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Genetic subdivision, glacial refugia and postglacial recolonization in the golden-striped salamander, *Chioglossa lusitanica* (Amphibia: Urodela)

J. ALEXANDRINO,*† E. FROUFE,† J. W. ARNTZEN*† and N. FERRAND*†
Departamento de Zoologia e Antropologia, Faculdade de Ciências, Universidade do Porto, Praça Gomes Teixeira, 4050 Porto, Portugal, †Centro de Estudos de Ciência Animal, ICETA, Campus Agrário de Vairão, Rua do Monte, Crasto, Vairão, 4480 Vila do Conde, Portugal

Abstract

The golden-striped salamander (*Chioglossa lusitanica*) is an ecologically specialized species, endemic to north-western Iberia. Patterns of genetic variation were assessed at seven polymorphic enzyme loci and one mitochondrial DNA (mtDNA) marker (cytochrome *b*) in 17 populations across its range. Estimates of enzyme genetic diversity revealed a high degree of genetic subdivision (F_{ST} = 0.68), mainly attributable to the existence of two groups of populations. The groups were located, respectively, north and south of the Mondego River, indicating that this river coincided with a major historical barrier to gene flow. A significant decrease in genetic variability from the Mondego northwards was associated with the Douro and Minho rivers. mtDNA sequence variation revealed a congruent pattern of two haplotype groups (*d* = 2.2%), with a geographical distribution resembling that of allozymes. The pattern and depth of genetic variation is consistent with the following hypotheses: (i) subdivision of an ancestral range of the species prior to the middle Pleistocene; (ii) secondary contact between populations representing historical refugia; (iii) relatively recent range expansion giving rise to the northern part of the species range; and (iv) loss of genetic variation through founder effects during range expansion across major rivers.

Keywords: allozymes, Amphibia, *Chioglossa lusitanica*, genetic subdivision, glacial refugia, golden-striped salamander, mtDNA, postglacial range expansion

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Introduction

Most organisms are distributed in a more or less spatially structured array of populations. This structure depends on the interaction between the ecological requirements of the species, its inherent ability for dispersal, and habitat availability. However, the genetic structure of populations is not only determined by current population dynamics, but also by historical patterns of gene flow, thus raising difficulties in separating the two factors. The smaller the influence of current population dynamics on the pattern of genetic variation the easier it will be to assess the history of populations and use it, along with information on past geological and climatic events, to reconstruct the historical biogeography of a species.

Salamanders are organisms with low mobility and strict ecological requirements, often subdivided into genetically isolated populations and, therefore, potentially suitable for studies of historical biogeography (Slatkin 1981; Larson *et al*. 1984). Indeed, recent work on *Ambystoma* salamanders confirmed the importance of historical events in shaping the present-day distribution of genetic variation (Phillips 1994; Templeton *et al*. 1995). The golden-striped salamander, *Chioglossa lusitanica* Bocage 1864, is a peculiar streamside species with a distribution restricted to north-western Iberia. These salamanders are generally found in forested, low elevation mountainous areas, with high precipitation and mild winters, and exhibit a suite of morpho-physiological traits that determine a strong dependence on brook vicinities and other moist habitats. This may

Correspondence: João Alexandrino. Present address: †Centro de Estudos em Ciência Animal, ICETA, Campus Agrário de Vairão, Rua do Monte, Crasto, Vairão, 4480 Vila do Conde, Portugal. Fax: +351252 661780, E-mail: jmalexan@mail.icav.up.pt

result in a highly directional dispersal ability with gene flow occurring parallel rather than perpendicular to watercourses, through adults and juveniles following moist brook embankments and through larval drift (Arntzen 1981, 1994). In spite of the ecological constraints, the dense network of mountain streams in north-western Iberia renders its distribution continuous if considered on a 10-km square grid scale (Sequeira *et al*. 1996). However, large streams and rivers do not constitute a regular habitat for *C. lusitanica* and could form barriers to gene flow roughly proportional to their size.

Our aims are to investigate patterns of genetic variation in *C. lusitanica* in order to reconstruct its historical biogeography and to evaluate implications for conservation. Preliminary work covering four populations and 21 allozyme loci revealed a high level of genetic substructuring (Alexandrino *et al*. 1997). Here we document the spatial distribution of allozyme and mtDNA variation in populations across the whole range of *C. lusitanica*, to examine the predictions that: (i) rivers are instrumental in shaping genetic structure in this species; and (ii) patterns of genetic diversity reflect the history of populations. We found evidence supportive of those predictions, allowing us to develop hypotheses that may explain the historical biogeography of the golden-striped salamander, notwithstanding a certain lack of knowledge concerning the north-western Iberian palaeoecology.

Materials and methods

Salamanders were collected from 17 localities across north-western Iberia covering the species range (Fig. 1). Sample size was between 12 and 107. The tail end of each salamander was removed mimicking natural autotomy and frozen at –70 °C. A tissue extract was obtained by grinding tail tissue in distilled water (1:3 w/v). The homogenate was centrifuged at 15 000 **g** for 7 min at 0 °C. The supernatant was decanted and applied to either starch gels for electrophoresis (SGE) or to polyacrylamide gels for isoelectric focusing (IEF). Peptidase A (PEP-A), peptidase C (PEP-C), phosphogluconate dehydrogenase (PGD), alcohol dehydrogenase (ADH) and enzymes previously shown to be polymorphic (phosphoglucomutase 1, PGM-1; peptidase B, PEP-B; and peptidase D, PEP-D; Alexandrino *et al*. 1997) were scored for all populations. Alleles of ADH were resolved by isoelectric focusing a pH gradient established by the 1:1:3 mixture 5–6, 5–8 and 3.5–10 pharmalytes (Pharmacia) at a final concentration of 6% (v/v). Phosphoric acid 0.25 M and sodium hydroxide 1 M were used as anodal and cathodal electrode solutions, respectively. Gels were prefocused for 1 h. An 8-μL sample of tissue extract was applied to the gel using a silicone strip (Serva) placed at 15 mm from the cathode. Focusing was then performed for 3 h. Enzymatic detection of ADH

