Chapter XX

Historical biogeography and conservation of the golden-striped salamander (*Chioglossa lustanica*) in northwestern Iberia: integrating ecological, phenotypic and phylogeographic data

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Keywords

allozymes, *Chioglossa lusitanica*, ecological modeling, evolutionary significant units, golden-striped salamander, glacial refugia, mitochondrial DNA, phenotypic variation, post-glacial range expansion

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Abstract The Golden-striped salamander (*Chioglossa lusitanica*) is an endemic species inhabiting streamside habitats in mountainous areas in northwestern Iberian Peninsula. This salamandrid is listed in the IUCN Red Data Book as a threatened species. The combination of bioclimatic modeling of the species distribution and multivariate analysis of genetic and phenotypic data strengthens previous hypotheses concerning the historical biogeography of *C. lusitanica*: the Pleistocene subdivision of the species' range and a process of postglacial re-colonization. Discrepancies between bioclimatic modeling predictions and the present-day distribution suggest that the species may still be expanding its range northwards. We propose the identification of two distinct units for the conservation of the species and suggest that this information should be taken into account in defining key areas for conservation in the Iberian Peninsula.

Introduction

Fluctuations in spatial distribution and abundance are commonplace for many plant and animal species. The biotic effects of Pleistocene glaciations exemplify how climate changes influence species distributions – by alternately inducing southward range contractions with northward expansions (Hewitt 1996, 1999, 2000). Southern Europe contained refugia where many species survived during glacial periods and thus represents a center of origin for many post-glacial re-colonization's. The geographic patterns resulting from these processes differ with the varying dispersal abilities and ecological requirements of each species (Hewitt 1996; Taberlet *et al.* 1998).

Patterns of species distribution and diversity have been represented through plotting presence/absence data on grid systems at various scales. Spatial modeling techniques may predict the distribution of species (Walker 1990; Pereira & Itami 1991; Brito *et al.* 1996) and individuals (Austin *et al.* 1996), be used to estimate suitable habitat (Mladenoff & Sickley 1998) or aid in the design of conservation plans (Velázquez & Bocco 1994). One important limitation of these models is that they fail to capture historical patterns of population persistence.

The geographical distribution of genetic diversity in species may be used to reconstruct historical biogeographies (Avise 1994, 1998; Bermingham & Moritz 1998). Phylogeography seeks to reveal historical biogeography of species through i) qualitative spatial association of alleles with geography, and ii) quantitative estimates of historical population size (Avise 2000, Emerson *et al.* 2001, Hare 2001, Templeton 2002). Ideally, phylogeographic inferences should be accompanied by evidence from independent sources such as the fossil record or paleo-ecology. Recently, a novel approach was explored using paleo-climatological models of species distributions in conjunction with phylogeography (Hugall *et al.* 2002).

One important caveat of phylogeographic studies is that they have mostly relied on single locus data, usually from mitochondrial DNA. Because nuclear gene based research is only now emerging (Hare 2001), multi-locus allele frequency data such as those obtained from allozyme polymorphism studies are invaluable in phylogeographic inference. Multivariate analysis of allele frequency data (e.g. data from allozyme variation) may summarize variation at several genes with a few independent synthetic variables that can be represented in geographical maps (Menozzi *et al.* 1978).Such analysis in humans has been used to construct maps that helped reveal demic expansions and determine centers of origin (Cavalli-Sforza *et al.* 1993, 1994)(Cavalli-Sforza 1993). While this approach has been applied extensively in human studies (Menozzi *et al.* 1978; Piazza *et al.* 1981; Cavalli-Sforza *et al.* 1993, 1994; Bosch *et al.* 1997) it has rarely been used on other organisms (Guinand & Easteal 1996, Le Corre *et al.* 1998)

