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The limits of mtDNA phylogeography: complex patterns of population history in a highly structured Iberian lizard are only revealed by the use of nuclear markers

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Abstract

Phylogeographic analyses based on the sole use of the mitochondrial DNA (mtDNA) molecule reveal only a small part of the evolutionary history of a species or a set of related species. In this study, we have combined the application of slow- and fast-evolving nuclear markers (proteins and microsatellites, respectively) together with the analysis of two-gene genealogies to further understand the history of the Iberian endemic Schreiber's green lizard, *Lacerta schreiberi*, a species for which a well established phylogeographical scenario is available. In sharp contrast with the observation of four divergent and almost allopatric mtDNA clades, our nuclear data revealed how two groups of populations diverged, persisted and began to admix along the mountains of the Iberian Central System. In addition, the combination of mtDNA and nuclear data showed how the core area of the species distribution responded to ice ages, first by relatively old processes of population expansion to the south followed by episodes of contraction that are at the origin of present-day isolates, and more recently by a postglacial expansion to the Iberian Northwest where new habitats were made available after climatic amelioration. Taken together with recently published results for a variety of other organisms, our results suggest that complex processes of fragmentation, expansion and admixture can only be properly addressed through the use of several and complementary types of molecular markers. Finally, we also suggest that southern European refugia are both hotspots and melting pots of genetic diversity.

Keywords: admixture, expansion, fragmentation, *Lacerta schreiberi*, mtDNA phylogeography, nuclear markers, refugia

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Introduction

The Quaternary ice ages left a remarkable genetic imprint on present-day biodiversity (Hewitt 2000). While its relative importance in boreal, temperate and tropical areas is still a matter of intense debate (Willis & Whittaker 2000; Hewitt 2004), the quantity and quality of information now available for Europe has resulted in a number of important generalizations describing patterns of molecular variation across a variety of plant and animal species (Hewitt 1996, 1999; Taberlet *et al*. 1998). It is now clear that major peninsulas like Iberia, Italy and the Balkans (and also the Caucasus, for which only few data are available) allowed the persistence of many species in the south through repeated ice ages, promoting divergence between refuges, because of their isolation, and the accumulation of genetic diversity within refuges, because of their persistence (Hewitt 2000; Weiss & Ferrand 2007). It is also well established that these peninsulas functioned as sources for the rapid recolonization of deglaciated central and

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§ We would like to dedicate this paper to Prof. Godfrey Hewitt due to his insightful and outstanding work in the study of hybrid zones, that predicted the patterns and processes we are now describing for many species in Southern European Refugia.

northern regions, resulting in the so-called northern purity as genetic diversity was lost in a series of founder events during expansions (Hewitt 1996, 2001; for a different perspective see Bilton *et al*. 1998). Finally, the combination of occupied refugia, postglacial expansion rates and the location of geographical barriers determined the occurrence of major clusters of recent hybrid zones in Scandinavia, central Europe and the Alps (Taberlet *et al*. 1998; Hewitt 2000). In the last few years, however, some of these emergent features characterizing European biodiversity have been challenged as being too simplistic (Widmer & Lexer 2001). In particular, Comps *et al*. (2001) showed that heterozygosity — but not allelic richness — was higher in colonizing populations due to the admixture of divergent genomes and suggested that similar patterns could eventually be found in other organisms. In fact, this pattern was recently described for a variety of European trees and shrubs by Petit *et al*. (2003), who claimed that glacial refugia are hotspots but not melting pots of genetic diversity. There is therefore a need to further investigate the genetic structure of natural populations in species endemic to glacial refugia in order to get a solid comparison between populations that persisted in the south with those more thoroughly studied that were able to spread into central and northern Europe.

Our current understanding of the present genetic structure of populations and species is mostly derived from the growing discipline of phylogeography (Avise 2000). The underlying goal of this approach is the analysis of gene trees in geographically explicit contexts, an idea that gained enormous popularity in the last two decades and that created a deviation from the more traditional field of population genetics (Hey & Machado 2003). However, phylogeography has, essentially, relied on data from nonrecombining mitochondrial DNA (mtDNA), which has important limitations that are now widely recognized. The primary reason for these limitations results from the unavoidable fact that the analysis of mtDNA effectively corresponds to the study of a single locus. This means that even when using rigorous and sophisticated statistical frameworks combining phylogenetic, coalescent, demographic and nested-clade analyses in the study of the natural history of populations (see for example Althoff & Pellmyr 2002, and Pfenninger & Posada 2002) the total dependence on mtDNA as a marker will give us only a small part of the history (Zhang & Hewitt 2003). Essentially, this is because a mtDNA tree may be different from the population or species tree due to the effects of natural selection (see, for example, Weinreich & Rand 2000 and Bazin *et al*. 2006), introgression (Chan & Levin 2005; Melo-Ferreira *et al*. 2005) or the wide stochastic variance that characterizes a sample of gene trees collected from a set of populations or species (Hey & Machado 2003). Recently, both Knowles & Maddison (2002) and Chikhi & Bruford (2005) emphasized the need to avoid over-interpretations of phylogeographic data and, together with Hey & Machado (2003), advocate the merging of gene-tree based methods with the more traditional and mathematical based field of summary statistics. This will certainly be necessary if we want to address more complex questions and further develop the fields of phylogeography and population genetics (Zhang & Hewitt 2003). In this respect, the recent study by Magri *et al*. (2006) on the palaeoecology and genetics of the European beech (*Fagus sylvatica*) constitutes a beautiful example of how the use of different types of molecular markers can challenge previous interpretations and reveal a new and complex perspective on the history of this tree species.

The Iberian Peninsula has been described as a major refugium during Pleistocene glaciations (Taberlet *et al*. 1998; Hewitt 2000), but also as a place of speciation and endemism (Bilton *et al*. 1998). Although these perspectives have sometimes been viewed as antagonistic, they are actually complementary interpretations of the tremendous complexity of the many events that occurred in the past in southern Europe. In fact, the cyclic fluctuations of the climate during the last three million years have been promoting repeated contraction, fragmentation, expansion and admixture in many species that have persisted in the south for many ice ages (Hewitt 2000). Accordingly, we may expect that the complex demographic and evolutionary patterns that characterize those species will be revealed only through the combination of different types of molecular markers together with the explicit consideration of various historical scenarios. An example of this was recently described by Alexandrino *et al*. (2000, 2002, 2007) and Sequeira *et al*. (2005, 2008), who reported a complex and unexpected natural history of the populations of the golden-striped salamander (*Chioglossa lusitanica*) by combining information from mtDNA and nuclear markers with inferences based on recent climatological data (Teixeira *et al*. 2001; Teixeira & Arntzen 2002).

