

## Chapter V

# Refugia within refugia: patterns of phylogeographic concordance in the Iberian Peninsula

Africa Gómez and David H. Lunt

\*E-mail: a.gomez@hull.ac.uk

### Abstract.

The Iberian Peninsula was one of the most important Pleistocene glacial refugia in Europe. A number of recent studies have documented the phylogeography of Iberian taxa and their relationship to more widely distributed species that expanded from this southern European refugium. We use a comparative approach to review the literature that challenges the paradigm of Iberia as a single refuge during Pleistocene glacial maxima and instead supports the occurrence of several Iberian refugia for a range of flora and fauna. Some patterns of phylogeographic concordance were found between the refugial areas identified by different case studies and these broadly overlapped with previously recognized areas of high endemism in the Iberian Peninsula. Such patterns help to illustrate the internal complexity of the Iberian Peninsula as a glacial refugium, and show that for many species, populations with a high degree of genetic structure have existed throughout the Pleistocene. Importantly, the occurrence of these 'refugia-within-refugia' may confound the interpretation of phylogeographic patterns of European species, and can misleadingly support the occurrence of northern refugia. We discuss these and other consequences, especially when a limited number of samples from the southern European refugia are used.

**Keywords:** comparative phylogeography, phylogeography, areas of endemism, glacial refugia, Pleistocene, Ice Ages

### Introduction

The Iberian Peninsula was one of the most important Pleistocene glacial refugia in the European subcontinent (Hewitt 1999, 2001). This claim is well supported by several lines of evidence. The persistence of temperate species throughout the Ice Ages is provided by paleontological, palynological and paleolimnological data (Huntley & Birks 1983; Bennett *et al.* 1991). The high level of endemism in both Iberian plants and animals (Gómez-Campo *et al.* 1984; Doadrio 1988; Moreno Saiz *et al.* 1998; Ribera 2000; García-Barros *et al.* 2002) indirectly suggests *in situ* long-term survival, differentiation and

speciation. Additionally, an increasing number of phylogeographic studies of European flora and fauna depict the Iberian Peninsula not only as a cradle for genetic differentiation, but also a species repository for the northern latitudes of Europe after the Pleistocene Ice Ages. Thus, since the last reviews on European phylogeography (Comes & Kadereit 1998; Taberlet *et al.* 1998; Hewitt 1999), many species have been added to the wide array of organisms known to have colonized western and northern Europe from the Iberian Peninsula after the Ice Ages, including mammals such as the roe deer (*Capreolus capreolus*, Vernesi *et al.* 2002), woodmouse (*Apodemus sylvaticus*, Michaux *et al.* 1998, 2003) and field voles (*Microtus agrestis*, Jaarola & Searle 2002); birds (chaffinch, *Fringilla coelebs*, Griswold & Baker 2002), reptiles (pond turtle, *Emys orbicularis*, Lenk *et al.* 1999) amphibians (natterjack toad, *Bufo calamita*, Beebee & Rowe 2000) and plants (ivy, *Hedera* ssp., Grivet & Petit 2002) to name a few.

In spite of its geographically isolated position on the westernmost point of Europe, several characteristics favored survival in the Iberian Peninsula throughout the Pleistocene. First, the Iberian Peninsula possesses high physiographic complexity, with several large mountain ranges primarily oriented east-west. This mountain range orientation offers the highest microclimatic scope, and allows survival of populations by altitudinal shifts, tracking suitable microclimates up or down mountains as the general climate worsens or ameliorates (Hewitt 1996). Second, due to its geographical position, the Iberian Peninsula is under the influence of both the North Atlantic and the Mediterranean, and enjoys a wide range of climates, including desert, Mediterranean, Alpine, and Atlantic. Interestingly, these very same characteristics, together with its large area (580 000 km<sup>2</sup>), make it unlikely that Iberia offered a single homogeneous and continuous refugial area throughout the Pleistocene. Instead, the differential distribution and fragmented nature of suitable habitats favor the occurrence of multiple glacial refugia isolated from one another by the harsh climate of the high central Iberian plateau.

Phylogeographic concordance refers to the non-random patterns of similarity in the geographic distribution of evolutionary lineages of codistributed species or species complexes (Avice 2000). Phylogeographic concordance has parallels to historical biogeography since it can reveal the role of vicariance in structuring biotas (Avice 2000; Riddle *et al.* 2000). Comparative phylogeography can shed light on the role of geography in speciation, the associations between climate cycles and species distributions, and can help to identify biodiversity hotspots and inform conservation policies (Avice 2000; Hewitt 2000; Riddle *et al.* 2000). Insights provided by comparative phylogeography can additionally aid sampling design when planning future studies. Comparative phylogeographic analyses have yielded numerous novel insights into the development of regional historical patterns of genetic lineages (Bernatchez & Wilson 1998; Schneider *et al.* 1998; Walker & Avice 1998; Riddle *et al.* 2000; Arbogast & Kenagy 2001). In Europe, the location of the

major refugia, the main postglacial colonization routes and the suture zones for terrestrial species have been well established and show concordant patterns (Hewitt 1996, 1999; Taberlet 1998). Given the above, and the recent surge of phylogeographic studies centered in the Iberian Peninsula, it is opportune to undertake a comparative phylogeographic analysis on this area in order to understand the number and location of glacial refugia present, point to areas where more work is needed, and contribute to clarify the broader picture of European phylogeography.

Here, using a comparative phylogeographic approach, we: i) review phylogeographic and biogeographic evidence indicating the occurrence of multiple glacial refugia in Iberia, ii) investigate the patterns of concordance found, and iii) discuss the consequences of these findings for European phylogeographic research and historical biogeography. We have excluded marine taxa, and the Balearic Islands, focusing on mainland species. We review both single species and species complexes (with largely parapatric distributions) for a geographic component to their genetic diversity. We note that both the literature in general and this review are biased towards species with low dispersal abilities or limited gene flow, which are most likely to reflect historical discontinuities. We primarily review and discuss case studies with extensive sampling of the Iberian Peninsula. We are also aware that expansions into Iberia from other regions may give the appearance of multiple refugia and concentrate on endemics and systems for which there is adequate sampling outside of Iberia. Since different organismal dispersal and habitat requirements can lead to distinct patterns of phylogeographic structuring, we discuss freshwater/amphibian, terrestrial and lacustrine organisms in separate sections. Throughout the review we use the existence of this Type I phylogeographic structure (Avice 2000) as evidence of multiple refugia in the Iberian Peninsula.

## **Freshwater/amphibian habitats**

### **1. Fish**

Freshwater fish tend to show particularly clear phylogeographic structure as they do not normally disperse between river basins, and thus the distribution of their lineages tends to reflect the history of river drainages instead of contemporary dispersal (see reviews in Bernatchez & Wilson 1998; Avice 2000). The 11 main river basins in the Iberian Peninsula formed from the Upper-Oligocene to the Pliocene, together with the rise of the main mountain ranges. The current network of river systems was already formed by the Quaternary, and the general absence of canals between rivers has maintained the natural distribution of native fish barring intentional human introductions (Doadrio

1988). The Pyrenees constitute a formidable barrier for freshwater fishes as no river drainage crosses them, and, covered by an extensive ice cap, they were an even stronger barrier during glaciations. Therefore, due to its isolation, the Iberian fish fauna is not as species rich as that of central Europe (Bănărescu 1991; Elvira 1995). Not surprisingly over 45% of Iberia's native fish species are endemic. This high level of endemism is shared with the other southern European peninsulas, particularly the Balkans (Bănărescu 1991). Thus, Iberia behaved more as a cradle for freshwater fish endemics than as a refugium for the rest of Europe. Here we will illustrate the phylogeography of Iberian fishes with two relatively well-studied Iberian species complexes.