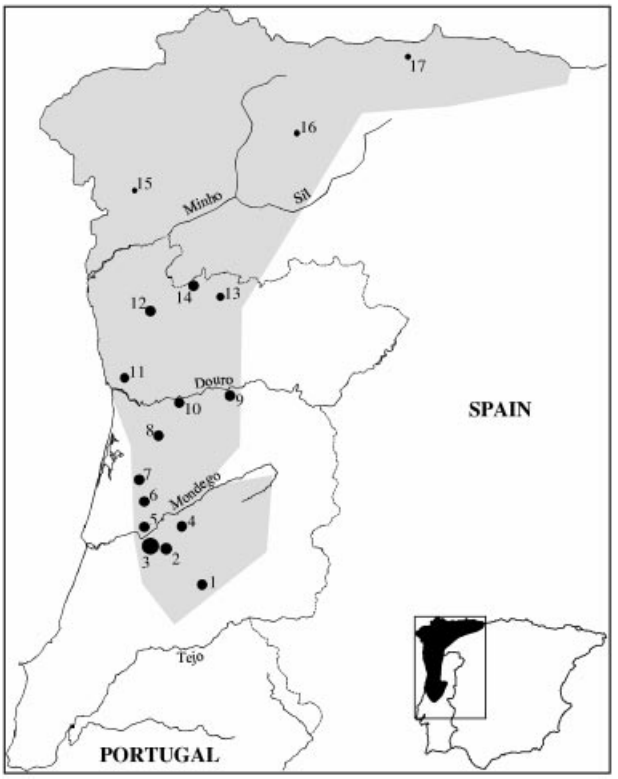

Fig. 1 The distribution of *Chioglossa lusitanica* in the north-western Iberian Peninsula (shaded areas, from Arntzen 1999) and the study localities: 1, Muradal, Foz de Giraldo (MU); 2, Lousã, C. Pêra (LCP); 3, Lousã, Vilarinho (LVI); 4, Açor, Margaraça (AC); 5,Várzeas (VA); 6, Buçaco (BU); 7, Saide (SA); 8, Covelo (C); 9, Tarouca (T); 10, Montemuro (M); 11,Valongo (V); 12, Bom Jesus (B); 13, Barroso (BA); 14, Gerês (G); 15, Pontevedra (PO); 16, Fonsagrada (FS); 17, Salas (SAL). Dots representing localities are sized proportionally to the level of expected heterozygosity.

was carried out following Harris & Hopkinson (1976). Protocols for the other enzymes were those of Alexandrino *et al*. (1997).

Allelic frequencies were determined by direct gene counting. Private alleles were defined as those observed in a single population. The Genetic Data Analysis computer program (Lewis & Zaykin 1999) was used: (i) to calculate the average number of alleles (A) and average expected heterozygosity (H_E); (ii) to assess deviations from hypothesized Hardy–Weinberg equilibrium (by exact probability following Fisher's method in a Markov-chain procedure); and (iii) to estimate Wright's F-statistics F, θ and f (estimators of F_T, F_{ST} and F_{IS}, respectively) according to Weir & Cockerham (1984), with associated bootstrap estimates for determination of the 95% confidence interval (CI). Hierarchical cluster analysis of populations (UPGMA) was carried out using PHYLIP version 3.5 (Felsenstein 1993) with Cavalli-Sforza & Edwards (1967) chord genetic distances. Bootstrap replication values

were determined to assess the strength of support from the data to the UPGMA-dendrogram. Non-hierarchical cluster analysis was carried out by multidimensional scaling (Lessa 1990) with the genetic distances using STATISTICA (StatSoft 1993).

DNA was extracted from alcohol-preserved tissue following the protocol of Sambrook *et al*. (1989). An 840-bp fragment of the Cyt *b* mtDNA gene was amplified via polymerase chain reaction (PCR) using the primers MVZ15 (Moritz *et al*. 1992; Tan & Wake 1995) and cytb702 (5′-GGCAAATAGGAAGTATCATTCTG-3 Moritz *et al*. 1992, modified). Samples were amplified through 35 cycles of denaturing for 90 s at 92 °C, annealing for 60 s at 54 °C and extending for 90 s at 72 °C. Sequences were determined by automated sequencing in the ABI PRISM 310 Genetic Analyser (PE Applied Biosystems) following the ABI PRISM BigDye Terminator Cycle Sequencing protocols. Sequences of 329 bp, corresponding to 16 309 (5′ end) and 16 637 (3′ end) of the *Xenopus laevis* mtDNA (Roe *et al*. 1985) were obtained for 44 individuals (two to four specimens per population).

A phylogenetic analysis of mtDNA haplotypes with published sequence data for *Mertensiella caucasica* (Tarkhnishvili *et al*. 2000) and *Salamandra salamandra* (García-París *et al*. 1998) as outgroups, was carried out with PAUP version 3.1 (Swofford 1993). A heuristic search with equal weighting for different character types (i.e. codon positions or transition/transversions) yielded a single tree and, because no homoplasy was observed within the ingroup, a single maximum parsimony network. Hierarchical cluster analysis of haplotypes (UPGMA) was carried out with Kimura two-parameter estimates of sequence divergence.

The significance of association between genetic variation and ecological and geographical distances was tested by partial Mantel tests (RT version 2.0; Manly 1996). Pairwise genetic distance, heterozygosity difference (absolute pairwise difference between H_E values for populations) and allelic difference (absolute pairwise difference between *A* values for populations), for allozymes, and average haplotype sequence divergence (between haplotypes for each pair of populations), for mtDNA data, were used as dependent variables. Geographic distance was measured on 1:100 000 maps. Ecological distance was defined as the number of major rivers separating pairs of populations. The major rivers considered were those completely crossing the species range, i.e. Mondego, Douro and Minho/Sil (Fig. 1).

Results

Allozyme variation

Allozyme polymorphisms. Genetic polymorphisms were newly described in four enzyme systems: ADH, PEP-A, PEP-C and PGD. Zymograms showed separate zones of activity for all enzymes. Polymorphisms were interpreted as determined by codominant alleles and the enzymatic systems examined as controlled by four structural loci. Two alleles were observed at *Pep-A*, three at *Pep-C* and *PGD*, and four at *ADH*. Four alleles (*PGM-1*1FV*, *PGM-1*1SV*, *PGM-1*5* and *PEP-B*4*) were newly described at loci known to be polymorphic (Alexandrino *et al*. 1997), bringing the total at 28 alleles over seven loci. Statistically significant deviations from expected phenotypic distributions assuming the Hardy–Weinberg equilibrium were not observed. Enzyme phenotypes corresponded to expectations based on their reported quaternary structure in amphibians and other vertebrates (Harris & Hopkinson 1976; Wright & Richards 1982; Frick 1983; Matson 1989). Allele frequencies and measures of genetic variability are given in Table 1.

Allelic distribution and heterozygosity. Substantial differences in the allelic distributions were observed between populations south and north of the Mondego River, to which five loci contributed (*ADH*, *PEP-C*, *PEP-D*, *PGD*, *PGM-1*). The loci *ADH* and *PEP-D* had alleles at high frequency in southern populations that were not observed in northern populations (*PEP-D*2* and *ADH*2*). The other three loci showed a similar but less differentiated pattern, with either alleles typical for southern populations present at low frequency in northern populations (*PGM-1*3F* in populations 5 and 6, and *PEP-C*2* in population 12), or with an allele at low frequency restricted to (*PGD*2*) or shared with (*PGM-1*1F*) southern populations. Yet other alleles were found south of the Mondego only, at low frequency (*PEP-B*4* and *PGM-1*3S*).

