

Research Article

**Phylogeographic insights of the lowland species *Cheirolophus sempervirens* in the southwestern Iberian Peninsula**

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**Abstract** The southwestern Iberian Peninsula is an important biogeographic region, showing high biodiversity levels and hosting several putative glacial refugia for European flora. Here, we study the genetic diversity and structure of the Mediterranean, thermophilous plant *Cheirolophus sempervirens* (Asteraceae) across its whole distribution range in SW Iberia, as a tool to disentangle some of the general biogeographic patterns shaping this southern refugia hotspot. Null genetic diversity was observed in the cpDNA sequencing screening. Nonetheless, AFLP data revealed high levels of among-population genetic differentiation correlated to their geographic location. Our results suggest longer species persistence in southern Iberian refugia during glacial periods and subsequent founder effects northwards due to colonizations in warmer stages (i.e. the southern richness to northern purity pattern). Additionally, our phylogeographic analyses indicate the presence of two separate genetic lineages within *Ch. sempervirens*, supporting the hypothesis of multiple minor refugia for SW Iberia in agreement with the refugia within refugia model.

**Key words:** AFLP, genetic diversity, genetic structure, glacial refugia, Mediterranean, Pleistocene climatic oscillations, southern Europe.

The Iberian Peninsula is a widely recognized hotspot of biodiversity (Médail & Quezél, 1999), and represents a key area from a biogeographic point of view (Nieto Feliner, 2014). In this way, the scientific community has largely proven the significant role played by this region as a major refugium for flora and fauna during Pleistocene glaciations (Taberlet et al., 1998; Hewitt, 2011). Until recently, it was widely agreed that the entire Iberia acted -similarly to other Mediterranean peninsulas such as the Italian or the Balkan- as a single refugium for biodiversity during the coldest periods of climatic oscillations (e.g. Hewitt, 2001). However, in recent times, the idea of multiple refugia occurring within the Iberian Peninsula (i.e. the refugia within refugia model) has been largely supported by several works reviewing phylogeographic patterns of different plant and animal taxa (e.g. Olalde et al., 2002; Gómez & Lunt, 2007; Médail & Diadema, 2009). Indeed, even considering multiple independent refugia would not adequately reflect the complexity of the biogeographic processes that occurred in Southern European peninsulas during Pleistocene climatic oscillations (Nieto Feliner, 2011), and the relationship among minor refugia nested within the so-called larger refugia (e.g. big peninsulas) still remains under discussion (Hewitt, 2001).

Many studies dealing with the effects of Pleistocene glaciations in European flora have focused on temperate or alpine species that survived in Mediterranean refugia during colder periods, but whose distribution range after Ice Ages extended across the continent, including glaciated areas (e.g. Taberlet et al., 1998, Comps et al., 2001; Weiss & Ferrand, 2007; Sanz et al., 2014). Only recently, the evolutionary history of plant species exclusively occurring along the European southern peninsulas (e.g. in the Balkan (Surina et al., 2011; Grdiša et al., 2014) or the Italian (Španiel et al., 2011; Hardion et al., 2014)) has been investigated, deeply contributing to better understanding the biogeographic patterns that influenced these refugia hotspots during Pleistocene climatic oscillations. Focusing on the Iberian Peninsula, similar phylogeographic studies have lately zoomed in endemic and subendemic taxa from the Pyrenees (e.g. Segarra-Moragues et al., 2007), the

Central Range (e.g. García-Fernández et al., 2013) or the Mediterranean coast (e.g. Garnatje et al., 2013), all of them considered refugia-rich areas according to the recent reviews by Gómez & Lunt (2007) or Médail & Diadema (2009) (Fig. 1). These fine-scale works have been useful to reveal a significant diversity of processes and patterns occurring on Mediterranean minor refugia during climatic oscillations, comparatively more complex than those observed in non-Mediterranean refugia (Nieto-Feliner, 2014). The southwestern half of the Iberian Peninsula (hereafter SW Iberia) also hosts multiple regional Pleistocene refugia according to the aforementioned review studies (see Fig. 1). In addition, many endemic species (García-Barros et al., 2002) – as well as numerous subendemic plants whose only Eurasian populations occur on this particular area (Rodríguez-Sánchez et al., 2008) – inhabit along the SW coast of Spain and Portugal. However, very scarce phylogeographic attention has been paid on this region to date, so the effects of climatic oscillations on the flora from SW Iberia are still poorly understood.

