

High genetic diversity within island-like peripheral populations of *Pedicularis sceptrum-carolinum*, a species with a northern geographic distribution

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Isolated and island-like populations at the periphery of a geographic range of a given species are usually predicted to have low genetic diversity due to founder effect, habitat fragmentation, and bottleneck and/or inbreeding. As for parasitic plants, they may be more vulnerable to environmental and demographic stochasticities, habitat degradation, and genetic limitation because of their specialized life-history strategies depending on i.e. host plants. *Pedicularis sceptrum-carolinum* is a hemiparasitic species with a strongly fragmented geographic range in Eurasia whose small, isolated, island-like populations are scattered at the periphery of its geographic range. I studied its genetic diversity patterns at the western periphery of the species' range (Poland) using AFLP markers in order to unravel how isolation, population size and life-history traits (i.e. type of reproduction) influence its population genetic structure. Despite the geographic isolation among the four investigated populations (ca. 35–350 km), and irrespective of their small population sizes (14–50 individuals) and areas (6–100 m²) they preserved relatively high genetic diversity ($\text{Frag}_{\text{poly}} = 46.7\%–54.6\%$, Shannon's $I = 0.222–0.241$) in comparison with other polyploid, long-lived and outcrossing perennials, and significantly higher than that in the other *Pedicularis* species. Among the factors generating such high genetic diversity is the polyploid origin of this species. Additionally, sexual reproduction, the breeding system, and seed dispersal seem responsible for the patterns of within-population spatial genetic structure. The moderate genetic differentiation among populations ($F_{\text{ST}} = 0.154$) and the evidence of recent genetic admixture of populations, as well as genetic similarity among all investigated individuals suggested that gene flow may be relatively high and multi-directional, reflecting recent range expansion of the study species in Europe. I considered that the estimates of genetic differentiation supported the possibility of repeated colonization from different source populations.

Introduction

The process of range expansion after the last glacial maximum in the northern biota of Eurasia and North America usually involved founder effects and loss of genetic variation (Hewitt 1996, 2000). The geographically peripheral populations, or populations in recently inhabited areas, often showed lower levels of genetic variation as compared with populations from the centre of distribution or refugia (Lesica & Allendorf 1995, Eckert *et al.* 2008). On the other hand, such reduced levels of genetic variation were not always found in marginal populations, and this fact could be explained by occasional repeated inter-population gene flow, such as in wind-pollinated tree species (Langerkrantz & Ryman 1990). In such cases, no clear spatial trends in within-population genetic diversity across geographic species' ranges can be expected. The existence of historical and contemporary gene flow is also a key factor that can prevent strong genetic differentiation among peripheral populations (Kirkpatrick & Barton 1997) and also allows the recovery of genetic diversity after a recent genetic bottleneck, founder effect and/or inbreeding (Hansson *et al.* 2000, Keller *et al.* 2001).

However, the genetic diversity patterns in peripheral populations also depend on other factors, including the type of reproduction, breeding systems, seed dispersal, population size as well as habitat fragmentation, environmental heterogeneity and human activity (Jump & Peñuelas 2006, Bizoux & Mahy 2007). Alsos *et al.* (2012) stressed that loss of genetic diversity in plants with a northern distribution range due to climate warming depends on dispersal adaptations and the growth forms of the plant species. The short distance-dispersed herbs are expected to enter the extinction vortex more rapidly than the long distance-dispersal woody species. Both theoretical and empirical studies also showed that habitat fragmentation can erode the genetic diversity of populations due to decreased population size and inter-population connectivity (Johansson *et al.* 2007). After fragmentation, small populations and lower genetic diversity lead to genetic drift, higher risks of inbreeding, lower evolutionary potential and, consequently, to higher risk of extinction (Young *et al.* 1996). Therefore, knowl-