The golden-striped salamander, *C. lusitanica*, is an Iberian endemic listed in the IUCN Red Data Book. Its range is restricted to the northwestern corner of the Iberian Peninsula, where it lives around small brooks in fairly mountainous terrain. We recently analyzed allozyme and mitochondrial DNA variation and uncovered two genetically distinct groups of populations that are geographically separated by the river Mondego in central Portugal (Alexandrino et. al. 1997, 2000, 2002). The two groups represent lineages that separated in the early Pleistocene, probably as a result of climate change in combination with local environmental conditions. A secondary contact zone between the groups was formed post-glacially. We further inferred that the northern part of the present range was colonized from a refuge located between the Mondego and Douro rivers, and that major rivers such as the Douro and the Minho acted as barriers to dispersal, lowering genetic diversity through sequential bottlenecking of northward expanding populations.

We combine ecological models of the distribution of *C. lusitanica* (Teixeira *et al.* 2001) with both mtDNA data and synthetic genetic maps constructed from multivariate analysis of allozyme genetic data. Our aims are i) to discuss hypothesized historical biogeographic scenarios, and ii) to propose areas for the long-term conservation of the species based on present habitat suitability and historical population persistence.

Debate in the field of conservation genetics has revolved around the relative importance of molecular versus quantitative genetic and phenotypic characterization of diversity and the resulting assignment of systematic management units. It was recently suggested that greater clarity would be achieved by partitioning genetic diversity into two components: that arising from adaptive evolution and that resulting from long-term historical isolation (Moritz 2002). The former can be estimated through analysis of phenotypic variation, while the latter is readily assayed through molecular phylogeography. Both approaches have their place, but measure different components of intraspecific diversity. It has been suggested that long-term historical isolation and persistence of populations should be given more emphasis in conservation because the genetic variation arising through such processes represents an irreplaceable component of intraspecific biodiversity. As phenotypic diversity is also highly relevant to conservation efforts, we also compare overall patterns of genetic and morphological variation in *C. lusitanica*.

Materials and methods

The distribution model

The spatial model used to predict the distribution of *C. lusitanica* was recently constructed based on presence/absence data in Portugal and a set of environmental parameters, using logistic regression (Teixeira *et al*. 2001). The probability of the species' occurrence across the Iberian Peninsula was estimated with a 93% success rate, based on total annual precipitation (PRET), slope (SLOP), altitude (ALTI), and mean July temperatures (TJUL), $g(x) = -0.087 + 0.131 \times \mathrm{PRET} + 0.063 \times \mathrm{SLOP} - 0.063 \times \mathrm{ALTI} - 0.052 \times \mathrm{TJUL}$) (Teixeira *et al*. 2001).

Allozyme data

Genetic data consisted of allele frequencies at six polymorphic allozyme loci (PGM1, PEPB, PEPC, PEPD, ADH and PGD) scored from 17 populations distributed across the entire species' range (Alexandrino *et al*. 2000). The most common allele at five nearly di-allelic loci (*PEPB*1*, *PEPC*1*, *PEPD*1*, *PGD*1* and *ADH*1*) and four out of five alleles at the highly polymorphic PGM1 locus (*PGM1*1F*, *PGM1*1S*, *PGM1*2* and *PGM1*3F*) were used for PCA. This selection of alleles emphasizes the major components of variation, and the exclusion of rare alleles (frequency <0.05) decreases the effect of sampling error. Allele frequencies were spatially interpolated by a linear distance weighting model (kriging default) to generate 400 allele frequency values regularly distributed within a grid delimited by parallels 39-44° N and meridians 4-9° W, using the Surfer 6.0 geostatistical software (Golden Software 1996). Interpolated allele frequencies for each allele were then used as input variables in a principal component analysis with the software package Statistica/w 4.5 (StatSoft 1993) The factor scores for the first two principal components (PC) were used to construct geographical maps with the kriging interpolation procedure in Surfer 6.0 (Golden Software 1996). Following Menozzi *et al.* (1978), maps were overlaid with a weighting function reflecting the percent of total variance explained, for the area within the *C. lusitanica* range. The software used for the manipulation of maps was Idrisi for Windows v. 2.0 (Eastman, 1997).

