Short Communication

Speciation in mountains: phylogeography and phylogeny of the rock lizards genus *Iberolacerta* (Reptilia: Lacertidae)

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1. Introduction

The vertebrate faunas in Southern Europe have a higher specific richness than in central and northern Europe and a high level of endemism (see, e.g., Baquero and Telleria, 2001; Meliadou and Troumbis, 1997), both probably related to the presence of faunal refugia during the Pleistocene glacial episodes (Hewitt, 1996, 1999; Taberlet et al., 1998). This scenario implies that the Pleistocene glacial oscillations are responsible for the current geographic distribution of species, but not that speciation events producing the contemporary species occurred at the same time. Indeed, genetic divergences between temperate vertebrate species imply that most diverged before the Pleistocene (Avise et al., 1998; Johns and Avise, 1998). Under this hypothesis, the high levels of species richness and endemism in Southern Europe would result from lower extinction rates in these areas during glacial maxima (refuge effect) combined with limited dispersal capabilities of non-avian vertebrates which prevented some of them to colonise Northern and Central Europe. A non-exclusive hypothesis relates the higher species richness in southern European peninsulas to the complex topography and history of these areas, their numerous mountains providing separated habitats where populations would be isolated following colonisation events (García-Barros et al., 2002).

The Iberian rock lizards of the genus *Iberolacerta* Arribas, 1997 constitute a promising model to evaluate these biogeographical scenarios, since they are restricted to mountains in the Western Mediterranean area (Fig. 1). The monophyly of this genus is well established (Arribas, 1999a, Fu, 2000; Harris et al., 1998), but the systematic status and evolutionary relationships of several taxa are still largely unsettled. According to Arribas (1996, 1999a, 2001) the genus includes six species (see Fig. 1). The systematic uncertainties concern the validity of the Pyrenean species (sometimes lumped under *Iberolacerta bonnali*), the specific status of *Iberolacerta cyreni* (traditionally treated as a subspecies of *Iberolacerta monticola*) and the validity of the subspecies within *I. cyreni* and *I. monticola* (see, e.g., Almeida et al., 2002; Arnold and Ovenden, 2002; Peréz-Mellado, 1998a, Pleguezuelos, 1997; Salvador and Pleguezuelos, 2002). This lack of consensus has important effects on establishing the conservation priorities for these taxa, especially since some of them have very restricted ranges and are prime candidates for regular monitoring.

In this paper, we use mitochondrial DNA sequences to investigate evolutionary relationships among *Iberolacerta* taxa. The first objective is to clarify the systematics of the genus *Iberolacerta* and help identifying meaningful conservation units. The second objective is to reconstruct the history of this genus and determine whether Pleistocene climatic oscillations were involved in creating the diversity in this genus or in allowing its persistence in southern Europe.

2. Materials and methods

Muscle samples or tail tips were obtained from 73 individuals (specimens in collection or live individuals)

