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The legacy of ice ages in mountain species: post-glacial colonization of mountain tops rather than current range fragmentation determines mitochondrial genetic diversity in an endemic Pyrenean rock lizard

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ABSTRACT

Aim The genetic impact of Quaternary climatic fluctuations on mountain endemic species has rarely been investigated. The Pyrenean rock lizard (*Iberolacerta bonnali*) is restricted to alpine habitats in the Pyrenees where it exhibits a highly fragmented distribution between massifs and between habitats within massifs. Using mitochondrial DNA markers, we set out: (1) to test whether several evolutionary units exist within the species; (2) to understand how the species persisted through the Last Glacial Maximum and whether the current range fragmentation originates from distribution shifts after the Last Glacial Maximum or from more ancient events; and (3) to investigate whether current mitochondrial diversity reflects past population history or current habitat fragmentation.

Location The Pyrenees in south-western France and northern Spain.

Methods We used variation in the hypervariable left domain of the mitochondrial control region of 146 lizards collected in 15 localities, supplemented by cytochrome *b* sequences downloaded from GenBank to cover most of the species' distribution range. Measures of population genetic diversity were contrasted with population isolation inferred from topography. Classical (*F*-statistics) and coalescence-based methods were used to assess the level of gene flow and estimate divergence time between populations. We used coalescence-based simulations to test the congruence of our genetic data with a scenario of simultaneous divergence of current populations.

Results Coalescence-based analyses suggested that these peripheral populations diverged simultaneously at the end of the last glacial episode when their habitats became isolated on mountain summits. High mitochondrial diversity was found in peripheral, isolated populations, while the populations from the core of the species' range were genetically impoverished. Where mitochondrial diversity has been retained, populations within the same massif exhibited high levels of genetic differentiation.

Main conclusions As suggested for many other mountain species, the Pyrenean rock lizard survived glacial maxima through short-distance range shifts instead of migration or contraction in distant southern refugia. Most of the main Pyrenean range has apparently been re-colonized from a single or a few source populations, resulting in a loss of genetic diversity in re-colonized areas. As a result, current levels of intra-population mitochondrial diversity are better explained by post-glacial population history than by current habitat fragmentation. Genetic population differentiation within massifs implies severe reduction in female-mediated gene flow between patches of habitats.

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Keywords

Conservation genetics, Europe, genetic population structure, *Iberolacerta bonnali*, mitochondrial control region, phylogeography, Pleistocene glaciation, Pyrenees, reptiles.

INTRODUCTION

In Europe, many temperate species experienced considerable range expansion after the Last Glacial Maximum (LGM) about 23,000 to 18,000 years ago. During glacial periods, arctic tundra ecosystems covered much of northern and central Europe while parts of the high mountain systems of the south of Europe were glaciated (Webb & Bartlein, 1992; Dansgaard *et al.*, 1993; Roy *et al.*, 1996; Hewitt, 2004; Schmitt, 2007). In these periods, many populations adapted to temperate climates were forced to retreat to southern refugia, mainly situated in the peninsulas of Iberia, Italy and the Balkans and near the Caucasus and the Caspian Sea (e.g. Orth *et al.*, 2002; Hewitt, 2004; Schmitt, 2007), but also around the Pyrenees and the French Mediterranean Basin (e.g. Vogel *et al.*, 1999; Guillaume *et al.*, 2000; Kotlík & Berrebi, 2001; Palmé & Vendramin, 2002; Pfenninger & Posada, 2002; Schmitt, 2007) or in central Europe (Schmitt, 2007). As a result, Pleistocene climate changes have shaped the pattern of geographical distribution, demographic history and genetic diversity in many Holarctic species (Hewitt, 1999, 2004; Soltis *et al.*, 2006; Schmitt, 2007; Stewart *et al.*, 2010).

The impacts of Pleistocene range expansions on genetic diversity have largely been studied with regard to lowland northern and temperate species, but a series of papers have recently examined the effects of Pleistocene glaciations on mountain organisms. In Europe, most studies have concentrated on alpine plants and butterflies and suggest that many species persisted through the last Ice Age in nearby down-slope refugia rather than distant southern refugia (e.g. Kadereit *et al.*, 2004; Schönswetter *et al.*, 2005; Albach *et al.*, 2006; Bettin *et al.*, 2007; Ehrich *et al.*, 2007; Haubrich & Schmitt, 2007; Schmitt, 2007; Puşcaş *et al.*, 2008; Ronikier *et al.*, 2008; Schmitt & Haubrich, 2008; Stewart *et al.*, 2010). There seem to be fewer published studies dealing with the Quaternary history of Pyrenean mountain populations (but see Allegrucci *et al.*, 2005; Vila *et al.*, 2005; Puşcaş *et al.*, 2008; Schmitt *et al.*, 2006; Schmitt & Haubrich, 2008) and none for Pyrenean endemics, which makes it difficult to understand the origin of the high incidence of endemism in this region.

In this paper we use mitochondrial DNA (mtDNA) variation to elucidate the recent history of the Pyrenean rock lizard, *Iberolacerta bonnali* (Lantz, 1927), a Pyrenean endemic inhabiting rocky habitats (boulders and rocky slopes) at elevations between 1600 and 3300 m a.s.l. (Arribas, 2000). Mitochondrial DNA offers several advantages over other genetic markers to reconstruct the recent history of populations (Zink & Barrowclough, 2008; Barrowclough & Zink, 2009), and is thus still the favoured marker for many phylogeographic studies. However, a number of well-known confounding factors can weaken inferences concerning population history (e.g. Funk & Omland, 2003; Renoult *et al.*, 2009) and especially diversity (Galtier *et al.*, 2009) based on mtDNA data.

The genus *Iberolacerta* includes eight species restricted to the mountains of central and north-western Spain, the Pyrenees and the south-eastern Alps (Arribas *et al.*, 2006). Speciation within *Iberolacerta* has been dated to the end of the Tertiary (Carranza *et al.*, 2004; Crochet *et al.*, 2004) but how these species survived the climatic oscillations of the Quaternary remains unstudied. The current distribution of *I. bonnali* is fragmented at two spatial scales. Firstly, it inhabits several distinct, and sometimes disconnected, massifs (Arribas, 2000; Pottier, 2007; see Fig. 1). Secondly, within each massif, populations are highly fragmented due to the patchy occurrence of their rocky habitats, to intervening areas of low or high elevation unsuitable for the species, and to the fact that many, seemingly favourable, patches of habitats are apparently unoccupied (Arribas, 2000; authors' pers. obs.). These landscape features are expected to influence both the genetic differentiation between populations and the genetic diversity within populations by restricting gene flow among habitat patches.

Palaeoecology suggests that, during the LGM, large parts of the Pyrenean mountains were covered with ice (e.g. Bordonau *et al.*, 1992; García-Ruiz *et al.*, 2003; and map in Arribas, 2004, p. 56). The lower limit of permanent snow cover was about 1000 m lower than today, i.e. between approximately 1200 and 1600 m a.s.l. in the current range of *I. bonnali*, with the western half of the Pyrenees being subject to the harshest climatic conditions. In addition, glaciers occupied most of the valleys on the northern slopes, whereas the southern slopes were less affected by ice cover (Arribas, 2004). As a consequence, most of the current populations of *I. bonnali* are located in areas that were unsuitable for the species during the LGM. This is especially true for populations currently located in the central Pyrenean chain or in the western portion of the species' range. Therefore, this lizard constitutes a valuable model for understanding how Pleistocene climatic oscillations influenced the population history and current distribution of genetic diversity in a southern European mountain endemic vertebrate.

