The LINNEAN SOCIETY of London BOTANICAL Journal of the Linnean Society

Botanical Journal of the Linnean Society, 2013, **171**, 751–763. With 2 figures

Genetic diversity of nectar-rewarding *Platanthera chlorantha* and nectarless *Cephalanthera rubra*

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Received 25 April 2012; revised 21 September 2012; accepted for publication 28 December 2012

We examined the genetic diversity of two orchid species, the nectar-rewarding *Platanthera chlorantha* and the nectarless *Cephalanthera rubra*, in north-eastern Poland. We found lower differences in genetic diversity between the species than we expected. The level of genetic variation at species level was lower in *C. rubra* ($P_{POL} = 14\%$, $A = 1.14$ and $H_E = 0.060$) than in *P. chlorantha* ($P_{POL} = 25.5\%$, $A = 1.35$ and $H_E = 0.078$). In the majority of populations of both species a high proportion of unique genotypes was noted. The overall F_{ST} values for all populations were moderate and similar for both species (*P. chlorantha*: 0.251, $P < 0.001$; *C. rubra*: 0.267, $P < 0.001$). No relationship was found between genetic and geographical distances in either species ($P > 0.05$, Mantel test). We discuss the breeding systems, small population size and population subdivision as the most important factors affecting the genetic diversity of this species. We suggest that conservation programmes should be initiated to maintain or even increase the fitness and genetic variation of populations of both species. *Botanical Journal of the Linnean Society*, 2013, **171**, 751–763.

ADDITIONAL KEYWORDS: allozymes – genotypic diversity – Orchidaceae – small populations.

INTRODUCTION

The biological properties of species are stressed as one of the most important factors shaping the genetic diversity of plants (Loveless & Hamrick, 1984; Hamrick & Godt, 1989, 1997; Scacchi *et al*., 1991; Brzosko, Ratkiewicz & Wróblewska, 2002a; Brzosko, Wróblewska & Ratkiewicz, 2002b; Pellegrino *et al*., 2006; Duffy *et al*., 2009; Brzosko *et al*., 2011). Such properties include a complex of traits, for example type of reproduction (generative/vegetative), pollination mechanisms and breeding systems. Most data indicate that species reproduce in a generative manner and that cross-pollinated and self-compatible species possess higher levels of genetic variation (Hamrick & Godt, 1989; Wong & Sun, 1999; Bänziger, Sun & Luo, 2008; Honnay & Jacquemyn, 2008). It should be noted that biological traits constitute a potential of species which is realized in different ways in different populations because the traits can be modified under environmental conditions where populations occur. For example, when pollinators are abundant in a plant population autogamy is not activated, whereas a lack of pollinators can have the effect of increasing autogamy. In clonal plants the ratio between vegetative and generative reproduction depends on soil properties, light or the abundance of other plant species; under unfavourable habitat conditions for the germination of seeds, vegetative spread may prevail. Differentiation of biological properties with respect to environmental factors is reflected in the demography and genetic variation of populations (Callaghan, 1988; Kull, 1995, 1998; Brzosko, 2002; Brzosko & Wróblewska, 2003). The relationship between changes in biological traits and demography or genetic diversity is easily noticeable and well documented in Orchidaceae (Scacchi, De Angelis & Corbo, 1991; Sun & Wong, 2001; Brzosko *et al*., 2002b; Cozzolino & Widmer, 2005). Members of this family are suitable subjects for such analyses because they represent a wide spectrum of life-history traits (Cozzolino & Widmer, 2005; Tremblay *et al*., 2005). The variety of flower structure and adaptation to pollination are noteworthy and often focus the attention of researchers. Some orchid species attract pollinators by different rewards, whereas others do so by

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deception. The most effective among the rewards offered by orchids is nectar (e.g. Neiland & Wilcock, 1998; Pedron *et al*., 2012) and the secretion of nectar has demographic and genetic consequences. The reproductive success of nectar-producing orchids is significantly higher than that of non-rewarding orchids and those offering other rewards (Neiland & Wilcock, 1998). Jacquemyn *et al*. (2007) showed that nectarless pollination results in a fitness cost, with decreasing fruit set and seedling recruitment rates when the population size decreases. Although nectar is more attractive and more effective, it may cause pollen flow distances to decrease, resulting in more self-pollinations and inbreeding depression (Waser & Price, 1982, 1983; Bawa & Webb, 1983; Peakall, 1989; Burd, 1995; Hodges, 1995). On the other hand, the lack of, or less attractive, rewards may increase pollen movement but result in outbreeding depression (Waser & Price, 1983) or may reduce visitation frequencies and decrease fruit production (Hessing, 1988). The common expectation is that the absence of a reward in orchid species reduces the levels of autogamy or geitonogamy (Johnson & Nilsson, 1999), although only some results of field observations and experiments display such a pattern.

The manner of seed production influences the level of genetic variation in populations, which, in turn, influences their persistence in time, especially in changed environments. Intensive autogamy or crossing between relatives decreases the level of genetic variation in populations (Hodges, 1995; Brzosko & Wróblewska, 2003; Frankham, Ballou & Briscoe, 2003; Honnay & Jacquemyn, 2008). The commonly held view is that species with higher levels of genetic diversity are more effective in changing habitats, because their populations possess better adaptability. Thus, higher genetic diversity increases fitness and decreases the probability of extinction. In contrast to the above-mentioned statements are the observations of Jacquemyn *et al*. (2005); they compared the extinction risk of nectar-producing versus nectarless orchids differing in reproductive parameters in Belgium and the Netherlands over a 50-year period and did not find any difference between these types of species.

