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Extensive genetic differentiation at a small geographical scale: reduced seed dispersal in a narrow endemic marsh orchid, *Anacamptis robusta*

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The endangered orchid *Anacamptis robusta* is a narrowly endemic species restricted to fragmented marsh habitats on Majorca, Spain. To investigate the effects of habitat fragmentation, we quantified genetic diversity and levels of seed exchange among all living metapopulation units of this species. A hypervariable plastid minisatellite was analysed in 1882 individuals and used to estimate genetic diversity, structure, and levels of seed dispersion. High levels of genetic isolation were detected, indicated by low effective migration values between metapopulation units and high genetic differentiation. Bayesian inferences of population growth confirmed previous results of overall population reduction. Comparison of haplotypes found in adult and juvenile plants confirmed reduced seed dispersal among patches. Given the small effective population size and strong population structuring with low exchange of migrants, demographic stochasticity is likely to be the greatest threat to the long-term persistence of this species. However, high values of genetic diversity were observed in almost all metapopulation units, suggesting that the initial colonization process probably involved seed immigration from multiple sources. The genetic survey presented here provides vital information for the future effective management of this rare orchid.

ADDITIONAL KEYWORDS: gene exchange – habitat fragmentation – metapopulation – Orchidaceae – orchid conservation – population dynamics – seed dispersion.

INTRODUCTION

Species dispersal and colonization are determining ecological processes that affect the dynamics of populations and ecosystems in a changing world (Couvet, 2002). More recently, human activity has led to extensive habitat fragmentation and disturbance and such anthropogenic factors may affect population distribution range shifts and long-term persistence (Young, Boyle & Brown, 1996; Aguilar *et al.*, 2006). The spatial isolation of populations may restrict connectivity, leading to low levels of gene flow between fragments, with subsequently greater genetic structure and lower genetic diversity in remnant populations (Honnay & Jacquemyn, 2007). Gene exchange and diversity are also affected by recurrent extinction and recolonization events, as observed in metapopulations. In this scenario, the rates of gene exchange and dispersal ability are important parameters for inferring the demographic viability of a metapopulation (Giles & Goudet, 1997; Pannell & Charlesworth, 2000). Furthermore, increased spatial isolation and reduced

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population sizes can lead to reduced fitness and survival rates (Leimu *et al.*, 2006). However, we still lack basic information about the local dispersal ability and population dynamics for most species, including many rare and endangered species that are in urgent need of conservation.

One way to determine the effects of population fragmentation is to study the landscape scale of spatial genetic structure (He *et al.*, 2010; Binks, Millar & Byrne, 2015a, b). Studies performed with different plant species and at different spatial scales have clarified the role of seed and pollen dispersal in shaping the current patterns of genetic structure observed in highly fragmented habitats, such as islands (Mayol *et al.*, 2012; García-Verdugo *et al.*, 2014). At landscape scales, genetic differentiation among populations can increase as a function of distance due to limited gene exchange by pollen (Jacquemyn *et al.*, 2004) and seeds (Trapnell & Hamrick, 2004). In this regard, nuclear markers are used as a proxy for estimates of pollen-mediated gene exchange and uniparental inherited markers, such as plastid loci, are used to estimate gene exchange by seed dispersion.

Orchids are model organisms for studies aiming to estimate levels of population fragmentation and gene exchange. The number of founding individuals of a population is relatively easy to track since seeds from one fruit are often full-siblings (i.e. a single pollinium can fertilize all ovules of an orchid flower; Cafasso, Widmer & Cozzolino, 2005). Therefore, orchid colonization and population dynamics, for example whether the population came from *in situ* regeneration of the colonists or experienced continued seed immigrations, can be detected by examining gene flow within and among populations (Trapnell & Hamrick, 2004; Chung, Nason & Chung, 2011). The traditional view that the minute, dust-like, and wind-dispersed orchid seeds are able to travel over long distances has not been confirmed by recent studies using molecular genetic data (Peakall & Beattie, 1996; Trapnell & Hamrick, 2004; Chung, Nason & Chung, 2005; Jacquemyn *et al.*, 2006). Indeed, these studies found significant values of spatial genetic structure, explained by limited seed-dispersal distances, sometimes of only a few meters (Trapnell & Hamrick, 2004; Chung *et al.*, 2005). As a result, populations characterized by limited seed dispersal and reduced seed output are expected to increase their genetic differentiation, even at small spatial scales (Trapnell *et al.*, 2013) whether or not this effect is mitigated by higher levels of outcrossing for deceptive orchids (Cozzolino & Widmer, 2005). Furthermore, the low levels of gene exchange detected among orchid populations can challenge management strategies based on the expectation that seeds of rare and endangered species have a high dispersal ability (Chung *et al.*, 2011).

In this study, we took advantage of employing an hypervariable maternally inherited plastid marker (Cozzolino *et al.*, 2003a) for investigating genetic structure and colonization patterns of populations in the rare and threatened *Anacamptis robusta* (T. Stephenson) R.M.Bateman (Orchidaceae). The natural habitats of this terrestrial orchid typically consist of a mosaic of water-rich marshes and dry grasslands, which were formed as a result of recent human management initiatives of the watershed (Herrero, 2013; MXR and SC, personal observations). The distribution of orchid plants is discontinuous among marshes and dry grasslands and different patches can be recognized in the study area (*c.* 4 km^2). Preliminary data suggest that *A. robusta* is able to propagate in marsh and dry grassland habitats, but long-term census analysis (1991–2012) indicates a decrease in population sizes, a trend that could probably affect the long-term survivorship and persistence of this species. The main questions of this study are as follows. (1) What are the patterns of genetic diversity and differentiation among metapopulation units? (2) Is there any evidence of significant genetic structure? and (3) How do processes such as gene flow and population growth differ among geographically close metapopulation units?

