The potential genetic consequences of seed mixtures

Tsipe Aavik[1], Peter Edwards[2], Rolf Holderegger[1], Regula Billeter[2]

[1] Swiss Federal Research Institute WSL, Zürcherstrasse 111, CH-8903 Birmensdorf
[2] Institute for Integrative Biology, ETH Zürich

Scientific summary

Sowing with commercial wildflower seed mixtures is a common restoration practice in areas with impoverished species pools. The potential genetic consequences of using seed mixtures, however, are poorly understood and often not considered in practical restoration. One of the key objectives of such restoration measures is to enhance connectivity among isolated natural and semi-natural habitats, e. g. by establishing new habitat patches and (sown) populations. Nevertheless, improvement to functional habitat connectivity is rarely assessed when evaluating restoration success. Furthermore, the impact of landscape structure on habitat connectivity is seldom examined.

Our study area was located in an intensively managed agricultural landscape in the Oberaargau area, Switzerland. A few years before our study took place, several extensively managed grasslands were recreated in this landscape. Restoration activities included sowing with commercially produced wildflower seed mixtures. We studied the genetic diversity and structure of nine sown and 17 naturally occurring populations of *Lychnis flos-cuculi*, which is an insect-pollinated grassland species. We found that sown and natural populations had similar gene diversity and allelic richness. Inbreeding, by contrast, was significantly higher in sown populations. Source populations, where the seeds for propagation in seed companies were collected, may have been small and/or inbred resulting in higher inbreeding in sown populations. Inbreeding can also be caused by repeated regeneration of the same seed stock over several cycles at the seed company. We also found that sown populations were genetically very distinct from natural ones despite the fact that source populations of sown plants originated from the same seed zone as the restored sites. These results suggest that seeds for propagation should be collected from numerous individuals in large and non-isolated populations nearby restored sites. Stocks for the production of seed mixtures should only be propagated for a small number of generations to avoid negative effects such as inbreeding and loss of local adaptation.

To study the effect of provenance, genetic diversity and composition on plant fitness, we measured the fitness of the study populations of *L. flos-cuculi*. In addition, we established an experiment in the study area as well as in an experimental garden in Zürich by sowing seeds originating from natural populations, sown populations and seed companies. We recorded the establishment, survival and fitness of the experimental plants. We detected no significant effect of genetic diversity on the fitness of study plants, which suggests that neutral genetic diversity examined in the present study may not have a direct relationship with the adaptive genetic variation.

In order to evaluate functional connectivity among restored and remnant grasslands, we examined contemporary gene flow patterns of *L. flos-cuculi* using assignment tests and first-generation migrant tests. Assignment tests, which reflect gene flow during several generations, revealed high gene flow among the natural populations of *L. flos-cuculi*. By contrast, little gene flow occurred between sown and natural populations. Furthermore, we detected only a few first-generation migrants among sown and natural populations as well as among natural populations, which indicates insufficient spatial connectivity of extensively managed grasslands in this landscape. Alternatively, gene flow occurred more often than detected, but lower adaptation of sown genotypes to local environmental conditions or higher inbreeding observed in these populations could have impeded establishment.

Additionally, we examined the effects of Euclidian distance, cumulative elevation change and the proportion of various landscape elements (forest, settlements, ditch verges, agricultural land) in corridors (cor-

ridor width of 50, 100, 200, 300, 500, 1000 m) between the natural populations of *L. flos-cuculi* on gene flow among those populations. Only forest had a significant positive effect on the genetic differentiation F_{ST}. Forest inhibits gene flow among the study populations most probably through hampering the movement of pollinators.

The findings of the current study are of high significance for nature conservation and ecological restoration. In particular, we suggest that more attention should be paid to the genetic quality of seed mixtures used in ecological habitat restoration. In addition, the evaluation of restoration measures should include assessing the improvement to connectivity of habitats. Our findings suggest that enhancing spatial connectivity through restoration measures does not necessarily increase functional connectivity in short term.

