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Recent evolutionary history of the Iberian endemic lizards *Podarcis bocagei* (Seoane, 1884) and *Podarcis carbonelli* Pérez-Mellado, 1981 (Squamata: Lacertidae) revealed by allozyme and microsatellite markers

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Podarcis bocagei and *Podarcis carbonelli* are two species of wall lizards endemic to the western Iberian Peninsula. A detailed phylogeographical study based on mitochondrial DNA (mtDNA) variation has shown that they responded differently to the Quaternary climatic oscillations. These differences have been attributed to their distribution patterns: *P. bocagei* is distributed in the north of the Peninsula and in a continuous fashion, whereas *P. carbonelli* has a more southern and fragmented distribution. In this study, we assessed whether nuclear markers reveal similar evolutionary patterns to those inferred from mtDNA variation. We studied a battery of allozyme and microsatellite loci in a geographically representative set of individuals from both species. For each species we evaluated overall levels of differentiation, patterns of geographical variation in genetic diversity, genetic relationships amongst localities, and applied model-based individual multilocus genotype clustering approaches to detect hidden population structure. Our results for *P. bocagei* are highly concordant with the phylogeographical scenario inferred from mtDNA variation: we found very low levels of population differentiation, consistent with survival in a single glacial refugium, and detected signatures of a rapid demographic and geographical expansion. The analyses of nuclear markers furthermore helped to identify a probable refugial area, as well as expansion routes. Additionally, in concordance with observations based on mtDNA variation, a low level of population differentiation was observed in *P. carbonelli,* but this was significantly higher than in *P. bocagei*. However, the geographical basis for differentiation in *P. carbonelli* is highly inconsistent between mtDNA and nuclear markers, suggesting a complex, albeit recent, history of fragmentation. A recent reduction of this species' distribution has probably erased the signatures of glacial isolation and post-glacial expansion that are normally found in other Iberian species, suggesting that the currently observed pattern of genetic differentiation in this species was shaped more by recent genetic drift than by the Pleistocene climatic oscillations.

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INTRODUCTION

Pleistocene climatic oscillations left considerable signatures on the genetic structure of temperate organisms (Hewitt, 1996, 1999, 2000). This period was characterized by an alternation of cold (glacial) and warm (interglacial) stages, which dramatically shifted habitat conditions across the globe over short periods of time. Although these changes increased extinction rates, many species were able to survive by moving along with the changing habitat. The genetic consequences of such range contractions and expansions have been well characterized in many organisms. Amongst the patterns that most commonly emerge are signatures of long-term isolation and persistence in distinct refugia, leading to high levels of population subdivision and, in a more debatable perspective, even speciation (e.g. Avise, Walker & Johns, 1998). In the case of European-wide distributed temperate taxa, for example, a common pattern is the presence of highly divergent lineages, signatures of glacial isolation, in distinct southern European peninsulas, and evidence for post-glacial recolonization of northern Europe. Classical examples of such scenarios are, for example, the grasshopper *Chorthippus paralellus* (Cooper, Ibrahim & Hewitt, 1995), the hedgehog *Erinaceus europaeus* (Santucci, Emerson & Hewitt, 1998), and the brown bear *Ursus arctos* (Taberlet & Bouvet, 1994); reviewed in Taberlet *et al*. (1998) and Hewitt (1999, 2000). As a result of post-glacial expansion from these source populations, northern areas often show depleted levels of genetic diversity, a paradigm known as 'northern purity vs. southern richness' (Hewitt, 1996, 2000). However, cases have also been documented in which northern areas show evidence of high levels of genetic diversity because of the mixing of waves expanding from different sources (Comps *et al*., 2001; Petit *et al*., 2003). In a number of species, these differentiated lineages establish hybrid suture zones, the location and orientation of which often reveal remarkably similar patterns amongst different organisms (Taberlet *et al*., 1998; Hewitt, 2000).

Adding to their important role in functioning as repositories of European-wide genetic variability, southern Peninsulas mirror at a smaller scale the processes that have been documented at a larger scale (isolation in different refugia, post-glacial expansion, and secondary contact). This 'refugia-within-refugia' model has been particularly well documented in the Iberian Peninsula. Various species have been shown to present highly complex population structure consistent with isolation in distinct glacial refugia (Comes & Abbott, 1998; Alexandrino *et al*., 2000; Branco, Ferrand & Monnerot, 2000; Paulo *et al*., 2001, amongst many others; reviewed in Gómez & Lunt, 2007). Accordingly, strong signatures of post-glacial demographic expansion and establishment of secondary contact zones have also been detected (Alexandrino *et al*., 2000; Branco *et al*., 2002; García-París *et al*., 2003; Sequeira *et al*., 2005, 2008; Godinho *et al*., 2006a, b; Godinho, Crespo & Ferrand, 2008). Although most studies indicate a high level of fragmentation, consistent with long-term survival in relatively stable refugia, the depth of divergence amongst lineages varies widely amongst species; furthermore, other endemics reveal low genetic diversity and substructure, and a shallow coalescent history, indicating lower persistence levels across the Quaternary glaciations (e.g. Pinho, Harris & Ferrand, 2007a). Rather than failing to corroborate the paradigm of multiple refugia within Iberia, these cases illustrate the variety of species-specific responses to the same climatic phenomenon.

The objects of this study, the Iberian endemic wall lizards *Podarcis bocagei* and *Podarcis carbonelli*, are good examples of species exhibiting shallow population subdivision, at least from a mitochondrial DNA perspective (Pinho *et al*., 2007a). This lack of phylogeographical structure is extreme in the case of *P. bocagei*, with an inferred coalescence time of around 70 000–100 000 years, and has been related to its northern distribution within the Iberian Peninsula. In fact, species with natural distribution ranges at more northern latitudes are expected to have experienced more severe habitat changes in response to Pleistocene climatic oscillations than species distributed in the south, affecting persistence levels throughout the Ice Ages. Accordingly, the signature of population demographic recovery is also expected to be stronger in these northern localities than in southern localities that were allowed to maintain a relatively high long-term effective population size throughout glacial cycles. However, the endangered *P. carbonelli* (Sá-Sousa, Pérez-Mellado & Martínez-Solano, 2008) is distributed in central and southern Iberia and shows higher levels of genetic variation and subdivision, although reduced when compared to those observed within other Iberian lizard species (e.g. Paulo *et al*., 2001).

In this study we investigate in more detail the evolutionary history of these two species using two different sets of nuclear markers: allozymes and microsatellites. We use these data to test whether the scenarios inferred from mitochondrial DNA variation are validated from an independent source of information. Specifically, we were interested in (1) evaluating whether the apparent low degree of genetic substructure (when compared to that observed in several other species) is reflected by a lack of subdivision in nuclear loci; (2) testing hypotheses related to the partitioning of genetic variability in both species, namely whether mtDNA-defined clusters in *P. carbon-*

elli correspond to distinct entities from a nuclear gene perspective; (3) validating the scenarios of post-glacial expansion inferred for *P. bocagei* and some geographical regions in *P. carbonelli*; (4) comparing the patterns of genetic diversity observed in both species, testing whether the differences in their distribution (*P. bocagei* is distributed northerly to *P. carbonelli* and has a continuous distribution range, whereas *P. carbonelli* shows a much more fragmented range and at least one geographical isolate) are reflected in different evolutionary scenarios.

