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High clonal and low sexual reproduction in fragmented populations of *Astragalus arenarius* (Fabaceae) at the northern edge of its geographic range

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Populations of plant species at their geographic range limits often suffer from low levels of genetic diversity caused by fragmentation, genetic drift, restricted gene flow, inbreeding or vegetative reproduction. We assessed the reproduction mode, allozyme diversity, differentiation and spatial genotypic structure in four Estonian remnant subpopulations and three small and isolated Latvian populations of *Astragalus arenarius* (Fabaceae) at the northern edge of the species' range. Plants of all populations had a clear clonal structure with same multilocus genotypes (MLGs) growing sequentially and alternately with different MLGs. The number of MLGs among populations varied between 1 and 13, with the longest genets extending over 30 m. The populations were highly differentiated with no common MLGs shared. Sexual reproduction in the studied populations was severely reduced. The generative reproduction was modest and variable in different years. Our results show that the small fragmented populations of *A. arenarius* have a low genotypic richness. Extensive clonal propagation of the few surviving genets has evidently contributed to their persistence. Conservation measures for the endangered populations are discussed.

Introduction

As a result of habitat loss, many plant species at their geographical and ecological margins frequently grow in small and isolated populations. Such populations are subjected to temporarily variable extreme environmental conditions (Levin 1970, Pamilo & Savolainen 1999, Hardie & Hutchings 2010). Numerous studies have shown that small and isolated populations formed through habitat fragmentation frequently have reduced genetic diversity, causing lower long-term persistence in fluctuating and harsh environments (Ellstrand & Elam 1993, Young *et al*. 1996, Honnay & Jacquemyn 2007 and references therein).

Insect-pollinated perennials are especially endangered due to deficiency of pollinators, low availability of compatible mates, and reduced reproductive success (Glemin *et al*. 2008, Kolb 2008, Young & Pickup 2010). Such populations are under a serious extinction risk due

to decreased reproductive performance and offspring fitness caused by increased selfing, accompanied by increased individual homozygosity and decreased fecundity resulting from inbreeding depression (Buza *et al*. 2000, Keller & Waller 2002, García-Fernández *et al*. 2012). Therefore, species limited to few small populations at their range margins are often included in the regional Red Books, and their population ecology, demography and genetic diversity require detailed studies in order to elaborate appropriate conservation measures (Lesica & Allendorf 1995, Tigerstedt 1997, Petit *et al*. 1998, IUCN 2010).

We selected *Astragalus arenarius* (Fabaceae) for this study because the species reaches its northern distribution limit in Estonia and is currently known from only one location in south-central Estonia. The species is classified as endangered in the *Estonian Red List of Threatened Species* (cf. http://elurikkus.ut.ee/kirjeldus.php?id=19839&rank=70&id_puu=19839&rank_puu=70&lang=eng). It is naturally distributed throughout central and eastern Europe, extending to NW European Russia (Fig. 1), but is considered threatened and included in the national Red Books of Germany, Czech Republic, European Russia, Sweden and Ukraine (Smekalova *et al*. 2012). In Finland, north of Estonia, the species is considered a casual alien, found only before 1951 (Hämet-Ahti *et al*. 1998). In Sweden, the species grows only on calcareous sand-steppe fragments of the Skåne province in the southernmost part of the country (Tyler 2003). In Latvia, south of Estonia, the species has a scattered distribution in northern pine forests and western seaside dunes (Kuusk *et al*. 2003). *Astragalus arenarius* is a perennial insect-pollinated herb, growing in dry, sandy, pine woodlands, preferring gaps with an open sand layer (sand dunes, road verges, abandoned railways), and also in continental sandy or calcareous grasslands.