In another study of an Iberian endemic, Paulo *et al*. (2001) revealed that the populations of the Schreiber's green lizard *Lacerta schreiberi* started their divergence in the late Pliocene and persisted in separate locations throughout the Pleistocene. They further suggested that additional fragmentations happened during the Pleistocene and described a total of four fully allopatric clades. Finally, they used nested-clade analysis to assess the history of colonization of this lizard (Paulo *et al*. 2002). However, inferences on its population history were limited because both studies were based on the single use of the mtDNA cyt *b* gene. In our project, we used 10 polymorphic proteins, four hypervariable microsatellites and two nuclear genealogies to further explore the evolutionary history of *L. schreiberi*. In particular, we were motivated by the remarkable geographical distribution of this species, that combines a continuous area in the north with isolated populations in central and southern Iberia, and addressed the following questions:

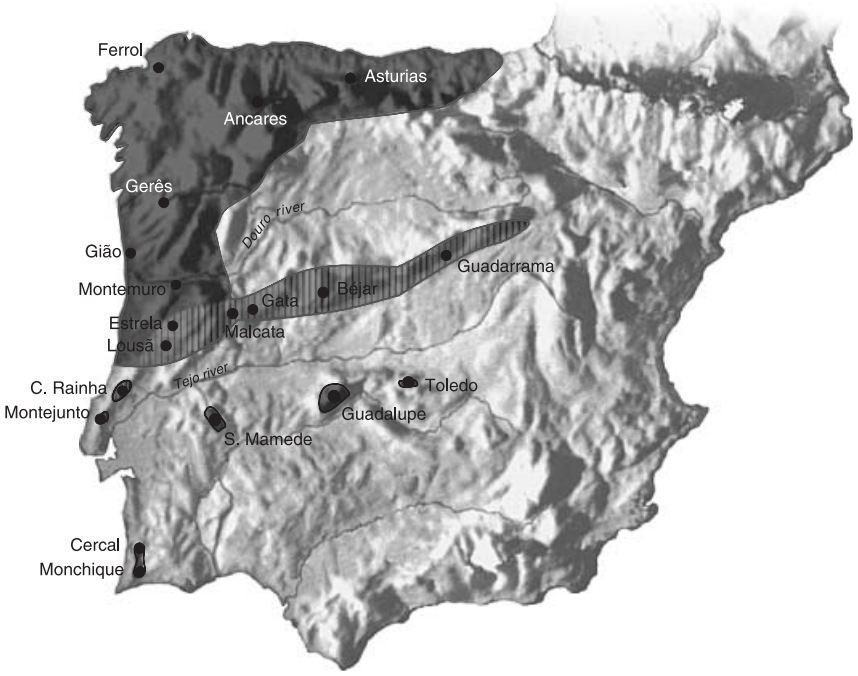

Fig. 1 Sampling locations of *Lacerta schreiberi* in the Iberian Peninsula. Dark grey represents the current distribution area of *Lacerta schreiberi*. The Iberian Central System is highlighted with vertical lines.

1 Do the nuclear markers have a molecular signature corresponding to that of mtDNA?
2 Do the nuclear data suggest strong genetic discontinuities between the populations with the previously described allopatric mtDNA lineages or, on the contrary, support the occurrence of gene flow?
3 Is it possible to reconstruct the population expansion that produced the present-day central and southern isolates?

Our results indicate that the evolutionary history of *L. schreiberi* during the ice ages was shaped by complex patterns of population fragmentation, expansion and admixture that were not detected using mtDNA alone.

Materials and methods

Sampling

We collected 374 individuals by hand and by noosing between 1994 and 2002 from 19 sites that represent the whole distribution range of *Lacerta schreiberi*, including the northwestern continuous area and the central and southern population isolates (Fig. 1). All lizards were immediately released after cutting a tail tip, from which DNA was extracted using standard protocols (Sambrook *et al*. 1989) and blood collected with the help of a capillary. Both blood samples and tail tips were conserved at –70 °C for subsequent electrophoretic analysis.

Molecular methods

A total of 16 nuclear polymorphic loci were screened for all individuals, including ten proteins, four microsatellites and two single copy nuclear genes. The allozyme loci selected for this study were *Ck1*, *Me*, *Mpi*, *PepB*, *PepD*, *Pgd*, *Pgi* and *Pgm* (Shaklee *et al*. 1990), and two plasma proteins named *Px2* and *Px3*. A detailed description of the electrophoretic and isoelectric focusing techniques used in the analysis of those proteins can be found in Godinho *et al*. (2003). The four microsatellite markers included three loci (La3, La6 and La9) originally characterized for *Lacerta agilis* (Gullberg *et al*. 1997) and one locus (Lv-4-α) characterized for *Lacerta vivipara* (Boudjemadi *et al*. 1999). This option was based on a previous sequencing of two alleles at each microsatellite locus to confirm the presence of simple repeat arrays. Polymerase chain reactions (PCR) were carried out in 10 μL containing 20 ng of DNA, 5 pmol of each primer, 0.10 nmol of each dNTP, 1 unit of Taq polymerase and 1.5 nmol $MgCl_2$, and cycling conditions were according to the original publications. PCR products were analysed by electrophoresis in 6% denaturing polyacrylamide gels using a size standard of 10 bp and visualized using silver staining. Nucleotide polymorphism at β-fibrinogen intron 7 (*β-fibint7*) and the single exon of the C-*mos* gene was analysed by a combination of single-strand conformation polymorphism analysis (SSCP) screening and sequencing of resolved alleles, as described in Godinho *et al*. (2006a) and Godinho *et al*. (2006b), respectively.

Data analysis

Allele frequencies and measures of genetic variation such as heterozygosity, variance in allele size (in the case of microsatellites) and mean number of alleles were calculated using GENETIX version 4.0 and MS-ANALYZER version 2.32 (Belkhir *et al*. 2000; Dieringer & Schlötterer 2003). Tests for differences among groups of populations were performed using FSTAT version 2.9.1 (Goudet 2000). Tests for Hardy–Weinberg equilibrium and linkage disequilibrium were performed for all populations using the ARLEQUIN version 2.000 (Schneider *et al*. 2000) and GENEPOP version 3.1 (Raymond & Rousset 1995) programs. Fu & Li (1993) and Tajima (1989) neutrality tests were performed for the single copy nuclear genes and were not significant which allowed the inclusion of these loci in the following analyses.

