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Conservation genetics of the rare Iberian endemic *Cheirolophus uliginosus* (Asteraceae)

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Running title: Conservation genetics of *Cheirolophus uliginosus*

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Abstract

Cheirolophus uliginosus is a rare species, endemic to the south-western Iberian Peninsula and listed as a characteristic taxon from the temperate Atlantic wet heaths, a priority habitat for conservation by the European Union. The conservation status of this species in most of its distribution area is poorly known. However, in recent times the disappearance of populations and a reduction in the number of individuals on some of them has been noticed. In this context, we analysed the effects of population size on genetic diversity, revealing that genetic erosion and inbreeding depression could be having a significant impact on smaller populations. Furthermore, we studied the patterns of genetic structure and variability at the species level, finding a strikingly low within-population diversity and high among-population genetic differentiation. Finally, the genetic structure analyses suggested a long and complex phylogeographic history of *Ch. uliginosus* in the region, in agreement with the climate relict status proposed for this species.

Keywords: AFLP-cpDNA-endangered species-genetic diversity-genetic structure-Habitats Directive-phylogeography-population genetics-rare species

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Introduction

Wet heathlands are extraordinarily valuable habitats because of the biodiversity they harbor and the important ecological functions and services that they provide (Webb, 1998; ALFA, 2004; Muñoz *et al*., 2012). Like many other wetland habitats, these are also very sensitive ecosystems to natural and anthropogenic disturbances (Cristofoli, Monty & Mahy, 2010; LePage, 2011). For those reasons, temperate Atlantic wet heaths with *Erica ciliaris* L. and *Erica tetralix* L. are ranked as priority habitats for conservation by the European Union (Habitats Directive 92/43/EEC, code 4020). These humid heathlands typically occur along the Atlantic shores of Spain, Portugal, France and some localities in south-west England (MacSharry, 2009). In Portugal and south-western Spain they represent a naturally fragmented distribution (Ojeda, 2009), occupying a few scattered and rare areas where the specific environmental conditions required for this ecosystem take place at such lower latitudes. From the point of view of biodiversity value, these southern wet heaths are particularly interesting, showing comparatively higher species richness and a larger amount of narrow endemics than in the northern representatives (Andres & Ojeda, 2002). However, given the high dependence on water availability as well as their occurrence in the Mediterranean climate, the Iberian heathlands are recognised as especially vulnerable to global change (Schröter *et al*., 2005). For this reason, studying the diversity, phylogeographical patterns and the conservation biology of the endemic flora that constitutes these threatened habitats can help understanding their importance and vulnerability.

Cheirolophus uliginosus (Brot.) Dostál (Asteraceae) is listed as a characteristic species of temperate Atlantic wet heaths according to the Interpretation Manual of European Union Habitats–EUR28 (2013). However, the geographic distribution of *Ch. uliginosus* is limited to the south-western and western Iberian Peninsula (Ruiz de Clavijo & Devesa, 2013), so that this species could be considered as a distinctive component of the Mediterranean-climate wet heathland flora. This Iberian endemic plant is a hemicryptophyte –the only herbaceous member of the genus– showing vegetative reproduction through rhizomes and an allogamous breeding system (Bañares *et al*., 2010). Flowers are hermaphroditic, arranged in capitula and most likely pollinated by generalist hymenoptera. Seeds have a deciduous pappus and they are gravity-dispersed. The natural rarity of the habitat where *Ch. uliginosus* occurs has probably prevented this species from becoming as widespread as other more generalist relatives (e.g. *Ch.*

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sempervirens Pomel) (Susanna, 1993). In addition, these ecosystems are under increasing anthropogenic pressures, such as forest planting, grazing, urbanization, drying out, pollution, mismanagement of water levels and competition with ruderal vegetation (ALFA, 2004; Ojeda, 2009). Consequently, numerous populations of *Ch. uliginosus* cited during the early and middle 20th century and compiled by Rivas-Martínez *et al*. (1980) or Susanna (1993), have not been confirmed during recent field surveys or have undergone a significant reduction, which has led to extinction in some cases. Besides, primary consequences of such habitat fragmentation and reduction of population size are increased inbreeding rates and genetic drift (Frankham, Ballou & Briscoe, 2002). Notwithstanding, this species is not yet included in the current Spanish legislation of endangered species (Catálogo Español de Especies Amenazadas, Real Decreto 139/2011), but it is classified in the Spanish red book of threatened vascular flora (Bañares *et al*., 2010) as CR ("Critically Endangered") according to IUCN criteria. In Portugal, where most of the populations of *Ch. uliginosus* occur, its conservation status is poorly known since no red list of threatened vascular flora exists for this country. Nevertheless, according to the Sociedade Portuguesa de Botânica, it is recognised as a rare species (Porto *et al*., 2010).

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The genus *Cheirolophus* Cass. has been comprehensively studied from an evolutionary point of view (Garnatje *et al.*, 1998, 2012; Garnatje, Garcia & Canela, 2007; Susanna, Garnatje & Garcia-Jacas, 1999; Vitales *et al.*, 2014a). In a recent phylogenetic reconstruction (Vitales *et al*., 2014a), *Ch. uliginosus* is partially resolved as an early-diverged lineage of the genus, suggesting a climate relict status for this species in the Atlantic coast of the Iberian Peninsula. Additional molecular cytogenetic studies (Garnatje *et al.,* 2012) support the significant evolutionary distinctiveness of this species and hence highlighting its important conservation value (Cadotte & Davies, 2010). At the same time, in a previous study focusing on the reproductive features of this species (Vitales *et al*., 2013), a preliminary genetic survey evidenced certain inter-population variability in some plastid DNA regions suggesting that plastid markers could be also a helpful complement to understand the phylogeographic history of this rare species. DNA fingerprinting methods –e.g. amplified fragment length polymorphism (Vos *et al*., 1995; AFLP)– have also proven helpful in studying the phylogeography and population genetics in different *Cheirolophus* species (Garnatje *et al*., 2013; Vitales *et al*., 2014b).

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In the present work we use both plastid DNA sequences and AFLP markers to study *Ch. uliginosus* from a conservation genetics perspective and to gain better understanding of the phylogeographic history of this endemic species to the southern heathlands of the Iberian Peninsula. Specifically, we aim to: i) examine the genetic diversity at the population and species levels, checking whether small populations are being affected by a particular loss of genetic diversity; ii) discuss the patterns of genetic structure among populations in relation to the early and ancient evolutionary history of *Ch. uliginosus* in the context of the habitat where it occurs; and iii) infer conservation management strategies for this species according to the results obtained.

Materials and methods

Sampling strategy

Cheirolophus uliginosus was sampled from 17 populations located in the south-western Iberian Peninsula. These were all the populations in the area we were able to find consulting the herbarium records and experts in the local flora (E. Sánchez-Gullón, V. Girón, J. Paiva & M. Porto, pers. comm.). Details of locations, collectors and herbarium vouchers of each population are listed in Table S1 and Fig. 1. To avoid sampling clones, we only collected individuals placed at a minimum distance of 5 meters from each other. Rhizomes of *Ch. uliginosus* are small and vegetative reproduction is expected to occur just a few centimetres away from the mother plant. In the case of some small populations, the reduced number of individuals limited the distance among sampled plants as well as the optimal sampling size. Leaf tissue was immediately dried in silica gel and stored at room temperature until DNA extraction.