The aim of this study was to provide information on the reproduction mode, genetic diversity, spatial structure and differentiation of the populations of *A. arenarius* and to discuss measures for the conservation of the severely decreased Estonian population and other similar populations (e.g. in Latvia). Although we found no data about clonality of *A. arenarius* in the literature, we suppose that it may be a clonal

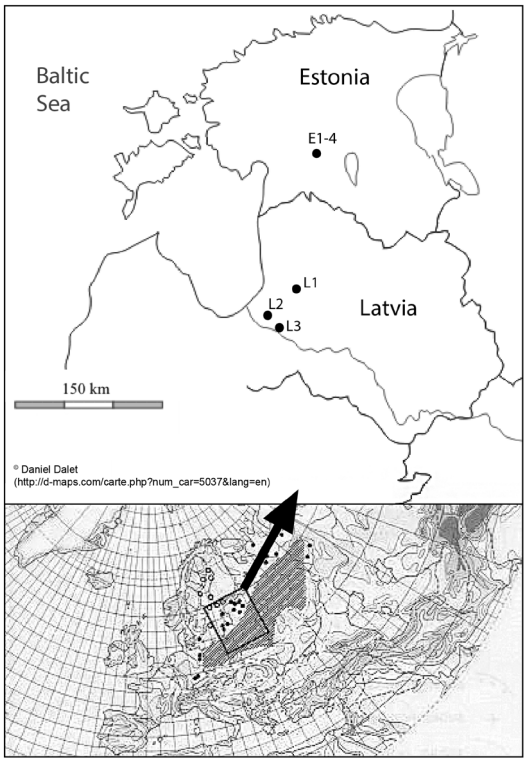

Fig. 1. Geographic distribution of *Astragalus arenarius* (Hultén & Fries 1986) and locations of populations studied in Estonia and Latvia.

species, given that up to about 85% of European forest perennial herbs are clonal and combine sexual and vegetative reproduction (Klimeš *et al*. 1997: fig. 7). We hypothesize that generative reproduction, genetic and genotypic diversity may be reduced in such small and isolated populations, especially in the presence of extensive clonal reproduction. We addressed the following specific questions: (1) Does *A. arenarius* reproduce clonally and, if it does, to what degree? (2) What is the extent of genetic diversity and differentiation among populations? (3) What are the implications for conservation of the critically decreased populations?

Material and methods

Study sites and sampling

The sole currently known Estonian population of *A. arenarius* is located in south-central Estonia

in the Soomaa National Park. This habitat was first described in 1936 within a 4 × 5 km area on three sand dunes formed on the coast of the post-glacial Baltic Ice Lake about 12 600–10 300 BP (Tamsalu 1940). *Astragalus arenarius* was abundant on bare sand or among heather under a sparse young pine forest growing on dunes after a forest fire in 1902 (Tamsalu 1940). This habitat is now an old-growth pine forest, and the ground is covered by a thick and continuous moss layer lacking *A. arenarius*. The only remaining habitats for *A. arenarius* are forest roadsides, where the plants are subjected to crushing by vehicles. The population has drastically decreased during 2006–2011, from 1936 ramets to 400 (our pers. obs.). The remaining population consists of four small stretches along four connected roads, designated as isolated local subpopulations E1, E2, E3 and E4 (Table 1), which are spatially separated from each other by 500–1500 m.

The studied Latvian populations are located in north-central Latvia (Fig. 1). L1 is a linear population along a forest roadside, L2 is a spreading population on an abandoned railway, and L3 is a small, nonlinear population along a roadside (Table 1). All three populations are small and isolated, growing on sandy habitat fragments in pine forest.

For allozyme analyses, young apical leaves from individual plants were collected sequentially by sampling all ramets in the populations in order to obtain data to characterize the expected clonality. Where it was possible to determine that ramets close to each other belonged to the same genet, we sampled only one of them. In Cekule (L2) the material was collected along two transects through the population.

Table 1. Sample sites and codes of the sites, type (shape) of *Astragalus arenarius* populations, size of populations and number of individual shoots in population.