MATERIAL AND METHODS

Study species

Anacamptis robusta is a terrestrial orchid endemic to Mallorca (Spain) in the western Mediterranean Sea, with a distribution range restricted to the wetland of the s'Albufera de Mallorca Natural Reserve in the north of the island (Supporting information, Fig. S1). This species occurs mainly in marshes with dense vegetation, but it is also occasionally found in open dry grasslands surrounding the marshes. As a consequence of its narrow distribution, *A. robusta* had experienced great population fluctuations during recent years (Herrero, 2013), mainly due to drainage of marshes resulting from various human activities in historical and recent times. Indeed, from 1991 to 2012, a significant decrease in orchid abundance was detected within the Natural Reserve limits (mean rates of population reduction per year = 7.82%, Herrero, 2013).

The flowers produce no nectar reward, but they have large showy petals and a long spur for attracting nectar-seeking bees (Ren *et al.*, 2014). High levels of fruit set were observed in *A. robusta* (Ren *et al.*, 2014), in contrast with the results observed in other Mediterranean food-deceptive orchids (Cozzolino & Widmer, 2005). The species easily colonize new sites, but also disappear quickly when habitat conditions change due to altered drainage levels or grazing (Herrero, 2013).

STUDY SITE AND SAMPLING STRATEGY

To investigate genetic structure and patterns of gene exchange among populations of *A. robusta*, we adopted a broadly based metapopulation approach that views assemblages of patchy distributed individuals as discrete entities in space that interact via gene flow (Hanski, 1998; He *et al.*, 2010). Following this definition, we considered each discontinuous orchid patch as a metapopulation unit (Fig. 1).

Sampling was performed in all known metapopulation units in dry grasslands and marshes and at different distances from each other (48–3571 m apart). All sampling sites were georeferenced and the type of habitat (dry grassland or marsh) was recorded. To explore the haplotype composition across different generations, all adults and juveniles (young plantlets without inflorescences) were sampled in a metapopulation unit from a dry grassland site (C6) and the spatial location of all adults and juveniles was mapped. Dense vegetation and high water level prevented us from sampling juvenile plants from the marsh sites. Samples from 1882 specimens distributed in ten metapopulation units, plus 166 juveniles from metapopulation unit C6, were collected. Leaf material was collected from all plants and immediately stored into plastic bags with silica gel. A voucher was deposited in the Herbarium of the Universitat de les Illes Balears (*Conesa & Cardona 15786*).

PLASTID MINISATELLITE ANALYSIS

Total DNA was extracted from 5 mg dried leaf material according to Doyle & Doyle (1987). The occurrence of a tandem repeat in the plastid genome of *A. robusta* involves a 16-basepair repeat unit in the $tRNA^{LEU}$ intron (Cafasso *et al.*, 2001; Cozzolino *et al.*, 2003a). Variation in repeat numbers of this plastid DNA minisatellite locus is extensive and has been used to estimate genetic variation even among individuals within populations (Cozzolino et al., 2003b, c). Polymerase chain reaction (PCR) amplification of the $tRNA^{LEU}$ intron was carried out using two specific primers and reaction conditions as described in Cozzolino *et al.* (2003b). The forward primer was dye-labelled, and length variation in the amplification products was resolved on an ABI 3130 capillary DNA sequencer with Liz-500 as internal size standard (Life Technologies).