MATERIAL AND METHODS

SAMPLING

We sampled individuals from a total of eight localities ($N = 176$) for *P. bocagei* and nine ($N = 157$) for *P. carbonelli*. Samples consisted of a portion of tail tissue obtained from the lizards' natural autotomy. All individuals were released after sample collection. Samples were stored frozen at –80 °C (for allozyme analyses) and in 96% ethanol (for DNA extraction). Sampling details (location and sample sizes) are given in Table 1 and Figure 1. Individuals from all sampled localities were included in a previous article addressing the mitochondrial DNA phylogeography of these two species (Pinho *et al*., 2007a). Some of the data included in this work (allozyme data for the *P. bocagei* localities of Madalena, Vairão, Zimão, and A Coruña and for the *P. carbonelli* localities of Villasrubias, Esmoriz, Aveiro, S. Pedro de Moel, and Playa de Rompeculos; partial microsatellite data sets for Zimão, Vairão, Madalena, Esmoriz, and Aveiro) have also been used in previous publications (Pinho, Harris & Ferrand, 2003, 2007b, Pinho, Ferrand & Harris, 2004a; Pinho *et al*., 2004b, 2009)

DATA COLLECTION

Allozymes

Tissue extraction, protein separation, and enzymatic detection of all loci followed the procedures given in Pinho *et al*. (2003). Variation at a total of ten allozyme loci was screened by means of conventional starch gel electrophoresis (*GOT, GPI, IDH, MPI, PEPA, PEPD, 6-PGD*) and isoelectric focusing (*LDH-2, PEPB, PGM*).

Microsatellites

DNA was extracted following standard procedures (Sambrook, Fritsch & Maniatis, 1989). For *P. bocagei* individuals, we studied six dinucleotide microsatellite

Table 1. Sampling details for this study

Species	Locality ID	Locality	Country	Sample size	
				Allozymes	Microsatellites
Podarcis bocagei					
	Mad	Madalena	Portugal	22*	25*
	Vair	Vairão	Portugal	33*	27*
	Bra	Braga	Portugal	11	21
	Zim	Zimão	Portugal	18*	18*
	Mon	Montesinho	Portugal	30*	29
	San	Sanxenxo	Spain	10	10
	Cor	A Coruña	Spain	16*	16
	Sar	Sarria	Spain	20	20
				160	166
Podarcis carbonelli					
	LA	La Alberca	Spain	16	16
	VR	Villasrubias	Spain	21*	22
	SE	Serra da Estrela	Portugal	15	15
	Esm	Esmoriz	Portugal	18*	18*
	Av	Aveiro	Portugal	17*	17*
	SPM	S. Pedro de Moel	Portugal	22*	22
	CR	Cabo Raso	Portugal	19	19
	MC	Monte Clérigo	Portugal	16	16
	PR	Playa del Rompeculos	Spain	12*	12
				156	157

Sample codes refer to those in Fig. 1.
*Includes previously published data (Pinho *et al*., 2003, 2004a, b, 2007b, 2009).

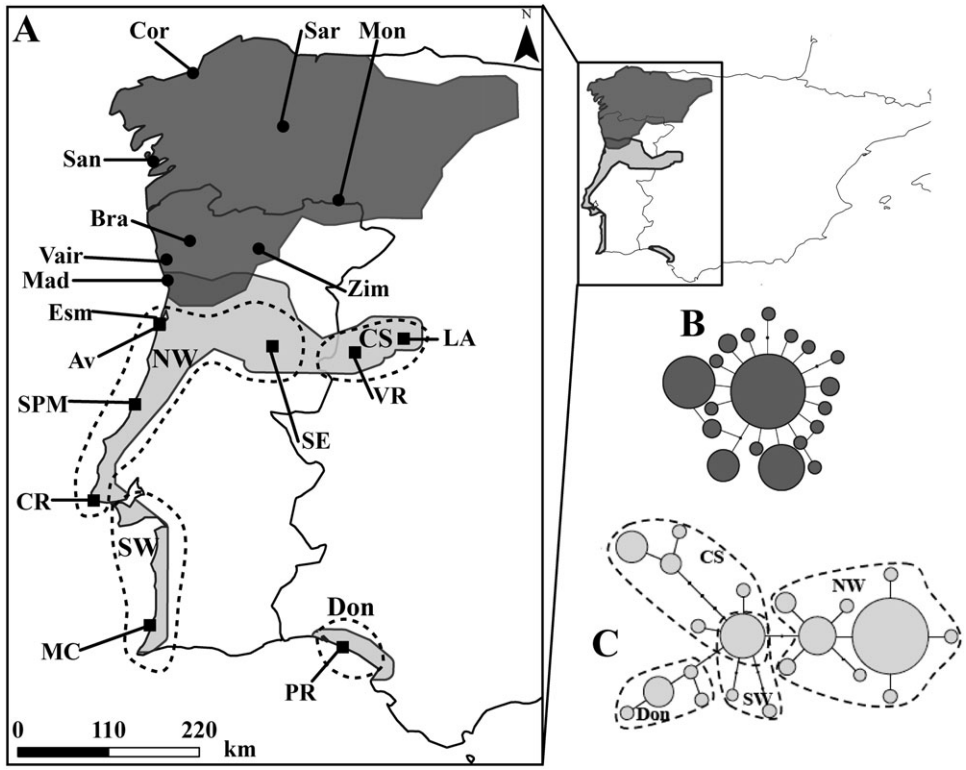

Figure 1. Distribution and localities analysed in this study for *Podarcis bocagei* (dark grey, circles) and *Podarcis carbonelli* (light grey, squares) (A), and median-joining networks of mtDNA haplotypes for *P. bocagei* (B) and *P. carbonelli* (C) reproduced from Pinho *et al*., 2007a. Dashed lines indicate the mtDNA groups and corresponding geographical distributions in *P. carbonelli*. Locality codes and sampling details are given in Table 1. Notice that the distribution shown for *P. carbonelli* is based on a 20 km buffer of occupied 10 × 10 km UTM (Universal Transverse Mercator) squares and is therefore amplified compared to that in Pinho *et al*., 2007a. This was necessary in order to visualize geographical patterns of genetic variation (see Material and methods).

loci from the battery of nine developed for this species by Pinho *et al*. (2004a) (*Pb11, Pb37, Pb47, Pb50, Pb66*, and *Pb73*). Only a subset of these (*Pb11, Pb47, Pb50, Pb66*) were analysed in *P. carbonelli*. These loci were amplified according to the described conditions with the exception of the annealing temperature, which was lowered to 53 °C in all cases in order to amplify difficult samples. The electrophoretic separation of the amplified fragments was carried out in 6% denaturing polyacrylamide gels and silver stained as described in Pinho *et al*. (2004b).

Analytical methods

Within species variation

We used FSTAT (Goudet, 2001) to calculate allele frequencies for each locus in each sampling locality, as well as evaluating putative deviations from Hardy–Weinberg (HW) and linkage equilibria. In order to compare genetic diversity across localities of each species, FSTAT was also used to compute allelic richness (*sensu* El Mousadik & Petit, 1996; Petit, El Mousadik & Pons, 1998) and unbiased gene diversity (Nei, 1987) per locus per locality. To investigate the significance of the differences in diversity measures between certain groups of sampling locations (see Results), we used permutation tests. We performed 10 000 permutations of the per locality values and compared the observed diversity differences to permuted ones, using the probability of observing a higher or equal difference between group means as an approximate *P*-value (see Adams & Anthony, 1996; Adams & Rohlf, 2000 for similar approaches). Permutation analyses were performed using the Microsoft Excel add-in PopTools v. 3.0.6 (Hood, 2008).

ARLEQUIN v. 3.1 (Excoffier, Laval & Schneider, 2005) was used to estimate F_{ST} values for each marker in each species, and their significance assessed using 10 000 permutations. Genetic relationships amongst localities of each species were estimated using neighbor-joining (NJ, Saitou & Nei, 1987) trees based on Cavalli-Sforza & Edwards' (1967) chord distance obtained from the combined allozyme and microsatellite data sets, using PHYLIP

3.6 (Felsenstein, 1993). Bootstrap support was evaluated through 1000 pseudoreplicates.

