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ARTICLE *in* PERSPECTIVES IN PLANT ECOLOGY EVOLUTION AND SYSTEMATICS · FEBRUARY 2015

Impact Factor: 3.61 · DOI: 10.1016/j.ppees.2014.09.003

Perspectives in Plant Ecology, Evolution and Systematics 17 (2015) 54–65

Contents lists available at ScienceDirect

Perspectives in Plant Ecology, Evolution and Systematics

journal homepage: www.elsevier.com/locate/ppees

Research article

A multi-faceted approach for assessing evolutionary significant conservation units in the endangered *Omphalodes littoralis* subsp. *gallaecica* (Boraginaceae)

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ARTICLE INFO

Article history:
Received 28 November 2013
Received in revised form
14 September 2014
Accepted 16 September 2014
Available online 20 September 2014

Keywords:
Genetic structure
Phenotypic traits
Selfing
Rare plant
Reciprocal transplants
Boraginaceae

ABSTRACT

Genetic diversity is now regarded as a key component of biodiversity and its assessment has become a frequent addition to conservation studies. However, due to practical limitations, most studies assess genetic variation using neutral markers while the variability of evolutionary relevant quantitative traits is typically overlooked. Here, we simultaneously assessed neutral and quantitative variation in an endangered plant to identify the mechanism behind their spatial arrangement and to propose conservation guidelines for maximizing mid- to long-term survival. *Omphalodes littoralis* subsp. *gallaecica* is a self-fertilizing therophyte with an extremely narrow and fragmented distribution. Regardless of the marker set (non-coding sequences of cpDNA or Amplified Fragment Length Polymorphism loci), the five extant populations of *O. littoralis* subsp. *gallaecica* showed minimal to none neutral genetic diversity and a lack of gene flow between them. Moreover, genetic structure was identical in samples collected on two consecutive years suggesting that the seed bank cannot buffer against genetic loss. High rates of self-fertilization together with a strongly fragmented distribution and recurrent bottlenecks seem the likely mechanisms that may have led to a dramatic loss of genetic variation in a classic scenario drawn by genetic drift. Despite the extremely narrow distribution range, reciprocal transplant experiments revealed that the populations differed in several quantitative traits and that these differences likely have a genetic basis. Nevertheless, the pattern of differences among populations did not fit the expectations of local adaptation. Instead, phenotypic variation seemed another outcome of genetic drift with important implications for conservation because each population should be designated as an independent evolutionary significant unit (ESU). Our study evidences the benefits of combining neutral markers with appropriate assessments of phenotype variation, and shows that even endemics with extremely narrow ranges can contain multiple conservation units.

Introduction

Designing and implementing appropriate measure that enhance the long-term survival of populations is a major challenge in conservation (Ellstrand and Elam, 1993). In this regard, the genetic structure of endangered populations has become a primary focus of research since theory predicts that intraspecific genetic variation is pivotal for the persistence of species (Ouborg et al., 2006). Under the premise that populations may achieve their greatest evolutionary potential by maximizing their genetic diversity, conservation efforts often aim to preserve the most divergent populations and/or those displaying the largest levels of variation (Moritz, 1994).

Due to practical limitations, the genetic structure is usually assessed with neutral molecular markers even if their suitability for conservation purposes has been repeatedly questioned (Landguth and Balkenhol, 2012; Reed and Frankham, 2001). Instead, quantitative traits are those of most concern for conservation because they are directly related to the species' fitness (Frankham et al., 2010). As natural selection act directly on phenotypes, not on genotypes, these traits reflect the species' ability to undergo adaptive evolution as well as the consequences of inbreeding and outbreeding on reproductive fitness (Allendorf and Luikart, 2012). Unfortunately, current evidence suggests that neutral variation may not be an accurate indicator of quantitative variation; consequently, making

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http://dx.doi.org/10.1016/j.ppees.2014.09.003
1433-8319/

decisions based only on genetic differences detected by neutral markers is not without risk (Frankham et al., 2010; Hedrick, 2001; Landguth and Balkenhol, 2012; Reed and Frankham, 2003). Nevertheless, knowledge of neutral diversity levels is useful for determining genetic relationships among individuals, among populations (gene flow and population structure), or the demographic history of the species (Reed and Frankham, 2001). In this context, a multifaceted approach that combines neutral and phenotypic data should provide a more comprehensive picture of the genetic structure, eventually leading to better conservation management.

Phenotypic variation among individuals results from the interaction between genotype and environment (Kawecki and Ebert, 2004). In the absence of other forces, populations are expected to develop traits that provide an advantage under their local environment resulting in a pattern where resident genotypes are better fitted to their local conditions than genotypes from other habitats (Williams, 1996). This pattern is known as local adaptation (Ashton and Mitchell, 1989). Nevertheless, local adaptation may be hindered by gene flow, confounded by genetic drift, and constrained by a lack of genetic variation (Lenormand, 2002). Disentangling whether the variation observed in phenotypes results from genetic differences, from environmental influence or from both forces acting additively is challenging because genotypes cannot be directly inferred from observed phenotypes (Frankham et al., 2010). Instead, reciprocal transplants are required to evaluate the relative contribution of phenotypic plasticity and genetics (Kawecki and Ebert, 2004).

From a conservation perspective, rare and/or endemic plants are of great concern because of their intrinsic characteristics: small population size, habitat specificity, and geographic isolation (Frankham et al., 2010). These features can be detrimental for the evolutionary potential of the species due to low genetic diversity, strong genetic drift, and inbreeding depression (Cole, 2003; Frankham et al., 2010; Höglund, 2009; Willi et al., 2006). However, rarity is only one of several factors known to have an impact on the species' genetic structure. Life history traits, particularly life form and breeding system, have long been recognized as greatly influencing the distribution pattern of genetic diversity in plant populations (Hamrick and Godt, 1996). Namely, selfing species can maintain lower levels of genetic diversity and higher levels of differentiation among populations compared to outcrossers (Hamrick and Godt, 1996; Nybom, 2004).

The small annual *Omphalodes littoralis* subsp. *gallaecica* (Boraginaceae) M. Laínz (1971) is a rare herb (total occupancy <100,000 m^2) restricted to coastal dune systems in northwest Iberian Peninsula (Romero Buján, 2005; Serrano and Carbajal, 2011). In the last decades, its populations experience continuous decline due to the threats faced by its sensitive habitat (Bañares et al., 2004); as a result, its current distribution is extremely fragmented and today the plant is known to occur in just five dune systems. Because of this rarity, *O. littoralis* subsp. *gallaecica* is cataloged as "endangered" by both the IUCN and the Spanish Catalogue of Threatened Species (Serrano and Carbajal, 2011; Ministerio de Medio Ambiente y Medio Rural y Marino, 2011), and listed as a priority species in the EU Habitats Directive (92/43/EEC, Annex II). Additionally, its habitat is considered as a Site of Community Importance (SCI) within the Natura 2000 network. *O. littoralis* subsp. *gallaecica* is a non-clonal, self-compatible plant and autogamy has been suggested as the most probable mechanism of reproduction (Bañares et al., 2004). Nevertheless, insects are often attracted to its flowers suggesting that in some cases it might reproduce by outcrossing (R. Retuerto, pers. comm.). Flowering period is very short, from March to April, and the ephemeral flowers last less than three days (Romero Buján, 2005). Each fruit develops four seeds that are mainly dispersed by exozoochory (Bañares et al., 2004). The seeds have specialized hooks that easily attach to the animal's fur (Bañares et al., 2004). Seed germination is high (R. Retuerto, pers. comm.) suggesting a small, viable seed bank. Population fluctuate greatly between years, multiplying or dividing by up to ten the number of their individuals (Bañares et al., 2004). The total number of individuals has been reported to be below 140,000 for the species with estimates per site ranging from 740 (our XN site) to 43,000 (our PC and DN sites) (Serrano and Carbajal, 2006). However, these estimates were published as part of the information provided in the IUCN Red List with no further detail on how they were obtained.