### The *Luciobarbus* group

The subgenus *Luciobarbus* colonized the Iberian Peninsula from North Africa and gave rise to a complex of six endemic species (Machordom & Doadrio 2001; Doadrio *et al.* 2002). The phylogeography of these barbels has been investigated using mtDNA sequence variation (Zardoya & Doadrio 1998; Callejas & Ochando 2000; Machordom & Doadrio 2001; Doadrio *et al.* 2002). Analyses revealed a clear-cut geographic distribution of the different species, with one mainly Mediterranean clade, including *B. graellsii*, *B. guirraonis* and *B. microcephalus*, one southern clade of *B. sclateri* and one mainly Atlantic with *B. bocagei* and *B. comizo* (Figure 1). In the first two clades, species are distributed according to the main river basins, although some cases of sympatry due to secondary contact are present in the Tajo and Guadiana basins, most likely due to episodes of headwater river capture. The average sequence divergence among these seven species varies from less than 0.5% to 6.5%, and hybridization events have been reported in areas of sympatry (Callejas & Ochando 2000). Application of molecular clocks calibrated with the opening of the Gibraltar straits suggests that the radiation of these Iberian barbels began in the mid Pliocene and continued throughout the Pleistocene, with distinct lineages existing in separate river basin refugia (Machordom & Doadrio 2001).

### The *Leuciscus* group

Several Iberian endemic species of the genus *Leuciscus*, mostly with parapatric distribution ranges, have been described. *Leuciscus carolitertii* inhabits northern Atlantic rivers, excluding the Cantabrian basin. The sister species *L. pyrenaicus* inhabits the southern Atlantic and Mediterranean Iberian rivers. Both species co-occur in the Tajo River, probably as a consequence of a river capture. An mtDNA analysis of populations of these species revealed four main lineages (Brito *et al.* 1997; Zardoya & Doadrio 1998), two of them coincident with the already described species. The other two highly divergent lineages are restricted to small river drainages in the southernmost tip of Portugal

and they were proposed to be new species, *L. aradensis* and *L. torgalensis* (Figure 1) (Coelho *et al.* 1998). These species, with sequence divergences between 5 and 11% were estimated to have radiated between 2 and 3 mya, probably following the partition of river drainages from the Pliocene to the early Pleistocene (Brito *et al.* 1997).

### The Brown Trout

The brown trout, *Salmo trutta*, is possibly the best studied European freshwater fish from a genetic and phylogeographic perspective (Antunes *et al.* 2001). Due to its anadromous life history, at least for some populations, the phylogeographic history of brown trout does not compare well to other Iberian fish. Admixture between different lineages is common, due to multiple colonizations and secondary contact zones (Ball-Llosera *et al.* 2002), therefore there is not a clear concordance between the distribution of genetic lineages and current river drainages. Of the five main genetic lineages found in the global distribution range of this species (Bernatchez 2001), three (the Atlantic, the Mediterranean, and the Adriatic) are naturally present in the Iberian Peninsula. These lineages display a large amount of genetic diversity (Machordom *et al.* 2000; Suárez *et al.* 2001). In the Atlantic rivers, Iberian brown trout display clear genetic subdivisions and populations show evidence of genetic isolation for at least 200 000 years (Weiss *et al.* 2000), pointing to the occurrence of a complex of glacial refugia in the area. In the Mediterranean clade, four main subgroups, probably the result of allopatry during the Pleistocene have been identified, but secondary admixture and the effects of drift have contributed to a complex current distribution of lineages (Ball-Llosera *et al.* 2002). In summary, brown trout display a high level of complexity in the Iberian Peninsula, pointing to the presence of several Iberian refugia. However, and in a similar fashion to other freshwater fish, brown trout lineages did not contribute to the most recent waves of postglacial colonization in northern Europe (Weiss *et al.* 2000). Even for a species that presumably has the ability to colonize across saline habitats, the Iberian Peninsula is nonetheless significant as a complex of refugia.

### Other fish

Other phylogeography studies in Iberian fish indicate that refugial populations and local endemic species remained in several of the main Iberian river basins throughout the Pleistocene. The final configuration of the current drainage system seems to have been the historical process that generated barriers of gene flow isolating populations and initiating the process of differentiation in *Chondrostoma lemmingii*, *C. lusitanicum*, and *Anaecypris hispanica* (Carmona *et al.* 2000; Alves *et al.* 2001; Mesquita *et al.* 2001) (see Table 1). Another Iberian endemic fish, *Aphanius iberus*, which inhabits salt

marshes, also shows deep phylogeographic patterns coinciding with the Atlantic and Mediterranean drainages (Perdices *et al.* 2001). In summary, the main river catchments, including the Guadalquivir, Guadiana, Tajo, Ebro and Duero, and a number of minor ones (significantly the southern Portuguese Mira and Arade rivers) seem to have harbored distinct fish lineages in suitable habitats throughout the Pleistocene's multiple Ice Ages.

## 2. Amphibians

Of the 28 native Iberian amphibian species, eight are endemic (Vargas *et al.* 1998), but the number of described species and subspecies is growing (García-París & Martínez-Solano 2001). Due to their mobility over land, amphibians are less confined to river basins as fishes, but they tend to show strong genetic variation among populations, and their patterns of genetic diversity do still tend to reflect historical rather than contemporary processes (see review in Avise 2000). Several of the endemic species have restricted ranges (around the Pyrenees *Rana pyrenaica* and *Euproctus asper*, in western Iberia *Rana iberica*, and *Triturus boscai*) suggesting long-term population persistence in these areas.

### The *Discoglossus* toads

García-París and Jockusch (1999) investigated the phylogeography of the two Iberian *Discoglossus* endemics: *D. jeanneae* and *D. galganoi* (Figure 1), with a sampling design that aimed to pinpoint the contact zone between these morphologically very similar toads. Cytochrome b sequence divergence between these two species was 8.6%, and suggested that the two lineages started to diverge before 5 mya. The western species *D. galganoi* showed higher and geographically structured intraspecific diversity than *D. jeanneae*, with haplotypes south and north the Duero River estimated to have diverged over 1 mya (Figure 1) (García-París & Jockusch 1999; Martínez-Solano, pers. comm.). As for *D. jeanneae*, two very closely related haplotypes were found, which might suggest a recent episode of range expansion, and then a contraction, which left the patchy distribution we find today. The location of the refugium from which *D. jeanneae* expanded has been suggested to be in or nearby the Betic ranges (Barbadillo 1987).

### The midwife toads

The midwife toads (genus *Alytes*) are thought to have evolved in the Iberian Peninsula in the late Miocene. Three mainland Iberian species, plus an endemic of the Balearic Islands are currently recognized. Two of the Iberian species are endemic, the other one, *A. obstetricans* is also present in western



Europe and a small region in North Africa. The phylogenetic and biogeographic relationships of these taxa were examined by Arntzen & García-París (1995) using morphological and allozyme variation. *Alytes cisternasii* is a quite divergent species that differentiated in the western half of the Iberian Peninsula. *Alytes dickhilleni* is present in the Betic mountains and it is thought to have evolved in this area (Arntzen & García-París 1995) coinciding with the opening of the Betic Straits and their isolation in the Betic-Riffian massif (5–6 mya). *Alytes obstetricans* presents a high level of geographic variation, which has led to the description of four subspecies with parapatric distributions based on allozyme and morphological differences (Arntzen & García-París 1995; García-París & Martínez-Solano 2001). More recently phylogeographic analyses using mtDNA sequence variation have established geographic differentiation in Portugal indicating the occurrence of multiple Pleistocene refugia (Fonseca *et al.* 2003). The extent to which these subspecies hybridize and the distribution limits need to be investigated further, but sequence divergence between them suggests that the radiation in *A. obstetricans* happened around 3.5 mya (Arntzen & García-París 1995; Fonseca *et al.* 2003). The location of putative glacial refugia is not yet known, although it seems reasonable to assume that each subspecies evolved in isolated Iberian refugia during glacial periods, and that range expansion happened during the Holocene, resulting in the contact zones observed today.

### The golden-striped salamander

The golden-striped salamander, *Chioglossa lusitanica*, inhabits streams in humid forested areas in northwestern Iberia. A detailed allozyme and mtDNA variation study revealed a high degree of genetic subdivision (Figure 1), with populations south of the Mondego River being quite distinct from the northern populations (Alexandrino *et al.* 2000). The phylogeography of this salamander was further investigated over its entire geographical range using cytochrome b sequence variation (Alexandrino *et al.* 2002) and the pattern found (Figure 1) suggested the occurrence of a minimum of two refugia. The authors suggest that the level of divergence between the two main lineages (1.5%) reflects divergence from the late Pliocene to early Pleistocene (3 to 1.5 mya).