The first aim of this paper was to examine the impact of between- and within-massif habitat fragmentation on the genetic connectivity of the populations. Our goal was to determine at what geographic scale populations constitute management units (MUs *sensu* Moritz, 1994) and whether there are unrecognized basal units of biodiversity [such as

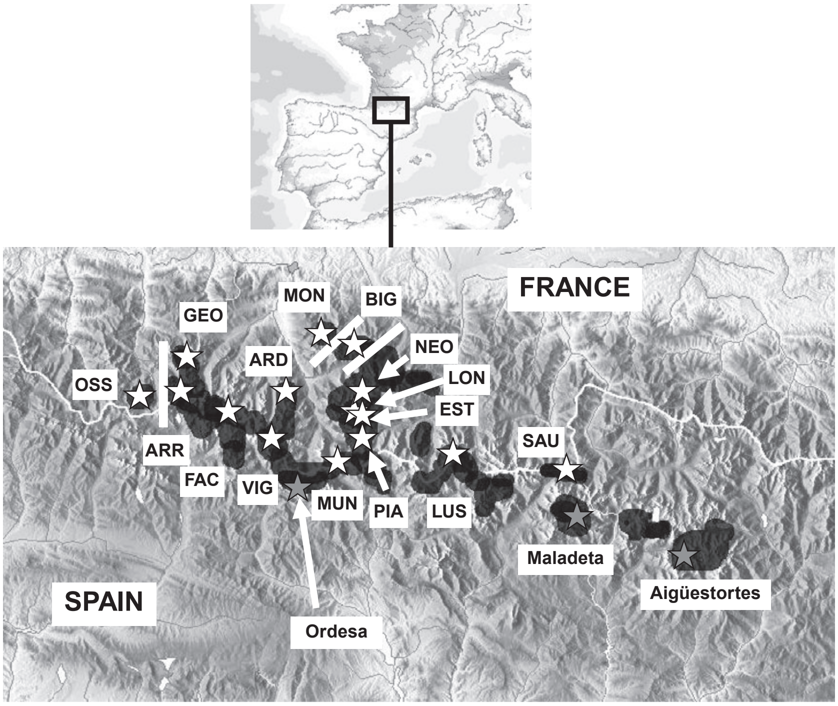

Figure 1 Approximate distribution range of the Pyrenean rock lizard (*Iberolacerta bonnali*) (based on published and unpublished data) and sampled localities (stars). Population names (see Table 1): OSS, Pic du Midi d'Ossau; ARR, Pic d'Arriel; GEO, Pic de Geougue d'Arre; FAC, Pic de la Fache; VIG, Pic de Vignemale; MUN, Pic de la Munia; SAU, Pic de Sauvegarde; LUS, Pic de Lustou; PIA, Piau Engaly; EST, Pic d'Estaragne; LON, Pic Long; NEO, Pic de Néouvielle; ARD, Pic d'Ardiden; BIG, Pic du Midi de Bigorre; MON, Pic de Montaigu. The thin white line is the French–Spanish border. Thick white bars indicate inferred range fragmentation (see Introduction). Grey stars are samples for which GenBank cytochrome *b* sequences only are available.

evolutionarily significant units (ESUs) *sensu* Moritz, 1994] within *I. bonnali*. Carranza *et al.* (2004) identified two lineages in the species, supported by mtDNA and nuclear DNA sequences. Unfortunately, the number of specimens they analysed was very low. Secondly, we wanted to separate the effects of current range fragmentation and history on the distribution of intra-population mitochondrial diversity in *I. bonnali*. If current levels of female gene flow are the main factor determining mitochondrial diversity, we would expect a lower diversity in isolated peripheral massifs. Finally, we wanted to know how *I. bonnali* survived the LGM by comparing the current pattern of genetic diversity with expectations based on the three possible scenarios detailed below.

1. The first scenario is a southward shift of the distribution during the LGM towards refugia located south of the current range (southern Pyrenean slopes or foothills). This latitudinal range shift corresponds to the general model of species persistence in southern refugia (see above) although the species' hardiness and affinity to rocky substratum make it unlikely that it moved a long distance from the Pyrenees. Under this latitudinal range shift scenario, we would expect mitochondrial diversity to decrease from the south to the north of the current range.
2. The second scenario involves short-distance movements to down-slope glacial refugia located on either side of the Pyrenees, as recently found in the Alps and the Pyrenees for several plant and Lepidoptera species (e.g. Schönswetter *et al.*, 2005; Schmitt *et al.*, 2006; Haubrich & Schmitt, 2007; Ronikier *et al.*, 2008; Varga & Schmitt, 2008; Schmitt, 2009). Under this elevational range shift scenario, we would expect mitochondrial diversity to decrease with distance from the foothills and population divergence to be posterior to the end of the LGM when lower-elevation habitat became unsuitable with climate warming (in the Pyrenees probably 15,000 to 10,000 years ago; Arribas, 2004).
3. Finally, the species might have survived in several *in situ* refugia within the central Pyrenean range in unglaciated habitats, as found for a few Alpine plants (Bettin *et al.*, 2007). Under this scenario, no prediction can be made about the spatial distribution of mitochondrial diversity but population divergence would have been initiated at the beginning of the LGM, earlier than 30,000 and probably around 50,000 years ago (García-Ruiz *et al.*, 2003; Arribas, 2004).

These three main scenarios are not mutually exclusive.

MATERIALS AND METHODS

Sample collection and population information

The known range of the species can be broadly separated into three zones: (1) the central Pyrenean chain, between the Ossau

Massif in the west and the Vénasque area in the east, on both sides of the chain but always close to the French–Spanish border; (2) the northern peripheral massifs, a series of massifs in France isolated from each other and the central Pyrenean chain; and (3) the Aigüestortes–Maladeta range, composed of two massifs (Aigüestortes and Maladeta) on the south (Spanish) side of the chain, which are connected by a high ridge but are separated from the central Pyrenean chain by deep valleys, and a pass where the species is not currently known but could conceivably occur (Fig. 1). Except for the Aigüestortes–Maladeta range, there are no populations outside the central Pyrenean chain on the southern slopes (Arribas, 2000; Pottier, 2007).

We collected tail tips from 146 individuals of *I. bonnali* in 15 localities of the central Pyrenean chain and the northern peripheral massifs (see Table 1) in the summer of 2000 and 2001. This sampling was supplemented by eight cytochrome *b* (cyt *b*) sequences downloaded from GenBank (see Carranza *et al.*, 2004, for accession numbers) representing populations Ordesa, Maladeta and Aigüestortes in Table 1. These eight cyt *b* sequences are our only samples for the Aigüestortes–Maladeta range. We sequenced a hypervariable control region segment (see below) in all the 146 individuals we sampled ourselves, and in some of them (see below) we also sequenced the same cyt *b* fragment that was sequenced by Carranza *et al.* (2004) to compare with sequences downloaded from GenBank. Adding the sequences retrieved from GenBank to our own sampling, we had genetic data from most of the current distribution range of *I. bonnali* (Table 1, Fig. 1). The locations of all populations are given in Table 1.

Among the 15 populations sequenced for the control region, nine localities (from ARR in the west to SAU in the east, central Pyrenean chain and its northern spurs: GEO and ARD) are located within the main continuous part of the species range and are connected by suitable habitats (see Table 1 and Fig. 1). Three localities (NEO, LON, EST) in the Néouvielle range are clearly set apart from the main Pyrenean chain but are also connected to the main species' range by suitable habitats. In contrast, the BIG population is only connected to the Néouvielle Massif by grassy slopes with little suitable rocky habitat where the species has not been found to date, and the OSS and MON populations are currently completely isolated by low-elevation, unsuitable habitats (authors' pers. obs.). These last three populations are thus considered as isolated throughout (see Table 1).