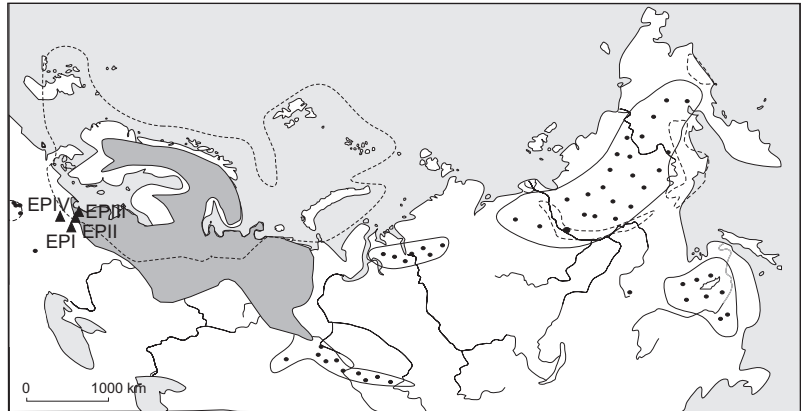
edge on species traits and population history may thus help us forecast which species are at risk.

Pedicularis sceptrum-carolinum (Orobanchaceae), moor-king, is a hemiparasitic species with a strongly fragmented geographic range in Eurasia (Fig. 1) due to the restricted distribution of mesotrophic mires and human activity (Minayeva *et al.* 2009). It reaches the limit of its continuous distribution in Europe, and isolated, island-like populations are scattered in the Alps and Carpathians, as well as in the central part of the continent. The natural habitats of *P. sceptrum-carolinum* include the shores of oligotrophic lakes, rivers, peat-covered areas and moist meadows (Stoicovici 1984). Due to changes in land-use practices and natural succession on peatlands, natural habitats of *P. sceptrum-carolinum* are decreasing in Europe (Magnez 2003). As a result, the European populations are often small (1–100 individuals per population) and isolated from each other. This species is classified as extinct (Holub & Procházka 2000), or as extremely rare and endangered in red data books and red data lists in Europe (Maglocký & Feráková 1993, Ludwig & Schnittler 1996, Niklfeld & Schrott-Ehrendorfer 1999, Kaźmierczakowa & Zarzycki 2001, Mirek *et al.* 2006).

According to Krogulevich (1976), as well as Saggoo and Srivastava (2009), the species is a tetraploid, with the somatic chromosome number $2n = 4x = 32$. A division to subspecies has been proposed for *P. sceptrum-carolinum* (Li 1949, Hong *et al.* 1998). In this paper, the investigated samples represented *P. sceptrum-carolinum* subsp. *sceptrum-carolinum*.

The surveys of Macior (1982) and Macior and Tang (1997) showed that *Pedicularis* species all over the world are pollinated almost exclusively by bumblebees. *Pedicularis sceptrum-carolinum*'s flower structure is different from the other *Pedicularis* species and promotes pollination by insects. The upper and the lower lip adhere to one another, and they are slightly overlapping (Fig. 2). Only the strongest insects are able to push the lower lip down to get to the nectar at the bottom of the tube, or to the pollen. The fact that the flowers are so difficult for most insects to get inside is probably why it has such poor seed production. Kampny (1995) stated

Fig. 1. Locations of the sampled populations of *Pedicularis sceptrum-carolinum* (triangles, for the population codes see Table 1), geographical range of the species (dark shading), marginal island populations (black dots) and maximum extent of ice sheet during the last glacial maximum (dotted lines).



that different strategies, such as dichogamy, have evolved to encourage cross-pollination in *P. sceptrum-carolinum*. My own observations (unpubl. data) of the Polish populations confirmed that vegetative reproduction was possible but occurred rarely. Adventitious buds grow at the bottom of the shoot as daughter plants, forming small clumps.

Here, I employ AFLP molecular markers to test the hypothesis that the highly fragmented and small *P. sceptrum-carolinum* populations at the western edge of its range (central Europe, Poland) will reveal general patterns of low genetic diversity within and restricted gene flow among them. Furthermore, despite the flower structure of this species promoting outcrossing, the populations could be vulnerable to a loss of genetic diversity if selfing and/or bi-parental inbreeding increased due to habitat isolation and small population sizes. Because of inbreeding and limited seed dispersal, kinship groups of individuals can also be established at the population scale. Finally, I conclude with a discussion of the conservation strategies for the western peripheral *P. sceptrum-carolinum* populations.