Populations located between the Mondego and the Douro (populations 5–10) were characterized by possession at moderate to high frequency (0.28–0.82) of the *PEP-B*2* allele, that was otherwise only detected in population 11 at low frequency. Populations 9 and 10, adjacent to the Douro, are further characterized by a high frequency of the allele *PGM-1*2*. Portuguese populations located north of the river Douro and all Spanish populations showed little genetic variation (average H_E 0.050 and 0.004, respectively), with up to nine alleles over seven loci. Eleven private alleles were detected and nine of these were found in populations from between Douro and Mondego. One low frequency allele was shared between two populations north and south of the Mondego (Table 1). Expected heterozygosity ranged from low values for populations at the northern and southern ends of the species range to a maximum of 0.27 at locality 3 in the Lousã mountains (Table 1, Fig. 1). A significantly negative relationship was observed between H_E and distance along a south-north axis ($r^2 = 0.82$, $P < 0.05$), in

Table 1 Allele frequency, sample size (*N*), average number of alleles (*A*) and average expected heterozygosity (H_E) at seven polymorphic loci in 17 populations of *Chioglossa lusitanica*

			Populations																
Locus		Allele	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
	N		28	36	31	33	25	47	29	26	17	21	24	14	17	23	18	12	25
ADH		1	—	0.46	0.34	—	1.00	0.99	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		2	1.00	0.54	0.66	1.00	—	—	—	—	—	—	—	—	—	—	—	—	—
		3	—	—	—	—	—	0.01	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	—	—	0.02	—	—	—	—	—	—	—	—	—
	N		28	26	28	21	24	26	20	21	17	21	23	14	17	21	17	12	26
PEPA		1	1.00	1.00	1.00	1.00	1.00	0.90	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		2	—	—	—	—	—	0.10	—	—	—	—	—	—	—	—	—	—	—
	N		26	79	31	33	21	48	33	48	17	44	45	14	17	30	18	12	26
PEPB		1	1.00	0.99	0.98	1.00	0.55	0.51	0.76	0.55	0.18	0.23	0.95	1.00	1.00	1.00	1.00	1.00	1.00
		2	—	—	—	—	0.43	0.49	0.24	0.45	0.82	0.77	0.05	—	—	—	—	—	—
		3	—	0.01	—	—	0.02	—	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	0.02	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		28	25	20	13	17	17	18	21	15	21	22	11	16	20	18	12	22
PEPC		1	—	0.12	0.33	—	0.97	1.00	1.00	1.00	1.00	1.00	1.00	0.73	1.00	1.00	1.00	1.00	1.00
		2	1.00	0.88	0.67	1.00	—	—	—	—	—	—	—	0.27	—	—	—	—	—
		3	—	—	—	—	0.03	—	—	—	—	—	—	—	—	—	—	—	—
	N		28	83	34	31	27	47	39	21	17	44	45	14	17	26	17	12	26
PEPD		1	0.02	0.01	0.32	0.07	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		2	0.98	0.99	0.68	0.93	—	—	—	—	—	—	—	—	—	—	—	—	—
	N		16	26	23	21	24	29	20	27	17	21	23	13	17	18	18	12	15
PGD		1	0.87	0.94	0.93	0.90	1.00	1.00	1.00	1.00	1.00	0.98	1.00	1.00	1.00	1.00	1.00	1.00	1.00
		2	0.13	0.06	0.07	0.10	—	—	—	—	—	—	—	—	—	—	—	—	—
		3	—	—	—	—	—	—	—	—	—	0.02	—	—	—	—	—	—	—
	N		27	83	44	33	25	47	34	48	17	58	107	14	16	33	18	12	26
PGM1		1F	—	0.02	0.09	—	0.82	0.84	0.77	0.45	0.44	0.28	0.46	0.36	0.34	0.63	1.00	0.96	1.00
		1S	—	—	—	—	—	0.12	0.22	0.53	—	0.09	0.54	0.64	0.66	0.37	—	—	—
		2	—	—	—	—	—	—	—	0.01	0.56	0.63	—	—	—	—	—	—	—
		3F	1.00	0.94	0.73	1.00	0.18	0.01	—	—	—	—	—	—	—	—	—	—	—
		3S	—	0.04	0.18	—	—	—	—	—	—	—	—	—	—	—	—	—	—
		3V	—	—	—	—	—	0.02	—	—	—	—	—	—	—	—	—	—	—
		4	—	—	—	—	—	0.01	—	—	—	—	—	—	—	—	—	—	—
		1FV	—	—	—	—	—	—	—	0.01	—	—	—	—	—	—	—	—	—
		1SV	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0.04	—
		5	—	—	—	—	—	—	0.01	—	—	—	—	—	—	—	—	—	—
A			1.29	2.00	2.00	1.29	1.57	2.00	1.43	1.71	1.29	1.57	1.29	1.29	1.14	1.14	1.00	1.14	1.00
H_E			0.041	0.140	0.273	0.042	0.124	0.143	0.105	0.149	0.112	0.131	0.083	0.122	0.065	0.066	0.000	0.011	0.000

the group of populations north of the Mondego (Fig. 2). A similar result was obtained for *A* and geographical distance ($r^2 = 0.64$, $P < 0.05$).

Genetic differentiation. *F*-statistics were estimated at F (F_{IT}) = 0.679 ± 0.097 (CI = 0.538–0.848) θ(F_{ST}) = 0.678 ± 0.088 (CI = 0.533–0.845) and f (F_{IS}) = – 0.005 ± 0.048 (CI = – 0.051–0.210). These values were significantly different from zero except for the latter one. All loci revealed a high contribution to the overall θ with the exception of the less polymorphic *PEP-A* and *PGD* (results not shown).

Multidimensional scaling of genetic distances resolved 17 populations of *Chioglossa lusitanica* in a coordinate system of which we present the first and second dimension (Fig. 3). Visual inspection of the bivariate plot revealed two clusters consisting of populations 1–4 (group 1) and populations 5–17 (group 2). The latter group can arbitrarily be subdivided in three subgroups. Hierarchical clustering in a UPGMA-dendrogram produced the same two

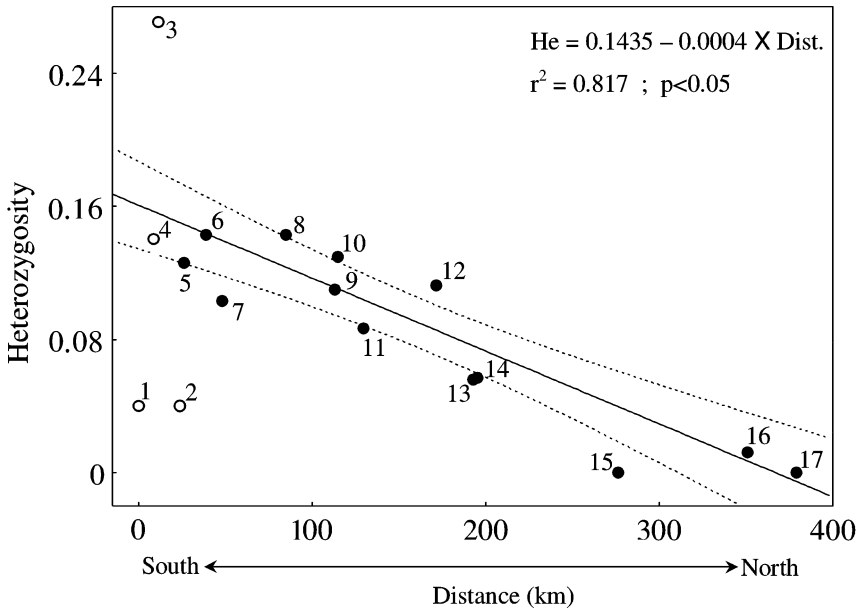

Fig. 2 Regression of expected enzyme heterozygosity of group 2 populations of *Chioglossa lusitanica* against the distance from the southernmost locality (locality 1) along a South-North axis. The interrupted lines represent the bounds of the 95% confidence limits of the regression line. Group 1 populations (open symbols) are included for comparison.