### The fire salamander

Nine out of the sixteen described subspecies of the highly polytypic European fire salamander *Salamandra salamandra* occur in the Iberian Peninsula (Alcobendas *et al.* 1996; García-París *et al.* 2003). These morphologically recognized subspecies can be assigned to four mtDNA lineages, each of them apparently of different age (Dopazo *et al.* 1998; Steinfartz *et al.* 2000; García-París *et al.* 2003). First, a quite distinct and basal clade encompasses a single subspecies, *S. s. longirostris*, occurring in the western Betic Ranges of

southern Spain. This clade, with 6.3% sequence divergence from the rest, evolved in isolation 2–4 mya, with a putative refugium existing throughout most of the Pleistocene around the Betic Ranges (Steinfartz *et al.* 2000). A second, quite genetically complex clade, corresponds to *S. s. bernardezi*, which occurs in Picos de Europa, and is characterized by its viviparous reproduction (Dopazo *et al.* 1998). The situation of this clade is peculiar as its closest relative is a ovoviviparous subspecies found in the southernmost tip of Italy and the large genetic divergence between the Iberian and Italian subclades suggests that the ancestor of this clade was much more widespread in a past interglacial, and that both subclades have survived at or close to their current distribution ranges for at least several glacial cycles (Steinfartz *et al.* 2000). Therefore, viviparity is likely to have arisen in the ancestor of the Iberian subclade, possibly in a glacial cycle when the populations of the clade retreated to the Picos de Europa ranges, where viviparity was favored by the lack of suitable aquatic habitat for larvae. A third, geographically subdivided mtDNA lineage coincides with three recognized subspecies, one in the Algarve area (*crespoi*), another in the central mountain ranges (*almanzoris*) and the third north of the Guadalquivir River (*morenica*), thus further indicating more recent refugia for this salamander (García-París *et al.* 1998, 2003). Finally, the fourth mtDNA clade, comprising three subspecies, *fastuosa*, *gal-laica* and *terrestris* is widespread in the Iberian Peninsula and also occurs in most of Europe. Despite its relatively low diversity, suggesting relatively recent colonization of the area, this clade exhibits substantial geographic structure with evidence of past fragmentation in the Iberian Peninsula, and the level of diversity is consistent with occupation of the Iberian Peninsula for at least one glacial cycle (García-París *et al.* 2003). The joint study of life history, allozymes and mtDNA variation revealed the complex population interactions between this clade, comprising both ovoviviparous and viviparous populations, and the northern viviparous populations of Picos de Europa (García-París *et al.* 2003). In summary, we can conclude that *S. salamandra* survived in the Iberian Peninsula in several glacial refugia, throughout the Pleistocene in the Betic Ranges, through several glacial cycles in Picos de Europa and at least throughout the last glacial cycle in several other refugia.

### Other amphibians

The marbled newt *Triturus marmoratus*, distributed in Iberia and western France, shows evidence of two Iberian refugia: *T. m. pygmaeus* is restricted to the southwestern part of the Iberian Peninsula and *T. m. marmoratus* to the northern half and most of France (García-París *et al.* 1993). Both subspecies are over 4% divergent in their mtDNA, which suggests independent evolution since the Pliocene (Wallis & Arntzen 1989). In addition, *Triturus boscai*, an endemic species inhabiting the western half of the Iberian Peninsula comprises two population groups showing chromosomal incompatibilities and



hybrid sterility (Herrero 1991). The distribution of the subspecies of *Triturus helveticus* is also suggestive of past population fragmentation, although a phylogeographic analysis is needed.

In conclusion, most of the amphibian taxa investigated show evidence of multiple Iberian mtDNA lineages that are geographically structured indicating survival in different Pleistocene glacial refugia. Several of these species show compelling phylogeographic concordance with a putative refugium located in or near the southern Betic Ranges (*Salamandra salamandra longirostris*, *Discoglossus jeanneae*, *Alytes dickhilleni*). The Atlantic side of the Peninsula also seems to have served as refugium or refugia for multiple species, and may often have limited the distribution of other taxa possibly due to its high humidity (*Chioglossa lusitanica*, *Alytes cisternasii*, *A. obstetricans boscai*, *Triturus boscai*, *Discoglossus galganoi*). Finally, several endemic amphibian species have restricted ranges suggestive of relict habitat in the present conditions following more extensive distributions in the past. Interestingly, such relictual ranges often coincide with areas known for their high level of endemism in plants.

### 3. Terrestrial habitats

#### 3.1. Reptiles

Reptiles share the low mobility of amphibians but are often associated with drier and warmer climates. Glacial advances are very likely to have limited their distribution to the southernmost reaches of the European continent, and there, fragmentation of populations in suitable habitats should have led to geographic structuring. Of the 38 reptile species in the Iberian Peninsula eight are endemic (21%), and, as in amphibians, the number of described species is growing (Andreu *et al.* 1998). Several endemic species have restricted ranges indicating geographic structuring: *Podarcis bocagei* is distributed in northwestern Iberia, *Lacerta schreiberi* and *L. monticola* are restricted to northwestern Iberia and Central System mountains, *Lacerta bonnali* occurs in the Pyrenees, and *Algyroides marchi* occurs in the eastern end of the Betic ranges. A subspecies of the widespread *Lacerta lepida* (also present in France), *L. l. nevadensis* occurs in the Betic Ranges of southern Spain. Three published studies will be presented here as case studies.

#### The Iberian wall lizards

The small Iberian wall lizard, *Podarcis hispanica*, has been shown to be a species complex containing several lineages with a minimum of six species, several still undescribed (Harris & Sa-Sousa 2001, 2002) (see Figure 1). The

range of sequence divergence between the lineages (9.5 to 15.2%) indicates a pre-Pleistocene divergence. Most of the lineages are parapatric, although the geographic distribution and contact zones between them are only well known for western Iberia (Harris & Sa-Sousa 2001). In the cases investigated, interbreeding does not seem to happen, despite high morphological similarity. The genetic and geographic pattern found indicates survival of isolated populations in more than one Iberian refugium.

### The Schreiber's lizard

The lizard *Lacerta schreiberi* is an Iberian endemic with a preference for humid temperate forests and mountain river valleys. The distribution range and the deep phylogeographic structure revealed by cytochrome b sequence variation is shown in Figure 1 (Paulo *et al.* 2001). Despite a remarkable morphological uniformity, the two major clades found, inland and coastal, display a sequence divergence of 4.7%, suggesting divergence before 2 mya (Paulo *et al.* 2001, 2002). Each of these clades was subdivided in a northern and a southern lineage, with divergence times of 1 and 0.6 mya, and none of the clades overlap geographically. This pattern suggests survival in separate glacial refugia, with a northern coastal, southern coastal, Central System and Montes de Toledo refugia. The phylogeography and diversification time frame of this lizard, particularly the coastal lineages, is remarkably concordant with the salamander *Chioglossa lusitanica* (Paulo *et al.* 2001; see also Alexandrino *et al.* this volume).

### The viviparous lizard

The western oviparous form of the viviparous lacertid lizard *Zootoca vivipara*, displays geographic variation in sex-linked allozyme alleles and mtDNA (cytochrome b), with two maternal lineages, one distributed in the central and eastern Pyrenees and Aquitaine, and the other in Picos de Europa and southern slopes of the western Pyrenees (Figure 1) (Guillaume *et al.* 2000; Surget-Groba *et al.* 2001). Both lineages meet in a contact zone in the upper Ossau valley and Aquitaine (Guillaume *et al.* 2000; Surget-Groba *et al.* 2001). These authors explained their results as suggesting geographic isolation of the viviparous populations in two refugia, one near Picos de Europa and the other near the Pyrenees during glacial periods, and subsequent expansion with recent secondary contact during the Holocene.

### The viperine snake

Some recent studies support the presence of several Iberian refugia in other Iberian reptiles. The Viperine snake, *Natrix maura*, which colonized western Europe from Iberia, has been shown to comprise several mtDNA European

lineages with a 1.3% sequence divergence (Guicking *et al.* 2002). All the western European lineages are present in Iberia, which presents the highest genetic diversity, as well as the most ancestral haplotypes. The current preliminary data does not reveal the location of the putative Iberian refugia, but the data is suggestive that France and western Italy were colonized via two routes, one through the east and one through the west of the Pyrenees.

### 3.2. Mammals

There are 118 described mammal species in the Iberian Peninsula – and the highest level of endemism for mammals in Europe (Baquero & Tellería 2001). However, the number of studies centered on Iberian mammals is quite small, most probably due to the difficulties involved in obtaining samples.