State of the art (pre-ENHANCE)

Sowing with commercial seed mixtures has become a common practice for restoring species diversity in areas with impoverished species pools (Kiehl *et al.* 2010). In spite of the increasingly broad application of this measure all over Europe, the genetic composition and diversity of plant populations originating from commercial seed mixtures has received practically no attention. Furthermore, the potential influence of gene exchange between sown and local natural populations on the genetic properties and subsequent fitness of species is largely unknown. If seeds from seed mixtures are characterised by lower genetic diversity, which is often related to lower fitness in case of self-incompatible species (Leimu *et al.* 2006), then sowing commercial seed mixtures in large quantities may become detrimental to natural populations by “polluting” the natural gene pool with genotypes exhibiting lower fitness. Gene exchange among plants from seed mixtures and local populations may result in outbreeding depression, which can also have negative consequences for plant fitness (Montalvo and Ellstrand 2001). Additionally, seeds of non-local origin may be poorly adapted to the environmental conditions at the restoration site, and therefore exhibit significantly lower fitness compared to local natural populations (Bischoff *et al.* 2006). Furthermore, the *ex-situ* propagation of seeds in gardens favours genotypes that are well suited to garden conditions but not necessarily to those in the restored habitat (Ensslin *et al.* 2011). To decrease the negative effects of using unsuitable seeds, conservation biologists have suggested sowing seeds, which originate from the same seed zone as the restored site (Vander Mijnsbrugge *et al.* 2010). However, seed zones are mostly defined on the basis of climatic and bio-geographical data, while there is no information on how these zones correspond to natural patterns of genetic variation (Kramer and Havens 2009). There is also insufficient data about the effects of repeated propagation on the genetic variation of seed stocks.

The success of ecological restoration measures is often judged by the increase in the number and abundance of plant species. In practical conservation, the area and spatial connectivity of habitats are frequently considered as indicators of restoration success. However, there is limited knowledge, how restoration measures influence functional connectivity among plant populations. Direct estimation of functional connectivity of plants (e.g. by tracking the movement of seeds or pollen) is complicated, and can only be done for certain plant species (Sork 1984; Van Geert *et al.* 2010). Furthermore, ecological methods may strongly misjudge the extent and amount of seed and pollen flow (Kamm *et al.* 2009). Genetic methods offer an alternative means of estimating functional connectivity by assessing historical as well as contemporary gene flow (Lowe and Allendorf 2010). Regrettably, genetic methods have rarely been applied for studying the functional connectivity of plants in response to restoration measures.

Genetic connectivity among plant populations is mostly predicted as a function of Euclidian distance between populations (Honnay *et al.* 2007; Jacquemyn *et al.* 2007; Mix *et al.* 2006). However, genetic structuring may also be influenced by landscape characteristics between populations and not by geographic distance alone (Holderegger and Wagner 2008). Landscape genetic approaches, which combine the methods of population genetics and landscape ecology, have increasingly been applied to study the effect of landscape properties on the dispersal of individuals. However, most landscape genetic studies are focused on vertebrata, while only about 15 % of studies deal with plants (Holderegger *et al.* 2010; Storfer *et al.* 2010).

Motivation and research questions for the project

Little is known about the genetic diversity and composition of commercially produced seed mixtures used in ecological restoration. Therefore, our first aim was to examine whether natural and sown populations of the study species differ in regard to their genetic characteristics. In particular, we wanted to know if there are any differences in the genetic diversity (measured as gene diversity, allelic richness, observed heterozygosity, inbreeding) and structure of sown and natural populations. Because genetic diversity may have an effect on the fitness of plants, we also examined the relationship between the genetic properties and plant fitness of sown and naturally occurring populations.

The assessment of restoration effectiveness does usually not encompass evaluating the improvements to functional connectivity. Our study system of sown and natural plant populations enabled us to examine whether and how much gene flow occurred among restored and natural populations since restoration. In one of the two study regions, half of the populations had been restored by sowing wildflower seed mixtures eight years before the study; in the other region, two populations had been sown three years prior to our sampling, while most populations were of natural origin. Thus we could also compare the effect of age and amount of restored populations on gene flow.

Few studies have examined the effect of landscape structure on the gene flow among plant populations, whilst most studies concentrate on animals. In the present study, we firstly examined how much the spatial connectivity of habitats (measured by geographic distance) does mirror the functional connectivity among populations (measured as genetic differentiation F_{ST}). Secondly, we studied the effect of various landscape elements on gene flow among the study populations.