Site	Code	Population		No. of shoots
		Type	Area	
Latvia				
Silciems	L1	linear	70 m	~100
Cekule	L2	nonlinear	30 × 15 m	~300
Ogre	L3	nonlinear	3 × 10 m	30
Estonia				
Ruunaraipe	E1	linear	17 m	32
Valgeraba	E2	linear	45 m	~60
Sauga	E3	linear	15 m	40
Miiliaugu	E4	linear	6 m	25

Allozyme analyses

Eight enzymes were preliminarily assayed. The following four enzymes displayed sufficient band resolution to allow for allozyme interpretation: aspartate aminotransferase (AAT, EC 2.6.1.1), superoxide dismutase (SOD, EC 1.15.1.1), phosphoglucoisomerase (PGI, EC 5.3.1.9), and leucine aminopeptidase (LAP, EC 3.4.11.1.). Phosphoglucomutase (PGM, EC 2.7.5.1), esterase (EST, EC 3.1.1.1.), 6-phosphogluconate dehydrogenase (6PGD, EC 1.1.1.44) and NADH dehydrogenase (stained as menadione reductase MNR, EC 1.6.99.3) showed either overly complex banding (EST) or poorly resolved faint bands that could not be interpreted genetically. Leaves collected from individual plants were homogenised in a 0.05 M Tris (hydroxymethyl) aminomethane (Tris)–0.01 M EDTA buffer containing 5 mM cysteine. After adding 20–50 mg of sucrose–Sephadex G200 mixture (4:1) to increase viscosity, the extracts were subjected to electrophoresis in vertical polyacryamide gel slabs (120 × 70 × 2 mm). The following three gel-buffer systems modified from Jaaska (1997) were applied to different enzymes to attain better band resolution:

Gel 1: 7.5% acrylamide, 0.3% *N*,*N*´-bisacrylamide (Bis), 0.125 M Tris, 0.1 M HCl; applied for AAT and PGI.
Gel 2: 10% acrylamide, 0.3% Bis, 0.125 M Tris, 0.25 M HCl; applied for SOD.
Gel 3: 10% acrylamide, 0.3% Bis, 0.125 M Tris, 0.1 M HCl; applied for LAP.

N,*N*,*N*´,*N*´-tetramethylethylenediamine (0.05 ml), 1 ml of riboflavine (0.5 mg ml^{-1}) and 1 ml of ammonium persulfate (1 mg ml^{-1}) were added per 100 ml of the gel mixture to initiate and catalyse polymerization between two daylight fluorescent bulbs over a period of 1 h. The upper catholyte consisted of 80 mM 2-alanine with 10 mM Tris. The lower anode buffer

was 0.1 M Tris with 0.02 M acetic acid, and it was used repeatedly while pH remained over 7. After electrophoresis, the gels were stained for isozymes by applying standard histochemical methods described by Wendel and Weeden (1989) with minor modifications. For LAP staining, gels were incubated for 20 min at 35 °C in 0.2 M maleate buffer of pH 5.6 containing 1 mg ml^{-1} L-leucyl-2-naphthylamide hydrochloride substrate, 4 mM $MnCl_2$ and 0.1% detergent Triton X-100, followed by adding about 10 mg of the diazo dye Fast Black K (Sigma) dissolved in 0.2 ml *N*,*N*-dimethyl formamide. Electrophoretic isozyme phenotypes (zymograms) were genetically interpreted as one-banded homozygotes and two- or three-banded heterozygotes, taking into account the known monomeric versus dimeric structures of enzymes (Wendel & Weeden 1989).