To test hypotheses related to the geographical partition of genetic variation we used ARLEQUIN to perform hierarchical analyses of molecular variance (AMOVA, Excoffier, Smouse & Quattro, 1992) using the combined data sets. In these analyses we partitioned each data set into several combinations of subsets, searching for structures that produced the highest fraction of variation amongst groups (that is, that maximized Φ_{CT}). We tested hypotheses related to the influence of geographical barriers, mitochondrial DNA-defined subdivision, and relevant geographical clusters in the total genetic diversity. As we were aiming at detecting genetic substructure concordant with geographical partitions, we focused mainly on clusters involving geographically close localities. In order to visualize geographical patterns of genetic variation while incorporating information from all markers simultaneously, we used GENETIX v. 4.05.2 (Belkhir *et al*., 1996–2004) to perform a factorial correspondence analysis (FCA) of allele frequencies of sampling localities for each species separately. In continuation, we used the Geostatistical Analyst extension of ArcMap 9.3 (Environmental Systems Research Institute, Inc., 2008) to interpolate factor scores observed in sampling localities across the distribution range of each species. We used the inverse distance weighted exact algorithm with a power of two to obtain synthetic maps of genetic variation (Hanotte *et al*., 2002; Manel *et al*., 2003).

These analyses used sampling localities as operational units. To explore further the patterns of intraspecific variability we used the model-based individual multilocus genotype clustering method implemented in the software STRUCTURE 2.1 (Pritchard, Stephens & Donnelly, 2000). By doing so we were able to search for hidden population structure and evaluate how it is distributed across geographical space. For these analyses, in order to minimize missing data, we used only individuals analysed for both classes of markers, a total of 150 *P. bocagei* and 151 *P. carbonelli* individuals. The parameter settings included the assumption of admixture and a correlated allele frequencies model, which assumes that allele frequencies are similar across localities and that variation amongst them arose mainly by genetic drift rather than mutation. Although this model seemed appropriate for our data given both the shallow substructure inferred for both species from mtDNA variation and the low differentiation amongst localities detected in this study (see Results), we repeated the runs without assuming correlated allele frequencies given that the choice of the model might strongly influence the outcome of the clustering algorithm (Serre & Pääbo, 2004; Rosenberg *et al*., 2005). We tested scenarios assuming a number of populations (K) varying from one to ten or 11 (in *P. bocagei* and *P. carbonelli*, respectively, representing the actual number of sampled sites plus two). STRUCTURE was run for 700 000 steps and the first 200 000 discarded as burn-in. For each value of K, five independent replicates of the Markov chain Monte Carlo were conducted.

We used the software DISTRUCT (Rosenberg, 2002) to obtain graphical representations of the inferred genetic structure. In order to choose the value of K that most accurately characterized each data set from amongst the various possible values, we used the method described by Evanno, Regnaut & Goudet (2005), which searches for a mode in the distribution of ΔK, a quantity related to the second order rate of change of the log probability of the data.

Comparison between species

The above analyses were performed for both species separately. An important goal of this study was also to evaluate whether differentiation and diversity are different overall in both species (as shown for mitochondrial DNA variation). For these analyses, we recalculated pairwise F_{ST} and diversity measures in *P. bocagei* using only the common panel of four markers that were also analysed in *P. carbonelli* (which provided very similar results in terms of overall genetic differentiation amongst populations to those obtained using the full data set). Allelic richness for both allozymes and microsatellites was also re-estimated in *P. carbonelli* so that the used rarefaction size ($N = 9$ individuals) was common to both species and the results could be directly compared. To test for significance of differences between the two species in the measures of interest (allelic richness, gene diversity, and pairwise F_{ST} for each class of markers), we used the same permutation approach described earlier by randomly swapping populations between species. In practice, this corresponds to shuffling diversity values (option 'SHUFFLE' in PopTools) and shuffling F_{ST} matrix vectors (option 'SHUFFLEDISTANCE').

RESULTS

Genetic diversity

Allelic frequencies for each locality and marker are given in Tables S1 and S2 for *P. bocagei* and *P. carbonelli*, respectively.

All ten allozyme loci studied were polymorphic in *P. bocagei*, whereas in *P. carbonelli* locus *GPI* was found to be monomorphic and was therefore not included in analyses of differentiation within this species. A total of 31 alleles was detected in the ten polymorphic loci

Table 2. Mean diversity measures (gene diversity and allelic richness) across localities of *Podarcis bocagei* (based on a data set of ten allozymes and six microsatellites) and of *Podarcis carbonelli* (based on ten allozyme and four microsatellite loci)

		Mean diversity measures across loci			
		Allozymes		Microsatellites	
Species	Locality	Gene diversity	Allelic richness	Gene diversity	Allelic richness
P. bocagei					
	Madalena	0.224	1.849	0.857	7.685
	Vairão	0.195	1.940	0.903	9.283
	Braga	0.242	1.974	0.894	9.745
	Zimão	0.127	1.478	0.891	9.055
	Montesinho	0.180	1.752	0.905	9.531
	Sanxenxo	0.102	1.290	0.773	7.277
	A Coruña	0.109	1.260	0.700	5.667
	Sarria	0.153	1.450	0.866	7.942
P. carbonelli					
	La Alberca	0.229	1.753	0.613	5.145
	Villasrubias	0.186	1.704	0.835	8.279
	Serra da Estrela	0.274	2.043	0.761	8.523
	Esmoriz	0.243	1.990	0.798	7.208
	Aveiro	0.256	2.022	0.659	5.542
	S. Pedro de Moel	0.211	2.041	0.809	8.966
	Cabo Raso	0.282	2.017	0.758	6.077
	Monte Clérigo	0.111	1.422	0.678	4.906
	Playa del Rompeculos	0.208	1.567	0.871	9.745

in *P. bocagei*, with variability levels ranging from two (in *GPI, IDH, PEPB*) to four (in *LDH-2, PEPA, PEPD, PGD*) alleles per locus. Thirty-six alleles were detected in the studied localities of *P. carbonelli* amongst the nine polymorphic loci, ranging from two allelic variants in *IDH* and *MPI* to six alleles observed in *PEPD*. Microsatellite loci showed high levels of variation, with a minimum of 19 alleles observed per locus in *P. bocagei* (*Pb11, Pb50*) and 15 in *P. carbonelli* (*Pb47, Pb66*), whereas the loci with the highest number of alleles detected were *Pb37* in *P. bocagei* (36 alleles) and *Pb50* in *P. carbonelli* (31 alleles). With the exception of *Pb11*, all loci analysed showed both even and odd allele sizes. This suggests that insertions or deletions in the flanking regions may have occurred. Taking these results together with the multimodal distribution of overall allele frequencies in most markers, this implies that a strict stepwise mutation model of evolution (Ohta & Kimura, 1973) cannot be properly applied to our microsatellite data sets. We thus abstained from performing analyses that assume this model to compute divergence or differentiation amongst localities.

Apart from loci *Pb37* and *Pb50* in the *P. bocagei* locality of Montesinho, no loci were found to be in HW disequilibrium (after Bonferroni correction). All loci were in linkage equilibrium across localities.