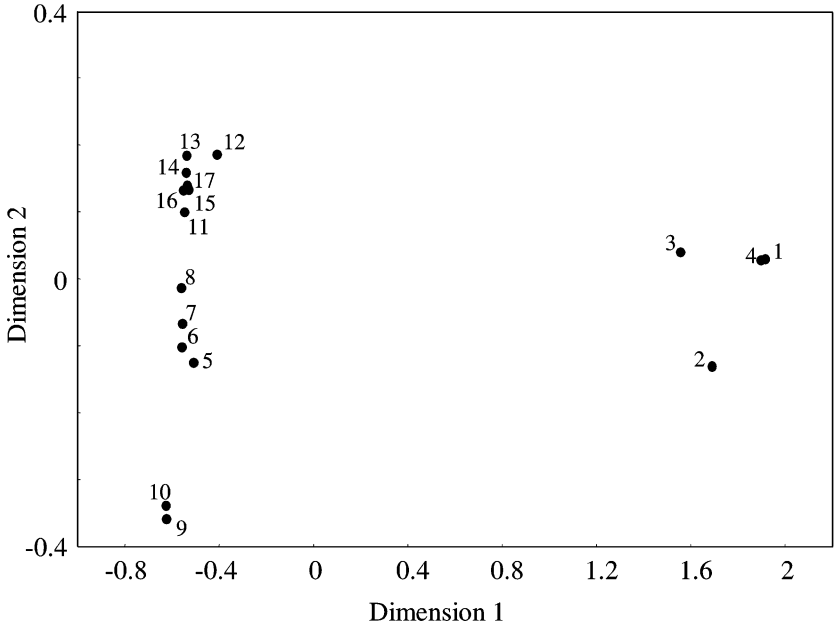

Fig. 3 Scatterplot of scores along the first and second dimensions obtained by multidimensional scaling of enzyme genetic distances for 17 populations of *Chioglossa lusitanica* (see text for details). Note that the axes have different scales.

major groups, the separate status of which was strongly supported by the bootstrap analysis (Fig. 4). Within group 2 populations four UPGMA-clusters were found with varying levels of bootstrap support (55–100%). On basis of the combined data (Figs 1, 3 and 4) we distinguish the following groups for practical purposes: group 2a (populations 5–8); group 2b (populations 9 and 10); and group 2c (populations 11–17). Together groups 2a and 2b comprise the study populations located between the Mondego and the Douro, and group 2c comprises the populations north of the Douro.

Previously published data for populations 2, 10, 11 and 14 (Alexandrino *et al*. 1997) were taken into account to calculate Nei's (1978) genetic distance over 22 loci. The genetic distance between group 1 (represented by population 2) and group 2 (represented by populations 10, 11 and 14) averaged at 0.154 ± 0.023.

mtDNA variation

Fifteen variable positions defined 13 distinct haplotypes with sequence divergence ranging from 0.3% to 3.2% and

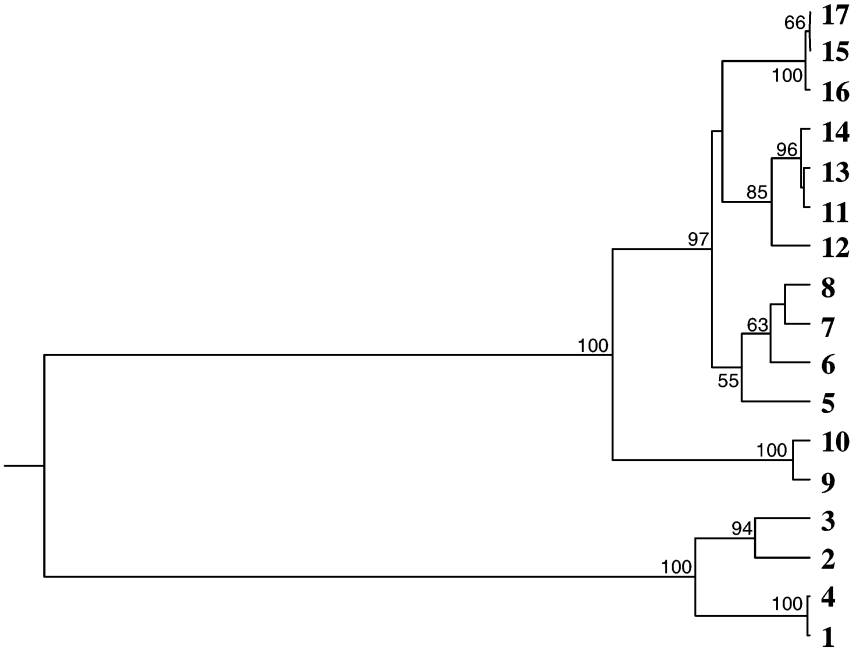

Fig. 4 A UPGMA dendrogram based on Cavalli-Sforza & Edwards (1967) chord enzyme genetic distances for 17 populations of *Chioglossa lusitanica*, with percent bootstrap replication scores > 50 indicated.