The number of individuals in each population was visually counted (Table 1). However, as this species can reproduce vegetatively, the accurate determination of population size at the genet level (i.e. group of genetically identical individuals) may be difficult (e.g. Luijten *et al*., 1996). Indeed, during fieldwork, it was difficult to distinguish between individuals of *Ch. uliginosus* occurring in dense clusters of rosettes. In addition, in several populations, poor accessibility and/or leafy vegetation did not allow visual contact with all individuals so the approximate size was estimated according to apparent density and extension of populations.

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DNA isolation, AFLP fingerprinting and DNA sequencing

Total genomic DNA was extracted from 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) in accordance with the modified AFLP® Plant Mapping Protocol of PE Applied Biosystems Inc. using EcoRI and MseI with 500 ng of isolated genomic DNA per sample. After a preliminary 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 GeneScanTM 500 ROXTM Size Standard per sample. Amplified fragments were scored 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, following the recommendations for high quality AFLP development (Crawford, Koscinski & Keyghobadi, 2012). In addition, those individuals that did not produce scorable patterns for all three primer combinations were excluded. Finally, 122 out of 175 attempted individuals (70 %) produced scorable and reproducible patterns for all three primer combinations and were consequently analysed.

For DNA sequencing, we conducted a previous screening test involving nuclear (ITS and ETS) and plastid (*rpoB-trnD*, *rps16-trnK*, *rpl32-trnL* and *trnS-trnC*) markers that were sequenced for a few individuals of different and distanced populations. Of the six regions tested, we selected the ones providing the highest levels of polymorphism: *rpl32-trnL* and *trnS-trnC*. Both regions were amplified and sequenced following protocols in Vitales *et al.* (2013), except for ~~some~~ two accessions that were obtained from GenBank (see Table S2). The number of individuals finally analysed per

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population ranged between four and 13 due to the plant material availability and PCR success (Table 1). Nucleotide sequences were edited using Chromas LITE v. 2.01 (Technelysium Pty, Tewantin, Australia) and subsequently aligned manually with BioEdit v. 7.0.5.3 (Hall, 1999).

Data analysis

The use of AFLP data (dominant markers) for estimating allelic frequencies implies the consideration of an outcrossing mating system and near random mating. This means that those populations would be under Hardy-Weinberg equilibrium (Lynch & Clarke, 1994). *Cheirolophus* has a predominantly outcrossing mating system and is pollinated by generalist insects, so one expects near-random mating in the studied populations. Although the sampling strategy was designed to avoid the collection of clone individuals, this issue was investigated using the function Clones in the AFLPdat software (Ehrich, 2006). A histogram of the number of pair-wise differences among individuals within populations was constructed to explore the occurrence of several plants belonging to the same clonal lineage. In this case, the distribution of pairwise differences within populations is expected to be bimodal. While the main peak represents the pair-wise differences among genotypes, the second peak at low values may represent the differences among plants belonging to a single clonal lineage (Ehrich *et al.,* 2008). The number of genotypes per population and the Nei's gene diversity among genotypes (excluding the putative clones) was also measured using the same software.

To estimate genetic diversity in each population, the following parameters were calculated: a) private alleles (*Npriv*); b) rare alleles (where present in < 10% of the samples); and c) Nei's unbiased heterozygosity within populations (*Hj*) and average gene diversity within populations (*Hw*) calculated using TFPGA v. 1.3 (Miller, 1997). A rarefacted measure of Nei's unbiased heterozygosity was also estimated, randomly resampling the populations to $N = 4$ and recalculating the index [*Hj* (4)] with the same software. Further measures of genetic diversity were estimated through: (i) the band richness (*Br*), which is the number of phenotypes expected at each locus, and can be interpreted as an analogue of the allelic richness, ranging from 1 to 2 (Coart *et al.*, 2005); and (ii) the percentage of polymorphic loci (*PLP*) with a significance of 1% (P =

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0.99). *Br* and *PLP* indices were calculated according to the rarefaction method of Hurlbert (Petit & Mousadik, 1998), and conditioned to the smallest population size (N = 4) with the software AFLPDIV v. 1.0. The frequency-down-weighted marker values (*DW*) index of Schönswetter & Tribsch (2005) was calculated as ratio of means, making the measure less sensitive to large differences in sample size between localities, using AFLPDAT (Ehrich, 2006). ~~Linear~~ Simple linear regression analyses were performed with R software (R Development Core Team, 2015) to study the effect of population size (explanatory variable) on genetic variation (dependent variable). Population size was log-transformed in all analyses. The significance of linear regressions was tested with an analysis of variance approach and the *p*-values –were adjusted using FDR method (Benjamini & Hochberg, 1995) to correct for multiple comparisons. Total gene diversity in the species (*Ht*) and the unbiased derived estimate θ_I (analogue of Wright's F_{ST} coefficient) were calculated using Hickory (Holsinger, Lewis & Dey, 2002). Pairwise F_{ST} values were estimated for each pair of populations studied with AFLP SURV v. 1.0 (Weir & Cockerham, 1984). Significance was evaluated through 10000 permutations. In addition, estimates of inbreeding coefficient were calculated with the software I4A (Chybicki *et al.,* 2011). The measures were obtained after 60,000 steps, after 10,000 burnin steps, as recommended by the author. Because the method requires initial guesses on the priors, analyses were conducted starting from three initial sets of parameters [α=β= (0.1,1,5)] to avoid a dependence of final results on these guesses.

Population genetic structure revealed by AFLP was investigated using phylogenetic and clustering 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. Bayesian clustering analyses were carried out using STRUCTURE v. 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 (from K = 1 to 17), using a burn-in period of 10^5 generations and run lengths of 5×10^5. To estimate the number of genetic groups (K) we selected the K value that maximizes the probability of the data $L(K)$. We also considered the criterion proposed by Evanno, Regnaut & Goudet (2005) to estimate the best value of K for our data set, based on the rate of change in the probability between successive K values, ΔK. Bayesian analyses of the genetic structure

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6 were also conducted with BAPS (Bayesian Analysis of Population Structure; Corander & Marttinen, 2006), which uses stochastic optimization instead of Markov chain Monte Carlo to find optimal partition. We performed a mixture analysis of individuals with the geographic origin of the samples used as an informative prior (spatial clustering of individuals) or without this prior (clustering of individuals). BAPS simulations were run with the maximal number of groups (K) set at the number of sampled populations in each species. Each run was replicated 10 times, and the results were averaged according to the resultant likelihood scores. The output of the mixture analyses were used as input for population admixture analysis (Corander & Marttinen, 2006), with the default settings in order to detect admixture between clusters. Finally, we conducted AMOVA analyses by using ARLEQUIN v. 3.5 (Excoffier, Laval & Schneider, 2005) to estimate genetic differentiation following an alternative and widely used non-Bayesian approach that does not assume Hardy-Weinberg equilibrium or independence of markers. Three independent AMOVA analyses were carried out i) without taking regional structure into account (i.e. among and within population variance only), ii) considering the clusters proposed by STRUCTURE and iii) considering the clusters proposed by BAPS.

To further characterize the spatial genetic distribution of *Ch. uliginosus*, we performed Mantel tests and a spatial autocorrelation test to evaluate the existence of isolation by distance patterns. In order to execute these analyses, genetic distance matrices were constructed with F_{ST} values between populations, and geographical matrices were calculated by the spatial distance (X and Y coordinates) between populations using ArcGIS v. 9.1 (ESRI, Redlands, CA, USA). Mantel tests were performed on ARLEQUIN v. 3.5 with 100000 permutations and considering a p-value limit of 0.05. The additional spatial genetic structure calculation was estimated with SPAGeDi software (Hardy & Vekemans, 2002) considering the kinship multilocus coefficient (F_{ij}) with dominant markers. Inbreeding coefficient (F_{IS}) was set according to the estimate obtained with the software I4A (other values were also used in a preliminary stage, but results were similar), and 20000 permutations were run for the test.