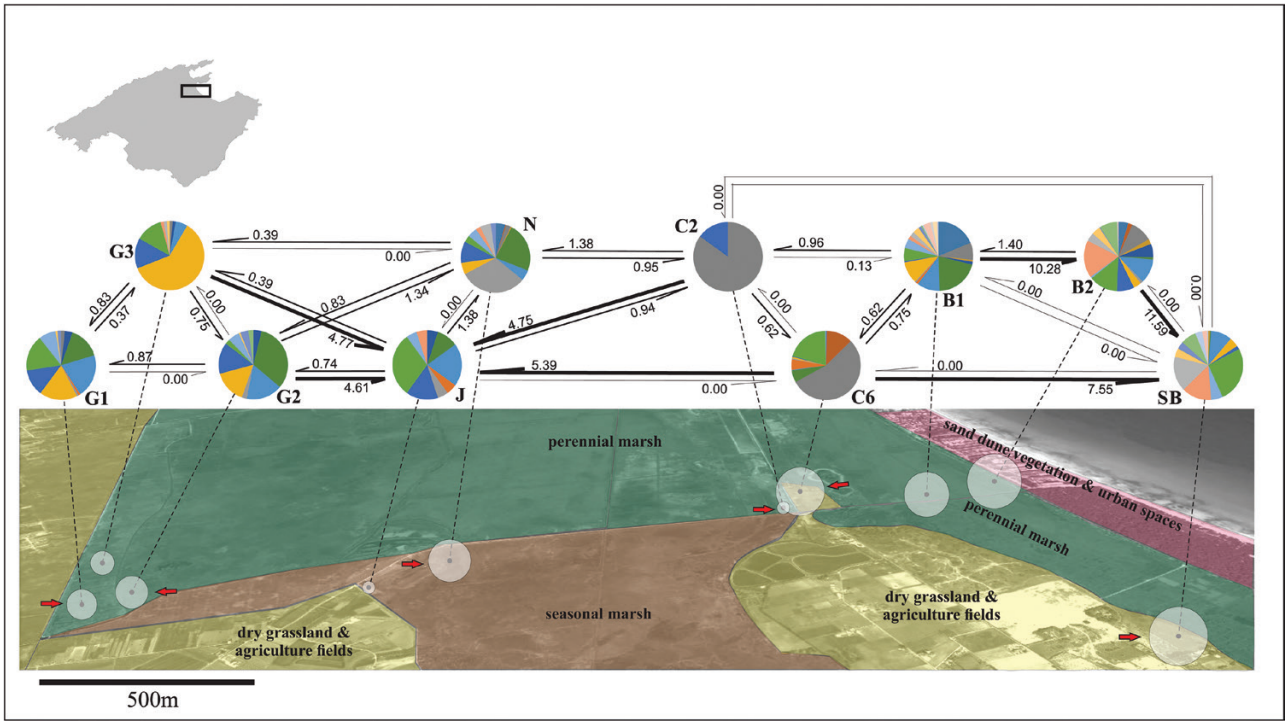

Figure 1. Geographical distribution of *Anacamptis robusta* metapopulation units sampled in this study. The effective number of migrants (*Nem*) between adjacent metapopulation units of *A. robusta* is indicated by arrows between sites. The thickness is proportional to the *Nem* values. Pie charts indicate haplotype frequencies within metapopulation units; white circles are proportional to sample sizes. Red arrows indicate metapopulation units in which consistent negative values of population growth (g) were detected (see Table 1 for details).

Genetic diversity, structure, and demography of populations

Minisatellite repeat number variation was used to define haplotypes. All metapopulation units sampled were characterized for levels of diversity using the number of haplotypes detected, haplotype diversity, and haplotype richness, estimated using the software CONTRIB v. 1.02 (available at https://www6.bordeaux-aquitaine.inra.fr/biogeco/Production-scientifique/Logiciels/Contrib-Permut/Contrib). Estimates of haplotype richness were corrected for differences in sample size using the rarefaction method described by Petit, El Mousadik & Pons (1998).

Genetic differentiation among metapopulation units was determined based on the standardized measure of genetic differentiation Φ'_{PT} (Meirmans, 2006). This measure of genetic differentiation is independent on the amount of within-population genetic variation (Meirmans, 2006). The hypothesis that metapopulation units are differentiated because of isolation-by-distance was tested by assessing the correlation between pairwise geographical distances with pairwise values of Φ'_{PT} using a Mantel test. Correlation significance was estimated after performing 10 000 permutations between pairwise geographical distance and pairwise genetic differentiation matrices. All analyses were performed using the program GenAlEx 6.502 (Peakall & Smouse, 2006, 2012).

A previous population census has identified a continuous and significant decrease in population size of *A. robusta* within the Natural Reserve limits. Accordingly, we analysed our data using Lamarc 2.1.9 (Kuhner, 2006) to estimate population growth (g). The analysis was conducted using a Bayesian search with priors for $g = -100.0$ and 100.0. Four adaptively heated Markov chains (with temperatures of 1.0, 1.5, 3.0 and 10 000.0) were utilized, with 50 000 recorded trees (with the first 10 000 discarded as burn-in) and a sampling increment of 20.

Because gene flow is a crucial process influencing small and/or isolated populations, estimating gene flow is critical to explain current patterns of genetic structure. Theta ($N_e\mu$ for maternal inherited loci, with N_e = effective population size and μ = mutation rate) and the effective number of migrants (N_em for maternal inherited loci, with m = migration rate) were estimated under a coalescent framework using the program Migrate-n 3.6.4 (Beerli & Felsenstein, 2001; Beerli, 2006). Both theta and effective number of migrants were estimated using methods specially designed for uniparental markers (Migrate-n 3.6.4 documentation). The stepwise mutation model was used following previous information reported by Cozzolino *et al.* (2003a). Starting values were calculated using F_{ST}, and we used model averaging to estimate migration rates and θ values. Migrate-n analyses were conducted using a static heating strategy with four short chains (with temperature values of 1.0, 1.5, 3.0, and 1.0×10^6) and a single long chain with 50 000 recorded steps, an increment of 50 and 20 000 steps discarded as burn-in. We used ten concurrent chains (replicates). Stationarity of the Markov chain was assessed by examining the effective sample size for each parameter.

Juveniles were analysed separately for genetic diversity estimates, the same as those used to analyse adult plants. To visualize the spatial distribution patterns of juveniles of different haplotypes relative to the adults, we mapped the adults and juveniles for each haplotype and calculated the matrix plot of juvenile frequencies in different distance intervals to their adults using the program PAST Version 3 (Hammer, Tharper & Ryan, 2001). Genetic diversity estimates (number of haplotypes, effective number of haplotypes, and haplotype diversity) were calculated for all adults and juveniles using the program GenAlEx 6.501.

RESULTS

Broad-scale patterns of genetic diversity and demography

Among 1882 sampled *A. robusta* adult plants, we detected 23 length variant haplotypes (Table 1; Supporting information, Table S1). The number of haplotypes ranged from two (C2) to 22 (B2) and all haplotypes were found in more than one metapopulation unit, except haplotype H22 (Supporting information, Table S1). Haplotypes H7 and H12 were found in nine out of ten metapopulation units, and haplotype H11 was present in all metapopulation units. Haplotype diversity ranged from 0.268 to 0.907 and haplotype richness ranged from 1.000 to 9.048 (Table 1).

High genetic differentiation was observed among metapopulation units ($\Phi'_{PT} = 0.695$), with pairwise Φ'_{PT} values among metapopulation units ranging from 0.038 to 0.996 (Supporting information, Table S2). Approximately 66% of Φ'_{PT} values were >0.500 and only 7% of Φ'_{PT} values were <0.200. Isolation by distance among metapopulation units was not detected ($P = 0.156$).

