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Genetic structure and seed germination in Portuguese populations of *Cheirolophus uliginosus* (Asteraceae): Implications for conservation strategies

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Abstract

Genetic structure and seed germination in Portuguese populations of *Cheirolophus uliginosus* (Asteraceae): Implications for conservation strategies.— *Cheirolophus uliginosus* is a threatened species, endemic to the Atlantic coast of the Iberian Peninsula, where it occupies a few restricted localities. In our study we analysed the patterns of cpDNA haplotypes variation and reproductive success—germinability—among seven Portuguese populations of varying size. The aim was to examine the reproductive performance of *Ch. uliginosus* related to genetic structure and population size. The results showed very low within-population variability of cpDNA markers. Our study indicates that the germination rate is significantly reduced in small populations (< 50 plants) whereas medium (50–250 individuals) and large-sized (> 250 individuals) do not show any constraint. In the search for plausible causes explaining the lower germination success in the smallest populations, ecological concerns and genetic isolation must be taken into account. Besides, in large-sized populations of *Ch. uliginosus* (> 250 plants) a higher incidence of predispersal seed predation was observed, maybe affecting their sexual reproductive response. Finally, smaller populations—presenting a reduced reproductive success—contain also the most evolutionary distant haplotypes, so their conservation should be a priority.

Key words: germination rate; habitat loss; haplotype structure; seed predation; small populations.

Resumen

Estructura genética y germinación de semillas en poblaciones portuguesas de *Cheirolophus uliginosus* (*Asteraceae*): Implicaciones para su conservación.— *Cheirolophus uliginosus* es una especie amenazada endémica de la costa atlántica de la península ibérica, donde ocupa unas pocas y reducidas localidades. En nuestro estudio, analizamos los patrones de variación de los haplotipos de ADN cloroplástico y el éxito reproductivo —capacidad germinativa— en siete poblaciones portuguesas de diferente tamaño. El éxito reproductivo de *Ch. uliginosus* se ha examinado en relación con la estructura genética y el tamaño de sus poblaciones. Los resultados indican una variabilidad intrapoblacional muy baja para los marcadores cloroplásticos utilizados. Nuestro estudio muestra una tasa de germinación significativamente reducida en las poblaciones pequeñas (< 50 individuos) respecto a aquellas de tamaño mediano (50-250 individuos) o grande (> 250 individuos). Para explicar este fenómeno, se deben tomar en consideración las limitaciones ecológicas y el aislamiento genético. Por otro lado, en las poblaciones de *Ch. uliginosus* de mayor tamaño (> 250 individuos) se ha observado una incidencia más acusada de la depredación de semillas antes de su dispersión, lo cual podría estar afectando a su respuesta reproductiva. Finalmente, las poblaciones más pequeñas —que presentan un reducido éxito reproductivo— contienen los haplotipos más distantes evolutivamente y su conservación debería ser, por tanto, prioritaria.

Palabras clave: depredación de semillas; estructura haplotípica; pérdida de hábitat; poblaciones pequeñas; tasa de germinación.

INTRODUCTION

Habitat loss is one of the main forces causing plant population decline and extinction (Brooks *et al.*, 2002). This phenomenon has a strong impact on small and isolated plant populations, leading to genetic deterioration and greater sensitivity to non-genetic forces such as environmental and demographic stochasticity (Gilpin & Soulé, 1986). These dynamics may result in an Allee effect, predicting that the primary outcome of a reduction in population size is a potential erosion in the fitness (Allee, 1931; Groom, 1998; Bataillon & Kirkpatrick, 2000; Higgins & Lynch, 2001). Many studies (e.g. Menges, 1991; Lamont *et al*., 1993; Fischer & Matthies, 1998; Paschke *et al.*, 2002; Hensen & Oberprieler, 2005) have compared certain fitness parameters, mainly seed production and germinability, between plants from small and large populations, finding a clear relationship between reproductive success and population size. Alternatively, other authors (Ouborg & van Treuren, 1995; Morgan, 1999; Costin *et al.*, 2001; Rabasa *et al.*, 2009) point to the existence of several factors that may mask or limit the impact of population size on reproduction capacity. Identifying factors involved in fitness response is a prerequisite for designing management plans to increase the size and persistence of endangered populations, thereby decreasing the probability of extinction (Pavlik, 1994).

