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RESEARCH ARTICLE

Genetic variability, population size and reproduction potential in *Ligularia sibirica* (L.) populations in Estonia

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Abstract *Ligularia sibirica* (L.) Cass. (Asteraceae) is a EU Habitats Directive Annex II plant species that has suffered a lot from human-caused major changes in quality and availability of habitats in Estonia. The aim of this study was to find out if the observed decline in population size is reflected in the amount of genetic variation and fertility in remnant populations of this species. AFLP technique was used for that purpose. Genetic diversity within populations was assessed as the percentage of polymorphic loci in a given population and average gene diversity over loci. The degree of genetic differentiation among populations and genetic differentiation between pairs of populations was estimated. The amount of viable seeds per flower stem was compared among populations and between years (2007 and 2008). Average genetic diversity over loci and proportion of polymorphic loci in *L. sibirica* populations were significantly correlated with population size, suggesting the action of genetic drift and/or inbreeding. No correlation was found between genetic and geographic distances. Natural barriers like forests may have been efficiently preventing seed migration even between geographically closer populations. Results of this study suggest that genetic erosion could be partially responsible for the lower fitness in smaller populations of this species.

Keywords AFLP · Genetic diversity · *Ligularia sibirica* · Population genetic structure · Seed production · Small population

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Introduction

Populations of many endangered plant species have decreased in size and become isolated because of the changes in land use during the last century (e.g. Brook et al. 2008; Hobbs and Yates 2003). However, the impairment of habitat conditions is often accompanied by changes in demographic processes (Schleuning and Matthies 2009) and increased genetic stochasticity, i.e. genetic drift (Ouborg et al. 2006). As the effective population size diminishes, random loss and fixation of alleles through drift result in the loss of genetic variation and differentiation of populations (Ellstrand and Elam 1993). This, in turn, could lead to reduced fitness and further reduce the population size and thus predestine a population into a vortex of extinction (Ellstrand and Elam 1993; Gilpin and Soulé 1986).

The predicted relationship between plant population size and genetic diversity has been documented in numerous studies (Dittbrenner et al. 2005; Fischer and Matthies 1998; Gaudeul et al. 2000; Hensen et al. 2005; Hensen and Oberprieler 2005; van Treuren et al. 1991; Vergeer et al. 2003), whereas some studies reveal no such correlation (Bachmann and Hensen 2007; Hensen et al. 2010; Kahmen and Poschlod 2000; Pluess and Stöcklin 2004; Tero et al. 2003). The negative effect of small population size on genetic variation is often stronger in predominantly outcrossing and self-incompatible plant species (Honnay and Jacquemyn 2007; Leimu et al. 2006), possibly in connection with reduced pollinator activity with decreased number of flowering plants (Kwak et al. 1998). Genetically eroded populations can be "rescued" by efficient gene flow between them owing to migration of pollen and seeds (Ellstrand and Elam 1993; Loveless and Hamrick 1984).

Reduction in population size is often accompanied with frequent matings among relatives, leading to heightened

homozygosity and inbreeding depression (Frankham et al. 2010). Several traits related to plant fitness are shown to be affected by declining population size: e.g., seed production decreased with population size of rare *Gentianella germanica* (Fischer and Matthies 1998) and *Dracocephalum austriacum* (Dostálek et al. 2010), plants produced fewer and lighter seeds in smaller populations of *Dictamnus albus* (Hensen and Oberprieler 2005) and *Pulsatilla vulgaris* (Hensen et al. 2005); germination was less successful in smaller populations of *Silene regia* (Menges 1991) and *Succisa pratensis* (Vergeer et al. 2003). The negative effect of low genetic variation and small population size on plant fitness and thus increased probability of extinction was recently confirmed by meta-analysis based on studies published between 1987 and 2005 (Leimu et al. 2006). Therefore, assessing the survival probabilities of endangered plant species, it is important to carry out studies where both plant performance and genetic aspects are analyzed.