Marker selection

Preliminary sequencing of 17 individuals of *I. bonnali* with the two fragments used in Crochet *et al.* (2004) (approximately 1030 bp of cyt *b* and 450 bp of the control region) revealed a complete absence of variable sites in these two fragments. We

Table 1 Pyrenean rock lizard (*Iberolacerta bonnali*) samples. Sequences from the Aigüestortes–Maladeta range and the Ordesa population were obtained from GenBank and correspond to samples used in Carranza *et al.* (2004) (see Materials and Methods). Arrows indicate isolated populations.

Geographic area	Sites sampled	Sample size	Latitude/longitude	Elevation (m a.s.l.)
Central Pyrenean chain				
Massif du Pic d'Arriel (ARR)	Col d'Arrious	10	42°50′50″ N/0°20′42″ W	2350
Massif du Géougue d'Arre (GEO)	Vallon d'Anglas	10	42°55′46″ N/0°20′00″ W	2200
Massif de la Fache (FAC)	Pla de Loubosso	10	42°48′46″ N/0°12′54″ W	2100
Massif du Vignemale (VIG)	Oulettes d'Ossoue	10	42°45′43″ N/0°06′26″ W	1850
Massif de la Munia-Mont Perdu (MUN)	Vallon d'Estaubé	13	42°44′06″ N/0°02′44″ E	1750
Massif du Pic de Sauvegarde (SAU)	Boums de Venasque	11	42°41′58″ N/0°38′11″ E	2350
Massif du Pic de Lustou (LUS)	Vallon de La Pez	12	42°43′36″ N/0°22′44″ E	1780
Massif du Pic d'Ardiden (ARD)	Grand Lac d'Ardiden	6	42°50′47″ N/0°03′58″ W	2450
Piau Engaly (PIA)	Vallon de Badet	10	42°47′04″ N/0°08′16″ E	1850
⇒ Massif du Pic du Midi d'Ossau (OSS)	Col de Peyreget	10	42°50′10″ N/0°26′25″ W	2300
Ordesa (from Carranza *et al.*, 2004)	???	2	approx. 42°40′ N/0°01′ E	???
Northern peripheral massifs				
Massif du Pic Long-Pic d'Estaragne (EST)	Vallon d'Estaragne	10	42°48′18″ N/0°08′20″ E	2400
Massif du Pic Long-Pic d'Estaragne (LON)	Barrage Cap de Long	10	42°49′08″ N/0°08′21″ E	2200
Massif du Pic de Néouvielle (NEO)	Lac d'Aubert	10	42°50′19″ N/0°08′30″ E	2150
⇒ Massif du Pic du Midi de Bigorre (BIG)	Lac d'Aouda	4	42°55′46″ N/0°06′59″ E	2230
⇒ Massif du Pic de Montaigu (MON)	Col d'Era Osque	10	42°58′30″ N/0°03′14″ E	2000
Aigüestortes–Maladeta range				
Maladeta (=Possets, from Carranza *et al.*, 2004)	Estany de Llauset	3	42°35′00″ N/0°41′24″ E	2100
Aigüestortes (from Carranza *et al.*, 2004)	Port de Rus	3	42°29′15″ N/0°55′21″ E	2600
Outgroups				
Iberolacerta aranica (ARA)	Val d'Aran (Spain)	2	42°48′06″ N/0°50′47″ E	2250
Iberolacerta aurelioi (AUR)	Soulcem Valley (France)	6	42°36′59″ N/1°26′58″ E	2200

thus looked for intra-specific variation in other control region fragments (left and right domain) which are known to be highly variable in many vertebrate species. Using squamate sequences downloaded from GenBank, we designed primers in conserved regions that allowed us to amplify the tRNA-Thr and tRNA-Pro genes and more than 1100 bp of the control region in our species, leaving out around 400 bp at the end of the right domain (based on alignment with the complete *Teira dugesii* control region sequence, GenBank accession number AY147879). We sequenced this long fragment in 12 specimens (six yielding incomplete sequences) randomly chosen from five populations: the only variable sites identified were in the left domain of the control region corresponding to the hypervariable region described in *T. dugesii* (see Brehm *et al.*, 2003).

We thus designated primers BONF (5′-CTT-CAC-TTC-TGT-CTC-TTA-AAC-G-3′) and BONR (5′-CTG-AAT-GTA-AGA-TTA-TGG-AGG-G-3′) to amplify a fragment in the left domain between tRNA-Pro and the conserved central domain corresponding to positions 1126–1630 of the GenBank *T. dugesii* control region sequence. This fragment includes a variable number of 35 bp tandem repeats similar to the ones described in the extreme left domain of *T. dugesii* by Brehm *et al.* (2003). Its length thus varied between 465 and 570 bp in our individuals.

After alignment of the sequences of our 146 individuals, elimination of the tandem repeats that were not present in all individuals and elimination of the regions adjacent to the primers that were not available for all individuals we obtained a 400-bp data set that we used in all analyses. This fragment of 400 bp (called CR1 hereafter) thus includes all the variable sites identified in more than 2300 bp of cytochome *b*, two tRNA genes and a control region except for one site situated very close to the forward primer. Additional sequencing of the cyt *b* in specimens carrying different CR1 haplotypes confirmed that the variation observed in CR1 was not associated with sequence polymorphism in this gene.

DNA extraction, polymerase chain reaction and sequencing

Samples were stored individually in 95% ethanol at room temperature before processing. DNA was extracted by complete digestion of a minute piece of muscle in 400 μL of 5% Chelex 100 (Bio-Rad, Hercules, CA, USA) with 1 mg mL^{-1} Proteinase K followed by boiling for 25 min. Contamination was monitored by including two extraction blanks in every extraction round.

Polymerase chain reaction (PCR) amplifications were performed in a 50-μL reaction volume containing 5 μL DNA solution, 5 μL 10× buffer (TRIS-HCl 100 mm + KCl 500 mm), 3 μL MgCl_2, 2 μL deoxynucleotides (dNTP), 2 μL of each primer and 0.2 units *Taq* DNA polymerase. PCRs started with an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55.2 °C for 30 s and extension at 72 °C for 1 min. All reactions were finished with a final extension at 72 °C for 2 min.

The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Hilden, Germany). We sequenced only one strand of the purified DNA using the sequencing kit Big Dye (Applied Biosystems, Foster City, CA, USA) and the amplification primer BONR. All sequencing reactions consisted of 25 cycles of 30 s denaturation at 96 °C, 15 s annealing at 50 °C and 3 min elongation at 60 °C and were electrophoresed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) following recommended procedures. Sequences were edited, assembled and aligned using the software BioEdit v. 5.0.6 (Hall, 1999). Chromatographs were visually checked to confirm the presence of all point mutations found in a single individual and to resolve some ambiguities of the automated reading.

To assess the level of divergence between the populations from the central Pyrenean chain/northern peripheral massifs and the Maladeta–Aigüestortes populations, we used cyt *b* sequences of *I. bonnali* previously published in Crochet *et al.* (2004) (two specimens from the FAC population and two from the ARR population) and in Carranza *et al.* (2004) (two specimens from Ordesa, three from Maladeta and three from Aigüestortes), complemented by sequences obtained for this study (two from BIG and two from LUS populations).

Within-population analyses

The haplotype diversity ($h \pm \text{SD}$), nucleotide diversity ($\pi \pm \text{SD}$) and mean number of pairwise differences ($k \pm \text{SD}$) were estimated on mtDNA data using arlequin v. 2.0 (Schneider *et al.*, 2000). Based on the findings of Ramos-Onsins & Rozas (2002), we tested the hypothesis of demographic stability of the populations with the F_S statistics of Fu (1997) and the R_2 test of Ramos-Onsins & Rozas (2002). These tests were performed separately for each population that contained more than one haplotype with the program DnaSP v. 5 (Librado & Rozas, 2009). The significance of these tests was assessed by comparing the values of these test statistics with their empirical distribution generated from 1000 data sets simulated under a constant population size hypothesis with the 'coalescent simulations' module of DnaSP.