Cheirolophus uliginosus (Brot.) Dostál is a hemicryptophyte with a habitat preference for the wetlands of the southwestern and western Iberian Peninsula (Fig. 1A), differing from the remaining representatives of the genus as it is the only herbaceous one. As a species closely associated with either permanent or intermittent water streams, it is very vulnerable to fluctuations in the water table. In relation to this water dependence, extensive plantations of eucalypts (*Eucalyptus globulus* Labill.) and crops such as corn (*Zea mays* L.), especially in Portugal, and strawberry [*Fragaria* ×*ananassa* (Weston) Duchesne ex Rozier] fields, nearby Doñana National Park (Andalusia, Spain), represent excessive water consumption leading to a decrease in the availability of this resource (Casermeiro *et al.*, 2002) for wild species. Watercourses are scarce and this, together with an increase in grazing and subsequent land nitrification, allows wetlands to be colonized by brambles (*Rubus ulmifolius* Schott) and ferns [*Pteridium aquilinum* (L.) Kuhn], which represent an excessive competition for *Ch. uliginosus* (Bañares *et al.*, 2009). Furthermore, flower heads of this species, as well as those from other congeners, are usually infested by insects, destroying achenes and capitula (Bañares *et al.*, 2009).

Presumably as a result of the above-mentioned threats, numerous localities cited during the early and middle 20th century by Rivas-Martínez *et al.* (1980) or Susanna (1993) have subsequently been revisited and have either not been rediscovered or have recently undergone a significant reduction in the number of individuals and/or extension. In fact, this species is included in the Spanish red book of threatened vascular flora (Bañares *et al.*, 2009) and classified as CR ("Critically Endangered") according to IUCN criteria. In contrast, since there does not exist any red list of threatened vascular flora in Portugal, there is a lack of knowledge about the conservation status of the species in this country. Given that Spanish populations of *Ch. uliginosus*—occurring in Andalusia and Extremadura regions—have already been the subject of specific conservation studies (Palacios-González *et al.*, 2008; Bañares *et al.*, 2009), we decided to focus our investigation on the lesser known Portuguese populations.

DNA-sequence analysis has a long-standing history in population and conservation biology research (for reviews, see Schlötterer, 2004; Höglund, 2009). Particularly, chloroplast DNA (cpDNA) haplotyping has been extensively used to infer genetic structure and diversity in endangered plant populations (Soltis *et al.*, 1992; El Mousadik & Petit, 1996; Ueno *et al.*, 2005; Saeki & Murakami, 2009; Zhao *et al.*, 2012). In our work, the molecular approach arises as a preliminary survey of genetic structure in *Ch. uliginosus* that may help us to interpret the results derived from reproduction components.

The present study investigates (1) the population genetic structure of *Ch. uliginosus* by using chloroplast haplotypes, (2) the actual population size, (3) the germinability of the seeds, and (4) the predispersal predation of the seeds. These data may help to find out the causes of the observed demographic decline and propose conservation strategies for this endangered species.