Values of gene diversity and allelic richness are given in Table 2. In *P. bocagei*, a trend suggesting a decrease of genetic diversity from southern to northern localities was observed in both classes of markers. This lower diversity in the north is statistically significant: comparing Portuguese (Madalena, Vairão, Braga, Zimão, Montesinho) to Galician (Sanxenxo, Sarria, A Coruña) localities revealed significantly lower diversity in the latter (either gene diversity or allelic richness) in both allozymes and microsatellites ($P < 0.05$). In *P. carbonelli*, however, there appears to be no such observable trend. In particular, because a scenario of demographic expansion was observed for the mtDNA lineage occupying the north-western region of the species' distribution, we would expect a decrease in genetic variability similar to the one observed in *P. bocagei*. However, no significant differences in diversity measures were observed between the southern (Cabo Raso and S. Pedro de Moel) and the northern (Aveiro, Esmoriz, Serra da Estrela) localities within this region. The locality of Monte Clérigo consistently exhibits low diversity levels in both classes of markers.

Genetic differentiation amongst localities

Overall F_{ST} estimates for all markers reveal relatively low, although statistically significant, levels of differentiation in both species, with an average of 0.1056 and 0.0695 for allozymes and microsatellites, respectively, in *P. bocagei,* and 0.1757 and 0.1079 for both types of markers in *P. carbonelli* (Table 3).

NJ trees depicting relationships amongst localities are shown in Figure S1A (*P. bocagei*) and 1B (*P. carbonelli*). It should be noted that because our phylograms are not rooted, their interpretation in terms of relationships amongst localities is problematic and we refrain from analysing them in more detail; however, these graphs support a shallow level of intraspecific differentiation in both species, as long internal branches (that would be suggestive of differentiation amongst groups of populations) are not observed.

Table 3. Overall genetic differentiation (F_{st}) in *Podarcis bocagei* and *Podarcis carbonelli* using allozymes, microsatellites, and mitochondrial DNA

	P. bocagei	*P. carbonelli*
Allozymes		
Got1	0.0565**	0.1117***
Gpi	0.0232	0.0000
Idh	0.0571**	0.5354***
Ldh2	0.1311***	0.1987***
Mpi	0.1684***	0.1844***
PepA	0.0897***	0.2019***
PepB	0.0855**	0.1392***
PepD	0.0432**	0.1043***
Pgd	0.2456***	0.1148***
Pgm	0.0307	0.043*
All	0.1056***	0.1714***
Microsatellites		
Pb11	0.1659***	0.1757***
Pb37	0.0568***	–
Pb47	0.0515***	0.1022***
Pb50	0.0447***	0.0795***
Pb66	0.0543***	0.0784***
Pb73	0.0477***	–
All	0.0695***	0.1079***
Nuclear markers		
All	0.0753***	0.1381***
Mitochondrial DNA		
Nucleotide	0.7036***	0.8348***
Haplotype	0.6039***	0.6334***

Mitochondrial DNA data were obtained from Pinho *et al*., 2007a. Only data from the same populations analysed in this study were used for comparison. Overall F_{st}s were computed using nucleotide distances amongst haplotypes ('nucleotide') and only considering haplotypic frequencies, independently of the nucleotide variation ('haplotype').
*, significant ($P < 0.05$); **, significant ($P < 0.01$); ***, significant ($P < 0.001$); –, not analysed.

We also conducted AMOVAs to test hypotheses regarding the partition of genetic variability in both species. We did not intend to perform an exhaustive search of all possible combinations of localities within species. Instead, we were expecting to find signatures of some relationship between genetic variability and geographical groups and therefore focused on groups involving geographically close localities. The results for data partitions producing maximum values of Φ_{CT} plus important hypotheses to be tested (the influence of the Douro river in *P. bocagei* and mtDNA-based partition in *P. carbonelli*) are given in Table S3. In both species only shallow genetic variation was found amongst groups. In *P. bocagei* the highest Φ_{CT} values (> 0.08) were obtained when Galician localities (especially A Coruña, but also Sanxenxo and Sarria albeit at lower levels) were placed in separate groups from the remaining localities, suggesting that they are somewhat derived in the context of *P. bocagei* genetic variability. Grouping the localities of Vairão and Braga and considering each of the remaining localities as belonging to a distinct group also produced a significant and relatively high Φ_{CT} (0.086). To test the influence of the Douro river, a well-known biogeographical barrier in the context of north-western Iberian herpetofauna (e.g. Sequeira *et al*., 2008), we also tested the differentiation between Madalena and all the other localities, which yielded a very low and nonsignificant Φ_{CT} of 0.023. In *P. carbonelli*, none of the tested groups exhibited higher levels of variation amongst them than the variance observed between ungrouped sampled localities. The highest value of Φ_{CT} (0.102) is found when all localities are considered separately with the exception of Esmoriz and Serra da Estrela. Within this species, four geographical groups were detected at the mtDNA level, although some haplotypes were shared amongst them (Pinho *et al*., 2007a). We also tested the significance of such partitioning of genetic variability on the data sets. Only 3.64% of the total genetic variation could be ascribed to differences amongst these groups. Also based on mitochondrial DNA variation, we tested partitions involving the grouping of the localities of Villasrubias and Monte Clérigo because, despite their distant geographical situation, these localities share one haplotype (haplotype C4 in Pinho *et al*., 2007a). None of these hypotheses produced significant Φ_{CT} values (results not shown).

Synthetic maps produced using the results obtained for the first three axes of the FCAs for both species are shown in Figure 2. In *P. bocagei*, axes 1, 2, and 3 account for 21.74, 19.16, and 15.05%, respectively, of the total variation in allele frequencies. Visual

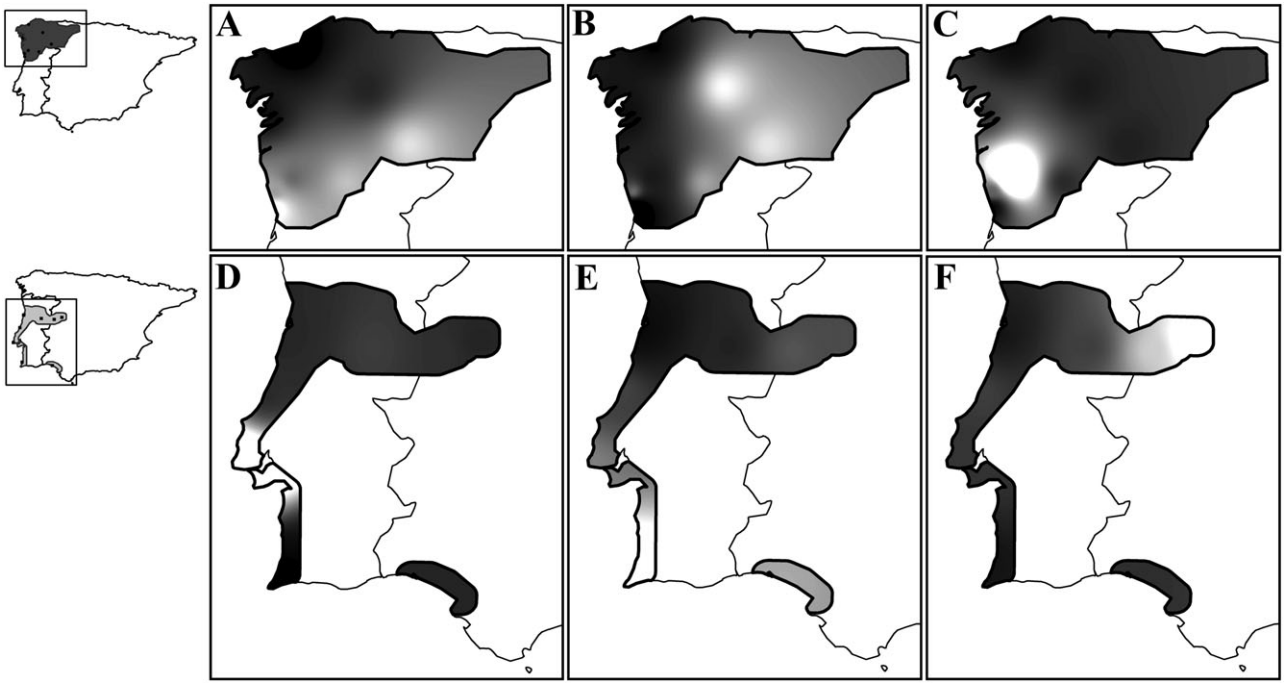

Figure 2. Synthetic maps showing patterns of geographical variation in allele frequencies across species distribution ranges based on the inversed distance weighted interpolation of the values calculated for each locality of *Podarcis bocagei* (top) and *Podarcis carbonelli* (bottom) for the first (A, D), second (B, E), and third (C, F) axes of factorial correspondence analyses. White represents higher and black lower factor score values.