The Nei's standard genetic distance was calculated between all pairs of populations and the matrix was converted into a dendrogram using the Neighbour-Joining algorithm (Saitou & Nei 1987) provided with the PHYLIP version 3.5 software package (Felsenstein 1995) and graphically displayed with TREEVIEW version 1.6. (Page 1996). A bootstrap analysis was performed using the program Boot3 (Múrias A, unpublished results) that is implemented in the PHYLIP package. The mtDNA sequences described by Paulo *et al*. (2001), as well as the nuclear sequences reported for the *β-fibint7* (Godinho *et al*. 2006a) and the C-*mos* genes (Godinho *et al*. 2006b), were reanalysed and used to construct median-joining networks with the NETWORK version 3.1 software (Röhl 2000). The *β-fibint7* and the C-*mos* networks were rooted using sequences obtained in four closely related *Lacerta* species (Godinho *et al*. 2005). We used the Analysis of Molecular Variance (AMOVA) implemented in the ARLEQUIN version 2.000 program (Schneider *et al*. 2000) to examine the hierarchical population structure in *L. schreiberi*. In particular, we were interested in partitioning total variance components into those derived within and among four a *priori* defined groups corresponding to the populations that exhibit the four mtDNA allopatric clades described in Paulo *et al*. (2001) and further examined by Godinho (2004). Unbiased estimates of population differentiation (F_{ST}) were obtained by calculating pairwise values (Weir & Cockerham 1984) using the FSTAT 2.9.1 program (Goudet 2000). The significance of pairwise F_{ST} values was tested by permuting (10^4 times) genotypes among populations. We also calculated pairwise F_{ST} values for groups of populations clustered according to their mtDNA lineage. AMOVA and F_{ST} analyses were conducted using both all the nuclear markers pooled together and independently for each type of nuclear marker.

Genotype data for the 16 loci were used to estimate individual admixture values with the STRUCTURE program (Pritchard *et al*. 2000). The program was run assuming two parental populations ($k = 2$) but neither flagging them nor defining the population of origin for each individual. Three different replicas were performed. While values of $k > 2$ could have been explored, it was the focus of this study to analyse the dynamics of admixture between two divergent genomes that were initially identified on the basis of mtDNA differentiation. Hence, no further partitions of the data were considered. The ALPHAPROPSD parameter was set to 0.2 in order to stabilize the estimate of α (the Dirichlet parameter for the degree of admixture) throughout the run. We chose a burn-in length of 30 000 steps to minimize the effect of the starting configuration followed by 10^5 MCMC iterations. For each individual, a mean value of q_{ki} (the proportion of individual *i*'s genome that originated from population *k*) was estimated and plotted in a graph according to its population of origin and corresponding mtDNA lineage.

Results

Measures of genetic diversity at 16 nuclear loci

A total of 16 nuclear loci were analysed in 19 *Lacerta schreiberi* populations encompassing the whole distribution area of the species. On average, populations located on the Iberian Central System (a series of mountain ranges that cross the peninsula from the west to the east) exhibit the highest values of both heterozygosity and mean number of alleles for proteins, microsatellites and single copy nuclear polymorphisms (scnp), whereas a trend for a decrease in variability was observed to the north, in the continuous distribution area of this lizard, and to the south, in the population isolates (Table 1). Nevertheless, comparison of average expected heterozygosity among groups of populations were not significant ($P > 0.1$). The central Spanish isolates of Guadalupe and Toledo are remarkable because they almost lack protein variability and also show depleted levels of diversity in their microsatellites and scnp. These results confirm a previous and more limited analysis of *L. schreiberi* populations in which the samples collected in the Iberian Central System mountains of Lousã-Estrela (Portugal) and Béjar-Guadarrama (Spain) were consistently the most variable ones (Godinho *et al*. 2003).

Estimating population differentiation

To examine population differentiation, we initially performed an analysis of molecular variance (AMOVA; Excoffier *et al*. 1992) to determine the partitioning of variation among populations and geographical groups as defined by the four mtDNA clades described by Paulo *et al*. (2001) and named A_1, A_2, B_1 and B_2 by Godinho *et al*. (2006a). We found evidence for strong population differentiation as revealed by the highly significant components of variance observed between populations (24.4%) and among mtDNA

Table 1 Genetic diversity parameters in 19 populations of *Lacerta schreiberi*: Expected heterozygosity (H_E), variance (V, for microsatellites) and mean number of alleles per locus (n_a). *P* — proteins; M — microsatellites; scnp — single copy nuclear polymorphisms

Populations	*mtDNA lineage*	H_E *P*	H_E M	H_E scnp	V	n_a *P*	n_a M	n_a scnp
Continuous distribution area								
Iberian Northwest								
Ancares	A_1	0.061	0.367	0.189	1.60	1.29	3.75	2.00
Asturias	A_1	0.064	0.402	0.215	0.55	1.24	3.25	2.00
Ferrol	A_1	0.075	0.333	0.186	0.44	1.21	3.50	2.00
Gerês	A_1	0.075	0.468	0.402	1.00	1.32	4.25	2.00
Gião	A_1	0.060	0.419	0.420	1.33	1.37	5.00	2.50
Montemuro	A_1	0.072	0.539	0.444	5.04	1.41	4.00	3.00
Average		0.068	0.421	0.309	1.66	1.31	3.96	2.25
Portuguese Central System								
Estrela	A_1	0.083	0.691	0.446	4.68	1.47	6.00	3.50
Lousã	A_1	0.095	0.665	0.635	5.40	1.38	5.00	4.00
Malcata	A_1/B_1	0.094	0.578	0.674	4.03	1.47	5.50	4.50
Average		0.091	0.645	0.585	4.70	1.44	5.50	4.00
Spanish Central System								
Gata	B_1	0.082	0.555	0.621	4.83	1.47	4.50	4.50
Béjar	B_1	0.139	0.708	0.232	3.56	1.63	5.75	2.00
Guadarrama	B_1	0.082	0.412	0.459	1.47	1.41	3.75	2.00
Average		0.082	0.511	0.410	2.83	1.39	4.52	2.83
Central isolates								
S. Mamede	A_1	0.069	0.302	0.429	0.65	1.37	4.25	3.50
Guadalupe	B_2	0.029	0.406	0.237	2.31	1.06	4.00	2.50
Toledo	B_2	0.000	0.373	0.000	2.17	1.00	2.00	1.00
Average		0.033	0.360	0.222	1.71	1.14	3.42	2.33
Coastal isolates								
C. Rainha	A_2	0.100	0.430	0.376	5.43	1.37	4.50	3.00
Montejunto	A_2	0.096	0.536	0.550	4.62	1.32	4.50	3.50
Average		0.098	0.483	0.463	5.02	1.34	4.50	3.25
Southern isolates								
Monchique	A_2	0.061	0.308	0.357	2.97	1.21	2.50	2.50
Cercal	A_2	0.045	0.336	0.228	0.69	1.16	4.25	1.50
Average		0.053	0.322	0.292	1.83	1.18	3.38	2.00

defined groups (18.1%) compared to a moderate fraction (57.5%) attributed to differences within populations. When analysing the three types of nuclear markers separately, the pattern is highly congruent.

