THE ROYAL SOCIETY

doi 10.1098/rspb.2001.1706

The persistence of Pliocene populations through the Pleistocene climatic cycles: evidence from the phylogeography of an Iberian lizard

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Ancient climatic fluctuations have caused changes in the demography and distribution of many species. The genetic differentiation between populations of the same species and of sister species is often attributed largely to the more recent Pleistocene fluctuations. Recent interpretations, which implicate earlier episodes, have proved controversial. We address the timing of genetic divergence in the Iberian lizard *Lacerta schreiberi* by studying the phylogeography of the cytochrome *b* sequence. The species has a remarkable morphological uniformity, yet our evidence suggests that earlier events in the Pliocene initiated the main divergence between populations. This interpretation implies that the different populations survived through the Pleistocene in separate localities. This conclusion is robust to different molecular clock calibrations. The persistence of earlier differentiation through the Pleistocene has wide implications for our understanding of Pleistocene refugia in this species and, by extension, to the biogeography of the whole region.

Keywords: phylogeography; range expansion; mitochondrial DNA; Pleistocene glaciations; glacial refugia

1. INTRODUCTION

The Northern Hemisphere started to be affected by ice sheets *ca*. 2.5 million years (Myr) ago, but major climatic oscillations with a period of 100 000 years only started *ca*. 0.7 Myr ago (Webb & Bartlein 1992). This pattern generated the sequence of glacial and interglacial periods and evidence from numerous species suggests that southern parts of Europe served as glacial refugia. Particularly implicated are the peninsulas of Iberia, Italy and the Balkans and possibly areas near the Caucasus and Caspian Sea (Hewitt 2000). Phylogenetic studies of many North European species suggest that the Iberian Peninsula provided the major western refuge from which large areas were recolonized (Hewitt 1996).

Even at the height of the last ice age, one of the more intense glaciations, which lasted until 18 000 years before the present (BP), the Iberian Peninsula maintained a comparatively high diversity of habitats, some with temperate characteristics (Huntley & Birks 1983; Zagwijn 1992), allowing species to find refugia and persist throughout the climatic cycles. The study of genetic variation within species currently inhabiting the region should provide insights into the recent history of post-glacial expansion and the location of refugia. If the rate of genetic divergence between haplotypes can be calibrated, it may be possible to identify species with a 'shallow divergence' history where the genetic signal of any early events has been substantially erased during the range changes of the latest Pleistocene glaciations. Other species will show deeper divergence containing more divergent haplotypes, which have persisted from more ancient periods. This persistence through the Pleistocene era may have been due to larger population sizes through the intervening climatic fluctuations or to population subdivision or to chance.

These considerations can be seen in interpretations of avian evolution (Klicka & Zink 1997; Avise & Walker 1998) and vertebrates more generally (Avise *et al*. 1998). Klicka & Zink (1997) argued for a deep history of speciation in North American songbirds, questioning hypotheses suggesting Late Pleistocene origin. However, there may be an important role for Pleistocene conditions in initiating or modifying pre-existing variety, at least for mammals and birds. Avise *et al*. (1998) also conclude that, for poikilotherms, the interpretation is strongly dependent on the calibration of the molecular clock, as there are greater uncertainties about evolutionary rates in these taxa.

In this paper we consider the origin and phylogeography of an Iberian lizard species. We calibrate the molecular clock using a range of related species and address the depth of history revealed in the cytochrome *b* phylogeny. We will draw inferences about the events during the Pleistocene era, which, we believe, will have shaped the evolution of a wide range of other species in addition to this lizard.

