The ecology, genetics and evolution of two *Saxifraga* species with different fragmentation histories

Dissertation

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Tag der mündlichen Prüfung am 25.11.2015.

I am the family face;
Flesh perishes, I live on,
Projecting trait and trace
Through time to time anon,
And leaping from place to place
Over Oblivion.

…

Extract from *Heredity*, Thomas Hardy

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Chapter 1

General Introduction

BACKGROUND

Over the past century, the increasing urbanisation and the intensification of agriculture have resulted in the destruction, degradation and fragmentation of natural and semi-natural habitats threatening species around the globe. Many species have thus become 'new rares' that were formerly more common, but now have smaller and more isolated populations (Huenneke 1991). The ecological consequences of the habitat fragmentation process are a reduction of the size of populations, increased isolation and reduced gene flow between populations, and more pronounced edge effects (Oostermeijer 2003). The recent decline of formerly common species may be more rapid than their evolutionary responses, and this makes them more susceptible to the negative effects of ongoing habitat fragmentation (Huenneke 1991, Brigham 2003). In contrast, 'naturally rare' species, that occur in a narrow range of habitats and within geographically restricted areas (Rabinowitz 1981), have existed in naturally fragmented habitats over long periods of time, and are therefore expected to be somewhat adapted to fragmentation (Brigham 2003).

Fragmented populations face a number of challenges which increase their risk of extinction (Young et al. 1996). First, they are more sensitive to the effects of environmental and demographic stochasticity (Matthies 2004). Second, small and isolated populations are less genetically diverse and lose alleles from one generation to the next. The erosion of genetic diversity over time is called drift and it may reduce the evolutionary potential of a population to adapt to environmental changes compromising its persistence in the long term (Schemske et al. 1994, Young et al. 1996, Willi et al. 2006, Aguilar et al. 2008, Weber and Kolb 2014, Mittell et al. 2015). Third, in fragmented populations, changes in pollinator behavior and reduced availability of mates may increase self-pollination and pollinations between closely related individuals, resulting in increased inbreeding (Mustajärvi et al. 2001, Honnay et al. 2005). By increasing the frequency of homozygous genotypes in the offspring generation inbreeding may increase the expression of deleterious alleles reducing the fitness of offspring (Falconer and Mackay 1996, Husband and Schemske 1996, Fischer and Matthies 1998a, Keller et al. 2002, Leimu et al. 2006, Wagenius et al. 2010). Accordingly, numerous empirical studies have detected a positive correlation between population size and plant fitness (Leimu et al. 2006, Angeloni et al. 2011). Inbreeding may also reduce the adaptive plasticity of plants in response to changes in the environment (Fischer et al. 2000, Kéry et al. 2000). Moreover, inbred plants may be less fit under stressful conditions (Armbruster and Reed 2005, Fox and Reed 2010, Cheptou and Donohue 2011, but see Angeloni et al. 2011). Because of its negative effects on plant fitness, increased inbreeding in fragmented populations is a major concern for conservation. Inbreeding is also of great interest to evolutionary plant biologists, because

variation among genotypes in inbreeding depression is thought to be a major factor in the evolution of plant breeding systems (Holsinger 1988, Uyenoyama et al. 1993, Schultz and Willis 1995, Picó et al. 2004).

Fragmentation may increase local adaptation, because the risk that maladapted genes are transferred into the population from outside is reduced. However, the process of selection operates best in larger populations with sufficient genetic potential to evolve, while in small and isolated populations it may be less effective due to enhanced drift and limited evolutionary potential (Gravuer et al. 2005, Johansson et al. 2007). Molecular genetic measures of genetic diversity are commonly used in conservation genetic studies even though they are poor predictors of a species' evolutionary potential to respond to future environmental change (eg. Reed and Frankham 2001, Leinonen et al. 2008, Mittell et al. 2015). Conservation genetic studies should therefore use quantitative adaptive traits to predict the evolutionary potential of populations, i.e. the ability of populations and species to withstand or adapt to biotic and abiotic change, such as predicted climate change (Brigham 2003, Edwards 2015).

To alleviate the negative effects of fragmentation such as increased drift and inbreeding, conservation actions may retain or artificially increase gene flow among the remnant populations (Keller and Waller 2002, Hufford and Mazer 2003). Although increased fitness of the hybrids from crosses among populations has been demonstrated for a number of plant species (Vergeer et al. 2004, Erickson and Fenster 2006, Willi et al. 2007), outbreeding may not always be beneficial. There may be outbreeding depression as a result of the dilution of local adaptation or the breakup of coadapted gene complexes (Hufford and Mazer 2003). Genetic incompatibility may only show in the second generation, but second generation outbreeding effects have been rarely investigated (eg. Fenster and Galloway 2000, Hufford and Mazer 2003, Willi et al. 2007, Volis and Zhang 2010).

