Genetica (2011) 139:1095–1105
DOI 10.1007/s10709-011-9609-6

Salamandra salamandra (Amphibia: Caudata: Salamandridae) in Portugal: not all black and yellow

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Received: 8 July 2011 / Accepted: 5 September 2011 / Published online: 22 September 2011

Abstract The fire salamander complex is quite diverse in the Iberian Peninsula where nine subspecies of *Salamandra salamandra* are currently recognized. Here, we analysed the geographical distribution of the subspecies *S. s. gallaica* and *S. s. crespoi* using partial sequences of the mitochondrial cytochrome *b* gene of 168 individuals from 12 locations in Portugal. Our results support the existence of a deep lineage divergence between the two subspecies, with non-overlapping geographical distributions except in two contact zones: one in Sesimbra on the western coast, and another in Alcoutim on the southeastern border with Spain. Moreover, *S. s. crespoi* displays signs of gene flow among the sampled locations whereas *S. s. gallaica* shows evidence of some restriction to gene flow. Present-day genetic make-up of *S. s. gallaica* and *S. s. crespoi* is a result of past historical events, fine-tuned by contemporary Iberian geoclimate. Humid mountain areas were found to harbour increased genetic diversity possibly acting as past refugia during drier interglacial periods. To analyse wider geographical patterns and lineage splitting events within *S. salamandra* we performed a Bayesian dating analysis completing our data set with previously published sequences. The observed divergences were associated to successive biogeographic scenarios, and to other Iberian species showing similar trends.

Keywords Amphibians · Salamander · Evolution · Phylogeography · Mitochondrial DNA

Introduction

During the evolutionary history of a species, range expansions, contractions and population fragmentations followed by secondary contacts, may be deeply affected by climate and by geomorphological changes (Hewitt 2004). Such events can severely impact demography and, affect gene flow and in many instances, can be inferred from the present genetic structure of extant populations (Schmitt et al. 2006; Seddon et al. 2001; Tollefsrud et al. 2009). Complex evolutionary histories may be expected to be of particular relevance in areas where geography and geomorphology promoted the maintenance of refugia during extreme events (Gómez and Lunt 2007). The recolonization of the landscape by individuals from different refugia may lead to areas of secondary contact, thereby creating "hybrid" zones of pivotal importance in microevolutionary processes and speciation (Alexandrino et al. 2005; Kuchta et al. 2009).

The establishment of the geographical delimitation and boundaries at inter and intra-specific levels are critical to better understanding the evolution of organisms. This is

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especially important for groups with low dispersal capabilities, such as amphibians (Staub et al. 1995) that are highly affected by habitat loss and fragmentation (Cushman 2006). The demographic consequences of habitat fragmentation are increases in genetic drift and homozygosity due to declines in effective population size and population connectivity (Johansson et al. 2007), thus impacting the genetic makeup of species by eroding neutral and adaptive genetic diversity. Loss of genetic diversity may reduce adaptability, reproductive rates, and survival, i.e., a decrease in fitness (Frankham 2003; Charlesworth and Charlesworth 1999). The loss of local, isolated amphibian populations has serious consequences for local biodiversity, resulting in a partial view of the evolutionary history of a given lineage.

The Iberian Peninsula harbors the highest diversity of *Salamandra salamandra* in Europe, where nine subspecies are currently recognized (Gasser 1978; Joger and Steinfarz 1994; Steinfartz et al. 2000; Veith 1994). The term subspecies is used for populations that display slight morphological differences in allopatry and evidence of intergradation (morphological intermediacy) in contact zones with other conspecific populations (Wilson and Brown 1953). Previously published results (García-París et al. 2003; Steinfartz et al. 2000), identified two main clades: (1) the central, western and eastern Europe largely unresolved clade including *S. s. gallaica* and other subspecies (García-París et al. 2003; Steinfartz et al. 2000), and (2) the southern Iberian clade that groups *S. s. crespoi, S. s. morenica* and *S. s. longirostris*. These three subspecies hypothetically diverged from a refugium in southern Spain during the Pliocene (García-Paris et al. 1998). The sister relationship found between *S. s. morenica* and *S. s. crespoi* to the exclusion of *S. s. longirostris* presumably depicts the vicariant effect of the Guadalquivir River basin formed during the Pliocene due to the uplift of the Iberian Peninsula (García-Paris et al. 1998).

So far, no records of sympatry between *S. s. galaica* and *S. s. crespoi* have been reported in Portugal with most of the available information indicating a general center-northern distribution for *S. s. gallaica*, and a southern distribution for *S. s. crespoi* (Almeida et al. 2004; Malkmus 2004; Rebelo 2008; Steinfartz et al. 2000). These two subspecies are morphologically similar and juveniles are difficult to unequivocally distinguish. *S. s. gallaica* adults are generally smaller with respect to the other subspecies exhibiting few large yellow spots with occasional red flecks, whereas *S. s. crespoi* has many small and irregular yellowish spots.

In this study, we build on previously published studies at the wider geographical level (García-Paris et al. 1998; Steinfartz et al. 2000) to focus on a particular area of interest by increasing sample coverage at a finer scale where the putative subspecies *S. s. crespoi* and *S. s. gallaica* may share habitats. We comparatively assessed the spatial distribution and genetic differentiation establishing the geographical distribution and limits of each subspecies using mitochondrial DNA (mtDNA) sequence data of 168 individuals from 12 locations in Portugal. We aim to: (1) identify putative contact zones and to infer possible demographic events that may have contributed to the occurrence of the two forms of *S. salamandra* in Portugal; (2) understand the wider regional patterns of the species in Iberia by combining previously published results with new data, and (3) propose evolutionary hypothesis within a biogeographical scenario to explain the present-day genetic structure of the two subspecies.