(a) *The study species*

Schreiber's green lizard (*Lacerta schreiberi*) is an Iberian Peninsula endemic with a distribution on the northwest and central mountain systems and some isolated populations in central and southern Spain and Portugal (figure 1). Compared with other Iberian lizards, *L. schreiberi* has a

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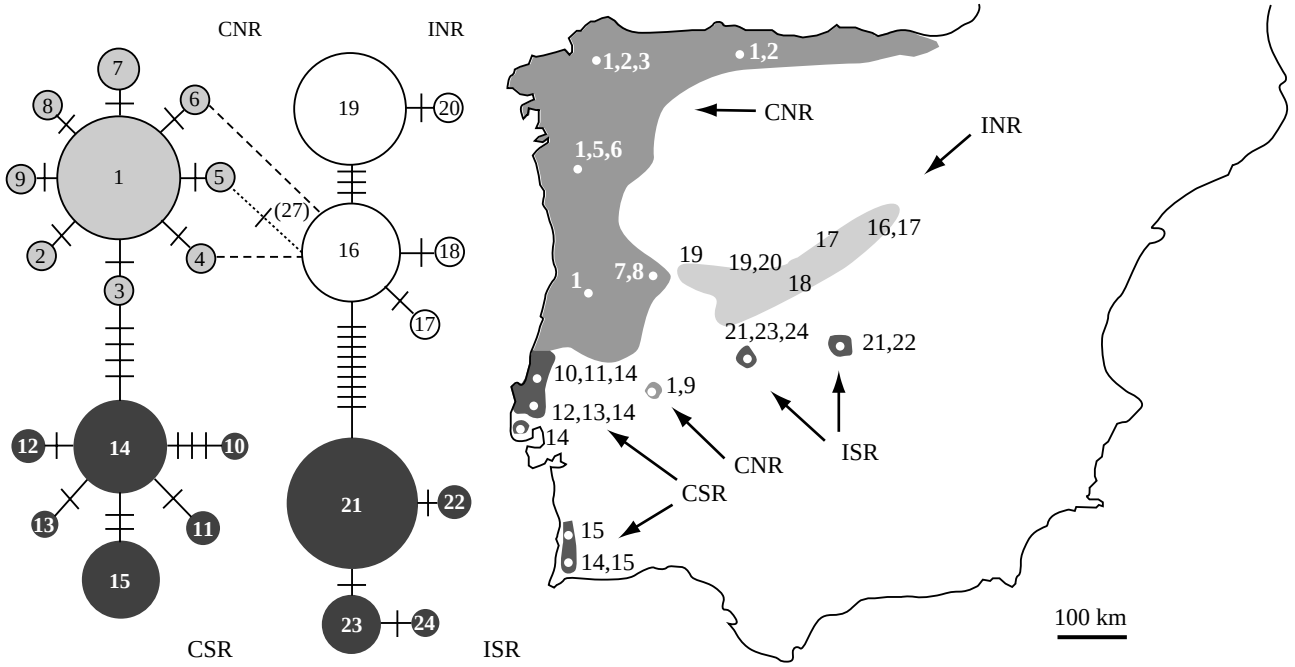

Figure 1. Map of the Iberian Peninsula with the distribution of the four clades and minimum spanning network of the 24 haplotypes detected. CNR, coastal northern region; CSR, coastal southern region; INR, inland northern region; ISR, inland southern region. The map and network have the same colour and numbering schemes for the haplotypes. White dots on the map are sample points. The areas of the circles in the network are proportional to haplotype frequency (range 1–13). Bars on the connecting lines between haplotypes indicate the minimum number of substitutions, with the exception of the connection between the coastal and inland regions where the number is given (27). Dashed lines represent alternative links between these two main clades.

preference for habitats with lower annual sunlight hours and higher precipitation, which results in a narrow ecological range. The species is restricted to river corridors in southern and central isolates, usually in mountains. The species has a less restricted, continuous distribution in deciduous forest or humid mountain habitats, which are predominantly in the northwest of its range (Brito *et al*. 1999*a*).

Several authors have tried to date and place *L. schreiberi*'s origin and the post-glacial colonization routes that would explain its present distribution. The consensus of opinion is that the species originated in northwestern Iberia by allopatric speciation from a common ancestor with *Lacerta viridis*. This separation could be explained by the Late Pleistocene glaciations, in particular the last, the Wurm, which probably isolated the ancestral population from the other European populations. The species could have colonized the eastern mountain systems from the northwestern glacial refuge and also the central and southern coastal areas of Portugal (Marco 1994). The current isolated populations in central and southern mountain areas are therefore thought to be residual populations from an ancient larger and connected distribution.

