Ann. Bot. Fennici 40: 309–315 ISSN 0003-3847
Helsinki 29 October 2003

Low allozymic variation in two island populations of *Listera ovata* (Orchidaceae) from NE Poland

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Received 11 Sep. 2002, revised version received 24 Jan. 2003, accepted 28 Jan. 2003

Brzosko, E. & Wróblewska, A. 2003: Low allozymic variation in two island populations of *Listera ovata* (Orchidaceae) from NE Poland. — *Ann. Bot. Fennici* 40: 309–315.

Two populations of the orchid species *Listera ovata* localised on mineral islands in the Biebrza National Park (NE Poland) were genetically examined with 32 allozyme loci. Genetic diversity was low (P = 9.4%; A = 1.09; H_o = 0.058). A moderate but significant genetic differentiation among the two populations was found (F_{ST} = 0.101). The observed low genetic variation was most likely a result of small population size and genetic drift. Because the populations are on islands and have a low allozymic variation, they are prone to extinction. Loss of any single genotype would lead to a substantial reduction in the overall genetic diversity of the populations.

Key words: allozymes, genetic diversity, island populations, *Listera ovata*, Orchidaceae

Introduction

One of the main goals in conservation biology is to assess levels of genetic variation, and how they are partitioned in endangered plant populations (Hamrick & Godt 1996). Due to the significance of this information for conservation purposes the causes of genetic structure must also be considered. The level of genetic variation indicates possible adaptation to environmental changes and the potential for long-term survival of species (Ellstrand & Elam 1993).

Breeding system, past levels of gene flow among populations, the actual sizes of populations, geographic distributions, isolation, historical events, and human impact may all affect the levels of genetic diversity within a given species and its populations (Loveless & Hamrick 1984, Hamrick & Godt 1989). The low levels of genetic diversity found in many rare and endangered species (Frankham 1995; but *see* also Ellstrand & Elam 1993, Gitzendanner & Soltis 2000) suggest that genetic drift and/or inbreeding lead to random fixation of alleles and reduced heterozygosity in a small isolated population (Evans *et al*. 2001).

Habitat fragmentation or historical isolation are predicted to cause a loss of genetic diversity and to increase divergence among populations (Young *et al*. 1996). This may cause local population extinction, although small populations are susceptible to a wide variety of factors, both ecological and genetic (Whitlock 2000). Frankham (1997) suggested that island populations are prone to extinction due to their genetic paucity.

Although genetic assessments of orchid species are scarce (Ackerman 1998), recent data indicate that the levels of genetic variation found

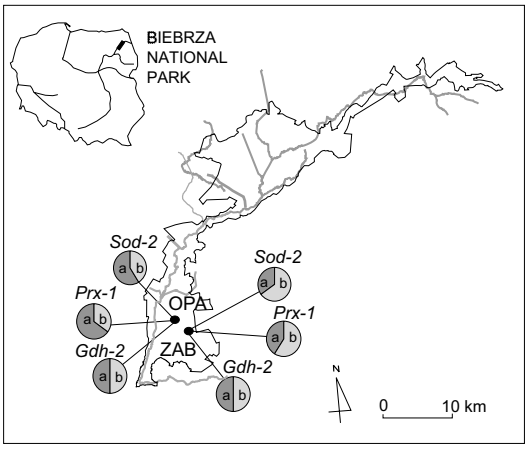

Fig. 1. Localities of *Listera ovata* populations and allele frequencies at three polymorphic loci: *Gdh-2*, *Sod-2* and *Prx-1*.

within orchids are diverse. Species and populations within species may sometimes differ substantially in genetic diversity.

Listera ovata is a shade-tolerant, long-lived (> 80 years; Hutchings 1989) forest herb (Tamm 1972) and common in Poland. The general distribution area of the species covers Europe and reaches eastern Siberia. The yellow-green flowers are on a flexible flowering stalk and outcrossing by wasps seems to be the main pollination mechanism (Ackerman & Mesler 1979). The fruits contain an average of 1240 seeds per capsule (Willems & Melser 1998). Tamm (1972) noted that recruitment from seeds is low and that most propagation is vegetative. *Listera ovata* is listed as endangered in Poland, although its geographical range is wide.

We carried out an isozyme study addressing the following questions: (1) What are the levels of genetic variation within two populations of *Listera ovata* in different habitats? (2) What is the degree of genetic differentiation between isolated populations?