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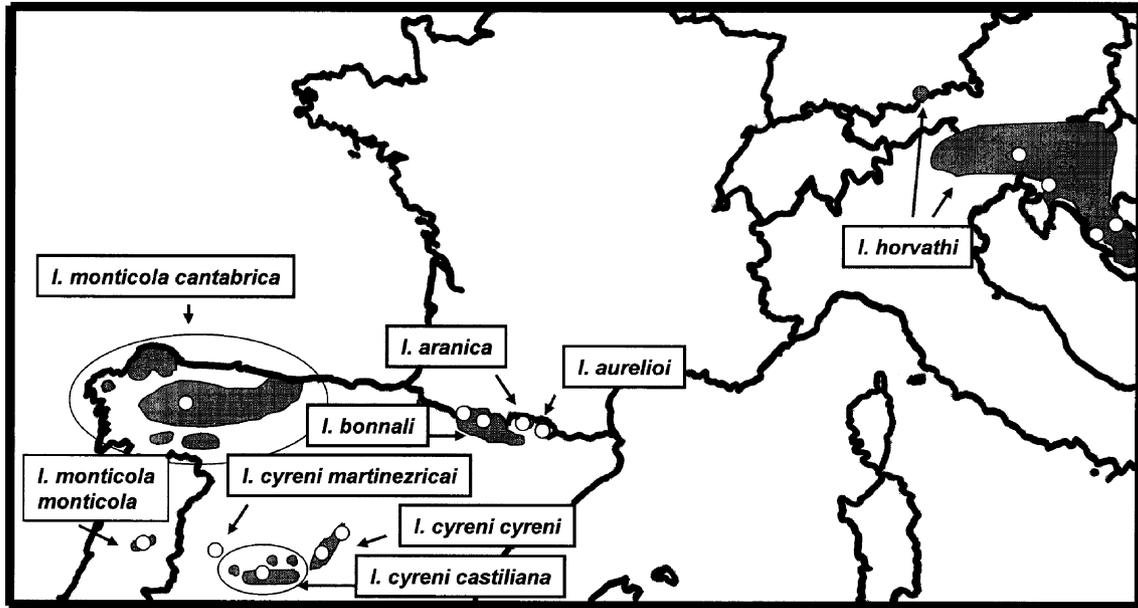
Fig. 1. Approximate range of *Iberolacerta* taxa (black areas) with sampling localities (white dots) according to the systematic hypotheses of Arribas (see Section 1). Distribution based on Arribas (1999b, 2000, 2001), Bischoff (1984), Gasc et al. (1997), Pleguezuelos (1997), and Tiedemann (1997). *I. cyreni cyreni* (Müller and Hellmich, 1937): Sierra de Guadarrama; *I. cyreni castiliana* (Arribas, 1996): Sierra de Gredos, de Béjar, de la Serrota, and de la Paramera; *I. cyreni martinezricai* (Arribas, 1996): Sierra de la Peña de Francia; *I. monticola monticola* (Boulenger, 1905): Serra da Estrela; *I. monticola cantabrica* (Mertens, 1929): North-West Spain; *I. bonnali* (Lantz, 1927): western and central Pyrenees; *I. aranica* (Arribas, 1993): central Pyrenees; *Iberolacerta aurelioi* (Arribas, 1994): eastern Pyrenees; *I. horvathi* (Méhely, 1904): south-eastern Alps and north-western Balkan mountains.

representing all known taxa of *Iberolacerta* (Table 1, Fig. 1, voucher details available on request). DNA was extracted either by a complete digestion of a minute piece of muscle in 400 μl of 5% Chelex 100 (Biorad, Hercules, USA) with 1 mg/ml proteinase K followed by a 10 min boiling (these extracts were diluted 50 times for use as PCR templates) or using Qiaamp tissue extraction kit (Qiagen, Santa Clarita, CA) following the supplier's procedure. Polymerase chain reaction (PCR) amplifications followed standard procedures. Sequencing reactions were conduced using the amplification primers with ABI dye terminator chemistry (Applied Biosystem, Forester City, CA, USA) following the standard ABI cycle sequencing protocol and were analysed on an ABI Prism 310 Genetic Analyzer following recommended procedures.

Genetic diversity within taxa and partition of genetic variation among taxa were investigated using a control region fragment which was sequenced for all specimens. This 460 bp segment (the CR segment) corresponds to part of the central and right domain (from position 1902 or 1903 to position 2337 of the *Teira dugesii* control region complete sequence, Accession No. AY147879). To increase resolution of the phylogenetic relationships among taxa, a 1033 bp fragment of the cytochrome *b* gene (the *cytb* segment) was sequenced from two representatives of most control region haplotypes (see Section 3). It starts at position 14,216 and ends at position 15,248 of the *Eumeces egregius* complete mitochondrial genome