Analysis of population structure and gene flow

Population genetic structure was assessed with an analysis of molecular variance (AMOVA) using a Tamura and Nei genetic distance model to estimate Φ_{ST} values. The significance of Φ_{ST} was tested by permutation of haplotypes between populations (1000 permutations). We also estimated gene flow with the coalescence-based method of migrate v. 1.5.1. (Beerli, 2002), which releases several assumptions of the island model used to infer migration from *F*-statistics values such as Φ_{ST}. Results were not qualitatively different from the Φ_{ST}-values approach and will not be further discussed. Last, we also used Reynolds' genetic distance estimated from Φ_{ST}-values given by arlequin [$D = -\log(1 - \Phi_{ST})$] to generate a matrix of genetic distances among populations based on differentiation in haplotype frequencies and distance between haplotypes.

Phylogenetic analysis

To infer phylogenetic relationships between mtDNA control region or cyt *b* haplotypes we first used MEGA v. 5 (available at http://www.megasoftware.net/; see Kumar *et al.*, 2008) to determine the appropriate model of substitution. For both cyt *b* and the control region the selected model was a simple HKY model. We then ran maximum-likelihood (ML) analyses with the selected model and 1000 bootstrap replications in MEGA v. 5. All trees were rooted using homologous sequences of two closely related species (see Table 1).

As intra-specific genealogies are typically multifurcating and descendant genes coexist with their ancestor, genetic relationships among haplotypes can be better represented in the form of a network. Therefore, a minimum spanning network was also implemented in ARLEQUIN for the CR1 haplotypes, assigning equal weights to all variable sites and with default values for the epsilon parameter ($\varepsilon = 0$).

Dating past fragmentation

We used the program MDIV (Nielsen & Wakeley, 2001) to date isolation between populations and hence the timing of range fragmentation (T). This analysis was performed on the populations of the main Pyrenean clade (see Results) for which we have CR1 sequence data, excluding populations that were fixed for a single haplotype and population EST because we estimated a substantial level of gene flow with LON (see below). We also excluded population ARR that our results place in the part of the range which has been re-colonized after the range fragmentation. We thus retained 15 pairs of populations (all possible pairs among the following six populations: LUS, ARD, LON, NEO, BIG and MON, see Fig. 1).

We set the migration parameter M to zero because: (1) the assumption of no migration is the most reasonable for many population pairs given topography (see Introduction) and population structure results, and (2) we anticipated an increase in the reliability of the estimates of the parameter of interest, T. We checked if this assumption of no gene flow was reasonable on a limited number of population pairs and found that the distribution of the parameter M indeed often peaked at zero, then decreased regularly, which is indicative of no gene flow between populations.

For each pair of populations, we thus ran the program with the following settings: migration parameter M set to zero, 5,000,000 cycles for the Markov chain length, 500,000 cycles of burn-in. Values of $T_{\max}$ (the maximum value for T) were determined for each pair of populations separately by running the program three to four times with different random seeds to get an idea of the estimated distribution of T. $T_{\max}$ was then chosen to be close to, but higher than, the maximum values of T estimated by MDIV over these three to four preliminary runs.

The standard version of MDIV returns the posterior distributions of three parameters: $M = 2N_e m$ (m = migration rate); $T = t/(2N_e)$ and $\theta = 4N_e\mu$, where t is the divergence time in number of generations, N_e is the gene effective population size estimated by the posterior distribution of θ and μ is the rate of mutation per sequence per generation. As t needs to be computed as $T\theta/2\mu$, there is no straightforward way to get the confidence interval of t. We thus used a modified version of MDIV tailored for us by R. Nielsen, Departments of Integrative Biology and Statistics, University of California, Berkeley (USA), which also returns the posterior distribution of the product $T\theta$.

A calibration point for the divergence rate in this lizard or in closely related species is not available. To calculate divergence time t from the parameters estimated by MDIV, we first used cyt *b* and CR1 sequences of the three species *I. bonnali*, *Iberolacerta aranica* and *Iberolacerta aurelioi* (Crochet *et al.*, 2004) to determine that CR1 evolves 2.75 times faster than cyt *b* in these species. We then selected three values of cyt *b* divergence rate encompassing the realistic range of variation in these lizards: 1.5, 2 and 2.5% divergence per million years (Myr^{-1}), respectively (see Crochet *et al.*, 2004). We thus used three different values of μ derived from 4.125, 5.5 and 6.875% divergence Myr^{-1} for the CR1 fragment.

We used the same approach to date the divergence time of the Maladeta–Aigüestortes clade from the Pyrenean clade based on cyt *b* sequences. We used the same three values of cyt *b* divergence rate and the same parameter values as above.

Simultaneous vicariance

The hypothesis of simultaneous post-glacial vicariance of the peripheral massifs (LUS, ARD, LON, NEO, BIG, MON) was tested using a simulation approach. Two values of the supposed time of divergence between populations (t_{simult}) were used for the simulations. The first one was obtained from MDIV runs (see above): the average divergence resulting from the 15 estimates was taken as the time of supposed simultaneous divergence (t_{simult}). MDIV outputs were also used to obtain an estimate of the effective population size of each population (N_e), by averaging the estimates resulting from the five inferences in which each population is involved (scenario 'Gen' in Table 2). With the second value of t_{simult}, we explored the hypothesis that divergence occurred between the beginning and the end of the warming period at the end of the last glacial episode (i.e. between 32 and 11 ka, see above). Because the last glacial period apparently terminated *c.* 15 ka in the Pyrenees, this date was used as a fixed point for most simulations (scenario 'Biog' in Table 2).

We then simulated the divergence of our six populations t_{simult} generations ago with SIMCOAL v. 1.0 (Excoffier *et al.*, 2000), exploring a wide range of plausible conditions. In particular, we investigated the possibility of a large ancestral population during the last glacial episode (details of simulations in Table 2). We used the variance of π_{net}, $\text{var}(\pi_{\text{net}})$, measured from the matrix of the 15 pairwise measures of π_{net},

Table 2 Testing for simultaneous vicariance in the Pyrenean rock lizard (*Iberolacerta bonnali*).

Run	Scenario*	G	μ	t_{simult}	N_{cur}	N_{anc}	$Stat_{obs}$	$Stat_{sim}$	P-value
1	Gen	12	1	54.7	2524 (±520)	1	0.65	0.28 (0.00–1.02)	0.12
2	Gen	12	3	18.2	842 (±173)	1	0.65	0.36 (0.00–1.30)	0.19
3	Gen	8	2	27.3	1892 (±390)	1	0.65	0.65 (0.01–2.92)	0.37
4	Biog	12	2	11	250	10	0.65	0.72 (0.00–3.79)	0.38
5	Biog	11	2	11	250	10	0.65	0.80 (0.00–3.59)	0.34
6	Biog	12	2	32	250	10	0.65	1.40 (0.02–5.23)	0.60
7	Biog	11	2	32	250	10	0.65	0.95 (0.11–4.11)	0.46
8	Biog	12	2	15	100	10	0.65	0.46 (0.00–1.51)	0.29
9	Biog	11	2	15	100	10	0.65	0.40 (0.00–1.55)	0.14
10	Biog	12	2	15	1500	10	0.65	4.33 (0.13–28.70)	0.71
11	Biog	12	2	15	100	50	0.65	1.74 (0.00–7.37)	0.60
12	Biog	12	2	15	250	100	0.65	22.93 (0.94–116.50)	0.98

*Scenario: ‘Gen’ when parameters for simulations (one run = 100 simulations) were drawn from MDIV outputs, ‘Biog’ when parameters were chosen to capture our biogeographic hypothesis (see Materials and Methods).