Materials and methods

We sampled a total of 168 individuals from 12 locations (Fig. 1; Table 1) during 2003, 2004, and 2008 covering the entire range distribution of *S. salamandra* in Portugal. All adults were released after a sample 0.5 cm was cut off from the tip of the tail. Larvae were sampled whole. DNA was extracted from 1 to 2 mm of the tail tips of adults and larvae using a modified NaCl method (Sambrook and Russell 2001). A fragment of the cytochrome *b* region of the mitochondrial DNA (mtDNA) was amplified using primers MVZ 15 and MVZ 16 (Moritz et al. 1992). PCR reactions were performed in a total volume of 25 μL, containing 0.4 μM of each primer, 750 μM of dNTP's, 5 μL of 5× GoTaq Flexi buffer (pH 8.5, 1.5 mM of $MgCl_2$), 0.2 units of GoTaq DNA polymerase (Promega) and ca. 50 ng of extracted DNA. The PCR profile consisted of 3 min of denaturation at 95°C, followed by 40 cycles of denaturation, annealing and elongation (45 s at 95°C, 45 s at 48°C, 1 min at 72°C), and a final elongation step of 10 min at 72°C. Samples were purified by ethanol-sodium acetate precipitation and forward sequencing was carried out on the ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems) using chemistry from Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) at the Centre for Marine Sciences, Faro, Portugal. For this reaction, 5–20 ng of the purified PCR product were used with 4 μL of pre-mix and 2 μL BigDye (both vs. 3.1, Applied Biosystems), 1 μL primer (10 mm), and up to 20 μL distilled water. After an initial denaturation by rapid thermal ramp to 96°C, for 1 min, 25 PCR cycles (96°C for 10 s, 50°C for 5 s and 60°C for 4 min) were performed (according to manufacturer's instructions, http://www.ibt.lt/sc/files/BDTv3.1_Protocol_04337035.pdf).

Population genetic structure

Estimation of all genetic parameters was performed using DnaSP version 5 (Rozas et al. 2003) and ARLEQUIN

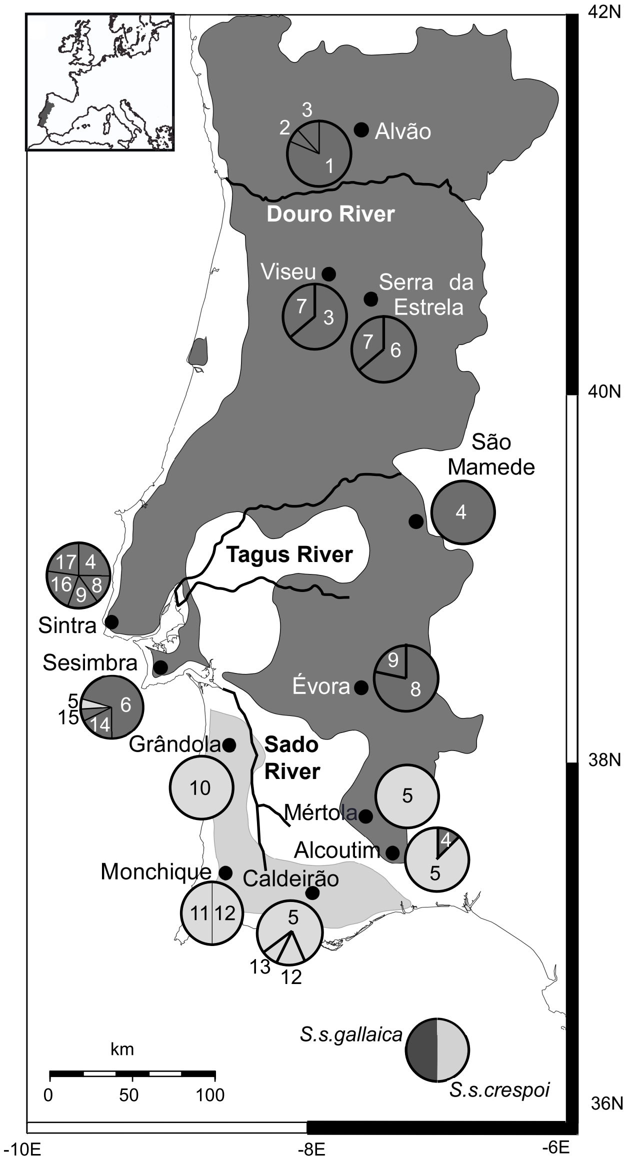

Fig. 1 Haplotype diversity in *S. s. gallaica* and *S. s. crespoi* (size of slices represent proportion of haplotypes indicated by number), and locality groups as defined in Table 1. *Dark grey* indicates *S. s. gallaica* and *light grey S. s. crespoi* according to the known distribution of the two subspecies of salamander existing in Portugal (Almeida et al. 2004)

version 3.5 (Excoffier and Lischer 2010). Nucleotide and haplotype diversity indexes (h and π and their standard deviation), as well as polymorphic and parsimony informative sites were calculated for each location. In order to compare haplotype diversity values, the statistics and asymptotic confidence intervals derived by Salicru et al. (1993) were used for both overall diversity comparison and pairwise comparisons between populations. Measures of genetic subdivision were measured by pairwise ΦST values among populations using ARLEQUIN 3.5 (Excoffier and Lischer 2010). To detect population groups (k) without the need of an a priori assumption of group composition that is necessary for AMOVA, we used the Spatial Analysis of Molecular Variance (SAMOVA) approach (Dupanloup et al. 2002). SAMOVA (ver. 1.0, http://web.unife.it/progetti/genetica/Isabelle/samova.html) employs a simulated annealing algorithm to identify groups of populations that are geographically homogeneous and maximally differentiated in terms of among-group component (conventional F-statistics, ΦST the analogous to θ, Cockerham 1973) of the overall genetic variance. The analyses were conducted by repeatedly increasing the number of groups (K). A total of 100 simulated annealing processes were used to determine optimal allocation of the geographic samples into groups.