Technical issues: material, methods and sampling

Study species

Lychnis flos-cuculi L. (*Silene flos-cuculi*; Caryophyllaceae) is a diploid polycarpic perennial herb (Fig. 1). It grows in moist, open habitats such as floodplains and calcareous fen meadows, and is distributed throughout Europe (Jalas 1986). *L. flos-cuculi* is an insect-pollinated species visited by a wide range of pollinators: Lepidoptera, Diptera and Hymenoptera (Van Rossum and Triest 2010). The species is self-compatible, but the protandrous flowers of *L. flos-cuculi* are predominantly outcrossed (Biere 1991). In addition to sexual reproduction, *L. flos-cuculi* forms vegetative rosettes from axillary stem buds. Plants overwinter as rosettes. In the second year, they produce stems which are 30–90 cm tall and flower between April and June. Ripe capsules contain an average of about 150 seeds. Seeds are released by vibrations of the stiffened stalks (Biere 1991).

Study populations and fieldwork

The study was carried out in an intensively managed agricultural landscape located in the Cantons of Bern and Aargau in Switzerland (Fig. 2). As part of a restoration programme, several new streams were created in the area between 2001 and 2003. The verges of these watercourses were sown with standard wildflower seed mixtures developed for extensively managed meadows or wet meadows, containing the study species *L. flos-cuculi* (UFA seed company, Winterthur). In 2006–2007, a few wet and mesotrophic grasslands were restored using the same type of seed mixtures. Most of these sown areas were managed as ecological compensation areas: no fertilizers were used and mowing took place once per year after June 15. In addition to nine sown populations of *L. flos-cuculi*, we found 17 naturally occurring populations of *L. flos-cuculi* in the study area (Tab 1).

In large populations, we sampled leaves from approximately 30 individuals for genetic analysis (Table 1). However, in small populations, fewer individuals could be sampled. Most of the populations at ditch verges and field margins were long and narrow. To take into account the spatial structure of these linear populations, we divided them into sectors of approximately 70–100 m. Within each sector, we collected the leaves of about 30 individuals, with a distance of approximately 2–3 meters between sampled plants.

(a) (b)

Fig. 1. The study species Lychnis flos-cuculi (a) and one of the restored habitats in the study area (b) hosting a sown population of *L. flos-cuculi*.

The largest linear population comprised 13 sectors (238 individuals were sampled). In total, 1413 individuals of *L. flos-cuculi* were sampled (813 in natural populations and 600 in sown populations). In each study population, soil samples were collected to estimate soil nitrogen and phosphor content. In addition, soil moisture was measured using the HydroSense Soil Water Measurement Systems CD620 (Campbell Scientific).

In order to study the effect of origin (sown, natural) and genetic characteristics on the fitness of study populations, we measured various fitness traits of approximately 30 randomly selected reproductive individuals in 20 of the study populations of *L. flos-cuculi* in spring 2011. We counted the number of flowers and stalks per plant and measured plant height. Ripe seeds were collected from 30 randomly selected individuals in each population. A hundred seeds per individual were weighed. Fifty seeds of 15 individuals per population were placed on filter paper in Petri dishes (in total 300 Petri dishes). Petri dishes were placed in a greenhouse at 20 °C with 16 hours of light and were regularly watered with tap water. The number of germinated seeds was counted after 30 days.

We established an experiment in the study area and in an experimental garden to examine the effect of provenance and genetic characteristics on plant fitness. The seeds from two natural populations (Natural 13, Natural 23) and two sown populations (Sown 1, Sown 3) were collected in summer of 2010 for the experiment. In addition, seeds of *L. flos-cuculi* were ordered from two seed companies in Switzerland (UFA seed company, CH-Wildblumen). In September 2010, the experiment was set up at two sites in the study area and in the experimental garden at ETH Hönggerberg in Zürich. In all sites, four blocks with the size of 2 x 3 m were established. Within each block, six plots with the size of 0.75 x 0.75 m were created by removing the above- and below-ground vegetation. In the experimental garden, 24 pots with the size of 0.74 x 0.56 x 0.37 m were filled with a mixture of soil and sand and were covered with a 10-cm layer of humus on top. At the beginning of October 2010, we sowed 200 seeds in each plot/pot. Every block contained a plot with seeds of different origin: two sown populations, two natural populations and two plots with seeds originating from two seed companies. At the end of May 2011, seedlings were counted and harvested so that in every plot ten seedlings remained (when possible). At the beginning of July, August and September, we recorded survival and measured the fitness of experimental plants (plant diameter, length of the longest leaf, height, number of stalks, number of flowers). At the beginning of September we collected the above-ground parts of plants for biomass measurements. Biomass samples were weighed after drying in an oven at 70 °C for 48–72 hours.