G, generation time (in years); μ, cytochrome *b* mutation rate (% divergence Myr^{-1}); N_{anc}, relative size of the ancestral population as compared with current ones, N_{cur}; t_{simult}, time of simulated divergence (ka); $Stat_{obs}$ corresponds to the actual value of var(π_{net}) (the test statistic); $Stat_{sim}$ corresponds to the mean (95% confidence interval) of the test statistic under the null hypothesis of simultaneous divergence.

as a phylogeographic summary statistic for testing simultaneous vicariance (following Hickerson *et al.*, 2006), where $\pi_{net} = \pi_b - \pi_w$ is the net average pairwise differences between two populations, π_b is the average pairwise differences between these populations and π_w is the average pairwise differences within populations. Each run of SimCoal consisted of 100 simulations, which were analysed using ARLEQUIN v. 3.1 to obtain 100 matrices of π_{net}, from which the distribution of var(π_{net}) under the null hypothesis of simultaneous divergence could be inferred. The *P*-value of the test was calculated as the percentage of simulations that yielded a larger value of var(π_{net}) than the actual one.

The generation time (GT) of *I. bonnali* was estimated using the approximate formula $GT = \alpha + s/(1 - s)$, where α is the mean age at first reproduction (here of females) and *s* the adult annual survival rate (Lande *et al.*, 2003). Arribas & Galan (2005) found α to be 5 years for females of Pyrenean *Iberolacerta*. An ongoing capture–mark–recapture study of *I. bonnali* (M.C., unpublished data) found $s = 0.86$ in this species, resulting in a GT of approximately 11 years. We thus used a generation time varying between 8 and 12 years to account for uncertainties in demographic parameters.

RESULTS

Phylogenetic analyses: two ESUs

The 307 bp cyt *b* sequences (described in previous studies: Carranza *et al.*, 2004; Crochet *et al.*, 2004) fall into two distinct groups in the ML tree (see Fig. 2a). The first group is a monophyletic clade (bootstrap support 99) comprising the specimens from the central Pyrenean chain and the northern peripheral massifs. The second group includes the sequences from the isolated Spanish Maladeta–Aigüestortes massifs, which are all very similar but are not retrieved as monophyletic. All the specimens from our own sampling had the same cyt *b* sequence, irrespective of their CR1 haplotype. We can thus be confident that all our CR1 haplotypes belong to the main Pyrenean clade. Our mtDNA data thus complement the results of Carranza *et al.* (2004) and support the existence of two ESUs in *I. bonnali*, corresponding to populations inhabiting (1) the main Pyrenean chain and the northern massifs and (2) the somewhat isolated Spanish massifs of Maladeta and Aigüestortes. The Maladeta–Aigüestortes populations have not been extensively sampled and have not been sequenced for the hypervariable CR1 segment, so they are not the subject of further detailed analyses.

The 146 CR1 sequences of the main Pyrenean ESU formed a 400-nucleotide alignment with eight polymorphic sites (three transversions and five transitions) defining 10 different haplotypes (see Appendix S1 in the Supporting Information, GenBank accession numbers DQ207392–DQ207403). The ML tree (Fig. 2b) resulted in a poorly supported topology with no deep divergence. The minimum spanning network (Fig. 3) did not support the existence of any major phylogeographic partition either. All the haplotypes sampled were at a maximum of five mutations from all the other haplotypes and were one step away from their closest neighbour. Based on the geographic origin of the CR1 haplotypes, no clear geographic structure is apparent in the haplotype tree.

Population variability and population size changes in the Pyrenean ESU

The number of haplotypes per population ranged from one in seven populations to five in LUS (Table 3 and Appendix S1; Figs 2 & 4). Five haplotypes were restricted to a single population; the other five were shared among several populations (Table 3, Fig. 4 and Appendix S1). Haplotype diversity ranged from 0 to 0.83 (average $h = 0.54$), nucleotide diversity ranged from 0 to 0.006 (average $\pi = 0.004$) and pairwise

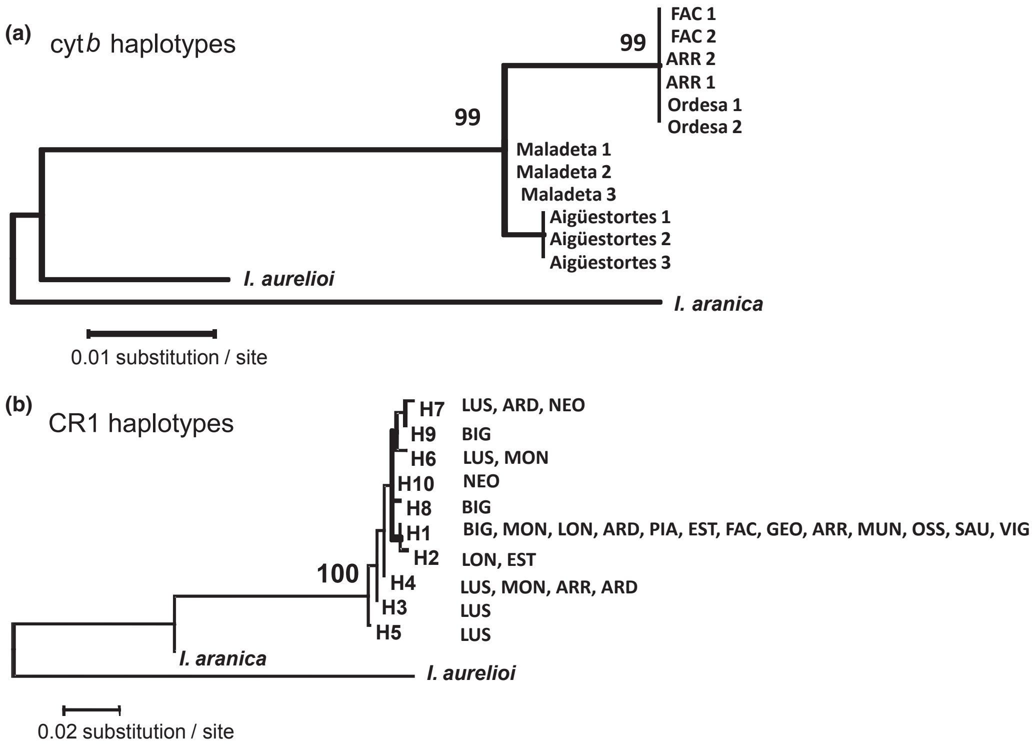

Figure 2 Maximum likelihood trees of (a) the cytochrome *b* haplotypes and (b) the control region (CR1) haplotypes of the Pyrenean rock lizard (*Iberolacerta bonnali*). Both trees are rooted with homologous sequences of *Iberolacerta aranica* and *Iberolacerta aurelioi*. Numbers above internodes indicate bootstrap values. Haplotype names and the populations where each haplotype was found are indicated at the tip of branches (legend as in Table 1).

divergence between haplotypes ranged from 0 to 2.5 (average $k = 1.48$).

Mitochondrial diversity estimates were especially low for the nine populations of the main Pyrenean range (the central chain) where only one haplotype was found (H1), except for one individual with haplotype H4 in ARR. These populations contain a reduced sample of the total mitochondrial diversity of the species, as both haplotypes are widespread in the other populations. The populations of the Néouvielle Massif, one population from the central chain (LUS) and one population from the northern spurs of the central chain (ARD) showed variable levels of haplotypic diversity ($0.20 < h < 0.67$). Two of the three isolated populations (MON, BIG) showed among the highest haplotypic diversity values ($0.51 < h < 0.83$), the highest values for nucleotide diversity and mean number of pairwise differences between haplotypes (Table 3). There was thus no general tendency for a lower diversity in the peripheral Néouvielle Massif (populations EST, LON and NEO) and in the isolated populations MON and BIG.