Species may be more or less susceptible to the effects of fragmentation depending on their life history traits. Outbreeding and wind-pollinated species may maintain a certain amount of gene flow among populations, thus preserving genetic diversity within populations and limiting the differentiation process among populations. Furthermore, plants with long generation times such as many plants show a delayed response to the fragmentation of their habitats (Aguilar et al. 2008). A meta-analysis did, however, not confirm a general influence of longevity on the genetic response of populations to fragmentation (Honnay and Jacquemyn 2007). Other life-history traits such as a seed bank, clonal growth or polyploidy may also buffer against the loss of alleles through genetic drift (Young et al. 1996, Young et al. 2000, Münzbergova et al. 2013, James and Jordan 2014, van der Meer and Jacquemyn 2015).

There is evidence that formerly common species are more susceptible to fragmentation than naturally rare species. In a comparative review, formerly common species more frequently showed an decrease of genetic diversity with declining population size than historically rare species (Brigham 2003) and a meta-analysis concluded that common species face a higher risk of genetic erosion than naturally rare species because they host comparatively higher genetic diversity (Aguilar et al. 2008). Increased inbreeding and its negative consequences on the performance of plants are predicted to be a major problem in formerly common species undergoing rapid fragmentation and the performance of plants is expected to decline more frequently in small populations of formerly common than of naturally rare species (Brigham 2003). Naturally rare species may have purged deleterious alleles because of repeated bottlenecks or due to small population sizes over extended periods of time, and thus be less affected by inbreeding depression (Ellstrand and Elam 1993, Brigham 2003, Angeloni et al. 2011). However, small populations also have a higher risk to accumulate mildly deleterious alleles via mutations (Lynch et al. 1995, Ellstrand and Elam 2003). Finally, there is some support that historically rare species are better able to maintain pollinator service (Brigham 2003) through various mechanisms such as large floral rewards that make insects fly long distances (Moran and Hopper 1987), the reliance on generalist pollinators that are attracted by the flowering neighbour species, or self-compatibility that reduces their dependance on pollinator visitors (Brigham 2003).

Conservation efforts should not ignore formerly common, recently fragmented and mainly outcrossing species because they are very susceptible to the effects of fragmentation (Aguilar et al. 2008). Conservation strategies can greatly benefit from quantitative genetic common garden studies and the studies on the effects of increased inbreeding resulting from fragmentation and of potential rescue effects by crosses among populations.

OUTLINE OF THE THESIS

I studied the consequences of habitat fragmentation on the molecular genetic diversity and on the evolutionary potential of populations, as well as on the neutral and adaptive genetic variation among populations in two congeneric species with different fragmentation histories. *Saxifraga granulata* is a species of dry mesophile grasslands that has become fragmented in recent decades due to the intensification of agricultural practices, while *Saxifraga rosacea* subsp. *sponhemica* (hereafter referred to as *S. sponhemica*) is a historically rare species and a putative ice age relict (Thorn 1960, Walter and Straka 1970), that grows in open, long-term fragmented rock-face and scree habitats. Both species are perennial, long-lived, and may reproduce clonally: *S. sponhemica* via rosettes and *S. granulata* via underground bulbils. The two species are self-compatible, and flowers of both species are protandrous, but ripen at different times within the same genet, which

allows geitonogamous pollination. I assumed that the formerly common *S. granulata* would be more sensitive to habitat fragmentation than its historically rare congener, and expected to see a reduction of genetic diversity with reduced population sizes, and a pronounced differentiation among populations due to drift. I furthermore explored the effects of increased inbreeding resulting from fragmentation and of outbreeding on the performance and the plasticity of two generations of offspring of the formerly common grassland plant *S. granulata.* The aim of this thesis was to advance the knowledge of the effects of fragmentation on the ecology, the genetics and the evolutionary biology of the species under investigation and to use the knowledge to formulate recommendations for appropriate and efficient conservation strategies of the species.

This thesis contains four studies:

In chapter 2 ('Genetic structure of *Saxifraga rosacea* subsp. *sponhemica*, a rare endemic rock plant of Central Europe'), RAPD markers are used to study the population genetic structure and diversity of 30 populations of different size across the whole distributional range of *S. sponhemica*. The genetic distances between each pair of populations are correlated with geographic distances to check for an isolation by distance pattern. The genetic differentiation among populations and the genetic diversity of populations are estimated and correlated with the size of the populations to study the effect of habitat fragmentation. Putatively non-neutral loci are identified and their frequencies in populations are correlated with the climate in the populations to detect signs of selection. Spatial autocorrelation analyses are performed to test for a significant spatial genetic structure within populations indicating restricted gene flow.