inspection of the maps reveals a north–south orientated gradient for the first axis (Fig. 2A), whereas the second shows instead an east–west gradient in allele frequencies (Fig. 2B). Finally, the third axis represents the differentiation of the Braga population (Fig. 2C). In *P. carbonelli*, the percentages of explained variance are 25.22, 20.38, and 14.26% for axes 1, 2, and 3, respectively. Contrary to *P. bocagei*, no gradual variation is observed in *P. carbonelli*, factorial axes representing rather local variation. The first axis illustrates the differentiation of the population of Cabo Raso (Fig. 2D), the second that of Monte Clérigo (Fig. 2E), and the third that of the La Alberca population (Fig. 2F).

Individual multilocus genotype clustering

Results obtained using the software STRUCTURE are presented in Figures 3 and 4 for *P. bocagei* and *P. carbonelli*, respectively, using a model with admixture and correlated allele frequencies. Results from runs employing an independent allele frequencies model produced highly similar results for both data sets, albeit with lower degrees of 'clusteredness' (*sensu* Rosenberg *et al*., 2005; results not shown). We present the partitions that for each value of K (from 2 to 5 because higher levels of subdivision produce complex and uninterpretable scenarios) showed the highest values of Ln probability. As STRUCTURE does not integrate over the possible values of K, we employed the method suggested by Evanno *et al*. (2005) to choose the number of partitions that better represented the variation present in the data sets; these results are also presented in Figures 3 and 4 and the chosen values of K represented with respect to geography.

In *P. bocagei*, the first layer of differentiation that appears (with $K = 2$) is that between Galician localities and southern ones; nevertheless, the discrimination is not perfect, with apparently admixed individuals in the localities of Vairão, Braga, and Sarria. Increasing the number of subpopulations ($K = 3$), A Coruña and Sanxenxo appear as almost 'pure' for one of the inferred clusters. A second cluster is present at higher proportions in individuals from Zimão and Montesinho; individuals from Sarria are highly admixed with respect to these first two clusters, with a predominance of the Galician component. A third cluster is more frequent in the locality of Madalena, with nearly all individuals 'pure' for this locality. Individuals from Vairão and, in particular, Braga present a high level of admixture amongst all three clusters. With $K = 4$ a clade formed mainly by individuals from Vairão and Sarria but also present in

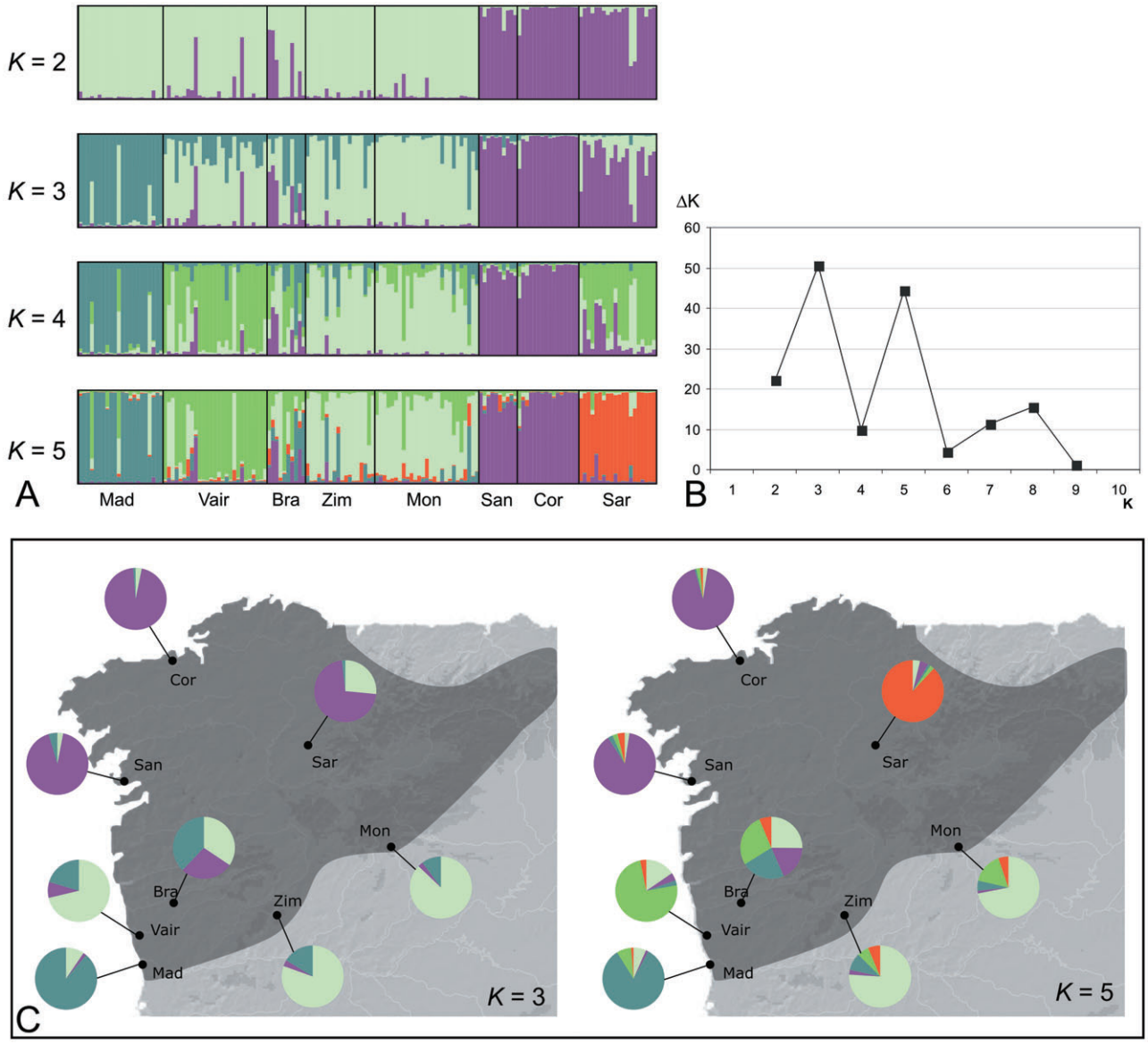

Figure 3. Individual multilocus genotype clustering analysis for *Podarcis bocagei*. A, inferred population structure from the number of clusters (K) = 2 to 5. These plots were obtained from the runs producing the highest values of Ln probability for each value of K, assuming correlated allele frequencies. In these plots, each individual is represented by a column divided into K segments, the size of each corresponding to the individual's estimated membership fraction in each of the K clusters. See Table 1 for locality name abbreviations. B, variation of the value of ΔK with the number of clusters, following Evanno *et al*. (2005). C, pie charts representing the mean proportion of membership for $K = 3$ and 5 (chosen by the previous method) for each locality.

other localities emerges. With $K = 5$, the clusters roughly correspond to Madalena, Vairão, the two localities from the south-east (Zimão and Montesinho), the two western Galician localities (Sanxenxo and Coruña), and Sarria. Braga remains as admixed, with high proportions of the first four clusters. The test of Evanno *et al*. (Fig. 3B) produced inconclusive results, with both $K = 3$ and $K = 5$ receiving high support.