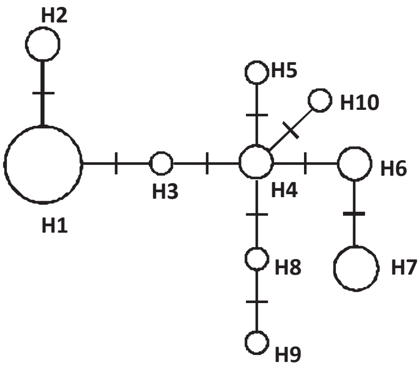

Figure 3 Median-joining network of Pyrenean rock lizard (*Iberolacerta bonnali*) haplotypes. The size of the node indicates the relative frequency of the corresponding haplotype in the whole data set. The vertical bar on the line connecting two haplotypes indicates that a single substitution separates them.

Values for Fu's F_S and Ramos-Onsins and Rozas' R_2 were all non-significant for the eight polymorphic populations ($P > 0.05$, Table 3), indicating no evidence of recent demographic expansions, although the power of these tests is limited given the small number of segregating sites and samples per population we used (see Ramos-Onsins & Rozas, 2002). These values cannot be computed for populations lacking DNA polymorphism.

Population structure and gene flow in the Pyrenean ESU

An AMOVA performed on 13 populations (populations BIG and ARD were excluded because of small sample sizes) showed that 80% of the total mtDNA genetic variance was distributed among populations ($\Phi_{ST} = 0.798$; $P < 0.0001$), demonstrating a strong genetic differentiation among populations except

Table 3 Population names, number of samples (n), control region haplotypes, haplotype diversity ($h \pm$ SD), nucleotide diversity ($\pi \pm$ SD), and values of Fu's F_S and Ramos-Onsins and Rozas' R_2 (none of them significant at the 5% level) for the Pyrenean rock lizard (*Iberolacerta bonnali*).

Populations	n	Haplotypes	h	π	R_2	F_S
Central Pyrenean chain						
Pic d'Arriel (ARR)	10	H1, H4	0.200 ± 0.154	0.001 ± 0.001	0.30	0.59
Géougue d'Arre (GEO)	10	H1	0.000	0.000		
Fache (FAC)	10	H1	0.000	0.000		
Vignemale (VIG)	10	H1	0.000	0.000		
Munia-Mont Perdu (MUN)	13	H1	0.000	0.000		
Pic de Sauvegarde (SAU)	11	H1	0.000	0.000		
Pic de Lustou (LUS)	12	H4, H6, H7, H3, H5	0.667 ± 0.141	0.005 ± 0.001	0.17	−0.45
Pic d'Ardiden (ARD)	6	H1, H4, H7	0.600 ± 0.215	0.004 ± 0.001	0.20	0.54
⇒ Pic du Midi d'Ossau (OSS)	10	H1	0.000	0.000		
Northern peripheral massifs						
Piau Engaly (PIA)	10	H1	0.000	0.000		
Pic d'Estaragne (EST)	10	H1, H2	0.533 ± 0.095	0.001 ± 0.000	0.28	1.03
Pic Long (LON)	10	H1, H2	0.533 ± 0.095	0.001 ± 0.000	0.28	1.03
Pic de Néouvielle (NEO)	10	H7, H10	0.200 ± 0.154	0.001 ± 0.001	0.30	0.59
⇒ Pic du Midi de Bigorre (BIG)	4	H1, H8, H9	0.833 ± 0.222	0.004 ± 0.001	0.20	−0.13
⇒ Pic de Montaigu (MON)	10	H1, H4, H6	0.511 ± 0.164	0.003 ± 0.001	0.17	0.52

among the populations that were fixed or nearly so for the haplotype H1 (Table 4). The similar haplotypic compositions of these eight populations cannot be explained by strong ongoing gene flow given the distance between these populations relative to the restricted dispersal of the species (see below), leaving a recent common origin as the most likely explanation.

In the remaining seven populations, haplotype H1 was present in only about half (EST + LON) or fewer (MON, BIG, ARD, LUS and NEO) of the specimens (Fig. 4). Taking into account the within-population genetic variability, the Φ_{ST} values (Table 4) were near the maximum possible, and indicated a (nearly) complete lack of gene flow among these seven populations, and between them and the main Pyrenean range (except the EST and LON populations; see Discussion).

In summary, two groups of populations can be distinguished on the basis of their haplotypic composition: (1) an undifferentiated group of populations from the main central Pyrenean range which are fixed or nearly fixed for the haplotype H1; (2) a group of populations with a higher level of within-population polymorphism (LON + EST, MON, BIG, ARD, LUS and NEO), which are all strongly differentiated from populations of the first group and from each other. Strong Φ_{ST} values among these populations and concordant results from MIGRATE (not shown) demonstrate that there is currently very little, if any, gene flow between

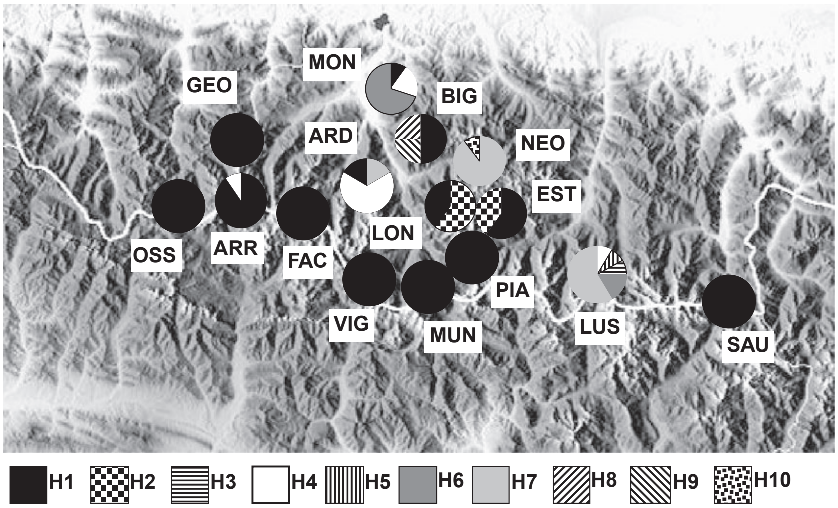

Figure 4 Frequency of each of the 10 haplotypes in the 15 Pyrenean rock lizard (*Iberolacerta bonnali*) populations sampled.

Table 4 Genetic differentiation between populations of the Pyrenean rock lizard (*Iberolacerta bonnali*) (13 populations with sample size ≥ 10). Pairwise Φ_{ST} values from control region (CR1) fragments are given below the diagonal and Reynold's distances between populations are given above the diagonal. See Table 1 for a definition of population codes.

Populations													
	OSS	ARR	GEO	FAC	VIG	MUN	SAU	LUS	PIA	EST	LON	NEO	MON
OSS	–	0.00	0.00	0.00	0.00	0.00	0.00	1.58	0.00	0.41	0.81	3.67	1.75
ARR	0.00	–	0.00	0.00	0.00	0.03	0.01	1.29	0.00	0.25	0.54	2.52	1.31
GEO	0.00	0.00	–	0.00	0.00	0.00	0.00	1.58	0.00	0.41	0.81	3.67	1.75
FAC	0.00	0.00	0.00	–	0.00	0.00	0.00	1.58	0.00	0.41	0.81	3.67	1.75
VIG	0.00	0.00	0.00	0.00	–	0.00	0.00	1.58	0.00	0.41	0.81	3.67	1.75
MUN	0.00	0.00	0.00	0.00	0.00	–	0.00	1.70	0.00	0.48	0.91	3.82	1.89
SAU	0.00	0.00	0.00	0.00	0.00	0.00	–	1.62	0.00	0.43	0.85	3.73	1.80
LUS	0.79*	0.73*	0.79*	0.79*	0.79*	0.79*	0.79*	–	1.58	1.39	1.45	0.16	0.21
PIA	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.79*	–	0.41	0.81	3.67	1.75
EST	0.33	0.22	0.33	0.33	0.33	0.33	0.33	0.75*	0.33	–	0.00	2.47	1.43
LON	0.56*	0.42*	0.56*	0.56*	0.56*	0.56*	0.56*	0.77*	0.56*	–0.03	–	2.51	1.49
NEO	0.97*	0.92*	0.97*	0.97*	0.97*	0.97*	0.97*	0.15*	0.97*	0.92*	0.92*	–	1.06
MON	0.83*	0.73*	0.83*	0.83*	0.83*	0.83*	0.83*	0.19*	0.83*	0.76*	0.78*	0.65*	–

*Significant values after correction for multiple tests.

these populations, even when they are in the same massif (NEO and LON + EST).