Despite the status of *O. littoralis* subsp. *gallaecica* as a species of conservation concern, its population genetics and the variation of its ecophysiological traits between populations have, to our knowledge, never been addressed. Here, we aim to fill this gap with an exhaustive molecular and phenotypic study of the five extant populations of this rare herb. We used sequences of the chloroplast DNA *trn*T-F region and genotypes derived from mostly-nuclear Amplified Fragment Length Polymorphism (AFLP) markers to address the following questions: (a) Is *O. littoralis* subsp. *gallaecica* genetically impoverished as it might be suggested by its life history traits? (b) Are its populations significantly differentiated from each other? (c) Given that *O. littoralis* subsp. *gallaecica* is a therophyte, are there significant between-year differences in its genetic structure? On the other hand, we performed a series of reciprocal transplant experiments to investigate the adaptive component of several quantitative traits related to fitness. Phenotypic variation was examined with an aim to answer: (d) Are there any phenotypic differences between populations? if so, (e) do these differences result from phenotypic plasticity or do they have a genetic basis? (f) Are they adaptive? Finally, we combined the molecular and phenotypic information to propose specific guidelines for the conservation of this endangered plant.

Materials and methods

Leaf sample collection and DNA extraction

Samples for genetic analyses were collected on two consecutive years (2009 and 2010). In March 2009, plants (31–34 per site) were randomly sampled along the whole area occupied by the species at each of the five dune systems currently inhabited by *O. littoralis* subsp. *gallaecica* (Fig. 1). Minimum and maximum distances by the coast between study sites were 21 km (PC-TC) and 153 km (DN-XN), respectively. In four out of the five locations (DN, BD, TC and XN), plants occurred in dune slacks behind the fore dune while in PC the individuals where located in a sandy slope hill. All sites were carefully inspected aiming to sample the whole area occupied by the species. At most sites (DN, BD, PC, XN), *O. littoralis* subsp. *gallaecica* was thinly spread over a rather similar area while in TC the plant was only detected in a smaller patch (see Table 1 for maximum distance between samples within site; minimum distance between sampled individuals was 20 cm). *O. littoralis* subsp. *gallaecica* occupied a continuous area in four sites (BD, PC, TC and XN) while in DN the plant was sampled at two subpopulations separated by approximately 150 m and located on different terrain: a beach trail and an abandoned man-made slope. One year later, the sampling was repeated at three of the systems (DN, BD, and XN). Sampling was non-destructive to meet the requirements of regional conservation authorities; only two-three leaves per individual were collected, dried in silica gel, and stored at −20 °C until DNA extraction. DNA was extracted using the Wizard Magnetic Kit (Promega) and DNA extracts were further purified with PowerClean DNA Clean-up Kit (Mobio, CA, USA) following manufacturers' protocols. The quality

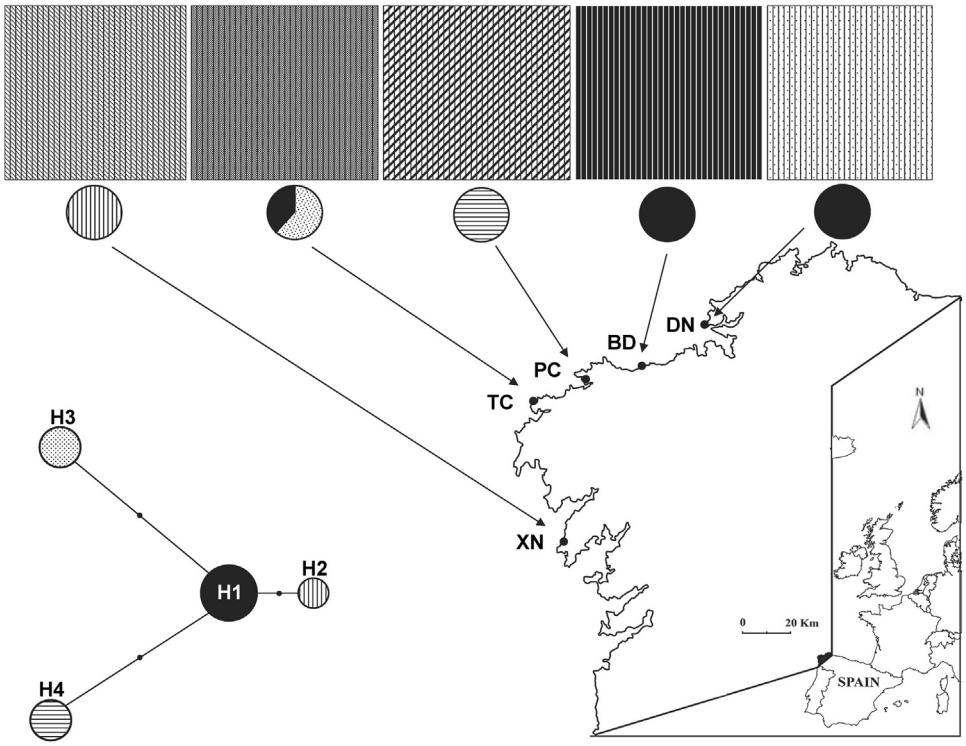

Fig. 1. Sampling sites, genetic structure based on AFLP genotypes, and cpDNA haplotypic network of *Omphalodes littoralis* subsp. *gallaecica*. Range occupancy is strongly fragmented into very small enclaves. Locations: Doniños (DN), Baldaio (DB), Ponteceso (PC), Trece (TC) and Xuño (XN). The histogram on the top depicts individual assignment by an admixture analysis performed for an optimal number of 5 genetic clusters (probability = 0.9996) using AFLP genotype data. Each vertical bar represents one individual with patterns indicating the probability of assignment to each cluster. Pie charts show the relative abundance of four cpDNA haplotypes (H1–H4) in each population; patterns match the haplotype median-joining network shown on the bottom-left. Circle size in the network is proportional to haplotype frequency across populations; black-dots indicate mutational steps.

of the extracted DNA and negative controls were systematically checked on 1.5% agarose gels.

AFLP analyses

Since AFLP performance can be sensitive to reaction conditions (Bonin et al., 2004), we used several control measures to guarantee the reproducibility of our AFLP fingerprints. First, selective primer combinations were chosen after screening twenty-four pairs of primers with three selective bases on 20 individuals (4 individuals per sampling site). The entire process was repeated with new, independent DNA extractions of the same individuals to assess reproducibility. Nine primer combinations were chosen due to their reproducible and easily scorable profiles (EcoRI/TruI: TCA/CAT, TAC/CAT, TAC/CAA, TCC/CTG, TAG/CTG, TCT/CTA, TCT/CAT, TGC/CAG and TGC/CAT). Second, replicate DNA extractions were obtained for 10% of the individuals used in the study (evenly distributed among the 5 sampling sites) and run in parallel with the other DNA samples to monitor reproducibility. Samples and replicates were run in a blind-manner to avoid any bias during scoring. Individuals from each sampling site were evenly partitioned between the various 96-well plates used for PCR while replicates and originals were always run in separate plates; both samples and replicates were randomly distributed within plates. Third, a negative control with no sampled tissue added was included in each set of DNA extractions (24 samples) and went through the entire genotyping procedure. The estimated genotyping error (0.5%) was consistent with results of reproducibility tests conducted for AFLP both in plants and animals (Bonin et al., 2004); none of the individual loci exceeded the maximum acceptable error rate (10%) recommended by Bonin et al. (2007).

AFLP analyses were performed according to Vos et al. (1995) with minor modifications and using nonradioactive fluorescent dye-labeled primers. Approximately 250 ng of genomic DNA were

Table 1
Genetic diversity in *Omphalodes littoralis* subsp. *gallaecica* based on AFLP data.