Plastid DNA haplotypes of *Ch. uliginosus* were determined using the number and position of nucleotide substitutions and indels from the aligned sequences. A statistical parsimony haplotype network was also constructed using TCS v. 1.21 (Clement, Posada & Crandall, 2000). For this latter analysis, insertions/deletions longer than one base pair were re-coded as single base pair mutations, and sequence gaps were treated as a fifth

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character state. Total haplotype diversity (*Hd*), average haplotype diversity within populations (*Hs*) of Nei (1973) and genetic differentiation among populations (G_{ST}; Nei, 1973) were calculated for the combined matrix of plastid sequences using DNASP v. 5 (Librado & Rozas, 2009).

Results

AFLP profile, genetic diversity, structure and isolation by distance.

Initially, 183 alleles were obtained from automatic genotyping of *Ch. uliginosus* AFLP profiles. After manual correction, error rates calculation, elimination of small and troublesome alleles and low intensity peaks, a final matrix with 157 (85.8%) alleles was considered for subsequent analyses. The final data set showed an error rate of 2.3%. The distribution of the number of pairwise differences among plants within populations did not present a bimodal shape (Fig. 2), but certain increase in the frequency of individuals differing by zero alleles could be observed. Therefore, to explore the potential effect of clonality in our sampling, individuals showing the same genotype were tentatively considered as possible ramets belonging to the same clonal lineage. Fifteen putative clones were detected in populations DO1 (3), AG1 (6), AL1 (1), AL3 (4) and AA1 (1). Nei's gene diversity among genotypes (excluding the potential clones) ranged among $D = 0.0170$ for population AG1 to $D = 0.0960$ for population BM4 (Table 1), averaging 0.0561 ± 0.0251.

Additional population genetic diversity measures are shown in Table1. Average gene diversity per population (*Hw*) was 0.0572 ± 0.0279. Nei's unbiased heterozigosiy ranged (*Hj*) ranged from 0.0112 (AG1) to 0.1073 (BM4), whereas the rarified measure [*Hj*(4)] varied among 0.0109 (AG1) and 0.0928 (BM4). The percentage of polymorphic loci [*PLP*(4)] ranged from 22.9 (BM4) to 2.5 (AG1) among populations, and band richness [*Br*(4)] varied between 1.178 (BM4) and 1.017 (AG1). The frequency-down-weighted marker values (*DW*) demonstrated large variation among populations, ranging between 332.8 (AL3) and 141.9 (DO5). Private fragments were scarce across the studied populations; only four populations exhibited one or two of them (Table 1). Ten rare alleles were detected: five of them being exclusive of one population [i.e. AL2 (1); SP1 (2); AL3 (1); AG2 (1)], four of them shared by a couple of populations [i.e. AL2 and AL3 (3); DO3 and SP1 (1)] and another one shared by several northern populations (i.e. AA1, BM2, BM3, BM4 and BV1). We detected significant positive relationships

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between most of the genetic diversity indexes and the size of populations in *Ch. uliginosus* (*Hj*, $R^2 = 0.498$, $p < 0.05$; *Hj*(4), $R^2 = 0.3097$, $p < 0.05$; *PLP*(4), $R^2 = 0.481$, $p < 0.05$; *Br*(4), $R^2 = 0.357$, $p < 0.05$; *D*, $R^2 = 0.3113$, $p < 0.05$). Conversely, there was not a significant relationship between the rarity index *DW* and the population size in this species ($R^2 = -0.0463$; $p > 0.05$).

Total genetic diversity at the species level (*Ht*) was 0.208 and the average population differentiation due to genetic structure (Wright's F_{ST}) was 0.582 (SD = 0.022). All three inbreeding analyses executed with different alpha and beta prior distributions converged to almost the same posterior distributions of the inbreeding coefficient (mean value = 0.0169; Table ~~S2~~S3). Also the likelihood behaviour across different priors (e.g. similar average and standard error values of Log L) proved that the model was stable. Results of AMOVA analyses are depicted in Table 2. Genetic variation between the populations contributed at least 42.08% to overall genetic diversity in *Ch. uliginosus*. 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 of the different populations (r = 0.308, $p < 0.05$). Significant effects of isolation by distance in the kinship coefficient among the studied populations were also found with SPAGeDi (Fig. S1), especially at distance intervals up to 150 km, where populations were more similar than expected by random.

The Bayesian analysis of population genetic structure conducted with STRUCTURE for *Ch. uliginosus* dataset found the highest $L(K)$ and ΔK values for $K = 2$. This grouping separated Doñana populations (Andalusia) from the rest of the populations located in Portugal, showing high percentages of individual memberships for these predefined groups (Fig. ~~3B~~3A). Alternatively, BAPS results supported a more fragmented distribution, with $K = 4$ as the most plausible number of clusters (P = 0.97; Fig. S2 and Fig. ~~3A~~3B). This clustering analysis segregated: Doñana area (DO1-5) populations (Cluster I); south-western seaside populations (Algarve, AG1-2; Alentejo Litoral, AL1-2; and Setubal Península, SP1; Cluster II); population AL3 from inland Alentejo Litoral (Cluster III); and northern populations (Alto Alentejo, AA1; Baixo Mondengo, BM1-4 and Baixo Vouga, BV1; Cluster IV). The mixture analyses with or without spatially informative priors resulted in congruent assignment of individuals. No individual reassignments between the populations were observed. The hierarchical AMOVA analyses showed a significant differentiation among groups defined by both clustering

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approaches (Table 2), but the grouping proposed by BAPS explained better (24.86%) than STRUCTURE (21.96%) the overall genetic diversity found in the species. Although the phylogenetic network constructed with SplitsTree (Fig. 3A3B) resulted in a poorly resolved genetic structure of *Ch. uliginosus* populations, the four clusters proposed by BAPS could be identified.

The two plastid markers were successfully sequenced for 139 samples from 17 different populations (see Table S2 for GenBank accession numbers) resulting in an alignment of 1783 bp. We detected 16 polymorphic sites –including a 22 bp indel– representing ten different haplotypes (Fig. 1 and Table 1). All populations contained one single haplotype (*Hs* = 0), with the exception of the two large populations that held two (BM4, *Hs* = 0.282; and SP1, *Hs* = 0.536). Haplotypes C, D, F, H and J were each one restricted (i.e. private haplotypes) to a single population (Table 1; Fig. 1). Phylogenetic relationship between haplotypes inferred by the parsimony network is shown in Fig. 1. Different haplotypes of *Ch. uliginosus* distinguished from adjacent haplotypes by one, two or three evolutionary events (substitutions or indels) and extinct or unsampled haplotypes were represented as black dots in the parsimony network. Total haplotype diversity resulted to be considerably high (*Hd* = 0.759) and plastid DNA among-population differentiation was large (G_{ST} = 0.903).