Most of the metapopulation units growth values were negative (Table 1), indicating shrinkage. In fact, negative values not including 0 in the 95% credible intervals were observed for metapopulation units G1, G2, N, C6, C2, and SB, indicating a smaller population size in the present than in the past (Lamarc documentation). Bayesian estimates of N_em were low for most metapopulation unit pairs (Fig. 1: to reduce the complexity of the data set, only N_em estimates among adjacent metapopulation units are shown; the complete data set is available from authors upon request). By considering only the 38 N_em estimates calculated between adjacent metapopulation units, 27 values were below one (Fig. 1). Asymmetries in gene flow between

Table 1. Metapopulation units of *Anacamptis robusta* sampled with their identification code, habitat description and sample size (N), number of haplotypes (NH), haplotype diversity (HD), haplotype richness (HR), effective population size (Ne), and values of population growth (*g*), including 95% confidence intervals. Metapopulation units are indicated as shown on the map in Fig. 1

Units	Habitat	N	NH	*HD*	*HR*	N_e [1]	*g* [2]
B1	Perennial marsh	246	19	0.907	9.048	3.006	–95.03 (–100.48 to 12.67)
B2	Perennial marsh	359	22	0.887	8.553	5.165	2.3 (–69.42 to 2.38)
G1*	Perennial marsh	113	12	0.860	6.446	0.531	–92.38 (–99.03 to –28.28)
G2*	Perennial marsh	130	11	0.821	6.112	0.465	–92.69 (–100.23 to –14.56)
G3	Perennial marsh	71	9	0.602	4.083	0.237	–8.06 (–93.02 to 13.84)
N*	Seasonal marsh	220	14	0.827	6.959	0.856	–63.41 (–97.07 to –15.48)
J	Seasonal marsh	20	9	0.868	8.000	2.943	–62.10 (–98.63 to 54.82)
C6*	Dry grassland	285	11	0.644	3.758	0.390	–79.23 (–98.08 to –4.88)
C2*	Dry grassland	20	2	0.268	1.000	0.600	–97.78 (–100.26 to –48.24)
SB*	Dry grassland	418	17	0.864	7.541	4.809	–93.83 (–98.52 to –40.10)
Total		1882	23	0.919			

[1]Effective population sizes were calculated using the formula Ne = theta/μ, where μ = mutation rate (0.0032, calculated for minisatellite markers in *Anacamptis* Rich. (Cozzolino *et al.*, 2003); [2]Positive values of *g* indicate population growth and negative values indicate shrinkage, only if confidence intervals exclude zero.
*Population shrinkage detected.

metapopulation units were also evident, and only gene exchange between metapopulation units B1 and B2 showed values higher than one, in both directions.

COMPARISON BETWEEN ADULTS AND JUVENILES IN THE DRY GRASSLAND SITE

The juveniles showed similar patterns in genetic diversity and structure to the adults. In the examined 166 *A. robusta* juveniles, seven haplotypes were found. The haplotype diversity and effective number of haplotypes were higher in juvenile plants (0.729 and 3.68, respectively) than in adults (0.644 and 2.79, respectively). The most common haplotype among adults, H3, was also dominant among the juvenile plants, with >30% of juveniles carrying this haplotype. Two haplotypes, H7 and H9, were found in juveniles, but not in adults.

Most juveniles accumulated near the clusters of the adults (Fig. 2). Juveniles showed various distribution patterns relative to adults based on haplotype correspondence (Fig. 3). For example, for haplotype H12 juveniles were mostly found 5–6 m from the adults, whereas most juveniles with the H8 haplotype were not found further than 5 m from the adults carrying the same haplotype (Figs 2 and 3). Haplotypes H6 and H12 had a distribution origin of juveniles at 7–8 m and 5–6 m away from adults, respectively (Fig. 3).

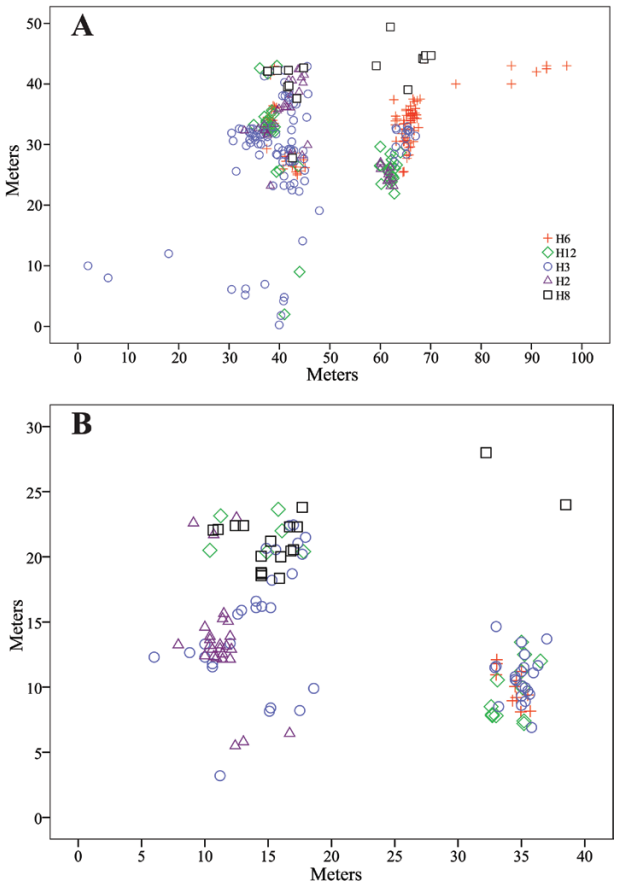

Figure 2. Spatial distributions of haplotypes found in adults (A) and juveniles (B) of *Anacamptis robusta* in the dry grassland C6.