Population	*N*	Max dist	PLP (%)	P_b	H_e (±SE)	H_{SW}	*G*	G_{eff}	*G*/*N*	G_d	*Eve*
DN	34	171	17 (20.99)	1	0.069 (±0.017)	0.104 (±0.025)	33	32.11	0.97	0.99	0.97
BD	34	111	0 (0.00)	2	0.000	0.000	1	1.00	0.03	0.00	0
PC	34	165	1 (1.23)	2	0.006 (±0.006)	0.008 (±0.008)	2	1.84	0.06	0.47	0.92
TC	30	39	3 (3.70)	2	0.011 (±0.008)	0.016 (±0.011)	3	2.76	0.10	0.66	0.92
XN	33	135	0 (0.00)	19	0.000	0.000	1	1.00	0.03	0.00	0
Total	165		81 (29.35)	26	0.356 (±0.016)	0.530 (±0.018)	40	8.42	0.24	0.89	0.21

N, number of individuals; Max dist, maximum distance between samples (in m); PLP, number and percentage of polymorphic loci (under 5% criterion, only segregating loci were retained for within population estimates); P_b, number of private bands (percentage for the total data set based on 276 scorable loci); H_e; expected heterozygosity (±standard error); H_{WS} Shannon-Weaver Index (±standard error); *G*, number of genotypes; G_{eff}, number of effectives genotypes; *G*/*N*, ratio between genotypes and number of individuals; G_d, genotypic diversity; *Eve*, evenness of the effective number of genotypes. Nei's gene diversity was calculated using segregating fragments only.

digested at 37 °C for 3 h in a final volume of 20 μl with 1.25 units of EcoRI and TruI (Fermentas) and 2× Tango Buffer (Fermentas). Digested DNA was ligated for 3 h at 37 °C to double-stranded adapters (50 pmols of adaptors E, 5′-CTCGTAGACTGCGTACC-3′ and 5′-AATTGGTACGCAGTCTAC-3′, and M, 5′-GACGATGAGTCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′) using 0.5 units of T4 DNA ligase (Fermentas). Then, 2 μl of the ligation product was pre-amplified with 0.3 μM of each single selective primer (EcoRI-T and TruI-C), 2.5 mM $MgCl_2$, PCR buffer 1× (Applied Biosystems), 0.8 μM dNTPs, 0.04 μg/μl BSA, 1 M Betaine and 0.4 units of Taq polymerase (Applied Biosystems) in a final volume of 20 μl. Amplification conditions were 2 min at 72 °C; 2 min at 94 °C; 20 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and a final extension of 30 min at 60 °C. Pre-amplification fragments were diluted 1:5 with Milli-Q water; 2.5 μl of the resulting solution were selectively amplified using 0.6 μM of the selective primers, 0.8 μM dNTPS, 2.5 mM $MgCl_2$, 0.04 μg/μl BSA, PCR Buffer 1× (Applied Biosystems) and 0.4 units of AmpliTaq Gold polymerase (Applied Biosystems) in a final volume of 10 μl. Selective amplification was performed as follows: 4 min at 95 °C; 12 of cycles of 30 s at 94 °C, 30 s at 65 °C (first cycle, then decreasing 0.7 °C for each of the last 11 cycles), and 2 min at 72 °C; 29 cycles of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C; and a final extension of 30 min at 72 °C. Digestion, ligation, and PCR reactions were performed in a PxE thermal cycler (Thermo Fisher Scientific Inc., Waltham, MA, USA). Selective amplification products were electrophoresed on an ABI 3130XL automated DNA (Applied Biosystems) sequencer with HD-500 as size standard (Applied Biosystems). Fragments from 70 to 400 bp were manually scored for presence/absence at each selected locus with the help of GeneMarker v.1.70 (SoftGenetics LLC, State College, PA, USA) following common recommendations (Bonin et al., 2005). Scores of the nine primer combinations were assembled into a single binary data matrix.

cpDNA sequencing

The *trnT-F* region was sequenced according to Taberlet et al. (1991) with minor modifications. PCR reactions for the intergenic spacer between *trn*T-*trn*L were performed in 25 μl using primers a and b (Taberlet et al., 1991), containing 1× reaction buffer, 2 mM $MgCl_2$, 0.2 of each dNTP, 0.5 μM of each primer, 1 μl of genomic DNA and 1.25 units of DNA polymerase (Applied Biosystems). The *trn*L intron and the intergenic spacer *trnL-trn*F were amplified using primers c-d and e-f, respectively. PCR mix incorporated 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 of each dNTP, 0.5 μM of each primer, 1 μl of genomic DNA and 0.35 units of DNA polymerase (Applied Biosystems). PCR profiles consisted of 2 min denaturation at 94 °C followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 50 °C and 90 s of extension at 72 °C with a final elongation of 3 min cycle at 72 °C. PCR products were visualized on 1.5% agarose gels and purified with 1 μl of Exonuclease I (20 U/μl) and 2 μl of FastAP (10 U/μl) (Fermentas). Purified PCR products were bi-directionally sequenced on an Automatic Sequencer 3730XL (Applied Biosystems, USA) following manufacturer's recommendations. Sequences were trimmed with CodonCode Aligner (CodonCode Co., MA, USA) and aligned using Clustal-W (Thompson et al., 1994) implemented in DnaSP v 5.0 (Librado and Rozas, 2009; Rozas et al., 2003).

Reciprocal transplants and phenotypic measures

In May and June 2009, seeds were collected from at least 40 randomly selected native plants growing in each of the five dune systems (sites). Seeds from each site were bulked and stored at 8 °C in a cool chamber until sowed in November 2009. At each and every site, reciprocal transplants were accomplished by sowing seeds from the five origins in 10 haphazardly selected small plots. Plots where arranged into three to four areas within each site, covering all the possible environmental variability. Before sowing, the first 10 cm of the top soil of each plot were carefully inspected and any native *O. littoralis* subsp. *gallaecica* seed was removed. Sowing plots consisted of shallowly buried plastic trays with 60 alveoli filled with local soil; alveoli (2 cm × 2 cm × 2 cm) were tagged according to the provenance of their seeds. Twelve seeds per origin were randomly sowed per tray (60 seed per tray; 600 seeds per site, 120 from each origin). Considering that sand dune species are reported to achieve maximum germination rates when buried 0.5–4 cm deep (Maun, 1994), seeds were sown two centimeters deep. The low depth of the alveoli allowed interactions among the root systems of the plants.

From the date of sowing until the end of the life cycle of *O. littoralis* subsp. *gallaecica* in late May-early June 2010 (precise date varies with provenance), the experimental sites were visited at least once per month to record germination, establishment, and survival. Visit frequency increased as necessary at the time of fruiting to collect the new seeds before dispersal. At the end of the growing season, plants were individually harvested, transported to the laboratory, and separated into roots, shoots and reproductive mass. Roots were washed and all plant material was oven-dried at 35 °C until the weight stabilized to the nearest 0.0001 g (Mettler AJ100, Griefensee, Switzerland). We measured root DW (dry weight), stem DW (combined stems and leaves), reproductive DW (calyxes and seeds), total DW (root plus all above-ground biomass), mean seed weight (mean DW of individual seeds), and number of seeds. All DW measurements were made to the nearest mg. We also recorded germination (proportion of seeds that germinated), maturement (proportion of germinated seeds that became established and reached adult plant size), and fructification (proportion of adult plants bearing fruits).

Statistical analyses of AFLP and cpDNA data

For data analyses, plants from each dune system were considered members of a putative population. With AFLP markers, genetic diversity was estimated with the help of GenAlex 6.41 (Peakall and Smouse, 2006) as the percentage of polymorphic bands (5% criterion), the expected heterozygosity (H_e) (equivalent to Nei's gene diversity), and the Shannon-Weaver Index (H_{SW}). For estimates of within population genetic diversity only segregating bands across all populations were considered. Private bands unique to a single population were also detected with GenAlex 6.41 (Peakall and Smouse, 2006). Since autogamy has been suggested as the most probable mechanism of reproduction of *O. littoralis* subsp. *gallaecica*, the former estimates were complemented with measures of genotypic diversity based on the frequency of distinct multi-locus genotypes. Potential clone mates, *i.e.* individuals with identical banding pattern, were identified with the software GenoType (Meirmans and Van Tienderen, 2004). Since rates for somatic mutations are difficult to determine for natural populations (Douhovnikoff and Dodd, 2003), the genotyping error rate estimated in our reproducibility tests was set as the threshold value for genotype detection (maximum distance between two individuals at which they are still assigned to the same genotype). Genotypic diversity was estimated with the help of GenoDive (Meirmans and Van Tienderen, 2004) as number of genotypes (G), effective number of genotypes ($G_{eff} = 1/\sum p_i^2$, where p_i is the frequency of each i genotype), proportion of distinguishable genotypes (G/N, where N is the number of individuals), genotypic diversity ($G_d = (n/n-1)\cdot(1-\sum p_i^2)$, where n is the sample size), and evenness ($Eve = G_{eff}/G$). The effective population size (N_e) was estimated for each population with Colony v 2.0.5 (Jones and Wang,

2010). Colony employs a single-sampled approach that uses sibships assignment analysis to estimate N_e, using the multilocus genotypes of a sample of offspring taken at random from a single cohort in a population (Wang, 2009). Colony has been shown to perform well with both simulated and empirical data (Beebee, 2009; Phillipsen et al., 2011; Wang, 2005). We run our AFLP data assuming a monoecious plant with polygamy and inbreeding under the full likely-hood model with high precision and uniform prior for shibship sizes. Furthermore, for monoecious species under the inbreeding model, Colony estimates the probability that each offspring comes from selfing fertilization.