Material and methods

Plant material

The study was based on samples comprising 67 individuals representing four populations (tagged EPI–EPIV) of *P. sceptrum-carolinum* in Poland (Table 1 and Fig. 1). Despite the fact

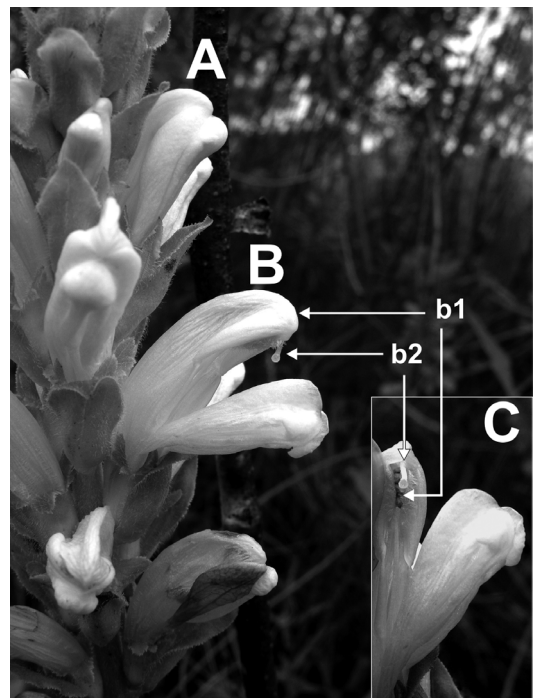


Fig. 2. Flowering shoot of *Pedicularis sceptrum-carolinum* with the upper and lower lips of the flower adhered to one another, slightly overlapping (A). Manually opened flower (B and C) with hidden anthers inside the upper lip (b1) and visible stigma outside the upper lip (b2).

that *P. sceptrum-carolinum* rarely regenerates clonally, one leaf sample was taken from clumps and/or from single shoots within each population in order to avoid the effects of population sub-structure. In Poland the areas covered by *P. sceptrum-carolinum* populations vary greatly, from

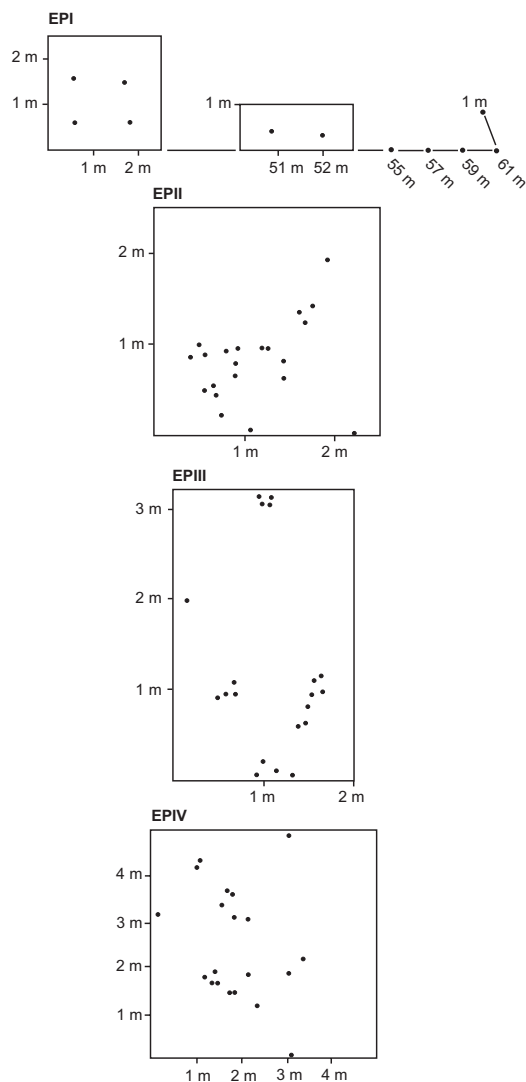


Fig. 3. Population areas and spatial distribution of individuals within four *Pedicularis sceptrum-carolinum* populations (black dot = shoot).

one patch of extremely small size, ca. 6–25 m² (EPII–EPIV) to ca. 100 m² (EPI) (Fig. 3). In all

populations, each sample was mapped on the grid coordinate system, using measuring tapes, to calculate the distance from a reference point. Genomic DNA was extracted from dry leaf tissues with the Genomic Mini AX Plant kit (A & A Biotechnology, Poland), and then the samples were genotyped for AFLP markers.