The effects of Quaternary climatic oscillations in the flora of southern European refugia have been proposed to be less drastic – and therefore more complex – than in northern regions, where extinction was the dominant process (Birks & Willis, 2008; Nieto Feliner, 2011). In this regard, thermophilous plants are considered to be particularly interesting for the study of the climatically-buffered Mediterranean refugia, since they are likely to be more severely affected by glaciations than cold-adapted taxa (Hewitt, 2000; Stewart et al., 2010). Recent biogeographical works investigating the complex effects of climatic oscillations on various lowland southern refugia successfully employed thermophilous plants (e.g. *Arundo plinii* Turra (Hardion et al., 2014); *Cheirolophus intybaceus* (Lam.) Dostál (Garnatje et al., 2013); *Tanacetum cinerariifolium* Sch.Bip. (Grdiša et al., 2014)) as models of study. Thermophilous species from lowland or coastal habitats are likely to respond to climatic oscillations with geographical (mostly latitudinal) range shifts – contributing to the so-called southern richness to northern purity pattern (hereafter SR-NP; Hewitt,

2000) genetic diversity pattern. In contrast, cold-resistant or alpine taxa are believed to respond with elevation range shifts limited by their island-like environment (i.e. “island in the sky”, Hewitt, 2001), resulting on genetic structure distributed in different isolated refugia with equivalent diversity levels (Surina et al., 2011). Consequently, lowland and coastal flora from Mediterranean regions such as SW Iberia should fit better to the SR-NP genetic pattern rather than to the equivalent isolated refugia pattern. However, the response to this phylogeographical and microevolutionary processes in southern European species yet remains poorly studied (Hewitt, 2011).

*Cheirolophus sempervirens* (L.) Pomel (Asteraceae) is a species particularly well suited for testing biogeographical hypotheses related to the effects of Pleistocene climatic oscillations in lowland flora from SW Iberia. This is a thermophylous shrub with a narrow cold tolerance, inhabiting humid valleys and montane stage with clear maritime influence (Susanna, 1991). The species is a subendemism occurring exclusively in the SW Iberian Peninsula, excepting for a few isolated populations cited from the northern mountains of Algeria. Interestingly, the distribution of *Ch. sempervirens* in the Iberian Peninsula shows a linear distribution, following moist and warm locations close to the coast from Malaga to Coimbra (see Fig. 1) and extending along 500 km of latitudinal range. Additional isolated locations have been cited in more inland regions –i.e. Salamanca- or in northern latitudes – i.e. Galicia – of the Iberian Peninsula, but these citations are thought to be either misidentifications (Susanna, 1991) or have not been found in recent field surveys (D. Vitales, pers. comm.). Similarly to the rest of the members of the genus, *Ch. sempervirens* has an outcrossing pollination and produces seeds that disperse by gravity very close to the mother plant (Ruiz de Clavijo & Devesa, 2013). The evolutionary history of *Cheirolophus* has been recently studied (Vitales et al., 2014b) and – as reported for other Mediterranean taxa (e.g. Migliore et al., 2012; Besnard et al., 2013; Fiz-Palacios & Valcárcel, 2013) – the time-calibrated

phylogeny describes the first divergence of the main Mediterranean lineages of the genus close to the onset of the Mediterranean climate (ca. 3.1 Ma, late Pliocene) and their diversification during the Quaternary (around 1.3 Ma). The divergence among *Cheirolophus sempervirens* and its closely related taxa *Ch. mauritanicus* (Font Quer) Susanna from northern Morocco – considered *Ch. sempervirens* subsp. *mauritanicus* (Font Quer) M. Ibn Tattou by other authors (Fennane & Ibn Tattou, 1998) – was reported to occur very recently (ca. 80.000 years ago). Therefore, we may hypothesize that the genetic diversity showed by *Ch. sempervirens* should have been shaped by the climatic fluctuations of the last glacial period (0.126–0.0117 Ma), affecting the Mediterranean region during this episode of earth history (Thompson, 2005).

In this context, the main goal of our study was to analyze the phylogeography of *Ch. sempervirens* across its whole distribution range in SW Iberia as a complement to disentangle some general biogeographic patterns affecting this southern refugia hotspot. Specifically, we proposed to: i) test whether *Ch. sempervirens* shows signs of the SR-NP genetic diversity pattern reported, based on other plant taxa, in other southern European refugia, and ii) investigate whether the phylogeographical structure of this thermophilous species fits the refugia within refugia model proposed for the Iberian Peninsula.