Comparisons with three closely-related central and southern European species, *L. viridis*, *Lacerta agilis* and *Lacerta trilineata*, provide an indication of the period during which ancestral *L. schreiberi* has persisted in the region. Lutz & Mayer (1985) used micro-complement fixation, a technique based on the immunological properties of albumin antisera, to estimate a divergence time of 3–4 Myr between *L. viridis* and *L. trilineata* and 6–7 Myr between *L. agilis* and *L. viridis*. Since *L. schreiberi* is morphologically more similar to *L. viridis* than to *L. agilis*, a plausible time of origin for *L. schreiberi* is around 5 Myr ago, with a range of between 4 and 10 Myr ago (Barbadillo *et al*. 1997).

2. METHODS

Eighty-three samples were collected throughout the species' geographical range (figure 1). The three closest species to *L. schreiberi* were used as outgroups. The samples of *L. viridis* are from Hungary, those of *L. agilis* are from Germany and those of *Lacerta biliniata* are from northern Spain. All animals were sampled by tail clipping under licence, and immediately released. Both strands of a 663-bp fragment of the cytochrome *b* gene of mitochondrial DNA were amplified using modified versions of the published primers L14841 (5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′) and H15551 (5′-AAATAGGAAGTATCACTCTGGTTT-3′) (Kocher *et al*. 1989; Moritz *et al*. 1992). We used standard polymerase chain reaction protocols and both strands were run on an ABI Prism 377 automatic sequencer.

In order to calibrate the molecular clock applicable to this data set, four published cytochrome *b* data sets were used: one from Canary skinks (*Chalcides viridanus*) (Brown & Pestano 1998), two from the Canary lizard (*Gallotia galloti*) (Thorpe *et al*. 1994; González *et al*. 1996) and one from gekkonid lizards (*Tarentola*) (Carranza *et al*. 2000). The calibration was performed using the most probable age for two Canary islands, i.e. El Hierro (1 Myr) and La Palma (2 Myr) (Juan *et al*. 2000) and assuming rapid colonization. Phylogenetic analysis was performed using PAUP* 4.0.b4a (Swofford 2000). Modeltest 3.0 software (Posada & Crandall 1998) associated with PAUP* 4.0 was used for choosing the most plausible evolutionary model for

Table 1. Percentage sequence divergence between species and clades of *L. schreiberi* calculated by the Kimura two-parameter model.

(Columns show the estimated times of divergence (Myr) under the assumption of a slow rate of substitution (1.70%) and standard (2.00%) and fast rates (2.85%) of substitution.)

		% sequence divergence	divergence times		
			slow	standard	fast
coastal region	inland region	4.67	2.75	2.34	1.64
inland northern region	inland southern region	1.65	0.97	0.82	0.57
coastal northern region	coastal southern region	1.06	0.62	0.53	0.37
L. schreiberi	*L. viridis*	17.20	10.11	8.60	6.03
L. viridis	*L. bilineata*	6.54	3.84	3.27	2.29

the different data sets. Molecular clock assumptions were tested by a likelihood ratio test (Huelsenbeck & Crandall 1997). The Kimura (1980) two-parameter distance was calculated for all datasets and used to estimate divergence times. The corrected net genetic distance between two populations was calculated according to Edwards (1997). Measures of genetic diversity, mismatch analyses, minimum spanning trees and AMOVA (Excoffier *et al.* 1992) results were obtained using Arlequin 2.0 (Schneider *et al.* 2000). Fixation indices were used according to Ecoffier *et al.* (1992). A *G*-test was performed to test the goodness of fit between the observed distribution of pairwise genetic differences and the Poisson distribution, which is expected for an expanding population (Slatkin & Hudson 1991).