The analyses of pairwise F_{ST} values confirmed the extent of population differentiation and most of the comparisons were statistically significant (Table S1). However, populations sampled in the northwestern part of the continuous distribution area of *L. schreiberi* (including the mountains of Montemuro, Lousã and Estrela) revealed a number of nonsignificant pairwise F_{ST} comparisons, suggesting the occurrence of extensive gene flow between these populations. This observation is in agreement with the lowest Φ_{ST} value reported by Paulo *et al*. (2001) for these populations based on mtDNA data and with their suggestion of a recent postglacial expansion in this region of the Iberian Peninsula.

When average F_{ST} values are calculated for all 19 populations, as well as within and between mtDNA defined groups, the lowest value is observed precisely in the Northwestern populations, exhibiting mtDNA lineage A_1, thus suggesting less differentiation in this geographical area (Table 2). On the other hand, comparisons between groups indicate similar F_{ST} values for the pairs A_1/B_1 and A_1/A_2, but considerably higher values for the pairs A_2/B_2 and B_1/B_2 (Table 2). A possible explanation for this result relates with the presence of fixed alleles at almost all loci for Toledo and Guadalupe populations, the two inland isolates that constitute group B_2. In general, the F_{ST} values obtained from different molecular markers were in broad agreement.

Comparing population trees with gene trees

The genetic relationships between the 19 lizard populations analysed in our study are summarized in the NJ tree constructed from a matrix of Nei's genetic distances (Fig. 2). While both other types of genetic distances (Dc, Cavalli-Sforza

Table 2 F-statistics between and within mtDNA defined groups of populations

	Proteins	Microsatellites	C-*mos*	*β-fibint7*	All loci
Within groups					
All populations	0.440	0.386	0.431	0.388	0.371
mtDNA A_1	0.224	0.278	0.436	0.103	0.131
mtDNA A_2	0.156	0.204	0.335	0.338	0.212
mtDNA B_1	0.258	0.253	0.015	0.212	0.234
mtDNA B_2	0.778	0.241	—	0.280	0.331
Between groups					
mtDNA A_1/B_1	0.205	0.226	0.232	0.400	0.234
mtDNA A_2/B_2	0.565	0.459	0.149	0.421	0.489
mtDNA A_1/A_2	0.205	0.222	0.131	0.317	0.222
mtDNA B_1/B_2	0.387	0.284	0.278	0.597	0.355
mtDNA A_1/B_2	0.321	0.382	0.345	0.084	0.334
mtDNA A_2/B_1	0.189	0.295	0.130	0.432	0.263

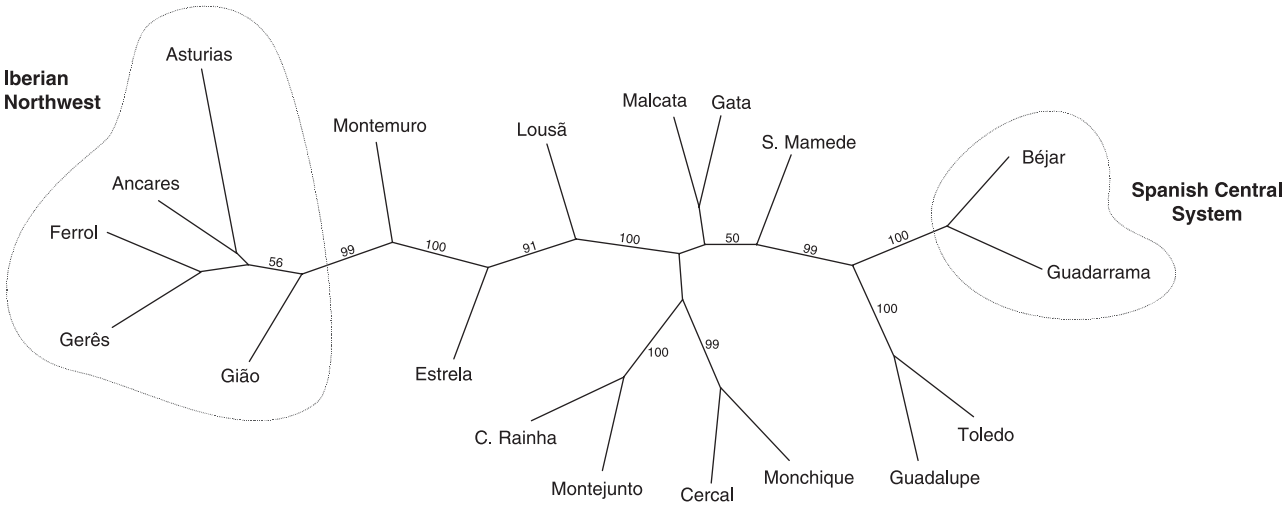

Fig. 2 Neighbour-Joining tree based on Nei (1972) genetic distance for a set of 10 protein loci, four microsatellites and two scnp in 19 populations of *Lacerta schreiberi*. Representative populations of the two most divergent population groups (Iberian Northwest and Spanish Central System) are indicated.

& Edwards 1967; and Da, Nei *et al.* 1983), as well as tree building procedures (likelihood and Bayesian) were also applied to the data, results were consistently similar. Interestingly, the populations of the Iberian Northwest are most distant from the Spanish Central System group, whereas the geographically close mountains of Malcata and Gata, and also the more distant central and southern Iberian isolates, occupy intermediate positions in the cladogram. This observation is surprising given that all isolated populations with the exception of S. Mamede exhibited derived mtDNA lineages (Paulo *et al*. 2001).

To compare our population tree with gene trees obtained from the same set of populations, we reanalysed the data reported by Paulo *et al*. (2001) for the mtDNA cyt *b*, Godinho *et al*. (2006a) for the *β-fibint7*, and Godinho *et al*. (2006b) for the C-*mos* nuclear genes and constructed median-joining networks (Fig. 3). Both nuclear gene trees display the molecular signature corresponding to the deep divergence observed between the two major mtDNA lineages, A and B. NJ trees of the C-*mos* and the *β-fibint7* haplotypes exhibit high bootstrap values (88% in both cases) supporting their first split (results not shown). The more recent divergence of mtDNA sublineages A_1/A_2 and B_1/B_2 is not captured by the C-*mos* nuclear gene, probably due to its low variability. In contrast, the split between A_1 and A_2 is clearly detected in the *β-fibint7* gene tree (the NJ tree constructed for *β-fibint7* shows a bootstrap value of 96% supporting the clade that clusters haplotypes E and F; results not shown), but not the split between B_1 and B_2. Although stochastic variance could eventually explain this observation, Godinho *et al*.