Table 2 Variable positions in the 329 bp segment of the cytochrome *b* gene defining 13 different haplotypes and their distribution across 17 populations of *Chioglossa lusitanica*. Nucleotide positions in boldface indicate aminoacid replacement substitutions. Species included for outgroup comparison are *Salamandra salamandra* (*Ssc*, *Ssm*) and *Mertensiella caucasica* (*Mc*A, *Mc*B1)

	Nucleotide position															Populations															
		5	1 0	1 1	**1 5**	1 7	1 8	**1 9**	2 2	2 2	2 5	2 6	2 7	2 9	3 1																
Haplotypes	3	4	2	4	**2**	5	3	**3**	2	5	2	7	6	4	8	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
h1	t	t	a	t	g	t	t	a	t	t	t	a	a	a	c	—	—	1	—	1	—	—	—	—	—	—	—	—	—	—	—	—
h2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	t	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
h3	.	.	.	.	.	.	.	g	.	.	.	.	.	.	.	3	2	1	—	1	1	—	—	—	—	—	—	—	—	—	—	—
h4	c	.	.	.	.	.	.	g	.	.	.	.	.	.	.	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
h5	.	.	.	.	.	.	.	g	.	c	.	.	.	.	.	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—
h6	.	.	.	.	.	.	c	g	.	c	.	.	.	.	.	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
h7	.	.	.	.	.	.	.	g	.	c	.	.	.	t	.	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
h8	.	.	.	.	c	.	.	g	.	c	.	.	.	.	.	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
h9	.	c	g	c	.	.	.	g	c	c	.	.	.	.	.	—	—	—	—	—	1	—	2	—	1	1	2	2	4	2	2	2
h10	.	c	g	c	.	.	.	g	c	c	.	.	g	.	.	—	—	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—
h11	.	c	g	c	.	c	.	g	c	c	.	.	g	.	.	—	—	—	—	—	—	—	—	2	2	—	—	—	—	—	—	—
h12	.	c	g	c	.	.	.	g	c	c	.	g	g	.	.	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—
h13	.	c	g	c	.	.	.	g	c	c	c	g	g	.	.	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—
Ssc	c	a	.	a	c	.	a	g	c	.	c	.	.	.	.																	
Ssm	c	a	.	a	.	.	a	g	c	.	.	.	.	.	.																	
*Mc*A	c	a	.	a	.	c	c	.	.	.	c	.	.	t	t																	
*Mc*B1	c	a	.	a	.	c	c	.	.	.	.	g	.	g	.																	

an overall nucleotide diversity of 1.4%. Two nucleotide substitutions were observed at the first codon position, one at the second position, and 12 at the third position, underlying two aminoacid replacements (Table 2).

Two sets of haplotypes were evident from the maximum parsimony network (Fig. 5A) and an UPGMA-dendrogram (results not shown). Haplotypes h1–h8 (group A) and haplotypes h9–h13 (group B) differed by a minimum of four nucleotide substitutions and 2.2% average sequence divergence (d). Within group A and group B, haplotypes were separated by single substitutions.

The phylogenetic analysis revealed a single most parsimonious tree with a total length of 147. Two monophyletic groups were found at the level of the root, one consisting

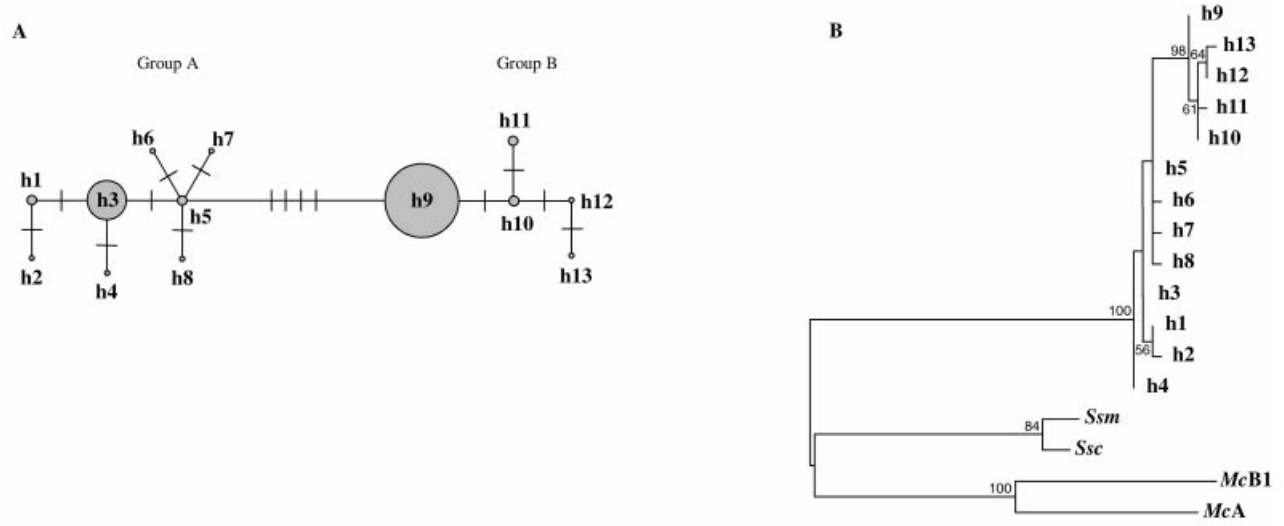

Fig. 5 (A) Maximum parsimony network of cytochrome *b* haplotypes observed in *Chioglossa lusitanica*. Circle size is proportional to the observed haplotype frequencies. (B) Single most parsimonious tree for 13 mtDNA cyt*b* haplotypes from *Chioglossa lusitanica* and four outgroup haplotypes from *Salamandra salamandra* (*Ssc*, *Ssm*) and *Mertensiella caucasica* (*Mc*A, *Mc*B1). Percent bootstrap replication scores > 50 are indicated on each branch.

Table 3 Partial Mantel test for association between genetic differentiation and geographical and ecological (river) distance in *Chioglossa lusitanica*. In the right hand panel ecological distance is partitioned over the three rivers constituting hypothesized barriers to gene flow. Tests are carried out over 17 populations (upper panel) and over 'group 2' populations (lower panel).

	Causal hypothesis (independent variables)					
Genetic differentiation (dependent variable)	Geographical distance	Ecological distance	Geographical distance	Ecological distance		
				Mondego	Douro	Minho
17 populations						
Enzyme genetic distance	ns	***	ns	***	ns	ns
mtDNA distance	ns	**	**	**	ns	ns
Group 2						
Enzyme genetic distance	ns	*	ns	na	**	ns
Heterozygosity difference	*	***	ns	na	**	**
Allelic difference	ns	*	ns	na	**	ns
mtDNA distance	ns	ns	ns	na	ns	ns

na – not applicable, ns – not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

of haplotype h4, and the other consisting of the remaining 12 haplotypes. Group B was defined by three synapomorphic character states (haplotypes h9–h13), with a high (98%) bootstrap replication score. No unambiguous support (< 56%) was found for the placement of the root, as long as group B remained monophyletic (Fig. 5B).

The geographical distribution of haplotypes was distinctly nonrandom. Haplotype h1 was observed in populations 3 and 5, haplotypes h2–h8 were observed in populations 1–5 and haplotypes h9–h13 were observed in populations 7–17. Only population 6 possessed haplotypes of the B as well as the non-B group (haplotypes h9 and h10 and haplotype 3, respectively; Table 2). The most frequently observed haplotype (h9, $n = 19$) was found in all populations north of the Mondego (except for populations 5 and 7), and was the only one detected in six out of seven populations north of the Douro. The other most frequent haplotype was found in populations adjacent to the Mondego (h3, $n = 8$; distance from Mondego < 55 km).

Association between genetic variation and geographical distance

Ecological distance and not geographical distance was significantly associated with enzyme genetic distance between populations (Table 3). When ecological distance

was partitioned into its components (Mondego, Douro and Minho/Sil), the Mondego and not the other rivers were significantly associated with genetic distance. mtDNA divergence between populations was associated with ecological distance, the Mondego river in particular and, ambiguously, with geographical distance also.