3. RESULTS

For the purpose of calibration of a provisional molecular clock, three different rates were used: a value of 2% sequence divergence per Myr as the standard rate for homeotherms, which also coincides with the average value of our estimations for the *Gallotia* datasets, a slow rate of 1.70% as the most probable rate and a fast rate of 2.85%. The last two values are obtained from a close species, the ocellated lizard, assuming two different evolutionary histories (Paulo 2001). Founder events can cause divergence times to be overestimated and, consequently, high rates should be treated with caution. The likelihood ratio test shows no significant difference between the log-likelihood of phylogenetic trees with and without the molecular clock enforced ($2\delta = 22$ and $p > 0.05$, assuming $2\delta \sim \chi^2_{24}$) and the HKY85 model (Hasegawa *et al.* 1985) with a transition:transversion rate of 5.60 and a shape parameter of 0.25. Twenty-four haplotypes were detected and sequences were deposited in GenBank (accession numbers AF372103–AF372126 and AF373032–AF373034).

(a) *Genetic differentiation*

The levels of sequence divergence between these species of green lizards range between 15.5 and 18.5%, except for the divergence between *L. viridis* and *L. bilineata*, which shows a much smaller value of just 6.5% suggesting a much more recent origin (table 1)

Two main clades were identified within *L. schreiberi*, each one being divided into two secondary clades that do not overlap geographically (figure 1). The inland region comprises two monophyletic subclades, one in the central system Bejar–Gredos–Guadarrama Mountains (inland northern region) and the other in the Toledo–Guadalupe Mountains (inland southern region). The coastal region also has two reciprocal monophyletic subclades, one covering the central and northern part of Portugal and Spain (coastal northern region) and the other ranging from central to southern Portugal, which is formed by several small isolated populations (coastal southern region).

Phylogenetic analyses reveal that each of these clades has high bootstrap support. The values for the maximum likelihood trees are 100% for the two main clades and for the inland northern region and inland southern region subclades. The values are 77 and 58% for the coastal northern region and coastal southern region subclades, respectively. The coastal clade has a mean sequence divergence of 4.6% from the inland clade. The differences between the northern and southern parts of each main clade are much smaller, 1.6% for the inland clade and 1.0% for the coastal clade (table 1 and figures 1 and 3). The net genetic distances, taking into account the intra-clade variability (Edwards 1997), are 3.8, 1.3 and 0.7%, respectively. Table 1 also shows the divergence times between species and clades estimated under the three selected rates for the molecular clock.

(b) *The geographical distribution of diversity*

Detailed information on the distribution of samples and haplotypes among the regions is shown in table 2 along with the nucleotide diversity and fixation indices. The coastal region has half the nucleotide diversity of the inland region, and *ca.* 92% of the total genetic variation is explained by the differences between the four regions. This differentiation between the regions is reflected in the lower ϕ_{ST} values observed within each of the four regions compared with the values in the hierarchical analyses of combined regions (table 2). Notice that the high ϕ_{SC} value for the inland region is mainly due to the high differentiation between the populations within the inland northern region.

A detailed analysis of each of the four regions reveals different evolutionary histories. Two of them, the coastal northern region and inland southern region, show much lower nucleotide diversity, low ϕ_{ST} values (table 2) and a unimodal mismatch distribution of pairwise differences (figure 2). The inland southern region distribution fits the Poisson distribution characteristic of a simple population expansion, whereas the coastal northern region differs significantly ($p < 0.01$) (figure 2). The two other regions, the coastal southern region and inland northern region, have much higher nucleotide diversity, a higher ϕ_{ST} value

Table 2. Summary of mtDNA variation within the species, clades and subclades.