The partitioning of the genetic diversity and the occurrence of differences between years were evaluated by the analysis of molecular variance (AMOVA) (Excoffier et al., 1992) implemented in GenAlex 6.41 (Peakall and Smouse, 2006). Its significance was tested by 9999 random permutations of individuals among populations/generations and genetic variation was expressed as Φ_{PT}, an analog of F_{ST}. Population structure was further investigated by calculating the pairwise simple-matching dissimilarities between populations and depicted in a Principal Coordinates Analysis (PCoA) as in Kloda et al. (2008). Also, the correlation between pairwise Φ_{PT} statistics and log-transformed geographic distances was assessed with the Mantel test (10,000 bootstrap randomizations) implemented in the Isolation by Distance Web Service (Jensen et al., 2005). Finally, the arrangement of genetic differentiation was further investigated with the individual-based Bayesian approach implemented in BAPS 5.3 (Corander et al., 2008). The option for clustering of individuals was run 3 times for each of $K = 1–20$. The optimal partition determined by the program was used to estimate the levels of genetic admixture of each individual (with 200 reference individuals simulated for each genetic group and each original individual analyzed 20 times).

The *trn*T-*trn*F region of the cpDNA amplified in this study contains two intergenic spacers, *trn*T-*trn*L and *trn*L-*trn*F, and the *trn*L intron (Taberlet et al., 1991). Given the non-recombinant nature of cpDNA, the three fragments were combined into a single sequence for each individual. The various distinct haplotypes found in our data set were identified with the help of Geneious v.6.1.6 (Biomatters Ltd., Auckland, New Zealand). The phylogenetic relationships between haplotypes were inferred using the median-joining algorithm implemented in Network 4.6 (Bandelt et al., 1999). For the phylogeographic reconstruction, indels were treated as a fifth state, *i.e.* multi-position gaps were upweight compared with ungapped positions (Simmons et al., 2007; Ogden and Rosenberg, 2007). Population diversity estimated as haplotype diversity (H_d) and nucleotide diversity (π) was calculated with Arlequin 3.5 (Excoffier et al., 2005) while intra-population genetic diversity (h_s) and total genetic diversity (h_t) were estimated using Permut (Pons and Petit, 1996). The contribution of each population to the total haplotype diversity (C_T) and the total haplotypic richness (C_T^r) were estimated with Contrib (Petit et al., 1998). C_T and C_T^r were partitioned into two components, the contribution due to a population's own level of diversity (C_S and C_S^r), and its differentiation from other populations (C_D and C_D^r), respectively.

Population structure was again estimated by an analysis of molecular variance (AMOVA) based on haplotype frequencies (Excoffier et al., 1992) and its significance assessed by calculating the F_{ST} statistic (after 1023 permutations) (Excoffier et al., 2005). Permut (Pons and Petit, 1996) was used to estimate the G_{ST} statistic, based on haplotype frequencies, and N_{ST} values, based on both haplotype frequencies and distances between haplotypes (number of mutational steps). Significantly higher values of N_{ST} compare with G_{ST} suggest the presence of phylogeographic structure. Finally, the correlation between geographic and genetic distance was inferred using a Mantel test implemented in the IBD web service (Bohonak, 2002) and its significance was determined after 10,000 randomizations.

Statistical analyses of phenotypic traits

The various phenotypic traits (root DW, stem DW, reproductive DW, total DW, mean seed DW and number of seeds) measured in the reciprocal transplant experiments were analyzed with an analysis of the variance (ANOVA) using the general linear model implemented in package *nlme* of the statistical software R v. 3.0.1 (R Development Core Team, 2013). Given the controversy about the pattern that should be taken as diagnostic for local adaptation in reciprocal transplants, we followed the two criteria proposed by Kawecki and Ebert (2004). First, we tested the "*local vs. foreign*" hypothesis that compares demes within habitats: should local adaptation occur, "*local*" demes are expected to perform better than demes from other habitats ("*foreign*" demes). Second, we tested the "*home vs. away*" criterion that compares a deme's fitness across habitats: should local adaptation occur, demes should perform better when growing at their own habitat ("*home*") than at others ("*away*"). Although both criteria were examined, the "*local vs. foreign*" test provides more convincing evidence of local adaptation because the "*home vs. away*" test may confound the effects of divergent selection with intrinsic differences in habitat quality (Kawecki and Ebert, 2004).

To test both criteria, we designed two dummy variables "*local vs. foreign*" and "*home vs. away*" that identified those seeds growing at their site of origin. Initially, general linear models were run with area, origin, and site treated as fixed factors, and tray treated as a random term; the models also included the interaction between site and origin. Whenever area showed no significant effect, the analyses were repeated taking both tray and area as random terms, being tray nested within area. Contrasts for "*local vs. foreign*" and "*home vs. away*" were based on the posterior distributions of the model parameters using the MCMC method (Venables and Ripley, 2002) using package *gmodels*. The goodness of fit of the model was examined by plotting the residuals *vs.* the fitted responses from the model. Root DW, stem DW, reproductive DW, and total DW had to be log-transformed to meet the requirements of the model. Similarly, the number of seeds was square root transformed since the latter is more adequate for counts.

Differences in germination, maturement, and fructification success were assessed with Chi-squared tests (Pearson, 1900) using Statgraphics Centurion v16.1.11 (StatPoint Technologies Inc.). The proportion of individuals reaching each stage was compared among seeds of the various origins on a per transplant site basis. Whenever significant differences were detected, the pattern of the standardized residuals was examined to detect provenances with anomalous rates (Quinn and Keouggh, 2002).

We also estimated the phenotypic variability of root DW, stem DW, reproductive DW, total DW, mean seed DW and number of seeds. We considered plasticity at the population level as an average across individuals from each origin (Richards et al., 2006; Valladares et al., 2006). Plasticity was quantified for each origin with the simplified relative distance plasticity index ($RDPI_s$) (Valladares et al., 2006). $RDPI_s$ ranges from 0 (no plasticity) to 1 (maximal plasticity) and is calculated as the absolute phenotypic distances between the means of same origin in two different environments (transplant sites), divided by the sum of the two mean phenotypes within a given environment. In our case, with five transplant sites the number of $RDPI_s$ values was 10 for each origin and differences between origins in $RDPI_s$ were evaluated with one-way ANOVA using Statgraphics Centurion v16.1.11.

Results

Genetic diversity and structure

A total of 276 reproducible AFLP markers were scored in the 165 individuals sampled in 2009. Eighty-one (29.35%) loci were segregating for the whole data set and were retained for population's diversity estimates. Overall, 26 private bands were detected in all populations: one in population DN; two in BD, PC, and TC each; and 19 in XN (Table 1). Estimates of total genetic diversity for the species (H_e = 0.356; H_{SW} = 0.530) were one or two orders of magnitude higher than the values observed at individual populations where diversity was consistently low. The various indices of genetic diversity were correlated across populations: diversity was low at DN (20.99% polymorphic loci, H_e = 0.069, H_{SW} = 0.104), very low at PC and TC (1.23% polymorphic loci, H_e = 0.006, H_{SW} = 0.008 and 3.70% polymorphic loci, H_e = 0.011, H_{SW} = 0.016, respectively), and zero at BD and XN.

The 165 individuals only produced 40 distinct genotypes (G_{eff} = 8.42, G/N = 0.24); moreover, most individuals shared just seven genotypes, explaining the low evenness recorded at species level (*Eve* = 0.21). Nevertheless, none of the genotypes detected in the study occurred in more than one population so that each local deme had a distinctive set of AFLP genotypes. Genotypic diversity echoed the changes between populations seen above for genetic diversity. However, while genetic diversity was consistently low across populations, genotypic diversity in DN could be described as high because almost every individual sampled at this site exhibited a distinct genotype (G = 33, G_{eff} = 32.11, G/N = 0.97, G_d = 0.99). In contrast, most of the individuals sampled at the other four dune systems shared just one (BD, XN) or a very few (two in PC, three in TC) genotypes producing very low estimates of the G/N ratio at these sites (<0.10). Nonetheless, the high evenness recorded at PC and TC (0.92) indicates that the few haplotypes found on these sites were evenly partitioned among individuals. As for genotype diversity, the estimated effective population size (N_e) showed the highest value in DN (N_e = 16, 95% IC = 8–33), while TC and PC displayed intermediate N_e values (in TC N_e = 6, 95% IC = 3–21; in PC N_e = 4, 95% IC = 1–12) and eventually, since all individuals in BD and XN are identical it was not possible to infer the N_e with Colony. Nevertheless, the expected N_e would range from 1, assuming selfing matting, to 3 for outcrossing with polygamy. Selfing estimates greatly varied among populations ranging from a low 0.320 in DN to a high 0.925 in BD and XN, with TC and PC displaying intermediate values (0.644 and 0.647, respectively).