Genetic data analysis

Genetic diversity for each population at the genet level was assessed using the number of alleles per polymorphic locus (A_p), the effective number of alleles (A_e), Nei's unbiased estimate of expected heterozygosity (H_e) and observed heterozygosity (H_o) using the POPGENE 1.31 program (Yeh *et al*. 1999). Based on the number and frequency of alleles in each genotype, genetic diversity within populations was estimated with Shannon's diversity index (H'). To evaluate the distribution of genetic diversity among and within populations, analysis of molecular variance (AMOVA) (Excoffier *et al*. 1992) was computed with the program GENOTYPE/GENODIVE (Meirmans & Van Tienderen 2004). Genetic differentiation among the populations and subpopulations at the genet level was quantified by Nei's unbiased genetic distance (D_N) adjusted for small sample sizes (Nei 1978) and subjected to UPGMA (unweighted pair-group method) using the TFPGA software ver. 1.3 (Miller 1997).

For the quantitative characterization of clonal diversity in populations and subpopulations, we determined genotypic (clonal) richness (R) as $R = (G - 1)/(N - 1)$, where G is the number of genotypes and N is the number of sampled ramets in a population (Dorken & Eckert 2001). Each distinct multilocus genotype (MLG), based on the four polymorphic isozyme loci, is assumed to correspond to a separate clone. Simpson diversity index (D) as a measure of clonal diversity, the effective number of genotypes (G_e) and the evenness of the effective number of genotypes (E) were calculated with GENOTYPE/GENODIVE. D values range from zero in a population composed of a single genotype to one in a population in which each individual sampled represents a different genotype. Genotypic evenness E ranges from zero in populations dominated by only few genotypes to one in populations with an even distribution of clones.

Results

Genetic diversity and differentiation

Six isozyme loci of four enzymes, *Aat-1*, *Aat-2*, *Lap-1*, *Pgi-2, Pgi-3* and *Sod-1* produced PAGE phenotypes (zymograms) with distinct bands that enabled their genetic interpretation. In total, the data were evaluated for 185 plants from seven populations belonging to 29 genets. Five of the six loci were polymorphic; only *Pgi-3* was monomorphic in all populations. Genetic diversity estimates (Table 2) varied considerably in the Estonian and Latvian populations. The mean numbers of alleles per polymorphic locus were 2.7 and 2.2 in Latvian and Estonian populations, respectively. Mean heterozygosity for all loci in the populations was 0.35. Notably, L3, consisting of only one genet, exhibited even higher H_o (0.33) than L1 with 13 genets (H_o = 0.28), indicating a relatively high heterozygosity of the sole surviving genet of this population. By contrast, the Estonian monoclonal E4 had a much lower H_o (0.17) than E1 and E2, with five and four genets, respectively. The unique genets of the monoclonal populations L3 and E4 differed thus about twice in the individual heterozygosity, with H_o values 0.33 and 0.17, respectively.

Genetic diversity expressed by Shannon's diversity index (H') was highest in L1 (H' = 0.58) and lowest in E4 (H' = 0.12) (Table 2). Similar results were obtained from the H_e data, which assume that populations are in the Hardy-Weinberg (HW) equilibrium of random mating.

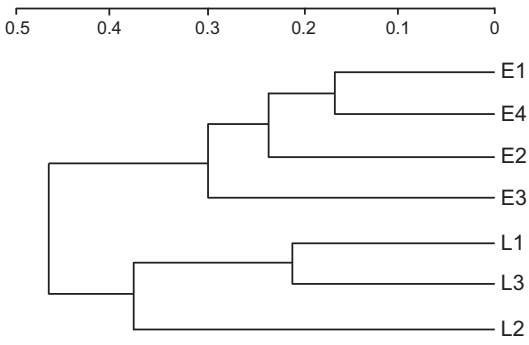

Fig. 2. UPGMA dendrogram of genetic differentiation between the Estonian subpopulations and Latvian populations of *Astragalus arenarius* based on Nei's unbiased genetic distances.

A comparison of H_o and H_e, however, showed considerable deviation from the theoretical equilibrium in most populations towards deficiency or excess of heterozygotes, caused presumably by high clonality with a low number of genets with different individual heterozygosities in the populations. H_o thus provided a real measure of genetic diversity in the studied non-equilibrium populations, whereas H_e and H' reflect expectations for theoretical cases in the HW equilibrium.