(Genbank Accession No. AB016606). Primers for the CR segment were DL3F 5′-GGCCTCTGGTTAATGGGTT AGTTAC-3′ (1901) and DL4R 5′-AATTGTTGGTAGG GGGGTAGG-3′ (2339). Primers for the *cytb* segment were CTBF 5′-TCCTTTATTGACCTCCCAAC-3′ (14,200), CTBR 5′-CATTTGAGGAGTTTATTTTC-3′ (15,249), DL3R 5′-GTAACTAACCCATTAACCAGA GGCC-3′, the reverse of DL3F, CTBIF 5′-GAAACTGG CTCAAATAATCC-3′ (14,755), CTBIR 5′-GGGTGGA ATGGAATTTTATCT-3′ (14,777), CTBImonF 5′-CAC CTCCTTTTTCTCCACGAAA-3′ (14,739), CTBIhorF 5′-CACCTACTTTTCCTTCACGAAA-3′ (14,739), and the degenerated primer CTBImodF 5′- CACCTACTYT TTCTYCACGAAA-3′ (14,739). An F at this end of the primer name indicates forward primer, an R reverse primer, and numbers in parentheses correspond to the position of the 3′ end of the primer in the *T. dugesii* sequence for the CR primers or the *E. egregius* sequence for the *cytb* primers. For the *cytb* segment, the following primers combinations were used: *bonnali*, CTBF + CTBIR and CTBIF + CTBR, *aranica* and *aurelioi*, CTBF + CTBIR and CTBImodF + CTBR, *cyreni* and *castiliana*, CTBF + CTBIR and CTBIF + CTBR, *martinezricai*, *monticola*, and *cantabrica*, CTBF + CTBIR and CTBImonF + CTBR, *horvathi*, CTBF + CTBIR and CTBIhorF + DL3R.

We used as outgroups the Lacertidae species *Zootoca vivipara* (Accession No. U69834 for the cytochrome *b* and AF290402 for the control region) and *T. dugesii*

Table 1
Origin of specimens, sample size per locality for the CR haplotypes and the *cytb* haplotypes and mtDNA haplotypes found in each locality

Taxon-abbreviation	Locality	Sample size for the CR (sample size for the *cytb*)	name of CR haplotypes (number of specimens)
monticola MON	Upper part of the Vale glaciário do Zezere, Serra da Estrela, Portugal	7 (2)	monticola (7)
	Dam 8 km SSE Seia, Serra da Estrela	3	monticola (3)
cantabrica CAN	Piornedo de Ancarès, Sierra de Ancarès, Spain	2 (1)	cantabrica-1 (2)
	Refugio de Ancarès, Sierra de Ancarès	5 (3)	cantabrica-1 (2) cantabrica-2 (3)
	Portelo de Ancarès, Sierra de Ancarès	5	cantabrica-1 (1) cantabrica-2 (4)
castiliana CAS	12 km SW Hoyos del Espino, Sierra de Gredos, Spain	4 (2)	castiliana (4)
cyreni CYR	Puerto de Navafria, Sierra de Guadarrama, Spain	1 (1)	cyreni (1)
	Puerto de Navacerrada, Sierra de Guadarrama	7 (1)	cyreni (7)
martinezricai MAR	Summit of the Sierra de la Peña de Francia, Spain	1 (1)	martinezricai (1)
aranica ARA	4 km SSE Estany de Liat, Val d'Aran, Spain	9 (2)	aranica (9)
	Estany de Liat	2	aranica (2)
aurelioi AUR	Soulcem valley, Ariège, France	6 (2)	aurelioi (6)
	Port de Rat, Andorra/France	1	aurelioi (1)
bonnali BON	Pla de Loubosso, Massif de la Fache, Hautes-Pyrénées, France	2 (2)	bonnali (2)
	Col d'Arrious, Massif du Pic d'Arriel, Pyrénées Atlantiques., France	3 (2)	bonnali (3)
	Vallon d'Estaragne, Massif du Pic Long – Néouvielle, Hautes-Pyrénées, France	4	bonnali (4)
	Vallon d'Anglas, Massif du Géougue d'Arre, Pyrénées Atlantiques., France.	1	bonnali (1)
horvathi HOR	Velebit Mountains, 6 km after Stirovaca toward Krasno Polje, Croatia.	3 (1)	horvathi-2 (3)
	Porto del Canson de Lanza, near Tarvisio, Italy	2 (2)	horvathi-1 (2)
	Plitvice lakes, Croatia	2	horvathi-1 (2)
	Rakov Skocjan, NE Postojna, Slovenia.	2	horvathi-1 (2)