In the case of such species as orchids, besides biological properties, sizes of populations and their geographical distribution, including isolation, should be taken into account as determinants of genetic diversity. Population size is a fundamental parameter in determining the importance of natural selection and genetic drift for population differentiation and isolated populations appear to be more sensitive to drift than continuous populations (Tremblay & Ackerman, 2001). Existing data in the literature provide a great deal of evidence that small and isolated populations show lower levels of genetic variation within populations and higher differentiation between them when compared with large and more continuously dispersed populations. Loss of genetic diversity, with other factors such as pollinator limitation, often decrease the long- and short-term viability of small and isolated populations.

Long-term conservation plans more often concern extremely rare and the most endangered species. Good practice should also include those species which, although represented by a relatively large number of populations and not currently classified as rare, show low levels of genetic variation. A decrease in the genetic variation of populations could be an important signal reflecting a threat of extinction of a population or species. Therefore, knowledge about the genetic diversity of a species is stressed strongly in modern conservation strategies (Blackmore, Gibby & Rae, 2011; McNeely, 2011). From a conservation point of view, information about genetic diversity has a predictive value for the development of appropriate and more effective conservation plans (Frankham *et al*., 2003; Pillon *et al.,* 2007; Chung, 2009). For population/species preservation it is important to initiate adequate action at the appropriate moment.

In the context of the above-mentioned problems we tested genetic variation in populations and genetic differentiation between populations of a nectar-producing *Platanthera chlorantha* Cust. ex Rchb. and nectarless *Cephalanthera rubra* (L.) Rich., both of which have extremely small populations in north-eastern Poland.

MATERIALS AND METHODS

Study species

Both species have relatively wide distribution areas. *Platanthera chlorantha* occurs across Europe from Britain and Ireland and central Scandinavia to Spain, Italy, Cyprus and Greece (Hultén & Fries, 1986; Vakhrameeva *et al*., 2009). The border of its distribution usually coincides with the border of deciduous forests (Vakhrameeva *et al*., 2009). This species predominantly inhabits broad-leaved forests, often growing at their edges or along paths and roads in average to significant shade, but in prolonged shady conditions it disappears (Vakhrameeva *et al*., 2009). It grows on dry to moist soils, but never in swampy soils. It is rare in north-eastern Poland, and populations consist of fewer than 50 shoots. Elsewhere, it also grows as solitary plants or in small groups, and large aggregations are rare (Vakhrameeva *et al*., 2009). Flowering in northern Europe occurs in June–July. Flowering shoots have 10–20 white, greenish, nocturnally fragrant flowers, each with a slender,

nectariferous spur. This nectar-rewarding orchid is mainly pollinated by moths from Noctuidae and Sphingidae (Nilsson, 1983). Flowering shoots represent 5–52% of the population in various parts of the geographical range. Fruit set generally ranges from 30 to 95%, but under unfavourable conditions it is lower (10%; Vakhrameeva & Zagulskii, 1995). The number of seeds per fruit ranges from 3580 to 14 320.

Cephalanthera rubra extends from Europe to Iran. The northernmost localities are in southern Finland, where it is rare and seldom flowers (Tuulik, 1998). It is fairly shade tolerant, growing mainly in moderate shade but sometimes in full light (Vakhrameeva *et al*., 2009). Processes leading to increased shading of the forest floor are a natural cause of the disappearance of the species. *Cephalanthera rubra* grows on various soils. It is primarily a forest species, growing in broad-leaved and mixed birch–pine forest and among bushes. In Poland *C. rubra* is known from approximately 300 localities, but only half of these have been confirmed (Kaźmierczakowa & Zarzycki, 2001; Fig. 1). Populations of this species usually comprise from one to 20 plants (occasionally > 100 plants). It is rhizomatous and perennial. The pink flowers are on a flexible flowering stalk. Flowering period occurs in June and at the beginning of July. *Chelostoma campanularum* and *Ch. fuliginosum* (Megachilidae) are the main pollinators of *C. rubra* (Nilsson, 1983; Szlachetko & Skakuj, 1996; Vakhrameeva *et al*., 2009), although Newman *et al*. (2007) questioned *Ch. campanularum* as an effective pollinator of this species, due to its small size. Fruiting is rarer in shade (10–30%) than in favourable light conditions (50–70%; Vakhrameeva *et al*., 2009). Kaźmierczakowa & Zarzycki (2001) noted that reproduction from seeds predominates in this species and vegetative propagation is sporadic, and Micheneau *et al*. (2010) demonstrated multiple plastid haplotypes in some small populations. Other observations, however, stress the role of vegetative reproduction (Scacchi *et al*., 1991; Brzosko & Wróblewska, 2003; Vakhrameeva *et al*., 2009).