Within group 2 populations, genetic distance was significantly associated with ecological distance, as defined by the Douro river. Heterozygosity difference was associated with both Douro and Minho, and allelic difference was associated with the Douro. No significant association was found between mtDNA sequence divergence and any of the independent variables.

Discussion

Patterns of genetic diversity

Variation at seven polymorphic nuclear loci and the mtDNA cytochrome *b* cytoplasmic locus revealed remarkable levels of genetic substructuring in the salamander *Chioglossa lusitanica*. The observed overall enzyme genetic diversity was mainly attributable to differences between populations and largely partitioned over two spatially coherent groups of populations, distributed to the south (group 1) and to the north (group 2) of the Mondego River. A deep divergence was observed between group 1 and group 2 populations that were geographically close. Support for further subdivision within the northern group was spatially consistent but statistically weak with the exception of populations adjacent to the Douro River (group 2b). Estimates of F_{ST} θ (= 0.68) were high compared to other organisms in general (Avise 1994) and just below the highest F_{ST} value (0.80) reported among 22 species of salamanders (Larson *et al*. 1984). All loci, with the exception of two essentially uninformative ones (*PEP-A* and *PGD*), consistently described genetic variation partitioned between group 1 and group 2 populations. The high F_{ST} value is, therefore, more likely to reflect isolation for extensive periods of time than continuing low levels of gene flow per generation (Larson 1984; Larson *et al*. 1984).

Diverse values of genetic variability were observed both across group 1 and group 2 populations. Expected heterozygosity ranged from zero to 0.149, with the exception of population 3 (group 1) that exhibited an unusually high value (0.273). This resulted from intermediate allelic frequencies at otherwise nearly diagnostic loci, suggesting a recent admixture of genetically differentiated populations. Within group 2 populations, a marked decrease of genetic diversity was detected from south to north, both as measured by allelic diversity and observed heterozygosity. Moreover, both allelic diversity and heterozygosity decreased in steps coinciding with the rivers Douro and Minho/Sil, showing parallel variation at all loci, with the exception of *PGD* that is monomorphic within group 2 populations.

The geographical distribution of the mtDNA variants was generally concordant with the pattern shown by the enzyme nuclear loci. Two genetically different mtDNA haplotype groups (A and B) were observed in *C. lusitanica*. Unambiguous support was found for a monophyletic group of mtDNA haplotypes (group B), contrasting with uncertainty on the monophyly of the others (group A) and precluding the reconstruction of a hypothetical common ancestor. Doubts on position of the root notwithstanding, the geographical distribution of the group B haplotypes closely matched that of group 2 populations as defined by the enzyme data, suggesting a process of long-term independent evolution of this group 2–group B lineage.

The observed concordance of spatial genetic variation is more likely to result from the historical biogeography of the species than from current population dynamics or selection. Two mechanisms are invoked to explain the observations: (i) an historical fragmentation in the south of the species range; and (ii) a relatively recent range expansion resulting in (a) a decrease of genetic variation to the north and (b) an increase of genetic variation in the Mondego area upon secondary contact of populations.

Historical biogeography

Glacial refugia. The pattern of genetic variation suggests the former isolation of at least two population units in the southern part of the present-day species range. Following a 'molecular clock' argument and using clock calibrations for allozymes (0.05–0.1 *DNei*/MY; Maxson & Maxson 1979; Thorpe 1982; Beerli *et al*. 1996) and cytochrome *b* in the Salamandridae (0.8% sequence divergence/MY; Tan & Wake 1995; Caccone *et al*. 1997) the observed differentiation between groups (*DNei* = 0.15, *d* = 2.2%) would correspond with a genetic isolation of 1.5–3 MY, i.e. from the late Pliocene/early Pleistocene onwards. Given the timeframe involved, the major climatic and environmental changes that occurred during the Pleistocene appear to have determined the history of *C. lusitanica*. Unfortunately, the Pleistocene glaciations are not well documented for the Iberian Peninsula (except for some glaciated areas in northern and central Iberia — Ribeiro *et al*. 1987; Dias 1997; Vieira & Cordeiro 1998). The present-day distribution of *C. lusitanica* is associated with a temperate climate and covering areas historically dominated by deciduous forest. It is, therefore, not unlikely that the species followed a southward regression similar to that of the deciduous forest as reconstructed for western Iberia by Zagwijn (1992).

The two described population groups are separated by the Mondego suggesting that this river constituted a barrier to gene flow. Alternatively or additionally, dispersal

between the groups may have been limited by a zone of unfavourable habitat associated with the river. The present-day distribution shows a marked constriction in this very area (Fig. 1), where the Mondego valley is wide and constitutes a band of flat and dry landscape. Under adverse climatic conditions during the Pleistocene this may have contributed to a south-north separation as suggested by bioclimatic modelling (Teixeira 1999).

The hypothesized refugia differ in the number of alleles that they support, being low in the southern refugium and high in the northern one. A more substantial loss of allelic diversity may have been caused by strong population bottlenecking (Leberg 1992) in a comparatively small southern refugial population. Additionally, the northern group may have been composed of several small refugial populations that, not being separated one from the other by major rivers, merged upon amelioration of the climate and resulting range expansions. The presence of a variety of private alleles in populations in between Mondego and Douro supports the hypothesis of multiple refugia in this area. The Montemuro region may have been one of these as evidenced by the high frequency of the alleles that characterize group 2b (*PEP-B*2* and *PGM-1*2*).

Range expansion and postglacial recolonization. The present-day distribution of *C. lusitanica* is continuous across the Mondego river (Fig. 1). In combination with the identified refugia this suggests range expansion and secondary contact upon climatic amelioration following the Pleistocene. The presence of alleles characteristic for group 2 in a group 1 population (*ADH*1*, *PEP-C*1*, *PEP-D*1* in population 3 from Lousã Mountains) suggests gene flow between the groups and helps to explain the high heterozygosity of that population (Fig. 2). Similar observations were not made for neighbouring populations (e.g. population 2, also from the Lousã mountains) south of the Mondego, indicating that introgression has been spatially restricted. The presence of the allele *PGM-1*3F* and of non-B haplotypes in populations 5 and 6 suggests that introgression on the northern side of Mondego has also taken place. Differential introgression of genetic markers is not uncommon and has been observed both between nuclear loci and between nuclear and cytoplasmic loci (e.g. Hunt & Selander 1973; Gyllensten & Wilson 1987; for a review see Arnold 1997). To address this issue in *C. lusitanica* would require an analysis at finer spatial scale documenting the position and shape of the contact zone.

In view of the long-term (1.5–3.0 MY) independent evolution of two *C. lusitanica* lineages and the level of genetic differentiation achieved, it is tempting to speculate about their taxonomic status. A broad secondary contact zone, implying extensive introgressive hybridization, would be incompatible with species recognition under the biological species concept, but not incompatible with subspecies recognition, irrespective of the shape (clinal or stepped) the morphological variation takes (J. Alexandrino, unpublished data).