A haplotype network was constructed using Network v4.5 (fluxus-engineering.com). Median networks containing all possible equally short trees were simplified by running the maximum parsimony calculation option to eliminate superfluous nodes and links (Polzin and Daneshmand 2003). Patterns of isolation by distance in the data were assessed with Mantel tests (Mantel 1967). A corrected pairwise nucleotide distance matrix among haplotypes was used to test for correlation with a geographical distance matrix.

Molecular clock dating analysis

We added 86 cytochrome *b* sequences from the GeneBank produced by García-París et al. (2003) to the 168 sequences. This enlarged set will allow a better understanding of the wider regional evolutionary patterns of the species in Iberia. Sequences were collapsed into haplotypes with Collapse v.1.5 (Posada 2008a) yielding a final 326 bp-dataset with 31 taxa including the two outgroups *Chioglossa lusitanica* and *Mertensiella caucasica*. Alignment of the nucleotide sequences was performed with GENEIOUS version 5.1 (Drummond et al. 2010) and verified by eye in order to maximize positional homology.

Divergence times of the main cladogenetic events in the Salamandridae phylogeny were estimated using a relaxed molecular clock Bayesian approach as implemented in BEAST version 1.4.8 (Drummond and Rambaut 2007). This methodology uses probabilistic calibration priors instead of point calibrations, allowing the incorporation of fossil uncertainties (Drummond et al. 2006). We used the GTR+ evolutionary model indicated by jModeltest (Posada 2008b) and the Yule speciation model that assumes a constant rate of speciation, following a pure birth–death process (Yule 1925) as suggested in Drummond et al. (2006). The calibration of the tree was provided by placing a LogNormal prior distribution on the node corresponding to the divergence between *Chioglossa lusitanica* and *Mertensiella caucasica*. According to the fossil

Table 1 Sampled locations, code, corresponding coordinates, and matching number of individuals collected on each location (N). Locations with less than 5 individuals (Grândola, Mértola, Monchique and Viseu) were excluded from haplotype and nucleotide diversity estimations

Locality	Code	Geographical coordinates	N	Diversity indexes							
				H	PS	PIS	K	*h*	SD	π	SD
Alvão	ALV	41°23′N; 7°48W	18	3	2	1	0.32	0.31	0.13	0.0005	0.0002
Viseu	VIS	40°40′N; 7°54′W	3	2	7	0	4.67	–	–	–	–
Serra da Estrela	EST	40°24′N; 7°31′W	20	2	9	9	4.31	0.48	0.07	0.0063	0.0009
São Mamede	MAM	39°19′N; 7°18′W	19	1	0	0	0.00	0.00	0.00	0.0000	0.0000
Sintra	SIN	38°47′N; 9°26′W	36	5	16	16	7.00	0.81	0.02	0.0101	0.0005
Sesimbra	SES	38°27′N; 9°01′W	20	4	29	1	3.20	0.49	0.12	0.0046	0.0034
Évora	EVO	38°25′N; 7°40′W	11	2	1	1	0.33	0.33	0.15	0.0005	0.0002
Alcoutim	ALC	37°33′N; 7°28′W	11	2	29	0	5.27	0.18	0.14	0.0076	0.0060
Caldeirão	CAL	37°20′N; 7°58′W	25	3	3	3	0.79	0.35	0.11	0.0012	0.0004
Grândola	GRA	38°06′N; 8° 37′W	1	1	0	0	–	–	–	–	–
Mértola	MER	37°37′N; 7°40′W	2	1	0	0	–	–	–	–	–
Monchique	MON	37°19′N; 8°33′W	2	2	1	0	–	–	–	–	–
Clade 1			127	12	24	22	6.12	0.87	0.01	0.0089	0.0004
Clade 2			41	5	3	3	0.74	0.35	0.09	0.0011	0.0003
Total sample			168	17	43	41	13.42	0.89	0.01	0.0195	0.0009

Corresponding diversity indexes to each location, number of detected haplotypes (*H*), polymorphic sites (*PS*), parsimony informative sites (PIS), average number of nucleotide differences (*K*), haplotype diversity (*h*) and corresponding standard deviation (*SD*), nucleotide diversity (π) and standard deviation (*SD*). The clades reflect the grouping of haplotypes identified in the haplotype network

record, the divergence between these two taxa occurred at least at 23 million years (MY) ago (Ivanov 2008). Estimates are accompanied by 95% High Posterior Density (HPD) within square brackets.

Results

Population genetic structure

A 690-bp sequence of the 5′-end of the mitochondrial control region from 168 individuals across the 12 localities, revealed 17 haplotypes defined by 43 segregating sites of which 41 were parsimony-informative. Twenty-nine individuals did not amplify this fragment. Two clades were clearly defined based on 17 diagnostic mutations that are essentially non-random: Clade 1 grouping mostly center-northern locations, and Clade 2 grouping mostly southern locations (Fig. 1; Table 2).