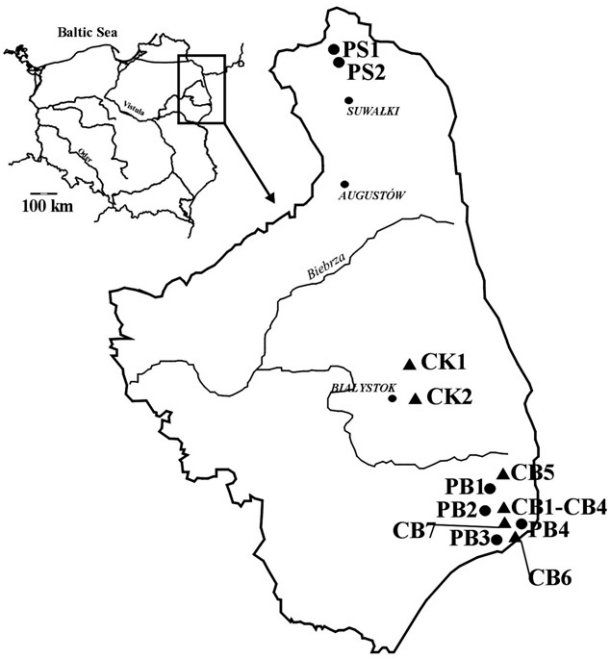

Figure 1. Locations of *Platanthera chlorantha* (PS1 and PS2, PB1–PB4, circles) and *Cephalanthera rubra* (CK1 and CK2, CB1–CB7, triangles) populations in north-east Poland.

Study area and sampling

We investigated six *P. chlorantha* and nine *C. rubra* populations in north-eastern Poland (Białowieża and Knyszyńska Primeval Forest, Szeszupa river valley) in natural, semi-natural and anthropogenic communities of national and landscape parks, reserves and protected areas, such as Natura 2000 sites (Fig. 1). Despite the fact that they are located in protected areas, many occur on railway embankments, along roads and paths in forests or in clearings.

The sampling procedure depended on the population size. Leaf samples from almost all ramets within populations of each species were taken (except population PS2; Table 1); no samples were taken from damaged or very young individuals. One hundred and ninety-eight samples from *P. chlorantha* and 95 samples from *C. rubra* were collected. Leaf tissue was kept on ice until it could be stored at –80 °C, pending allozyme analysis. All collected samples were used for allozyme analysis.

Allozyme polymorphism

Homogenates were prepared by grinding the leaves in a buffer with 2-mercaptoethanol (1%, v/v). Electrophoresis was carried out on 10% starch gels and Titan III cellulose acetate plates (Helena Laboratories, Beaumont, TX, USA) following standard electrophoretic procedures. Fifteen loci (*Adh*, *Gdh*, *Got-1*, *Got-2*, *Idh-1*, *Idh-2*, *Mdh-1*, *Mdh-2*, *Me*, *Pgi*, *Pgm*, *6Pgd*, *Skd*, *Sod*, *Tpi*) in *P. chlorantha* and 16 loci in *C. rubra* (*Adh*, *Got-1*, *Got-2*, *Gdh*, *Idh-1*, *Idh-2*, *Mdh-1*, *Mdh-2*, *Me*, *6Pgd*, *Pgi*, *Pgm*, *Skd*, *Sod*, *Tpi-1*, *Tpi-2*) were investigated. Two electrode/gel buffer systems were used to resolve enzyme systems: GDH and GOT (10% lithium-borate horizontal starch gel at pH 8.2/8.3) and MDH, SKD and TPI (10% histidine-citrate buffer at pH 7.0/7.0). Enzyme activity staining followed Soltis & Soltis (1989). The other enzyme systems (ADH, IDH, ME, 6PGD, PGI, PGM, SOD) were screened using Titan III cellulose acetate plates,

Table 1. Characteristics of *Platanthera chlorantha* and *Cephalanthera rubra* populations in north-east Poland

	N	N_S	N_G/N_W	P_{POL} (%)	A	H_O	H_E	F_{IS}	G	G/N_S	G_U	G_U (%)
Platanthera chlorantha												
PS1	43	43	40/3	20.0	1.33	0.071	0.061	–0.151*	10	0.23	4	40
PS2	42	131	58/73	20.0	1.20	0.025	0.029	0.125**	4	0.09	2	50
PB1	36	36	13/23	33.3	1.60	0.109	0.127	0.155**	27	0.75	20	74
PB2	8	8	5/3	20.0	1.20	0.075	0.061	–0.167	5	0.62	2	40
PB3	37	37	12/25	26.7	1.40	0.078	0.094	0.192**	24	0.65	17	71
PB4	32	32	15/17	33.3	1.40	0.077	0.097	0.219*	21	0.66	15	71
Species	198#	287#		25.5	1.35	0.072	0.078	0.062***	69#	0.50	60#	57.6
Cepahalnathera rubra												
CK1	29	24	22/7	12.5	1.13	0.005	0.028	0.489*	3	0.13	2	66.7
CK2	6	6	4/2	25.0	1.25	0.115	0.109	–0.095	4	0.67	4	100.0
CB1	5	5	3/2	6.3	1.06	0.063	0.031	–1.000	1	0.20	0	0
CB2	9	9	4/5	6.3	1.06	0.031	0.063	–1.000	1	0.11	0	0
CB3	5	5	4/1	6.3	1.06	0.038	0.026	–0.333	2	0.40	1	50.0
CB4	5	5	2/3	6.3	1.06	0.031	0.063	–1.000	1	0.20	0	0
CB5	33	32	8/25	37.5	1.38	0.129	0.116	–0.103*	11	0.34	11	100.0
CB6	3	3	1/2	6.3	1.06	0.063	0.038	–1.000	1	0.33	1	100.0
CB7	7	7	0/7	18.8	1.19	0.071	0.059	0.000*	2	0.29	2	100.0
Species	95#	95#		14.0	1.14	0.060	0.060	–0.449***	23#	0.30	21#	57.4

N, population size; N_S, number of samples analysed; N_G/N_W, number of generative ramets/number of vegetative ramets; P_{POL}, percentage of polymorphic loci; A, mean number of alleles per locus; H_O, observed heterozygosity; H_E, expected heterozygosity; F_{IS}, inbreeding coefficient; G, number of genotypes, G/N_S, clonal diversity; G_U, number of unique genotypes; G_U %, percentage of unique genotypes (Fischer's exact test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$); #, sum of parameters.

which were resolved using Tris-glycine buffer at pH 8.6 and Tris-citrate buffer at pH 7.6 (Richardson, Adams & Baverstock, 1986). The enzyme staining recipes were based on Soltis & Soltis (1989) and Richardson *et al*. (1986), with modifications.