Ligularia sibirica (L.) Cass. (Asteraceae) is a rare and endangered plant species everywhere it is found in Europe. In Estonia, its populations have primarily suffered from human-caused changes in quality and availability of habitats (primarily overgrowth by trees and shrubs coinciding with worsened light conditions and increased competition, as well as the drainage of former wet habitats). The number of localities of this rare plant has decreased from 18 in 1969 to 9 (Kukk 2003; Kukk and Kull 2005) and most of its populations continue to decline. In several populations the decrease in the number of juveniles and generative plants has been reported (Kukk 2003). In some populations, there have been attempts to restore the habitat and in some others to reinforce the population size by introducing ex-situ nourished new individuals originating from the same populations (M. Sammul, pers. inform.). The success of these actions has been inconsistent, however, in order to evaluate the potential of a success of conservation actions, it is essential to first understand the intrinsic genetic and demographic hindrances to population growth.

The main objective of this study was to find out if the observed decline in population size has induced a decrease in the amount of genetic variation as well as reproductive plant performance in the populations of *L. sibirica.* The amplified fragment length polymorphism (AFLP) technique was selected for its ability to create a large amount of markers in a short time without any previous knowledge of genome of plant species (Meudt and Clarke 2007). Specifically, we asked the following questions: (i) what is the overall level of genetic diversity and how is it distributed within and among populations of *L. sibirica*? (ii) is there an association between within-population genetic diversity and population size? (iii) is the plant fitness affected by population size and genetic variation? On the basis of our results, we proposed the guidelines for reinforcement of populations of *L. sibirica*.

Materials and methods

Study species

Ligularia sibirica (L.) Cass. is a tall (50–150 cm) perennial herb, the life-span of its genet can be more than 10 years. It has a short slowly growing rhizome, kidney-shaped long-stalked lower leaves and much smaller stalkless upper leaves. In Estonia, it flowers at the end of July and in August, forming 1–8 erect flowering stems with numerous (10–30; sometimes even 50) yellow flower heads. This species is mainly insect-pollinated but is capable of self-fertilization (M. Sammul, pers. obs.). The seeds are supplied with pappus and mature in late Aug. or in Sept.

The main distribution area of *L. sibirica* is in Russia, extending from the European part of Russia over Siberia to the Far East (Hultén and Fries 1986). In Europe, this species is considered to be a postglacial relict, occurring in isolated localities in Estonia, Latvia, Ukraine, Poland, Croatia, Romania, Czech and Slovak Republics, Hungary, Bulgaria, Austria and France (Hegi 1987; Hultén and Fries 1986). *L. sibirica* is listed in the Annex II of the EU Habitats Directive (Council Directive 92/43/EEC, 1992). In Estonia, *L. sibirica* is at the north-western edge of its main distribution range. Typical habitats here for this species are paludified grasslands, floodplain grasslands, forest plains and spring fens (Kukk 2003).

Sampling of populations, seeds and germination

Seven populations differing largely in number of *L. sibirica* were included in the study: Anne, Jõhvi, Õisu, Sootaga, Tagula, Pressi and Väägvere (Fig. 1). Two other populations, Kikaste and Ädise, were left out because of the very small size of these populations (consisting of only some remaining vegetative individuals). The sizes of these populations were estimated through counting the number of tussocks, which in most cases corresponds to the number of genets. For AFLP analysis, we collected 20 healthy leaves per population from randomly selected generative plants in summer 2009 (except in Anne where we found only seven plants) and stored in silica-gel bags. In 2007 and 2008, we collected the seeds from 15 to 25 randomly selected plants in all populations except in Anne and Jõhvi, where fewer generative plants were available. In 2008, Pressi population could not be sampled. We regarded seeds that were completely filled and fully developed as viable in contrast to empty, non-developed seeds and those damaged by insect larvae or other parasites. The number of viable seeds per flower stem was used as one measure for plant fitness.

The germination rates were estimated using seeds collected in 2007. For most populations, we used the healthy-looking seeds from five plants (except for Jõhvi and Anne