Mitochondrial DNA

Genetic data consisted of mtDNA haplotypes scored from the same 17 populations as noted above (Alexandrino *et al.* 2002). A geographic map summarizing mtDNA variation was constructed by overlaying the results from Nested Clade Analysis (NCA) (Templeton 1998) and population expansion tests (see Alexandrino *et al.* 2002).

Multivariate analysis of phenotypic data

Morphometric and dorsal color pattern variation in *C. lusitanica* were previously described (Alexandrino 2000; Alexandrino *et al.* submitted). Data for eight morphometric measurements in 18 populations were analyzed for males and females separately using principal component analysis (PCA). Colour pattern data for 420 individuals classified into six distinct types was analyzed by Correspondence Analysis (CA). Trend surface maps were generated for both PCA and CA axes by kriging under default settings in Surfer 6.0 (Golden Software 1996) geo-statistical software.

Results and discussion

Genetic diversity

The first axis from the PCA based on allozyme frequencies explained 70% of the total variation, showing a south-to-north cline in variation from Serra do Muradal in central Portugal to Salas in northern Spain (***Table 1***; ***Figure 1A***). The steep geographic gradient along the Mondego valley likely reflects recent secondary contact between two formerly isolated groups and implies the existence of both southern and northern glacial refugia (Alexandrino *et al*. 2000, 2002). The second axis explained 25% of the total variation (***Figure 1B***). It shows a diffusion gradient, suggesting range expansion from a center of origin following genetic isolation (Menozzi *et al*. 1978).

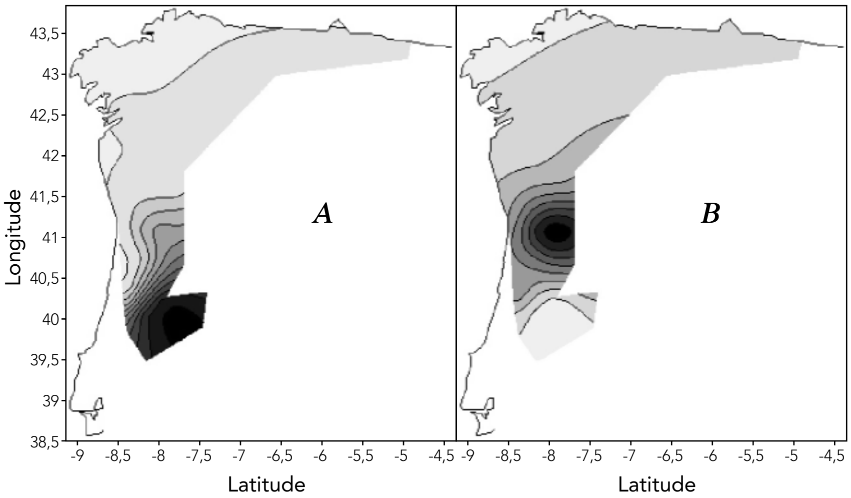

Figure 1*. Principal Component Analysis of 9 independent allele frequencies at the loci PGM1, PEPB, PEPC, PEPD, PGD and ADH in 17 populations of Chioglossa lusitanica across its documented range (Arntzen, 1999)*: ***A***) Synthetic map for the first principal component, representing 70% of the total variation; ***B***) Synthetic map for the second principal component, representing 25% of the total variation.

***Table 1**. Factor loadings for the first and second Principal Component (PC) axis for nine allele frequencies observed at six allozyme loci in 17 populations of Chioglossa lusitanica (Alexandrino et al. 2000).*

Allele	PC 1	PC 2
PGM1*1F	0.88	0.15
PGM1*1S	0.79	0.48
PGM1*2	0.01	-0.96
PGM1*3F	-0.97	0.09
PEPB*1	-0.05	0.96
PEPC*1	0.96	-0.14
PEPD*1	0.97	-0.02
PGD*1	0.94	-0.01
ADH*1	0.98	-0.05
Variance explained	**70 %**	**25 %**