(Accession Nos. Z48037 and AY147879 for the cytochrome *b* and AY147879 for the control region). Distances between haplotypes or between groups were computed in MEGA (Kumar et al., 2001) using a Kimura 2-parameter model with gamma distribution of the substitution rates, using the shape parameter obtained from PAUP* as explained below. Prior to phylogenetic analyses, we assessed congruence in phylogenetic signal between the *cytb* and CR segments with a partition-homogeneity test (Farris et al., 1994) in PAUP* version 4.0b8 (Swofford, 1998). The following options were used: parsimony-informative characters only, all characters had equal weight, 10,000 replicates, with one random addition sequence per replicate. Tree searches were conducted with maximum parsimony (MP) and maximum likelihood (ML) methods. MP searches were made with MEGA using the Max Mini Branch and Bound option with equal weight for all changes. Node support was evaluated by bootstrap with 1000 replications. For the ML tree, we first used the program MODELTEST version 3.0 (Posada and Crandall, 1998) to select the best model of DNA evolution. The selected model was a General Time Reversible model (GTR model, Rodríguez et al., 1990) with a gamma distribution of the substitution rates and a proportion of invariable sites. The ML tree was obtained with PAUP* using a loop approach as in Delsuc et al. (2002). Node support was evaluated by bootstrap (500 replications), repeating this tree searching procedure on each bootstrapped data set. Alternative phylogenetic hypothesis were compared with the Shimodaira and Hasegawa (1999) test as implemented in PAUP*. Because no calibration point was available in this genus, estimates of divergence times were obtained by applying a range of absolute substitution rates for reptilian cytochrome *b* (from Brown et al., 2000; Carranza et al., 2002; Paulo et al., 2001). Based on these values, cytochrome *b* sequences of *Iberolacerta* might evolve at a rate comprised between 1.5 and 2.5% divergence per million years (Myr). Estimations of divergence time

were thus computed as: (i) a low value obtained by using the fastest rate (in combination with the lowest genetic distance between groups for poorly resolved nodes), (ii) an average value obtained by using the average rate of 2% per Myr (in combination with the mean between groups genetic distance based on the ML topology for poorly resolved nodes), and (iii) an upper value obtained by using the slowest rate (in combination with the highest genetic distance between groups for poorly resolved nodes). Distances were obtained from MEGA as explained above, using the cytochrome b sequences only.

3. Results and discussion

Sequences are deposited in GenBank (Accession Nos. AY267232–267242 for the cytb and AY267243–267253 for the CR segments). In most taxa, all specimens had the same CR haplotypes. In *horvathi* and *cantabrica*, two haplotypes were found (Table 1 and Fig. 2A). The cytb segment was sequenced in two representatives of each CR haplotype except "martinezricai" (only one specimen available), "monticola" (only one specimen gave good results for the complete cytb segment) and "horvathi-2" (only 560 bp available corresponding to CTBF + CTBIR). These cytochrome b sequences revealed additional differences between haplotypes but no new haplotype. Using both CR and cytb sequences, different haplotypes found in the same taxon always formed monophyletic clades (Fig. 2B, results not shown for "horvathi-1" and "horvathi-2" because of partial sequences). The partition-homogeneity test indicated that cytb and CR segments had congruent phylogenetic signal (ILD, $p = 0.19$) and they were thus combined for subsequent phylogenetic analyses. After alignment of our sequences with the *Z. vivipara* and *T. dugesii* sequences and suppression of the sites with indels, a composite alignment of 1454 sites (1033 for the cytb and 421 for the CR segments) was available in every taxon.