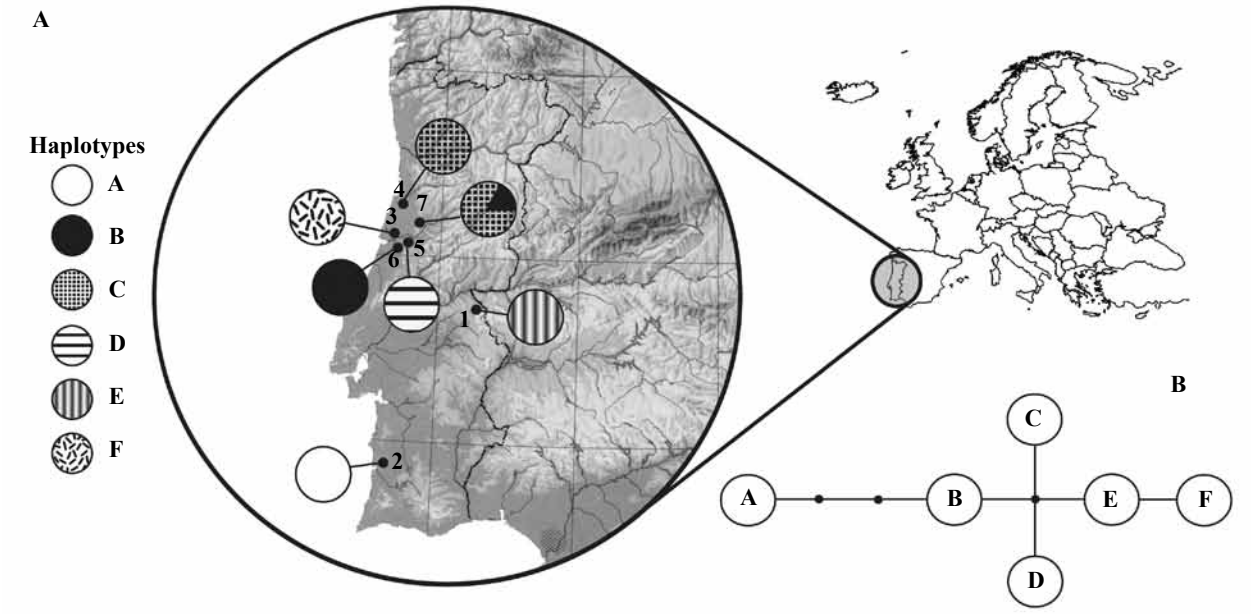

Figure 1. (A), geographical distribution of cpDNA haplotypes and studied populations of *Ch. uliginosus*. The population numbers correspond to those in Table 1, and the pie charts represent the percentage of each haplotype in each population; (B), statistical parsimony network showing relationships of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

MATERIALS AND METHODS

Sampling strategy

The sampling strategy was designed to cover a large range of the distribution of *Ch. uliginosus* to detect intra- and inter-population variability. Two field collections were carried out during the flowering season in 2009. Seven populations of *Ch. uliginosus* were sampled in Portugal (Fig. 1A), located within an altitudinal range from 0 to 705 m a.s.l. Detailed information of each locality and herbarium voucher is summarized in Table 1.

The number of individuals per population was not always precisely counted, because in dense populations it was difficult to distinguish between different individuals in dense clusters of rosettes. As *Ch. uliginosus* also reproduces vegetatively, the accurate estimation of population size at the genet level (i.e. group of genetically identical individuals) may be very difficult (e.g. Luijten *et al.*, 1996). Alternatively, population size was categorised adapting the IUCN criteria for scarce and declining populations (IUCN, 2011): small populations < 50 individuals, medium-sized populations with 50–250 individuals, and large populations > 250 individuals. The number of individuals of small-sized populations was visually counted one by one (Table 1). For larger 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.

For genetic analyses, we collected healthy leaves from each population studied in this work. We sampled one leaf per plant covering the entire population area—up to 13 individuals per population separated by more than one meter where possible— resulting in a total of 57 samples (see Table 1). Leaf material was immediately dried in silica gel and stored at room temperature (20–25ºC) until DNA extraction.

Achenes were taken from three to 20 plants per population depending on population size and taking into account the distribution of individuals. Seeds were dried in silica gel and stored at room temperature. The sampling of capitula was conducted during the autumn in populations 3–7 from Centro Region (Portugal). Approximately 40 flower heads were randomly collected from 20 individuals for each population. Capitula were also dried in silica

gel and stored at room temperature. Unfortunately, we were unable to sample capitula from the smallest populations [Reguengo (1) and Almograve (2)].

Germination tests

Full-sized, healthy-looking achenes were incubated at 20°C with a 12 h day-night photoperiod in Petri dishes with filter paper soaked with distilled water to saturation, following the recommendations of Garnatje (1995). Viable seeds are easily distinguished from unviable seeds (aborted seeds) by their colour and shape. The number of tested achenes per population ranged between 14 and 40 according to the available achene stock (with a maximum of 20 achenes per Petri dish). Germination was considered to be successful when a 1 mm-long radicle emerged from the achene; each achene that germinated was removed from the Petri dish. The number of achenes germinating was recorded daily during one month. In order to compare possible differences of germination vigour due to seasonality of achene field collections, germination tests of achenes from population 4 collected in August and November were carried out.

State of capitula

Randomly sampled flower heads from populations 3 to 7 of *Ch. uliginosus* were dissected and classified in three different categories according to their status: (1) good condition, (2) capitula abortion and (3) predated capitula. Flower heads were recorded as predated when they had direct evidence of damage caused by insects such as wounds, holes or occurrence of insect larvae inside the seeds. Incompletely developed capitula were recorded as aborted only when there was no evidence of insect damage; otherwise they were recorded as predated. The occurrence of predatory insects was also reported and specimens were subsequently preserved in 70% ethanol and determined taxonomically.