Statistical analysis

The data matrix of individuals was analysed using the TFPGA package (Miller, 1997), FSTAT 2.9.3 (Goudet, 2001) and GENEPOP 3.2 (Raymond & Rousset, 1995) for calculation of standard measures of allozyme diversity: allelic frequencies, percentage of polymorphic loci (P_{POL}), number of alleles per locus (A), genetic diversity (i.e. observed H_O and expected heterozygosity H_E) and inbreeding coefficient (F_{IS}). The occurrence of unique alleles was used to describe population distinctiveness (Slatkin, 1985). Deviations from Hardy–Weinberg expectations were tested for the population by the Markov chain method (GENEPOP).

Parameters of within-population genotypic diversity were also estimated. Three different measures of clonal diversity were used: number of observed genotypes (G), number of genotypes unique to a single population (G_U) and the probability that the next ramet sampled would be a different genotype (G/N_S; where N_S is the number of ramets sampled). The relationships between parameters of genetic (P_{POL}, A, H_O and F_{IS}) and population size were tested with Spearman's pairwise rank correlations (StatSoft, 1995).

Genetic differentiation across seven regions in Poland was tested by hierarchical analysis of F_{xy} (ARLEQUIN 3.11, Excoffier, Laval & Schneider, 2005). F statistics were calculated to quantify levels of genetic differentiation between pairs of populations (F_{ST}) and to assess population subdivision (Weir & Cockerham, 1984; GENEPOP). We used the Mantel test to examine the pairwise relationships between $F_{ST}/(1 - F_{ST})$ and logarithms of geographical distance between all populations and between populations within regions (TFPGA). To investigate spatial patterns of genetic variation, evidence of group distinctness was obtained with groups separated by principal component analysis (PCA) of allozyme gene frequency data, using PCAGEN version 1.2 (Goudet, 2001).

RESULTS

Genetic variation

Platanthera chlorantha

Five polymorphic loci out of 15 were resolved in *P. chlorantha* (*Mdh-1*, *Mdh-2*, *6Pgd*, *Pgi*, *Pgm*), but not all were variable in the six populations studied. Allele frequency at different loci varied between the populations studied. Only one unique allele, Pgm^c in population PB3, was noted (Appendix 1). The level of polymorphism was lower in Szeszupa valley (20%) than in Białowieża Primeval Forest (33.3%). Population-level estimates of the mean number of alleles per locus (A) ranged from 1.2 in populations PB2 and PS2 to 1.6 in population PB1. Levels of expected heterozygosity (H_E) ranged from 0.029 to 0.127, and in most cases they were similar to observed heterozygosity (H_O) (Table 1). The lowest values of heterozygosity, both observed and expected, were noted in the largest population, PS2. Deviation from Hardy–Weinberg equilibrium was found in almost all populations, where a significant overabundance of homozygotes was usually detected (Table 1). No relationship between genetic variation and population size was found ($P > 0.05$; Spearman's rank correlation).

Cephalanthera rubra

Eight of 16 analysed loci were polymorphic in nine *C. rubra* populations. In five populations (all from Białowieża Forest) only one polymorphic locus was detected (Appendix 2). Two unique alleles, Pgi^a and Pgi^b, were identified (Appendix 2). The percentage of polymorphic loci ranged from 6.3 to 37.5%. (Table 1). The mean number of alleles per locus ranged from 1.06 to 1.38. Values of observed heterozygosity (H_O) and expected heterozygosity (H_E) were identical at the species level but were differentiated in the populations studied (Table 1). Despite negative values of F_{IS} being noted in the majority of populations, a significant deviation from Hardy–Weinberg equilibrium was found in population CK1, with an overabundance of homozygotes, and in CB5, with an overabundance of heterozygotes (Table 1). A relationship between the level of polymorphism and population size was found ($r = 0.68$, $P < 0.05$; Spearman's rank correlation).

Clonal diversity

Platanthera chlorantha

We detected 69 distinct multilocus genotypes of *P. chlorantha* among 198 ramets sampled (Table 1). The lowest number (four) and the highest number (27) of genotypes were found in the largest population (PS2) and population PB1, respectively. A high proportion of unique multilocus genotypes for a given population was noted (40–74%). The probability of finding a new genet was usually high, with the exception of the largest population, PS2 ($G/N_S = 0.09$, Table 1).

Cephalanthera rubra

Only 23 multilocus genotypes were detected among 95 ramets sampled in nine *C. rubra* populations. In four populations all multilocus genotypes were unique for

a given population, whereas in three populations unique genotypes were not observed (Table 1). In three populations out of four in which only one genotype was noted it was the same multilocus genotype. The probability of finding a new genet (G/N_S) varied from 0.11 to 0.67 (Table 1).