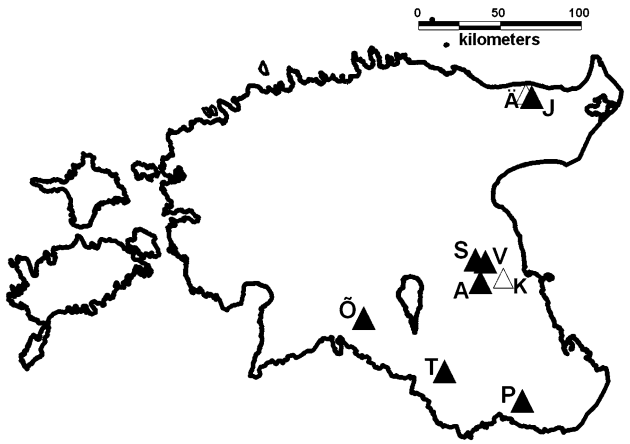

Fig. 1 Location of seven studied *L. sibirica* populations in Estonia. *Filled symbols*: sampled populations. *A* Anne, *J* Jõhvi, *V* Väägvere, *S* Sootaga, *T* Tagula, *P* Pressi, *Õ* Õisu. *Empty symbols*: remnant non-sampled populations. *Ä* Ädise, *K* Kikaste

where we used seeds from four and three plants, respectively), the total number of seeds per population ranged between 64 and 960. Germination was carried out in Petri dishes on wet filter paper in the laboratory at fluctuating day/night length of 14/10 h and temperature of 27/17 °C.

AFLP analysis

DNA was extracted from approximately 100 mg of dried leaf material using CTAB procedure according to Doyle and Doyle (1987). We checked the quality of obtained DNA on a 1.5 % agarose gel and excluded samples with poor DNA quality from further analysis. AFLP procedures were performed as described by Vos et al. (1995) with slight modifications. Instead of 500 ng of genomic DNA, 300 ng was restricted with 1 U*Tru1*I (isoschizomer of *Mse*I) at 65 °C for 2 h, followed by 5 U *Eco*RI digestion (both restrictases from Fermentas, Lithuania) and ligation of double-stranded oligonucleotide adapters with 1 U of T4 DNA-Ligase (Naxo, Estonia) in 2× Tango™ buffer (also provided by Fermentas) at 20 °C overnight. Preselective amplification was performed using primer pairs with a single selective nucleotide, *Eco*RI-A and *Mse*I-C. For selective amplification, we tested 16 primer combinations with three selective nucleotides on five individuals. We selected three primer pairs that amplified most readable AFLP profiles: *Eco*RI-ACT(FAM)-*Mse*I-CAT; *Eco*RI-AGC(NED)-*Mse*I-CAG; *Eco*RI-AGG (JOE)-*Mse*I-CAC. Separation of amplification products was performed by capillary electrophoresis on an ABI3130 Genetic Analyzer (Applied Biosystems, USA). GeneScan 500 Rox™Size Standard (Applied Biosystems, USA) was loaded in each lane. To estimate the reproducibility of the genotyping process, we compared the profiles from five replicates originating from independent DNA extractions which yielded the average reproducibility of 97.9 %. Negative controls were run at each step of the process to check for contaminations.

Data analysis

AFLP electropherograms were scored as present (1) or absent (0) using software GeneMapper® 4.0 (Applied Biosystems, USA). All profiles were checked visually and only these AFLP fragments that could be scored unambiguously were included in the analysis. The amount of polymorphic fragments and these present only in one population (private bands) was determined. We excluded fragments that were monomorphic across all individuals, samples with odd profiles and AFLP fragments present or absent only in one individual to reduce genotypic errors (Bonin et al. 2004). Because of the slight shift between peaks among the small size AFLP fragments and the assumption that smaller fragments are more likely to be homoplasious (Paris et al. 2010; Vekemans et al. 2002), we used the size range of 90–500 base pairs.

To visualize the general patterns among populations, the presence/absence matrix was subjected to a principal coordinates analysis (PCoA) based on Jaccard's coefficient of similarity using the software PCO (Anderson 2003). Jaccard's coefficient does not take into account shared band absences and is therefore adequate for analyzing dominant markers such as AFLP (Duarte et al. 1999).