(Number of samples (n), number of haplotypes (n_H), nucleotide diversity (π), standard deviation (s.d.) and fixation indices (ϕ_{ST}, ϕ_{CT} and ϕ_{SC}).)

region	n	n_H	$\pi \pm$ s.d.	ϕ_{ST}	ϕ_{CT}	ϕ_{SC}
total	83	24	0.02572 ± 0.00500	0.971	0.928	0.603
coastal region	43	15	0.00550 ± 0.00037	0.845	0.754	0.369
inland region	40	9	0.00864 ± 0.00035	0.961	0.800	0.806
coastal northern region	23	9	0.00128 ± 0.00027	0.225	—	—
coastal southern region	20	6	0.00295 ± 0.00051	0.444	—	—
inland northern region	20	5	0.00283 ± 0.00027	0.909	—	—
inland southern region	20	4	0.00090 ± 0.00026	0.317	—	—

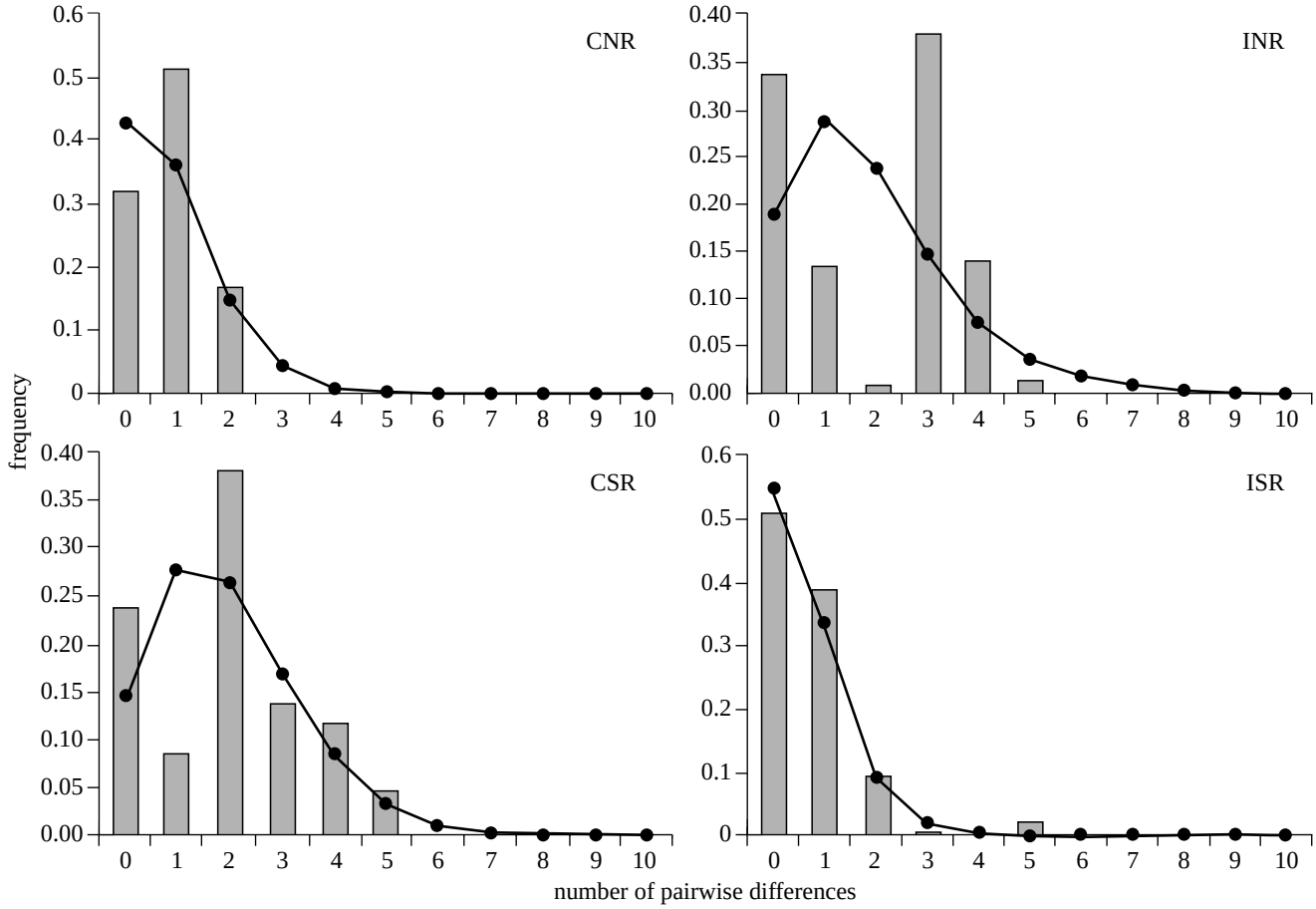

Figure 2. Observed distribution (bars) of pairwise differences among individuals within each clade and the expected Poisson distribution under an expanding population model (line). CNR, coastal northern region; CSR, coastal southern region; INR, inland northern region; ISR, inland southern region. The fit of observed data distribution to Poisson distribution expectation was tested with a *G*-test. Only the inland southern region fitted to Poisson distribution expectations.

(table 2) and a bimodal mismatch distribution, which is significantly different from the Poisson distribution (figure 2).

4. DISCUSSION

We will first consider the inferences that can be drawn from the timings implied by the genetic distances between the clades. The branching patterns and geographical distributions of haplotypes within each clade will then be used to investigate the demographic history of each region before examining the wider implications for the ecological history of the Iberian Peninsula.

(a) *Genetic distances and timing*