Genetic differentiation was extremely high and almost reached the theoretical limit of one (Φ_{PT} = 0.963, P < 0.0001), indicating that nearly all the genetic variation (96%) was due to differences between populations. Pairwise comparisons were likewise high and significant (Φ_{PT} > 0.79 and P < 0.05 after Bonferroni correction for each and every pairwise comparison). The most diverse population, DN, displayed the lowest pairwise Φ_{PT} values while the southernmost and relatively isolated XN showed the highest differentiation (Φ_{PT} > 0.94). A PCoA plot based on genetic distances among individuals (95.50% of variation explained by the first two axes, Fig. 2) revealed the three well-resolved groups that seemed consistent with the geographical placement of their population of origin. Thus, the genotype found at the southernmost site XN (33 individuals with identical AFLP genotype) was clearly separated from those recorded at other sites, echoing the very high pairwise Φ_{PT} values estimated for this population. Likewise, the remaining four demes were arranged into two groups of geographically consecutive sites (BD-DN and PC-TC, respectively). Despite the apparent correlation between genetic distance and geographical position suggested by the PCoA, the Mantel test found no evidence of isolation-by-distance (r = 0.0462, Mantel P = 0.5323). As for changes over time, when the same set of AFLP markers was scored in samples collected one year later at three of the sites (DN, BD and XN), the genetic structure and diversity were nearly identical to those obtained in 2009 to the point that AMOVA revealed non-significant differences between years (Φ_{PT} = −0.009, P = 0.931). The individual-based Bayesian analysis corroborated the results obtained with the population-based approaches confirming that most of the genetic variation occurred among populations. In BAPS, the optimal partition identified five genetic groups that perfectly matched the five sampling populations (log-likelihood value = −1267.78, probability for 5 clusters = 0.9996). Moreover, no sign of genetic admixture was detected for any individual (Fig. 1).

Among the three non-coding fragments sequenced for the *trn*T-*trn*F region, only the intergenic spacer *trn*T-*trn*L was polymorphic. Therefore, the *trn*L intron and the intergenic spacer *trn*L-*trn*F were excluded from further analyses. The alignment of the *trn*T-trnF fragment resulted in a final consensus sequence of 762 bp. Sequences were rich in A and T nucleotides, with A/T content of 73.80%, in accordance with the nucleotide composition of non-coding chloroplast regions (Kelchner, 2000). The four haplotypes detected (GenBank accession number KM516104 – KM516107) were arranged in a star-like shape parsimony network with haplotype H1 in a central position and haplotypes H2–H4 in the tips. Haplotype H2 was separated by one mutational step while H3

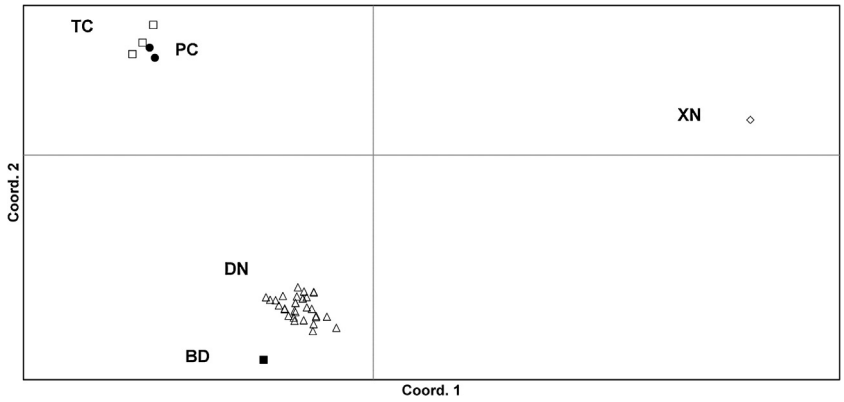

Fig. 2. Principal Coordinates Analysis calculated from simple-matching pairwise distances between individuals of *Omphalodes littoralis* subsp. *gallaecica* collected at five dune systems and scored with 81 segregating AFLP loci. Individuals coded by sampling site: TC, open squares; PC, filled circles; BD, filled squares; DN, open triangles; XN, open diamonds. Individuals with identical AFLP genotype appear as a single symbol. Together, coordinates 1 and 2 explain 95.50% of the total variation.

Table 2
Genetic diversity measures of *Omphalodes littoralis* subsp. *gallaecica* based on cpDNA.

Population	*N*	*S*	*H*	H_d (SD)	$\pi \times 10^2$ (SD)
DN	32	0	1	0.000 (0.000)	0.000 (0.000)
BD	31	0	1	0.000 (0.000)	0.000 (0.000)
PC	32	0	1	0.000 (0.000)	0.000 (0.000)
TC	31	22	2	0.473 (0.054)	1.386 (0.723)
XN	32	0	1	0.000 (0.000)	0.000 (0.000)
Total	158	34	4	0.687 (0.023)	1.154 (0.593)

N, number of sampled individuals; *S*, number of segregating sites; *H*, number of haplotypes; H_d, haplotypic diversity; and $\pi \times 10^2$, nucleotide diversity.

and H4 were distanced from H1 by two relatively large indels (22 bp delection and 11 bp insertion respectively). Haplotype H1 was found in nearly 47% of the individuals in populations DN, BD and TC, while H2–H4 were restricted to XN, PC and TC, respectively.

Estimates of total genetic diversity for the species based on cpDNA were $H_d = 0.687$, $\pi \times 10^2 = 1.154$, $h_s = 0.095$ and $h_t = 0.829$ respectively (Table 2). Population diversity was even lower than that recorded with AFLP. Four out of five populations were dominated by a single haplotype and their within population diversity was zero. Interestingly, the set of demes with no cpDNA variation included DN, the only site where almost each individual displayed a distinctive AFLP genotype. On the other hand, the only location with two haplotypes (TC) exhibited intermediate to high values of haplotypic and nucleotide diversity ($H_d = 0.473$, $\pi \times 10^2 = 1.386$) because its two haplotypes were evenly partitioned among individuals. TC had also produced three AFLP genotypes and cpDNA haplotype was significantly correlated with AFLP genotype (likelihood ratio test, $P < 0.0001$): haplotype H3 only occurred in plants with the same AFLP genotype while the widespread H1 mostly, but not exclusively, coincided with the other two AFLP genotypes. As for the contribution to haplotypic diversity and richness, some populations clearly contributed more than others (Fig. 3). Three populations contained all the cpDNA haplotypes detected in the species and, consequently, they were the only ones with a positive total contribution to haplotypic diversity (PC, XN) and richness (PC, XN, and TC). Their positive contribution was mostly due to their differentiation from other populations (components C_D and $C_D{}^r$) rather than to their own level of diversity (components C_S and $C_S{}^r$). The latter reflects the fact that each population was mostly (TC) or totally (PC, XN) dominated by a private cpDNA haplotype. In comparison, the contribution of the two northernmost populations (BD and DN) was from negative (diversity, C_T) to negligible (richness, $C_T{}^r$) because they only contained the widespread haplotype H1 that also occurred in TC.

As seen with the AFLP genotypes, AMOVA revealed that most of the cpDNA variation (80.44%) was due to differences among populations, rendering a very high and significant F_{ST} estimate (0.804, $P < 0.001$). Also, F_{ST} values were always high and significant except for the comparison DN-BD, two populations dominated by the same haplotype (H1). No evidence of phylogeographic structure was detected because the magnitude of population differentiation inferred from haplotype frequencies ($G_{ST} = 0.886$) was not significantly different ($P > 0.05$ after 1000 permutations) from the level inferred taking haplotype divergence into account ($N_{ST} = 0.873$). Likewise, the Mantel test found no support to an isolation by distance pattern ($r = 0.048$; $P = 0.515$ after 10,000 randomizations).

