panmixia test. The individuals from LP formed their own genetic cluster, and this was also the population with the least genetic diversity in the Lepini Mountains. The uniqueness of this site is surprising because it is less than 300 m away from either CI or CN. However, we also found differences in life-history traits at LP compared to the adjacent sites (Angelini, 2006). The LP females breed only once per year, whereas at the adjacent sites, they breed in the spring and in the fall. The two rocky springfed ponds that form LP are located on an exposed outcrop, whereas at the adjacent sites, trees and shrubs provide more protection. We found no evidence of a genetic bottleneck in this population (nor in any other), nor evidence of a founder effect for LP, but gene flow into LP is severely reduced. It will be interesting to determine which ecological or behavioral factors are responsible for the contrasting features, such as time of mating. Although the overall diversity of the *cytb* haplotypes is low among the Lepini populations, some populations have private mitochondrial haplotypes that they do not share with their nearest neighboring populations (e.g. at CA, CP and CN). The lack of association between genetic and geographic distances in the Lepini, even when only considering populations from either mountain chain, could be taken as support of rather recent expansion into this mountain chain. However, the genetic diversity of *S. perspicillata* was higher in the Lepini than, for example, sites at the northern edge of the range that they are likely to have occupied more recently. Furthermore, the Valle Latina has separated the Lepini from its surroundings since the Plio-Pleistocene and possibly isolated the Lepini, preventing this region from acting as a source or a sink.

5. Conclusion

This study is the first to employ microsatellite analysis and multilocus dating to a genus distributed across the Italian Peninsula. We obtained a detailed picture of the phylogeographical patterns of *Salamandrina*, as well as on the population genetic structure at different geographical scales, which allowed us to draw conclusions about the possible underlying processes generating major biogeographical patterns. At the smallest geographical scale, the microsatellite data revealed significant genetic structure. Sequence data are in support of the northern species, *S. perspicillata*, having survived glacial periods in a comparatively large refugium from which it has expanded recently, whereas the pattern of genetic diversity found in the southern species, *S. terdigitata*, would be supported by survival of glacial periods in smaller and more isolated refugia.

Although our estimate of species divergence has a broad confidence interval, it clearly shows that multilocus divergence dating can differ substantially from those based on mtDNA only. Accordingly, future studies based on a multilocus framework can potentially reveal estimates that are different from those currently acknowledged, consequently changing our understanding about the historical biogeography of the study region. To investigate the potential contact zone of the species, dense population-level microsatellite analysis will help delineating this area and also to shed light on the degree of past and ongoing gene flow and potential species-biased processes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.08.015.

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