These two clades conform to the previously described existence of two subspecies of *S. salamandra*, *S. s. gallaica* in the center-northern Clade 1 and *S. s. crespoi* in the southern Clade 2. The best-represented haplotypes, with 27, 29, and 33 individuals have a widespread distribution in the center and south of Portugal (Table 2). Locations with less than 5 individuals (Grândola, Mértola, Monchique, Viseu) were excluded from diversity estimations. Haplotype diversity values (Table 1) varied from 0 to 0.81 and were significantly different among populations according to the χ^2 test developed by Salicru et al. (1993) ($\chi^2 = 75.21$, $P < 0.001$). Pairwise comparisons between localities showed that Sintra haplotype diversity values were significantly higher than those found in all other populations ($P < 0.003$), and São Mamede values were significantly lower than those found in all other populations ($P < 0.017$) to the exception of Alcoutim. Nucleotide diversities were generally low; locations could be divided in two groups, one with values ranging from 0 to 0.12% (Alvão, São Mamede, Évora, Grândola and Caldeirão) and the other with values ranging from 0.46 to 1.01% (Serra da Estrela, Sesimbra, Alcoutim and Sintra).

Statistical parsimony analysis under a 95% probability of connections consisted of two unconnected networks (Fig. 2) that reflect the two-subspecies pattern encountered. Further support for the same groups was found with SAMOVA by conducting repeatedly increases in the number of groups (*K*). The statistically significant maximum ΦCT value of 0.771 (*P* value < 0.01) was reached with $K = 2$.

Differentiation among the locations of the center-northern region and the southern groups was high with a ΦST value of 0.886 (*P* value < 0.001). Pairwise ΦST values between Clade 1 locations were all statistically significant, while the same comparison in Clade 2 yielded only non-significant results (not shown). The hypothesis of isolation by distance for the geographical distribution of haplotypes evaluated with Mantel test was not supported for any clade (Clade 1:

Table 2 Different haplotypes detected (H) and corresponding variable positions identified in the 690 bp fragment along with the number of individuals which bear each haplotype (N), and locations where they were encountered

		Nucleotide positions of the citochrome b fragment																																												
Clade	H	6	24	54	114	145	168	175	183	186	190	226	228	234	309	339	381	390	402	405	412	418	438	453	456	459	471	509	513	531	537	538	574	579	582	594	624	636	640	660	661	663	672	681	N	Populations
Clade 1	1	C	T	C	C	A	C	A	C	T	G	C	A	T	C	T	T	C	T	C	G	C	C	C	A	C	T	C	G	C	T	C	G	T	C	T	C	T	A	G	G	A	G	C	15	Alvão
	2	-	T	-	-	-	-	-	-	-	-	C	-	T	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	1	Alvão
	3	-	-	-	-	-	-	-	-	-	-	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	4	Alvão; Viseu
	4	-	C	-	-	-	-	-	-	-	-	A	-	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	-	-	-	-	-	-	-	-	-	-	29	Alcoutim; Sintra; São Mamede
	6	-	-	-	-	-	-	-	-	-	-	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	27	Serra da Estrela; Sesimbra
	7	-	-	-	-	-	-	-	-	-	-	C	-	T	T	C	-	T	-	T	-	-	-	-	-	-	-	-	-	-	-	-	T	T	-	C	-	-	-	-	-	G	G	-	8	Serra da Estrela; Viseu
	8	-	-	-	-	-	-	-	-	-	-	C	-	T	-	C	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	T	-	T	T	-	-	-	G	-	-	-	-	-	15	Evora; Sintra
	9	-	-	-	-	-	-	-	-	-	-	C	-	T	-	C	-	-	C	-	-	T	-	-	-	-	-	-	-	-	-	T	A	T	T	-	-	-	G	-	-	-	-	-	7	Evora; Sintra
	14	-	-	-	-	-	-	-	-	-	A	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4	Sesimbra
	15	-	-	-	-	-	-	-	-	-	A	C	-	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	Sesimbra
	16	-	-	-	-	-	-	-	-	-	-	C	-	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	-	-	T	-	-	-	A	G	A	-	8	Sintra
	17	-	-	-	-	-	-	-	-	-	-	C	-	T	T	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	T	-	-	-	-	-	-	A	G	A	-	8	Sintra
Clade 2	5	T	-	A	T	-	G	G	T	C	-	C	C	T	-	C	C	-	-	-	-	T	T	T	G	T	C	-	A	-	C	-	A	T	-	C	T	C	G	A	A	-	-	A	33	Alcoutim; Mertola; Caldeirão; Sesimbra
	10	-	-	-	-	-	-	G	-	-	-	C	-	T	-	C	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	A	T	-	C	T	-	G	-	A	-	-	-	1	Grândola
	11	-	-	-	-	-	-	G	-	-	-	C	-	T	-	C	-	-	-	-	-	T	-	-	-	-	-	T	-	-	-	-	A	T	-	C	T	-	G	-	A	-	-	-	1	Monchique
	12	-	-	-	-	-	-	-	-	-	-	C	-	T	-	C	-	-	-	-	-	T	-	-	-	-	-	T	-	-	-	-	A	T	-	C	T	-	G	-	A	-	-	-	4	Monchique; Caldeirão
	13	-	-	-	-	-	A	G	-	-	-	C	-	T	-	C	-	-	-	-	-	T	-	-	-	-	-	-	-	-	-	-	A	T	-	C	T	-	G	-	A	-	-	-	2	Caldeirão