DISCUSSION

The different levels of genetic diversity, the negative demographic signs detected and the historical records

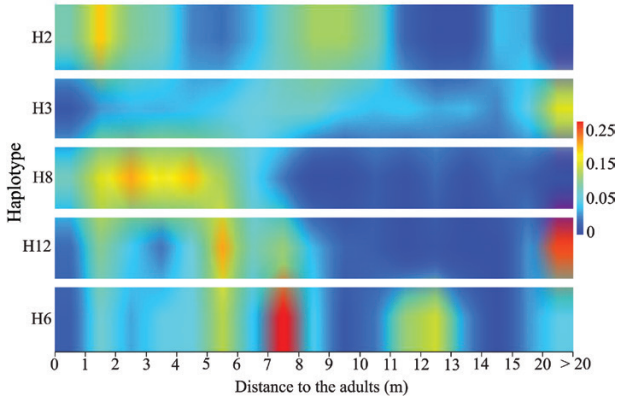

Figure 3. Distribution frequency of juveniles of *Anacamptis robusta* relative to the distances to their adults in the dry grassland C6.

of population decline suggest a dynamic scenario of extinction/recolonization events among metapopulation units of *A. robusta*. Moreover, the low levels of gene exchange observed in *A. robusta* suggest reduced seed dispersal which, consequently, may have contributed to the high plastid genetic differentiation observed among metapopulation units. Despite the high levels of population isolation and reduced seed dispersal, genetic diversity was high in most metapopulation units (Table 1). The hypervariable plastid marker used in this study, coupled with the extensive sampling of plants (1882 specimens), enabled us to conduct an unprecedented population genetic study, using genetic information from the plastid genome. Our data not only inform us about the genetic diversity and demography of this narrow endemic species, but they also contain important information about the mechanisms that may be responsible for shaping the current patterns of genetic structure, which are of great importance for conservation management.

Processes affecting genetic differentiation and demography among adjacent populations

Multiple lines of evidence confirm the strong genetic differentiation of populations separated by a few hundred meters (Fig. 1; Supporting information, Table S2). The overall plastid genetic differentiation found among *A. robusta* metapopulation units is in agreement with the results observed in other genetic studies using plastid markers (McCauley *et al.*, 1996; Levy & Neal, 1999). The plastid genetic differentiation reported here is similar to the values observed in naturally fragmented populations, separated by strong environmental barriers, for example in inselberg populations (Pinheiro *et al.*, 2014), patchy epiphyte communities (Trapnell *et al.*, 2013), and oceanic islands (Mayol *et al.*, 2012). Long-term fragmented populations may suffer the effects of genetic erosion through random genetic drift and increased levels of inbreeding (Ellstrand, 2014; Nistelberger *et al.*, 2015). However, since high levels of genetic diversity are observed in almost all metapopulation units (Table 1), the role of genetic drift is, at least, controversial in *A. robusta*. According to Binks *et al.* (2015a), rare species may preserve high levels of genetic diversity due to large population sizes and a combination of sexual and asexual reproduction. Species cohesion in *A. robusta* is probably maintained by pollen-mediated gene flow, which often shows higher values than seed dispersal in flowering plants (Petit *et al.*, 2005). In this context, the analysis of nuclear markers would fail to reveal population structure among metapopulation units in *A. robusta*. Pollinators visit flowers of *A. robusta* intensively, contributing to high levels of fruit set (Ren *et al.*, 2014) and potentially carrying pollen among metapopulation units. Indeed, values of population genetic differentiation based on nuclear markers are often low among orchid populations, indicating extensive gene flow by pollen (Phillips, Dixon & Peakall, 2012).

Apparently, the connectivity of populations is severely limited by the low levels of historic genetic exchange due to limited seed dispersal (Fig. 1). Even in populations a few meters apart, N_em values are below 1, a parameter often regarded as the minimum required for species and population cohesion. The high number of different haplotypes found in each metapopulation unit suggests that occasional seed dispersion events among units may still occur. Indeed, two haplotypes found among juveniles were not present among adult plants in the C6 metapopulation unit, suggesting dispersal from different sites. The high level of genetic variability found in almost all metapopulation units indicates different seed immigration events from multiple sources, as observed in other studies (reviewed by Hamrick & Trapnell, 2011). Continuous seed dispersal from multiple sources would result in high levels of gene exchange among units, since most recruits would be the products of immigration from different sites. This is not the case, as low gene exchange was detected with a maternally inherited marker as our plastid locus (Figs 1 and 2). Thus, the number of recruits from different sources may decrease in advanced stages of population colonization, when most recruits are from the progeny of the original founders. The gene flow estimates calculated here are historic and thus a product of past events of seed dispersal. The analysis of progenies using highly informative nuclear markers could clarify how contemporaneous gene exchange is affected by recent population fragmentation, which is potentially caused by different strategies of vegetation management, as described by Herrero (2013).

Different successional stages can significantly change the connectivity among populations in a

metapopulation scenario. In recently founded and expanding populations, a higher frequency of recruits from adjacent sites (Paggi *et al.*, 2010; Hamrick & Trapnell, 2011) and a strong spatial genetic structure are expected due to the low densities of adult plants (Chung, Nason & Chung, 2007). In subsequent successional stages, a significant spatial genetic structure persists only in long-lived perennials showing limited seed dispersal (Erickson & Hamrick, 2003), such as *A. robusta*. The selection of genetic-related individuals or fine-scale genetic interactions of mycorrhizal associations may further increase and favour the survival of spatial aggregates of relatives (Jacquemyn *et al.*, 2012), limiting recruitment and consequently gene exchange by seeds with adjacent sites. This scenario is also congruent with the hypothesis that, even if high levels of dispersion may initially occur, only a few selected genotypes can successfully establish in a new patch (i.e. a strong genotype-soil interaction) and then colonize it with pre-adapted genetic-related individuals (i.e. their progeny) (Schmitt & Gamble, 1990). A long-term study comparing metapopulation units submitted to different successional stages would clarify this question.