In chapter 3 ('Divergent selection along climatic gradients in a rare central European endemic species, *Saxifraga sponhemica'*) the genetic variation within and among 22 populations from the whole distribution area of the species are estimated, using both RAPD-markers and quantitative genetic traits of seed families grown in a common garden. The quantitative genetic differentiation among populations is compared with the molecular genetic variation among populations to evaluate the importance of selection relative to that of drift. As additional methods to detect divergent selection, trait means are correlated with climatic variables of the sites and the quantitative genetic distances between each pair of populations are correlated with the geographic and the climatic distances. We test whether the evolutionary potential of populations is related to the size of the populations, or to their molecular genetic diversity and investigate relationships between population means of fitness related traits and the genetic diversity of a population to test for inbreeding depression.

Chapter 4 ('Effects of recent habitat fragmentation on molecular and quantitative genetic variation of the grassland plant *Saxifraga granulata*') reports the result of a joint study on molecular and quantitative genetic variation within and among 19 populations of *S. granulata* from a restricted geographic area in Luxembourg and neighbouring Germany. The study estimates the relative contributions of selection and of drift to the overall genetic variation among populations by comparing quantitative adaptive and neutral molecular genetic variation. Moreover, correlation analyses between population size, molecular and quantitative genetic diversities and plant fitness traits are conducted to check for effects of genetic drift in populations. Clonal diversity, clonal spread and small-scale spatial genetic structure are studied within two small sample plots.

In Chapter 5 ('Effects of inbreeding and interpopulation crosses on performance and plasticity of two generations of offspring of a declining grassland plant') two generations of offspring from manual self-pollinations and within or between-population crosses were grown in the common garden. Plant traits related to the reproduction and the performance of the offspring were measured to estimate the magnitude of inbreeding and outbreeding depression, as well as heterosis effects. The susceptibilities to inbreeding or outbreeding depression of different seed families are examined to evaluate if the population had the potential to evolve towards increased or even complete selfing or outcrossing. Moreover, the first generation of offspring was subjected to a fertilization and two stress treatments (competition and defoliation) to investigate whether the effects of inbreeding and interpopulation crosses depend on environmental conditions.

Chapter 2

Genetic structure of *Saxifraga rosacea* subsp. *sponhemica*, a rare endemic rock plant of Central Europe

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with Diethart Matthies, Sylvie Hermant and Guy Colling

ABSTRACT

We used RAPD markers to study the population genetic structure and diversity of *Saxifraga rosacea* subsp. *sponhemica*, a rare Central European endemic rock plant with a highly disjunct distribution. Because of strong isolation current gene flow between populations is very low or absent. However, an isolation by distance pattern of genetic differentiation suggested historical gene flow during the last glaciation when suitable habitats for *S. sponhemica* were much more abundant. In most populations, considerable genetic variability has been preserved due to the longevity of *S. sponhemica*. Our results suggest that long-lived plant species can maintain historic genetic patterns despite small size and strong isolation of populations. Several RAPD loci were identified to be non-neutral and their frequencies correlated with climatic gradients, indicating natural selection. Adaptive genetic variation could be important for adaptation to environmental changes like ongoing climate change. The taxon does not appear to be genetically threatened in the short term, but populations are threatened by habitat destruction. The establishment of new populations in suitable habitats with seeds from the same region may be a suitable conservation measure avoiding potential maladaptation due to local adaptation.

Introduction

Species have undergone important range contractions and expansions during the glacial and interglacial periods of the Pleistocene (Hewitt 1996). During the glaciations, the Central European lowlands were covered by steppe-tundra vegetation suitable for cold-adapted plant species, which were then widely distributed (’t Mannetje 2007). In the postglacial warming period, these species migrated to the cold, previously inhospitable alpine or arctic regions, but some remnant populations survived in lowland habitats with suitable conditions. A disjunct distribution in combination with a habitat type that had already existed during the glaciation is often considered to be an indicator for the glacial relict status of populations (Walter and Straka 1970). Cliffs are a typical habitat type that has existed and remained stable since glaciations, because it was hardly affected by forest recolonisation in the postglacial period or by human activity during the Holocene. Because cliffs are naturally rare and fragmented in lowland Europe, glacial relicts occurring on cliffs are suitable model species to study the effects of long-term fragmentation (Tang et al. 2010).