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Discussion

Genetic diversity patterns within* Ch. uliginosus *populations

Declining gene diversity is a typical pattern found in rare plant species generally showing small population sizes (see Frankham *et al*., 2002 for a review). According to our AFLP results, average within-population gene diversity in *Ch. uliginosus* (*Hw* = 0.0572) is extremely low; notably below the values reported for endemic plant species using dominant markers [mean *Hw* = 0.20 in (Nybom, 2004)]. We have also found that *Ch. uliginosus* exhibits a significantly lower within-population diversity than its widespread Mediterranean congener *Ch. intybaceus* (Lam.) Dostál (*Hw* = 0.134; Garnatje *et al*., 2013). Gene diversity has been reported to be lower in data sets containing clones relative to clone-corrected data (Ellstrand & Roose, 1987; McLellan *et al.,* 1997). However, we did not found significant differences between the overall

gene diversity within populations ($Hw = 0.0572 \pm 0.0279$) and the gene diversity among genotypes ($D = 0.0561 \pm 0.0251$), so an underestimation of genetic diversity due to sampling of clonal individuals can be rejected. We tried to avoid collecting the same genetic individual twice and indeed putative clonal individuals were only identified in small sized populations where the distance among sampled plants was limited. Therefore, our study also indicates that single genets of *Ch. uliginosus* are not able to grow > 5 m in diameter, as we assumed when setting our sampling design.

The genetic diversity of *Ch. uliginosus* has resulted to be significantly lower in smaller populations than in larger ones. All the studied genetic diversity indexes –including rarefacted and clone corrected ones– have shown a significant positive relationship with population size. In contrast, we have not found an association among the genetic rarity index *DW* and the size of populations, reinforcing the hypothesis that genetic diversity indexes are better indicators of contemporary demographic processes than rarity ones, which may perform better to explain phylogeographic patterns (Comps et al., 2001; Widmer & Lexer, 2001; Paun *et al.*, 2008). Plastid DNA diversity recovered in our study is not large enough to construct statistically supported inferences at the intrapopulation level. Nevertheless, we observe that the only populations showing haplotypic diversity are the two large-sized ones (BM4, $Hs = 0.282$; and SP1, $Hs = 0.536$), therefore suggesting an effect of population size also in the plastid genetic diversity of *Ch. uliginosus*. The relationship between genetic variation and population size has already been reported in numerous studies involving different plant species and employing diverse molecular markers (Ellstrand & Elam, 1993; Frankham *et al*., 2002; Jadwiszczak *et al*., 2012; Ilves *et al*., 2013). Bottlenecks, genetic drift and inbreeding are usually the main causes proposed to explain the reduction of genetic diversity levels (Soulé, 1986). In this way, vegetative reproduction reported in the species may have acted as an enhancer of genetic drift by further reducing the effective size of local populations (Chung & Kang, 1996; Jones & Gliddon, 1999).

In some cases the genetic erosion can also lead to a reduction in plant performance in small populations (Reed & Frankham, 2003). Certainly, lower plant performance in small *Ch. uliginosus* populations has already been documented in previous research (Vitales *et al*., 2013). This former study showed that seed germination rate was significantly reduced in small populations, whereas medium and large populations did not show any noticeable germination constraint. Correlation between small population

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size and reduction in reproductive fitness traits –such as germination capacity– has been reported in several studies (see Reed, 2005 for a review) as a consequence of inbreeding depression. In the case of *Ch. uliginosus*, factors related to demographic stochasticity (such as pollen limitation) and habitat deterioration (see the Conservation remarks section) could also be detrimental, leading to reduced plant performance in small populations (Vergeer *et al*., 2003). However, given the significantly lower genetic diversity found in the smaller populations of this species, inbreeding depression appears to be the most likely explanation for the fitness reduction reported by Vitales *et al*. (2013).

***Among-population genetic differentiation in* Ch. uliginosus**

Heterozygosity at the whole species level (Ht = 0.208) in *Ch. uliginosus* does not seem to be as impoverished as it is at the population level, especially when compared with total heterozygosity in the common *Ch. intybaceus* (Ht = 0.211; Garnatje *et al*., 2013). These results are in agreement with AMOVA analysis, suggesting that much of the genetic diversity in *Ch. uliginosus* is distributed among the different populations. Certainly, this species shows high levels of genetic differentiation among populations (Wright's F_{ST} = 0.582), far greater than the values found by Nybom (2004) for endemic species analysed using dominant markers (mean Wright's F_{ST} = 0.26). These patterns of genetic diversity showed by AFLP data totally agree with those found in plastid DNA markers. Our survey based on two plastid DNA spacers (*rpl*32-*trn*L and *trn*S-*trn*C) revealed ten different haplotypes, similar to the values found in other more widespread Mediterranean species (e.g. Quintela-Sabarís *et al*., 2011; Mráz *et al*., 2012). Consequently, *Ch. uliginosus* shows as well considerable plastid diversity at the species level (Hd = 0.759). In contrast, all except two populations had a single fixed haplotype, which implies overall low within-population diversity and high genetic differentiation among populations (G_{ST} = 0.903). Thus, both AFLP and plastid DNA data suggest that *Ch. uliginosus* shows notably low intra-population heterozygosity but substantial inter-population diversity, being a large proportion of the genetic variance found in the species attributed to differences between populations.

A high degree of among-population genetic differentiation is –like the declining within-population diversity– a characteristic pattern observed in rare and endangered plants

(see Hamrick & Godt, 1996; Gitzendanner & Soltis, 2000; or Cole, 2003 for a review). Small population size, restricted distribution range, geographical isolation, reproduction features and limited seed dispersal have been reported as common factors contributing to the low genetic diversity and high population genetic differentiation in several species (e.g. Mousadik & Petit, 1996; Gong *et al.,* 2010; Lauterbach, Ristow & Gemeinholzer, 2011; Kolb & Durka, 2013). In the case of *Ch. uliginosus*, several of these elements may be shaping the genetic variability of this species. First, as it has been stated above, we must consider the reduced number of individuals found in most of the sampled populations. In addition, according to the historical citations of *Ch. uliginosus* collated by Susanna (1993), the distribution of this species seems to be originally disrupted as a consequence of the natural fragmentation of the habitat it occupies. Finally, the significant correlation found among genetic and geographical distances (Mantel test) certainly suggests that *Ch. uliginosus* is influenced by limited gene flow. Pollen exchange between populations of *Ch. uliginosus* separated by more than 10 km is not likely since generalist pollinators do not regularly travel such long distances (Kwak, Velterop & van Andel, 1998; Pasquet *et al*., 2008). The dispersal ability of seeds in this species is also restricted by lack of morphological adaptation for wind or animal transport, their dispersal occurring simply by gravity (Bañares *et al*., 2010). Thus, regular gene flow among the majority of the populations –in most cases separated by more than 10 km according to our field survey– must be limited. In summary, several factors such as small population sizes, natural habitat fragmentation, intrinsic low gene flow abilities and/or vegetative reproduction possibly contribute to the strikingly high genetic differentiation observed in this species.

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Genetic structure and phylogeography of **Ch. uliginosus**

In this study, four groups of populations are proposed according to the genetic clustering inferred by BAPS (Fig. ~~3A~~3B), and no genetic admixture has been observed among individuals belonging to different groups. A similar geographic distribution of genetic diversity, identifying the same four clusters of populations, is also recovered by Neighbor-Net analysis (Fig 3A). AMOVA analysis implemented taking into account the genetic structure found by BAPS supports as well a significant and notable differentiation among those groups (Table 2). Some of these clusters (e.g. clusters II and

IV) contain populations distanced by > 100 km, far beyond what regular gene flow in *Ch. uliginosus* is able to connect. Spatial autocorrelation test also indicate that populations separated by distances up to 150 km are more similar to each other than expected by random sampling (Fig. S1). These results suggest that other factors apart from current gene flow alone must be shaping the genetic structure observed in this species. Therefore, the observed subdivision of genetic diversity may be better explained by a pre-existing genetic structure related to the ancient phylogeographic history of this species.