PC1 and PC2 in combination clearly show the two differentiated population groups, to the south and north of the Mondego River (***Figure 2***). We infer that the northern range expanded from a glacial refuge in the Serra de Montemuro, northwards to occupy most of Galicia and Asturias in Spain and southwards to the Mondego River valley. The genetic uniformity exhibited in populations in the northernmost range of the species, suggests that these territories were either recently colonized or reflect founder effects (Alexandrino *et al*. 2000, 2002). Two divergent mtDNA lineages, distributed south and north of the Mondego valley, were observed in populations of *C. lusitanica*, implying the same past fragmentation processes promoting divergence at nuclear allozyme loci (***Figure 3***) (Alexandrino *et al*. 2000, 2002). Levels of local population mtDNA diversity revealed that populations near the river valley had a more stable demography in the past when compared with other populations to the south and to the north which may have undergone demographic and range expansions (Alexandrino *et al*. 2002).

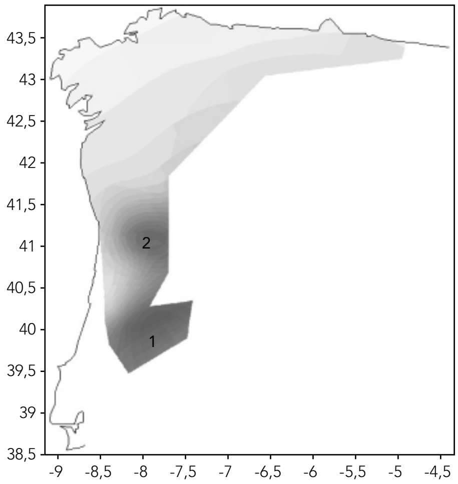

***Figure 2**. Superimposition of maps A and B of Figure 1, weighting the variance explained by each of the two PCs.* The red colour was used for the first principal component and the blue colour for the second principal component. The two colors correspond to two distinct evolutionary lineages in *Chioglossa lusitanica*.

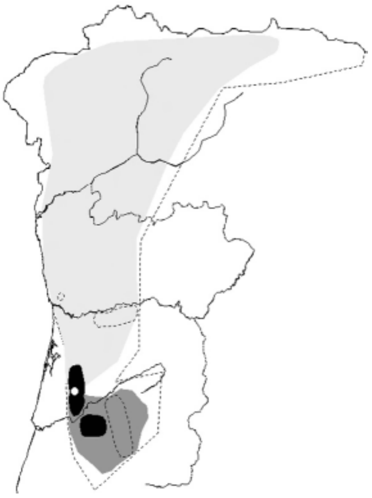

***Figure 3**. Geographic distribution of the two mtDNA clades observed in Chioglossa lusitanica.* The two clades are represented with distinct gray shading. The two entire clades and dotted-line areas may have undergone recent population expansions while black shaded areas represent historically more stable populations, according to mtDNA diversity analysis (see text for details)

Phenotypic vs. genetic diversity

Morphometric variation in *C. lusitanica* was found to be consistent with documented genetic differentiation. Populations south of the Mondego River are characterized by shorter digits than populations to the north, as revealed by PCA (***Figure 4A***) (Alexandrino 2000; Alexandrino *et al.* unpublished). However, a finer scaled assessment reveals a stepped south-to-north cline of increasing limb, toe- and finger length. We suggest that both historical isolation (vicariance) and selection account for the observed variation. Short appendages, with a low volume to surface ratio, may represent an adaptation to xeric environments (Nevo 1972; Lee 1993). *C. lusitanica* is a terrestrial streamside salamander extremely dependent on moist habitats and indeed the level of annual precipitation is the main predictor of its range in Portugal (Teixeira *et al.*, 2001). Given that southern populations appear to occupy a more xeric environment than northern populations (Arntzen & Alexandrino in press) and assuming that rainfall gradients in the past paralleled those found today, selection could have produced the documented (stepped) clines. Neither the pattern nor variability in colour was associated with group membership or with geographic distances between populations (Alexandrino 2000; Alexandrino *et al.* unpublished). However, colour pattern variability was higher within the contact zone than elsewhere, suggesting that the mixing of differentiated gene pools increased phenotypic variation. Two additional phenotypic characters show concordance with genetic variation within the northern population group. First, a south-to-north decrease was observed in genetic and colour pattern variability (***Figure 4B***). The processes of sequential bottlenecking and drift invoked to explain the decrease in genetic variation (Alexandrino *et al.*, 2000) appear equally applicable to morphological variation. Secondly, the dominance of an otherwise rare colour type in populations immediately south of the Douro River may reflect a separate historical refuge, as suggested by the presence of unique nuclear and mtDNA alleles (Alexandrino *et al.* 2000; Alexandrino *et al.* 2002). Overall, however, the genetic subdivision of *C. lusitanica* is not matched by an equally pronounced morphological differentiation. Selection operating along environmental gradients appears to be more important in shaping phenotypic diversity than genetic isolation.