Phenotypic analysis

Some trays were lost due to vandalism in three out of the five studied populations, meaning that while in DN and BD all trays reached the end of the experiment (*i.e.* 10 trays) in XN, PC and TC only 4, 7 and 8 trays remained until the end. Overall, 884 individuals remained until the end of the experiment that were used for the phenotypic analysis (184 growing in DN, 282 in BD, 132 in PC, 180 in TC and 106 in XN). The general linear model analyses showed that the partition of trays into several areas per site had no significant influence on the values of the various phenotypic traits with the only exception of mean seed DW and that the interaction between site and origin was not significant for all studied traits (Table 3). Therefore, all general linear model analyses were repeated with area as random term, except for mean seed DW. These analyses revealed significant differences between transplant sites for most variables suggesting that our plants performed better in some dune systems than in others. An examination of the mean values recorded at each transplant site revealed no obvious pattern (Fig. 4), although several variables (seed number, reproductive DW, total DW) seem to have reached higher values in the two southernmost sites.

Provenance (origin) also had a significant influence on phenotype indicating that part of the variation seen at the various

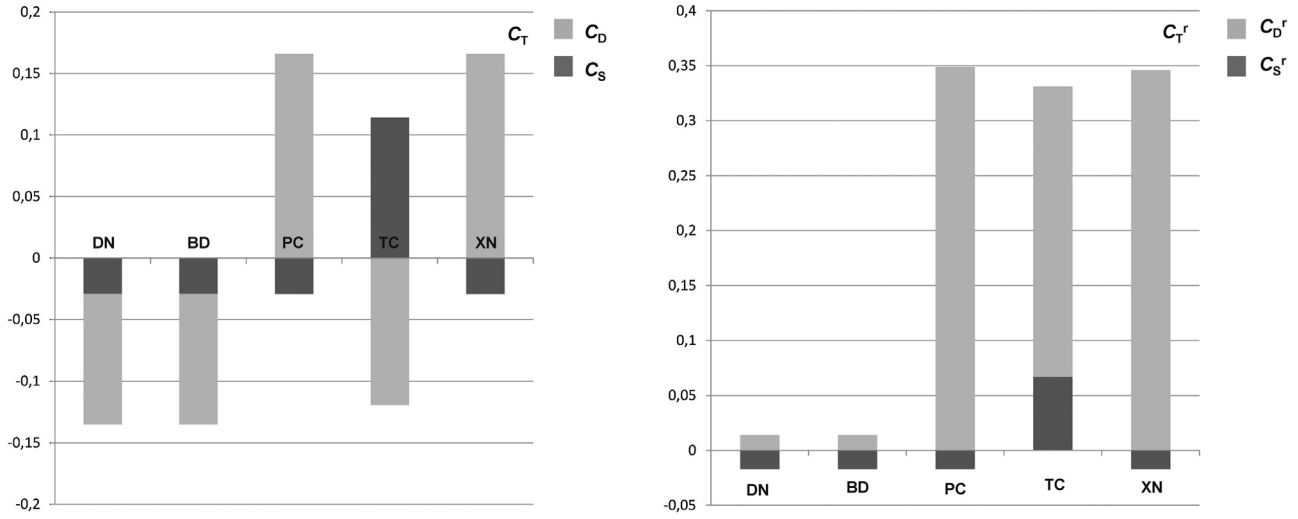

Fig. 3. Contribution to total cpDNA haplotype diversity (left, C_T) and haplotypic richness (right, $C_T{}^r$) of each population of *Omphalodes littoralis* subsp. *gallaecica*. Gray and black bars represent the contribution due to the diversity (C_S and $C_S{}^r$) and differentiation (C_D and $C_D{}^r$) of each population.

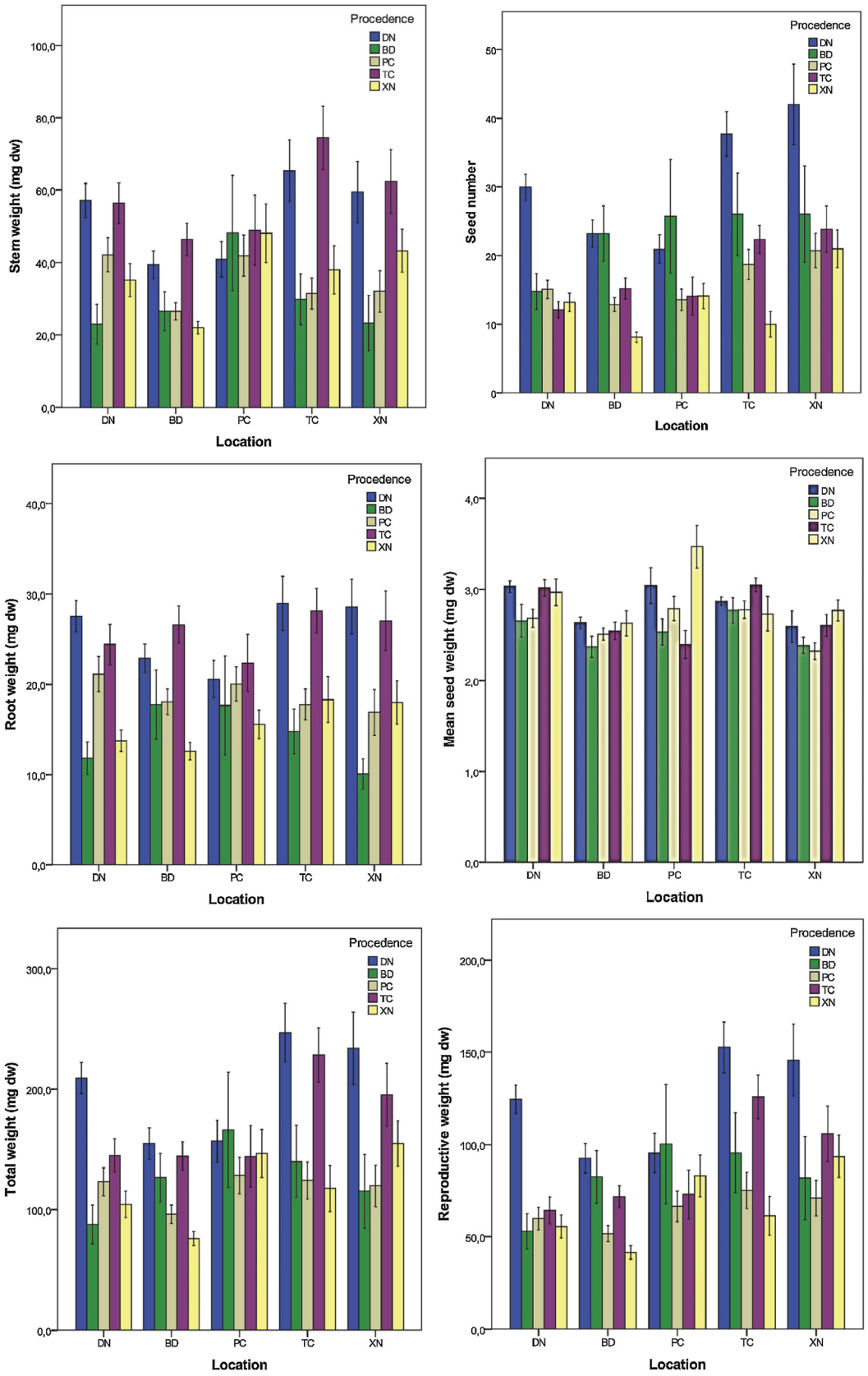

Fig. 4. Mean for the quantitative traits studied in *Omphalodes littoralis* subsp. *gallaecica*. Axis *Y* indicates the value of the studied phenotypic trait (from upper-left to the right-bottom: Seed number, Mean seed DW, Reproductive DW, Stem DW, Root DW and Total DW). Axis *X* represents the location of growth. For each location all possible origins are represented with colors (blue for DN, green for BD, gray for PC, purple for TC and yellow for XN). Each vertical bar represents the mean for a given phenotypic trait for a deme growing in a certain location and with a specific origin. The standard error is indicated in each vertical bar.

Table 3
General linear model results and MCMC contrasts for criteria "*local vs. foreign*" and "*home vs. away*" in the quantitative traits investigated in reciprocal transplants of *Omphalodes littoralis* subsp. *gallaecica*. See test for further details.