Open and hand pollination

Open and hand pollination treatments were performed in the EPII population from July to August in 2011 (Table 2). I carried out one pollination experiment on open inflorescences and three pollination experiments on bagged inflorescences. The first treatment was (1) open pollination ($n = 117$ flowers), in which all insects had access to the flowers (open inflorescence without a nylon mesh bag). The three pollination treatments on the inflorescences bagged throughout blooming were as follows: (2) spontaneous self-pollination ($n = 53$ flowers) to determine the probability of autogamy without the participation of pollinators (insects were excluded by a nylon mesh bag); (3) induced self-pollination ($n = 13$ flowers) to indicate the presence and level of self-compatibility (hand pollination with pollen of the same flower); (4) induced xenogamy ($n = 15$ flowers) as cross pollination (hand pollination with the pollen of several other plants). The position of the flower on each inflorescence was numbered from the bottom up. In each flower in treatment 4, anthers were removed before hand pollination to prevent self-pollination. Pollination was done quickly after emasculation to avoid the influence of pollinia removal on fertilization potential. All inflorescences were quickly rebagged after the 2nd, 3rd and 4th treatments. The fruit set consistency is a commonly used cri-

Table 1. Characteristics of four *Pedicularis sceptrum-carolinum* populations (AFLP loci) at the western periphery of the species' range (Poland). No. = population number, N = approximate population size (individuals), n = number of samples analyzed, $\text{Frag}_{\text{poly}}$ = proportion of polymorphic fragments, I = Shannon's diversity index ($\pm$ SD).

No.	Location	Code	N	n	$\text{Frag}_{\text{poly}}$ (%)	I ($\pm$ SD)
1	Chelm	EPI	~14	10	46.7	0.220 ($\pm$ 0.254)
2	Lipsk	EPII	~30	20	54.6	0.231 ($\pm$ 0.269)
3	Wigry	EPIII	~30	18	54.3	0.239 ($\pm$ 0.225)
4	Wólka Bodzechowska	EPIV	~50	19	51.7	0.241 ($\pm$ 0.260)

terion to estimate reproductive success in plants. Because of the low number of flowering shoots in the EPII populations (18 in 2011), all shoots were taken into the pollination treatments.

AFLP procedure

I followed the AFLP procedure by Vos *et al.* (1995), but modified it according to the Applied Biosystems protocol (AFLP™ Plant Mapping). First, 12 primer pair combinations were tested in four selected samples. The fluorescence-labelled selective amplification products were mixed with 500 Liz labelled size standard (Applied Biosystems) and run on an ABI 3130. From this analysis, I chose three primer combinations that gave polymorphic, clear, reproducible fragments of homogeneous intensity (i.e. *Eco*R1-ACA/*Mse*I-CAC; *Eco*R1-AGC/*Mse*I-CAG; *Eco*R1-ACG/*Mse*I-CAA). Variable fragments in the 70–500 bp size range were scored as present (1) or absent (0) using GENEMAPPER 4.0 (Applied Biosystems). To test the repeatability of the AFLP results, three individuals from each population were completely replicated starting from the restriction/ligation reaction of AFLP. Potential resampling of clones was checked with AFLPDAT R-script (Ehrich 2006), but was of insignificant importance and thus not corrected for.

AFLP analysis

To infer population structure and assign individuals to populations I used the model-based clustering method described by Pritchard *et al.* (2000), as implemented in STRUCTURE ver. 2.3.3. The AFLP data sets were coded with a

top row indicating 1 as the recessive allele in STRUCTURE 2.3.3 available also for studies using dominant markers for polyploids (Falush *et al.* 2007). In this study, the data were analyzed with an admixture model with correlated allele frequencies, elaborated by Falush *et al.* (2003). Ten replicates were run for all possible values of the maximum number of clusters (*K*) up to *K* = 4. Following the recommendations of Evanno *et al.* (2005), I calculated the ad hoc statistic ΔK based on the rate of change in the log likelihood of data between consecutive *K* values. All runs were based on 500 000 iterations after a burn-in of 100 000 iterations. The STRUCTURE assignment test takes into account the source population of the sample. I set the option GENSBACK = 3, which would test each individual for evidence of ancestry from any of the four populations for three generations. I set MIGRPRIOR = 0.001, which assumes that the probability of a sample's ancestry being strictly from its predefined population is very high (0.999).