The distribution of haplotypes among the population clusters supports the hypothesis that those genetic clusters may have a long evolutionary history. For instance, different and unconnected haplotypes can be found within northern (BAPS Cluster IV) or south-western (Cluster II) populations, indicating that both groups retain a large amount of the genetic diversity found in *Ch. uliginosus*. This pattern also suggests that both clusters of populations possess a long evolutionary history, sufficient to generate and maintain the broad variability observed in the relatively slowly evolving plastid DNA (Lynch, Koskella & Schaack, 2006). The case of Doñana populations (DO1-5, Cluster I) and AL3 (Cluster III) seems to be different. Populations within both clusters share the same haplotype A (Fig. 1), suggesting that any of the two genetic groups could have been originated from a recent long-distance-dispersal or a contemporary fragmentation event. However, DO populations not only form a genetic group according to BAPS (Cluster I) but they also constitute the only separate cluster defined by STRUCTURE (Fig. ~~3B~~3A) and a clearly separate group according to the Neighbor-Net analysis (Fig. ~~3A~~3B). These results point to a relatively ancient isolation of Andalusian populations. Meanwhile, population AL3 possesses the largest value for the frequency-down-weighted marker (*DW*) in relation to the other sampled *Ch. uliginosus* populations (Table 1). This genetic rarity index is expected to be high in long-term isolated populations where rare markers should accumulate due to mutations (Schönswetter & Tribsch, 2005; Lihová, Kudoh & Marhold, 2010), thus suggesting that AL3 population could also be well the result of a long evolutionary history. Therefore, attending to our data, the main genetic and geographic subdivision of *Ch. uliginosus* does not seem to be the result of recent fragmentation, extinction or colonisation events, but it could be the result of an ancient and independent evolutionary history in different isolated groups of populations.

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According to recent phylogenetic and molecular cytogenetic studies of the genus (Garnatje *et al.,* 2012; Vitales *et al.,* 2014a; Vitales *et al.,* in prep.), *Ch. uliginosus* has been proposed to be an early diverged species within *Cheirolophus*. These authors hypothesised that this species may possess a long in-situ evolutionary history in the humid Atlantic coast of the Iberian Peninsula, where the lineage probably arrived seeking refuge from the progressive aridification of the Mediterranean climate during the Plio-Pleistocene (Thompson, 2005). Subsequently, *Ch. uliginosus* must have survived to the climatic oscillations associated to Pleistocene glaciations that deeply affected the European and Mediterranean floras (Weiss & Ferrand, 2007). The long-term ability of this species to persist throughout repeated episodes of climate oscillation has been associated to both intrinsic (e.g. asexual propagation) and extrinsic (e.g. particularly stable ecological habitats) features (Hampe & Jump, 2011). In this way, the geographic distribution of the four genetic clusters of *Ch. uliginosus* populations proposed by AFLP analyses virtually overlaps with different putative glacial refugia previously identified by Médail & Diadema (2009) in the region (i.e. Beira Litoral, Extremadura, Algarve and Cadiz regions). The narrow ecological niche of this species might concur with particularly stable areas from a climatic point of view, possibly contributing to the survival of this climate relict species in separated groups with long and independent evolutionary histories. The particular phylogeographic pattern showed by *Ch. uliginosus* might be the result of this ancient segregation in different groups of populations.

Conservation remarks

Our results demonstrate the need for accurate censuses of *Ch. uliginosus* populations, particularly in Portugal, where no previous data about the conservation status of the species exist. In relation to the conservation of the smallest populations of this species, showing particularly low values of genetic diversity, additional measures could be adopted to avoid the effects of possible inbreeding depression and the resultant risk of extinction. The conservation of populations DO1, AG1, AA1 and AL1, showing very small population sizes (< 50 individuals) and the lowest values of genetic diversity should be a priority. First, seed collection –conducted according with the genetic structure detected in our results– and storage in germplasm banks will ensure the

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maintenance of the genetic diversity even if they eventually disappear. Moreover, specific demographic monitoring on these extremely small populations should be carried out to evaluate the risk derived from inbreeding depression on their long-term survival. If conservation managers decide to reinforce these impoverished populations with individuals from other localities to increase their heterozygosity levels and overcome the effects of inbreeding depression, our results may serve to choose the best candidate populations to transfer some individuals based on their genetic closeness.

From the point of view of the protection of the species as a whole, considering that much of the genetic variability showed by this species is distributed among populations, we believe that conservation measures must focus on the preservation of the maximum number of different populations. At least all the plastid DNA haplotypes and the genetic clusters inferred by AFLP data should be well represented when defining conservation strategies for *Ch. uliginosus*. Due to the valuable ecosystem where this species typically occurs, most of the populations are included in natural protected areas (see Table S1). However, some of these habitats are still affected by threats derived from the human activity occurring inside or next to the protected areas [e.g. agriculture and overexploitation of water resources in Sudoeste Alentejano e Costa Vicentina and Doñana natural parks (Bañares *et al.,* 2010; LPN, 2011)]. In addition, other populations are located in areas without any legal protection so their conservation cannot be currently guaranteed in the long term. In these latter cases, the creation of botanical reserves –small protected areas for wild plants already working on Portugal (Laguna, 2001)– may be appropriate for the conservation of these currently unprotected populations of *Ch. uliginosus*. Finally, attending to the suggested refugial role of these southern heathlands in the conservation of the genetic diversity of this species –together with the occurrence of other evolutionary and floristic interesting taxa usually sharing the same habitat of *Ch. uliginosus* (e.g. *Euphorbia uliginosa* Welw. ex Boiss.; *Genista ancistrocarpa* Spach)–, we agree with Ojeda (2009) about the particular ecologic and biogeographic value of temperate Atlantic wet heaths from the Iberian Peninsula, thus deserving further protection and supplementary studies.

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The authors declare that they have no conflicts of interest in the research.

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Figure legends

Figure 1~~.~~ ~~(~~A~~)~~. Geographical distribution of cpDNA haplotypes and studied populations of *Ch. uliginosus*. The population codes correspond to those in Table 1, and the pie charts represent the percentage of each haplotype in each population. ~~(~~B~~)~~. Statistical parsimony network showing relationships of the ten plastid haplotypes. Each line between haplotypes indicates a mutational step, and black dots represent extinct or unsampled haplotypes

Figure 2. Distribution of the number of pairwise differences among *Cheirolophus uliginosus* plants within populations.

Figure 3. A~~–~~. Bayesian estimation of genetic structure within *Cheirolophus uliginosus* inferred from AFLP according to the best model proposed by STRUCTURE (K = 2).~~Neighbor-Net of AFLP data obtained from the 17 sampled populations of *Ch. uliginosus*. Colour coding profiles delimitate the different clusters assigned by BAPS.~~ B~~–~~. Neighbor-Net of AFLP data obtained from the 17 sampled populations of *Ch. uliginosus*. Colour coding profiles delimitate the different clusters assigned by BAPS.~~Bayesian estimation of genetic structure within *Cheirolophus uliginosus* inferred from AFLP according to the best model proposed by STRUCTURE (K = 2).~~

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Figure S1. Spatial autocorrelation analyses based in SPaGeDi results, comparing kinship coefficient (y axis) with spatial distance lags (x axis). Solid lines indicate the mean kinship coefficient per distant class and dashed lines the limits of its 95% confident limit.