Population divergence time

The divergence time between the two cyt *b* clades was estimated using MDIV as *c.* 380–630 ka (Table 5), with a wide confidence interval which, however, does not overlap the time of the last glacial cycle. Divergence of the two ESUs corresponding to the Pyrenees and Maladeta–Aigüestortes massifs thus pre-dates the last glacial cycle.

Among the Pyrenean (and northern massif) populations, estimated divergence times varied substantially and had wide confidence intervals, as should be expected given the random nature of single-locus coalescence processes. Minimum divergence times were around 2.5–4 ka and maximum divergence times around 75–130 ka (depending on divergence rate, see Table 5). Based on the consensual rate of 2% divergence Myr^{-1}

Table 5 Divergence time estimates (in years, usually rounded to the nearest 1000 years) obtained with MDIV between pairs of populations of the Pyrenean rock lizard (*Iberolacerta bonnali*) based on control region (CR1) sequences (except for the Maladeta/Aigüestortes–Pyrenees pair, which was based on cytochrome *b* sequences). The three columns correspond to three values of cytochrome *b* divergence rate (see Materials and Methods). For each pair of populations and each value of mtDNA divergence rate, we provide the MDIV estimate in bold between the lower and upper bound of the 95% confidence interval. See Table 1 for a definition of population codes.

Pairs of populations	1.5% Myr^{-1}	2% Myr^{-1}	2.5% Myr^{-1}
LUS–ARD	2000–**9000**–48,000	1000–**7000**–36,000	1000–**5000**–29,000
LUS–LON	49,000–**128,000**–391,000	37,000–**96,000**–293,000	30,000–**76,800**–235,000
LUS–NEO	3000–**14,000**–73,000	2000–**10,000**–55,000	2000–**8000**–44,000
LUS–BIG	32,000–**92,000**–317,000	24,000–**69,000**–238,000	19,000–**55,000**–190,000
LUS–MON	5000–**14,000**–62,000	3000–**10,000**–45,000	3000–**8000**–38,000
ARD–LON	11,000–**35,000**–218,000	8000–**26,000**–164,000	6000–**21,000**–131,000
ARD–NEO	4000–**14,000**–67,000	3000–**10,000**–50,000	2000–**8000**–40,000
ARD–BIG	1000–**7000**–57,000	1000–**5000**–42,000	1000–**4000**–34,000
ARD–MON	1000–**4000**–33,000	1000–**3000**–24,000	500–**2000**–20,000
LON–NEO	27,000–**90,000**–304,000	20,000–**67,000**–228,000	16,000–**54,000**–183,000
LON–BIG	11,000–**37,000**–265,000	9000–**28,000**–199,000	7000–**22,000**–159,000
LON–MON	11,000–**31,000**–210,000	8000–**23,000**–157,000	7000–**19,000**–126,000
NEO–BIG	7000–**20,000**–95,000	6000–**15,000**–71,000	4000–**12,000**–57,000
NEO–MON	8000–**23,000**–95,000	6000–**17,000**–71,000	5000–**14,000**–57,000
BIG–MON	4000–**15,000**–86,000	3000–**11,000**–64,000	2000–9**000**–52,000
Mean inter-population divergence			
Pyrenees	**35,000**	**27,000**	**21,000**
Maladeta/Aigüestortes–Pyrenees	228,000–**632,000**–1,726,000	171,000–**474,000**–1,294,000	136,800–**379,000**–1,035,000

for cyt *b*, most divergences (9 out of 15) were thus dated after the end of the LGM.

Simultaneous vicariance

The null hypothesis of simultaneous divergence of the six peripheral populations could not be rejected in any of the runs (Table 2), as the actual value of var(π_{net}) was never significantly greater than the baseline level of variance expected by coalescence stochasticity alone (i.e. all *P*-values >0.10). This result does not appear to be sensitive to the choice of the mutation rate, generation time or current and ancestral population sizes.

DISCUSSION

Habitat fragmentation and genetic connectivity: ESUs and MUs

Carranza *et al.* (2004) identified two lineages in *I. bonnali*, strongly supported by mitochondrial and nuclear sequence data. To include their sequences and ours in the same dataset we had to restrict our analysis to a short (300 bp) segment of mtDNA. Although the support for the two lineages is weaker in our dataset (see Fig. 2), we increased considerably the geographical coverage of their sampling. It is now obvious that most of the species range is inhabited by a widespread northern ESU (represented in Carranza *et al.*, 2004, by only two individuals from a single locality) and that the southern ESU has a very restricted range. Since Maladeta and Aigüestortes populations (included in the southern ESU) are also somewhat differentiated from other Pyrenean populations in morphology (Arribas, 1993, 2008), formal nomenclatural action to name the southern ESU could be helpful in promote their conservation. Within the northern ESU (main Pyrenean chain and northern massifs) there is no well-supported clade among CR haplotypes and no geographically coherent group of haplotypes (Fig. 2).

Populations that exhibit mtDNA polymorphism are usually strongly differentiated genetically, even at a small geographic scale (Fig. 4, Table 4). The only exceptions are the two populations LON and EST, which are very close to each other (1.5 km, see Fig. 1) and, most importantly, occupy the same slope and are connected by rocky areas at suitable elevation (G.P. and M.C., pers. obs.). Their very similar haplotype frequencies suggest extensive gene flow between them (cf. Table 4). On the other hand, these two populations are separated from the NEO population (which is only 2 km away) by two geographical barriers: a low forested valley and a high-elevation ridge. The strong genetic differentiation between NEO and LON/EST populations (Fig. 3) indicates that these barriers strongly reduce gene flow. We found similar gene flow restriction (Table 4, Fig. 4) between populations PIA and LON, which are separated by less than 3 km but are situated in different valleys.

Genetic variation in the Pyrenean rock lizard is thus strongly structured geographically, even at small geographic scales, and gene flow seems to be strongly reduced by low-elevation valleys or high-elevation ridges. As long as current climatic conditions are maintained, natural re-colonization of extinct local populations across unfavourable habitats would thus seem to be an infrequent process. This probably explains the patchy distribution of the species in favourable areas, with many seemingly suitable patches of habitats apparently unoccupied. Local sets of populations of the species should thus probably be treated as independent MUs.

Distribution of mitochondrial genetic variability: no effect of isolation

Habitat fragmentation and isolation can reduce genetic variability by decreasing local effective population size and by restricting gene flow (Frankham *et al.*, 2002; Keyghobadi, 2007), an effect observed in wild populations of birds (Bouzat *et al.*, 1998a,b; Oyler-McCance *et al.*, 1999), mammals (Wauters *et al.*, 1994; Trizio *et al.*, 2005), fish (Laroche & Durand, 2004), reptiles (Madsen *et al.*, 1996) and insects (Krauss *et al.*, 2004; Schmitt *et al.*, 2005).