## **Material and Methods**

### **Sampling strategy**

*Cheirolophus sempervirens* was sampled from 10 populations located on SW Iberian Peninsula, covering a most of the distribution range of the species. The number of analysed individuals per population ranged between 5 and 10 depending on population size and uneven success of laboratory procedures. Details of locations and number of sampled individuals of each population are listed in Table 1 and Fig. 1. Leaf material was immediately dried in silica-gel and stored at room temperature

(20 - 25°C) until DNA extraction.

### **DNA isolation, AFLP fingerprinting and DNA sequencing**

Total genomic DNA was extracted from fragments of silica-gel-dried leaf tissue following the protocol of Doyle & Doyle (1987) with slight modifications. DNA samples were cleaned using QIAquick columns (Qiagen, Valencia, CA, USA) and their quality and DNA concentration was determined using NanoDrop ND-1000 spectrophotometry (ThermoScientific, Wilmington, DE, USA).

The AFLP technique was carried out following the protocol described in Vos et al. (1995) with some modifications (see Vitales et al., 2014a). After a primer trial involving 12 selective primers, three primer pairs were finally chosen: EcoRI-AC/MseI-CTT; EcoRI-AG/MseI-CTC; and EcoRI-AT/MseI-CAG. The success of each step was tested by running the PCR products on a 1.5% agarose gel. Fragments were run on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with 10 µL High Dye (deionized formamide) and 0.2 µL GeneScan™ 500 ROXTM Size Standard per sample. Amplified fragments were genotyped using GeneMarker®AFLP/Genotyping software (version 1.9; SoftGenetics, LLC, State College, PA, USA). AFLP error rates were calculated following Bonin et al. (2004). Twenty random samples per primer combination were replicated to ensure reproducibility, repeating all parts of the AFLP protocol. All alleles with an error rate >5% were eliminated. In addition, those individuals that did not produce scorable patterns for all three primer combinations were also excluded. Out of the 93 attempted individuals, 85 (91 %) were retained in further data analysis.

We also conducted a screening test for DNA sequencing involving four highly variable chloroplast markers (*rpoB-trnD*, *rps16-trnK*, *rpl32-trnL* and *trnS-trnC*), which were sequenced for a few individuals of different populations. All these regions were newly amplified and sequenced for nine individuals from Andalusia, Algarve, Centro and Coimbra regions following protocols in

Vitales et al. (2013).

## Data analysis

Based on AFLP data, the unbiased heterozygosity within populations ( $H_j$ ), the average gene diversity within populations ( $H_w$ ), and total gene diversity in the species ( $H_t$ ) were calculated using the software TFPGA v. 1.3 (Miller, 1997). Additionally, the band richness ( $Br$ ) (Coart et al., 2005) and the percentage of polymorphic loci ( $PLP$ ) with a significance of 1% ( $P = 0.99$ ) were calculated according to the rarefaction method of Hurlbert (Petit et al., 1998), and conditioned to the smallest population size ( $N = 5$ ) with the software AFLPDIV v. 1.0. We also conducted measures of genetic rarity: the number of private alleles in each population; and the frequency-down-weighted marker values ( $DW$ ) index of Schönswetter & Tribsch (2005), calculated as ratio of means –which makes the measure less sensitive to differences in sample size between localities– using AFLPDAT (Ehrich, 2006). R software (R Development Core Team, 2015) was used to perform Spearman rank correlation analyses between the genetic diversity ( $H_j$ ) of populations and their latitude, as well as between the  $DW$  index of populations and their latitude. Pairwise  $F_{ST}$  values were estimated for each pair of populations studied with AFLP SURV 1.0 (Weir & Cockerham, 1984). Significance was evaluated through 10000 permutations. Finally, we conducted AMOVA analyses by using ARLEQUIN 3.5 (Excoffier et al., 2005) to estimate genetic differentiation attributable to population subdivision. To further characterize the spatial genetic distribution in this species, we performed Mantel tests based on genetic distance matrices constructed with  $F_{ST}$  values between populations and on geographical matrices calculated by the Euclidean distance (X and Y coordinates) between populations using ArcGIS 9.1 (ESRI, Redlands, CA, USA). Mantel tests were performed on ARLEQUIN 3.5 with 100000 permutations and considering a p-value limit of 0.05.