Introducing a two-locality structure into the *P. carbonelli* data set, Cabo Raso and Monte Clérigo appear as a group versus all other localities. The locality of Playa del Rompeculos remains, however, admixed with respect to these two clusters, as well as some individuals from Villasrubias. With $K = 3$, Cabo Raso becomes clearly detached from all other localities; a second cluster is formed by individuals mainly from the localities of Monte Clérigo and La Alberca, but is

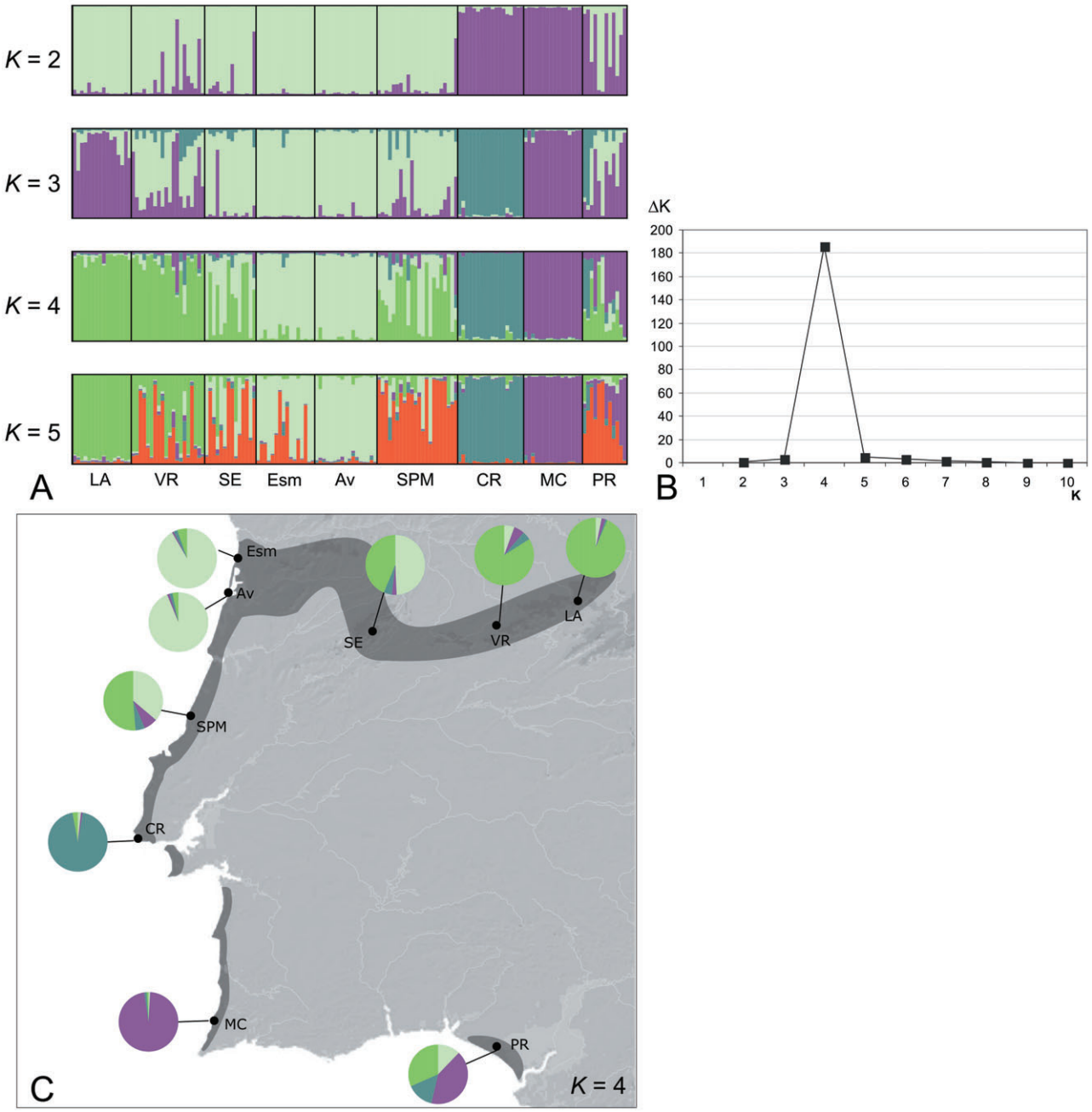

Figure 4. Individual multilocus genotype clustering analysis for *Podarcis carbonelli*. A, inferred population structure from the number of clusters (K) = 2 to 5. These plots were obtained from the runs producing the highest values of Ln probability for each value of K, assuming correlated allele frequencies. In these plots, each individual is represented by a column divided into K segments, the size of each corresponding to the individual's estimated membership fraction in each of the K clusters. See Table 1 for locality name abbreviations. B, variation of the value of ΔK with the number of clusters, following Evanno *et al*. (2005). C, pie charts representing the mean proportion of membership for $K = 4$ (chosen by the previous method) for each locality.

also represented in Playa del Rompeculos, Villasrubias and, to a lesser extent, S. Pedro de Moel. A third cluster is widely distributed amongst all localities (except Cabo Raso and Monte Clérigo), with high levels of homogeneity particularly in the localities of Serra da Estrela, Aveiro, and Esmoriz. With $K = 4$, individuals from Cabo Raso fall once again in a cluster of their own; individuals from Monte Clérigo

likewise, although this cluster is also represented in some individuals from Playa del Rompeculos. Individuals from La Alberca and Villasrubias on the one hand, and from Aveiro and Esmoriz, on the other hand, are placed with a low degree of admixture in a third and a fourth cluster, respectively. The localities of Serra da Estrela and S. Pedro de Moel appear admixed between these two clusters. Increasing the number of localities to five, La Alberca, Aveiro, Cabo Raso, and Monte Clérigo each constitute a highly homogenous cluster; a fifth group appears at high frequency in the genome of individuals from S. Pedro de Moel and also with admixture from other clusters in the localities of Villasrubias, Serra da Estrela, Esmoriz, and Playa del Rompeculos. For this species, results from Evanno *et al.*'s (2005) test show a clear mode for $K = 4$.

Comparison between species

A summary of an overall comparison between *P. bocagei* and *P. carbonelli* in terms of mean diversity of sampling localities and pairwise differentiation values is presented in Table 4. We were interested in testing the hypothesis that the values observed in *P. carbonelli* were significantly higher than those in *P. bocagei* to evaluate whether the patterns reported for mtDNA are confirmed using nuclear markers.

An interesting trend is the remarkable difference observed between the two classes of markers. On the one hand, when comparisons involved the allozyme data set, *P. carbonelli* showed higher values for the three measures being compared [although one of these comparisons – for allelic richness – is not significant ($P = 0.075$)]. On the other hand, the microsatellite data set consistently showed higher diversity values in *P. bocagei* (but not significantly higher). Average pairwise F_{ST} for microsatellite loci is higher in *P. carbonelli*, although these differences are not significant.

These results should be taken with a cautionary note. Although they cross-amplify in *P. carbonelli*, the microsatellite loci were developed for *P. bocagei*. Therefore it is not unlikely that the comparisons involving these markers could be affected by some kind of ascertainment bias.