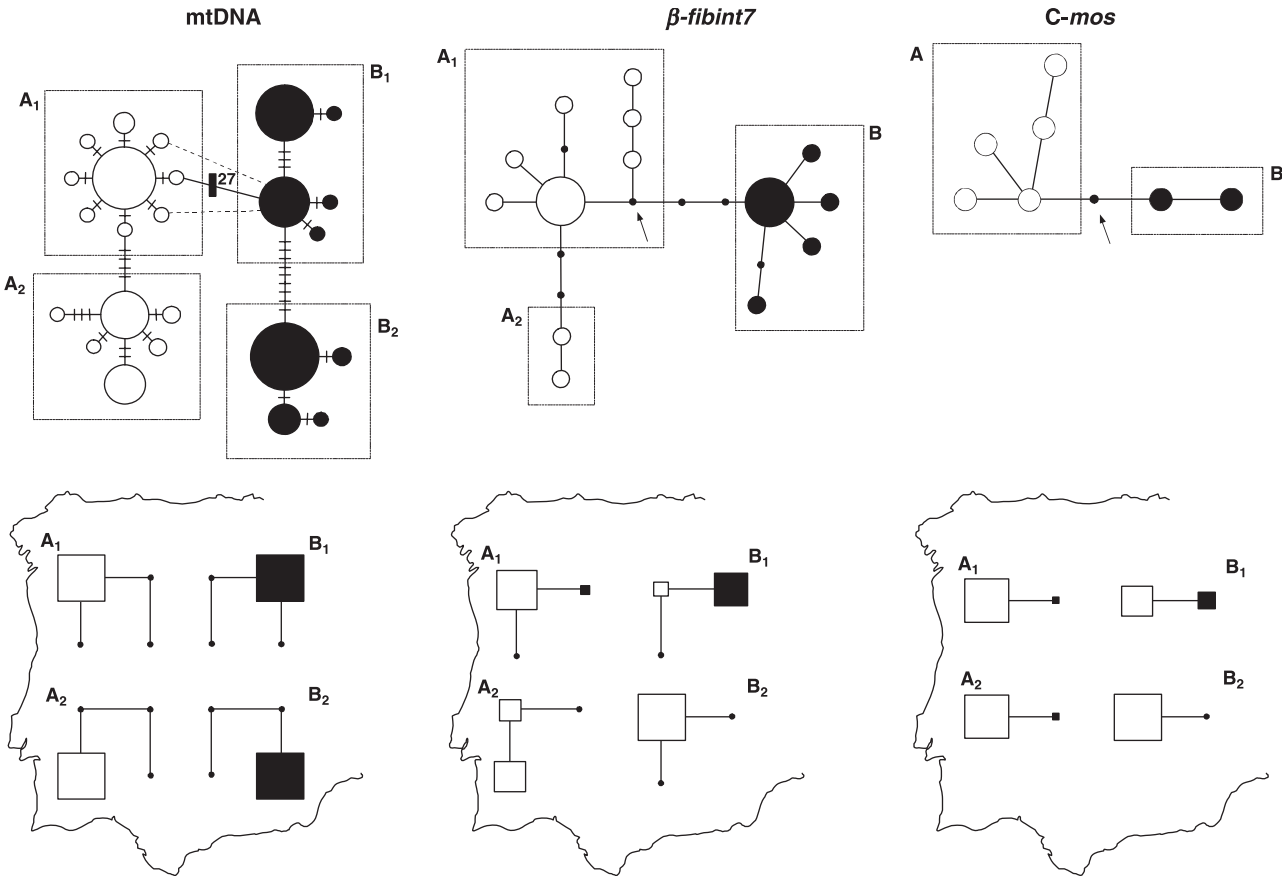

Fig. 3 Median-joining networks obtained for mtDNA (Paulo *et al*. 2001) and the nuclear markers *β-fibint7* and C-*mos* (Godinho *et al*. 2006a and 2006b). Boxes represent the partitions of nuclear gene trees that may correspond to the allopatric mtDNA lineages and sublineages indicated on the left. Black points correspond to missing haploytpes and arrows indicate the root of the networks. mtDNA lineages A and B are represented in white and black, respectively, and 27 is the number of mutations separating the two clades. In the maps of the Iberian Peninsula below each network, the geographic distribution of the major clades has been summarized. The geographic range has been divided into four regions, and the proportion of each clade in each region is represented by the area of the corresponding square (arranged as in the network above it).

(2006a) suggested that recent gene flow from a western region is a more likely alternative interpretation of the data. Finally, while the four mtDNA clades are essentially allopatric, haplotypes at both C-*mos* and *β-fibint7* are shared by many populations. In the first case, the distribution of C-*mos* alleles indicates the occurrence of incomplete lineage sorting, but in the second case the divergent *β-fibint7* haplotypes A and H coexist in the populations that are geographically close to the mtDNA contact zone, thus suggesting the occurrence of gene flow between two formerly isolated populations (Godinho *et al*. 2006a).

Evaluating admixture patterns

The determination of individual admixture estimates for each of 10 different *L. schreiberi* populations across the continuous distribution area is illustrated in Fig. 4. Considering $k = 2$ (the two major population groups exhibiting the deep divergent and allopatric mtDNA lineages A and B), we found evidence for extensive nuclear admixture along a considerable geographical area for both microsatellites and proteins (Fig. 4a, b). While both the populations north of the river Douro (Ferrol, Gerês and Gião) and in the eastern part of the Spanish Central System (Guadarrama) lack any sign of admixture, all other geographically intermediate populations (Montemuro, Estrela, Lousã, Malcata, Gata and Béjar) show evidence for a continued and necessarily old process of admixture. This is in sharp contrast with previously described mtDNA data surveyed by a restriction fragment length polymorphism (RFLP) strategy that indicated a very limited introgression of mtDNA lineages A and B (Godinho *et al*. 2001, 2006a; Fig. 4). The narrower gradient for microsatellites might be due to the greater precision in allocating admixture proportions using microsatellites (Fig. 4a, b), due to their higher heterozygosity (Bamshad *et al*. 2003). However, a weighted logistic