Genetic diversity within populations was assessed as (i) the percentage of polymorphic loci in a given population and (ii) average gene diversity over loci as defined by the software Arlequin 3.5.1.2 (Excoffier and Lischer 2010). To measure the degree of genetic differentiation among populations, we used a Bayesian approach presented by Holsinger et al. (2002) to calculate F_{ST} analogue $\theta^{(II)}$ by the software Hickory 1.1 (Holsinger and Wallace 2004). This method is appropriate for analysing data derived from dominant markers because neither are assumptions about Hardy–Weinberg equilibrium made nor is previous knowledge about the level of inbreeding in populations needed. Alternative models implemented in this program correspond to different hypotheses: there is no inbreeding ($f = 0$ model); there is no genetic structure ($\theta^{(II)} = 0$); neither $\theta^{(II)}$ nor f are known (full model). In addition to these three models, f-free model provides $\theta^{(II)}$ values without attempting to estimate f. To test the consistency of results, all models were run three times with default sampling parameters (burn-in = 50,000, sample = 250,000, thin = 50). Another analogue of F_{ST}, namely Φ_{ST} was calculated using analysis of molecular variance (AMOVA) by the software Arlequin 3.5.1.2 (Excoffier and Lischer 2010). AMOVA is based on squared Euclidean distances among molecular phenotypes and

enables to partition genetic variation within and among populations (Excoffier et al. 1992). In addition to overall Φ_{ST}, genetic differentiation between pairs of populations (pairwise Φ_{ST}) was estimated. Using the zt 1.1 software (Bonnet and Van de Peer 2002), a Mantel test was performed to find out whether the matrix of genetic distances based on linearised pairwise Φ_{ST} values was correlated with the matrix of log-transformed geographical distances. In both AMOVA analysis and Mantel test, the significance levels were calculated by conducting permutation procedures (1,000 permutations).

We tested the differences in the (log-transformed) amount of viable seeds per flower stem among populations and years (2007 and 2008) by two-way ANOVA without replicates. To investigate the relationship between population size, genetic diversity and plant fitness characters, we calculated non-parametric Spearman correlation coefficients r_S using R version 2.11.1 (R Development Core Team 2007).

Results

AFLP pattern and genetic diversity

AFLP analysis was successfully performed on 85 plants from 7 populations, resulting in a unique AFLP banding pattern for every individual. Three primer pairs generated in total 258 scorable fragments, 199 (77 %) of which were polymorphic. The mean number of fragments per AFLP profile were (±SD): 28(±2.7) for *Eco*RI-ACT(FAM)-*Mse*I-CAT, 45(±4.4) for *Eco*RI-AGC(NED)-*Mse*I-CAG and 40(±6.1) for *Eco*RI-AGG(JOE)-*Mse*I-CAC. The proportion of polymorphic loci varied between 24 and 67 %, being lowest in Anne where no private alleles were found and highest in Tagula where the number of private bands was also highest (Table 1). Mean gene diversity over loci of all populations was 0.167, varying between 0.109 and 0.230. There was a strong correlation between proportion of polymorphic loci and average gene diversity over loci (Spearman rank correlation coefficient $r_S = 0.96$, $p < 0.001$). Both estimates of within-population genetic diversity correlated significantly with approximate population size ($r_S = 0.78$, $p < 0.05$ for average gene diversity over loci and log population size and $r_S = 0.88$, $p < 0.05$ for proportion of polymorphic loci and log population size).

Genetic structure

In PCoA, individuals within populations were mainly grouped together, although there was some overlap in the ordination space: plants from Jõhvi and Väägvere showed a strong genetic similarity to each other, also individuals from Sootaga and Tagula were clustered together (Fig. 2). The three geographically closest populations—Sootaga, Anne and Väägvere—did not group together in PCoA; although Anne and Väägvere were genetically quite similar, Sootaga took place at the opposite end along PCoA 2-axis. Plants in Õisu population formed a distinct cluster which was completely separate from other populations. This general pattern in PCoA was supported by the Mantel test, which revealed no significant correlations between genetic and geographic distances ($r_m = 0.19, p = 0.28$, 1,000 permutations; Fig. 3).

Bayesian analysis of the population structure resulted in similar $\theta^{(II)}$ values for different models. According to the deviance information criterion (DIC), the full model proved to be the most suitable for the data, having the lowest DIC value (3,292.05) and yielding estimates of 0.398 (SD 0.032) and 0.704 (SD 0.031) for $\theta^{(II)}$ and f, respectively. Considerably worse fitted the data in the $f = 0$ model (no inbreeding) which had a difference of more than 100 DIC units from the full model (DIC 3,397.37), providing strong evidence for the occurrence of inbreeding in *L. sibirica* populations. The comparison of posterior distributions showed no significant difference between $\theta^{(II)}$ estimates in these models (0.0060 with 95 % credible intervals −0.0765; 0.0637), indicating that the obtained $\theta^{(II)}$ was not affected by the evaluating inbreeding coefficient in the full model.