Genetic differentiation among populations

Platanthera chlorantha

The variance components of AMOVA were the highest within populations (72.2%, $P < 0.001$). There was no statistically significant variation between the two regions in *P. chorantha* (13.8%, $P > 0.05$). The overall F_{ST} value for all *P. chlorantha* populations was moderate (0.251, $P < 0.001$). Pairwise comparison of F_{ST} values revealed significant differentiation between all *P. chlorantha* population pairs, ranging from 0.006 between PB3 and PB4 ($P < 0.01$) to 0.658 between PB2 and PS2 ($P < 0.001$). The highest F_{ST} values were observed between PS2 and the other *P. chorantha* populations (Appendix 3). No relationship was found between genetic and geographical distances ($r^2 = 0.31$, $P > 0.05$, Mantel test). The same structure was evident from the separate PCA ordination in which population PS2 was clearly separate from the others (Fig. 2A).

Cephalanthera rubra

The molecular variation, with division into two regions, was –4.3%, although this was statistically significant ($P < 0.05$). The majority of the variation was partitioned within populations (73.3%, $P < 0.001$). A significant differentiation between *C. rubra* populations was found ($F_{ST} = 0.267$, $P < 0.001$). The F_{ST} values between population pairs varied greatly and ranged from zero to 0.734 (Appendix 3). As with *P. chlorantha*, values of genetic differentiation were not correlated with geographical distance ($P > 0.05$, Mantel test). PCA analyses confirmed the high genetic differentiation of populations CB5, CB6 and CB7 of *C. rubra* (Fig. 2B).

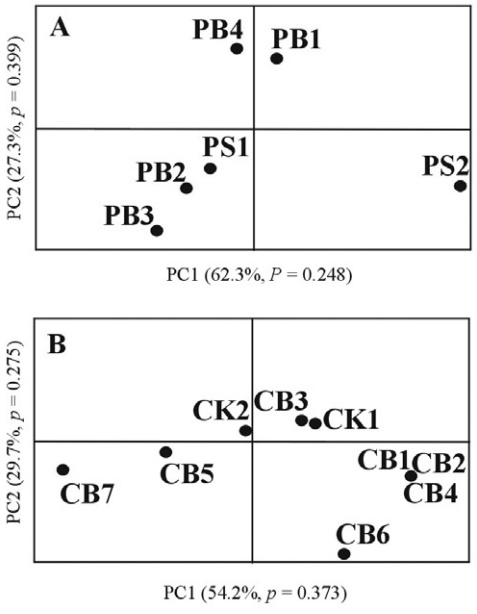

Figure 2. Principal component analysis (PCA) plot showing the genetic distances among *Platanthera chlorantha* (A) and *Cephalanthera rubra* (B) populations. *P* values for PC1 and PC2 axes were obtained by randomization test.

DISCUSSION

We found lower differences in genetic diversity between nectar-rewarding *P. chlorantha* and deceptive *C. rubra* in north-eastern Poland than expected. Moreover, the genetic variation in populations of both orchid species was relatively low. The parameters of genetic variation of *P. chlorantha* represent one of the lowest values among electrophoretically tested species of *Platanthera* Rich. (Cowden, 1993; Wallace, 2002, 2003; Brzosko *et al*., 2009). In the case of *C. rubra*, the level of genetic variation was much lower than those from our former study (Brzosko & Wróblewska, 2003) and those given by Scacchi *et al*. (1991) for Italian populations. Our estimates of genetic variation were also lower than the results reported by Hamrick & Godt (1989) for all plant taxa, for monocots, long-lived herbaceous perennials, species with widespread geographical ranges, outbreeding species and species with a reproduction mode that is both sexual and asexual. In comparison with other orchids, the genetic variation of *P. chlorantha* and *C. rubra* was moderate. Many orchid species have higher levels of genetic variation [e.g. *Epipactis helleborine* (L.) Crantz, *Cypripedium calceolus* L. (Hollingsworth & Dickson, 1997; Brzosko *et al*., 2002b; Brzosko, Wróblewska & Tałałaj, 2004); *Goodyera procera* Hook. (Wong & Sun, 1999)]. Some orchid species had lower levels or even a lack of genetic variation [*Platanthera leucophaea* (Nutt.) Lindl. (Wallace, 2002); *Neottia ovata* (L.) Bluff & Fingerh. (Brzosko & Wróblewska, 2003, 2012); *Cypripedium arietinum* R.Br. (Bornbusch *et al*., 1994; Case, 1994); *Neottianthe cucullata* (L.) Schltr., *Amitostigma gracile* (Blume) Schltr. and *Pogonia minor* (Makino) Makino (Chung, 2009)].

The first cause of the low level of genetic variation within populations, common for both species, is probably their small size. Excluding one population of *P. chlorantha* (PS2), the remaining populations comprised fewer than 50 shoots, with fewer than 10 in 7/9 *C. rubra* populations. According to Frankel, Brown & Burdon (1995) this is not enough to retain sufficient allelic richness (although relatively large populations

may also be genetically depauperate; e.g. Pedersen *et al*., 2012). The clear relationship between population size and polymorphism in *C. rubra* has been documented. Moreover, three populations from Biebrza National Park, with higher levels of genetic variation (Brzosko & Wróblewska, 2003), were much larger than those from this study. The influence of small population size on the level of genetic variation, especially of rare and endangered species, has been well documented (Frankham *et al*., 2003; Leimu *et al*., 2006; Chung & Chung, 2007).