Figure S2. Bayesian estimation of genetic structure within *Cheirolophus uliginosus* inferred from AFLP according to the best model proposed by BAPS ($K = 4$).

Table S1. Supporting information of the sampled populations, including their geographic coordinates, collectors, voucher codes and natural protected areas where they are located.Supporting information of the sampled populations, including their geographic coordinates, the collectors, the voucher codes and the natural protected areas where they are located.

Table S2. GenBank accession numbers for the two plastid DNA regions sequenced. The sample codes are composed by the population code (see Table 1) and the individual number.

Table S2S3. Average inbreeding coefficient (F) estimates based on AFLP markers for the study populations.

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Table1. Sampling information and genetic diversity indexes assessed. Population code, locality (see Table S1 for details), estimated population size, number of analysed individuals with AFLP and cpDNA [*N* (AFLP) and *N* (cpDNA)], number of genotypes [*N* (geno)] and genetic diversity indexes assessed in 17 populations of *Cheirolophus uliginosus*. Genetic indexes: gene diversity among genotypes (*D*); heterozygosity (*Hj*); heterozigosity rarefacted to four individuals [Hj(4)]; frequency-down-weighted marker values index (*DW*); band richness for a standardised sample size of four [*Br*(4)]; percentage of polymorphic loci for a standardised sample size of four [*PLP*(4)]; number of private alleles (*Npriv*); haplotypes found and average haplotype diversity within populations (*Hs*).

Population code	Locality	Estimated size	*N* (AFLP)	*N* (geno)	*D*	*Hj*	*Hj* (4)	*Br* (4)	*PLP* (4)	*DW* (means)	*Npriv*	*N* (cpDNA)	Haplotypes	*Hs*
DO1	Doñana: La Rocina	10	5	3	0.0212	0.0131	0.0144	1.029	0.032	150.900	0	5	A	0
DO2	Doñana: Palacio Acebrón trail	100	8	8	0.0569	0.0675	0.0372	1.106	0.159	150.874	0	9	A	0
DO3	Doñana: Estero Domingo Rubio	300	9	9	0.0248	0.0326	0.02	1.046	0.07	155.464	0	10	A	0
DO4	Doñana: Estero Las Madres	50	5	5	0.0611	0.0573	0.0526	1.115	0.134	143.347	0	4	A	0
DO5	Doñana: Forestal house	200	9	9	0.0573	0.0776	0.043	1.108	0.185	141.884	0	7	A	0
AG1	Algarve, Faro, Ocedeixe south	30	6	3	0.0170	0.0112	0.0109	1.017	0.025	162.010	0	4	E	0
AG2	Algarve, Faro, Ocedeixe north	200	9	9	0.0485	0.0515	0.0373	1.088	0.121	230.385	1	11	E	0
AL1	Alentejo Litoral: Almograve creek	25	5	4	0.0403	0.0308	0.033	1.065	0.07	158.174	0	8	B	0
AL2	Alentejo Litoral: Almograve, beach dunes	75	11	11	0.0651	0.0764	0.0547	1.118	0.185	255.836	1	8	B	0
AL3	Alentejo Litoral, Alcácer do Sal, Arez	50	10	7	0.0522	0.0503	0.0433	1.093	0.115	332.794	1	10	A	0
SP1	Setubal Peninsula, Calhariz	400	10	10	0.0722	0.0833	0.0771	1.131	0.178	297.374	2	8	C/D	0.536
AA1	Alto Alentejo, Reguengo	4	4	3	0.0255	0.0187	0.0187	1.038	0.038	150.513	0	4	F	0
BM1	Baixo Mondego, Paul Madriz	300	4	4	0.0934	0.0802	0.0802	1.172	0.172	151.489	0	8	H	0
BM2	Baixo Mondego, Figueiró do Campo	100	4	4	0.0828	0.0644	0.0644	1.146	0.146	153.529	0	10	J	0
BM3	Baixo Mondego, Mata da Foja	40	5	5	0.0879	0.0855	0.0825	1.159	0.178	146.970	0	7	I	0
BM4	Baixo Mondego, Valdoeiro	1000	6	6	0.0960	0.1073	0.0928	1.178	0.229	147.001	0	13	G/H	0.282
BV1	Baixo Vouga: Fermentelos	100	12	12	0.0523	0.0651	0.0294	1.096	0.166	151.140	0	13	G	0

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Table 2. Analyses of molecular variance (AMOVA) of *Cheirolophus uliginosus* based on AFLP markers. Three independent AMOVA analyses were carried out: 1) without taking regional structure into account (i.e. among and within population variance only), 2) considering the clusters proposed by STRUCTURE and 3) considering the clusters proposed by BAPS.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Fixation indices	Percentage of variation	P
1. No population structure						
Among populations	16	422.69	3.11	0.42	42.08	<0.001
Within populations	105	449.56	4.28		57.92	<0.001
2. STRUCTURE clustering						
Among groups	1	116.05	1.85	0.22	21.96	<0.001
Among populations within groups	15	306.63	2.29	0.35	27.17	<0.001
Within populations	105	449.56	4.28	0.49	50.87	<0.001
3. BAPS clustering						
Among groups	3	223.82	1.95	0.25	24.86	<0.001
Among populations within groups	13	198.86	1.62	0.27	20.65	<0.001
Within populations	105	449.56	4.28	0.45	54.49	<0.001

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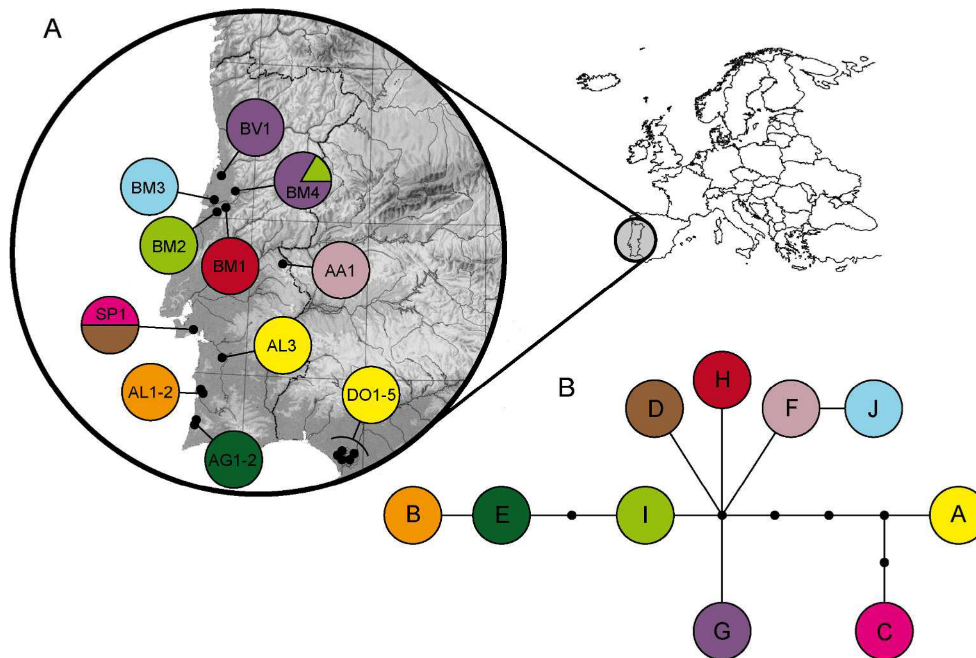