The UPGMA tree based on Nei's unbiased genetic distance D_N (Fig. 2), which characterizes genetic differentiation and partition of variation among populations, distinguished Estonian and Latvian populations into separate groups at mean $D_N = 0.466$, extending to 0.737 between E4 and L3. The mean D_N for the Latvian populations was 0.345 (range 0.206–0.375) and for the Estonian subpopulations 0.213 (range 0.160–0.309).

AMOVA based on Euclidean distances (Excoffier *et al*. 1992) indicated that in the Latvian populations, the genetic variation among populations (52%) was slightly greater than within populations. The genetic variance among Estonian subpopulations was slightly less than within the studied populations, with 40% of diversity partitioned among the four subpopulations (Table 3).

Clonal structure and diversity

Clonal richness, diversity and evenness of the four Estonian subpopulations and three Latvian populations were clear but variable (Table 4). A total of 29 genotypes were detected among the 185 ramets recorded in the populations. Three Estonian subpopulations and one Latvian population (L2) consisted of 2–5 genets. One Latvian population (L3) and one Estonian subpopulation (E4) had only one MLG (clone), suggesting growth exclusively by vegetative propagation. The greatest number of genets (13) and highest genotypic richness ($R = 0.30$) was recorded in the Latvian population L1. No genet was shared between localities, indicating extremely high genotypic differentiation that could be evaluated by the used polymorphic isozymes.

The spatial aggregation of ramets with the same isozyme MLGs indicates their belonging

Table 2. Genetic diversity of the populations of *Astragalus arenarius* based on polymorphic loci at the genet level. G = number of genets detected, A_p = number of alleles per polymorphic locus, A_e = effective number of alleles, H' = Shannon's index, H_o = observed heterozygosity, H_e = expected heterozygosity assuming random mating, F = Wright's fixation index, * = deviation from Hardy-Weinberg equilibrium at $p < 0.05$.

Population	G	A_p	A_e	H'	H_o	H_e	F
L1	13	2.3	1.7	0.58	0.28	0.36	0.22*
L2	3	1.8	1.7	0.50	0.39	0.32	–0.21*
L3	1	1.3	1.3	0.23	0.33	0.17	–0.94*
Latvia	17	2.7	1.9	0.70	0.30	0.42	
E1	5	2.0	1.7	0.54	0.40	0.35	–0.14*
E2	4	1.8	1.7	0.55	0.63	0.39	–0.62*
E3	2	1.7	1.5	0.40	0.25	0.27	0.07*
E4	1	1.2	1.2	0.12	0.17	0.08	–1.13*
Estonia	12	2.2	1.9	0.66	0.43	0.43	
Mean		1.7	1.5	0.42	0.35	0.28	
Estonia + Latvia		2.7	2.2	0.78	0.36	0.47	

to the same clone (Rhebergen *et al*. 1988). We indeed revealed that identical MLGs were spaced sequentially along all linear roadside populations (Table 5), suggesting their origin through clonal growth. The genet lengths varied widely: the longest genet spanned linearly over 30 m, and genets consisted of 1–40 ramets. The distance between ramets of the same genet varied mostly between 0.2 and 1.9 m, but the longest gap was 7.5 m for MLG L2-1 in transect II (L2; Table 5). Remarkably, only few unique genets were recorded intermingled among or between larger clonal genets (unique E1-4, E1-5, E2-2, L1-3, L1-9, L1-10 and L2-2, Table 5). We suppose that unique MLGs reflect genets which are derived through sexual reproduction from seeds, whereas adjacent ramets with two identical MLGs (E1-2, L1-7 and L1-11; Table 5) belong to a small genet that spreads clonally. The observed spatial distribution with only one to three unique MLGs per population indicates very limited sexual reproduction from seeds and extended vegetative reproduction of one or more genets consisting of multiple ramets. The four largest genets of linear road verge populations, E2-1, E2-3, L3-1 and E4-1, are composed of at least 17, 11, 22 and 14 ramets, respectively (Tables 4 and 5).