Apart from population size, other factors can shape the genetic diversity of both orchid species. One of the most important is the type of reproduction and breeding system. In a former paper on *C. rubra* we stressed the importance of vegetative reproduction in shaping the genetic structure of *C. rubra* populations. It was shown that shoots < 20 cm apart belonged to the same genetic individual, because *c.* 90% of shoots separated by such distances had an identical genotype (Brzosko & Wróblewska, 2003). Although the genotypic diversities reported in this paper are higher than from Biebrza National Park, *C. rubra* populations consisted of a small number of multilocus genotypes. Four populations had only one genotype and three of these shared the same genotype. An explanation for the domination of this genotype is that it is favoured under given environmental conditions. In the case of the *C. rubra* populations studied we suggest that the multilocus genotypes detected are those which are the most resistant in unfavourable habitats. They have survived in greatly changed and often disturbed places, as the majority of populations studied are on railway or road embankments or in clearings. The overabundance of heterozygotes found at the species level in *C. rubra* could indicate that only heterozygotes were able to survive in the extremely restricted habitats suitable for this species. Heterozygous individuals could be better adapted to change. Moreover, natural selection favours highly heterozygous individuals in some plant groups (Hamrick, 1987; Mitton, 1989). On the other hand, in some populations of both species an overabundance of homozygotes was noted. This could be the result of strong selection against heterozygotes (Wahlung effect), which is the inclusion of non-random mating or inbreeding (Murphy *et al*., 1996). The lowering of genetic variation due to more intense vegetative propagation has been observed in other orchids (Brzosko *et al*., 2002a, b; Pellegrino *et al*., 2006). In the case of *P. chlorantha* we excluded vegetative propagation as a factor shaping the genetic diversity of this species due to its life strategy.

Breeding system is another important factor affecting genetic parameters in plants. Scacchi *et al*. (1991) found that Italian populations of three *Cephalanthera* spp. with different breeding systems differed in levels of genetic variation, and Micheneau *et al*. (2010) obtained similar results with populations across Europe. The patterns of genetic variation in three wild orchids were apparently also related to their differences in breeding systems in studies conducted in Hong Kong by Sun & Wong (2001). In the case of *C. rubra*, the breeding system, both potential and realized, reduces the level of genetic variation of populations. *Cephalanthera rubra* attracts insects through deception and 'colour mimicry'. According to the literature, *Chelostoma campanularum* and *Ch. fuliginosum* (Megachilidae) are the main pollinators of *C. rubra* (Nilsson, 1983; Szlachetko & Skakuj, 1996), although in the light of the newest data of Newman *et al*. (2007) the size of *Ch. campanularum* is too small for it to be an effective pollinator of this orchid. Dependence on one main pollinator indicates high specialization in the pollination process. This could explain the lower level of genetic variation of *C. rubra* in comparison with *P. chlorantha*, which is pollinated by a greater number of pollinators. The more diverse relationships with pollinators are reflected in higher fruiting in *P. chlorantha*, up to 95% (Vakhrameeva & Zagulskii, 1995). The lower effectiveness of the reproductive success of *C. rubra* is connected to colour mimicry (insects pollinating *C. rubra* also pollinate species of *Campanula* L.). In the studies of Brzosko & Wróblewska (2003) and Tuulik (1998), observations were made that in habitats where many *Campanula* plants grow with *C. rubra*, fruiting of *C. rubra* was more frequent than in places without *Campanula* or in places where there were only single individuals. Generally, reproductive success in *C. rubra* is low. Newman *et al*. (2007) noted that over 10 years only one seed pod developed in response to natural pollination in the UK. In places where *C. rubra* occurs but *Campanula* spp. are absent, we can assume that seed production will be poor and sporadic. In the case of *C. rubra* we suggest that population size dictates the realization of breeding system. A low number of flowering shoots in populations, often representing one multilocus genotype, restricts, or reduces to zero, gene exchange within populations. In the population from Biebrza, in which generative shoots were more abundant, genetic variation was higher (Brzosko & Wróblewska, 2003). A small number of reproductive individuals existing in a restricted area (a few square metres) increases the probability of auto- and geitonogamy and crossing between relatives, decreasing the level of genetic variation in populations. The lower genetic variation of *C. rubra* populations can be explained by the fact that this nectarless species is less successful in fruit production than nectariferous *P. chlorantha*. This is in agreement with the theory and results of experimental studies (Neiland & Wilcock, 1998; Pellegrino *et al*.,

2006; Jacquemyn *et al*., 2007; Jacquemyn & Honnay, 2008). On the other hand, in populations with exclusively vegetative shoots or without pollinators these processes are not important in shaping the genetic variation of *C. rubra*, at least recently. If so, we can also assert that current levels of genetic variation of *C. rubra* represent a small fragment of the past genetic diversity of this species.