In order to assess levels of genetic diversity, I calculated the percentage of polymorphic fragments ($\text{Frag}_{\text{poly}}$) and Shannon's diversity index as $I = -\sum(p_i \ln p_i)$. This value was then averaged over the polymorphic loci. The F_{ST} -statistics of the four *P. sceptrum-carolinum* populations from Poland were compared using AMOVA using ARLEQUIN ver. 3.1 (Excoffier *et al.* 2005). Ninety-five percent confidence intervals were calculated for all F_{ST} -statistics by bootstrap resampling (9999 replicas). Genetic relationships among the 67 individuals were identified by principal component analysis (PCA) plotted with MVSP 3.0 (Kovach 1999). I tested the differences in average values of PC1 and PC2 between populations by one-way ANOVA with STATSOFT 5.0 (StatSoft Inc., Tulsa).

Table 2. Open pollination and hand pollination treatments in the EPII population of *Pedicularis sceptrum-carolinum* at the western periphery of its range (year 2011).

	Open pollination	Spontaneous autogamy	Induced autogamy	Induced xenogamy
Examined inflorescences	9	5	2	2
Examined flowers	117	53	13	15
Number of flowers ($\pm$ SD) per inflorescence	13.0 $\pm$ 8.3	10.6 $\pm$ 4.8	6.5 $\pm$ 3.5	7.5 $\pm$ 4.9
Number of fruits	20	0	10	12
Fruit set value ($\pm$ SD, %)	17.0 $\pm$ 3.1	0 $\pm$ 0.0	77.0 $\pm$ 2.8	80.0 $\pm$ 2.8

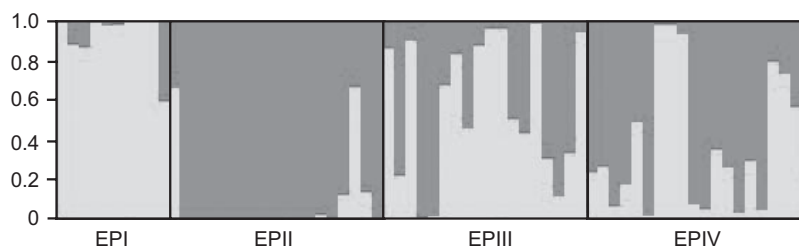


Fig. 4. Bayesian clustering result for the *Pedicularis sceptrum-carolinum* populations (for $K = 2$, each individual is represented by a vertical line partitioned into K segments), generated by STRUCTURE software calculated from AFLP markers.

I also examined the relationships between genetic distances on the basis of the AFLP data, and spatial distances between each pairwise combination of individuals within the four populations. I calculated the similarity coefficient following the method of Nei and Li (1979), which is based on that of Dice (1945). The Dice coefficients (D) were calculated using NTSYS ver. 2.0 (Rolf 1997) and then converted into distances as $1 - D$. A Mantel test for correlations of genetic and spatial distances between individuals within each of the four populations was then performed using GENALEX ver. 6. In this work, the distances were dependent on the area of the populations, and therefore 10-m intervals were selected in EPI, 0.2-m intervals in EPII, and 1-m intervals in the EPIII and EPIV populations.

Results

Hand pollination

Fruit set was noted only after open pollination, induced autogamy and induced xenogamy, but the rate of fruit set varied greatly among these treatments in the EPII population (Table 2). There was no fruit set from spontaneous autogamy (Table 2). The rate of fruit set in the open pollinated plants was relatively low (17%) in 2011. High rates of fruit set following induced autogamy (77%) and induced xenogamy (80%) were noted during one year of observation.