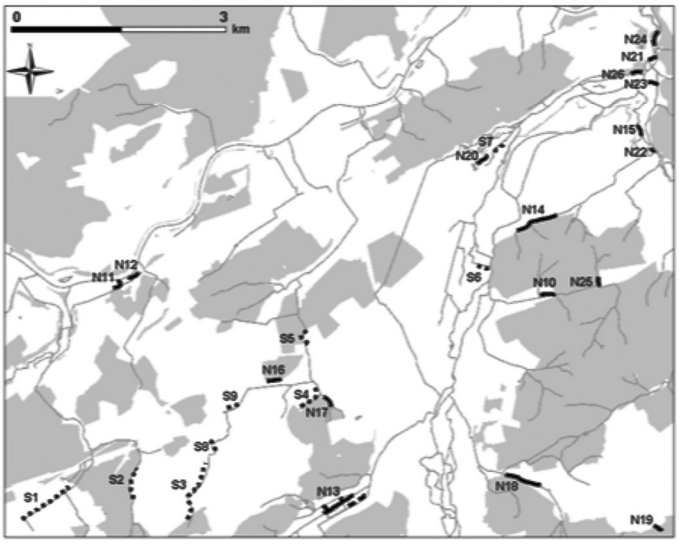


Fig. 2. Black continuous lines denote the locations of natural populations and black dashed lines the locations of sown populations of *Lychnis flos-cuculi.* Grey lines indicate ditches and other water bodies with flowing water; grey polygons show forested areas.

Genetic analysis

Plant material collected for genetic analysis was dried and kept in silica gel until used. DNA was extracted from 10 mg of dry leaf material using the Dneasy 96 Plant Kit (QIAGEN). We used three microsatellite markers developed for *L. flos-cuculi*: Cuculi 4, Cuculi 17, Cuculi 19 (Galeuchet *et al.* 2002), and, in addition, a selection of primers developed for *Silene latifolia* (Caryophyllacea; Moccia *et al.* 2009), a close relative of *L. flos-cuculi*: SL_eSSR13, SL_eSSR17 and SL_eSSR49. Polymerase chain reactions (PCR) were carried out as described in Aavik *et al.* (2012). PCR products were analyzed on an ABI 3730 automated sequencer (Applied Biosystems) using 400 ROXä size standard. Allele lengths were visualized and scored using GENEMAPPER 3.7 (Applied Biosystems).

Table 1. Origin (sown, natural), age, coordinates (E, N), population size, sample size, allelic richness (AR), gene diversity (HE), observed heterozygosity (HO) and inbreeding coefficient (FIS) of the study populations of Lychnis flos-cuculi in two study regions.

Location	Genetic population	Population age (years)	E	N	Pop. size	Sample size	A_R	H_E	H_O	F_{IS}
Region 1										
Sown 1	Sown I	8	621167	227643	1360	176	5.12	0.66	0.55	0.110
Sown 2	Sown I	8	622172	227874	2050	60	5.23	0.68	0.58	0.142
Sown 3	Sown I	8	623210	227882	1040	123	6	0.7	0.62	0.121
Sown 4	Sown I	8	624749	229019	600	75	5.46	0.68	0.58	0.119
Sown 5	Sown I	8	624628	229915	170	45	5.19	0.67	0.56	0.175
Sown 8	Sown II	8	623342	228322	2000	30	5.75	π0.7	0.65	0.068
Sown 9	Sown II	8	623570	228875	500	30	5.25	0.65	0.53	0.173
Natural 11	Natural 11	Natural	621919	230642	100	30	5.23	0.66	0.62	0.056
Natural 12	Natural 12	Natural	622202	230752	100	30	5.34	0.7	0.717	-0.026
Natural 13	Natural 13	Natural	625045	227516	4300	238	5.73	0.69	0.62	0.078
Natural 16	Natural 16	Natural	624406	229357	20	20	6	0.68	0.64	0.062
Natural 17	Natural 17	Natural	624908	229044	150	30	5.64	0.67	0.57	0.140
Natural 18	Natural 18	Natural	627475	227938	1170	89	5.22	0.66	0.63	0.063
Natural 19	Natural 19	Natural	629958	227380	15	15	3.26	0.51	0.35	0.309