Genetic analyses

Total genomic DNA was extracted from silica gel-dried tissue (*ca*. 10 mg) following the CTAB protocol of Doyle & Doyle (1987) with the modifications of Soltis *et al.* (1991) and Cullings (1992).

We conducted a previous screening test involving nuclear (ITS and ETS) and chloroplast (*rpoB-trnD*, *rps16-trnK*, *rpl32-trnL* and *trnS-trnC*) markers that were sequenced for a few individuals of different populations. Subsequently, we selected the regions that yielded the highest level of polymorphism: *rpl32-trnL* and *trnS-trnC*. The *rpl32-trnL* region was amplified and sequenced with rpl32f as forward primer and $trnL^{UAG}$ as reverse primer (Shaw *et al.*, 2007) referring to the PCR procedure described in the same publication. The *trnS-trnC* region was amplified and sequenced with $trnS^{GCU}$ as forward primer and $trnC^{GCAR}$ for reverse (Shaw *et al.*, 2005) with the following PCR conditions: 95ºC, 5 min; 30x (94ºC, 30 s; 62ºC, 1 min; 72ºC, 2 min); 72ºC, 5 min, and storage at 10ºC. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Hilden, Germany) or DNA Clean and Concentrator™-5 D4004 (Zymo Research, Orange, CA, USA) depending on the quality of the amplification. Direct sequencing of the amplified DNA segments was performed using the BigDye Terminator Cycle Sequencing v3.1 (PE Biosystems, Foster City, CA, USA) following the protocol recommended by the manufacturer. Nucleotide sequencing was carried out at the Centres Científics i Tecnològics of the University of Barcelona on an ABI PRISM 3700 DNA analyser (PE Biosystems, Foster City, CA, USA).

The number of individuals finally analysed per population range between three and 13 due to the plant material availability and the uneven success of extraction, amplification and sequencing procedures (Table 1). Locality 1 (Reguengo) presents only three plants, so all the individuals were sampled in this case. Regarding other sparsely sampled populations (i.e. Almograve and Mata da Foja), the difficult accessibility prevented getting more material. Nucleotide sequences were edited using Chromas LITE v2.01 (Technelysium Pty, Tewantin, Australia) and subsequently aligned manually with BioEdit v7.0.5.3 (Hall, 1999). Chloroplast DNA haplotypes 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 v1.21 (Clement *et al.*, 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 character state.

Data analyses

Statistical analyses of fitness-related parameters

Table 1. Studied populations of *Cheirolophus uliginosus* including localities, collection data, geographical coordinates and estimated population size. Herbarium vouchers are deposited at the Institut Botànic de Barcelona (BC). Localities 4 and 4′ correspond to the same population sampled in August and November, respectively.

No.	Locality	Date	Coordinates	Population size[1]	Haplotypes	*N*[2]	Number of tested achenes
1	Portugal, Alentejo: Reguengo, *Garnatje*, *Pellicer & Vitales* (BC)	2.08.2009	39°17′ N, 7°23′ W	(3 ind.) small	E	3	39
2	Portugal, Alentejo: Almograve, *Garnatje*, *Pellicer & Vitales* (BC)	31.07.2009	37°39′ N, 8°47′ W	(25 ind.) small	A	5	34
3	Portugal, Centro: Mata da Foja, *Paiva & Vitales* (BC)	2.11.2009	40°13′ N, 8°43′ W	(40 ind.) small	F	7	14
4	Portugal, Centro: Fermentelos, *Garnatje*, *Pellicer & Vitales* (BC)	4.08.2009	40°34′ N, 8°32′ W	medium	C	13	40
4′	Portugal, Centro: Fermentelos, *Paiva & Vitales* (BC)	2.11.2009					40
5	Portugal, Centro: Figueiró do Campo, *Paiva & Vitales* (BC)	3.11.2009	40°09′ N, 8°35′ W	medium	D	9	40
6	Portugal, Centro: Paul da Madriz, *Paiva & Vitales* (BC)	2.11.2009	40°07′ N, 8°37′ W	large	B	8	29
7	Portugal, Centro: Pampilhosa, Quinta do Valdoeiro, *Paiva & Vitales* (BC)	2.11.2009	40°21′ N, 8°25′ W	large	C (10 ind.) B (2 ind.)	12	20

[1] Size categories: small (< 50 individuals), medium (50–250 individuals) and large (> 250 individuals).
[2] No. of individuals studied for haplotypes.

were performed with the R software v2.7.0 (R Development Core Team, 2008). Prior to the analysis, data were tested for normality and homocedasticity and nonparametric statistics were applied when the assumptions of parametric tests were not met. The results of germination capacity tests were analysed within a generalised linear model (GLM) framework, assuming a binomial distribution and logit link function. Population size—i.e. small, medium and large size—and populations—nested to size categories—were used as independent variables of the GLM. In relation to the effect of seasonality on achene collection, data distribution did not fit a normal distribution, so a non-parametrical test (Mann-Whitney's U) was used to check whether data from summer and autumn field collections could be considered as the same statistical population.