Both methods of tree construction identified the same four main clades strongly supported by high bootstrap values (Fig. 2B). The first clade (Pyrenean clade) includes *bonnali*, *aranica*, and *aurelioi*, the second clade includes the closely related *cyreni* and *castiliana*, the third includes the closely related *cantabrica* and *monticola* together with the more distantly related *martinezricai*, and the fourth clade is made of *horvathi* alone. The *cyreni–castiliana* clade is identified as the closest relative of the *martinezricai–monticola* clade in the ML tree but with a low bootstrap score (Fig. 2B), while the MP tree recovers a closer relationship of the *cyreni–castiliana* clade with *horvathi* and the Pyrenean clade (results not shown). Given the discrepancies between the various methods and the lack of strong support under MP or ML methods, the relationships of the *cyreni–castiliana* clade are better considered as unresolved.
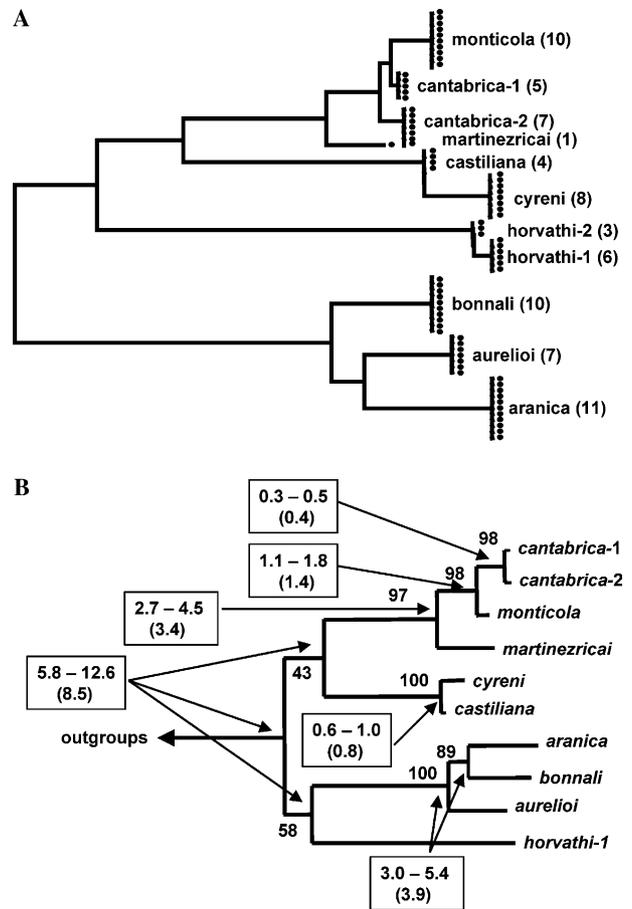

Fig. 2. (A) Phylogenetic tree of all 72 specimens of *Iberolacerta* (figured as a black dot) based on the CR segment only, with haplotype name and number of specimens for each haplotype (in parentheses). The tree is a neighbor-joining tree based on Kimura 2-parameter distance with gamma correction and "complete deletion" option obtained from MEGA. (B) Phylogenetic tree obtained by the ML method based on a composite alignment of control region and cytochrome b sequences. Numbers at nodes represent bootstrap values for these nodes. All haplotypes identified are shown on the tree except "horvathi-2" for which we had a partial sequence only. Numbers in boxes are low and high estimates of divergence time (in million years), with the average value given in brackets. Estimates for the divergence time of "horvathi-1" and "horvathi-2" based on partial sequences are 0.4–0.7 (0.5).

The position of *horvathi* is the same in all trees but bootstrap scores are always below 75 and this position is also considered as uncertain. The three undisputed species *Iberolacerta horvathi*, *I. bonnali*, and *I. monticola* thus fall within three of the four basal lineages of *Iberolacerta*. The *cyreni–castiliana* clade constitutes the fourth basal lineage and may not even form a monophyletic clade with *I. monticola*, which confirms that it should be treated as a distinct species *I. cyreni* (see Arribas, 1996).