Location	Genetic population	Population age (years)	E	N	Pop. size	Sample size	A_R	H_E	H_O	F_{IS}
Region 2										
Sown 6	Sown I	3	627183	230936	12	12	5.33	0.64	0.53	0.176
Sown 7	Sown I	3	627331	232740	300	49	6.10	0.71	0.65	0.067
Natural 10	Natural 10	Natural	628095	230481	430	61	5.79	0.66	0.66	0.026
Natural 14	Natural 14	Natural	627753	231501	650	60	5.31	0.69	0.63	0.058
Natural 15	Natural 15	Natural	629374	232675	100	30	5.02	0.66	0.62	0.056
Natural 20	Natural 20	Natural	627154	232378	400	59	5.38	0.67	0.64	0.054
Natural 21	Natural 21	Natural	629437	233871	260	31	4.14	0.63	0.64	-0.033
Natural 22	Natural 22	Natural	629526	232632	15	13	4.75	0.63	0.63	0.003
Natural 23	Natural 23	Natural	629581	233562	500	29	5.34	0.71	0.71	-0.002
Natural 24	Natural 24	Natural	629581	234200	70	30	5.74	0.69	0.65	0.063
Natural 25	Natural 25	Natural	628795	230672	100	17	4.04	0.57	0.55	0.041
Natural 26	Natural 26	Natural	629306	233697	150	31	5.12	0.71	0.72	-0.008

Data analyses

We calculated allelic richness A_R, gene diversity H_E, inbreeding coefficient F_{IS} and observed heterozygosity H_O of each study population using FSTAT 2.9.3.2 (Goudet 1995). The differences in H_E, A_R, H_O and F_{IS} between sown and natural populations using log-transformed population size as a covariate were tested with non-parametric distance-based (Euclidian) permutation tests implemented in R (*vegan* package; Oksanen, *et al.* 2008). The distribution of molecular variation among sown and natural populations, within groups and within populations was evaluated with the analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) implemented in ARLEQUIN 3.11. Individuals were clustered by applying the Bayesian Monte Carlo Markov Chain (MCMC) method implemented in STRUCTURE 2.3.3 (Pritchard *et al.* 2000).

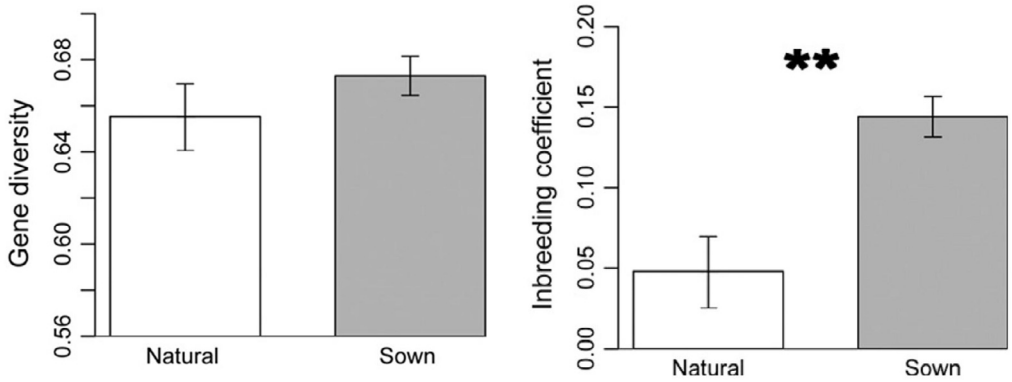
The effects of origin (sown, natural), genetic diversity and environmental variables on the fitness of study populations were analysed using linear mixed-effects models in R (packages *nlme* (Pinhero *et al.* 2012) and *lme4* (Bates *et al.* 2011)). The same models were used for studying the effect of seed origin (sown, natural, seed company) on the fitness of experimental plants in the study area and in an experimental garden.