Population genetic structure

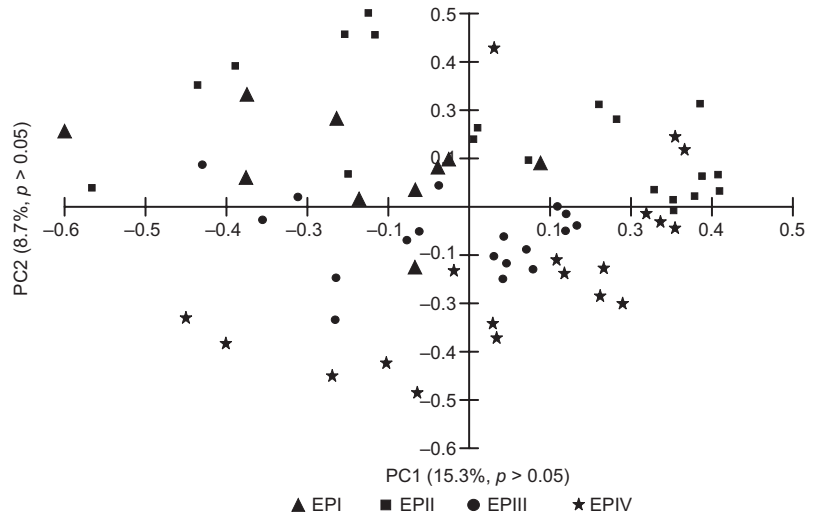
The AFLP analysis included 280 loci. The error rate was 3.1%. Stepwise clustering performed in STRUCTURE separated the samples into two

groups. At $K = 2$, clusters were not distinguished geographically or genetically from each other, but specimens from almost all populations represented a mixture of two diverse genetic backgrounds (Fig. 4). In the assignment analysis, 54 (85%) of the total of 67 individuals had recent ancestry only within their predefined populations. Thirteen individuals (three individuals in EPI, 5 in EPII, 2 in EPIII and 3 in EPIV) had a high probability ($p < 0.001$) of mixed ancestry.

There was a high percentage of polymorphic fragments ($\text{Frag}_{\text{poly}} = 46.7\% - 54.6\%$) and Shannon's diversity index ($I = 0.241 - 0.222$) was high too (Table 1). Each population had unique multilocus genotypes. An AMOVA indicated that variation among all populations was moderate ($F_{\text{ST}} = 0.154$), and all variance components were significantly greater than zero based on the permutation tests. The highest F_{ST} (0.211) was between EPI and EPII, and the lowest (0.130) between EPII and EPIII, located in northeastern Poland. As shown by AMOVA, the genetic variation partitioned within the four populations (84.5%, $p < 0.001$) was higher than among them (15.5%, $p < 0.001$). The Mantel test indicated a lack of relationship between genetic and geographic distances among the populations. PCA ordination, including all individuals, showed that in EPIV and EPIII multilocus genotypes had a non-random distribution and formed clear groups (Fig. 4). The genotypes from EPI and EPII mixed with each other and occupied a different plot in the PCA diagram. In PCA analysis the first factor explained 15.3% and the second 8.7% of overall variance ($p < 0.05$, Fig. 5).

On the smaller population spatial scales, weak relationships between genetic and spatial distances among pairs of individuals were found in two (EPIII and EPIV) out of the four populations (see Fig. 6).

Fig. 5. Principal component analysis (PCA) plot showing the genetic distances among 67 *Pedicularis sceptrum-carolinum* individuals from populations EPI–EPIV. *P* values for PC1 and PC2 axes were obtained using one-way ANOVA.



Discussion

The fragmentation and decrease of the size of *P. sceptrum-carolinum* populations at the western edge of the geographic range in Europe is occurring faster now than at any time in the past due to human activities (i.e. land melioration) rather than climatic changes (Magnes 2003; D. Wołkowycki pers. comm.). Despite the strong isolation between the four investigated populations (ca. 35–350 km), and irrespective of the small population sizes (14–50 individuals) and areas (6–100 m²), they preserved relatively high genetic diversity ($\text{Frag}_{\text{poly}} = 46.7\%–54.6\%$, $I = 0.222–0.241$) in comparison with other perennial and outcrossing polyploid species (Nyborg 2004). The diversity was also higher than in the other *Pedicularis* species studied so far. In *P. dasyantha*, the percentage of polymorphic loci (*P*) was 3% and the expected heterozygosity (H_e) was 0.016 (Odasz & Savolainen 1996); in *P. lanata* H_e was 0.028 (Philipp 1998); in *P. palustris* *P* was 43% (Schmidt & Jensen 2000); and in *P. fubishiae* *P* was 0% and H_e was 0.0 (Waller *et al.* 2006). Among the factors generating such high genetic diversity is probably the polyploid origin of *P. sceptrum-carolinum*. In polyploid plants, the generally higher level of genetic diversity than in diploids is supported because of their different modes of inheritance, which can allow them to adapt quickly