Although they are supported by a high bootstrap score (>80), the relationships among Pyrenean taxa suggested by ML methods (Fig. 2B) are not recovered by MP methods (results not shown) and are thus considered as tentative. The three Pyrenean taxa have distinct karyotypes (Odierna et al., 1996), allozymes

(Mayer and Arribas, 1996), morphology (Arribas, 1999b, 2000, 2001), and have reciprocally monophyletic mitochondrial DNA haplotypes (this study), indicating that they are now genetically fully isolated. Their amount of divergence for the *cytb* segment (7.4–8.2%) indicates that this isolation has been maintained during several million years. Since only 20 km without unsuitable habitats separate the known range of these taxa (from Arribas, 1999b, 2000, 2001) and given the evidence of range extension in *Iberolacerta* during the cold periods of the Quaternary (see below), they have probably been in contact repeatedly in the past. Their genetic isolation has thus certainly been maintained by some mechanisms of reproductive isolation. Indeed, Arribas (2001) hypothesised that the differences in hemipenis size between *aurelioi* and *aranica* could act as a prezygotic isolation mechanism, while karyological differences are potential factors of post-zygotic isolation. The amount of divergence between these three taxa combined with the complete reciprocal monophyly for different types of markers in spite of their very close geographic proximity indicate that they are best regarded as three valid biological species.

The main discrepancy between our mtDNA phylogeny and previous hypotheses on the evolution of *Iberolacerta* concern the position of *martinezricai*. The Shimodaira and Hasegawa test comparing our hypothesis (*martinezricai* as a sister taxon of the *monticola–cantabrica* clade) and the traditional hypothesis (*martinezricai* as the sister taxon of the *cyreni–castiliana* clade) indicates that the traditional position of *martinezricai* is significantly rejected by our data ($-\ln$ [likelihood of the unconstrained tree] $= 6257.36$; $-\ln$ [likelihood of the constrained tree] $= 6306.07$; $p < 0.001$). We have only one sample of *martinezricai*, but the small size of the remaining population (Arribas, 1999c) and the lack of extensive polymorphism in other taxa suggest that it can safely be regarded as representative of this population. This taxon being on present knowledge fully allopatric from other *Iberolacerta*, the only available information to assess its taxonomic status is its amount of genetic divergence. The sequence divergences of its *cytb* segment compared to its closest relatives *monticola* and *cantabrica* (6.0–7.5%) are just lower than the amount of divergence among the Pyrenean species and much higher than the highest intraspecific divergence in *Iberolacerta* (1.6% between *cyreni* and *castiliana*). Genetic divergence alone is not a very good indicator of systematic status, but the similar level of divergence between *martinezricai* and its closest relatives on one hand, and between the three biological species in the Pyrenees on the other hand, suggests that the most consistent treatment is to recognise *martinezricai* as another valid species of *Iberolacerta*, although further research is clearly needed to confirm this result.