#### The European rabbit

The rabbit, *Oryctolagus cuniculus*, is a native Iberian species with a natural distribution that in the Middle Ages reached the Loire River in France (Queney *et al.* 2001; see also Ferrand & Branco, this volume). Mitochondrial cytochrome b RFLP and sequence variation of Iberian rabbits revealed the occurrence of two clades with a strong geographic partitioning. One is located in southwestern Iberia and the other in the northeast, with an area of secondary contact crossing the Peninsula from the southeast to the northwest (Branco *et al.* 2000, 2002). The haplotype distribution is concordant with survival of rabbit populations in two glacial refugia and subsequent postglacial range expansion as climate ameliorated. These refugia are hypothesized to be in northeastern Spain, probably in the Mediterranean coast, and in the southernmost part of Spain, close to the Gibraltar straits. The two lineages presented a sequence divergence estimated to date at approximately 2 mya, or the Pliocene-Pleistocene boundary (Biju-Duval *et al.* 1991).

#### The Iberian hares

Three species of hare inhabit the Iberian Peninsula, the European hare, *Lepus europaeus*, and two endemic species, *L. castroviejoi* and *L. granatensis*. The restricted endemic *L. castroviejoi* inhabits the western side of Picos de Europa, *L. granatensis* inhabits most of the Iberian Peninsula south of the Ebro River, and *L. europaeus* inhabits the area north of the Ebro River and the eastern strip of the Cantabrian area. An RFLP study on mtDNA variation was undertaken using Iberian samples from these species (Pérez-Suárez *et al.* 1994). The three species were quite distinct in their mtDNA, and did not share any of the 11 haplotypes found, although only one or two populations were sampled for each species. The parapatric ranges and the level of sequence diver-

gence found between them (8-9%), suggests the isolation of these species in at least two Iberian refugia, one of them in or near Picos de Europa, and their independent evolution for 4.4 to 6 myr (Pérez-Suárez *et al.* 1994).

### Other mammals

Several other species of mammals have been focus of European phylogeographic analyses (Taberlet *et al.* 1998; Seddon *et al.* 2001; Vernesi *et al.* 2002; Michaux *et al.* 2003). However, the number of samples remains insufficient to draw any clear-cut patterns within the Iberian Peninsula. A recent exception is constituted by the field vole (Jaarola & Tegelstrom 1995; Jaarola & Searle 2002). The southern mtDNA lineage of this rodent was postulated to have recolonized southern France, Switzerland, Slovenia and Hungary from an Iberian refugium (Jaarola & Searle 2002). Detailed sampling in the Iberian Peninsula revealed two lineages, one of them restricted to Serra da Estrela in Portugal (Jaarola & Searle, pers. com.). The authors hypothesized that the field vole survived at least through the last glaciation in two Iberian refugia.

## 3.3. Invertebrates

Although there have been a number of phylogeographic studies of European invertebrates – biogeographical data is particularly good for insects – relatively few studies have included detailed sampling in Iberia. Two cases are presented below, and a third, the maritime pine scale insect is included later with its host.

### The leaf beetles of the *Timarcha goettingensis* complex

The beetles of the genus *Timarcha* are apterous insects favoring mountain regions distributed in central Europe and the northern half of the Iberian Peninsula. Nested clade analysis on mtDNA sequence variation on ten Iberian endemic species from the complex revealed the importance of population range expansions, but also of past population fragmentation in a number of Iberian refugia (Gómez-Zurita *et al.* 2000).

### The meadow grasshopper

The meadow grasshopper, *Chorthippus parallelus*, has become a European phylogeography icon since the early 1990s (Cooper & Hewitt 1993; Cooper *et al.* 1995; Lunt *et al.* 1998). The species is subdivided, with different lineages in the three southern peninsulas. Expansion from a Balkan refugium has recolonized most of Europe and a hybrid zone occurs in the Pyrenees between the Balkan and Iberian Peninsula subspecies. This hybrid zone has

been documented using sequence data, chromosomes, morphology, allozymes and behavior (see references in Hewitt 1993; Hewitt 1996, 1999, 2001). Sequence variation in an anonymous nuclear DNA sequence in European populations supported the separate origin for populations now found in France and Spain (Cooper & Hewitt 1993; Cooper *et al.* 1995). Additionally, the extensive geographic variation of the Spanish populations suggested the occurrence of at least two putative glacial refugia, one in the west, south of Picos de Europa, and the other possibly in the southeast (Hewitt 1996).

### 3.4. Plants

A number of European phylogeographic studies including the Iberian Peninsula present some evidence of Iberian refugia for plant taxa including ivy, white oaks, black alder, silver fir, *Aconitum lycoctonum*, and *Arabidopsis thaliana* (Konnert & Bergmann 1995; Ferris *et al.* 1998; King & Ferris 1998; Utelli *et al.* 1999; Muir *et al.* 2000; Sharbel *et al.* 2000; Grivet & Petit 2002). However, most of them include few Iberian localities, and therefore yield little information on the location and number of potential refugia within Iberia. The number of studies with extensive sampling in Iberia, has, however grown dramatically in recent years and here we review several of them.

#### The Scots Pine

The Scots pine, *Pinus sylvestris*, inhabits mountains in the Iberian Peninsula, usually in the altitudinal range of 1000 to 2000 m. It requires humid conditions, and it cannot withstand droughts. Several subspecies or races have been recognized in its natural distribution, including two in the Pyrenees (*P. s. catalaunica* and *P. s. pyrenaica*), another in the Iberian System, Maestrazgo, Central System and northern Portugal (*P. s. iberica*), and a relict subspecies in the south (Sierra de Baza, Sierra Nevada, *P. s. nevadensis*). Mitochondrial DNA and allozyme variation suggests that the Scots pine survived in the Iberian Peninsula during the Pleistocene glaciations, but most likely did not contribute to the postglacial colonization of northern Europe (Sinclair *et al.* 1999; Soranzo *et al.* 2000). Evidence of strong substructure in the Iberian Peninsula exists from mtDNA variation that coincides remarkably well with the recognized subspecies (Sinclair *et al.* 1999; Soranzo *et al.* 2000). This strongly suggests that the Scots pine survived in several refugia in Iberia: the Betic ranges, the Central System and the Pyrenees.

#### The maritime pine and its scale insect

The maritime pine, *Pinus pinaster*, forms scattered populations in the western Mediterranean basin. Allozyme analysis on twelve natural Iberian popula-

tions (Salvador *et al.* 2000) suggests the occurrence of an eastern refugium and a southern refugium possibly near or in the Betic ranges (see Martínez *et al.* this volume). Studies using cpDNA microsatellite markers (paternally transmitted in pines) failed to resolve the historical patterns of maritime pine in the Iberian peninsula (Vendramin *et al.* 1998).

The bast scale insect, *Matsucoccus feytaudi*, is a specific pest of maritime pine, so its phylogeography may also inform us about the history of the maritime pine. A phylogeographic study of this insect in its natural range revealed three mtDNA lineages termed: Western, found throughout most of the species distribution range; Andalusian in the Betic ranges, and Moroccan (Burban *et al.* 1999). The haplotype diversity of the different populations suggests colonization of western Europe from a refugium in the west of Iberia, possibly in Portugal. This phylogeographic structure is remarkably similar to the phylogeography of its host.

### The white oaks, *Quercus*

The deciduous oaks, *Quercus*, are widespread European forest trees, some of them of considerable economic importance. They are hermaphroditic and have heavy seeds (acorns), which are often edible, and disperse via birds. They produce abundant pollen that is dispersed via wind. Eight species are present in Europe. As oaks hybridize frequently, their maternally transmitted cpDNA haplotypes are often shared by several sympatric species (Dumolin-Lapègue *et al.* 1999). Although abundant information on the European phylogeography and population structure of the pedunculate oak, *Q. robur* and the sessile oak, *Q. petraea*, was available (Dumolin-Lapègue *et al.* 1997, 1999; Ferris *et al.* 1998) the sampling of the Iberian peninsula remained quite poor, and several endemic species had not been sampled. In the context of an ambitious, Europe-wide project, ample information of cpDNA variation has recently become available for the six species of Iberian white oaks (Olalde *et al.* 2002). The data suggests Iberian refugia for four species, *Q. robur*, *Q. faginea*, *Q. canariensis* and *Q. pyrenaica*, but the data is not conclusive for the other two species (Olalde *et al.* 2002; Petit *et al.* 2002). In all, four mitochondrial lineages were detected, although two of them were represented by a single haplotype. The four lineages had a mostly parapatric distribution, one of them present in the northwestern half of the peninsula, the second one southeastern, and the other two near the Pyrenees (Olalde *et al.* 2002). At least two refugia were detected, one southwestern, the other one in the Betic mountains area, and a third one on the Mediterranean coast near the Ebro basin (Petit *et al.* 2002). Some other smaller refugia in the north of the peninsula were also proposed (Olalde *et al.* 2002).