The genetic differentiation between the monophyletic *L. schreiberi* and the closest species *L. viridis/L. bilineata* suggests a more ancient origin than the Pleistocene era, *ca.* 8–10 Myr ago (table 1). This date is at the upper end of the range inferred from micro-complement fixation. The most plausible geological event that might explain this differentiation is the uprising of the Neopyrenees mountain chain during the Late Miocene period (Plaziat 1981). The isolated populations of Iberia could then have been the origin of *L. schreiberi*, while the continental populations might have given rise to the *L. viridis/L. bilineata* group. The less plausible 'fast rate' dates the divergence to

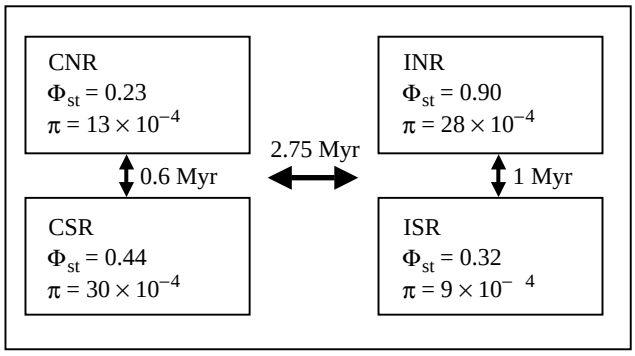

Figure 3. Summary of the genetic divergence within and between the four main regions and estimates of the time to the common ancestral mtDNA. CNR, coastal northern region; INR, inland northern region; CSR, coastal southern region; ISR, inland southern region.

the Messinian salinity crisis (5.3 Myr ago). The associated Kapitean–Optian Glaciation might have separated the populations on both sides of the Pyrenees.

The greatest difference from the expected pattern was the genetic divergence between the two main clades of *L. schreiberi*, which is much greater than anticipated, corresponding to a divergence time of 2.75 Myr ago (2.22 Myr ago if the net distance is used) according to our best calibration of the molecular clock. These timings imply divergence in the Late Pliocene era rather than the Pleistocene era. The two pairs of subclades have different divergence times. The inland subclades appear to have diverged *ca.* 1 Myr ago (0.78 Myr ago), while the coastal subclades probably diverged later during the extended cold phase of the Cromerian complex 0.6 Myr ago (0.4 Myr ago) when some residual populations appear to have been left isolated in a warmer valley.