Material and methods

Study sites and sampling

Our study was carried out in the Biebrza National Park, NE Poland. There are several hundred mineral islands among the widespread peat bogs of the Biebrza river valley. *Listera ovata* was found on only a few of the islands and on the majority of these represented only by a single individual. Two of these mineral islands, Zabudnik (ZAB) and Oparzelisko (OPA), were chosen for the study (Fig. 1) because they have a higher abundance of *L. ovata* individuals.

The two islands are about 4 km apart and differ in size, shape and vegetation cover. ZAB is a dune on subpeaty surface covered by fluvial soils, in the zone of peat bog eolians and river forms (Żurek 1991). This island is about 1.2 m above peat level and has an area of 5500 m^2. There is only a very small fragment of an oak–linden–hornbeam forest on the island, which is mainly covered by shrubs and reed (*Phragmites australis*) patches. The OPA population is a remnant of a river meander connected with the old-holocene Biebrza (Żurek 1991). It is less than 1 m above peat level and is about 5000 m^2 in area. The island is covered by a 15–20 years old birch forest with a typical herb layer of hornbeam forest.

Leaf samples were collected in 2001. In the ZAB population all 89 ramets present and in the OPA population 69 samples out of the 153 ramets present above-ground that year were sampled. The majority of OPA juveniles were excluded due to their small leaves (not enough for electrophoretic analyses). Moreover, after removing leaves from such small plants they might easily die. The presence of damaged plants in the OPA population also lowered the number of samples for genetic analyses. After excluding juvenile and damaged plants we were able to collect samples from 69 plants.

Allozyme analysis

For each sample, a fresh leaf tip (approximately 2 cm long) was placed in a tube and frozen in liquid nitrogen. Leaf tissue was subsequently grounded in an extraction buffer including 2-mercaptoethanol, except for the analysis of peroxidases (PRX), where we homogenised the tissue in water (Szweykowski & Odrzykowski 1990). Enzymes were separated on 11% starch gels in two discontinuous buffer systems, a Tris-

citrate–Lithium borate buffer system at pH 8.3 and a Histidine-citrate buffer system at pH 7.0 (Szweykowski & Odrzykowski 1990). The first system was used to resolve aconitase (*Aco*), alcohol dehydrogenase (*Adh*), aldolase (*Ald*), diaphorase (*Dia-1, Dia-2*), esterase (*Est-1, Est-2*), glutamate dehydrogenase (*Gdh-1, Gdh-2*), glutamate oxaloacetate transaminase (*Got*), hexokinase (*Hex*), lactate dehydrogenase (*Ldh*), malic enzyme (*Me*), mannose phosphate isomerase (*Mpi*), peptidase (*Pep-1, Pep-2*), peroxidase (*Prx-1, Prx-2, Prx-3, Prx-4*), 6-phosphogluconate dehydrogenase (*6Pgd*), phosphoglucose isomerase (*Pgi*), superoxide dismutase (*Sod-1, Sod-2*), and triose-phosphate izomerase (*Tpi*). The second buffer system was used for isocitric dehydrogenase (*Idh-1, Idh*-2), malate dehydrogenase NAD^+ (*Mdh-1, Mdh-2*), phosphoglucomutase (*Pgm*) and shikimate dehydrogenase (*Skd-1, Skd-2*). Stain recipes with minor modifications were taken from Soltis and Soltis (1989) and Szweykowski and Odrzykowski (1990).

Genetic variation

The following measures of genetic diversity were calculated using TFPGA (Miller 1997): percentage of polymorphic loci (*P*), mean number of alleles per locus (*A*) and the average observed (H_o) and expected (H_e) heterozygosities. Deviations from Hardy-Weinberg equilibrium (HWE) were tested using an exact test with a Markov chain algorithm. When multiple tests were performed, a Bonferroni correction was applied. *F*-statistics according to Weir and Cockerham (1984) were estimated either for each polymorphic locus or over all loci. As an indicator of the degree of differentiation among populations, F_{ST} (unbiased estimate θ) was calculated. Bootstrapping over loci was performed using 1000 replicates to generate 95% confidence intervals for F_{ST}. A multilocus fixation index (F_{IS}) was also calculated for each population and tested by permutation using FSTAT (Goudet 1995). The theoretical number of migrants exchanged between the two populations per generation (N_m) was estimated with the formula $N_m = (1 - F_{ST})/4F_{ST}$ (Wright 1951). In addition, Nei's (1978) unbiased genetic identity was calculated.