## Other plants

Analysis of cpDNA, allozyme and RAPD variation in the Iberian *Senecio gallicus* (Comes & Abbott 1998) revealed a complex phylogeographic pattern with coastal and inland groups. The coastal group with a high level of geographic variability, seems to be older, with putative glacial refugia in the southernmost tip of Iberia and SW Portugal, and has given rise to the inland group.

The hoary plantain, a common perennial plant of grasslands was investigated using RFLP on cpDNA (Van Dijk & Bakx-Schotman 1997). This plant is present in northern Spain, the Pyrenees and scattered localities in mountain areas of southern Spain. Of the nine haplotypes found, three are present in Iberia. A contact zone between two of these lineages (one diploid and the other tetraploid) occurs in the Pyrenees. The data suggests two refugia in Iberia.

The variation in chloroplast DNA in heather, *Calluna vulgaris*, is congruent with pollen records that suggest its survival in refugia in northern Spain (Picos de Europa) and southern France. In addition, a second Iberian refugium in the Pyrenees was suggested by the genetic data (Rendell & Ennos 2002).

Support for the occurrence of two Iberian refugia is provided by a genetic analysis of Holm oak, *Quercus ilex* (Lumaret *et al.* 2002). The distribution of the two cpDNA oak lineages in Iberia roughly coincides with the two recognized morphs, one distributed along the eastern Mediterranean coast and the second in the rest of Iberia suggesting the occurrence of two distinct southern refugia for this species.

## 4. Lacustrine fauna

### Rotifers

A recent phylogeographic appraisal in the Iberian Peninsula focused on the rotifer *Brachionus plicatilis*, a species complex distributed globally in salt lakes and coastal lagoons. Six of the species in the complex are present in Spain (Ortells *et al.* 2000; Gómez *et al.* 2002), the most common of them is *B. plicatilis* s.s. (Ciros-Pérez *et al.* 2001). Rotifers reproduce by parthenogenesis, but when the environment deteriorates they produce sexual resting eggs, which can be sampled from lake sediments. Using such samples of *B. plicatilis*, mtDNA sequence variation revealed 21 haplotypes. The haplotypes clustered in two groups with 2.8% divergence between them and a strong northern and southern geographic component overlapping in just two of the 18 lakes (Gómez *et al.* 2000) (Figure 1). The high genetic diversity and geographic structuring of the northern clade indicates that it had survived in the Iberian Peninsula for several glacial cycles. However, the southern lineage shows signs of recent expansion and although the presence of a second Iberian

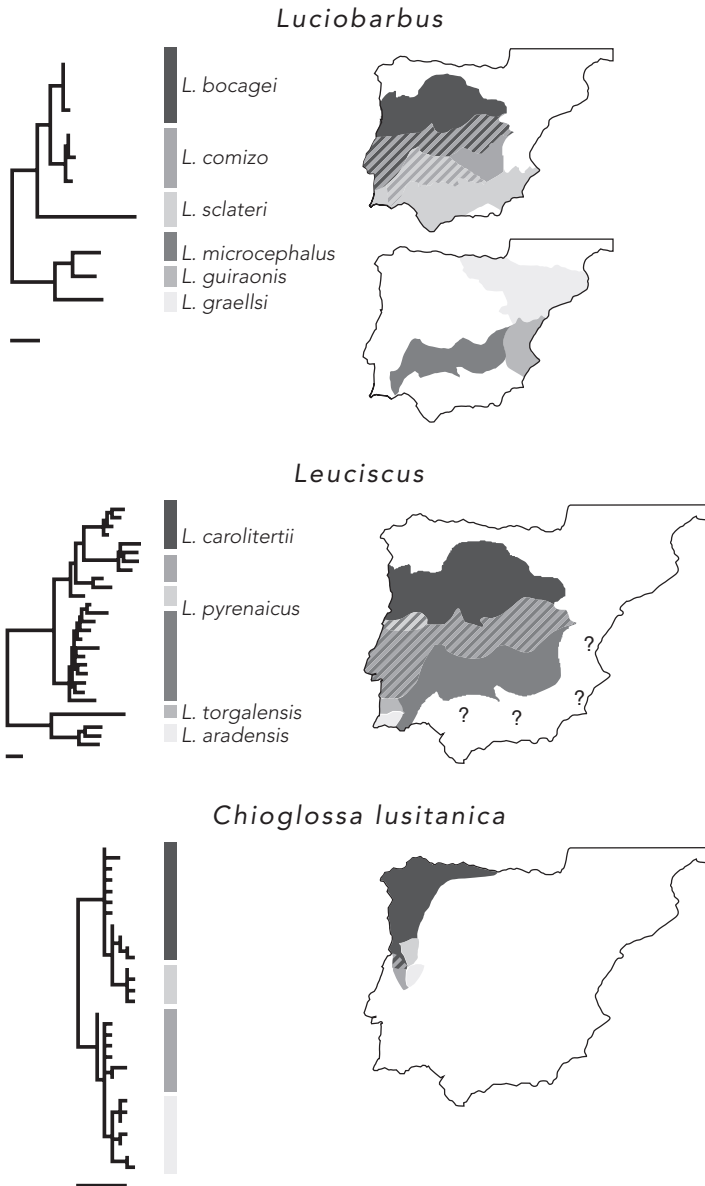
refuge is likely, colonization from northern Africa cannot be ruled out. The location of the hypothesized northern refugium would be in the Ebro river basin or on the northeastern Iberian coast.

A recent study confirmed the pattern of southern and northern refugia in another widespread species from the *Brachionus plicatilis* complex, *B.* 'Manjavacas' (Gómez *et al.*, unpublished data). In this species, sequence variation of the mitochondrial gene COI revealed seven haplotypes in two geographically segregated lineages (Figure 1).