DISCUSSION

Evolutionary history of *Podarcis bocagei*

In general, low levels of subdivision were found in nuclear markers across localities of *P. bocagei*. This is in accordance with evidence based on mitochondrial DNA differentiation. In fact, there is no sign of a strong geographical substructure that would be concordant with isolation and divergence in multiple glacial refugia, as has been described using nuclear markers in several other species from the northwestern Iberian Peninsula (e.g. Alexandrino *et al.*, 2000; Godinho *et al.*, 2008). Interestingly, levels of divergence based on nuclear multilocus data are much lower than those observed using mitochondrial DNA when the same localities and only allele (rather than nucleotide distance) data are considered [$\Phi_{ST(nuc)} = 0.07$ whereas $\Phi_{ST(mit)} = 0.60$]. This discrepancy probably reflects the different effective

Table 4. Comparison of overall diversity levels and differentiation between *Podarcis bocagei* and *Podarcis carbonelli*

	P. bocagei	*P. carbonelli*	*P*-value ($Pc > Pb$)
Diversity			
Mean allelic richness			
Allozymes	1.62 (0.29)	1.82 (0.22)	0.075
Microsatellites	7.84 (1.63)	6.84 (1.67)	0.891*
Mean gene diversity			
Allozymes	0.167 (0.053)	0.222 (0.052)	**0.020**
Microsatellites	0.824 (0.101)	0.754 (0.087)	0.924*
Differentiation			
Mean pairwise F_{ST}			
Allozymes	0.099 (0.062)	0.164 (0.081)	**0.044**
Microsatellites	0.084 (0.045)	0.109 (0.066)	0.266

The same panel of 14 markers were used for the computations, so the values are directly comparable. The values shown are means across localities of each species (diversity measures) or means across all pairwise comparisons (F_{ST}). Standard deviations are shown in parentheses. *P*-values were computed to test the hypothesis that *P. carbonelli* localities have on average significantly higher scores than *P. bocagei*. Significant values are shown in bold.

*As the mean observed in *P. bocagei* is higher than that in *P. carbonelli*, we also tested the more obvious hypothesis that these values were significantly higher ($Pb > Pc$). This hypothesis was rejected in both cases.

population sizes (and probably different overall variability levels) that characterize each class of markers.

The phylogeographical study of Pinho *et al*. (2007a), based solely on mtDNA, described a pattern consistent with an extremely rapid demographic growth from a single refugial source, but was inconclusive with respect to the location of the glacial refugium for *P. bocagei*. Nevertheless, from the magnitude of the demographic expansion inferred from mtDNA variation, we expect that this refugial region would be relatively small in comparison to the species' present-day distribution area. We may therefore assume that the majority of the species' current distribution range has been colonized after the most recent period of climate warming, that is, within the past 10 000 years. It is therefore surprising that based on a panel of 16 loci (including only a few highly variable markers) geographical-consistent genetic differentiation can be recovered within this short evolutionary time-frame. This genetic differentiation, albeit shallow, can be observed from multiple lines of evidence: AMOVA results, synthetic maps, and, in particular, the multilocus genotype individual clustering approach. All of these methods point, for example, to a moderate degree of differentiation of the Galician, northernmost localities, and to a close relationship between the two south-eastern localities (Zimão and Montesinho). In concordance with the findings based on mtDNA variation, we believe that this pattern of differentiation, which is particularly obvious in STRUCTURE analyses with $K = 3$, is a by-product of a recent post-glacial expansion. The differentiated population groups, rather than representing true 'clusters' in the sense that they result from independent evolutionary pathways, correspond to the extremes of clines deriving from the area that functioned as a glacial refugium. Corroborating this evidence, the central localities of Braga and, to a lesser extent, Vairão, present evidence of 'admixture'. Likewise, allele frequencies at some loci (e.g. *MPI*) show a clinal pattern. These particularities therefore identify the south-western region of the distribution as the source for geographical expansion and help pinpoint the probable location of the area to which this species was confined during the last glaciations. The dynamics of post-glacial expansion are furthermore documented by a marked reduction in variability levels in a northwards fashion, consistent with a scenario of 'northern purity' (Hewitt, 2000).

A much denser sampling scheme would be necessary to identify with precision the routes by which the expansion was carried out. Nevertheless, an interesting result is obtained by comparing mitochondrial DNA haplotype distribution with the nuclear data. A single mitochondrial DNA haplotype is shared amongst the localities from the north-eastern area of the species' distribution, including Sarria (Pinho *et al*., 2007a). This haplotype is also present at high frequencies in Montesinho, but at very low frequencies elsewhere. This suggests that colonization of the north-eastern areas of *P. bocagei*'s distribution occurred through the east. However, in STRUCTURE analyses individuals from Sarria usually have a large proportion of their genome assigned to the Galician cluster (along with Sanxenxo and A Coruña) rather than to the south-eastern cluster, as would be expected from mtDNA variation. These two apparently incompatible observations are reconciled by taking into consideration the signatures of admixture with the south-eastern clade presented by individuals of Sarria, thus suggesting a process of merging from two waves of expansion, one with a south-to-north and another with a west-to-east direction, which are also supported by the synthetic maps of genetic variation. This situation therefore mirrors, at a much shallower scale, the process of northern admixture of different expansion waves described by Comps *et al*. (2001).

Interestingly, the river Douro, which has been described as a major biogeographical break in other species with similar distributions (*Chioglossa lusitanica,* Alexandrino *et al*., 2000; Sequeira *et al*., 2008; *Lacerta schreiberi*, Godinho *et al*., 2008; probably *Discoglossus galganoi*, Martínez-Solano, 2004), does not appear to have significantly contributed to genetic differentiation within *P. bocagei*. Nevertheless, the sampling locality of Madalena (the only south of the Douro) stands out as mildly differentiated by emerging as a clade of its own in STRUCTURE analyses when three or more localities are enforced. It has been suggested (Sá-Sousa, 2001a) that the colonization of the area south of this river occurred in the past two-hundred years via human-built bridges. As the locality of Madalena is characterized by relatively high variability levels and moderate differentiation relative to other localities, our data are not consistent with the hypothesis of an extremely recent colonization and therefore do not support this suggestion.

Evolutionary history of *Podarcis carbonelli*

Although a scenario with some degree of isolation was inferred for *P. carbonelli* based on mitochondrial DNA variation (Pinho *et al*., 2007a), the observed level of differentiation is not consistent with early isolation within the first stages of the Pleistocene as inferred for many other species of the Iberian herpetofauna (Alexandrino *et al*., 2000; Paulo *et al*., 2001; Alexandrino, Arntzen & Ferrand, 2002). In fact, instead of deeply divergent lineages, only a few mutations were found amongst each haplotype group; moreover, these incipient lineages are still incompletely sorted con-

cerning geography, because the haplotype inferred to be ancestral to all the others still exists and is present in two distant localities (Villasrubias and Monte Clérigo). Applying a molecular clock, coalescence time for *P. carbonelli* mtDNA was estimated around 500 000–300 000 years ago (Pinho *et al*., 2007a).

According to allozymes and microsatellites, differentiation within this species is quite low when compared to mtDNA variation [$\Phi_{ST(nuc)} = 0.14$ whereas $\Phi_{ST(mit)} = 0.63$], a discordance that probably derives from the same factors already noted for *P. bocagei*. Surprisingly, we find that genetic variation in nuclear loci is not highly correlated to that in mtDNA. In fact, differentiation amongst the four groups described at the mtDNA level merely accounts for 3.64% of the total nuclear genetic variation observed in this species. Moreover, none of the possible geographical clusters that were tested yield higher levels of variability amongst groups than the ones observed amongst localities when these are considered individually. This is also suggested by synthetic maps of genetic variation, which provide evidence for the differentiation of particular populations instead of different groups. The relationships amongst localities suggested by mtDNA variation are also not clearly depicted on the individual clustering analyses; despite some degree of concordance between geographically close localities and clusters of individuals, these correspondences are not perfect, with some localities appearing as highly admixed. Although these results could be the outcome of complex patterns of historical fragmentation and admixture, an alternative explanation is that these analyses have instead identified localities or groups of closely related localities (such as Cabo Raso, Monte Clérigo, Aveiro + Esmoriz, and La Alberca + Villasrubias) that have experienced high levels of genetic drift. This interpretation thus suggests that localities that appear as admixed are probably those that were able to maintain higher effective population sizes throughout recent climatic changes. One such locality is the geographical isolate from southern Spain. Given the high variability levels detected, particularly in microsatellite loci, it is likely that it sustained high effective population sizes despite its apparent isolation. In contrast, the locality of Monte Clérigo shows depleted levels of genetic variability and was most likely strongly affected by genetic drift, possibly as a result of bottlenecks related to its marginal distribution.