Discussion

Genetic diversity and differentiation

We descibed the allozyme variability of *A. arenarius* from four Estonian subpopulations and three Latvian populations. Although the genetic diversity of several *Astragalus* species has been studied (Alexander *et al*. 2004, Baskauf & Burke 2009, Breinholt *et al*. 2009, Vicente *et al*. 2011), there are no data on the genetic structure of and variability within populations of *A. arenarius*. Despite the small and fragmented populations, *A. arenarius* was shown to have high genetic diversity. That diversity of the presum-

Table 3. Analysis of molecular variance (AMOVA) based on allele frequency data among 29 genets of *Astragalus arenarius* found in Latvian and Estonian populations.

	Sum of squares	df	Variance components	Percentage of total variance	*p*
Latvia					
Within populations	89.46	104	0.86	48.3	< 0.001
Among populations	128.13	2	0.92	51.7	< 0.001
Estonia					
Within populations	63.31	84	0.75	59.8	< 0.001
Among populations	99.43	3	0.51	40.2	< 0.001
Latvia + Estonia					
Within populations	152.77	188	0.81	73.2	< 0.001
Among populations	296.35	6	0.30	26.8	< 0.001

Table 4. Indices of clonal diversity. G_e = effective number of genotypes, E = evenness, D = Simpson index of diversity, R = genotypic richness.

Population	Sample size	Number of genotypes	G_e	E	D	R
L1	41	13	9.5	0.73	0.92	0.30
L2	44	3	1.2	0.38	0.13	0.05
L3	22	1	1.0	1.00	0	0
E1	17	5	3.0	0.61	0.71	0.25
E2	30	4	2.2	0.55	0.56	0.10
E3	17	2	1.6	0.81	0.40	0.06
E4	14	1	1.0	1.00	0	0
Mean		4.14	2.79	0.73	0.39	0.11

ably clonal *A. arenarius* is attributed to the long generation time and dominant clonal growth of highly heterozygous genets. Persistence of high genetic diversity in small forest-plant populations in changing landscapes has been described by other authors (Honnay *et al*. 2005). Izquierdo and Piñero (2000) found high genetic diversity in endemic *Aechmea tuitensis* despite its

Table 5. Spatial distribution of genets and ramets in four populations of *Astragalus arenarius*. In E1, E2 and L1 individuals along the linear populations are mapped. In L2 individuals from two transects (separated by 3 m) through the populations are mapped. Genets are indicated by numbers. *D* = distances, *n* = number of individuals. Genets consisting of only one ramet are set in boldface.