Population genetic structure revealed by AFLP was investigated using phylogenetic,



clustering and multivariate analysis. We used the Neighbor-Net method (Bryant & Moulton, 2004) carried out with SplitsTree v.4.10 (Huson & Bryant, 2006) to construct a distance-based network using the Jaccard coefficient (Jaccard, 1901), which is restricted to shared band presence rather than shared absence. A Neighbor-Joining (NJ) analysis of the same matrix, with 1000 bootstrap replicates, was also performed using SplitsTree 4.10. Bayesian clustering analyses were carried out using STRUCTURE 2.3 (Hubisz et al., 2009). We considered the admixture ancestry model and the correlated allele frequencies. Ten independent simulations were run for each possible number of genetic groups ( $K$ ) (from  $K = 1$  to 10), using a burn-in period of  $10^5$  generations and run lengths of  $5 \times 10^5$ . STRUCTURE HARVESTER (Earl & von Holdt, 2011) was employed to estimate the number of genetic groups ( $K$ ): we selected the  $K$  value that maximizes the probability of the data  $L(K)$  and we also considered the criterion proposed by (Evanno et al., 2005) based on the rate of change in the probability between successive  $K$  values,  $\Delta K$ . Similarities among individuals were also studied via Principal Coordinate Analysis (PCoA, Gower (1966)) using the Jaccard distance, in order to detect other possible relations that could not be visualized with assignment methods or phylogenetic analyses. This procedure was carried out with R software (R Development Core Team, 2015) using the vegan package (Oksanen et al., 2008).

## Results

Initially, 221 alleles were obtained from automatic genotyping of *Cheirolophus sempervirens* AFLP profiles. After manual correction, error rates calculation, elimination of small and troublesome alleles and low intensity peaks, a final matrix with 195 (88.2%) alleles was considered for subsequent analyses. The final data sets showed an error rate of 2.6%, which is below the maximum error rate percentage accepted for good AFLP reproducibility (5%) (Pompanon et al., 2005). None of the chloroplast regions analysed in the screening test yielded any variability among or within *Ch*.

*sempervirens* populations.

Population genetic diversity measures are shown in Table 1. Average gene diversity per population ( $H_w$ ) was  $0.0455 \pm 0.0245$ , being the results of the different genetic diversity index ( $H_j$ ,  $PLP$  and  $Br$ ) consistent among them. Private alleles were irregularly distributed across the studied populations: AND population showed five private fragments; ALG, ODE and MIL presented two; SET and COI showed one; and no private alleles were found in the rest of populations. The frequency-down-weighted marker values ( $DW$ ) also demonstrated considerable variation among populations, ranging between 567.466 (AND) and 164.459 (NAZ). Total genetic diversity for AFLP markers resulted to be  $H_t = 0.1602$ , and the AMOVA analysis revealed that most of the variability in *Ch. sempervirens* was attributable to differences among populations (73.62%,  $P < 0.001$ ; Table 2). Using the matrix of inter-population  $F_{ST}$  distances, and the matrix of geographical distances (in kilometres), the Mantel test indicated a significant correlation between genetic and geographical distances ( $r = 0.516$ ,  $P < 0.05$ ). Spearman rank correlation analyses revealed that within-population genetic diversity ( $H_j$ ) in *Ch. sempervirens* was not associated to the latitude of populations ( $r = -0.224$ ,  $P > 0.05$ , Fig. 2A;  $PLP$  and  $Br$  similar results, data not shown). In contrast, the same test indicated a significant negative correlation between the  $DW$  index and the latitudinal distribution of this species ( $r = -0.903$ ,  $P < 0.05$ ; Fig. 2B).

According to the Bayesian analysis of population genetic structure conducted with STRUCTURE, the populations of *Ch. sempervirens* showed the highest  $L(K)$  and  $\Delta K$  values for  $K = 2$  and  $K = 5$  (see Fig. S1). For  $K = 2$ , populations from south-western Portugal (Algarve and Alentejo Litoral) clustered separately from the rest of populations, whereas the one from Setubal Peninsula (SET) showed a considerable level of admixture among the two genetic groups (Fig. 3A). For  $K = 5$ , populations north of the river Tagus remained together while most of the others – excepting both populations located in Alentejo Litoral (ODE and MIL) which remained linked-constituted independent clusters (Fig. 3B). The Neighbor-Net (NN) and the Neighbor-Joining (NJ)

analyses showed considerably resolved phylogenetic reconstructions (NN, Fig. 4; NJ, Fig. S2), highly congruent with the genetic structure revealed by STRUCTURE.