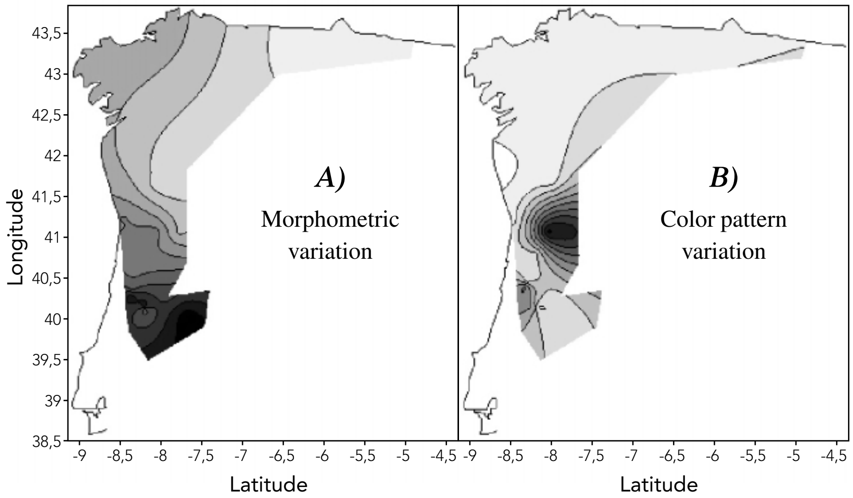

Figure 4**. Phenotypic diversity in Chioglossa lusitanica*: ***A) Trend surface map for morphometric variation generated by the kriging of mean factor scores of the first Principal Component axis (47% of total variation) of 18 populations of female *C. lusitanica*; ***B)*** Trend surface map for colour pattern variation generated by the kriging of mean factor scores of the first Correspondence Analysis axis (61% of total variation) of 20 populations of *C. lusitanica*.

Insights from the ecological models and comparison with *Lacerta schreiberi*

Ecological models for the past and present day distribution of *C. lusitanica* help to understand the historical biogeography of the species. First, the eastern Mondego valley receives low annual precipitation and is thus poor habitat exhibiting a correspondingly low probability for *C. lusitanica* occurrence according to the ecological model (***Figure 5A***). This region effectively separates southern and northern populations, at least in the central and eastern part of the species range. Under adverse climatic conditions of the Pleistocene, isolation may have been complete, supporting the hypothesis of vicariance across the Mondego river valley (Teixeira & Arntzen 2002).