In order to compare the frequencies of the three considered capitula variables among the different populations, a contingency table was created. The significance of the differences observed was analysed applying a chi-square test to the data. When the value of the expected frequency in any of the cases of contingence table was less than 5, Yates correction was applied in the chi-square calculation.

RESULTS

Germination tests

The percentages of seed germination in the populations studied are detailed in Fig. 2. Differences in germination vigour between the two batches from population 3 collected in August and November 2009 have not been found significant (U = 326.5,

$P > 0.05$), and therefore, both samples have been treated as the same statistic pool of data. The GLM analysis detected significant differences in the germination rates related to population size ($\chi^2 = 23.18$, d.f. = 11, $P < 0.05$). However, not all the levels of "population size" factor were statistically significant. Partial Wald Z-test checked the significance of each coefficient (i.e. levels of "size" factor) in the presence of the others. The significant differences in seed germination among size levels were largely due to small populations (Z-value = −3.388, $P < 0.05$) whereas the rest of coefficients did not have a significant effect on the model.

Evaluation of capitula predation

The results obtained from the classification of capitula according to their condition are summarized in percentages in Table 2. The chi-square test with Yates correction revealed significant differences between populations related to the capitula conditions ($\chi^2 = 25.26$, d.f. = 8, $P < 0.05$).

Among the insects found inside the capitula some were only determined as unidentified adults of Alydidae heteropterans, but in-depth taxonomical classification made it possible to determine adults and larvae of the curculionid coleopterans *Larinus leuzeae* H. Fabre (P. Gurrea, pers. comm.).

Haplotypic structure

Out of several plastid and nuclear DNA markers screened, only the *rpl32-trnL* intergenic cpDNA spacer (988 base pairs; bp) and the *trnS-trnC* intergenic cpDNA spacer (849 bp) showed a sufficient degree of variation, while other markers (*rpoB-trnD*, *rps16-trnK*, ITS, ETS) were either entirely invariable or exhibited only very few variable sites. The two chloroplast markers were finally sequenced for 57 samples from seven different populations resulting in an alignment of 1816 bp. We detected eight polymorphic sites—including a 22 bp indel—representing six different haplotypes (Fig. 1A–B and Table 3). All populations except one (population 7) contained one single haplotype. Haplotypes B and C were shared by more than one population while the rest of haplotypes (A, D, E and F) were each one restricted to a single population (private haplotypes). Phylogenetic relationships between haplotypes inferred by the parsimony network is shown in Fig. 1B. Different haplotypes of *Ch. uliginosus* distinguish from adjacent haplotypes by one, two or three evolutionary events (substitutions or indels) and extinct or unsampled haplotypes are represented as black dots in the parsimony network.

DISCUSSION

Genetic structure, population size, and fitness

The low variability of employed DNA markers and the limited extension of sampling prevent us from inferring accurate statements about the genetic diversity and structure of *Ch. uliginosus* populations. However, because this species was more common at the beginning of the twentieth century, and because its habitat is declining rapidly, we suggest that genetic drift and bottlenecks are likely to be the main causes for the loss of genetic variation in the small populations. Indeed, the sole population presenting two haplotypes—instead of only one—is precisely the population with a larger size (Pampilhosa; > 1000 individuals). Assuming that additional analyses are required to confirm this preliminary result, a positive association between genetic diversity and population size can be envisaged.

Our results also suggest that geographic distance is not shaping the genetic structure of populations in *Ch. uliginosus*. Two medium and large populations separated by only 5 km [(i.e. Figueiró do Campo (5) and Paul da Madriz (6)] do not share any cpDNA haplotype whereas Pampilhosa (7)—the only population showing two haplotypes that are shared—is separated from its nearest neighbouring populations by more than 25 km (Fig. 1A and Table 1). Furthermore, haplotypes evolutionarily distant, as in the case of B and F (Fig. 1B) are only 12 km away. Large cpDNA differentiation among spatially close populations may indicate ancient isolation and—at least for seeds—restricted gene flow. In any case, further analyses involving other molecular markers more sensitive for population genetics studies (i.e. AFLP) may help to confirm the accuracy of these preliminary hypotheses (Vitales *et al.*, unpubl. data).