The genetic composition of northern populations suggests that they were recently established and derived from a source located somewhere between the rivers Mondego and Douro. The range expansion proceeded to the north, following the habitat that had become newly available upon climatic amelioration. For group 1 populations range expansion to the south was problematic because of the specialized habitat requirements of the species, involving mountain brooks that are largely unavailable south of the inferred southern refugium.

The two-stepped loss of genetic variation coinciding with rivers Douro and Minho is a strong indication that rivers acted as barriers in the process of northern recolonization. The repeated founding of new populations by small numbers of animals would create, through drift, a pattern of genetic depauperation similar to the one we observed (Fig. 2). The river barrier effect can, however, by no means have been absolute because northerly range expansion has been successful. The drift of larvae, discharged from mountain streams into the rivers, may be common in this species (Thiesmeier 1994). Postmetamorphic salamanders must also be considered capable of crossing a river, accidentally or deliberately. More genetic markers than presently available would be required to investigate patterns of dispersal over a network of streams or to quantify salamander dispersal across and along rivers. Additionally, the availability of more highly variable genetic markers would alleviate the reduction of power that we experience in the analysis of populations with low variation at enzyme and mtDNA cytochrome *b* loci.

Implications for conservation

The effects of Pleistocene glaciations through fragmentation, isolation and differentiation, have been described throughout the northern hemisphere for a variety of organisms, including trees, insects, fish, salamanders and mammals (Highton & Webster 1976; Sage & Wolf 1986; Cooper *et al*. 1995; Dumolin-Lapègue *et al*. 1997; García-Marín *et al*. 1999). Most studies report the preservation of high levels of genetic diversity in populations derived from southern refugia and the loss of genetic diversity in northern recolonized areas (Larson 1984; Avise 1994; Hewitt 1996; Taberlet *et al*. 1998). However, few studies have addressed the consequences of Pleistocene climate change on the genetic architecture of organisms within any of the southern European refugia (Ragghianti & Wake 1986; Comes & Abbott 1998). The spatial pattern of genetic variation in the fire salamander, *Salamandra salamandra*, Linnaeus 1758, suggested the presence of Pleistocene refugia in northern Iberia (Alcobendas *et al*. 1996; Dopazo *et al*. 1998). Our conclusion that northern Iberian

populations resulted from a range expansion from the south argues against this scenario operating in *C. lusitanica*. Although *Salamandra* and *Chioglossa* are phylogenetically related (Titus & Larson 1995; Veith *et al*. 1998), they are ecologically dissimilar (Klewen 1991; Arntzen 1999) and biogeographically disparate histories are to be expected. The example of *C. lusitanica* provided the evidence for an unexpected natural history of populations on the Iberian Peninsula. The challenge is to evaluate to which extent other organisms with different, less explicit ecological requirements, show the same or similar patterns of fragmentation and dispersal. We anticipate that the diversity uncovered through such studies will have profound implications for the conservation of biodiversity in southern Europe.

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References

Alcobendas M, Dopazo H, Alberch P (1996) Geographic variation in allozymes of populations of *Salamandra salamandra* (Amphibia: Urodela) exhibiting distinct reproductive modes. *Journal of Evolutionary Biology*, **9**, 83–102.

Alexandrino J, Ferrand N, Arntzen JW (1997) Genetic variation in some populations of the golden-striped salamander, *Chioglossa lusitanica* (Amphibia: Urodela), in Portugal. *Biochemical Genetics*, **35**, 371–381.

Arnold ML (1997) Natural hybridization and evolution. Oxford University of Press, New York.

Arntzen JW (1981) Ecological observations on *Chioglossa lusitanica* (Caudata, Salamandridae). *Amphibia-Reptilia*, **1**, 187–203.

Arntzen JW (1994) Speedy salamanders: sedentariness and migration of *Chioglossa lusitanica*. *Revista Española Herpetologia*, **8**, 81–86.

Arntzen JW (1999) *Chioglossa lusitanica* Bocage, 1864 — Goldstreifensalamander, In: *Handbuch der Amphibien und Reptilien Europas* (eds K. Grossenbacher, B. Thiesmeier), pp. 301–321. AULA-Verlag, Wiesbaden.

Avise JC (1994) Molecular markers. *Natural History and Evolution* Chapman & Hall, London.

Beerli P, Hotz H, Uzzell T (1996) Geologically dated sea barriers calibrate a protein clock for Aegean water frogs. *Evolution*, **50**, 1676–1687.

Caccone A, Milinkovitch MC, Sbordoni V, Powell JR (1997) Mitochondrial DNA rates and biogeography in European newts (genus *Euproctus*). *Systematic Biology*, **46**, 126–144.

Cavalli-Sforza LL, Edwards AWF (1967) Phylogenetic analysis: Models and estimation procedures. *American Journal of Human Genetics*, **19**, 233–257.

Comes HP, Abbott RJ (1998) The relative importance of historical events and gene flow on the population structure of a Mediterranean ragwort *Senecio gallicus* (Asteraceae). *Evolution*, **52**, 355–367.

Cooper SJB, Ibrahim KM, Hewitt GH (1995) Postglacial expansion and genome subdivision in the European grasshopper *Chorthipus parallelus*. *Molecular Ecology*, **4**, 49–60.

Dias JMA (1997) Evolução da linha de costa, em Portugal, desde o último máximo glaciário até à actualidade: síntese dos conhecimentos. *Estudos do Quaternário*, **1**, 53–66.

Dopazo H, Boto L, Alberch P (1998) Mitochondrial DNA variability in viviparous and ovoviviparous populations of the urodele *Salamandra salamandra*. *Journal of Evolutionary Biology*, **11**, 365–378.

Dumolin-Lapègue S, Demesure B, Fineschi S, Le Corre V, Petit RJ (1997) Phylogeographic structure of white oaks throughout the European continent. *Genetics*, **146**, 1475–1487.

Felsenstein J (1993) *PHYLIP (Phylogeny Inference Package), Version 3.5. Distributed by the author*. University of Washington, Seattle.

Frick L (1983) An electrophoretic investigation of the cytosolic di- and tripeptidases of fish: molecular weights, substrate specificities, and tissue and phylogenetic distributions. *Biochemical Genetics*, **21**, 309.

García-Marín J, Utter FM, Pla C (1999) Postglacial colonization of brown trout in Europe based on distribution of allozyme variants. *Heredity*, **82**, 46–56.

García-París M, Alcobendas M, Alberch P (1998) Influence of the Guadalquivir river basin on the mitochondrial DNA evolution of *Salamandra salamandra* (Caudata: Salamandridae) from southern Spain. *Copeia*, **1998**, 173–176.

Gyllensten UB, Wilson AC (1987) Interspecific mitochondrial DNA transfer and the colonization of Scandinavia by mice. *Genetical Research Cambridge*, **49**, 25–29.