The PCoA using the first two principal coordinates explained 52.2% of the total variation in the data and confirmed several relationships detected in the phylogenetic and cluster analyses (Fig. S3). The first coordinate (accounting for 38.6% of the total variation) distinguished two main groups of species: the south-western populations occurring on Algarve and Alentejo Litoral clearly segregated from the rest of populations, being SET from Setubal Peninsula intermediate between the two groups. The second coordinate (representing 13.6% of variation) mainly segregated the population from Andalusia (AND) in a different cluster. The five genetic groups proposed by STRUCTURE could also be recognized in this PCoA analysis (Fig. S3).

## Discussion

The null genetic diversity observed in the cpDNA sequencing survey of *Cheirolophus sempervirens* could be related to the putative recent evolutionary history of this species (Vitales et al., 2014b). In contrast, AFLP analyses provided considerable information on the magnitude and pattern of genetic variation existing in ten populations covering the whole distribution range of *Ch. sempervirens* in SW Iberian Peninsula. This result reinforces the idea that AFLP is a particularly suitable tool to perform phylogeographic analyses when other molecular markers (such as cpDNA sequencing) provide insufficient information (Després et al., 2003; Meudt et al., 2007). Overall, the data revealed high levels of genetic differentiation among populations and low genetic diversity within populations. This pattern indicates a strong genetic structure among *Ch. sempervirens* populations, which seems to be correlated to their geographic distance according to the Mantel test.

### **Southern richness versus northern purity in southwestern Iberian Peninsula**

The results of our study on *Ch. sempervirens* support the hypothesis of longer species persistence in southern Iberian refugia, and founder effects in northward colonizations (i.e. the SR-NP pattern). A significant association between the genetic rarity index  $DW$  and the latitude of populations was clearly observed in our analyses (Fig. 2B). Similarly, private alleles were much more frequent in the five southernmost populations (12 private alleles in total) than in the five northern populations (only one private allele). The abundance of rare and private alleles has been proposed as a characteristic signal of populations with a long *in situ* history, most probably going back to the last glaciation (Schönswetter & Tribsch, 2005; Ehrich et al., 2008). Indeed, genetic rarity measures such as the  $DW$  or the private alleles have been successfully employed to infer the glacial refugia patterns in numerous plants from different parts of the globe (e.g Mráz et al. (2007) in the Alps; Pérez-Collazos et al. (2009) in the Iberian Peninsula; Tremetsberger et al. (2009) in South America; Li et al. (2011) in Asia). Conversely, intra-population genetic diversity indexes did not show any correlation with the latitude of populations in *Ch. sempervirens* ( $H_j$ , Fig. 2A;  $PLP$  and  $Br$ , data not shown). Genetic rarity indexes have been seen as better indicators of historical processes rather than genetic diversity indexes, which mirror contemporary processes such as connectivity of populations and population sizes (Comps et al., 2001; Widmer & Lexer, 2001; Paun et al., 2008). In this way, the irregular distribution of within-population genetic diversity measures observed in *Ch. sempervirens* could be explained by the differences in regional abundance and population size between the studied localities (D. Viales, pers. comm.).

A similar outcome suggesting the southern survival of the species during the last Pleistocene glaciations and the more recent formation of the northern populations was also supported by the genetic structure of *Ch. sempervirens*. The five genetic clusters proposed by STRUCTURE (Fig. 3B) and recovered as well in the PCoA (Fig. S3) and the networking (Fig. 4) analyses showed a marked SR-NP distribution pattern. Specifically, the southernmost populations constituted four of

these genetic groups (almost one group per population), whereas the five northern populations were grouped in one single genetic cluster. Populations located in refugial regions are expected to present greater genetic structuring than those located in recolonized areas (Hampe & Petit, 2005; but see also De Lafontaine et al. (2013) for a contrasting pattern). Indeed, the occurrence of larger number of genetic lineages in areas rich in glacial refugia has been reported in former studies analyzing the phylogeography of different plant species (e.g. Schönswetter et al., 2003; Picó et al., 2008). In summary, both within-population indexes (i.e. genetic rarity) and genetic structure inferences suggest that *Ch. sempervirens* from SW Iberia responded to Pleistocene climatic oscillations with latitudinal range shifts, somewhat mirroring the performance of lowland flora from other southern European refugia.