Despite the high levels of genetic diversity observed, a decrease in population growth was consistently detected in almost all metapopulation units (Table 1). Population growth can be severely affected by low levels of fruit set, a common characteristic in food-deceptive orchids (Tremblay *et al.*, 2005). However, a mean fruit set of 50.49% was found for the SB metapopulation unit by Ren *et al.* (2014), suggesting that fruit set and consequently seed production are not associated with negative signs of population growth. The census performed by Herrero (2013) also points to a continuous decrease in *A. robusta* populations. Accordingly, *A. robusta* populations recently experienced severe fluctuations due to habitat destruction (Herrero, 2013; Ren *et al.*, 2014), in agreement with a metapopulation scenario with recurrent extinction/recolonization events. The study performed by Jacquemyn *et al.* (2006) also detected higher population losses for orchids occurring in wet grasslands, which are more prone to extinction than species confined to forest habitats or calcareous grasslands. Herrero (2013) also reported the founding of new populations and high species turnover, highlighting the connection between *A. robusta* demography and the pattern expected for metapopulations (Giles & Goudet, 1997; Pannell & Charlesworth, 2000). To date, the different vegetation management strategies adopted over time in the region may be associated with metapopulation unit turnover. For example, fire and grazing are commonly used to decrease vegetation density and stimulate *A. robusta* colonization (Herrero, 2013). On the other hand, the lack of such initiatives increases native vegetation growth (establishment of tall grassland patches), which decrease the availability of suitable habitat and consequently the abundance of orchid populations in the studied area.

Differences between adults and juveniles in the dry grassland habitat

Despite the strong difference between sites, no obvious differences in genetic diversity or spatial genetic structure were observed between life-history stages (adult versus juveniles) within each dry grassland site in agreement with other studies on orchids (Peakall & Beattie, 1996; Chung *et al.*, 2005). Most of the haplotypes found in juveniles were also present in adult plants, with the exception of two haplotypes detected in juveniles. An initial phase of recruitment based on seed immigration from adjacent metapopulation units, followed the recruitment of the seeds from within the established metapopulation unit is suggested as an explanation of this pattern, as discussed above. The dominant haplotypes among adult plants also dominated most of the juvenile generation (Fig. 2), indicating that metapopulation C6 regenerates mainly from subsequent recruitment from the founders, without significant gene exchange with other sites. Tonsor *et al.* (1993) also found that genetic relatedness increases from adults to next generations (seedlings and seeds) in *Plantago lanceolata* L. (Plantaginaceae), which can be explained by continuous local seed dispersal of the colonists.

In *Orchis purpurea* Huds., Jacquemyn *et al.* (2006) found weaker or no spatial genetic structure among seedlings, which was attributed to the overlapping seed dispersal, random mating, and improvement of germination condition. The spatial distribution of *A. robusta* juveniles and adults in the dry grassland C6 (Fig. 2) indicates that juveniles of the same haplotype tend to form clusters overlapping largely with the adults. This pattern can be explained by the restricted seed dispersal and by adult plants accumulating appropriate fungi and suitable substrate for successful seed germination and seedlings establishment (Jacquemyn *et al.*, 2012). The spatial locations of juveniles relative to adults of the same haplotype further suggest that most juveniles are within 10 m of their adults (e.g. H6), whereas some haplotypes have two main distribution distance of juveniles from adults (e.g. H8, H12, and H2) (Fig. 3). These data indicate that distribution patterns of juveniles may vary considerably among haplotypes and sites, suggesting the spatial distribution of juveniles depends not only on the seed dispersal distance, but is also largely determined by successful of seed germination and seedling establishment (Jacquemyn *et al.*, 2006). Overall, these results suggest that population colonization history and local microenvironments in different habitats may significantly affect spatial

genetic structure and microevolutionary dynamics of orchid patches.

CONCLUSIONS

Many orchid species have suffered from increased levels of population fragmentation and consequently population declines, mainly in wetland habitats in Europe (Jacquemyn *et al.*, 2005). The low fruit set in deceptive orchids (Tremblay *et al.*, 2005) coupled with the limited seed dispersal observed in most species (Trapnell & Hamrick, 2004; Jacquemyn *et al.*, 2006) may further increase the negative effects of spatial isolation and low levels of gene exchange. Our results are in agreement with such expectations, indicating that urgent conservation measures are needed for *A. robusta*. The limited seed dispersal observed within metapopulation units mirrors the genetic isolation between adjacent units (Fig. 1). Negative population growth also suggests that the maintenance of viable populations in the long term may be problematic. Marsh and wetland habitats are particularly sensible to human interferences, such as drainage projects, and increased levels of biotic loss have been reported for this ecosystem (Amezaga, Santamaría & Green, 2002). Adjacent metapopulation units appear to differ in the level of seed immigration and probably can accumulate different genetic resources even at a small spatial scale. This unique potential for long-term storage of genetic variation is of vital significance for local survivorship of a rare and endangered species like *A. robusta*. Dry grasslands can provide an alternative habitat for seed storage and buffer the species from reduction or fragmentation of the marsh habitat and perhaps may act as 'stepping-stones' among isolated patches via seed dispersals (Hardy & Vekemans, 1999). Therefore, dry grasslands should also be taken into account when planning conservation and restoration methods for this endangered and narrow endemic orchid. Overall, the present study illustrates the importance of different types of habitats for population regeneration and micro-evolutionary dynamics in terrestrial orchids.

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REFERENCES

Aguilar R, Ashworth L, Galetto L, Aizen MA. 2006. Plant reproductive susceptibility to habitat fragmentation: review and synthesis through a meta-analysis. *Ecology Letters* **9:** 968–980.