Figure 1 (A). Geographical distribution of cpDNA haplotypes and studied populations of Ch. uliginosus. The population codes correspond to those in Table 1, and the pie charts represent the percentage of each haplotype in each population. (B) Statistical parsimony network showing relationships of the ten plastid haplotypes. Each line between haplotypes indicates a mutational step, and black dots represent extinct or unsampled haplotypes
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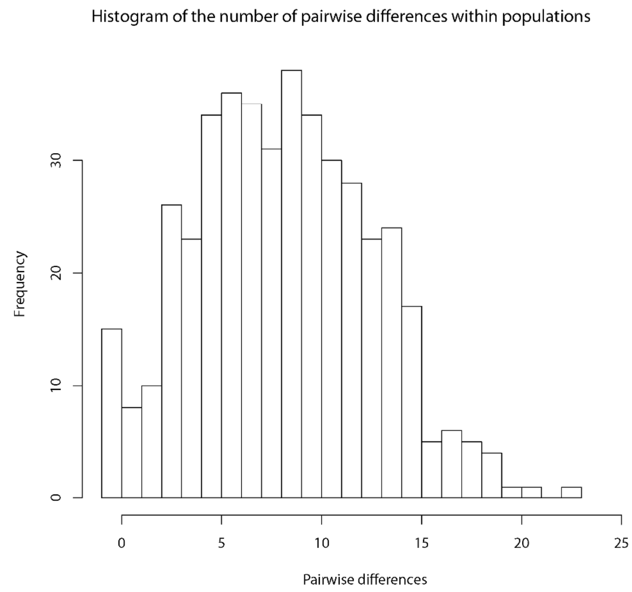

Figure 2. Distribution of the number of pairwise differences among Cheirolophus uliginosus plants within populations.
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A. Bayesian estimation of genetic structure within Cheirolophus uliginosus inferred from AFLP according to the best model proposed by STRUCTURE (K = 2). B. Neighbor-Net of AFLP data obtained from the 17 sampled populations of Ch. uliginosus. Colour coding profiles delimitate the different clusters assigned by BAPS.
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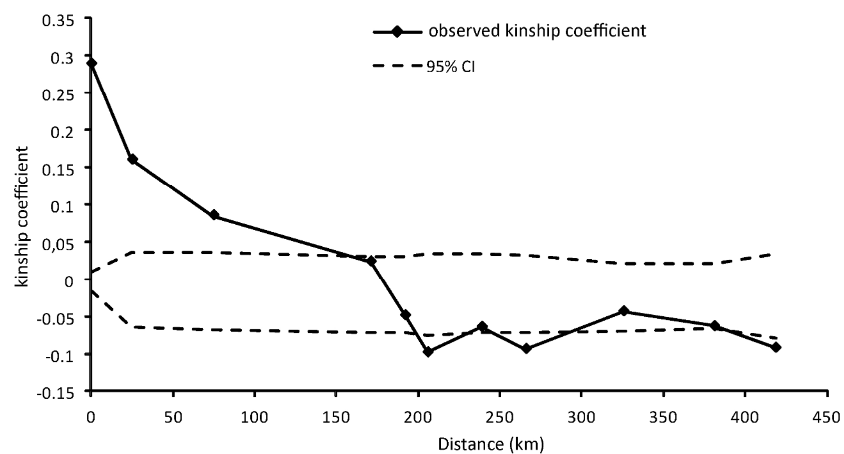

Figure S1. Spatial autocorrelation analyses based in SPaGeDi results, comparing kinship coefficient (y axis) with spatial distance lags (x axis). Solid lines indicate the mean kinship coefficient per distant class and dashed lines the limits of its 95% confident limit.
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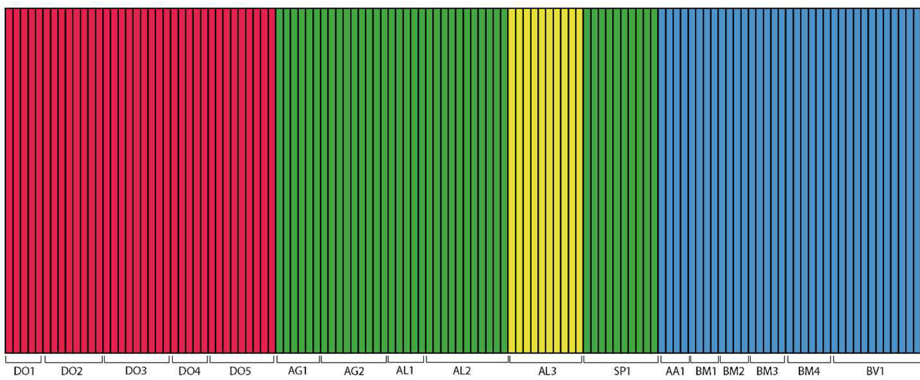

Bayesian estimation of genetic structure within Cheirolophus uliginosus inferred from AFLP according to the best model proposed by BAPS (K = 4).
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Table S1. Supporting information of the sampled populations, including their geographic coordinates, ~~the~~ collectors, ~~the~~ voucher codes and ~~the~~ natural protected areas where they are located.

Population	Locality	Collectors	Coordinates	Natural protected area	Herbarium voucher
DO1	Doñana: La Rocina	T. Garnatje, J. Pellicer, V. Girón & D. Vitales 29.07.09	37.14244 N 6.54258 W	Doñana National Park	BC640064
DO2	Doñana: Palacio Acebrón trail	T. Garnatje, J. Pellicer, V. Girón & D. Vitales 29.07.09	37.1435 N 6.54658 W	Doñana National Park	BC923494
DO3	Doñana: Estero Domingo Rubio	T. Garnatje, J. Pellicer, V. Girón & D. Vitales 29.07.09	37.20664 N 6.83575 W	Paraje Natural Estero Domingo Rubio	BC923500
DO4	Doñana: Estero Las Madres	T. Garnatje, J. Pellicer, V. Girón & D. Vitales 29.07.09	37.16042 N 6.82853 W	Paraje Natural Lagunas de Palos y Las Madres	BC923495
DO5	Doñana: Forestal house	T. Garnatje, J. Pellicer & D. Vitales 30.07.09	37.21903 N 6.57539 W	No protection	BC923496
AG1	Algarve: Faro, Ocedeixe south	O. Hidalgo & S. Vilandrau 02.09.11	37.43079 N 8.80628 W	Sudoeste Alentejano e Costa Vicentina Natural Park	BC923504
AG2	Algarve: Faro, Ocedeixe north	O. Hidalgo & S. Vilandrau 02.09.11	37.43471 N 8.80462 W	Sudoeste Alentejano e Costa Vicentina Natural Park	BC923502
AL1	Alentejo Litoral: Almograve creek	T. Garnatje, J. Pellicer & D. Vitales 31.07.09	37.651 N 8.78675 W	Sudoeste Alentejano e Costa Vicentina Natural Park	BC922633
AL2	Alentejo Litoral: Almograve. Beach dunes	O. Hidalgo & S. Vilandrau 02.09.11	37.65968 N 8.8014 W	Sudoeste Alentejano e Costa Vicentina	BC923506
AL3	Alentejo Litoral: Alcácer do Sal, Arez	O. Hidalgo & S. Vilandrau 01.09.11	38.28358 N 8.485 W	No protection	BC923505
SP1	Setubal Peninsula: Calhariz	O. Hidalgo & S. Vilandrau 01.09.11	38.46313 N 9.064 W	Arrábida Natural Park	BC923503
AA1	Alto Alentejo: Reguengo	T. Garnatje, J. Pellicer & D. Vitales 02.08.09	39.30108 N 7.40483 W	Serra de São Mamede Natural Park	BC923488
BM1	Baixo Mondego: Paul Madriz	J. Paiva & D. Vitales 03.11.09	40.12894 N 8.63131 W	Paul de Madriz Special Protection Area	BC923483
BM2	Baixo Mondego: Figueiró do Campo	J. Paiva & D. Vitales 03.11.09	40.14425 N 8.56975 W	No protection	BC923482
BM3	Baixo Mondego: Mata da Foja	J. Paiva & D. Vitales 02.11.09	40.21717 N 8.724 W	No protection	not yet assigned
BM4	Baixo Mondego: Valdoeiro	J. Paiva & D. Vitales 02.11.09	40.35325 N 8.42292 W	No protection	BC923484
BV1	Baixo Vouga: Fermentelos	T. Garnatje, J. Pellicer & D. Vitales 04.08.09	40.57131 N 8.53417 W	Ria de Aveiro Site of Communitary Importance	BC923491