(b) *The distribution of the diversity within each region*

Figure 3 summarizes the most likely timing of the main divergence events and distribution of genetic diversity using the data from tables 1 and 2. The present-day population sizes show surprisingly little relationship with the observed genetic diversity. The coastal northern region and inland southern region have genetic signatures characteristic of an expanding population (a low ϕ_{ST} value and genetic diversity, and unimodal mismatch distributions), yet it is the two northern populations that are flourishing at present (coastal northern region and inland northern region) with a population size conservatively estimated to exceed 1 million (Brito *et al.* 1999*b*). The southern populations are small and fragmented. There are fewer than 500 individuals remaining in the inland southern region and several thousands in the coastal southern region, but they are divided into fragmented populations along the coast.

The genetic patterns are more informative about past population demography. Consider the example of the coastal northern region. The star-like phylogeny implies increased effective size after a bottleneck, which may have been produced by founder events at the start of a range expansion. The wide geographical distribution and high frequency of internal (ancestral) alleles suggests a geographical expansion as well as an increase in population size. The low nucleotide diversity indicates that this expansion was recent. The current large population size will have reduced the effects of genetic drift and, therefore, preserved the genetic signal from the expansion, since mutation would be insufficient for subsequently establishing a mutation–drift equilibrium (Marjoram & Donnelly 1994).

Our interpretation of this genetic pattern implies that species with similar ecological requirements will show similar patterns. This is supported by a recent study of the golden-striped salamander (Alexandrino *et al.* 2000) which has a distribution similar to the coastal clade of *L. schreiberi*. It shows not only the same kind of north-western expansion, but also the same time-frame for the formation of the main clades in the Late Pliocene era.

Conversely, the data suggest different histories for the other regions. The relatively high nucleotide diversity in the coastal southern region implies that it underwent a more ancient expansion. The high ϕ_{ST} value is consistent with subsequent divergence under the action of drift in the small and fragmented populations. The current fragmented distribution may then be considered a set of interglacial refugia. The populations to the west and east of the inland northern region do not share haplotypes and show considerable differentiation. These results could be explained by multiple glacial refugia, with post-glacial expansion. The presence of common haplotypes between the two now isolated populations of the inland southern region reveals a more recent connection.

(c) *The persistence of Pliocene diversity*

The locations, ages and distributions of these four main clades and the genetic diversities within them do not fit with the expected species history that we outlined in § 1. That account assumed that the refugial areas were in southern or coastal regions where the climate was milder during the glaciations. Instead it appears that populations could persist in multiple inland refugia, presumably by moving between mountains during warm periods and river valleys during cool periods, tracking suitable habitats throughout the Late Pliocene and Pleistocene eras. Indeed, many of the proposed south European refugia for other species are not simply in warmer regions, but have a steep topography so that populations can respond to climatic fluctuations in a similar way (Hewitt 1996).

A few studies have carried out fine-scale phylogeographical analysis inside the Iberian Peninsula (e.g. Garcia-París & Jockusch 1999; Sinclair *et al.* 1999; Comes & Abbott 2000; Steinfartz *et al.* 2000). Each of these studies found considerable divergence within the peninsula. However, in one case the inland clades appear to be of recent origin from coastal populations (i.e. the ragwort *Senecio gallicus*) (Comes & Abbott 2000). We predict that further research into a wide range of species will also provide evidence of multiple refugia within the Iberian Peninsula. Populations previously thought to have been separated in the Pleistocene era may actually have a much deeper history with long-term persistence and separation of the descendant populations through the Pleistocene era. As in the case of the coastal southern region populations, such studies are likely to indicate that parts of the species' range, which are currently sparsely populated (particularly the 'southern edges') (Hewitt 2000), actually

represent important interglacial refugia, which have been centres of expansion during periods of climatic cooling.

This work was supported by a North Atlantic Treaty Organization Fellowship Portugal Invotan (03/A/97/PO) and by the Institute of Zoology, Zoological Society of London. We thank J. Pinheiro, J. Brito, R. Godinho, J. Gonzalez de la Vega and J. Barnestein for their assistance with the fieldwork, M. Kadwell and J. Groombridge for their laboratory work advice and T. Amann for the samples of *L. viridis* and *L. agilis*. All captures were performed under permit from the Portuguese Government and regional governments of Spain.

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