Amezaga JM, Santamaría L, Green AJ. 2002. Biotic wetland connectivity – supporting a new approach for wetland policy. *Acta Oecologica* **23:** 213–222.

Beerli P. 2006. Comparison of Bayesian and maximum likelihood inference of population genetic parameters. *Bioinformatics* **22:** 341–345.

Beerli P, Felsenstein J. 2001. Maximum likelihood estimation of a migration matrix and effective population sizes in n populations by using a coalescent approach. *Proceedings of the National Academy of Sciences of the United States of America* **98:** 4563–4568.

Binks RM, Millar MA, Byrne M. 2015a. Not all rare species are the same: contrasting patterns of genetic diversity and population structure in two narrow-range endemic sedges. *Biological Journal of the Linnean Society* **114:** 873–886.

Binks RM, Millar MA, Byrne M. 2015b. Contrasting patterns of clonality and fine-scale genetic structure in two rare sedges with differing geographic distributions. *Heredity* **115:** 235–242.

Cafasso D, Pellegrino G, Musacchio A, Widmer A, Cozzolino S. 2001. Characterization of a minisatellite repeat locus in the chloroplast genome of *Orchis palustris* (Orchidaceae). *Current Genetics* **39:** 394–398.

Cafasso D, Widmer A, Cozzolino S. 2005. Chloroplast DNA inheritance in the orchid *Anacamptis palustris* using single-seed polymerase chain reaction. *The Journal of Heredity* **96:** 66–70.

Chung MY, Nason JD, Chung MG. 2005. Spatial genetic structure in populations of the terrestrial orchid *Orchis cyclochila* (Orchidaceae). *Plant Systematics and Evolution* **254:** 209–219.

Chung MY, Nason JD, Chung MG. 2007. Effects of population succession on demographic and genetic processes: predictions and tests in the daylily *Hemerocallis thunbergii* (Liliaceae). *Molecular Ecology* **16:** 2816–2829.

Chung MY, Nason JD, Chung MG. 2011. Significant demographic and fine-scale genetic structure in expanding and senescing populations of the terrestrial orchid *Cymbidium goeringii* (Orchidaceae). *American Journal of Botany* **98:** 2027–2039.

Couvet D. 2002. Deleterious effects of restricted gene flow in fragmented populations. *Conservation Biology* **16:** 369–376.

Cozzolino S, Cafasso D, Pellegrino G, Musacchio A, Widmer A. 2003a. Molecular evolution of a plastid tandem repeat locus in an orchid lineage. *Journal of Molecular Evolution* **57** (Suppl. 1): S41–S49.

Cozzolino S, Cafasso D, Pellegrino G, Musacchio A, Widmer A. 2003b. Fine-scale phylogeographical analysis of Mediterranean *Anacamptis palustris* (Orchidaceae) populations based on chloroplast minisatellite and microsatellite variation. *Molecular Ecology* **12:** 2783–2792.

Cozzolino S, Noce ME, Musacchio A, Widmer A. 2003c. Variation at a chloroplast minisatellite locus reveals the signature of habitat fragmentation and genetic bottlenecks in the rare orchid *Anacamptis palustris* (Orchidaceae). *American Journal of Botany* **90:** 1681–1687.

Cozzolino S, Widmer A. 2005. Orchid diversity: an evolutionary consequence of deception? *Trends in Ecology & Evolution* **20:** 487–494.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin, Botanical Society of America* **19:** 11–15.

Ellstrand NC. 2014. Is gene flow the most important evolutionary force in plants? *American Journal of Botany* **101:** 737–753.

Erickson DL, Hamrick JL. 2003. Genetic and clonal diversity for *Myrica cerifera* along a spatiotemporal island chronosequence. *Heredity* **90:** 25–32.

García-Verdugo C, Baldwin BG, Fay MF, Caujapé-Castells J. 2014. Life history traits and patterns of diversification in oceanic archipelagos: a meta-analysis. *Botanical Journal of the Linnean Society* **174:** 334–348.

Giles, BE, Goudet, J. 1997. Genetic differentiation in *Silene dioica* metapopulations: estimation of spatiotemporal effects in a successional plant species. *American Naturalist* **149:** 507–526.

Hammer Ø, Tharper DA, Ryan PD. 2001. PAST: paleontological statistics software package for education and data analysis. *Palaeontologia Electronica* **4:** 1–9.

Hamrick JL, Trapnell DW. 2011. Using population genetic analyses to understand seed dispersal patterns. *Acta Oecologica* **37:** 641–649.

Hanski I. 1998. Metapopulation dynamics. *Nature* **396:** 41–49.

Hardy OJ, Vekemans X. 1999. Isolation by distance in a continuous population: reconciliation between spatial autocorrelation analysis and population genetics models. *Heredity* **83 (Pt 2):** 145–154.

He T, Lamont BB, Krauss SL, Enright NJ. 2010. Genetic connectivity and inter-population seed dispersal of *Banksia hookeriana* at the landscape scale. *Annals of Botany* **106:** 457–466.

Herrero C. 2013. *Evaluacion y trabajos de conservacion para la orquidea de prado (*Orchis palustris*). Memoria 2012.* Govern de les Illes Balears: Conselleria d'Agricultura, Medi Ambient i Territori.

Honnay O, Jacquemyn H. 2007. Susceptibility of common and rare plant species to the genetic consequences of habitat fragmentation. *Conservation Biology* **21:** 823–831.

Jacquemyn H, Brys R, Hermy M, Willems JH. 2005. Does nectar reward affect rarity and extinction probabilities of orchid species? An assessment using historical records from Belgium and the Netherlands. *Biological Conservation* **121:** 257–263.

Jacquemyn H, Brys R, Vandepitte K, Honnay O, Roldán-Ruiz I. 2006. Fine-scale genetic structure of life history stages in the food-deceptive orchid *Orchis purpurea*. *Molecular Ecology* **15:** 2801–2808.