### **Refugia within refugia in the southwestern Iberian Peninsula**

As reported for several organisms from worldwide distributed in different refugia hotspots (see Weiss & Ferrand, 2007; Shafer et al., 2010; Qiu et al., 2011 for some reviews) and particularly from Southern European refugia (Gómez & Lunt, 2007), the current genetic structure of *Ch. sempervirens* suggest the survival of the species in various glacial refugia across SW Iberia (i.e. the refugia within refugia model). This multiple refugia pattern is clearly depicted by one of the best clustering model ( $K = 2$ ) proposed by STRUCTURE (Fig. 3A) and supported as well by the other methodological approaches employed to study the phylogeography of *Ch. sempervirens*. At  $K = 2$ , the populations from Algarve and Alentejo Litoral (ALG, ODE and MIL) clustered in a differentiated group (lineage 1), a genetic segregation further supported with the high bootstrap (97%) assigned by the NJ analysis to the branch segregating those three southern Portuguese populations (Fig. 4). This phylogeographic split – together with the high genetic rarity levels showed by these populations – indicate that SW Iberian corner may be acting as an isolated glacial

refugium for *Ch. sempervirens*, a pattern already reported in other Iberian endemic plants such as *Senecio gallicus* Chaix (Comes & Abbott, 1998). The complementary genetic cluster at  $K = 2$  groups the five northernmost populations with the southernmost one from Malaga (Andalusia; AND), constituting an additional genetic lineage within this species (lineage 2). In this case, according to the higher genetic rarity values showed by Andalusian population (Table 1), the refugial area of this second phylogeographic lineage should be found in this southern region of Spain. Therefore, our data suggest that at least two independent refugia – probably located in southern Portugal and in southern Spain – may have played a primary role in the evolutionary history of *Ch. sempervirens* during Pleistocene climatic oscillations.

Notwithstanding, several authors have recently warned that the model of multiple independent refugia might be an oversimplification (Nieto Feliner, 2011; de Lafontaine et al., 2013), postulating that recurrent isolation and admixture processes would have shaped a more complex phylogeographic scenario in Pleistocene refugia hotspots. According as well to the  $K = 2$  model of STRUCTURE, the population from Setubal Peninsula (SET), located halfway from Algarve-Alentejo Litoral localities (lineage 1) and from the rest of northern populations (lineage 2), showed significant admixture between both genetic lineages. Likewise, the NN and the PCoA results also indicated that SET population shows an intermediate position between both main genetic clusters. On the one hand, this result may suggest that Tagus river may act in *Ch. sempervirens* both as a soft barrier and as a secondary contact zone of lineages diversified in different phases of climatic oscillation cycles. Indeed, river basins have been proposed as genetic barriers in other plants affected by Pleistocene glaciations in southern European refugia (e.g. Picó et al., 2008; Grdiša et al., 2014). On the other hand, intermediate genetic position and admixture clustering pattern in SET population could also be interpreted as this one being the source of northern populations, which subsequently could have lost part of their genetic diversity in stepping stone founder effects.

However, in this latter scenario, the genetic link between northern and AND populations would remain unexplained.

## Conclusions

Regarding the last advances in Mediterranean phylogeography, the existence of regional refugia with accurate delimitation (as proposed by Médail & Diadema (2009)) has been considered too simplistic by several authors (e.g. Nieto Feliner, 2011, 2014). According to our results, these fixed refugia are again discredited, because most of them include populations of *Ch. sempervirens* with a putative post-glacial origin (e.g. COI, FIG or SET) rather than populations with high conservation of genetic rarity (e.g. MIL, ODE, AND). Consequently, this representation of delimited universal refugia for every Mediterranean taxa does not seem to be applicable to *Ch. sempervirens*, supporting the individualistic response of species to changes in climate conditions (Stewart et al. 2010). In our work, the limited sampling has shed light into the general phylogeographic patterns showed by this lowland plant from SW Iberia, but prevent us to infer more precise details about its evolutionary history. Therefore, caution must be taken when hypothesizing on the mode and location of particular phylogeographic events that affected this (and other) species during the last Pleistocene glaciations. Further studies in the southwestern Iberian refugia will be necessary to test whether the phylogeographic history of *Ch. sempervirens* is idiosyncratic or represent more general patterns.