Results

Of the 32 loci examined, 29 loci (*Aco, Adh*, *Ald*, *Dia-1, Dia-2*, *Est-1, Est-2*, *Gdh-1, Got*, *Hex*, *Idh-1, Idh-2, Ldh*, *Mdh-1, Mdh-2, Me*, *Mpi*, *Pep-1, Pep-2*, *Pgi, Pgm*, *6Pgd*, *Prx-2, Prx-3, Prx-4, Skd-1, Skd-2, Sod-1, Tpi*) were monomorphic in the two populations studied. The remaining three (*Gdh-2, Sod-2, Prx-1*) were polymorphic (two alleles per locus) in both populations, resulting in only 1.09 alleles per locus (*A*). Allele frequencies showed significant differences among the two populations only for the enzyme systems *Sod-2* and *Prx-1* (Fisher's exact test, $P < 0.01$). In the OPA population alleles *Sod-2a and Prx-1a* dominated, while in the ZAB population *Sod-2b* and *Prx-1b* were more abundant (Table 1 and Fig. 1). The proportion of polymorphic loci was very low with ($P = 9.4\%$) in both populations. The average observed and expected heterozygosity values (over all loci) for the total *L. ovata* data set were 0.058 and 0.045, respectively. The average expected heterozygosity was the same for the two populations ($H_e = 0.045$) and observed heterozygosity was almost identical ($H_o = 0.059$ in ZAB and $H_o = 0.057$ in OPA). Allele frequencies consistent with Hardy-Weinberg proportions were found for *Sod-2* and *Prx-1* in both populations, while only heterozygotes were observed at *Gdh-2* in the whole data set. F_{IS} values were not significant in the two populations: there was a nonsignificant heterozygote excess at the *Sod-2* and *Prx-1* loci ($F_{IS} = 0.064$ and 0.094, respectively).

The genetic differentiation, calculated over the three polymorphic loci, among the two populations was moderate ($F_{ST} = 0.101$) but statistically significant ($P < 0.05$). Estimated level of gene flow among the populations was relatively high ($N_m = 2.2$). This was consistent with a high genetic identity between the populations ($I = 0.99$).

Table 1. Allele frequencies for two polymorphic isozyme loci in the two *Listera ovata* populations from Poland.

Locus and alleles		OPA ($n = 69$)	ZAB ($n = 89$)
Sod-2	*a*	0.654	0.410
	b	0.346	0.590
Prx-1	*a*	0.588	0.354
	b	0.412	0.646

Discussion

Genetic diversity within the *Listera ovata* populations from the Biebrza valley in Poland was very low. The percentage of polymorphic loci was only 9.4% and the mean number of alleles per locus was 1.09. In several orchid species higher values of these parameters have been detected (Schlegel *et al*. 1989, Scacchi *et al*. 1991, Rossi *et al*. 1992, Kull & Paaver 1997, Ackerman & Ward 1999, Wallace & Case 2000, Brzosko *et al*. 2002a, 2002b). Similar low values as in *L. ovata* have been found for *Encyclia cochleata* (P = 11%; Ortiz-Barney & Ackerman 1999), *Cypripedium acaule* (P = 5.3%–15.4%, A = 1.05–1.15; Bornbush *et al*. 1994), and for some *Goodyera procera* populations (P = 13%–33%, A = 1.1–1.3; Wong & Sun 1999). No genetic diversity at all (P = 0%) was documented in *Cypripedium arietinum* (Bornbush *et al*. 1994, Case 1994) and in *Cephalanthera damasonium* populations (Scacchi *et al*. 1991).

The factors that underlie the genetic structure of plant populations are very varied. Breeding system is one of the main factors and directly associated with the amount of genetic variation found in plant species (Hamrick & Godt 1989). According to those authors, selfing species and animal-pollinated mixed-mating species exhibit low values for several genetic parameters (Hamrick & Godt 1989). Despite the fact that autogamy was not observed in pollinators' exclosure experiment (Brzosko 2002b), genetic variation in *Listera ovata* is remarkably lower than even that reported by Hamrick and Godt (1989) for animal-pollinated perennial plants. The low, non-significant F_{IS} level in *L. ovata* did also not point to selfing and/or biparental inbreeding. A slightly higher F_{IS} value in the ZAB population, where vegetative propagation was more intensive, was noted (Brzosko 2002b). Detailed breeding studies that we plan to carry out will probably help explain the low levels of genetic variation in the *L. ovata* populations investigated.