The haplotypes are divided into the clades identified in the haplotype network. The light grey shaded positions highlight the diagnostic nucleotide positions that differentiate *S. s. gallaica* (Clade 1) from *S. s. crespoi* (Clade 2). Dots in Clade 1 haplotypes correspond to identical nucleotides to haplotype 1, and dots in Clade 2 haplotypes correspond to identical nucleotides to haplotype 5

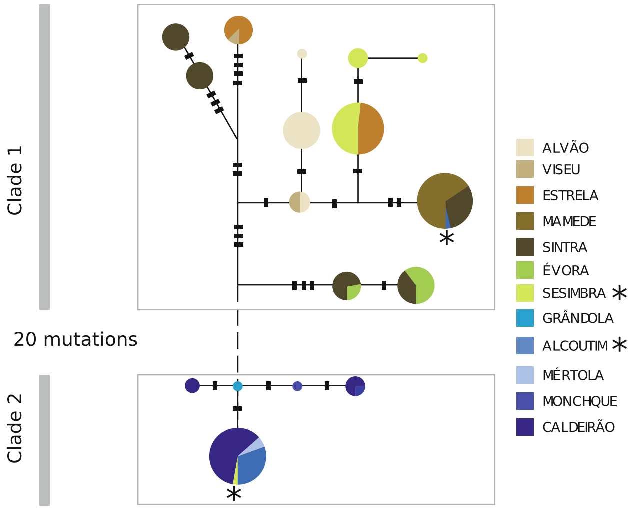

Fig. 2 Haplotype network and nested clade analysis for *S. salamandra* sequences obtained by statistical parsimony. The dimension of each *circle* is indicative of the haplotype absolute frequency (Table 2). *Solid lines* between haplotypes represent a single mutation. *Black rectangles* represent interior missing haplotypes. Clade 1 and Clade 2 correspond to *S. s. gallaica* and *S. s. crespoi*, respectively. *Asterisks* identify locations with a small number of individuals belonging to one clade while the majority belongs to the other clade

$r = 0.106$, $P = 0.26$; Clade 2: $r = 0.173$, $P = 0.23$). In two locations, Sesimbra and Alcoutim, individuals belonging to both genetic groups were found in sympatry. This is the first time that sympatry between *S. s. gallaica* and *S. s. crespoi* has been identified on the basis of genetic data.

Phylogenetic relationships of the major clades within the Iberian subspecies of *S. salamandra* using ML analysis of 326 aligned nucleotides from the mitochondrial cytochrome *b* gene combining 168 new sequences and all available Iberian *Salamandra* spp. GenBank sequences (results not shown) do not add to what was previously established (García-Paris et al. 1998; García-París et al. 2003; Steinfartz et al. 2000). The clade *S. s. crespoi* remained monoplyletic and the newly added *S. s. gallaica* sequences were polyphyletic.

Molecular clock dating analysis

According to the Bayesian dating analysis inferred with BEAST, the most recent common ancestor of the recovered three major clades (A and B include the center-northern specimens, and clade C the southern specimens) was estimated at 16 [95% highest posterior density interval (HPD):

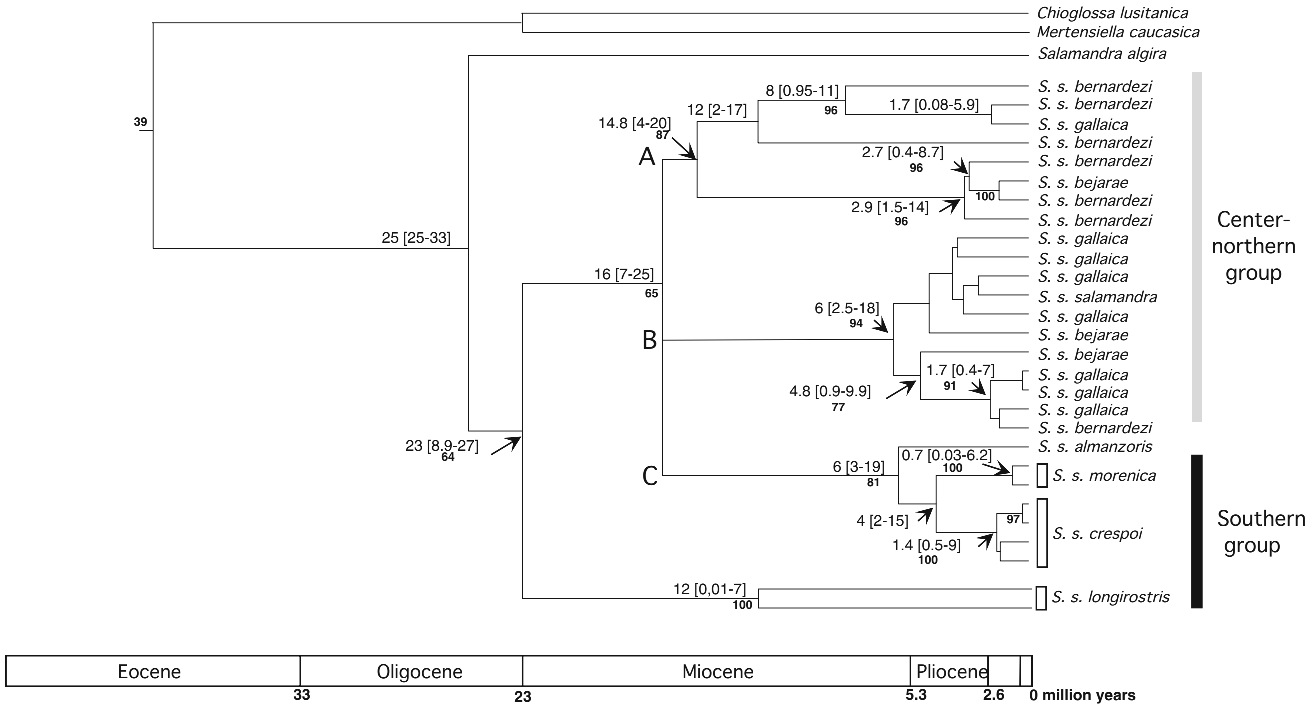

Fig. 3 Beast maximum clade credibility chronogram. Values *above* branches represent age estimates in million years and corresponding 95% highest posterior density intervals. Values *below* branches represent Bayesian posterior probabilities (only values above 75% are depicted)