Table 1 Population size, sample size, genetic diversity estimates, proportion of germinated seeds and mean amount of viable seeds per flower stem in the studied populations of *L. sibirica*

Site	Population size	No. of samples	Average gene diversity over loci	Proportion of polymorphic loci (%)	No. of private alleles	Germination % (SD)[a]	Mean amount of viable seeds (SD)[a]	
							2007	2008
Anne	7	7	0.109	24	0	41 (21)	24 (17)	9 (7)
Jõhvi	70	9	0.138	31	1	45 (17)	21(6)	57 (75)
Pressi	400	11	0.181	51	4	59 (30)	109 (98)	–
Väägvere	400	13	0.181	52	3	52 (9)	195 (145)	161 (83)
Õisu	600	17	0.115	38	2	65 (22)	22 (18)	52 (32)
Sootaga	1,000	13	0.217	63	3	73 (14)	93 (72)	149 (81)
Tagula	1,500	15	0.230	67	5	68 (22)	40 (35)	196 (99)

[a] Sample size for germination % and mean amount of viable seeds is as described in "Materials and methods"

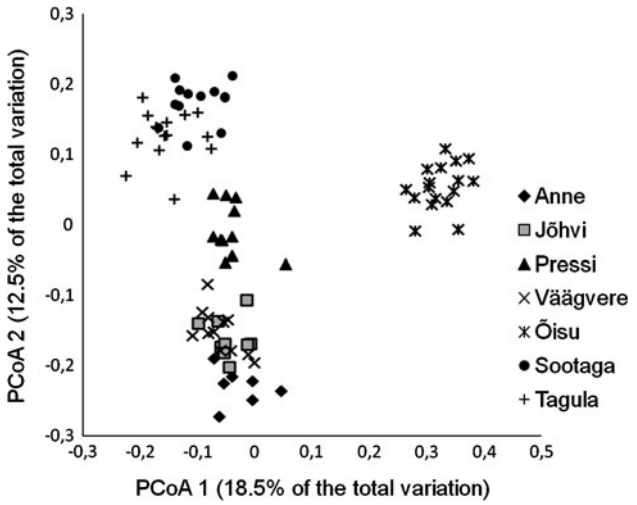

Fig. 2 Principal Coordinate Analysis (PCoA) of the 85 *L. sibirica* individuals based on Jaccard coefficient of similarity. X- and Y-axis explain 18.5 and 12.5 % of the total variation, respectively

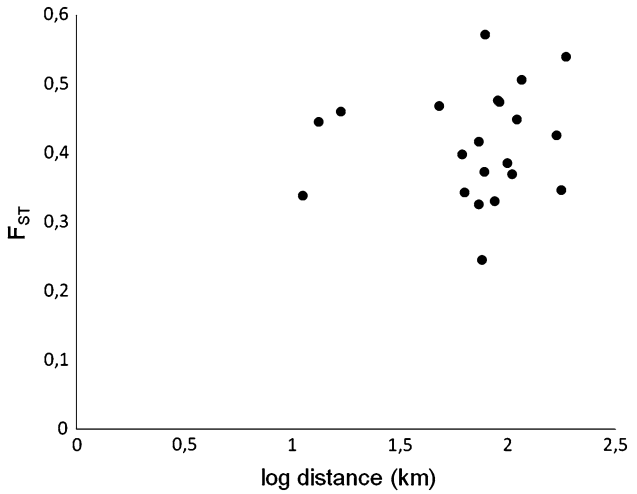

Fig. 3 The relationship between pairwise population F_{ST} values and logarithm of geographical distance (km). The relationship was not significant (Mantel test $r_m = 0.19$, $p = 0.28$, 1,000 permutations)

AMOVA analysis revealed similar levels of genetic differentiation among populations of *L. sibirica*: Φ_{ST} value was 0.41; 59 % of total molecular variation was attributable to the variation within populations and 41 % was due to the differences among populations. All values obtained in AMOVA were highly significant ($p < 0.001$, 1,000 permutations). Pairwise Φ_{ST} values varied between 0.57 and 0.25 (Table 2).