E1	*D* (m)	E2	*D* (m)	L1	*D* (m)	L2	*D* (m)
E1-1		E2-1		L1-1		L2-1	Transect I
E1-1	0.37	E2-1	1.37	L1-1	0.60	L2-1	1.00
E1-1	0.20	E2-1	0.40	L1-1	0.30	L2-1	0.33
E1-1	0.21	E2-1	0.27	L1-1	0.45	L2-1	0.30
E1-2	0.60	E2-1	0.80	L1-2	0.72	L2-1	2.10
E1-1	0.42	E2-1	3.70	L1-1	0.80	L2-1	0.30
E1-2	0.47	E2-1	0.30	L1-2	1.07	L2-1	0.40
E1-3	10.00	E2-1	0.15	L1-2	0.50	L2-1	3.00
E1-4	0.77	E2-1	0.35	L1-2	0.48	**L2-2**	0.25
E1-3	0.55	E2-1	0.90	L1-2	0.20	L2-3	0.50
E1-3	0.30	E2-1	0.43	L1-2	0.90	L2-3	1.00
E1-3	0.45	E2-1	1.40	L1-2	0.72	L2-3	3.60
E1-3	0.33	E2-1	0.95	**L1-3**	0.15	L2-3	0.20
E1-5	0.57	E2-1	0.70	L1-4	20.0	L2-3	0.44
E1-3	0.80	E2-1	0.47	L1-4	0.55	L2-1	0.60
E1-3	0.45	E2-1	1.40	L1-4	0.20	L2-1	0.25
E1-3	0.35	E2-1	4.75	L1-4	0.60	L2-1	2.50
		E2-2	4.32	L1-4	0.34	L2-1	0.40
n = 17		E2-3	5.95	L1-5	2.20	L2-1	0.38
		E2-3	2.30	L1-5	1.55	L2-1	0.26
		E2-3	0.76	L1-5	0.60	L2-1	Transect II
		E2-3	0.30	L1-6	1.07	L2-1	2.00
		E2-3	0.60	L1-6	2.45	L2-1	3.00
		E2-3	1.90	L1-6	0.20	L2-1	0.17
		E2-3	0.70	L1-6	0.54	L2-1	1.00
		E2-3	1.95	L1-6	0.17	L2-1	0.16
		E2-3	1.83	L1-6	0.65	L2-1	0.35
		E2-3	0.85	L1-7	10.00	L2-1	0.18
		E2-3	0.43	L1-7	1.27	L2-1	7.50
		E2-4	1.45	L1-8	0.40	L2-1	0.17
		n = 30		L1-8	1.10	L2-1	0.40
				L1-8	0.77	L2-1	0.27
				L1-9	2.95	L2-1	0.30
				L1-10	2.50	L2-1	0.25
				L1-11	9.00	L2-1	0.21
				L1-11	0.70	L2-1	0.27
				L1-12	18.00	L2-1	0.29
				L1-12	1.40	L2-1	0.29
				L1-12	2.50	L2-1	3.20
				L1-13	73.00	L2-1	0.39
				L1-13	6.00	L2-1	0.44
				n = 41		L2-1	0.21
						L2-1	0.54
						L2-1	0.42
						n = 44	

narrow geographic distribution, suggesting that vegetative reproduction might preserve genotypes. Genetic diversity among populations was slightly lower among Estonian subpopulations (40%) than among Latvian populations (52%). This trend is not unexpected as the four remnant subpopulations in Estonia are small, isolated fragments of a former larger population. However, the differentiation between the Estonian subpopulations is still remarkably high, with H_o ranging between 0.160–0.309 and with no shared genets, which means that genetic diversity with unique MLGs in all subpopulations has high conservation value.

Clonal structure and diversity

Our study is the first to characterize the clonal diversity, spatial structure and clone size variation in populations of *A. arenarius*. The results indicate that the populations studied are maintained mostly by extensive clonal growth with mostly low, but remarkably variable clonal richness and a high level of isozyme genetic divergence between populations and local subpopulations. The level of genotypic diversity *D* and richness *R* were highly variable in different populations (D = 0–0.9, R = 0–0.3). The occurrence of genets extending up to 30 m in length means that *A. arenarius* is a guerilla-type clonal species (Lovett-Doust 1981), able to spread extensively in favourable conditions. However, the presence of genets with highly variable sizes indicates that the clonal growth may depend on the heterogeneity of local soil conditions.

Extensive clonal growth causes reduced genetic diversity with a limited number of genets in small populations, leading to increased homozygosity through geitonogamous self-pollination and a low fecundity of seed progeny due to inbreeding depression (Handel 1985, Eckert 2000, Charpentier 2002). The observed consecutive placement of ramets with the same MLG in long, linear segments along narrow road verges will certainly facilitate geitonogamous self-pollination within the same clone. Furthermore, many studies have indicated decreased generative reproduction and dominant clonality in northern edge populations (e.g. Dorken & Eckert 2001, Broyles 1998), as we found for the sole Estonian remnant population of *A. arenarius*. Moreover, one of the three Latvian populations and one Estonian subpopulation consisted entirely of a single MLG, reflecting only clonal reproduction. We suppose that the harmful effects of traffic on plants and soil along the road verges will cause poor seedling recruitment and establishment of adult plants from the seed progeny, which will limit further genetic diversity, population growth and viability in worsening conditions.