The results of the germination tests suggest a significant heterogeneity in germination behaviour between *Ch. uliginosus* populations (Fig. 2). Similar results have been previously described for other species (see review in Baskin & Baskin, 1973), some of

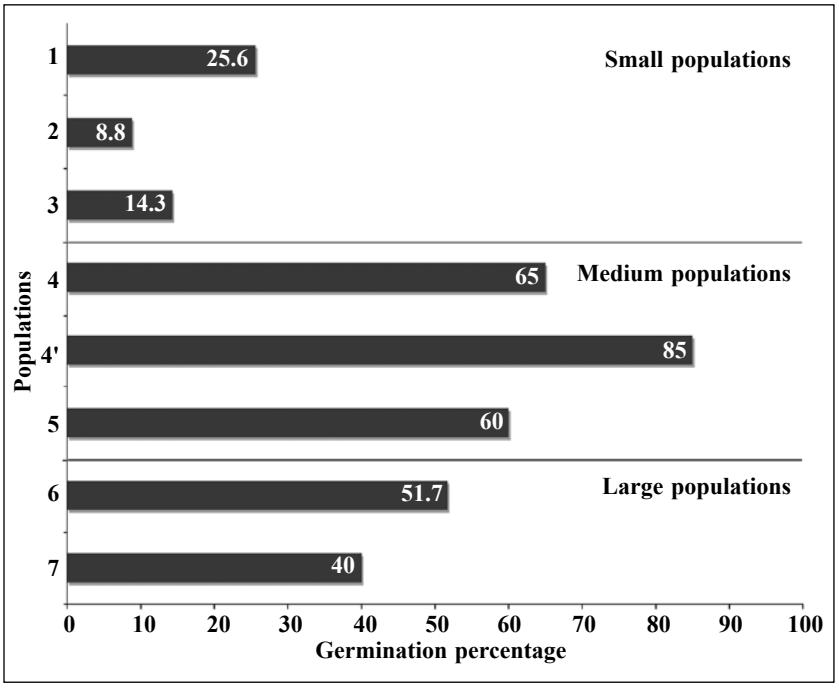

Figure 2. Germination rate of achenes (%) in *Ch. uliginosus* populations. Populations 4 and 4′ correspond to seeds from the same location (Fermentelos) collected in August and November respectively. The horizontal lines delimitate populations according to their size category [small (< 50 individuals), medium (50–250 individuals) and large (> 250 individuals)].

them attributing the inter-population differences to genetic factors and others proposing environmental factors as being of importance.

In *Ch. uliginosus*, reproductive fitness—measured by means of the percentage of germinated achenes—seems to follow a nonlinear pattern related to the number of individuals per population. Certainly, achenes from small-sized populations (1, 2 and 3), all of them displaying fewer than 50 individuals, showed the lowest germination percentages. Nonetheless, the largest germination rates were not found in large populations (> 250 individuals) but in medium-sized ones (50–250 individuals). The lack of a plain Allee effect (Stephens & Sutherland, 1999) on germination capacity has been severally reported in different species (see for instance Hauser & Loeschcke, 1994; Lammi *et al.*, 1999; Costin *et al.*, 2001; Le Cadre *et al.*, 2008) and factors causing unequal response to population size or density are multiple and difficult to disentangle (Vergeer *et al.*, 2003).

Table 2. Percentages of capitula condition in the studied populations**.** The estimated size per population (see Table 1) is indicated beside population number (in parentheses).

		Capitula condition		
Population number (estimated size)	**Number of evaluated capitula**	**Good condition (%)**	**Capitula abortion (%)**	**Predated capitula (%)**
3 (small)	20	55.00	10.00	35.00
4 (medium)	38	50.00	18.42	31.58
5 (medium)	33	51.51	0.00	48.48
6 (large)	31	32.26	0.00	67.74
7 (large)	40	25.00	7.50	67.50

Table 3. List of haplotypes found in the studied populations of *Cheirolophus uliginosus* with indication of nucleotide site variation within the chloroplast regions *rpl32-trnL* and *trnS-trnC*.