Seed production and germination

There were significant differences in viable seed production between populations ($F_{6,\ 227} = 23.8$, $p < 0.001$) and years ($F_{1,\ 227} = 8.7$, $p < 0.001$). In 2007, seeds were largely damaged and eaten in Tagula, Sootaga and Õisu which resulted in lower seed counts compared to the amount of viable seeds produced in 2008 in these populations (Table 1). Lowest seed numbers were measured in Anne for both years. Mean viable seed production in 2008 was positively correlated with both average gene diversity over loci and the proportion of polymorphic loci ($r_S = 0.94$, $p < 0.05$ and $r_S = 0.89$, $p < 0.05$, respectively), there was no correlation present in 2007. Correlations with population size were not significant in either year.

The germination % ranged between 41 and 73 % (Table 1), the average for all populations was 58 % (SD ± 22 %). Germination % was positively and significantly correlated with the proportion of polymorphic loci ($r_S = 0.82$, $p < 0.05$) and population size ($r_S = 0.95$, $p < 0.001$). The relationship between average gene diversity over loci and germination % was very close to being significant ($r_S = 0.74$, $p = 0.058$).

Discussion

Genetic diversity and population differentiation

Among several life-history traits, the breeding system is considered to be among the most important to determine the level of genetic variability and its distribution in populations of plant species (Duminil et al. 2007; Nybom and Bartish 2000). Plants with mixed-mating breeding system exhibit much lower levels of within-population gene diversity compared to outcrossing plants (Hamrick and Godt 1989; Nybom 2004). Genetic diversity in examined seven *L. sibirica* populations was relatively low (mean values 0.167 and 46.5 % for gene diversity and proportion of polymorphic loci, respectively) and slightly lower than the average for mixed-mating species measured by RAPD (0.18) presented by Nybom (2004). This implies that self-pollination or pollination between close relatives can play quite a substantial part in reproduction of this species. Intrapopulation diversity indices of the same range for endangered plant species with similar breeding system are obtained also by AFLP techniques, e.g., 0.134–0.234 for *Eryngium alpinum* (Gaudeul et al. 2000); 0.172–0.229 for *Silene chlorantha* (Lauterbach et al. 2011).

For outcrossing plants, most of total genetic variability is distributed among the individuals within a population, a smaller proportion of it is attributable to the variation between populations (Hamrick and Godt 1989). On average 27 % (Φ_{ST} 0.27) of RAPD diversity partitions between populations when the species is predominantly outbreeding, the mean value of Φ_{ST} is significantly higher (Φ_{ST} 0.4) for partially selfing plants (Nybom 2004). Thus, Φ_{ST} of 0.41

Table 2 Geographical distances (km; above the diagonal) and pairwise estimated Φ_{ST} values (below the diagonal) between seven populations of *L. sibirica*

	Anne	Jõhvi	Pressi	Väägvere	Õisu	Sootaga	Tagula
Anne	–	115.7	78.5	16.8	73.3	13.2	61.3
Jõhvi	0.506***	–	185.2	99.5	167.6	104.2	177.0
Pressi	0.460***	0.385***	–	90.0	109.8	91.5	48.0
Väägvere	0.398***	0.346***	0.373***	–	86.4	11.1	77.7
Õisu	0.571***	0.539***	0.476***	0.468***	–	75.9	62.9
Sootaga	0.416***	0.426***	0.330***	0.343***	0.448***	–	73.1
Tagula	0.445***	0.369***	0.338***	0.325***	0.474***	0.245***	–

*** Level of significance $p < 0.001$, based on 1,023 permutations

obtained in AMOVA in this study is in accordance with average for mixed-mating plants and together with the results of the Bayesian analysis ($\theta^{(II)}$ 0.398) indicates quite a high level of differentiation between *L. sibirica* populations. Much lower between-population diversity indices together with high variability within populations of *L. sibirica* was recently found in the Czech and Slovak Republic by using allozymes (Šmídová et al. 2010). They estimated the obtained F_{ST} value (0.179) as typical for predominantly outcrossing species as presented by Hamrick and Godt (1989). However, comparing results from studies using different marker systems like allozymes and genome-wide selectively neutral DNA markers (AFLPs and RAPDs) should be avoided. It has been suggested that differences in between-population estimates obtained both from allozyme and RAPD analysis of the same populations most likely show differences in rates of mutations at RAPD and allozyme loci rather than reflect different migration rates (Holsinger and Wallace 2004). Allozymes can also be subject to directional selection pressure which may enhance the action of genetic drift (Baruffi et al. 1995) or be influenced by balancing selection which maintains allozyme polymorphisms (Altukhov 1991).