7–25] MY (Fig. 3). Divergence between *S. s. morenica* and *S. s. crespoi* occurred at 4 [95% HPD: 2–15] MY. The splitting between *S. s. longirostris* and the three above-mentioned clades occurred at the beginning of the Miocene at 23 [95% HPD: 8.9–27] MY.

Discussion

Population genetic structure and contact zones

The major genetic break found between the center-northern and southern *S. salamandra* haplotypes (Fig. 1) is consistent with previous results based on the described range for the two *S. salamandra* subspecies in Portugal (Almeida et al. 2004; Malkmus 2004). In the present study, Clade 1 haplotypes displayed a geographical distribution compatible with the range of *S. s. gallaica* whereas Clade 2 corresponded to *S. s. crespoi* distribution (Fig. 2). In most locations, individuals were from a single clade. *S. s. crespoi* was detected in Mértola, where the use of morphological data previously also suggested its occurrence (Rebelo 2002). Moreover, genetic data clearly identified two locations, Sesimbra and Alcoutim in the center-western and southeastern edges of the distribution of the two subspecies, where the two clades are sympatric (Fig. 1; Table 2). The presence of *S. s. crespoi* was not previously recorded in these areas.

The existence of an asymmetrical distribution of haplotypes typical of each subspecies occurring in sympatry (Fig. 1) probably resulted from divergence in allopatry followed by secondary contact. Clades thought to arise via sympatric speciation must hold sister species (Bolnick and Fitzpatrick 2007), which is not the case of *S. s. gallaica* and *S. s. crespoi* (Fig. 3). Our results indicate the existence of two contact zones between *S. s. crespoi* and *S. s. gallaica* (see Figs. 1, 2). This raises the possibility of studies on differential flow of neutral and adaptive genes across this zone. The more recent disappearance of suitable habitats in the central/southern region of Portugal has prevented a widespread contact zone between both subspecies. Over thousands of years, the Alentejo region (mostly the hole in the distribution map, Fig. 1), originally a large woodland area, was transformed into a cultural savannah-like landscape caused by persistent human activity (Joffre et al. 1999; Vicente and Alés 2006; Surova and Pinto-correia 2008; Paleo 2010). Parwise ΦST results support both the existence of gene flow in *S. s. crespoi* between the sampled locations (Clade 2) and structure within *S. s. gallaica* (Clade 1). However, there is no statistical evidence of isolation by distance as evaluated by the Mantel test. The overall inference of restricted gene flow in Clade 1 may be explained by a combination of geographical distance, unsuitable habitat, and a high sea-level stand caused by a major marine introgression during the Pliocene (Cachão and Silva 2000). The contraction of the geographical distribution of *S. s. salamandra* to these locations might have generated higher haplotype diversity on each location. Large rivers such as Douro and Tagus probably prevented dispersal between the individuals among such distant locations. Douro has been recognized as an effective barrier to gene flow in other amphibians, e.g. *Chioglossa lusitanica* (Alexandrino et al. 2000) and Tagus was previously identified as a boundary promoting lineage diversification in *Discoglossus galganoi* (Martinez-Solano 2004).

In conclusion, the rejection of the null hypothesis of random geographic distribution of haplotypes within *S. salamandra* reflects the deep lineage divergence between the two subspecies, *S. s. crespoi* and *S. s. gallaica*, with non-overlapping geographical distributions except in the two contact zones Sesimbra and Alcoutim.

Dating analysis and biogeographic scenarios for *S. salamandra*

In order to understand the wider regional patterns of *S. salamandra* we included all available cytochrome *b* sequences in the GenBank, which resulted in a 326-bp data set from 31 taxa. Given the reduced number of nucleotide positions and the recovered Bayesian tree with unresolved relationships between the subspecies, dating estimates must be taken cautiously.

Previous work (García-Paris et al. 1998) assumed that the formation of the Guadalquivir River basin in southern Spain during the Pliocene triggered the divergence between *S. s. longirostris* and the clade including *S. s. crespoi* and *S. s. morenica*. This evolutionary scenario was based on a biogeographic event, the opening of the North-Betic straight, but no dating analysis was performed to support the claim. According to our Bayesian estimates, this splitting occurred at 4 MY, with a 95% HPD between 2 and 15 MY. The nature of the event that prompted the divergence between both lineages remains unclear. However, the Iberian Peninsula was characterized by a dry climate through the Zanclean period (5.3–3.6 MY) (Fauquette et al. 1999), which might have restricted the distribution of these amphibians to more humid areas such as Sierra Morena or Monchique, where they can be currently found.