Recent gene flow among sown and natural populations was estimated using assignment tests (Rannala and Mountain 1997) and first-generation migrant tests (Paetkau et al. 2004) implemented in GENECLASS 2.0 (Piry *et al.* 2004). In this analysis, we divided the study area into two regions being spatially separated by the town of Langenthal. Genetic differentiation F_{ST} among sown populations was very low. Thus, there was a high probability that assignment tests would place a migrant originating from any of the sown populations to a wrong source population due to high genetic similarity. We therefore pooled the sown populations within the same genetic cluster (according to STRUCTURE 2.3.3; Pritchard et al. 2000) together in assignment and first-generation migrant tests (“genetic population” in Table 1).

We carried out a corridor analysis to examine the effect of landscape variables on gene flow among the natural populations of *L. flos-cuculi*. Using ARCGIS 9.3.1 (ESRI), we calculated the amount of various landscape elements (agricultural land, settlements, forests, ditch verges) within corridors between populations (corridor widths of 50, 100, 200, 300, 500 and 1000 m). The effect of rank-transformed percentages of landscape elements within corridors on pairwise genetic differentiation F_{ST} among populations was estimated using multiple regression on distance matrices provided in R (package *ecodist*; Goslee and Urban 2007).

Innovation, gains, new insights and main results thanks to ENHANCE

Our first aim was to examine the genetic characteristics of sown and natural populations of *L. flos-cuculi*. Genetic analysis revealed no significant differences between gene diversity H_E and allelic richness A_R of sown and natural populations (Fig. 3a). However, sown populations were characterized by significantly lower observed heterozygosity H_O and correspondingly higher inbreeding coefficients F_{IS} in comparison with natural populations (Fig. 3b, Table 1). High inbreeding may have negative consequences for various aspects of fitness such as germination, survival and reproductive output (Hauser and Loeschcke 1995; Galeuchet *et al.* 2005), and can thus seriously jeopardize restoration success. Several reasons may have caused higher inbreeding in sown populations. First, the source populations, where the seeds were collected for propagation at the corresponding seed company, may have been small and inbred. Secondly, inbreeding in sown populations may have arisen when only a few source individuals were sampled causing genetic bottleneck effects and increasing the influence of genetic drift (Williams 2001). Thirdly, an increase in inbreeding can occur due to repeated regeneration of the same seed stock over several cycles (Schoen and Brown 2001), which is a common practice of seed companies. Consequently, to avoid inbreeding in seed mixtures, seeds for propagation should be collected from a substantial number of individuals in large and well connected populations. Additionally, seed stocks should be renewed after a few regeneration cycles.

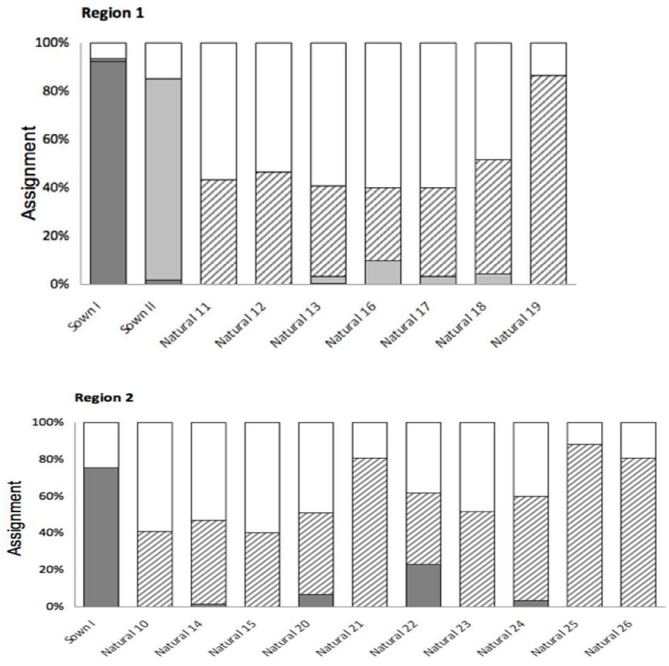


(a) (b)

Fig. 3. Mean (bars) and standard errors (whiskers) of gene diversity HE (a) and inbreeding coefficient FIS (b) in natural (white bars) and sown (grey bars) populations of *Lychnis flos-cuculi*. Asterisks denote a significant difference (**P < 0.01) between the two groups. (From Aavik *et al.* 2012).