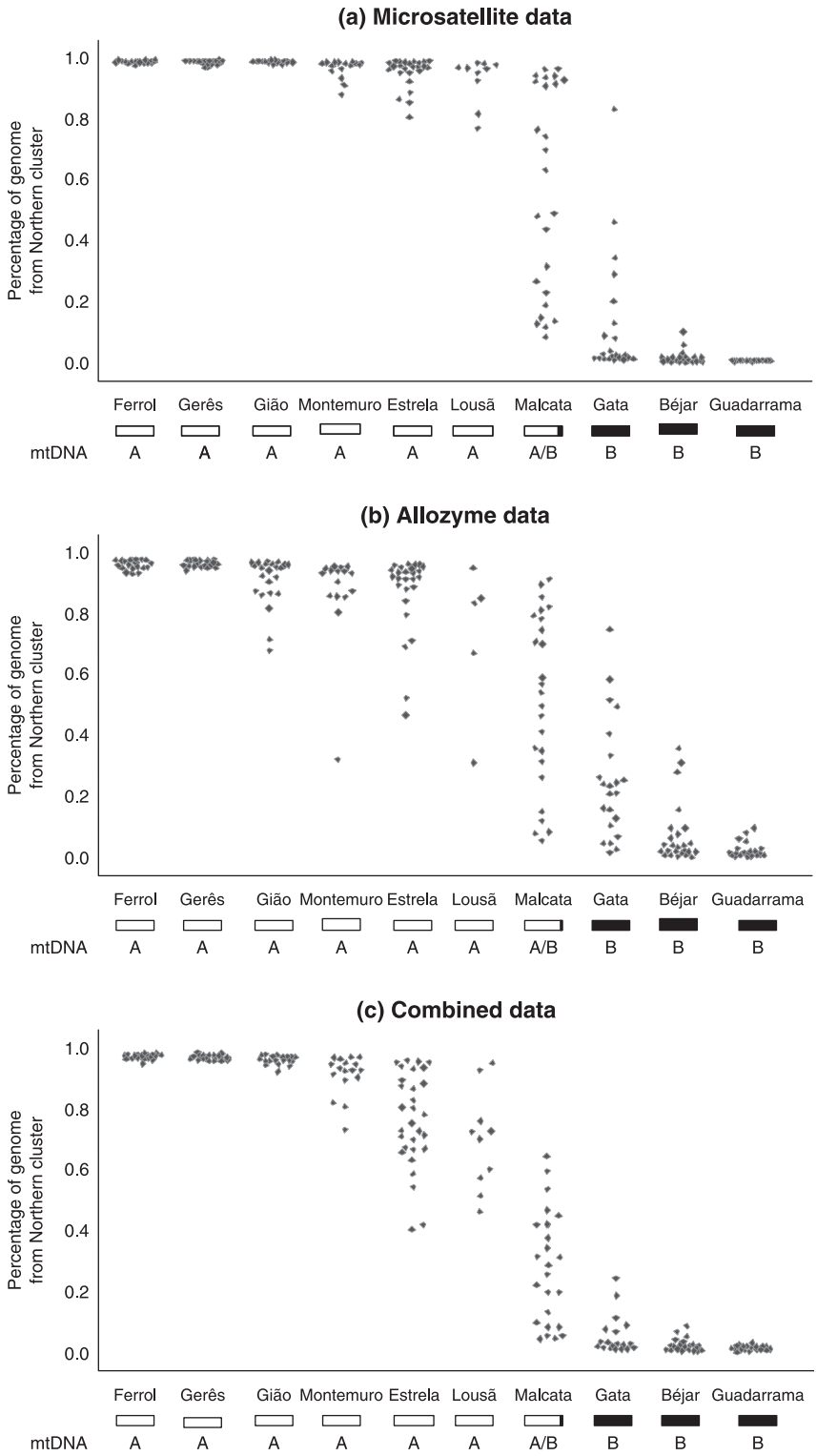

Fig. 4 Posterior mean estimates of the proportion of each individual's genome that originated from the Northern cluster, produced by STRUCTURE. The estimates are plotted along a geographical transect that crosses the putative hybrid zone. Dots represent each individual lizard in each population. Frequencies of mtDNA lineages A and B are also shown.

regression of the admixture proportions detected no significant difference in slope between the two categories of loci (results not shown).

A closer inspection of the allelic distribution profiles at selected microsatellite loci may further illuminate the dynamics of nuclear admixture between the two divergent genomes of *L. schreiberi*. (see Fig. S1 for the allelic distribution profiles of microsatellites La3 and Lv-4-α). Populations were clustered following a combination of geographical criteria and the results previously obtained with the

STRUCTURE program. Both microsatellites show evidence of two distinct and essentially nonoverlapping modal distributions characterizing the two most genetically distant groups of populations: those from the Iberian Northwest and those occurring in the Spanish Central System (Fig. 2 and Fig. S1). We can also infer from these data that the allelic distribution profiles exhibited by the populations sampled between the rivers Douro and Tejo (Montemuro, Estrela, Lousã, Malcata, C. Rainha and Montemuro) may be best explained by the combination of the two discontinuous size classes described above, thus supporting our interpretation of extensive gene flow between two formerly isolated groups of populations. Interestingly, different degrees of admixture are also detected in all isolated populations from central and southern Iberia.

Discussion

Our study of 16 nuclear markers revealed some concordant patterns with mtDNA and also indicates the Iberian Northwest and the Spanish Central System population groups as the more genetically divergent (Fig. 1). In addition, when our set of nuclear markers is split according to type (proteins, microsatellites and scnp) more information supported this interpretation. Two out of the four microsatellites used in this work showed alleles distributed in two disjunct size clusters that exhibit geographical coherence, strongly suggesting separate population histories for the Iberian Northwest and the Spanish Central System groups. An equivalent signature may be observed in the networks obtained for the C-*mos* and the *β-fibint7* genes, where missing haplotypes are close to (or even include) the root and separate the major haplotypes that characterize those two groups (Fig. 3). Populations of *L. schreiberi* evidently persisted in two geographically distinct areas in the Portuguese (western) and Spanish (eastern) parts of the Iberian Central System. Genetic divergence probably began as soon as intermediate populations became extinct and has continued throughout most of the subsequent history of these populations.

Extensive nuclear admixture across a sharp mtDNA contact zone

Given that the two population groups of *L. schreiberi* persisted through the Pleistocene climatic cycles in relatively close geographical areas and that they certainly responded to successive ice ages by repeated expansions and contractions, they probably experienced multiple episodes of admixture. While Paulo *et al*. (2002) described full allopatry for the two mtDNA lineages using a limited set of cyt *b* sequences, Godinho *et al*. (2006a) performed a fine-scale analysis of the putative contact zone through the RFLP analysis of the mtDNA molecule and found evidence for a single population exhibiting both mtDNA lineages. These results show that introgression of mtDNA lineages is limited and confined to a geographical region located between the populations of Malcata and Gata, which corresponds to a dry and Mediterranean mountainous area that divide the more rainy and continental systems of Lousã-Estrela (Portugal) and Béjar-Guadarrama (Spain).

In sharp contrast with this observation is our analysis of genetic introgression at nuclear loci, which shows a much wider zone of admixture than the mtDNA, centred at Malcata, the single location where the two divergent mtDNA lineages had been detected. Furthermore, intermediate populations consistently exhibited high levels of genetic diversity in all types of molecular markers used in this study (Table 1), indicating extensive gene flow across a west–east gradient defined by the mountains of the Iberian Central System.