A later study including northern lineages, yielded different phylogenetic relationships between *S. salamandra* subspecies (García-París et al. 2003). *S. s. longirostris* was retrieved in a basal position with respect to all subspecies included, instead of sharing a most recent common ancestor with both *S. s. crespoi* and *S. s. morenica*. Steinfartz et al. (2000) produced a phylogeny for the genus, including European, northern African, and Near East specimens and estimated the divergence between *S. s. longirostris* and the clade comprising both *S. s. morenica* and *S. s. crespoi* at the Pliocene. In the present study, we recovered *S. s. longirostris* in a basal position to all other Iberian subspecies (Fig. 3). Yet, our Bayesian estimates placed the separation between *S. s. longirostris* and the remaining *S. salamandra* much earlier at the Miocene (23 [95% HPD: 8.9–27] MY; Fig. 3). The disparity between estimates may result from using different genes and/or methodologies. We used cytochrome *b* sequences and a Bayesian relaxed molecular-clock approach whereas the topology from Steinfartz et al. (2000) was based on *D*-loop sequences, a distance method [neighbour joining; (Saitou and Nei 1987)] that does not incorporate any evolutionary model and uses fixed rates of divergence (0.8%/MY). Large discrepancies have been found in dating evolutionary events based on fixed mutation rates (Pulquério and Nichols 2006).

During the Miocene, a vast marine channel was opened in southern Iberia forming the North-Betic Strait, which allowed contact between the Atlantic Ocean and the Mediterranean Sea through the Betic Cordillera (Martín et al. 2009). In the Serravalian (15.9–13.8 MY), there was a wide-open marine passage limited by a southern platform, and during the Tortonian (11.6–7.2 MY) this passage was converted into a tidal-dominated strait (Martín et al. 2009). The Rifian corridors formed during the Miocene isolated the Betic-Rifian Massif from the main portion of Iberia (Martín et al. 2009). Therefore, the divergence of *S. s. longirostris* from the remaining *S. salamandra* may have been associated with the North-Betic Strait formation within our proposed time frame. This subspecies remains currently isolated from other southern lineages (*S. s. morenica* and *S. s. crespoi*) (see

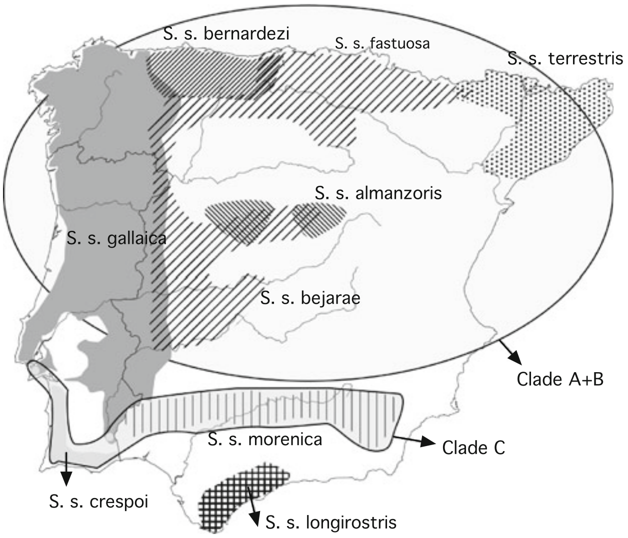

Fig. 4 Present-day distribution of the Iberian *Salamandra salamandra* subspecies based on information taken from García-París et al. (2003). The geographic distribution of *S. s. gallaica* and *S. s. crespoi* was modified according to the results obtained from this study

Fig. 4) most likely due to the Guadalquivir River Basin, originated in the Pliocene.

The divergence dates of the major clades (A, B, and C; Fig. 3) of *S. salamandra* subspecies from their most recent common ancestor was estimated at 16 [95% HPD: 7–25] MY (Fig. 3). During this timeframe, the tectonic activity in the Iberian Peninsula led to the drainage of most river systems to a large number of lacustrine endorheic (closed) basins in central Iberia (Altaba 1997; Calvo et al. 1993). These events lead to vicariant speciation in a large group of animals including e.g., midwife toads (Martinez-Solano et al. 2004) and freshwater snails (Altaba 1998). Fire-salamanders are unable to swim (Francis 1934) and these lacustrine environments may have formed insurmountable barriers to their dispersal, thus promoting the isolation and divergence of *S. salamandra* subspecies inhabiting the Iberian Peninsula. Both clades, A and B (Fig. 3), include specimens assigned to *S. s. bernadezi*, *S. s. gallaica*, and *S. s. bejarae* questioning the taxonomic validity of these three subspecies. The paraphyly of these subspecies was recovered in previous works (García-París et al. 2003; Steinfartz et al. 2000). Hence, with the present dataset no inferences regarding a plausible biogeographic scenario for *S. s. bernandezi*, *S. s. gallaica*, and *S. s. bejarae* can be established.