Harris H, Hopkinson DA (1976) *Handbook of Enzyme Electrophoresis in Human Genetics*. North-Holland Publishing Co, Amsterdam.

Hewitt GM (1996) Some genetic consequences of ice ages, and their role in divergence and speciation. *Biological Journal of the Linnean Society*, **58**, 247–276.

Highton R, Webster TP (1976) Geographic protein variation and divergence in populations of the salamander *Plethodon cinereus*. *Evolution*, **30**, 33–45.

Hunt WG, Selander RK (1973) Biochemical genetics of hibridisation in European house mice. *Heredity*, **31**, 11–33.

Klewen R (1991) *Die Landsalamander europas: 1*. A. Ziemsen-Verlag, Wittenberg Lutherstadt.

Larson A (1984) Neontological inferences of evolutionary pattern and process in the salamander family Plethodontidae. *Evolutionary Biology*, **17**, 119–217.

Larson A, Wake DB, Yanev KP (1984) Measuring gene flow among populations having high levels of genetic fragmentation. *Genetics*, **106**, 293–308.

Leberg PL (1992) Effects of population bottlenecks on genetic diversity as measured by allozyme electrophoresis. *Evolution*, **42**, 447–494.

Lessa E (1990) Multidimensional analysis of geographic genetic structure. *Systematic Zoology*, **39**, 242–252.

Lewis PO, Zaykin D (1999) *Genetic Data Analysis: Computer program for the analysis of allelic data, Version 1.0 (d12)*. Distributed at the internet at http://chee.unm.edu/gda/.

Manly BFJ (1996) *RT — A program for randomization testing, Version 2.0. Centre for applications of statistics and mathematics (CASM)*. University of Otago, New Zealand.

Matson RH (1989) Avian peptidase isozymes: tissue distributions, substrate affinities, and assignment of homology. *Biochemical Genetics*, **27**, 137–151.

Maxson LR, Maxson RD (1979) Comparative albumin and biochemical evolution in Plethodontid salamanders. *Evolution*, **33**, 1057–1062.

Moritz C, Schneider CJ, Wake DB (1992) Evolutionary relationships within the *Ensatina escholtzii* complex confirm the ring species interpretation. *Systematic Biology*, **41**, 273–291.

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics*, **89**, 583–590.

Phillips CA (1994) Geographic distribution of mitochondrial DNA variants and the historical biogeography of the spotted salamander, *Ambystoma maculatum*. *Evolution*, **48**, 597–607.

Ragghianti M, Wake D (1986) Genic variation and its evolutionary implications in the Italian newt, *Triturus italicus*. *Herpetologica*, **42**, 206–214.

Ribeiro O, Lautensach H, Daveau S (1987) *Geografia de Portugal. I. A posição geográfica e o território*. Edições João Sá da Costa. Lisboa.

Roe B, Ma D, Wilson R, Wong J (1985) The complete nucleotide sequence of the *Xenopus laevis* mitochondrial genome. *Journal of Biological Chemistry*, **260**, 9759–9774.

Sage RD, Wolff JO (1986) Pleistocene glaciations, fluctuating ranges and low genetic variability in a large mammal (*Ovis dali*). *Evolution*, **40**, 1092–1095.

Sambrook E, Fritsch F, Maniatis T (1989) *Molecular Cloning*. Cold Spring Harbor Press, Cold Spring Harbor, New York.

Sequeira F, Teixeira J, Alexandrino J, Lima V, Ferrand N (1996) Distribución de *Chioglossa lusitanica* en Portugal. *Boletin de la Asociacíon Herpetologica Española*, **7**, 7.

Slatkin M (1981) Estimating levels of gene flow in natural populations. *Genetics*, **99**, 323–335.

StatSoft (1993) *STATISTICA for Windows*, release 4.5. StatSoft Inc., Tulsa, USA.

Swofford DL (1993) *PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1*. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois.

Taberlet P, Fumagalli L, Wust-Saucy A, Cosson J (1998) Comparative phylogeography and postglacial colonization routes in Europe. *Molecular Ecology*, **7**, 453–464.

Tan AM, Wake DB (1995) MtDNA phylogeography of the California newt, *Taricha torosa* (Caudata, Salamandridae). *Molecular Phylogenetics and Evolution*, **4**, 383–394.

Tarkhnishvili DN, Thorpe RS, Arntzen JW (2000) Population genetic structure and geographical subdivision of the Caucasian salamander (*Mertensiella caucasica*). *Molecular Phylogenetics and Evolution*, **14**, 414–422.

Teixeira J (1999) *Biogeografia de Salamandra-lusitânica: utilização de Sistemas de Informação Geográfica na modelação da distribuição*. MSc Thesis. University of Porto, Portugal.

Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history: a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767–782.

Thiesmeier B (1994) Trophische Beziehungen und Habitatpräferenzen sympatrisch lebender *Salamandra salamandra* und *Chioglossa lusitanica*-Larven. *Abhandlungen und Berichte für Naturkunde*, **17**, 119–126.

Thorpe JP (1982) The molecular clock hypothesis: biochemical evolution, genetic differentiation and systematics. *Annual Review of Ecology and Systematics*, **13**, 139–168.

Titus TA, Larson A (1995) A molecular phylogenetic perspective on the evolutionary radiation of the salamander family Salamandridae. *Systematic Biology*, **44**, 125–151.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Wright DA, Richards CM (1982) Peptidase isozymes of the leopard frog *Rana pipiens*: properties and genetics. *Journal of Experimental Zoology*, **221**, 283–293.

Veith M, Steinfartz S, Zardoya R, Seitz A, Meyer A (1998) A molecular phylogeny of 'true' salamanders (family Salamandridae) and the evolution of terrestriality of reproductive modes. *Journal of Zoological Systematics and Evolutionary Research*, **36**, 7–16.

Vieira GT, Cordeiro AMR (1998) Geomorfologia periglaciária em Portugal: estado do conhecimento. *Procesos biofísicos actuales en medios fríos. Publicacions de la Universitat de Barcelona*, 347–371.

Zagwijn WH (1992) Migration of vegetation during the Quaternary in Europe. *Courier Forschunginstitut Senckenberg*, **153**, 9–20.

Our research group is interested in population genetics, biogeography and conservation, with the emphasis on vertebrate species of the Iberian Peninsula. The current study is part of the PhD thesis by João Alexandrino on the evolutionary biology of the endemic Iberian salamander *Chioglossa lusitanica*. Elsa Froufe worked on the project for her graduate thesis in Biology and has recently taken up a project on hybridizing newt species in Central Portugal. Pim Arntzen is a visiting scientist, working on the evolutionary biology, ecology and systematics of amphibians. Nuno Ferrand is Associate Professor and heads the Unit of Animal Genetics and Conservation, at the Centre for the Study of Animal Sciences. He is interested in a variety of questions in evolutionary and conservation genetics.