According to the IUCN guidelines (IUCN 2010), population size is measured as numbers of mature individuals. However, it should be emphasized that in clonal species counting adult stems (ramets) leads to overestimation of actual population sizes that should be measured by numbers of genetic individuals (genets) estimated with the use of appropriate molecular markers (Tepedino 2012 and references therein). The results of the current study exemplify this by showing that population sizes estimated by the number of MLGs are much lower than the number of adult shoots sampled.

Conservation implications

The Estonian population of *A. arenarius* is suitable for study because its habitat was described in detail when it was discovered about 75 years ago (Tamsalu 1940) and thus allows inference on changes over time. The monitoring data illustrate drastic changes in the population size and habitat conditions over time through natural successional changes in the habitat vegetation. The area is currently covered by an old-grown pine forest with a dense bryophyte understory without *A. arenarius*. The population has diminished to four small, isolated subpopulations limited to narrow stretches along verges of four connected forest roads at the southern edge of the former area. It is evident that natural succession of the pine forest has destroyed the native habitat needed for the survival of *A. arenarius*. The Estonian population of *A. arenarius* is thus a vivid example that mere presence in a Nature Reserve is inadequate for the maintenance of a plant species in its native habitat; special protection methods should be applied to preserve specific habitats

for viable populations of locally rare and endangered plant species.

Our results allow proposing management strategy needed to protect the population from extinction. Rapid decline in the number of population fragments and modest generative reproduction diminish the persistence of the sole population of *A. arenarius* in Estonia. In particular, forest roadsides as the only remaining habitat for the Estonian and for many Latvian populations of *A. arenarius* are unfavorable for sexual reproduction by seeds even in favorable years because of the trampling of soil, which results in damaged seedlings.

The information on the genetic diversity and its spatial distribution we provided is critically important to elaborate proper conservation methods. Our study revealed that the mostly vegetatively reproducing subpopulations and populations of *A. arenarius* are highly differentiated with no shared MLGs. The pronounced genetic differentiation suggests that they all should be protected. IUCN has also suggested that isolated subpopulations should be included in the Red List categorization system at the regional level (IUCN 2010).

Restoring habitat conditions for sexual reproduction of the insect-pollinated, outcrossing *A. arenarius* is a significant prerequisite to ensure the increase and maintenance of genetic diversity in the seed progeny by forming genotypes with new allelic combinations. The most effective way to regenerate a vital population would be to restore a suitable habitat and to transplant young plants grown from seeds collected from all four remaining subpopulations to combine the remaining genetic diversity. The removal of the moss layer at the selected restoration place is an important measure, but is insufficient to allow good germination, because the soil contains compounds excreted by mosses that are toxic to seedlings (Soudzilovskaia *et al*. 2011). In addition, moss spores allow faster recovery of a moss layer than seedling establishment, as shown by several studies of grassland species (e.g. Jeschke & Kiehl 2008, Soudzilovskaia *et al*. 2011 and references therein). Therefore, a recultivation of the soil layer is also suggested. Transplantation of young plants to restored habitat sites was found to be far more effective than sowing seeds (e.g. Reckinger *et al*. 2010). Given that the four remnant Estonian subpopulations contain few genets (each only 1–5; *see* Table 2) that are morphologically undistinguishable in the wild without laboratory analyses, transplantation of progeny generated from seeds would be preferable. Seedling recruitment of *A. arenarius* in nature is practically nonexistent, seeds from all subpopulations should be collected and germinated in the laboratory and transplanted into an appropriately restored habitat. It should be stressed that even with successful population restoration by transplanting young plants to a restored habitat, periodical removal of the renewing moss layer and forest would be needed in order to avoid the reoccurrence of habitat destruction and population decay through natural successional processes.

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