We thus anticipated that the isolated populations (MON, BIG and OSS) and the peripheral Néouvielle Massif would show a reduced haplotype diversity compared with the populations of the continuous main Pyrenean range. However, contrary to our predictions, two out of the three isolated populations (MON and BIG) and most of the Néouvielle peripheral populations had among the highest level of haplotype diversity (Table 3, Fig. 4). There is no indication that isolation has led to loss of mitochondrial diversity in the Pyrenean rock lizard. As loss of genetic diversity depends crucially on population size, and genetic variability is reduced significantly within isolated populations below some threshold value only (Johnson *et al.*, 2003), our results suggest that the isolated rock lizard populations have maintained effective female population sizes large enough to retain a significant part of the ancestral mitochondrial diversity.

Distribution of mitochondrial genetic variability: effects of Pleistocene glaciations

According to our estimates, the divergence of the two ESUs (Pyrenean and Maladeta-Aigüestortes) far pre-dates the LGM (see Table 5). This is corroborated by the estimates of even older divergence time than ours (1.2 Ma) by Carranza *et al.* (2004), partly based on other genes. Clearly, these two lineages persisted in different refugia during the LGM, one refugium north of the Pyrenees contributing to most of the current distribution of the species and one refugium south of the Pyrenees which did not expand much after the LGM. As we do not have access to the genetic variation in the Maladeta–Aigüestortes ESU, the following part of the text will only discuss the recent history of the northern lineage, which nevertheless embraces most of the species' range.

Palaeoclimatic data indicate that the species' distribution was more continuous during the colder periods of the Pleistocene, suggesting that the current fragmented distribution of the species is a consequence of post-glacial range fragmentation. This hypothesis is supported by our data: accounting for the variance in the coalescence process, we could not reject the hypothesis of simultaneous vicariance of the populations after the LGM (Table 2), and most divergence times were actually compatible with post-glacial fragmentation at the end of the LGM (Table 5).

Palaeoclimatic data also suggested that large parts of the current range of the species would have been unsuitable during the LGM. The spatial distribution of intra-population diversity (Table 3, Figs 1 & 4) supports this hypothesis: the populations of the north-central slopes and massifs have a large amount of polymorphism of mtDNA while the populations further west and east have no or very little polymorphism and carry a haplotype that is part of the diversity of the north-central populations. This pattern of reduced mitochondrial diversity is consistent with a strong bottleneck, as happens during range expansions (Hewitt, 1996; Le Corre & Kremer, 1998) and suggests that most of the main Pyrenean range has been recently re-colonized from a single glacial refugium.

Of the three possible scenarios for how the northern ESU of *I. bonnali* persisted during the LGM, we can exclude the 'southern refugia' hypothesis as there is no south–north gradient of mitochondrial diversity. It is more difficult to discriminate between the two remaining scenarios: refugia located at low elevation along the northern foothills or in unglaciated areas inside the current species' range. However, our data are compatible with a simultaneous vicariance of the current populations and the majority of estimated divergence times correspond to divergence after the LGM. In addition, the highest mitochondrial diversity is found in populations close to the foothills (ARD, LUS) or in peripheral massifs (EST, LON, NEO, BIG, MON) while most of the impoverished populations occupy either the central Pyrenean chain or the western end of the range, both areas that palaeoclimatic data identify as the least likely to have been occupied during the LGM (see Introduction). These arguments are more in line with persistence of the species in a nearly continuous range located around the northern-central part of the Pyrenean chain at lower elevation than today. While we favour this scenario of elevational range shift to the foothills, we acknowledge that our data do not allow the exclusion of more complex scenarios involving multiple refugia, including some in locally favourable microhabitats within the species' current range.

Can we confidently exclude a selective sweep?

Several issues can cloud the reconstruction of population history based on mitochondrial data. Firstly, the variance of the gene sorting process can generate discordance between gene trees and population trees (Degnan & Rosenberg, 2009). We took this phenomenon into account by comparing our observed data with a sample of data generated by explicitly simulating a coalescence process. Secondly, hybridization leading to mitochondrial introgression (e.g. Funk & Omland, 2003) could have affected our results. However, the recovery of two ESUs in *I. bonnali* is supported by the nuclear sequences reported in Carranza *et al.* (2004). The pattern of mtDNA variation in the northern Pyrenean ESU shows no sign of a divergent lineage that might suggest past introgression. In addition, we have sequenced mtDNA of the two congeneric taxa known in the Pyrenees, and detected no paraphyly or polyphyly in mtDNA lineages (as would be expected if introgression had happened in this group). We see no reason to suspect that our conclusions could have been affected by any of these phenomena. Thirdly, we acknowledge that the small number of variable sites in the CR1 data makes it impossible to recover accurately the phylogenetic relationships among the CR1 haplotypes. However, none of our conclusions rest on the phylogeny of the haplotypes, but on the geographic distribution of the haplotype diversity, which is not affected by the relationships among the haplotypes.

It is more difficult to exclude the possibility that the lack of mtDNA diversity in part of the species' range results from a selective sweep bringing haplotype H1 close to fixation in most populations from the central Pyrenean chain. After a long period when the neutrality of the mitochondrial DNA variation has been largely taken for granted, empirical evidence for the widespread consequences of selection on patterns of mtDNA diversity is now accumulating (reviewed in Galtier *et al.*, 2009), although it is not ubiquitous (e.g. McCusker & Bentzen, 2010). However, two lines of arguments add support to the neutral, historical explanation for the reduced polymorphisms in many central Pyrenean populations. Firstly, the haplotype H1 is also present in populations that show no sign of reduced mitochondrial diversity (see Fig. 4 and Appendix S1) but where ecological conditions are similar. Some of these diverse populations are not isolated from the populations where H1 is fixed. Therefore, we see no reason why if H1 had been fixed by a selective sweep in the central Pyrenean populations it would not have invaded more populations. Secondly, genetically impoverished populations are all situated either in the western part of the Pyrenees or close to the high central chain, precisely in those areas that have been more strongly affected by the LGM and where the Pyrenean rock lizard is extremely unlikely to have been able to maintain refugial populations. Historical explanations thus appear more parsimonious than selective explanations to explain the geographic pattern of mitochondrial diversity observed in this study.

CONCLUSIONS

The Pyrenean rock lizard persisted through the LGM in refugia located north and south of the Pyrenees, as recently documented in a growing number of alpine plants and insects whose glacial refugia have been identified at the periphery of the southern European mountains (reviewed in Schmitt, 2009). Pleistocene climate changes thus triggered short-distance, largely elevational range shifts rather than the usual latitudinal shifts of temperate species. This resulted in a counter-intuitive distribution of mitochondrial diversity: while small, isolated populations are usually expected to retain less genetic diversity than large or connected populations (Frankham *et al.*, 2002), genetic variability in our study has been influenced by recent history to the point where the currently isolated populations are among the most diverse genetically, probably because they are located closer to the glacial refugia. Therefore, mitochondrial diversity could be a poor indicator of global genetic diversity in our system, highlighting a need for a multilocus study of genetic connectivity.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Appendix S1 Variable positions in the mitochondrial DNA (mtDNA) control region segment and distribution of haplotypes in the 15 populations of the Pyrenean rock lizard (*Iberolacerta bonnali*).

BIOSKETCHES

This study is a joint project of the teams 'Biogeography and ecology of vertebrates' and 'Biometry and population biology' of the CEFE – UMR5175 lab. It is part of a series of studies focusing on the biogeography and conservation genetics of southern European reptiles.

Author contributions: P.-A.C. and M.C. designed the study; V.M. conducted most of the lab work and drafted the manuscript; P.-A.C., A.G. and A.-L.F. finished the writing; V.M., P.-A.C. and A.G. carried out the analysis; G.P. and M.C. performed the sampling.

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