	Site	Origin	Origin*Site	*Local vs. foreign*	*Home vs. away*
Seed number	***	***	NS	NS	NS
Mean seed weight (mg DW)[a]	**	***	NS	NS	NS
Reproductive weight (mg DW)	***	***	NS	NS	NS
Root weight (mg DW)	NS	***	NS	NS	NS
Stem weight (mg DW)	***	***	NS	NS	NS
Total weight (mg DW)	***	***	NS	NS	NS

NS: not significant.
** $P < 0.001$.
*** $P < 0.0001$.
[a] Indicates the only variable where the term area was significant ($P < 0.0001$).

traits must have a genetic basis. The plants from DN often yielded the highest mean estimates for most phenotypic traits regardless of the transplant site (Fig. 4). However, their outperformance seemed particularly pronounced when growing at their site of origin (at the north edge of the distribution range of the species) or when they had been transplanted to the two southernmost sites (TC, XN). In fact, DN plants produced more seeds and grew better (reproductive and total DW) at TC or XN than at home. Nonetheless, BD and TC plants reached mean biomass values (stem, root, and total DW) comparable to those of DN in several transplant sites. Despite the significant differences detected between sites and between origins, neither the "*local vs. foreign*" nor the "*home vs. away*" contrasts found significant differences for any quantitative trait, providing no support to the predictions of the hypothesis of local adaptation in *O. littoralis* subsp. *gallaecica* (Table 3). Germination rate and maturement success was very similar among origins across most of the transplants sites (Table S1). Germination was always high (55.8–86.7%) and most of the seedlings reached adult size (from 82.7 to 100%). Instead, fructification was significantly variable among origins regardless of the transplant site; BD consistently showed lower rates than the other origins (Fig. S1). The later was further confirmed analyzing the standardized residuals as BD always showed very high negative deviations (>3). Finally, we found no evidence of differences in the plasticity of the various origins for any of the phenotypic traits ($P > 0.05$ for each one-way ANOVA of $RDPI_s$ values).

Supplementary Fig. S1 and Table S1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ppees.2014.09.003.

Discussion

Taxa listed as endangered by the IUCN Red List of Threatened Species are considered to face a very high risk of extinction in the wild (IUCN, 2012). In the particular case of *O. littoralis* subsp. *gallaecica*, its status as endangered was granted attending to criteria of area of occupancy only: the plant occupies 10 hectares (well below the threshold of 500 km^2 used by IUCN for endangered species), this area of occupancy is in continuing decline due to many threats, and populations sizes fluctuate dramatically between years (Serrano and Carbajal, 2011). Leaving aside the fact that the plant possibly meets the IUCN's criteria for a higher level of risk (Critically Endangered), we have found new reasons for concern about the mid- to long-term survival of this dune dweller. Our results strongly support that effective population sizes are much smaller that census estimates. First, we found only 40 distinct genotypes among 165 genotyped individuals, with three quarters of them concentrated in a single local deme, thus most populations contained one or very few distinct genotypes. In fact, even the population with the highest number of genotypes showed very low genetic diversity indicating that its various genotypes were closely related to each other. Second, the estimates of N_e derived from the genotypes analysis are alarmingly low for all populations, even for DN were we found 33 AFLP genotypes in 34 individuals. Thus, we consider that the effective abundance of this endangered plant is much smaller than previously thought and should be considered a further reason for concern.

The low levels of within-population variation recorded in *O. littoralis* subsp. *gallaecica* are consistent with its life history traits (annual plant, putative high autogamy). Annual selfing taxa usually display the lowest levels of within-population variation (Nybom, 2004). Also, various comparative studies have found that narrow endemics are often less diverse than widespread taxa (Hamrick and Godt, 1990; Gitzendanner and Soltis, 2000; Cole, 2003). Despite the above, the diversity shown by most of the extant populations of *O. littoralis* subsp. *gallaecica* still is remarkably low. The estimates of H_e obtained with AFLP markers in four out of the five sites (range: 0.000–0.011) are one or two orders of magnitude below the average H_{pop} estimated for annuals and/or selfing plants using markers with the same mode of inheritance (Nybom, 2004). And the situation is even worse if we consider the variation displayed by the cpDNA because most populations seemingly contained a single haplotype.

The spatial arrangement of genetic variation is typically explained by contemporary (*e.g.* effective population size, gene flow) and historical (*e.g.* fragmentation, founder events) factors (Schaal et al., 2003). AFLP markers are typically associated with recent processes while cpDNA is more often related to ancient history (Avise, 2004). In the particular case of *O. littoralis* subsp. *gallaecica*, both AFLP and cpDNA suggest that gene flow must be very restricted. While acknowledging that caution must be exerted when drawing conclusions about gene flow based on Φ_{ST} (Whitlock and McCauley, 1999; Marko and Hart, 2011), the fact that an overwhelmingly majority of genetic variation was due to differences between populations is consistent with a scenario of restricted gene flow. Also, the occurrence of private AFLP markers at each and every population together with the fact that each population has its own AFLP genetic lineage in the Bayesian analysis suggest that the distribution of *O. littoralis* subsp. *gallaecica* was fragmented long time ago. Furthermore, the surprisingly high number of private loci detected in XN (29) suggests that XN may have been separated at an earlier time. In this regard, it is worth to note that XN is the southernmost population and it is 95 km away from the closest population (*i.e.* the largest distance between adjacent populations).

The conclusion of long-term maintained isolation is reinforced by the analysis of the cpDNA variability since most of the haplotypes detected were private to a single population and each population showed a distinct cpDNA composition except for the two northernmost sites (BD and DN). According to coalescent theory, central and widespread haplotypes such as H1 may be regarded as ancestral (Posada and Crandall, 2001). Thus, the occurrence of H1 in three non-adjacent populations possibly suggests that the various local demes might have been connected in a distant past. From a conservation perspective, the extreme fragmentation and isolation revealed by the lack of gene flow among local demes suggests that the genetic rescue of one population by others seems highly unlikely without external help.

The strong among-populations differentiation detected using markers with different mode of inheritance is again consistent with the life history traits of *O. littoralis* subsp. *gallaecica*. Selfing taxa are known to partition most of their genetic variation to differences between rather than within populations (Duminil et al., 2007). Together with the extremely low within-population diversity

showed before, this high differentiation among-populations suggests that this small plant could be reflecting the effects of genetic drift. The latter would be exacerbated if we recall that this narrow endemic typically shows strong fluctuations in population size indicating that the plant could have experienced recurrent bottlenecks over the years. The very low within-population diversity shown by *O. littoralis* subsp. *gallaecica* is a matter of concern. Populations with low genetic diversity can be threatened by stochasticity, even by relatively minor events, and are less capable to cope with environmental changes and/or stressful conditions (Frankham, 2005). Furthermore, small populations that fall below a certain effective size may enter an "extinction vortex" where reproductive dynamics favor inbreeding leading to lower reproduction, increased mortality, and smaller population sizes (Angeloni et al., 2011; Leimu et al., 2010).

While the large fluctuations in population size experienced by many annuals could compromise their genetic diversity, other attributes of their life cycle can act in the opposite direction. Some annual taxa have a large reservoir of viable seeds from which individuals may be drawn in the future (Levin, 1990). In these cases, a stable seed bank could have an important role buffering against genetic loss (McCue and Holtsford, 1998; Nunney, 2002). However, this seems not be the case in *O. littoralis* subsp. *gallaecica* as genetic diversity is consistently low over the years at most sites. Thus, the seed bank in *O. littoralis* subsp. *gallaecica* does not seem to act as a reservoir of hidden genetic diversity, adding further concern to the long-term persistence of the species.

An interesting result of our study is the finding that populations separated by just a few kilometers show statistically significant differences in their quantitative traits. While this variation could simply be a phenotypic response to subtle changes in the local environment of each site, our reciprocal transplant experiments indicate it actually involves a genetic component. Unlike what would be expected in a scenario of local adaptation, the individuals from one site (DN) often yielded the highest mean values for most of the phenotypic traits. BD and TC occasionally displayed mean values similar to those seen in plants from DN. However, plants from BD and TC did not show the consistent pattern of regular high values observed in plants from DN. Besides, BD plants showed conspicuously smaller fructification rates suggesting that the individuals from this origin might have lower fitness. Initially, there is no clear explanation to the better fitness of the plants from DN. The only obvious difference between DN and the other populations is that the former displays higher levels of within-population genetic diversity. Therefore, it seems tempting to speculate that the increased performance of its individuals could be related to the higher variation detected using neutral markers. While a correlation between neutral genetic diversity and fitness is far from universal, it is widely accepted that a lack of diversity can lead to the deleterious effects of inbreeding (Angeloni et al., 2011; Landguth and Balkenhol, 2012; Reed and Frankham, 2003). On the other hand, a closer look to Fig. 4 suggests that the particular environmental conditions of some sites may have a homogenizing effect on biomass traits. Thus, mean biomass values were always less variable in PC than in any of the other transplant sites. As mentioned before, PC is the only site where *O. littoralis* subsp. *gallaecica* grows on a sandy hill slope rather than on the fore dune. The mechanisms behind this homogenization remain unknown.