One explanation to high levels of differentiation among populations can be restricted or absent gene flow by pollen and seeds (Fischer and Matthies 1998; Hensen et al. 2010; Hogbin and Peakall 1999; Lauterbach et al. 2011; Schmidt and Jensen 2000). Pollen flow mediated by insects is generally limited since most pollinators can travel less than 1 km (Kwak et al. 1998) and they tend to visit neighbouring plants (Rasmussen and Brødsgaard 1992). Populations of *L. sibirica* in Estonia are most likely too far from each other to be connected by pollen exchange between populations. Gene flow by seeds is more realistic as seeds of this species are supplied with pappus and are adapted to dispersal by wind, and given the location of several populations on alluvial riverbanks they could also be distributed by flowing water. However, most of the seeds of this species are not considered to travel long distances, landing close to the parent plant (Kobiv 2005, Šmídová et al. 2010) and the small fraction which is carried high up and far by the strong wind is very likely to finally set down on an unsuitable environment where establishment is unlikely (Sheldon and Burrows 1973). As no correlation was found between genetic and geographical distances, it is possible that natural barriers like forests have been efficiently preventing seed migration even between geographically closer populations located on the same river system, Väägvere, Sootaga and Anne. Similarly, the high pairwise Φ_{ST} values (Table 2) imply limited gene exchange between *L. sibirica* populations. The highest Φ_{ST} values were obtained between Anne and Õisu (0.571) and Jõhvi and Õisu (0.539). Also closely situated populations were genetically quite differentiated (Φ_{ST} values between Anne, Sootaga and Väägvere ranging between 0.343 and 0.416). Comparable pattern was obtained in PCoA: plants from geographically closer populations were genetically not so similar and Õisu was placed completely separately from other populations (Fig. 2). The Õisu population is also conspicuous by its very low genetic diversity indices despite of its considerable size (Table 1). Although the first records about single *L. sibirica* plants in the Õisu area are from 1860, the main population was discovered in 2003 and its previous history is unknown. Therefore, it is not impossible that this population was founded by only a few individuals and a low level of genetic diversity has been maintained through insufficient gene exchange with other populations. In addition to that, almost all populations have maintained private alleles (Table 1), confirming the idea that populations of *L. sibirica* might experience very little migration between them and have been under the influence of random genetic drift.

Genetic diversity, population size and plant fitness

In *L. sibirica* populations, both average gene diversity over loci and proportion of polymorphic loci were significantly correlated with population size. This is in accordance with the theory that especially small isolated populations are