Based on well-supported nodes only, broad estimates of the timing of the differentiation events are given in Fig. 2B. The most parsimonious scenario for the evolution of *Iberolacerta* includes an initial phase of geographical expansion followed by fragmentation events leading to the isolation of the four main clades in the north-west Iberian mountains, in the Central Iberian mountains, in the Pyrenees and in the eastern Alps. Further divergence evolved within these four regions during subsequent isolation events. Based on the fact that the Iberian and Pyrenean mountains still harbour the most divergent clades, the genus probably originated from Iberia, the ancestor of *horvathi* reaching the Eastern Alps from the Pyrenees, as suggested by our mitochondrial DNA data. The monophyly of the Pyrenean species argues for their evolution within the Pyrenees, possibly promoted by a recent phase of intense orogenic activity resulting in a 1000 m raise of the Pyrenees during the last 6 my (J. P. Aguilar, pers. com.) which might have fragmented the range of the ancestor of the Pyrenean taxa. Which events lead to the isolation of the areas where the four main *Iberolacerta* clades diverged? Their current distribution is mainly determined by the presence of mountain ranges that formed before the Oligo-Miocene more than 22 million years ago (mya) (Autran and Dercourt, 1980), thus predating the initial divergence within *Iberolacerta* by at least 10 Myr (see Fig. 2B). It is possible that this initial fragmentation originates from the spread of the wall lizard of the genus *Podarcis*, isolating the ancestors of modern *Iberolacerta* in widely separated mountain ranges. Oliverio et al. (2000) estimated that the main diversification of *Podarcis* occurred between 16 and 10 mya, based on 0.5% divergence per Myr for 12S rDNA. This could fit our suggested scenario of *Iberolacerta* evolution, especially considering that their mtDNA evolution rate is at the low end of the range suggested for vertebrates. Arguments for competition between *Iberolacerta* and *Podarcis* can be found in the currently fragmented distribution of *Iberolacerta* species, which is included within the range of one or several species of *Podarcis*, with an altitudinal or ecological segregation between them (Arribas, 1999b, 2000, 2001; Bischoff, 1984; Pleguezuelos, 1997). Peréz-Mellado (1998b) suggested that one of the factors responsible for the fragmented distribution of *I. monticola* is ecological competition with *Podarcis*. Under this scenario, cold periods within the Pleistocene would have allowed the *Iberolacerta* taxa to outcompete *Podarcis* species over large areas of low-altitudes rocky habitats, connecting the isolated areas that many species inhabit today. Evidence for these recent connexions can be found in the genetic uniformity of our *horvathi* or *bonnali* specimens originating from currently isolated distant populations or in relict low-altitude populations of *monticola* in coastal North-Western Spain (Galán, 1982, 1999). During the warm interglacials, as is the case

now, various *Podarcis* species would exclude the *Ibero-lacerta* from these low altitude areas. Previous hypotheses on the evolution of *Iberolacerta* have postulated that the initial split between an eastern group (ancestor of *horvathi*) and a western group (ancestor of other species) occurred at the Plio-Pleistocene limit (around 1.6 mya), with further speciation events generated by climatic oscillations during the Quaternary (Arribas, 1999a, 2001). These scenarios of Quaternary speciation do not fit observed amount of genetic divergence between *Iberolacerta* species. As it has been previously shown for other vertebrates (Avise and Walker, 1998; Avise et al., 1998; Klicka and Zink, 1997), species diversity originates instead from older events, with Pleistocene divergence accounting for intraspecific phylogeographic units.

In conclusion, our results confirm the systematic hypotheses of Arribas (1996, 1999a, 2001) except for *martinezricai*, which is not a subspecies of *I. cyreni* as he proposed but a well-differentiated taxon, possibly a valid species, related to *I. monticola*. Considering the controversies surrounding the systematic of *Iberolacerta*, our results represent a valuable contribution to their conservation. For example, the conservation chapter about *I. monticola* in Peréz-Mellado (1998b) does not mention the existence of the isolated Peña de Francia population described as *martinezricai* by Arribas in 1996 since its validity and evolutionary distinctiveness was not widely recognised then, although *martinezricai* is one of the European reptile taxa in most urgent need for conservation. It is presently known from one mountain top only where the total population size is tentatively estimated at less than 50 individuals (Arribas, 1999c). Additionally, our results indicate that the diversity of the genus *Iberolacerta* originated well before the Pleistocene glacial oscillations and was promoted by the complex topography of Southern Europe. Mountains provided isolated habitats with limited possibilities of dispersal between them, possibly because of competition by species more adapted to lowlands, leading to the diversification of the genus by allopatric divergence during the end of the Tertiary. The current species were preserved through the climatic oscillations of the Quaternary thanks to the existence of glacial refugia within these southern European mountains.

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