Phenotypic plasticity can be a neutral answer toward the environment but it also has the potential to impact the population viability under environmental changes (Allendorf and Luikart, 2012). Under different environmental conditions, individuals with larger phenotypic plasticity could have a higher fitness that those with less phenotypic variability because they are more likely to adapt to the new conditions. In this regard, it could be hypothesized that the higher performance of some origins detected in our transplant experiments could be explained by a larger phenotypic variation. Instead, our observations do not support this hypothesis. We found no evidence of significant differences in the phenotypic plasticity of the various origins (*P*-values of the ANOVA *F*-tests ranged from 0.067 for total DW to 0.594 for stem DW), suggesting that in *O. littoralis* subsp. *gallaecica* there is no correlation between phenotypic variation and performance. Nevertheless, it should be recalled that our transplant sites possibly have very similar microclimatic conditions. Hence, it cannot be disregarded that our transplant experiments may have underestimated the actual phenotypic variation of the various origins. Further research testing the phenotypic plasticity under more contrasting conditions is warranted.

The higher genotypic diversity observed in DN ($G/N = 0.97$ compared to 0.03–0.10 in other sites) might indicate that this population has experienced sexual reproduction rather than autogamy. The latter would be in agreement with the lower estimates of selfing derived from our AFLP data. Furthermore, the apparent reduced levels of selfing at DN may explain why the seeds from this population often outperformed those from other provenances regardless of where they were grown. Due to genetic purge, inbreeding depression is supposed to have less impact in selfers than in obligated outcrossers (Crnokrak and Barret, 2002) but several studies have reported larger seed production and greater seed performance for plants reproduced by outcrossing rather than selfing (Bellusci et al., 2009; Sletvold et al., 2012). The reasons of why plants at this site may behave differently are difficult to grasp and we are left to speculate. Physical disturbance is the only distinctive feature of DN that we are aware of: DN experiences higher trampling by beach goers than other sites. However, a detailed examination of our data reveals that DN is fragmented in two subpopulations but only one of them is heavily affected by trampling (the subpopulation located on the access trail to the beach). If physical disturbance were behind the higher genetic diversity observed in DN, we would anticipate differences in the diversity recorded at each subpopulation. Instead, the two subpopulations show nearly identical amounts of diversity even though gene flow between them must be very low given their significant genetic differentiation (Φ_{PT}). Alternatively, DN may have experienced less, weaker bottlenecks that other populations resulting in a retention of higher genotypic diversity but further data would be required to prove this hypothesis.

Conventional wisdom assumes that self-compatible species are expected to display a strong adaptation to local conditions given their usually high levels of genetic differentiation (Leimu and Fischer, 2008). However, while the populations of *O. littoralis* subsp. *galaecica* are strongly isolated from each other, the patterns of quantitative differences detected in our reciprocal transplant experiments do not match the expectations under local adaptation. Instead, the inheritable differences in quantitative traits detected among populations must result from processes other than local adaptation. In the absence of gene flow, local adaptation can be confounded by genetic drift and/or constrained by a lack of genetic variation (Kawecki and Ebert, 2004). This might be the case of *O. littoralis* subsp. *galaecica* where the lack of evidence in support of local adaptation suggests that genetic drift might be responsible for the differences among demes in their quantitative traits.

From a conservation perspective, the criterion to select priority populations should consider its uniqueness and variation level with an emphasis on allelic richness (Petit et al., 1998). Our cpDNA analysis revealed that three out of five populations cover the complete genetic variation of the species (PC, TC and XN) and should be designated at least as MUs (management units *sensu* Moritz, 1994). However, our results also indicate that cpDNA contains only a portion of the genetic history of the species. The more variable

AFLP markers showed that each population belonged to a different genetic lineage. Moreover, the AFLP results also revealed that DN is the population with the largest genetic variation even though its cpDNA diversity is zero and totally redundant with other sites (the only haplotype detected in DN also occurs in BD and TC). Therefore, and unlike the cpDNA results, the AFLP markers indicate that each and every extant population of *O. littoralis* subsp. *gallaecica* should receive equal attention given their unique genetic composition; consequently, five rather than three conservation units should be designated, one per population. In fact, by a simple simulation exercise we can estimate the genetic loss derived from the disappearance of one population. Total gene diversity (H_e) decreases by 11.2–27.5% depending on which population is simulated to disappear. Eventually, it seems likewise reasonable to suggest that the five MUs should be designated as ESUs (evolutionary significant unit *sensu* Moritz, 1994) given the significant differences in inheritable quantitative traits detected among these populations. The proposal of five ESU is done while noticing that the differences in the quantitative traits among these ESUs are non-adaptive but a result of genetic drift. However, the occurrence of these differences indicates that the various local demes may have a different potential to evolve.

The typically recommended management action for ESUs is to treat them as separate conservation units (Crandall et al., 2000). Under this premise, translocating individuals between sites is strongly discouraged (Sletvold et al., 2012) and preserving seeds from each population in a germplasm bank seems as the only feasible management action to ensure the maintenance of *O. littoralis* subsp. *gallaecica*. Nonetheless, disregarding translocation without further discussion might not be very wise in the particular case of this plant. Our experiments show that the ecological differentiation between populations is non-adaptive and we also found evidence that higher genetic diversity within populations might be linked to an increased performance of their individuals. These two observations suggest that a sensible recommendation for *O. littoralis* subsp. *gallaecica* might involve increasing the genetic diversity of its populations (currently zero or near zero in most cases) by simultaneous translocations of individuals from various origins. However, the outcome of simultaneous translocations is difficult to anticipate. Regardless of where they grew, the plants from DN consistently outperformed those from other sources and displayed higher genotypic variation suggesting that this population may be of particularly relevance for the preservation for the species. However, it seems likewise plausible that seed/plants from DN could eventually end up displacing those from other sources in these hypothetically mixed populations. If so, the genetic diversity of the species would experience a net loss once most of the currently local lineages disappear. Additionally, seed translocations may seem appropriate only if seed banks are large enough to allow removing individuals without damaging source populations. The annually fluctuating population sizes observed in this plant might indicate that seed banks may be long-lived, serving as an insurance when environmental conditions for germination are not met in a given year. Unfortunately, there are no estimates about the size and long-term viability of the seed bank in this species. Altogether, we advise against the translocation of individuals between populations because, as mentioned above, the final result of this practice might entail a severe negative impact over the populations.

In summary, we have shown that by combining selfing with a strongly fragmented distribution, a narrow endemic plant can reach extremely low genetic variation within populations but high differentiation between local demes. Moreover, the various demes of *O. littoralis* subsp. *gallaecica* also differ in their quantitative traits and these differences have a genetic basis, contradicting the initial assumption that populations living in a very narrow range under similar environmental conditions should display a more homogeneous ecophysiology. Our reciprocal transplant experiments indicate that this variation in *O. littoralis* subsp. *gallaecica* cannot be attributed to local adaptation. Instead, high rates of self-fertilization together with recurrent bottlenecks caused by dramatic interannual fluctuations in population size may have led to a decrease in genetic diversity in a classic scenario drawn by genetic drift. Regardless of the mechanism behind the pattern, the current arrangement of genetic diversity is of some concern from a conservation perspective. Effective population sizes are much smaller than previously thought while the lack of gene flow among local demes suggests that if the plant disappears from one dune system, recolonization without assistance is highly unlikely. The plants from the only deme with moderate genetic diversity consistently outperformed those from other populations with minimal to zero diversity, suggesting that the latter might have diminished their ability to cope with the environment. We recommend that each population should be designated as an independent ESU because of their distinctive genetic and phenotypic make-up. Eventually, our study highlights that range size, geographic distance, and homogeneous environment may not be accurate indicators to delineated conservation strategies.

Acknowledgements

We would like to thank two anonymous referees for their helpful comments on an earlier version of the manuscript. Research supported by grant 07MDS031103PR and grant 00MDS006200PR (Xunta de Galicia). L. L. acknowledges support from Universidade da Coruña (contratos predoutorais UDC 2012).

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