Godinho (2004) made two interpretations of the data that are relevant for our analysis. First, a geostatistical analysis of the continuous distribution area of *L. schreiberi* indicated that the first and the second main principal components explaining the species variability were both associated with the region of Malcata-Gata mountains, defining a steep gradient and a centre of diffusion, respectively. While the steep gradient is certainly expected when two genomes have been diverging for a long period of time and then expand and contact, the centre of diffusion suggests that particular features characterizing the hybrid zone could be emerging (Arnold 1997). Second, a detailed analysis of nucleotide polymorphism of *β-fibint7* in *L. schreiberi* populations allowed the detection of a private haplotype in the Malcata population, which was the likely product of recombination between the two major haplotypes that characterize the putative parental populations of the Iberian Northwest and the Spanish Central System groups (Godinho *et al*. 2006a).

In light of this evidence, we suggest that after a long isolation period that may have started in late Pliocene (Paulo *et al*. 2001), the two divergent population groups of *L. schreiberi* have been (repeatedly) admixing during the last few ice ages, forming an old hybrid zone that extends from the regions immediately south of river Douro to the beginning of the Spanish Central System, in Béjar. Important demographic fluctuations during late Pleistocene climatic cycles may have been responsible for the limited introgression of mtDNA lineages when compared with nuclear markers, as could be expected due to differences in effective population size (Avise 2000). In addition, both male-mediated gene flow and cyto-nuclear genetic incompatibilities (see Buggs 2007) may also help to explain the observed discrepancy between mtDNA and nuclear markers. We will argue below that this peculiar situation, combining parapatric mtDNA lineages with an extensive and smooth gradient of nuclear admixture along a west–east transect,

will be instrumental in reconstructing the processes of population expansion and contraction that were responsible for the occurrence of central and southern isolated populations, as well as for the colonization of Northwestern Iberia.

Processes of population expansion and contraction

The central and southern isolated populations occurring along the Portuguese coast were found to have the derived mtDNA sublineage A_2, while the central Spanish isolates exhibited the derived mtDNA sublineages B_2 (Paulo *et al*. 2001). The single exception to this scenario is the inland central Portuguese population of S. Mamede, which exhibits the more ancestral mtDNA lineage A_1. Paulo *et al*. (2002) suggested that the observed patterns could be the outcome of a former widespread southern distribution during a Pleistocene glacial age followed by range contraction and the extinction of the intermediate populations when the climate warmed. Surprisingly, our Neighbour-Joining tree based on 16 nuclear markers show that all isolated populations occupy a central position in the cladogram, resulting in an apparent discrepancy with the mtDNA tree (Fig. 2). A closer inspection of Fig. 2 additionally reveals the remarkable fact that the population isolates branch off the tree in a perfect west–east transect, starting with the pair C. Rainha/Montejunto, followed by Monchique/Cercal, S. Mamede and finally Guadalupe/Toledo. Interestingly, the populations of Malcata and Gata that most likely define the boundaries of the mtDNA contact zone (Godinho *et al*. 2006a) and that show a high degree of genetic admixture (Fig. 4) are very close to the isolated populations (Fig. 1).

Our present results provide evidence that all isolated populations do show a certain degree of admixture between the two major groups of *L. schreiberi* depending on their geographical location. Accordingly, the coastal isolates of C. Rainha/Montejunto and Monchique/Cercal are genetically very close to the populations of the western part of the Iberian Central System, from which they received much influence, while the inland isolates of Toledo and Guadalupe are much closer to the populations of the eastern part of the Iberian Central System that were their most important source. The isolate of S. Mamede clusters with the Spanish populations when proteins are analysed (Godinho *et al*. 2003) but with Portuguese populations when microsatellites are considered (data not shown) and additionally exhibits the ancestral A_1 mtDNA lineage, revealing complex admixture processes that are concordant with its geographically intermediate location.

Following these observations, we suggest that the two divergent population groups of *L. schreiberi* established an old hybrid zone along the Iberian Central System that preceded the expansion to the south during a previous ice age. After population contraction and formation of the isolates, the almost allopatric distribution of the mtDNA lineages along the Iberian Central System left a molecular signature in the form of derived sublineages (A_2 in the western isolates and B_2 in the eastern isolates). On the other hand, the expansion to the north is recent and most likely after the last glaciation. This explains why all populations exhibit an ancestral mtDNA lineage (but see Paulo *et al*. 2002 for further details) and is congruent with similar colonisations of Northwestern Iberia observed in other species (Alexandrino *et al*. 2000, 2002; Pinho *et al*. 2007). In addition, as the postglacial expansion process was initiated close to the western end of the Iberian Central System transect, all populations north of the river Douro are the most derived when analysed at their nuclear markers (Fig. 1) and do not show signs of admixture with populations from the east.

In this context, two relevant published studies deserve a special reference. First, the seminal work of Hewitt (1996), where both persistence of isolated populations across ice ages and the establishment of long-term hybrid zones are remarkably anticipated for southern European refugia. Second, the recent review of Chikhi & Bruford (2005), where authors explicitly criticize phylogeographical studies because 'signals of population growth or decline are automatically attributed to the last (and best studied) glaciation events'. Our combination of protein polymorphisms, microsatellites and scnps together with mtDNA clearly showed a complex and unexpected natural history of a lizard species where population processes are necessarily associated to multiple glaciation events — including the last — and traditional limitations of mtDNA phylogeographical studies are removed. Finally, we also note that the combined use of fast- and slow-evolving molecular markers proved to be effective in a system where inferred evolutionary processes correspond to a considerable time frame, and the forces of mutation, selection and drift could have blurred past history.

Implications for the refugial debate

Many years of phylogeographic and population genetic studies lead to the general assumption that, in temperate areas, glacial refugia harbour higher levels of genetic diversity than regions colonized more recently, in postglacial times. Briefly, this prediction is based on the assumption that southern populations retain ancestral alleles, but that northern populations would be depleted of those alleles by founder events during the rapid colonization after climate amelioration. This assumption has recently been challenged by Comps *et al*. (2001) and Petit *et al*. (2003) who suggested that for many organisms the most diverse populations were not located in the south but at intermediate latitudes due to the secondary contact of divergent lineages. For this reason, Petit *et al*. (2003) stated that 'glacial refugia are hotspots but not melting pots of genetic diversity'.