A more important influence of auto- and geitonogamy and crossing between relatives in shaping genetic variation of populations may exist in *P. chlorantha*. Pollination of this type, between flowers on the same shoot or between relatives, is typical for species with inflorescences. Foraging pollinators should visit more flowers per inflorescence in species with nectar, which could increase geitonogamous self-fertilization. Because the reward, especially nectar, increases the abundance of pollinators and their activity, not only does selfing increase, but also the probability of crossing. Nilsson (1978), studying pollination biology in *P. chlorantha*, found that the behaviour of its pollinators restricts selfing and promotes crossing. Nilsson observed that moths pollinating this species usually removed one pollinarium during one visit. Moreover, Johnson & Nilsson (1999) noted that pollinators probed a mean of only 1.85 flowers per plant during seven visits in a natural population. The sectile character of the pollinarium means that pollen massulae can potentially be delivered to several stigmas/flowers (Maad & Nilsson, 2004). These facts increase the probability of gene exchange by pollen between different individuals, further maintaining or even increasing the levels of genetic variation of *P. chlorantha* populations. Johnson & Nilsson (1999) suggested that this nectar-producing orchid is highly outcrossing. They found that the first nine flowers probed by moths arriving with pollen were completely outcrossed, as a consequence of extensive pollen carryover and low pollinarium bending rates. Taking into account the above information, we suggest that the mean genetic variation and high inbreeding in *P. chlorantha* is a result of the balance between crossing and selfing and mostly depends on factors such as number and density, both of flowering shoots and insects. One example of the genetic answer to these factors could be population PS2. In this, the largest population, the lowest heterozygosity and the lowest genotypic diversity was noted. The cause of this could be the relatively high number of flowering shoots in comparison with other *P. chlorantha* populations in a restricted area (150 m^2). This promotes selfing and crossing between relatives. The situation in this population contrasts with the characteristics mentioned above concerning the biology of species and the behaviour of pollinators, promoting, according to other authors, outcrossing in this species. It also stresses the role of factors other than biological traits in shaping genetic variation in populations.

Among the factors shaping genetic structure of plants are historical factors (Karron *et al*., 1988; Case, 1994; Bingham & Ranker, 2000; Wallace & Case, 2000; Brzosko *et al*., 2004, 2009, 2011). Historical data show that the number and size of populations of *C. rubra* and *P. chlorantha* have both fallen over recent decades (Kaźmierczakowa & Zarzycki, 2001), and this is tightly connected to the history of the investigated areas. The extinction of many populations increased the distances between remnant populations, further increasing isolation among them. Suitable environments for the species studied are rare and spatially fragmented in north-eastern Poland due to human impacts. Human activity has increased the isolation of populations and in consequence decreased their genetic variation and increased differentiation between them (Young, Boyle & Brown, 1996; Kang, Jiang & Huang, 2005; Leimu *et al*., 2006). Many habitats in which populations of both studied orchids exist are not suitable for their development due to disturbance (edges of forests or along paths, roads or railways). Thus, it could be suggested that in such places we observed the remnants of former, larger, polymorphic populations. The sharp decrease in effective population size could be caused by bottlenecks. Indeed, the effect of a population bottleneck is a decrease in genetic diversity, promoting the effects of stochastic genetic drift over natural selection. In addition, repeated population bottlenecks can severely decrease reproductive fitness: deleterious alleles are able to accumulate especially when the time interval between bottlenecks does not allow the generation of new alleles through mutation. This could be the case of seven out of nine *C. rubra* populations and perhaps one population of *P. chlorantha*.

However, another possibility should be taken into consideration. We cannot exclude the possibility that the low levels of genetic variation, at least in some populations studied, are the result of founder effects. Due to the location of populations, especially in the case of *C. rubra*, along roads or railways, we can hypothesize that they could have been established by a small number of individuals from long distances, when roads were built.

The overall F_{ST} values for all populations were similar and moderate for both species (*P. chlorantha*: 0.251, $P < 0.001$; *C. rubra*: 0.267, $P < 0.001$). Alexandersson & Ågren (2000) also did not find a marked difference in genetic differentiation among populations between deceptive and rewarding orchid species, although their data set was relatively small (only three rewarding species were included). Our

results contrast with the statement of Cozzolino & Widmer (2005) that genetic differentiation among populations of rewarding species is higher (G_{ST} = 0.2–0.3) than deceptive species (G_{ST} = 0.10–0.15). Significant differentiation among populations of studied orchids indicates that gene flow among different populations, through both seeds and pollen, is limited, even between the less distant populations. Despite the widespread opinion that dust-like, wind-dispersed orchid seeds are dispersed over long distances, our unpublished data are in contrast to this view. In our field experiments, using seed traps in *C. rubra* populations on mineral islands in the Biebrza Valley, we found that > 90% of seeds dispersed < 2 m from fruiting shoots and the maximum distance at which seeds were trapped was 5 m (E. Brzosko *et al*., unpubl. data). Murren & Ellison (1998) and Machon *et al*. (2003) also observed restricted seed dispersal in *Brassovola nodosa* (L.) Lindl. and *Spiranthes spiralis* (L.) Chevall. Long-distance gene flow by pollen is also not possible, due to the restricted flight ranges of pollinators, which usually transport pollinia a few to several metres (Peakall & Beattie, 1996; Kropf & Renner, 2008 and references therein). Kropf & Renner (2008) found that the furthest transport in the bumblebee-pollinated *Dactylorhiza sambucina* (L.) Sóo (176 m) was the highest in Orchidaceae. Smaller insects than bumblebees forage over smaller areas (Kropf & Renner, 2008).

Thus, recent within-population processes play a key role in shaping the demographic and genetic structure of populations. The evidence for this is the high proportion of unique genotypes in particular populations. The levels at which demographic processes occur, especially in *C. rubra* populations, seems to be insufficient to maintain populations over a longer time. Lowered levels of genetic variation, at least in some of populations, may also reduce their ability to respond to environmental conditions and changes, and many authors state that populations with restricted size and genetic paucity are prone to extinction (Frankham, 1995; Young *et al*., 1996; Frankham *et al*., 2003; Tremblay *et al*., 2005).