Vegetative reproduction and clonal spread have a pronounced effect on the genetic structure of populations (Murawski & Hamrick 1990). For example, in a *Cypripedium calceolus* population with intensive vegetative reproduction low heterozygosity was observed, while in another population characterised by a high sexual recruitment from seeds, heterozygosity was high (Brzosko 2002a, Brzosko *et al*. 2002a, 2002b). The two *Listera ovata* populations differed especially in the effectiveness of sexual reproduction. In the OPA population, reproduction from seeds was more common, because the environmental conditions on this island are more favourable for seed germination (i.e. presence of safe sites) than in the ZAB population, where due to compactness of vegetation only vegetative propagation is possible (Brzosko 2002b). Hence, we expected that genetic variation in the ZAB population should be lower. However, values of genetic diversity (P, A and H_o) were almost identical in both populations. Thus, type of reproduction seems to be not an important factor shaping the genetic structure of *L. ovata* populations. Environmental conditions, different on the two mineral islands, also did not affect genetic differentiation.

From theoretical predictions and a number of empirical studies, smaller populations might be expected to show reduced levels of polymorphisms and allelic richness primarily due to random genetic drift (Barrett & Kohn 1991, Young *et al*. 1996). However, converse situations are sometimes recorded (Coates & Hamley 1999). Frankel *et al*. (1995) considered a population with an effective size of 50 individuals as the minimum capable to retain sufficient allelic richness, while an effective size of 500 individuals would be required to counteract the effects of genetic drift. In our *Listera ovata* populations the number of individuals did not exceed 500 (Brzosko 2002b). We conclude, therefore, that since autogamy did not lower the level of genetic variation and the type of reproduction or habitat conditions did not influence it either, the observed low genetic variation in the *L. ovata* populations is most likely a result of small population size. The populations may initially have been larger and reduction of population size may have caused a detectable loss in genetic variation. Alternatively, these populations might have always been small, although, historically, they were not isolated from other *L. ovata* populations (N_m = 2.2).

In deliberations of problems concerning levels of genetic variation we have discussed various explanations. Of course, one possible explana-

tion is that some species, by definition, possess lower and other species higher levels of genetic variation.

The genetic difference between populations *of Listera ovata* was smaller than the genetic variation within populations (F_{ST} = 0.101); 10% of genetic diversity was distributed between populations and the rest was within populations. The level of differentiation between the two *L. ovata* populations was higher than the mean for orchids (0.087; compiled by Hamrick & Godt 1996). On the other hand, Hamrick and Godt (1989) reported higher F_{ST} values for perennial (0.213), animal-pollinated outcrossed (0.197), animal-pollinated mixing-mating (0.216) and selfing species (0.510). The moderate level of differentiation between the two populations of *L. ovata* was probably the result of their close geographic proximity and a substantial gene flow, counteracting genetic drift (Wright 1951). Although N_m is the best available estimator of gene flow (Neigel 1997), it is impossible to distinguish between current and accumulated gene flow (Bossart & Powell 1998). With N_m values higher than one migrant per generation, we would expect that very little variation would be attributed to interpopulation differences (Chung 1995, Ackerman & Ward 1999). The high value of N_m in the *L. ovata* populations may represent a situation where gene flow is high and genetic drift plays a non-significant role in shaping the genetic structure of populations (cf. Wright 1951). Roughgarden (1996), on the other hand, suggested that in small populations genetic drift is important. Alternatively, gene flow by pollen or seed dispersal may homogenise historical patterns of differentiation and moderate the effects of genetic drift. Peakall and Beattie (1991) hypothesized that seed rather than pollen flow might be the major cause of the genetic structure in orchid species. Because seeds are numerous and very minute in *L. ovata* (a typical trait for terrestrial orchids), relatively long-distance dispersal by wind is likely.

Historical events, such as glaciation, might be an explanatory factor shaping the levels of genetic diversity of populations and species (Case 1993, 1994, Chung 1995, Bingham & Ranker 2000, Edwards & Sharitz 2000, Wallace & Case 2000). A low proportion of variable loci is not uncommon for plants in previously glaciated areas and is often explained by loss of alleles during the repeated founder events in post-glacial reimmigration. When a population was first established, a small and genetically isolated group may have been produced; often only a small amount of genetic variability was present in a colonizing group (Barrett & Husband 1989).

It seems that in some cases significant loss of genetic variation may occur before the endangered status of the species is apparent (Sydes & Peakall 1998). This may be true also for *Listera ovata*. To prevent this, an integrated genetic-demographic study should be made. There is a need to protect and preserve all genets, as the loss of any single genotype may lead to substantial reduction in the overall genetic diversity in *L. ovata* populations of the Biebrza National Park. It would be relevant to evaluate whether this low genetic diversity occurs in the Biebrza region only or whether it is a common feature in other *L. ovata* populations in Poland.

Acknowledgements

We are grateful to Mirosław Ratkiewicz for his comments on the manuscript. We also thank two anonymous reviewers for valuable suggestions. This work was supported by the Polish State Committee for Scientific Research (KBN grant No. 6P04C 10121).

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