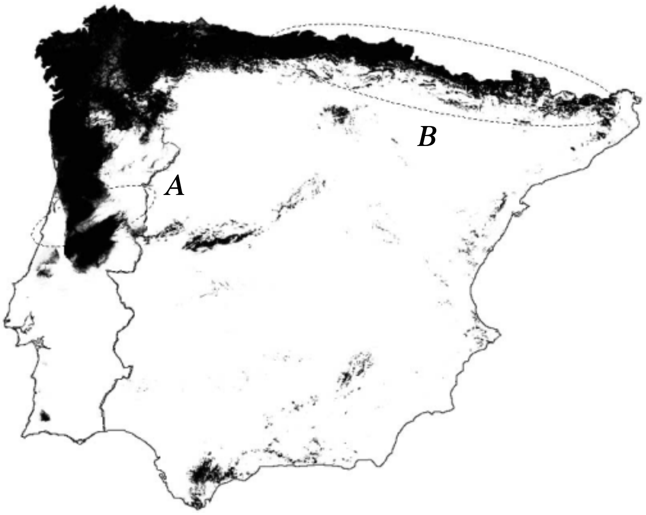

Figure 5**. Predictive distribution map of Chioglossa lusitanica in the Iberian Peninsula following the data presented in Teixeira et al. (2001). **A) Mondego valley; ***B)*** northeastern Spain, from eastern Cantabria to the Pyrenees, where *C. lusitanica* does not occur.

Second, a discrepancy exists between the current distribution of *C. lusitanica* and the model for northern Spain from eastern Cantabria to the Pyrenees (***Figure 5B***). Parameters invoked to explain species absence in this area have been soil type and the presence of a competing species (Vences 1997; Teixeira *et al.* 2001). However, the one Iberian amphibian that has similar habitat characteristics – *Euproctus asper* – and could possibly out compete *C. lusitanica*, is confined to the Pyrenees and thus the two species do not co-occur (Teixeira *et al.* 2001). An alternative explanation to a limiting ecological factor would be that *C. lusitanica* is still in the process of expanding its range. However, with the rafting of larvae (Thiesmeier 1994) and substantial migration distances of several hundreds of meters overnight (Arntzen 1981, 1994), low dispersal ability is an unlikely explanation for the unoccupied area.

Third, the documented distribution of *C. lusitanica* appears continuous across the range, with the exception of an isolated population in the Serra de Sintra (see below). However, several potentially suitable areas are shown in the model to the south of the current distribution (e.g. Monchique and the central Spanish system). These isolated mountain ranges may have been out of range for colonization, the local populations may have gone extinct, or species presence remains unrecorded. The Sintra population may have resulted from an introduction in the past century (Arntzen 1999). Its rediscovery and subsequent investigation may prove highly informative. Genetic variation across *C. lusitanica's* range is substantial, providing the possibility to distinguish between introduced and native occurrences. If introduced, it would show the survival of a population over six decades, confirming the general habitat suitability of the area, as predicted from the model. If native, it would indicate that *C. lusitanica* had a historical distribution that was more extensive than presently recognized.

Lacerta schreiberi has a range similar to that of *C. lusitanica* but occurs in several isolated mountain ranges of Portugal and central Spain (Brito *et al.* 1998). Ecological modeling applied to *L. schreiberi* (Brito *et al.* 1996) and the genetic structuring of populations (Paulo *et al.* 2002, Godinho *et al.* unpublished) suggests that the isolated populations resulted from range fragmentation due to climate warming since the last glacial maximum. This species, possessing broader ecological tolerance than *C. lusitanica* and possibly a higher dispersal ability, may have reached northeastern Iberia through postglacial recolonization (Paulo *et al.* 2002). *L. schreiberi* meets the congeneric *L. bilineata* in northern Spain close to the French border in a parapatric contact zone, the boundaries of which are maintained by interspecific competition (Barbadillo *et al.* 1999).

An historical biogeographical scenario

The joint interpretation of two independent data sets – biogeographical and genetic – strongly supports a vicariant scenario for the history of populations south and north of the Mondego River. Climatic change in the Pleistocene resulted in subdivision of the species range, with refugia located south and north of the river (***Figure 6A***). Sequential glacial and interglacial periods would have provoked recurrent range contraction and expansion (***Figures 6A & 6B***). After the last glacial maximum (18 kyr BP) range expansion shaped the present-day geographical distribution of *C. lusitanica*. In the south, a secondary contact zone resulted from the expansion of the two putative refugia and was observed near the Mondego valley (***Figure 6C***; Alexandrino *et al.* 2000).