Trends in western Iberian phylogeography

The Iberian Peninsula was stage of a suite of geological events and complex orography that contributed to shape and promote the present-day high endemicity of amphibians. Presently, Iberia is the most amphibian species-rich region in Europe (Martínez-Solano et al. 2005) with eight endemic species among twenty-eight native ones (Gómez and Lunt 2007). As mentioned above, the tectonic events occurring in the Iberian Peninsula during the Miocene shaped its oragraphy and current river basins (Altaba 1997). It is not therefore unexpected, that several species have emerged at the end of this period during the Betic and Messinian crisis. Yet, besides these impressive events during the Miocene more recent climatic changes also imprinted on the present-day distribution of genetic divergence. It seems clear that Pleistocene ice ages were probably responsible for the fragmentation of species and lineage splitting (Gonçalves et al. 2009; Recuero and García-París 2011). As the temperatures declined throughout the last ice ages, most of Europe's common deciduous forests were confined to some secluded locations in the Mediterranean Peninsulas with large regions being turned to steppe-tundra land (Deffontaine et al. 2005). During glacial periods, the Iberia experienced a deeper destruction of these forests (that were converted into arid cold steppe-tundra land) when compared to other Mediterranean peninsulas (Van Andel and Tzedakis 1996). Even through the interglacial periods, the climate was too dry for the forest cover to expand. Nevertheless, the present-day existence of a number of viable woodland areas in northern and southern Spain suggests that Iberian glacial refugia were more widespread than previously thought (Carrion et al. 2003). Yet, habitat fragmentation may have promoted many of the lineage splitting events that emerged from putative refugia (Teixeira 2007). Although Monchique was identified as an important refugium for the Iberian herpetofauna, namely *Lissotriton boscai* (Martinez-Solano et al. 2006), *Alytes obstetricans* (Fonseca et al. 2003), and *Lacerta schreiberi* (Paulo et al. 2001), the low sample size of *S. salamandra* ($N = 2$) did not allow a positive confirmation of this area as a refugium for this species. Other regions such as Sintra, and to a lesser extent Viseu and Serra da Estrela could have played a similar role as refugium for *S. s. gallaica*. During inter-glacial periods, these mountain areas were exposed to coastal northwestern winds producing high precipitation levels (Garzón et al. 2007; Griffiths 1996; Malkmus 2004), which would represent excellent habitats for amphibians.

Although species have individually reacted to past climatic changes, shared trends to diversification can be identified. Most of the Iberian endemic amphibians show signs of multiple divergence events and long-term vicariance but those patterns and processes can also be found in other species. A large number of widespread Iberian species, subspecies and species complexes, from plants (Rodríguez-Sánchez et al. 2010) to animals (Gómez and Lunt 2007), display a well-defined split between groups from the Mediterranean and the Atlantic provinces (*sensu* Sillero et al. 2009) of the Iberian Peninsula. Within amphibians, several examples illustrating this trend include the genus *Alytes* (Martinez-Solano et al. 2004); *Discoglossus galganoi* (Martinez-Solano 2004); *Lissotriton boscai* (Martinez-Solano et al. 2006); *Pelodytes punctatus* and *Pelodytes ibericus* (Veith et al. 2006); *Pleurodeles waltl* (Veith et al. 2004) and *Chioglossa lusitanica* (Alexandrino et al. 2000).

The so called "refugia-within-refugia" theory (Gómez and Lunt 2007), that proposes the existence of multiple glacial refugia within the larger refugia of the Iberian Peninsula, has been increasingly supported by an identical phylogeographic structure displayed in a broad spectra of taxa. An exhaustive list can be found in Gómez and Lunt (2007, and references therein). Recently, further evidence supporting this theory included the rotifer *Brachionus plicatilis* (Campillo et al. 2011), the grasshopper *Chorthippus parallelus* (Bella et al. 2007), the Mediterranean toad (*Alytes cisternasii*) (Gonçalves et al. 2009), the red-legged partridge (*Alectoris rufa*) (Ferrero et al. 2011), the field vole, *Microtus agrestis* (Jaarola and Searle 2003), and the southern water vole (*Arvicola sapidus*) (Centeno-Cuadros et al. 2009).

Some regions, including Iberian mountains such as Monchique in southern Portugal, the Cantabria in Northern Spain, and central Iberia, may have acted as main sources of refugia for several species being able to sustain viable populations throughout glacial periods. Warmer microclimates and altitude shifts generated by mountain ranges promoted a gradual habitat change determined by their specific response to climatic oscillations, which resulted in a better individual survival in these areas. The convergence of a number of intrinsic characteristics of the Iberian Peninsula such as irregular topography and climatic diversity produced conflicting outcomes. Habitat partitioning may have lead to the emergence of adaptive characteristics promoting diversification. In contrast, habitat fragmentation caused reduction of population sizes and ultimately, a decrease in the biodiversity. Although these divergent phylogeographic patterns may have a number of different explanations, they were all driven by common splitting events that affected several taxa. In general, our results agree with this model. Nevertheless, a single locus was analysed and we must acknowledge that gene trees might not necessarily reflect the whole history of *S. salamandra* in Iberia (Knowles and Kubatko 2010).

In conclusion, amphibian taxa display abundant evidence of multiple geographically structured lineages reflecting ancient divergences and long-term persistence in some refugial areas or represent more recent splits (for a comprehensive approach see Gonçalves et al. 2009). Although past historical events probably shaped the genetic structure and diversity of *S. s. gallaica* and *S. s.crespoi*, contemporary Iberian geoclimate (Sillero et al. 2009) has contributed to the present-day genetic make-up of these subspecies. Studies on Iberian endemic species are expected to reflect an increase of the diversity around the mountain ranges of Iberia given the existence of different climate regimes. Areas of sympatry already identified in Iberia should be further analysed to clarify phylogenetic relationships between subspecies and the underlying mechanisms of speciation.

Acknowledgments We are grateful to Sofia Caetano and Pedro Beja for an early conceptual and practical contribution to this work. Marta Valente provided the sequencing service. We thank Vítor Almada, and two anonymous referees for helpful comments on an early version of the manuscript and a third referee for a valuable comment on the last version. This work was supported by funding from the Portuguese National Science Foundation (Fundação para a Ciência e a Tecnologia—Portugal) to CCMAR (plurianual funding).

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