## Discussion

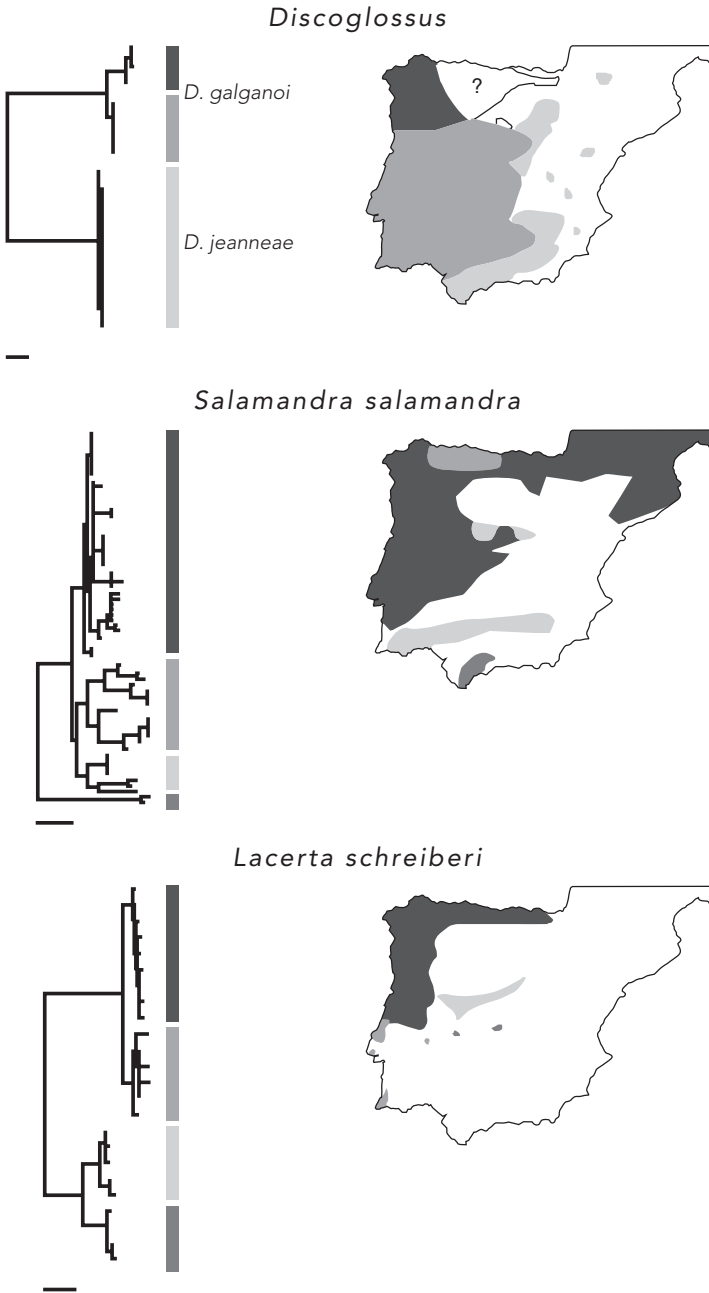
### Refugia within refugia

We have shown that many species and species complexes show strong genetic subdivisions in the Iberian Peninsula indicative of past population isolation. These taxa inhabit a range of habitats from rivers, humid forests and Mediterranean evergreen forests, to salt lakes and mountains. The current distribution of these lineages is largely parapatric and the genetic divergence between them is consistent with extended periods of isolation. These observations can be most easily interpreted by survival throughout the Pleistocene Ice Ages in separate glacial refugia (Table 1). To our knowledge, the earliest discussion on the occurrence of distinct isolated populations (refugia) within the southern Mediterranean peninsulas at the height of glacial advances was by Hewitt in his 1996 review (p. 265), as suggested by Cooper & Hewitt's (1993) results on *Chorthippus parallelus* and palynological data. However, in general, both palynological data and paleoclimatic reconstructions supported the paradigm of single refugia in the southern extremes of the Mediterranean peninsulas (see Olalde *et al.* 2002 for an excellent discussion), and evidence for the actual widespread occurrence of multiple refugia has only begun to accumulate and be acknowledged more recently, mostly based on phylogeographic information. The realization that many species display a strong population substructure within the Iberian glacial refugium, or were actually composed of completely isolated populations in distinct Iberian refugia has important consequences for the interpretation of European phylogeography and refugial diversity. The consequences of this 'refugia-within-refugia' scenario for the interpretation of European phylogeographic patterns need to be explored further. In our literature review we failed to find a single case in which a *detailed* sampling program recovered evidence for a single refugium in the Iberian Peninsula. This supports the idea that the presence of separate refugia is a general phenomenon, and reflects a common vicariant history of Iberian flora and fauna. We have deliberately avoided classifying the taxonomic level (e.g. populations, subspecies, species) at which to document phylogeographic structure.



**Figure 1.** Phylogeographic structure of some of the case studies discussed in the text. See main text for references. The scale bar under each tree represents 1% sequence divergence.

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*Figure 1. Continued.*

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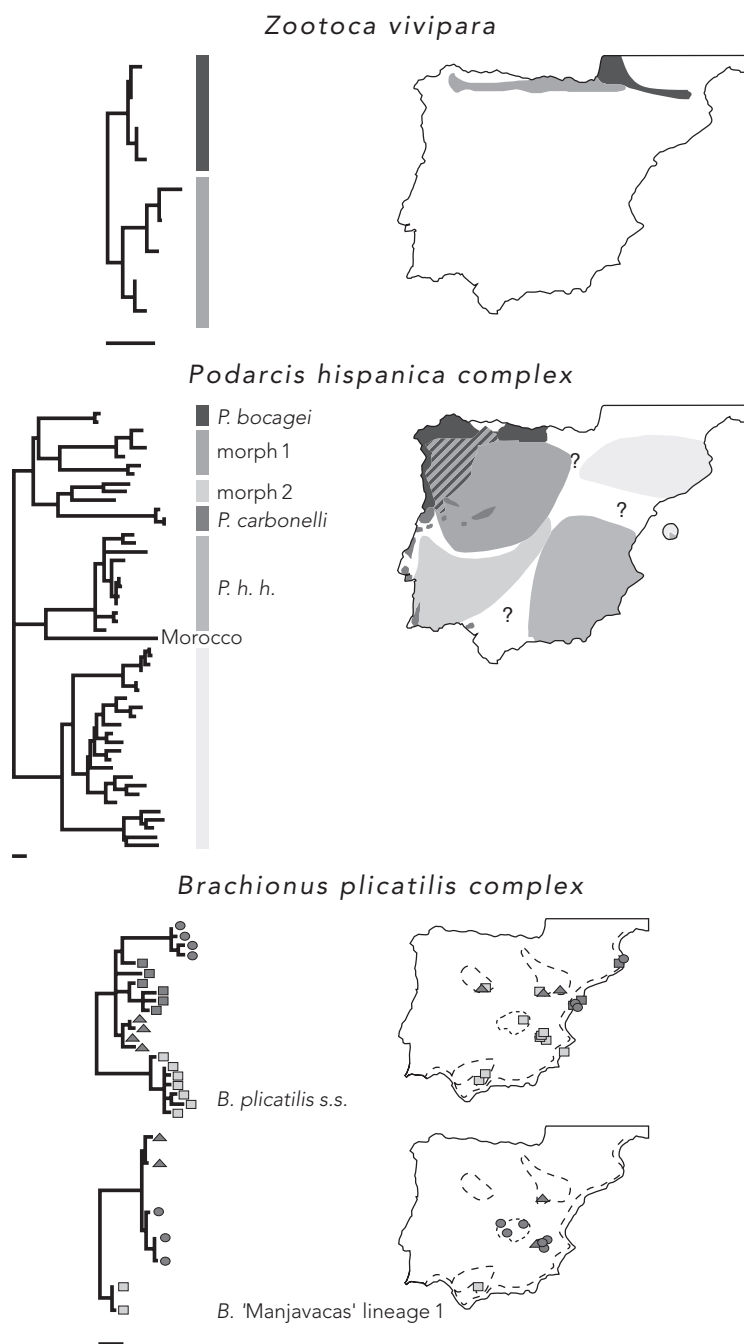


Figure 1. Continued.

**Table 1.** Species and species groups showing some evidence for two or more Iberian refugia. The putative number and location of refugia, and the methods used are indicated.

Taxon	Putative number and location of Iberian refugia	Remarks	Methods	Reference
<b>Plants</b>				
<i>Calluna vulgaris</i>	2 Northern, Pyrenees		RFLP cpDNA	Rendell & Ennos 2002
<i>Quercus ilex</i>	2 Eastern, southern		RFLP cpDNA	Lumaret <i>et al.</i> 2002
<i>Hedera complex</i>	3? <i>H. hibernica</i> , <i>H. helix</i> , <i>H. maderensis</i>	5 Iberian samples	RFLP cpDNA, ITS RFLP, microsat.	Grivet & Petit 2002
<i>Senecio gallicus</i>	SW Portugal, S Spain		RFLP cpDNA, Allozymes, RAPD	Comes & Abbot 1998, 2000
<i>Pinus pinaster</i>	2, East, Betic		Allozymes	Salvador <i>et al.</i> 2000
<i>Pinus sylvestris</i>	3, Pyrenees, Betic, Central		RFLP mtDNA	Soranzo <i>et al.</i> 2000; Sinclair <i>et al.</i> 1999
<i>Plantago media</i>	2?, C. Spain, Pyrenees	Reduced sampling in scattered southern populations	RFLP cpDNA	Van Dijk & Bakx-Schotman 1997
<i>Quercus</i> (white oaks)	2-4	6 species	RFLP cpDNA	Olalde <i>et al.</i> 2002; Petit <i>et al.</i> 2002
<b>Mammals</b>				
<i>Microtus agrestis</i>	2 Serra da Estrela and other		mtDNA	Jaarola <i>et al.</i> 2002, unpublished
<i>Apodemus sylvaticus</i> ?		Scattered sampling	mtDNA	Michaux <i>et al.</i> 2003
<i>Sorex coronatus</i> , <i>S. granarius</i>	Western, northern	Few samples from 2 populations.	mtDNA	Taberlet <i>et al.</i> 1994; Fumagalli <i>et al.</i> 1996
<i>Lepus sp.</i>	2-3, S Ebro R., Picos de Europa	1 or 2 populations sampled per species.	RFLP mtDNA	Pérez-Suárez <i>et al.</i> 1994
<i>Oryctolagus cuniculus</i>	2, SW and NE		RFLP mtDNA	Branco <i>et al.</i> 2000, 2002
<b>Reptiles and amphibians</b>				
<i>Lacerta schreiberi</i>	4		mtDNA	Paulo <i>et al.</i> 2001, 2002
<i>Podarcis hispanica</i>	>>2		mtDNA	Harris & Sa-Sousa 2000, 2002
<i>Zootoca vivipara</i>	2, Picos de Europa, Pyrenees		Allozymes, mtDNA	Guillaume <i>et al.</i> 2000; Surget-Groba <i>et al.</i> 2001
<i>Triturus marmoratus/pygmaeus</i>	2, Betic, North	Only 5 indivs. from 2 pops.	RFLP mtDNA	Wallis & Arntzen 1989
<i>Salamandra salamandra</i>	2, N and S		mtDNA	Steinfartz <i>et al.</i> 2000; Garcia-Paris <i>et al.</i> 2003
<i>Discoglossus</i>	2		mtDNA	García-París & Jockusch 1999
<i>Alytes</i>	>2		Allozymes, mtDNA	Arntzen & García-París 1995; Fonseca <i>et al.</i> 2003
<i>Chioglossa lusitanica</i>	2, NW, N Portugal		Allozymes, mtDNA	Alexandrino <i>et al.</i> 2000, 2002