Regions	***rpl32-trnL***						***trnS-trnC***	
Positions / Haplotypes	318	382	590–611[1]	644	653	951	1497	1794
A	A	T	+	G	A	C	C	T
B	A	T	–	G	A	A	A	T
C	A	C	–	G	C	A	A	T
D	A	T	–	G	C	A	A	C
E	A	T	–	A	C	A	A	T
F	T	T	–	A	C	A	A	T

[1] At the indel position, "+" means presence and "–" means absence of the fragment.

Our study indicates that reproductive success is severely reduced in small populations (< 50 plants) whereas medium and large-size ones (50–250 and > 250 individuals) do not show any fitness constraint. Reed (2005) pointed out that clearest effects of genetic erosion on fitness should start at some limited population size and it has been suggested by different authors that 50 individuals are required to retain reproductive fitness (Frankel & Soulé, 1981). Apart from this, other non-genetic mechanisms such as pollen scarcity (Berec *et al.*, 2007) may also be responsible for component Allee effects in reproduction. Furthermore, the three smallest populations of *Ch. uliginosus* grow under disadvantageous environmental conditions (D. Vitales, pers. obs.) that might increase stress on reproductive plants resulting in a reduced investment of maternal energy in the offspring (Oostermeijer *et al.*, 1995). In conclusion, either or both ecological and genetic deterioration could explain the lower germination success in small populations of this species.

Predispersal predation of seeds was expected to influence sexual reproduction, since it is a determinant factor in many Asteraceae, especially in the Cardueae tribe (Colas *et al*., 2001; Kolb *et al*., 2007). Interestingly, the extent of predation was not equally distributed among different populations: populations 6 and 7, both with more than 250 plants each, showed a significantly higher proportion of damaged seed and capitula than the small and medium-sized ones. Large host plant populations are more frequently colonized and seed predators may be more likely to persist when the food resource is more concentrated (Östergard & Ehrlén, 2005), this resulting in a major damage in reproductive output (Cunningham, 2000; Arvanitis *et al.*, 2007). Insects found inside *Ch. uliginosus* flower heads belong to taxonomic groups specialised in achene and capitulum consumption (Metcalf *et al.*, 2009) and a relationship between the presence of curculionid and hemiptera larvae and the damage to developing seeds has also been reported in other species (Louda, 1983; Fernández *et al.*, 2008). Therefore, the observed increase in seed predation in larger populations could explain the lower but not statistically significant decrease in germination capacity.

Implications for conservation and concluding remarks

Habitat loss and deterioration have resulted in a rapid decline of Portuguese *Ch. uliginosus* populations, probably also limiting the gene flow between them. Reproductive success in the small populations is clearly reduced and, therefore, recruitment in these populations is virtually absent at present (D. Vitales, pers. obs.). Thus, taking also into account the anthropogenic pressures in its habitat, the viability of small populations of *Ch. uliginosus* may be limited in the short term. The smallest populations contain the most evolutionarily-distant haplotypes and are, therefore, of especially high conservation value. In

order to preserve their distinctive genetic diversity, we recommend ex situ conservation strategies for these small populations (e.g. collection and storage of seeds in germplasm banks). Most of the studied populations are located within protected natural areas included in Nature 2000 network (ICNF, 2013) hence they benefit from some in situ protection. Unfortunately, populations of Mata da Foja (3), Figueiró do Campo (5) and Quinta do Valdoeiro (7) do not show any statutory protection so they should be considered as targets for in situ conservation measures.

Our study reinforces the modern idea on demography and conservation biology proposing that plant species respond somewhat individually to the effects of population decline and fragmentation (Costin *et al.*, 2001). Large populations of *Ch. uliginosus* were not reproductively advantaged in relation to medium-sized populations, at least when seed germination is used as an indicator of plant performance. As a moderate size may favour escape from seed predation (Janzen, 1971; Kéry *et al.*, 2000), medium-sized populations may achieve a better sexual reproduction performance than large ones. Besides, *Ch. uliginosus* could also be showing reproductive erosion signs, but only in populations below 50 individuals, this suggesting the existence of an asymptote in the relationship between population size and germination capacity in this species. Further studies including more populations varying in size as well as alternative molecular approaches such AFLPs (Vitales *et al.*, unpubl. data), may help to further elucidate the relationship between population size and reproductive success in *Ch. uliginosus*.

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