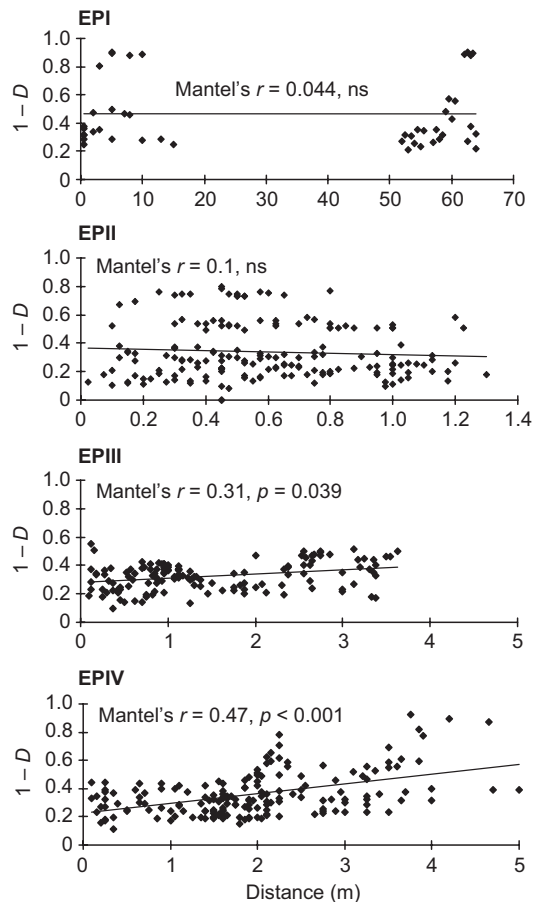


Fig. 6. Relationships between the Dice genetic distance and the spatial distance within four populations, * $p < 0.05$, ** $p < 0.001$. (D = Dice coefficient).

to changing environmental conditions, reducing the genetic drift effect (Soltis & Soltis 2000). Interesting conclusions were drawn by Husband *et al.* (2008), who analysed autopolyploids. They found that autopolyploids had a significantly higher outcrossing rate than allopolyploids, and that autopolyploids often have mixed or out-cross breeding systems. My own investigation (unpubl. data) of the karyotype of *P. sceptrum-carolinum* initially confirmed that it is autotetraploid, and its observed breeding system could be adequately captured in this model.

However, the four peripheral populations of this species appeared to be genotypically diverse, suggesting that clonal reproduction is rare and could instead occur on a small scale of a single maternal shoot. Therefore, within these peripheral populations, sexual reproduction, as well as the breeding system and seed dispersal, seem responsible for the patterns of within-population spatial genetic structure. The very high level of fruit set in the induced autogamy and induced xenogamy experiments, and low fruit set from open pollination, showed that *P. sceptrum-carolinum* is a self-compatible but pollinator-dependent species. A spatial separation of the stigma and anthers (Fig. 2) but also temporal separation via protogyny was observed in the species by Kampny (1995). In the present study, after the spontaneous autogamy treatment, “automatic” intraflower self-pollination was not observed (0% of fruit set). On the other hand, a high rate of fruit set following induced autogamy (77%) and induced xenogamy (80%, treatments at the same time) was noted, which may suggest that there is no protogyny in this species. Most likely the spatial separation of stigma and anthers explains the lack of spontaneous autogamy but there still may be occasional selfing. Pollinators of *P. sceptrum-carolinum*, such as bumblebees, are known to visit many flowers within the same inflorescence (thus promoting geitonogamy; Harder & Barrett 1996). The pattern of the floral visitations of bumblebees also causes cross-pollination, because bumblebees may also visit just one flower per plant and then fly to a flower of a different plant. This behaviour follows the model for the horizontal pollination sequence proposed by Harder and Wilson (1998). Therefore, the fine-scale genetic results suggested a