**Table 1.** *Continued.*

Taxon	Putative number and location of Iberian refugia	Remarks	Methods	Reference
<b>Fish</b>				
<i>Barbus</i> ( <i>Luciobarbus</i> )	6, most major river systems		mtDNA	Zardoya & Doadrio 1998; Machordom & Doadrio 2001; Callejas & Ochando 2000;
<i>Leuciscus</i>	4, North, South, Arade, Mira		mtDNA	Brito <i>et al.</i> 1997; Zardoya & Doadrio 1998
<i>Anaocypris hispanica</i>	4, Guadiana		mtDNA	Alves <i>et al.</i> 2001
<i>Chondrostoma lemingii</i>	4, Guadiana, Guadalquivir, Tajo, Duero		Allozymes, mtDNA	Carmona <i>et al.</i> 2000
<i>Chondrostoma lusitanicum</i>	>3, Mira-Arade, Sado, Tajo-Samarra		mtDNA RFLP	Mesquita <i>et al.</i> 2001
<i>Aphanius iberus</i>	2, Mediterranean, Atlantic		Allozymes, mtDNA	Perdices <i>et al.</i> 2001
<i>Salmo trutta</i>	>= 2, Duero, Tajo-Guadiana		mtDNA	Machordom <i>et al.</i> 2000; Suárez <i>et al.</i> 2001
<b>Invertebrates</b>				
<i>Timarcha goettingensis</i>	Several N		mtDNA	Gómez-Zurita <i>et al.</i> 2000
<i>Chorthippus parallelus erythropus</i>	2?	Only 4 pops. sampled	nDNA,	Cooper & Hewitt 1993; Cooper <i>et al.</i> 1995
<i>Brachionus plicatilis</i>	1-2, SW and NE		mtDNA	Gómez <i>et al.</i> 2000
<i>Brachionus</i> 'Manjavacas'	1-2	Limited sampling	mtDNA	Gómez <i>et al.</i> 2002
<i>Matsucoccus feytaudi</i>	2, W and Betic		MtDNA?	Burban <i>et al.</i> 1999

It is well established that genetic diversity has a hierarchical nature, and phylogeographic lineages are both part of higher level phylogenies as well as finer-scaled population structure (Avice 2000). In addition, taxonomic level is not standardized across taxa. Therefore we do not feel it especially useful (even if it were possible) to try to standardize a molecular clock and to determine which separations have their origins within the Pleistocene. Instead we view the lack of mixing of phylogeographic lineages as evidence for a physically separate organismal history throughout the Pleistocene even if the last common ancestor of the molecules studied predated this boundary. It is this lack of gene flow between phylogeographic lineages represented by geographically structured clades that points to distinct Pleistocene 'refugia-within-refugia.'

One implication of this refugia-within-refugia scenario is that the high genetic diversity of the south, that is, the contrast 'Northern-Purity, Southern-Richness' (reviewed in Hewitt 2000) will most likely be due to two levels of variation. First, the higher demographic stability of populations in the southern refugia and the loss of diversity associated with the colonization process in the north can lead to a higher intrapopulation polymorphism in the south (Hewitt 1996). Second, this diversity can be highly structured geographically due to allopatric differentiation between populations and therefore contribute to the higher diversity of southern areas (Guillaume *et al.* 2000). Since the rapid northward expansion with climatic amelioration will approximate serial bottlenecks, with the leading edge contributing disproportionately to the genetic composition of the northern populations (Ibrahim *et al.* 1996), not all of the southern diversity will be represented. This is especially significant if the diversity is structured into divergent allopatric lineages, as we suggest in this review, since loss of a lineage during the expansion will lose more biological information than a comparable level of unstructured diversity. Moreover, since different refugia will have different access to trans-Pyrenean dispersal routes, one lineage may effectively exclude a second from later northward expansion and reinforce the disparity between north and south. It may not be surprising therefore that there has not yet been a clear demonstration of separate Iberian lineages recolonizing Europe by distinct routes (east and west of the Pyrenees), although for the viperine snake *Natrix maura* there is some evidence that separate Iberian lineages might have dispersed around each edge of the Pyrenees (Guicking *et al.* 2002). Much more rigorous sampling designs are required to assess the potential generality of this double-colonization pattern for other taxa.

### Consequences of pre-Pleistocene differentiation

The consequences of the refugia-within-refugia scenario are made still more complex by the cyclical nature of the Pleistocene Ice Ages. It is quite possible that not all the refugia were equally suitable during each glaciation. In addition, and as suggested by the data reviewed, all the refugia were not suitable for all species. Although patterns of pre-Pleistocene differentiation must be taken into account to interpret the likely original causes of population differentiation, the fact that multiple populations that diverged prior to the Pleistocene are found today in the area suggests persistent availability of suitable habitat in or near the putative refugial areas through the Pleistocene.

### Consequences of inadequate sampling of southern refugia

Special consideration of the southern Mediterranean refugia has been recommended when attempting the reconstruction of European phylogeography patterns (Taberlet 1998). However, this recommendation is not always easy

to follow, due to problems associated with sampling particular taxa. In addition, some authors have explicitly assumed that single mtDNA sequences from particular geographical regions, including refugia, are good representatives of the genetic variability of such regions (e.g. Bilton *et al.* 1998). As the data reviewed here shows, this assumption is frequently violated. In addition, as the different lineages present in southern refugia need not be sister taxa (i.e. a mono-phyletic group) due to complex patterns of population range contractions and expansions throughout the different Ice Ages (Steinfartz *et al.* 2000), monophyly of a random sample from the putative refugium and of samples from postglacial recolonized areas cannot necessarily be assumed.

One particularly serious consequence of the failure to recognize the true phylogeographic structure of the southern peninsulas is the incorrect inference of northern refugia. In this context 'northern' refers to areas north of the Pyrenees or Alps. Studies may, for example, infer a refuge in southern France because this locality contains haplotypes very distinct from those sampled from Iberia, Italy or the Balkans. Without extensive sampling of Iberia, however, it would not be possible to distinguish a northern refugium scenario from range expansion into southern France from a second non-sampled Iberian refugium. Since Iberian multiple refugia have been shown to be so common, care must be taken to sample extensively in southern Europe before concluding that haplotype distributions indicate Ice Age survival in northern regions. Unfortunately, this is an aspect that studies on northern refugia fail to address (Bilton *et al.* 1998; Stewart & Lister 2001). Finally, as Mediterranean refugia often display high levels of genetic diversity, and, if the occurrence of several refugia within refugia in the south is common, biased conclusions could be drawn if sampling is poor in these areas, or misses whole areas containing putative refugia. These problems are more likely to be widespread in terrestrial organisms, where the Iberian Peninsula is more likely to have harbored the populations that served for the colonization of northern Europe, than for freshwater organisms, which are often endemic to the Peninsula. The European hedgehog can illustrate the problems that incomplete sampling of southern refugia can pose for the interpretation of European phylogeographic patterns. The hedgehog mtDNA lineage sampled in the Iberian Peninsula, one of the two presumed glacial refugia in western Europe, was involved in the colonization of France, the UK and Ireland (Santucci *et al.* 1998; Seddon *et al.* 2001). A recent study including additional samples and using a larger mtDNA fragment identified two strongly supported monophyletic lineages in this clade (Seddon *et al.* 2001). One of these lineages was present in Iberia and southern France, the other one in France, UK and Ireland, and this geographic distribution suggests survival in different glacial refugia. One of the refugia occupied by this clade seems to have been in the Iberian Peninsula, as most of the diversity, including basal haplotypes, is present there, but the location of the other one is unclear. Although the basal haplotypes of the latter group are present in northern France, the current absence