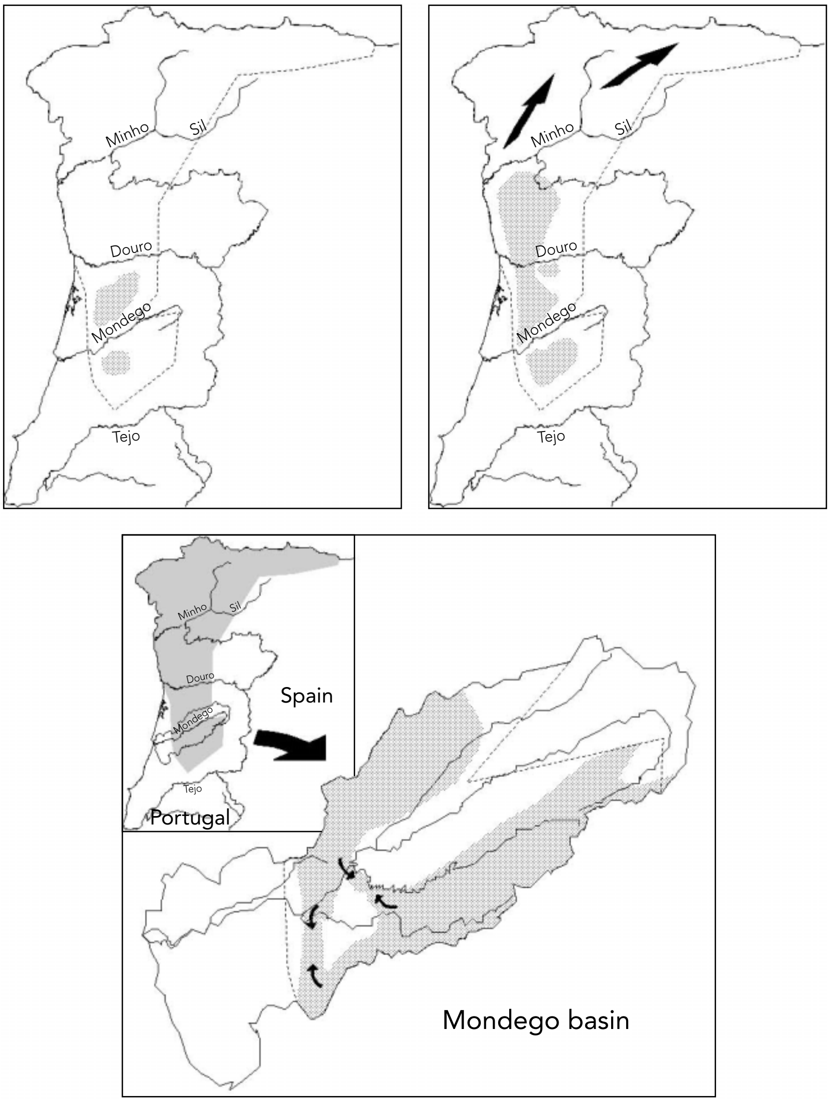

Figure 6. *Historical biogeographical scenario for Chioglossa lusitanica*: ***A)*** separation of two Pleistocene refugia (dotted areas). The present day species range is after Arntzen, (1999); ***B)*** post-glacial expansion from refugial areas (dotted areas and arrows), ***C)*** secondary contact limited by a zone of less favorable habitat near the valley of the Mondego river (undotted area within range).

The Mondego valley appears to have played an important role as a dispersal barrier for *C. lusitanica*, either serving as a complete barrier to gene flow during some period in the past, or as a zone of low population density that limited introgression between the two groups (***Figure 6C***). To the north, the stepwise decrease in genetic variability as measured by heterozygosity, allelic richness and haplotype diversity (Alexandrino *et al*. 2000) is consistent with a major postglacial colonization originating from a southern Douro refuge.