Sown populations of *L. flos-cuculi* were genetically very distinct from natural populations (Aavik *et al.* 2012), although they originated from the same seed zone as the restored site. Furthermore, amongst the nine sown populations, we could distinguish two very distinct gene pools, which most likely represent two different source populations used for seed propagation at the seed company. However, despite the genetic differences between sown and natural populations, measurements of population fitness as well as an experiment in the study area revealed no significant influence of gene diversity or inbreeding on plant fitness. Neutral genetic diversity examined in the present study may not have a direct relationship with the adaptive genetic variation (Reed and Frankham 2001), which could be one of the reasons for the lack of correlation between fitness and genetic diversity. It is, nevertheless, also possible that the studied range of inbreeding (F_{IS} = 0–0.15) and gene diversity (H_E = 0.57–0.71) was too narrow to detect a response of fitness.

The analysis of recent gene flow with assignment tests revealed that gene flow among sown and natural populations did occur, though at relatively low levels. Natural populations, by contrast, showed higher rates of gene flow during last generations (Fig. 4), which mirrors the common history of those populations. Nevertheless, we observed only a few first-generation migration events, not only between restored and natural populations, but also among natural populations. Low levels of gene flow are most likely caused by low spatial connectivity among populations: *L. flos-cuculi* grows in mesotrophic and moist grasslands, which cover a very small percentage of the study area. However, it is also possible that dispersal between restored and natural populations occurred more often than we could infer from our results, but the dispersed seeds did not germinate and/or seedlings did not survive due to lower adaptation to the environmental conditions in the study area. In conclusion, our findings suggest that despite restoration efforts, gene flow among spatially fragmented populations remained moderate.



(a) (b)

Fig. 4. Results of assignment tests depicting recent gene flow (during the last few generations) among populations of *Lychnis flos-cuculi* in Region 1 (a) and Region 2 (b). Bar charts indicate the proportion of individuals in each population assigned to the first sown gene pool (Sown I, dark grey), to the second sown gene pool (Sown II, light grey; not found in Region 2), to the same natural population where the individuals were sampled (striped bars), and to other natural populations in the region (white). (From Aavik *et al.* in prep).

Corridor analysis revealed that only forest had a significant effect on gene flow of *L. flos-cuculi.* A high percentage of forest between populations thus increases genetic differentiation F_{ST} among populations (Table 2). Gene flow in *L. flos-cuculi* can occur through insect-mediated pollen flow and seed dispersal. However, *L. flos-cuculi* does not have a specialized mechanism for seed dispersal. Hence, it is more likely that forest influences pollen flow among populations of *L. flos-cuculi* through impeding the movement of pollinators. Indeed, ecological studies have shown that the removal of forest between open areas can significantly enhance pollinator movement and consequent pollen flow (Tewksbury *et al.* 2002). Our findings indicate that landscape characteristics can be a better predictor of gene flow than geographic distance alone.

Table. 2. The results of multiple regression on distance matrix analysis on the effects of landscape variables on genetic differentiation F_{ST} among the populations of *Lychnis flos-cuculi* in Oberaargau, Switzerland, using different corridor widths. "+" refers to a positive effect of the variable on genetic differentiation; "n.s." marks non-significant relationships.

Corridor width	R2	Distance	Agricultural land	Forest	Ditch verges	Settlements
50 m	0.125	n.s.	n.s.	+ ($P < 0.01$)	n.s.	n.s.
100 m	0.134	n.s.	n.s.	+ ($P < 0.01$)	n.s.	n.s.
300 m	0.147	n.s.	n.s.	+ ($P < 0.01$)	n.s.	n.s.
500 m	0.119	n.s.	n.s.	+ ($P < 0.05$)	n.s.	n.s.
1000 m	0.121	+ ($P < 0.05$)	n.s.	n.s.	n.s.	n.s.

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