Such incongruent patterns are not easily reconciled in a simple evolutionary scenario. The lack of concordance between patterns of genetic differentiation inferred from mtDNA and nuclear genes may simply be a result of shallow and recent population structure in this species. When differentiation is recent, it is likely that distinct markers will lack concordance (Kuo & Avise, 2005), purely because of chance. In fact, both mtDNA and nuclear markers suggest a relative degree of concordance between genetic variation and geography, but in different ways depending on the marker. It has also been shown that apparent phylogeographical breaks in mtDNA may arise without this being caused by real isolation (Irwin, 2002). Another plausible hypothesis is that the evolutionary history of *P. carbonelli* corresponds to a complex sequence of events of fragmentation and admixture that left different signatures on different markers. Even though these hypotheses are not mutually exclusive, the present analyses do not allow a straightforward evaluation of the roles that allopatric fragmentation, genetic drift, and gene flow have played in this species' recent evolution. Additionally, unlike in *P. bocagei*, it is difficult to pinpoint areas that might have functioned as glacial refugia. The currently fragmented distribution of *P. carbonelli,* the uniqueness of its distribution in the context of Iberian herpetofauna, and the difficulties in modelling such a distribution (Sá-Sousa, 2001a) suggest that this species has probably had a much larger distribution area in the past and that it is still retracting. This possibility has led to its assessment as endangered (Sá-Sousa, Pérez-Mellado & Martínez-Solano, 2008). Our results agree with a scenario of fragmentation; it is possible that the genetic signatures of isolation, demographic recovery, and admixture usually associated with the alternation of glacial and interglacial stages have been erased or masked as a consequence of recent changes in the species distribution.

The mitochondrial DNA-based phylogeographical study carried out by Pinho *et al*. (2007a) detected a strong signature of demographic expansion within the north-western clade of *P. carbonelli*. This was particularly evident not only from statistical tests designed to detect demographic changes but also from a progressive loss of genetic variability in a northwards fashion. Given this, our preliminary expectation was that nuclear markers would reveal a similar pattern. Instead, we find no consistent decrease in levels of genetic variability. Which processes may underlie such discrepant results? A possible explanation resides in differences in current effective population sizes between putative source localities and recently colonized territories. In fact, *P. carbonelli* seems to be currently more abundant in the northern area of its distribution than in the south, where it becomes rare and more locally distributed (Sá-Sousa, 2001b; M. A. Carretero, A. Kaliontzopoulou, D. J. Harris, C. Pinho, pers. observ.). It is possible that, at the same time that the north-western clade of *P. carbonelli* expanded to the north, southern habitats became progressively less suitable and thus sustained lower effective population sizes and consequently lower diversity, whereas

the demographic expansion in the north compensated for the loss of diversity via genetic drift. A second scenario could be that of a slower expansion. Petit, Excoffier & Mayer (1999), for example, found no evidence of loss of variability in the post-glacial colonization of noctule bats, probably because the expansion was carried out in a slow fashion tracking forest expansion. In the case of *P. carbonelli*, the expansion would have to have been carried out at a speed that would allow drift to affect mtDNA but not nuclear markers because of different effective population sizes. A third scenario would imply extensive genetic admixture (from, for example, the Central System phylogroup) in northern localities but not in southern ones; however, we find no corroborating evidence for this using mtDNA. These hypotheses are purely demographic. However, scenarios that invoke natural selection cannot be ruled out. For example, a recent selective sweep in the mitochondrial genome of *P. carbonelli* could be responsible for the positive signature of demographic expansion, as both phenomena leave similar imprints on genetic variation.

Comparison between species

With this study, besides being interested in documenting the evidence for the evolutionary pathways of both species in response to climatic oscillations, we also wanted to test the hypothesis that the different distribution patterns (*P. bocagei* is distributed more northerly and continuously when compared to *P. carbonelli*) could leave different imprints on genetic variation (Pinho *et al*., 2007a). In order to do so, we tested both differences in overall diversity and in levels of differentiation between the two species. Our hypothesis was that *P. carbonelli*, being distributed in the south, would have experienced in a milder fashion the effects of the Pleistocene climatic oscillations, allowing for longer persistence of populations (leading to higher levels of differentiation) and for the maintenance of higher effective population sizes (leading to higher levels of diversity). Our results show that indeed *P. carbonelli* is more subdivided than *P. bocagei* (pairwise F_{ST} is higher for both types of markers, although only significantly higher for allozyme loci), thus conforming to the patterns documented for mtDNA (Pinho *et al*., 2007a). However, the fact that average pairwise F_{ST} is higher in *P. carbonelli* could result either from historical isolation or from recent fragmentation. To address this question, one would need to look at diversity. However, differences in diversity levels are not easy to interpret in a straightforward way. For microsatellites, *P. bocagei* actually bears higher levels of diversity than *P. carbonelli*. This could be explained by several factors: one is ascertainment bias because the markers were developed for *P. bocagei* (Pinho *et al*., 2004a). A second possibility is that of saturation. Microsatellites in general, and these in particular, have an extremely fast mutation rate, which implies that more historical phenomena, such as the ones we are trying to document, are easily erased from the patterns of genetic variation. Allozymes thus seem more appropriate to study differences in historical population sizes at this level, and from the analyses of these it is clear that *P. carbonelli* populations have on average higher diversity levels than in *P. bocagei*. Therefore, although some lack of statistical power prevents a more straightforward assessment, together with mtDNA results these results appear to fit our main predictions relating the species distribution patterns (latitude and continuity) with genetic variation.

Concluding remarks

This study highlights the need for the assessment of multiple loci prior to making inferences on the evolutionary history of organisms. Although general evolutionary trends were concordant between mtDNA and nuclear genes (e.g. low level of subdivision inferred for both species albeit higher levels of differentiation in *P. carbonelli;* a history of recent demographic and geographical expansion in *P. bocagei*), the study of mtDNA alone fails to detect significant particularities in the evolutionary history of both species. For example, the analyses of nuclear markers complemented those of mtDNA by pinpointing a probable refugial area and by detecting signatures of multiple expansion routes in *P. bocagei*. In *P. carbonelli*, although interpretation of the pattern of genetic variation in nuclear loci is not straightforward in the light of Pleistocene climatic alterations, mtDNA portrays an oversimplified history of isolation that does not reflect the necessarily complex dynamics of this species' evolution. In this particular case, the inclusion of more samples from the southern range of the species' distribution could eventually provide important cues for a better understanding of its evolutionary history and, eventually, help in the adoption of appropriate conservation measures for this endangered organism.

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SUPPORTING INFORMATION

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

Table S1. Allele frequencies in *P. bocagei* localities.
Table S2. Allele frequencies in *P. carbonelli* localities.
Table S3. Testing hypotheses concerning the geographic distribution of genetic variation in *P. bocagei* and *P. carbonelli*.
Figure S1. Neighbor-Joining trees depicting relationships between localities of *P. bocagei* (**A**) and *P. carbonelli* (**B**). Bootstrap values above 50% are shown.

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