susceptible to processes such as drift and inbreeding and also in agreement with high levels of differentiation found in *L. sibirica* populations. Lowered heterozygosity and inbreeding are shown to have a negative impact to plant fitness (Ellstrand and Elam 1993). Unfortunately, direct estimation of inbreeding is not possible from AFLP data. The *f* value derived from the Bayesian analysis is hardly reliable due to the dominant nature of the marker system and should be regarded with caution (Holsinger and Lewis 2003–2007). Nevertheless, since the Bayesian model with no inbreeding did not fit the data as well as the full model, the existence of inbreeding (and inbreeding depression) cannot be ruled out in smaller populations of *L. sibirica*. Therefore, lowered levels of genetic diversity and inbreeding depression can be considered as one factor influencing the seed production and germination ability of this species. However, since molecular markers such as AFLP are selectively neutral and may lose genetic variation at different speed compared to loci connected with fitness, the correlation between genetic diversity and fitness can be rather weak and explain only a small proportion of variation in fitness (Reed and Frankham 2003). Therefore, the loss of fitness should not be automatically inferred from the loss of genetic variation measured with molecular markers. Nevertheless, reductions in plant seed production in combination with AFLP or RAPD variation are described in several studies (Dittbrenner et al. 2005; Fischer and Matthies 1998; Hensen and Oberprieler 2005; Schmidt and Jensen 2000). In this study, germination percentage was in positive relationship with one estimate of genetic variability (proportion of polymorphic loci). Unfortunately, the sample size used in germination experiment was relatively small which could have influenced the relationships between germination percentage and genetic diversity estimates. We also found a strong positive correlation between the amount of viable seeds in 2008 and both genetic diversity estimates. In 2007, the correlation was missing: the inflorescences of most plants were seriously damaged by insect larvae in several populations (mostly in Tagula and Sootaga) which resulted in low levels of seed counts. Unfortunately, it was not possible to estimate the amount of developed seeds consumed by parasites. Therefore, the seed counts in these populations do not truly represent the plants potential to develop healthy, filled seeds and it is probable that the missing correlation in 2007 was conditioned by the observed damage reducing seed counts drastically in these populations. The levels of seed production in small populations with the lowest genetic diversity indices (Anne, Jõhvi and Õisu) were quite low even when the year was good (Table 1), showing the possibility that there can be a link between genetic factors and reproductive fitness in small populations of this species. While plants in large populations can buffer unsuccessful reproduction in a bad year by producing more seeds in a good year, small populations with reduced genetic diversity might have lost this property.

Environmental stress and poor habitat quality can be one possible factor contributing to the reduced plant performance in some populations of *L. sibirica.* Sub-optimal growth conditions are shown to lead to diminished maternal investment into the offspring, affecting reproductive success (Oostermeijer et al. 1994). In addition to direct effect of limited resources availability, it has been proved that the impact of inbreeding is more severe under stressful conditions (Armbruster and Reed 2005; Reed et al. 2002). Seed production can also be largely dependent on pollen availability (Byers 1995; Fischer and Matthies 1997). In *L. sibirica* populations, the influence of environment is most obvious in Anne where plants suffer from overly dry soil conditions, herbivores and competition with high-growing plant species (e.g., *Filipendula ulmaria*) which suppress the growth of *Ligularia* (K. Lanno, pers. obs.). Thus both environmental and genetic factors can explain the low reproductive success in this population.

Since it is not possible to disentangle the complex effect of genetic and various environmental factors, we cannot claim that genetic erosion is playing a major part in reproductive success in *L. sibirica* populations. However, the results of this study show that it could be one factor affecting plant fitness in smaller populations of this species.

Conservation implications

Populations of *L. sibirica* have suffered because of human-induced changes in their habitats. Therefore, there is a moral obligation to undo the harm. Reinforcement of populations by introducing new individuals could be one of the measures to support population recovery after the habitat has been restored. However, there are two different approaches to be considered. Firstly, as the isolated populations at the distribution margins are often genetically separated, one can argue that such distribution pattern should be preserved and considered as a natural value (Ehrlich 1988; Lesica and Allendorf 1995). In this case, plant material used for reinforcement must originate from the same population. On the other hand, if the population is genetically impoverished and inbreeding is one of the causes of lowered fitness, it may not be sufficient to overcome limitations caused by lack of genetic variability by using local plant material (Edmands 2007). In such cases reinforcement must be concentrated on introducing new genotypes (see Sackville Hamilton 2001 and Vander Mijnsbrugge et al. 2010 for further discussion).

Given that there were clear signs of isolation of populations of *L. sibirica* in this study we would argue for preserving the diversity of genetically different populations and

avoiding the mixing of genotypes from different populations. However, introducing new genotypes into the smaller populations may be unavoidable because of the possible link between small population size, small genetic diversity and lowered fitness revealed in this study. Based on our data, we suggest the introduction of new genotypes into *L. sibirica* populations in Estonia when population size is less than 100 genets and average gene diversity over loci is lower than 0.15. If one of these conditions is not met, only local plant material should be used in the population reinforcement.

Considerable isolation of populations of *L. sibirica* also imposes the need to conservationally treat them as isolated populations. This implies that we cannot handle the system as a metapopulation and in order to persist, each population individually must exceed the limit of a viable population size. We have detected both reduced genetic diversity as well as reduced plant fitness in smaller populations of this species. Thus, our data suggest that populations with less than 200 individuals need special attention as they may be prone to enter the extinction vortex.

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