The two studied species are rare, both in Poland as a whole and in the north-eastern part of the country. They are represented by a few populations containing a small number of individuals and these populations often exist in changed, unfavourable environments. In addition, they possess relatively low levels of genetic variation, as documented in this study. Taking these facts into account, we can assume that these populations are highly endangered. Therefore, special conservation programmes should be initiated to maintain or increase the fitness and genetic variation of populations of both species. We suggest, for example, the extension of the area of appropriate habitats for *P. chlorantha* and *C. rubra*. This could be done through the creation of open areas. Removing trees and shrubs could intensify flowering and fruiting, and could increase reproductive success and the level of genetic variation. In the case of *C. rubra* introduction of *Campanula* spp. in the vicinity of orchid populations could increase pollinator abundance, in turn improving reproductive parameters. Populations of both species consisted of only a few genotypes or even only one genotype. In the context of the number of multilocus genotypes, populations are extremely poor and thus especially endangered. They could be enriched with genotypes from other populations.

ACKNOWLEDGEMENTS

We thank Wojciech Adamowski, Adam Hermaniuk, Edyta Jermakowicz, Teresa Świerubska, Izabela Tałałaj and Dan Wołkowycki, who helped to collect samples in north-eastern Poland. This research was funded by a grant from the Polish Ministry of Science and Higher Education (2P04C 048 30).

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APPENDIX 1

ALLELE FREQUENCIES IN SIX *PLATANTHERA CHLORANTHA* POPULATIONS IN north-east POLAND (BOLD, UNIQUE ALLELE)

	PGM				*PGI*			*6PGD*		*MDH-1*		*MDH-2*		
	a	b	c	d	a	b	c	a	b	a	b	a	b	c
PS1	0.047	0.953	0.000	0.000	0.488	0.500	0.012	0.000	1.000	0.000	1.000	0.814	0.081	0.105
PS2	0.000	1.000	0.000	0.000	0.024	0.976	0.000	0.000	1.000	0.024	0.976	0.214	0.786	0.000
PB1	0.264	0.694	0.000	0.042	0.681	0.306	0.014	0.153	0.847	0.153	0.847	0.667	0.208	0.097
PB2	0.000	0.813	0.000	0.188	0.813	0.188	0.000	0.000	1.000	0.000	1.000	0.813	0.188	0.000
PB3	0.324	0.595	**0.081**	0.000	0.176	0.824	0.000	0.000	1.000	0.189	0.811	0.838	0.108	0.054
PB4	0.328	0.672	0.000	0.000	0.063	0.797	0.141	0.078	0.922	0.141	0.859	0.828	0.172	0.000

APPENDIX 2

ALLELE FREQUENCIES IN NINE *CEPHALANTHERA RUBRA* POPULATIONS IN north-east POLAND (BOLD, UNIQUE ALLELE; FOR POPULATION CODES SEE TABLE 1)

Loci/allele		CK1	CK2	CB1	CB2	CB3	CB4	CB5	CB6	CB7
IDH-1	a	0.000	0.170	0.000	1.000	0.000	0.000	0.100	0.000	0.000
	b	1.000	0.830	1.000	0.000	1.000	1.000	0.900	1.000	1.000
IDH-2	a	1.000	0.750	1.000	0.00	1.000	1.000	0.480	0.500	0.140
	b	0.000	0.250	0.000	1.000	0.000	0.000	0.520	0.500	0.860
MDH-2	a	0.750	1.000	1.000	1.000	0.700	1.000	0.650	1.000	0.500
	b	0.250	0.000	0.000	0.000	0.300	0.000	0.350	0.000	0.500
SKD	a	1.000	1.000	0.500	0.500	1.000	0.500	0.890	0.000	0.930
	b	0.000	0.000	0.500	0.500	0.000	0.500	0.110	1.000	0.070
6PGD	a	0.960	1.000	1.000	1.000	1.000	1.000	0.840	1.000	1.000
	b	0.040	0.000	0.000	0.000	0.000	0.000	0.160	0.000	0.000
PGI	a	1.000	**0.670**	1.000	1.000	1.000	1.000	1.000	1.000	1.000
	b	0.000	**0.330**	0.000	0.000	0.000	0.000	0.000	0.000	0.000
PGM	a	1.000	0.500	1.000	1.000	1.000	1.000	0.850	1.000	1.000
	b	0.000	0.500	0.000	0.000	0.000	0.000	0.150	0.000	0.000

APPENDIX 3

GENETIC DIFFERENTIATION (F_{ST}-VALUES) BETWEEN POPULATIONS OF *PLATANTHERA CHLORANTHA* AND *CEPHALANTHERA RUBRA* BASED ON ALLOZYMES (ALL VALUES STATISTICALLY SIGNIFICANT)

Platanthera chlorantha

	CK1	CK2	CB1	CB2	CB3	CB4	CB5	CB6
CK1	–							
CK2	0.356	–						
CB1	0.356	0	–					
CB2	0.373	0	0	–				
CB3	0.000	0.406	0.406	0.409	–			
CB4	0.717	0.500	0.500	0.500	0.734	–		
CB5	0.570	0.599	0.599	0.626	0.500	0.589	–	
CB6	0.392	0.343	0.343	0.401	0.278	0.489	0.416	–
CB7	0.196	0.230	0.230	0.258	0.128	0.316	0.069	0.175

Cepahalnthera rubra

	PS1	PS2	PB1	PB2	PB3
PS1	–				
PS2	0.093	–			
PB1	0.106	0.029	–		
PB2	0.165	0.147	0.262	–	
PB3	0.171	0.155	0.273	0.006	–
PB4	0.486	0.420	0.657	0.399	0.368