Jacquemyn H, Brys R, Honnay O, Roldán-Ruiz I, Lievens B, Wiegand T. 2012. Nonrandom spatial structuring of orchids in a hybrid zone of three *Orchis* species. *The New Phytologist* **193:** 454–464.

Jacquemyn H, Honnay O, Galbusera P, Roldán-Ruiz I. 2004. Genetic structure of the forest herb *Primula elatior* in a changing landscape. *Molecular Ecology* **13:** 211–219.

Kuhner MK. 2006. LAMARC 2.0: maximum likelihood and Bayesian estimation of population parameters. *Bioinformatics* **22:** 768–770.

Leimu R, Mutikainen P, Koricheva J, Fischer M. 2006. How general are positive relationships between plant population size, fitness and genetic variation? *Journal of Ecology* **94:** 942–952.

Levy F, Neal CL. 1999. Spatial and temporal genetic structure in chloroplast and allozyme markers in *Phacelia dubia* implicate genetic drift. *Heredity* **82** (Pt 4): 422–431.

Mayol M, Palau C, Rosselló JA, González-Martínez SC, Molins A, Riba M. 2012. Patterns of genetic variability and habitat occupancy in *Crepis triasii* (Asteraceae) at different spatial scales: insights on evolutionary processes leading to diversification in continental islands. *Annals of Botany* **109:** 429–441.

McCauley DE, Stevens JE, Peroni PA, Raveill JA. 1996. The spatial distribution of chloroplast DNA and allozyme polymorphisms within a population of *Silene alba* (Caryophyllaceae). *American Journal of Botany* **83:** 727–731.

Meirmans PG. 2006. Using the AMOVA framework to estimate a standardized genetic differentiation measure. *Evolution* **60:** 2399–2402.

Nistelberger HM, Byrne M, Coates D, Roberts JD. 2015. Genetic drift drives evolution in the bird-pollinated, terrestrial island endemic *Grevillea georgeana* (Proteaceae). *Botanical Journal of the Linnean Society* **178:** 155–168.

Paggi GM, Sampaio JAT, Bruxel M, Zanella CM, Goetze M, Büttow MV, Palma-Silva C, Bered F. 2010. Seed dispersal and population structure in *Vriesea gigantea*, a bromeliad from the Brazilian Atlantic Rainforest. *Botanical Journal of the Linnean Society* **164:** 317–325.

Pannell JR, Charlesworth B. 2000. Effects of metapopulation processes on measures of genetic diversity. *Philosophical Transactions of the Royal Society of London* **355:** 1851–1864.

Peakall R, Beattie AJ. 1996. Ecological and genetic consequences of pollination by sexual deception in the orchid *Caladenia tentaculata*. *Evolution* **50:** 2207–2220.

Peakall R, Smouse PE. 2006. GenAlEx 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6:** 288–295.

Peakall R, Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bioinformatics* **28:** 2537–2539.

Petit RJ, El Mousadik A, Pons O. 1998. Identifying populations for conservation on the basis of genetic markers. *Conservation Biology* **12:** 844–855.

Petit RJ, Duminil J, Fineschi S, Hampe A, Salvini D, Vendramin GG. 2005. Comparative organization of chloroplast, mitochondrial and nuclear diversity in plant populations. *Molecular Ecology* **14:** 689–701.

Phillips RD, Dixon KW, Peakall R. 2012. Low population genetic differentiation in the Orchidaceae: implications for the diversification of the family. *Molecular Ecology* **21:** 5208–5220.

Pinheiro F, Cozzolino S, Munt DD, Barros F, Félix LP, Fay MF, Palma-Silva C. 2014. Rock outcrop orchids reveal the genetic connectivity and diversity of inselbergs of northeastern Brazil. *BMC Evolutionary Biology* **14:** 49.

Ren MX, Cafasso D, Barone-Lumaga MR, Cozzolino S. 2014. Low pollination success of hybrids between nectar-rewarding and food-deceptive orchids. *Plant Systematics and Evolution* **300:** 1985–1993.

Schmitt J, Gamble SE. 1990. The effect of distance from the parental site on offspring performance and inbreeding depression in *Impatiens capensis*: a test of the local adaptation hypothesis. *Evolution* **44:** 2022–2030.

Tonsor SJ, Kalisz S, Fisher J, Holtsford TP. 1993. A life-history based study of population genetic structure: seed bank to adults in *Plantago lanceolata*. *Evolution* **47:** 833–843.

Trapnell DW, Hamrick JL. 2004. Partitioning nuclear and chloroplast variation at multiple spatial scales in the neotropical epiphytic orchid, *Laelia rubescens*. *Molecular Ecology* **13:** 2655–2666.

Trapnell DW, Hamrick JL, Ishibashi CD, Kartzinel TR. 2013. Genetic inference of epiphytic orchid colonization; it may only take one. *Molecular Ecology* **22:** 3680–3692.

Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN. 2005. Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biological Journal of the Linnean Society* **84:** 1–54.

Young A, Boyle T, Brown T. 1996. The population genetic consequences of habitat fragmentation for plants. *Trends in Ecology & Evolution* **11:** 413–418.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. *Anacamptis robusta* plant flowering (A) and different habitats where this species is found: dry grassland (B), seasonal marsh (C), perennial marsh (D), and a recently colonized habitat (E), which is the dry grassland at the SB metapopulation unit.

Table S1. Haplotype number, allele size, and frequency in each metapopulation unit of *Anacamptis robusta*. Sample size in each metapopulation unit is indicated in parentheses.

Table S2. Pairwise comparisons of Φ'_{PT} between metapopulation units of *Anacamptis robusta*. See Table 1 for metapopulation unit identification.