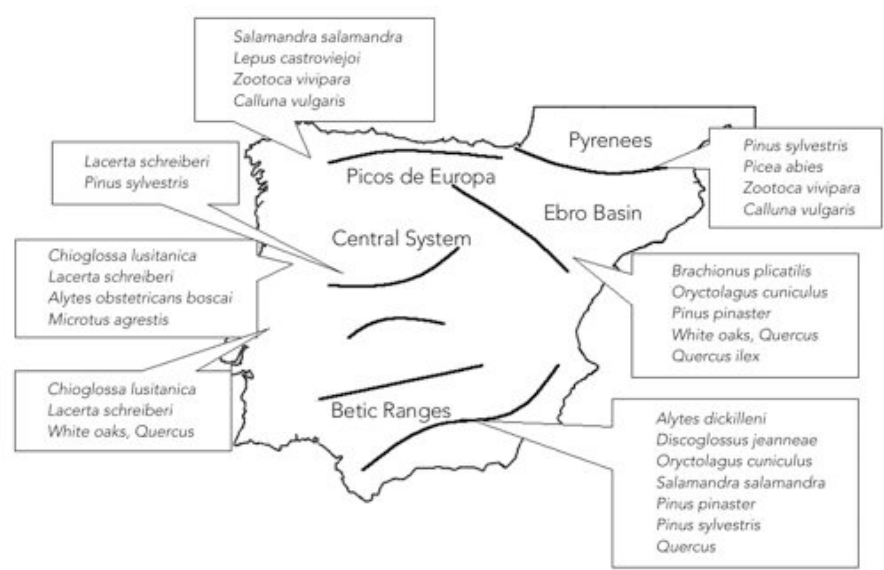
of samples from southwestern France and northeastern Spain precludes a conclusion about the location of the refugium for this clade though it could well be in Iberia. Although the occurrence of northern refugia should not be discarded *a priori*, and seems supported by fossil data in some species (Stewart & Lister 2001), exhaustive sampling of the southern distribution range where refugia are more likely, is imperative.

### Consequences of refugial extinction

The reduction or extinction of populations in putative refugia is another factor that can complicate interpretation in a refugia-within-refugia scenario. Distribution ranges and population sizes of many species have often contracted in historical times due to anthropogenic effects, as forests were cleared for farming or due to direct human exploitation, and also due to climate warming and the disappearance of suitable habitat in the Holocene, as documented for the brown bear (Taberlet & Bouvet 1994) and Atlantic salmon, *Salmo salar* (Consuegra *et al.* 2002). Therefore, refugial populations, especially along the southern fringes of their distribution, might have declined considerably to isolated relicts (Paulo *et al.* 2001) or even disappeared altogether during the Holocene. In any case, the remaining population relicts do not necessarily harbor the levels of genetic diversity expected for a refugium, as 1) they may have suffered genetic bottlenecks and loss of genetic diversity, and 2) an already extinct refugia within the Iberian Peninsula could have given rise to the currently more northern European population. If this scenario is suspected or documented, the only way ahead might be the analysis of ancient DNA in subfossil material or historical samples that can be compared with the DNA of the living descendants. Indeed, despite the limitations of ancient DNA analysis, the few phylogeographic studies that have used this technique have yielded surprising results (Leonard *et al.* 2000; Consuegra *et al.* 2002).

### Patterns of phylogeographic concordance: how many refugia and where?

Comparative phylogeography can help to pinpoint refugia common to several species, and serve as an aid to disclose cryptic events of historical vicariance. The suggestion of common refugial areas for distinct phylogeographic lineages (phylogeographic concordance aspect III according to Avise 2000) can be reinforced if such areas also show evidence of high endemism (phylogeographic concordance aspect IV, Avise 2000). Although we found considerable heterogeneity in the number and location of putative glacial refugia of the case studies investigated, this was most probably due to different habitat requirements. Yet clear patterns of phylogenetic congruence were apparent. For terrestrial taxa at least seven putative glacial refugia were recognized (Figure 2). In freshwater fish, the refugia coincide with the main river basins and a number of minor ones (Figure 3). Although most of the studies lack geo-



**Figure 2.** Map of the Iberian Peninsula showing the approximate location of putative inferred terrestrial refugia and the fauna that supports them.



**Figure 3.** Map of the Iberian Peninsula showing the location of river basins and the freshwater fish species that support their status as glacial refugia.

graphic precision pinpointing the localization of refugia, the suggested terrestrial refugia coincide quite strikingly with areas of endemism recognized by plant and animal biogeographers (Gómez-Campo *et al.* 1984; García-Barros *et al.* 2002), which tend to occur in the mountain ranges. Thus, the Betic ranges are pointed out as a putative refugium in three species of amphibians and reptiles, three trees and possibly the rabbit. This is not surprising, as the Betic ranges display the highest plant biodiversity and level of endemism in continental Europe (Gómez-Campo *et al.* 1984). One amphibian and one reptile species support the occurrence of two glacial refugia in Central Portugal. The Serra da Estrela in central Portugal is indeed an area rich in endemics (Gómez-Campo *et al.* 1984). In addition, an analysis of the distribution of western Iberian earthworm fauna suggests two biogeographical areas one north and the other south of the Mondego River (Rodríguez *et al.* 1997), a barrier found to separate approximately the current lineages of *Chioglossa lusitanica* and *Lacerta schreiberi*. The occurrence of a refugium in or near the Pyrenees is supported by a tree species, *Pinus sylvestris*, the heather *Calluna vulgaris*, the lizard *Zootoca vivipara* and the presence of a European refugium for the silver fir, *Abies alba* (Konnert & Bergmann 1995). The Pyrenees is indeed an area rich in animal and plant endemics, which suggests that in spite of being covered by an ice cap during glacial maxima, at least areas in or near the Pyrenean range served as glacial refugia (Gómez-Campo *et al.* 1984; García-Barros *et al.* 2002). Other areas of endemism also likely to have acted as glacial refugia are Picos de Europa and the Central System. An eastern refugium on the Mediterranean coast of Spain, possibly close to the Ebro valley, has been suggested for rotifers (Gómez *et al.* 2000), rabbits (Branco *et al.* 2000) *Quercus ilex* (Lumaret *et al.* 2002) and *Pinus pinaster* (Salvador *et al.* 2000). The Ebro River basin has indeed been recognized as a center for endemism for steppe fauna, and the continuity of its flora and fauna since the Tertiary is also well supported (Ribera & Blasco-Zumeta 1998). Further studies can help to delimit and possibly increase the number of separate glacial refugia for Iberian flora and fauna, and to sharpen our perception of which habitats were present.

## Conclusions and recommendations

To paraphrase Hewitt (1996), the Iberian Peninsula has been shown to be an excellent 'theatre for phylogeographic analysis'. In spite of its small size compared to other regions used for comparative phylogeography at a regional level, and the limited number of case studies available, the Iberian Peninsula clearly comprised a number of separate glacial refugia during the Pleistocene Ice Ages. Evidence of concordant patterns, suggesting shared regional refugia, was found, although more studies are needed to allow better analysis of these patterns. These putative refugia are remarkably congruent with centers of endemism for the current flora and fauna in Iberia, a conclusion that



fits well with the continuum between phylogeography and biogeography (Avice 2000). Neglecting the occurrence of multiple Iberian lineages can result in poor or biased sampling and lead to misleading conclusions when attempting European phylogeographic studies. Finally, the acknowledgement of the higher biodiversity awarded by the persistence of these separate refugia throughout the Pleistocene Ice Ages can be highly informative in the design of conservation areas in the Iberian Peninsula and can contribute to the understanding of the historical patterns that have given shape to the rich biodiversity of this corner of the planet.

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