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Table S2. GenBank accession numbers for the two plastid DNA regions sequenced. The sample codes are composed by the population code (see Table 1) and the individual number.

Code	*trnS-trnC*	*rpl32-trnL*
DO1-1	KR535628	KR535767
DO1-2	KR535629	KR535768
DO1-3	KR535630	KR535769
DO1-4	KR535631	KR535770
DO1-5	KR535632	KR535771
DO2-2	KR535689	KR535828
DO2-3	KR535690	KR535829
DO2-4	KR535691	KR535830
DO2-5	KR535692	KR535831
DO2-6	KR535693	KR535832
DO2-7	KR535694	KR535833
DO2-8	KR535695	KR535834
DO2-9	KR535696	KR535835
DO2-10	KR535688	KR535827
DO3-1	KR535697	KR535836
DO3-2	KR535699	KR535838
DO3-3	KR535700	KR535839
DO3-4	KR535701	KR535840
DO3-5	KR535702	KR535841
DO3-6	KR535703	KR535842
DO3-7	KR535704	KR535843
DO3-8	KR535705	KR535844
DO3-9	KR535706	KR535845
DO3-10	KR535698	KR535837
DO4-1	KR535707	KR535846
DO4-2	KR535708	KR535847
DO4-3	KR535709	KR535848
DO4-4	KR535710	KR535849
DO5-1	KR535711	KR535850
DO5-4	KR535712	KR535851
DO5-5	KR535713	KR535852
DO5-6	KR535714	KR535853
DO5-7	KR535715	KR535854
DO5-8	KR535716	KR535855
DO5-9	KR535717	KR535856
AG1-1	KR535763	KR535902
AG1-3	KR535764	KR535903
AG1-4	KR535766	KR535905
AG1-6	KR535765	KR535904
AG2-2	KR535758	KR535897
AG2-3	KR535759	KR535898
AG2-4	KR535760	KR535899
AG2-8	KR535761	KR535900

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Code	*trnS-trnC*	*rpl32-trnL*
AG2-9	KR535762	KR535901
AG2-10	KR535752	KR535891
AG2-11	KR535753	KR535892
AG2-12	KR535754	KR535893
AG2-14	KR535755	KR535894
AG2-16	KR535756	KR535895
AG2-17	KR535757	KR535896
AL1-1	KR535723	KR535862
AL1-2	KR535719	KR535858
AL1-3	KR535720	KR535859
AL1-4	KR535724	KR535863
AL1-5	KR535721	KR535860
AL1-6	KR535725	KR535864
AL1-8	KR535722	KR535861
AL1-10	KR535718	KR535857
AL2-1	KR535747	KR535886
AL2-2	KR535749	KR535888
AL2-3	KR535750	KR535889
AL2-5	KR535744	KR535883
AL2-7	KR535751	KR535890
AL2-8	KR535745	KR535884
AL2-9	KR535746	KR535885
AL2-11	KR535748	KR535887
AL3-1	KR535735	KR535874
AL3-2	KR535737	KR535876
AL3-3	KR535738	KR535877
AL3-4	KR535739	KR535878
AL3-5	KR535740	KR535879
AL3-6	KR535741	KR535880
AL3-7	KR535734	KR535873
AL3-8	KR535742	KR535881
AL3-9	KR535743	KR535882
AL3-10	KR535736	KR535875
SP1-1	KR535728	KR535867
SP1-3	KR535726	KR535865
SP1-4	KR535730	KR535869
SP1-5	KR535727	KR535866
SP1-6	KR535731	KR535870
SP1-7	KR535732	KR535871
SP1-9	KR535733	KR535872
SP1-10	KR535729	KR535868
AA1-1	KJ826359[1]	KJ826179[1]
AA1-2	KR535633	KR535772
AA1-3	KR535634	KR535773
AA1-4	KR535635	KR535774

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Code	*trnS-trnC*	*rpl32-trnL*
BM1-1	KR535670	KR535809
BM1-4	KR535671	KR535810
BM1-5	KR535672	KR535811
BM1-7	KR535673	KR535812
BM1-8	KR535674	KR535813
BM1-14	KR535675	KR535814
BM1-16	KR535676	KR535815
BM1-17	KR535677	KR535816
BM2-1	KR535678	KR535817
BM2-2	KR535679	KR535818
BM2-4	KR535680	KR535819
BM2-5	KR535681	KR535820
BM2-6	KR535682	KR535821
BM2-7	KR535683	KR535822
BM2-9	KR535684	KR535823
BM2-10	KR535685	KR535824
BM2-11	KR535686	KR535825
BM2-13	KR535687	KR535826
BM3-1	KR535663	KR535802
BM3-2	KR535664	KR535803
BM3-3	KR535665	KR535804
BM3-5	KR535666	KR535805
BM3-7	KR535667	KR535806
BM3-15	KR535668	KR535807
BM3-16	KR535669	KR535808
BM4-1	KR535650	KR535789
BM4-2	KR535652	KR535791
BM4-3	KR535653	KR535792
BM4-7	KR535654	KR535793
BM4-8	KR535655	KR535794
BM4-9	KR535656	KR535795
BM4-10	KR535651	KR535790
BM4-12	KR535657	KR535796
BM4-13	KR535658	KR535797
BM4-15	KR535659	KR535798
BM4-18	KR535660	KR535799
BM4-19	KR535661	KR535800
BM4-20	KR535662	KR535801
BV1-1	KR535637	KR535776
BV1-2	KR535639	KR535778
BV1-3	KR535640	KR535779
BV1-4	KR535641	KR535780
BV1-5	KR535642	KR535781
BV1-7	KR535643	KR535782
BV1-9	KR535644	KR535783

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Code	*trnS-trnC*	*rpl32-trnL*
BV1-10	KR535638	KR535777
BV1-14	KR535645	KR535784
BV1-15	KR535646	KR535785
BV1-17	KR535647	KR535786
BV1-18	KR535648	KR535787
BV1-19	KR535649	KR535788

[1] Sequences obtained from GenBank published by Vitales et al. (2013).

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Table S3. Average inbreeding coefficient (F) estimates based on AFLP markers for the study populations.

α, β	**F**	**95% CI**	**Log L (SD)**
0.1	0.0173	0.0011-0.0454	-2959.015 (13.636)
1	0.0165	0.0008-0.0443	-2959.443 (13.861)
5	0.0168	0.0007-0.0438	-2959.736 (14.005)
Mean	0.0169		

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