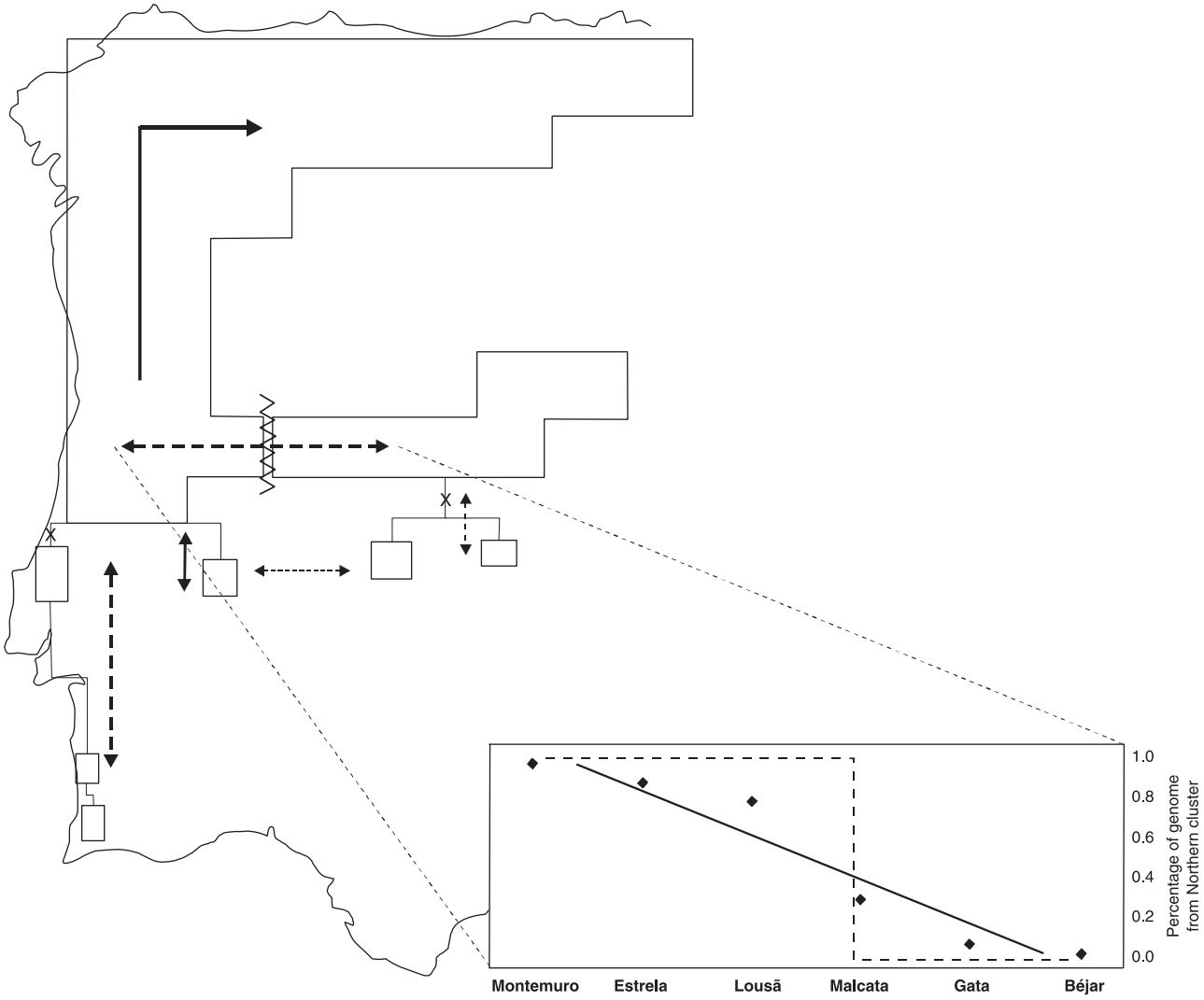

Fig. 5 Schematic representation of the geographic distribution of *Lacerta schreiberi* in Iberia and associated processes of population fragmentation, expansion and miscegenation. The separation of the two divergent mtDNA lineages A and B is represented by the symbol ʌʌ whereas the division of sublineages A_1/A_2 and B_1/B_2 is marked by the symbol X. Arrows vary in size and solid/dashed lines according to the intensity of gene flow between the different population groups inferred from Fst values. The inset corresponds to the core distribution area of the species and compares levels of mtDNA and nuclear admixture along a geographical gradient. Frequency of mtDNA lineages A and B are represented by a dashed line, while a solid line corresponds to the regression obtained from six point estimates of population admixture based on an individual estimated membership calculated from the Northern cluster.

The results of this study suggest that this perspective may be too simplistic. It is generally accepted that glacial refugia are occupied by rather homogeneous, albeit diverse, populations that are at the origin of cyclic and rapid recolonizations of deglaciated central and northern areas. However, as more data accumulate in the study of Iberian organisms, more evidence is available describing the complex patterns of contraction, fragmentation, persistence, expansion and admixture that characterize their evolutionary histories (Gómez & Lunt 2007). Our study of *L. schreiberi* revealed how two groups of populations have diverged, persisted and established a long-term admixture process along the mountains of the Iberian Central System, and how this core area of the species distribution responded to ice ages, either expanding to the south, either contracting again while leaving isolated populations behind, and simultaneously expanding to the north (Fig. 5; but see also Fig. 1 in Hewitt (1996), for a schematic anticipation of our present results in *L. schreiberi*).

Recent studies in a great variety of other species including the golden-striped salamander, *Chioglossa lusitanica* (Alexandrino *et al*. 2000, 2002, 2007; Sequeira *et al*. 2008), the fire salamander, *Salamandra salamandra* (García-París *et al*. 2003), the Bosca's newt, *L. boscai* (Martinez-Solano

et al. 2006), the wall lizards species complex, *Podarcis* spp. (Pinho *et al*. 2007), the worm lizard, *Blanus cinereus* (Albert *et al*. 2007) and the European rabbit, *Oryctolagus cuniculus* (Branco *et al*. 2000, 2002; Queney *et al*. 2001; Geraldes *et al*. 2006) additionally reveal the remarkable complexity of multiple evolutionary histories within the Iberian refugium, and do suggest that southern European peninsulas are hotspots *and* melting pots of genetic diversity.

The combined application of slow-evolving (proteins) and fast-evolving (microsatellites) nuclear markers together with the analysis of two gene genealogies in a well defined phylogeographic scenario provided new insights over previous studies of the evolutionary history of the Schreiber's green lizard, *L. schreiberi*. We believe that our investigation is a good example of how the complexity of the evolutionary processes that shaped the present-day patterns of genetic diversity in a lizard species can only be properly addressed through the use of several independent nuclear loci. It is expected that this study may encourage the use of diverse but complementary molecular markers together with the sophisticated statistical approaches that are now largely accessible, and that ultimately will improve our understanding of the natural history of populations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Amalgamated frequency distributions of microsatellites La3 (A) and Lv-4-α (B) in six population groups (Iberian Northwest, between Douro and Tejo, Spanish Central System, S. Mamede, Spanish central isolates and Portuguese southern isolates) of *Lacerta schreiberi*. p — number of amalgamated populations, n — number of individuals analysed. In the histograms, the x-axis represents allele sizes, while the y-axis shows allelic frequencies. Between Douro and Tejo corresponds to Portuguese Central System, Montemuro and Coastal isolates.

Table S1 Pairwise FST values (above the diagonal) derived from pooled data across all nuclear loci for the 19 populations of *Lacerta schreiberi*. ** means significance at the 5% nominal level. ns — non significant

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