Implications for conservation

Synthetic genetic maps complemented by ecological models of past and present day species distribution identified a southern and a northern center of genetic variation. These areas could be associated with Pleistocene glacial refugia. The population groups are distinguished by largely concordant variation across several nuclear and cytoplasmic genes, as well as morphological variation. This suggests a long-standing evolutionary divergence between groups. Introgression is limited and spatially restricted (Alexandrino 2000; Alexandrino *et al*. 2000). The management status of the two groups should be determined. The concept of Evolutionary Significant Units (ESU) was introduced to help answer such a question (Ryder 1986; Waples 1991; Moritz 1994). Following Peatkau (1999), we favor a holistic definition of ESU (Bernatchez 1995; Crandall *et al*. 2000) over more restrictive criteria that may be problematic at the intraspecific level. Accordingly, we support the recognition of two conservation management units in *C. lusitanica*. The observation that almost all genetic variation observed across the species range is also found between the Muradal Mountains and the Douro River suggests that this area should be central in conservation planning.

It is now well established that the Iberian Peninsula served as a major refuge during Pleistocene Ice Ages (Hewitt 1999). However, the consequences of this fact are still a matter of discussion. Some researchers emphasize the role this and other European peninsulas had as a source of postglacial migrations and associated re-colonizations at a continental scale for a variety of organisms (Hewitt 1999), while others suggest instead that those phenomena promoted long-term fragmentation followed by speciation and endemism (Bilton *et al.* 1998). Notwithstanding this controversy, it is clear that the present-day patterns of Iberian flora and fauna are not simply explained by these alternative models (see Gomez & Lunt, this volume). Research on the golden-striped salamander provides evidence for an unexpected natural history of populations and begs the question of whether other organisms with different, less explicit ecological requirements, show the same or similar patterns of fragmentation and dispersal. Parallel patterns of regional diversity in Iberia could have profound implications for conservation planning. Various researchers working on Iberian herpetofauna have indeed presented remarkably concordant results, some of which are listed as follows: 1) In *L. schreiberi*, two highly divergent mtDNA lineages were described, revealing an ancient split (Paulo *et al.* 2001). However, nuclear gene data obtained from both electrophoretic analysis of allozyme variation and the sequencing of a nuclear gene (Godinho *et al.* in press) showed highly discrepant patterns when compared with mtDNA, suggesting a more complex history of populations. The authors also predict processes of hybridization and admixture along contact zones between the two divergent mtDNA lineages, and a postglacial expansion to the north. 2) Similarly, two highly divergent mtDNA lineages are detected in the natterjack toad, *Bufo calamita*, (Harris, pers. comm.) and the marbled newt, *Triturus marmoratus*, (Arntzen, pers. comm.) separating the north and the south of Iberia, thus suggesting a common phylogeographic history. 3) Using genetic data, as well as external morphology and morphometrics, Sanchez-Herraiz *et al.* (2000) described a new Pelodytidae species (*Pelodytes ibericus*) from southern Iberian Peninsula (previously considered the parsley frog, *Pelodytes punctatus*). A contact zone and admixture is also predicted between the two taxa. This set of results in different amphibian and reptile species from the Iberian Peninsula clearly suggests that Pleistocene climatic oscillations associated with the high persistence times of species occupying a southern European refuge acted in combination to cause i) a pattern of fragmentation and deep subdivision in most Iberian amphibians and reptiles (Gomez & Lunt, this volume) that now generally show two evolutionary lineages , and ii) a post Würm expansion from glacial refugia, leading to the formation of secondary hybrid zones, and iii) a wide variety of scenarios of hybridization and admixture, some of which may be described as incipient or completed endemic speciation events.

Acknowledgements

This work was supported by the 'Instituto da Conservação da Natureza' (ICN), the 'LIFE' program of the European Community and the 'Junta Nacional de Investigação Científica e Tecnológica' (PRAXIS XXI/BD/5917/95 PhD grant to JA). Licensing was provided by ICN for Portugal and by the Galician and Asturian 'Junta de Medio Ambiente' for Spain. We thank Steve Weiss for comments and helpful suggestions that improved an earlier version of the manuscript.

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