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**Table 1** Sampling information and genetic diversity indexes of *Cheirolophus sempervirens* from the Iberian Peninsula. Code, locality, geographical coordinates, number of sampled individuals (*N*), and genetic diversity indexes assessed by AFLP in 10 populations of *Ch. sempervirens* from the Iberian Peninsula. Genetic indices: heterozygosity (*H<sub>j</sub>*); percentage of polymorphic loci (*PLP*); band

Code	Locality	Latitude °N	Longitude °W	<i>N</i>	<i>H<sub>j</sub></i>	<i>PLP</i>	<i>Br</i>	<i>DW</i>	Private alleles
AND	Andalusia: Málaga, Ronda	36.692	5.266	7	0.0321	0.077	1.064	567.466	5
ALG	Algarve: Aljezur	37.308	8.802	10	0.0340	0.082	1.060	436.645	2
ODE	Alentejo Litoral: Odemira	37.638	8.620	9	0.0743	0.168	1.124	359.830	2
MIL	Alentejo Litoral: Milfontes	37.726	8.769	10	0.0429	0.102	1.070	307.222	2
SET	Centro: Setubal, Sesimbra	38.458	9.113	9	0.0526	0.117	1.100	284.285	1
LIS	Centro: Lisboa, Sintra	38.788	9.403	5	0.0820	0.168	1.168	243.147	0
FOZ	Leiria: Foz do Arelho	39.428	9.187	9	0.0262	0.066	1.050	186.496	0
NAZ	Leiria: Nazaré	39.607	9.079	10	0.0072	0.020	1.016	164.459	0
COI	Baixo Mondego: Coimbra	40.185	8.436	6	0.0741	0.148	1.134	207.150	1
FIG	Baixo Mondego: Figueira da Foz	40.202	8.899	10	0.0304	0.082	1.064	199.991	0

richness [*Br*]; frequency-down-weighted marker values index (*DW*); and private alleles.

**Table 2** Analyses of molecular variance (AMOVA) of *Cheirolophus sempervirens* populations based on AFLP markers

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation	<i>P</i>
Among populations	9	805.76	10.15	73.62	<0.001
Within populations	75	272.80	3.64	26.38	<0.001
Total	84	1078.56	13.79		

## FIGURE LEGENDS

**Fig. 1.** Map of geographic distribution and sampled populations of *Cheirolophus sempervirens* in the Iberian Peninsula. Dashed line indicates distribution range of *Ch. sempervirens*. Shaded areas correspond to putative glacial refugia identified by Médail & Diadema (2009) in this region.

**Fig. 2. A,** The genetic diversity ( $H_j$ ) and **B,** the genetic rarity ( $DW$ ) of each sampled population of *Cheirolophus sempervirens* along their latitudinal distribution in the Iberian Peninsula, calculated using amplified fragment length polymorphism (AFLP). The Spearman rank correlation index and the p-value are also indicated.

**Fig. 3.** Bayesian estimation of genetic structure within *Cheirolophus sempervirens* inferred with STRUCTURE from AFLP data, according to the best models [(**A**)  $K = 2$  and (**B**)  $K = 5$ ] proposed by STRUCTURE HARVESTER.

**Fig. 4.** Neighbor-Net based on Jaccard distance obtained from 85 individuals of ten sampled populations of *Cheirolophus sempervirens*. Bootstrap values above 50% derived from a Neighbor-Joining analysis are given for the main branches.

**Fig. S1.** Plots with the estimates of the number of  $K$  groups based on the  $\Delta K$  statistic of Evanno et al. (2005) (**A**) and the mean likelihood  $Ln(K)$  calculated with STRUCTURE HARVESTER (**B**).

**Fig. S2.** Neighbor-Joining tree of 85 *Cheirolophus sempervirens* individuals from the 10 sampled populations. Bootstrap values greater than 50% that were obtained after 1000 permutations are indicated on the branches.

**Fig. S3.** Principal coordinates (PCoA) plot of AFLP data for the *Cheirolophus sempervirens* populations included in this study. Different symbols correspond to different populations as shown in the legend in the right side.