mixed-mating strategy within the *P. sceptrum-carolinum* populations at the western periphery of its range, balancing between the share of outbreeding and/or of biparental inbreeding and/or selfing. The breeding system together with limited dispersal has also contributed to the emergence of small kinship groups within populations (Vekemans & Hardy 2004). On the other hand, Ellstrand and Elam (1993) and Raijmann *et al.* (1994) reported that in animal-pollinated species, fragmentation may affect the breeding system through changes in the behaviour of pollinators, with consequences for the pollen transfer which have been ascribed to small population size, greater isolation and reduced density. As compared with wind-pollinated plants, species dependent on pollinators can be more susceptible to the effects of habitat fragmentation because of their reliance on animal behaviour to reproduce (Llorens *et al.* 2011). Moreover, the number of flowering shoots is important because as populations shrink they become more susceptible to suffering negative effects due to random events (Aguilar *et al.* 2008). Because of the lack of autofertility, *P. sceptrum-carolinum*, similar to *P. palustris* (Karrenberg & Jensen 2000), could be at risk of pollen limitation. Nevertheless, the high self-compatibility mechanism with a lack of autofertility in a few *Pedicularis* species (contrary to the other *Pedicularis* species in which autofertility and self-compatibility occur) might represent a phylogeographic constraint in this group (Karrenberg & Jensen 2000).

I detected moderate genetic differentiation among the populations ($F_{ST} = 0.154$), which indicates that gene flow among them was large enough to counteract the effects of genetic drift, in contradiction to the highest genetic differentiation among *P. palustris* ($F_{ST} = 0.05–0.89$) and *P. oederi* ($F_{ST} = 0.55$) populations (Schmidt & Jensen 2000, Alsos *et al.* 2012). Moderate genetic differentiation among populations, the evidence of recent local genetic admixture for populations, as well as the genetic similarity of individuals suggested that gene flow may be relatively high and multi-directional, reflecting the recent range expansion of *P. sceptrum-carolinum* in Europe. Alsos *et al.* (2012) stressed that seed dispersal adaptation appeared to be important in determining the rate of loss of genetic diversity

within plant species with a northern distribution. According to those authors, lower F_{ST} values were found in species with adaptation to long-distance dispersal than in species lacking such adaptations. *Pedicularis sceptrum-carolinum* seeds (as also those of its congeners) do not have morphological adaptations to long-distance dispersal, but the fruits and shoots are occasionally grazed by animals, which may be vectors of long-distance dispersal of seeds (Waller & Gawler 1987; own unpubl. data). Obviously, low genetic differentiation among populations does not only depend on adaptation to long-distance dispersal of seeds, but also on factors such as colonization processes, formation and establishment of populations, habitat fragmentation, and environmental heterogeneity. This result contradicts those of Alsos *et al.* (2012), and low genetic differentiation among the populations of *P. sceptrum-carolinum* is more surprising because during the last decade a strong decline in the population numbers and sizes has been observed in the whole Poland. Moreover, their genetic structure is not concordant with other marginal plant populations where founder effects (Pérez-Collazos *et al.* 2009, Lewandowska-Sabat *et al.* 2010, Lauterbach *et al.* 2011) or 'leading edge' model of colonization were observed (Hampe & Petit 2005). I consider that estimates of genetic differentiation were presented as relative measures of connectivity between patterns of single and rarely repeated colonization from the different source populations. Because of the strong geographic isolation of these populations, and the unique multilocus genotypes in each of them, they can act as discrete genetic units at the present time.

Although I found that genetic diversity was relatively high within the western peripheral populations of *P. sceptrum-carolinum*, the decline in the number and size of its populations is a common phenomenon in the whole Europe and Asia. The loss of its natural habitats and natural succession in the patches occupied by the species is a problem. Knowledge of its biology is still scarce, and therefore many questions, especially about the relationships between the host plants and this hemiparasite within different habitats, as well as the longevity of the seed bank, are still unanswered. Managing the populations of *P. sceptrum-carolinum*, as also those

of *P. palustris*, by cutting regimes can lead to the conservation of its typical habitats (Schmidt & Jensen 2000), and at the present time that can be considered the most appropriate method for the protection of this endangered plant.

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