The effects of habitat fragmentation on the genetics of plant populations have been a topic of many recent studies (reviewed by Young et al. 1996, Leimu et al. 2006, Honnay and Jacquemyn 2007). Many rare species have been found to harbour less genetic diversity than more widespread species (compilation by Hamrick and Godt 1990, Cole 2003, Nybom 2004) due to the loss of alleles through random genetic drift (e.g. Young et al. 1996, Frankham and Wilcken 2006, Yuan et al. 2012). Furthermore, reduced gene flow among isolated populations in fragmented habitats has led to strong genetic differentiation between populations of many rare species (e.g. Fischer and Matthies 1998, Šmídová et al. 2011, Wagner et al. 2011). Loss of genetic variation and genetic differentiation is expected to increase with time since fragmentation (Coates 1988, Gitzendanner and Soltis 2000, Zawko et al. 2001). Thus, ice age relict populations that have been fragmented for a long time are expected to show strong genetic differentiation and low genetic diversity. Strong genetic differentiation has been reported for isolated alpine relict populations, such as *Saxifraga* cernua (Bauert et al. 1998), *Erinus alpinus* (Stehlik et al. 2002) and for the lowland remnant populations of *Saxifraga* paniculata (Reisch et al. 2003). However, not all studies have found low genetic diversity in ice age relicts (Lutz et al. 2000, Reisch et al. 2003), presumably due to the longevity of the species buffering random genetic drift. Genetic variation has profound implications for species conservation (Schaal et al. 1991, Ellstrand and Elam 1993, Ouborg et al. 2006) and assessing genetic variation within and between populations is essential for efficient conservation measures for rare species. Loss of genetic variability and increased inbreeding in small populations (Young et al. 1996, Frankham et al. 2002) may result in reduced fitness of offspring (Ellstrand and Elam

1993, Keller and Waller 2002). In the long term, reduced genetic variation may lower the evolutionary potential of a species in the face of changing environmental conditions, such as ongoing climate change.

Genetic variation is often assessed by studying the variability of neutral markers. However, adaptive loci that are responding to environmental variation may provide more relevant information on the potential of populations for rapid adaptation (Hoffmann and Willi 2008, Manel et al. 2012). Recently developed genome scan methods allow detecting candidate loci under selection on the assumption that natural selection is a locus-specific force, which increases the frequencies of locally beneficial alleles in a population (Strasburg et al. 2012). The distribution of these candidate loci among populations may then be compared with the distribution of environmental factors, such as temperature or precipitation that affect adaptive genetic variation.

We studied the genetic population structure and diversity of the endangered, long-lived plant *Saxifraga rosacea* Moench subsp. *sponhemica* (C.C. Gmel.) D.A. Webb, an endemic of Central Europe. Because of its disjunct distribution and habitat type (screes and cliffs), the species is considered to be an ice age relict (Thorn 1960, Walter and Straka 1970). We used RAPD-markers to address the following questions (1) How is genetic variation distributed among regions, populations and individuals? Does the genetic distance between populations increase with geographic distance? (2) Are populations of *S. rosacea* subsp. *sponhemica* characterised by low genetic diversity and does genetic diversity increase with population size? (3) Are there loci putatively under selection and is their frequency related to climatic variables?

MATERIALS AND METHODS

Species and study sites

Saxifraga rosacea subsp. *sponhemica* (hereafter called by its synonym *S. sponhemica* C.C. Gmel) is an evergreen perennial that grows either in compact cushions, formed by short and suberect shoots or as loose mats, formed by procumbent and rather long shoots (Tutin et al. 1968). Cushion size is highly variable (1-100 cm) and the number of rosettes per plant ranges from 1 to over 600. Individual rosettes are semelparous, but the genets are iteroparous. *S. sponhemica* is able to spread sexually via seeds and vegetatively via rosettes (pers. observation, Hemp 1996). Demographic data indicate that genets of *S. sponhemica* can live for several decades (Decanter, pers. comm.).

The flowers of *S. sponhemica* are strongly protandrous (Webb and Gornall 1989), but flowers ripen at different times within the same genet, which allows geitonogamous pollination. Common pollinators are Diptera (Muscidae and Syrphidae) and Apidae (Webb

and Gornall 1989), we also observed some Coleoptera species as flower visitors. *S. sponhemica* has a mixed mating system with a selfing rate of about 46.8 % (Walisch unpubl.). *S. sponhemica* generally occurs on north to east facing rock faces, scree slopes and stone walls with no or little direct sunlight (Hemp 1996, pers. observation), which are fragmented habitats in lowland Europe. A few populations occur also on walls next to natural rock populations.

Saxifraga sponhemica has a disjunct distribution and occurs in the western part of its range in the Belgian Ardennes, the Luxembourg Oesling and the German Mid-Rhine region, with a few isolated populations in the French Jura, while in the east it occurs in the Bohemian low mountains (České středohoří) and in the Czech Bohemian Karst region (Českŷ kras), with isolated populations in the south of Moravia and in the Polish Sudetes (Webb and Gornall 1989, Fig. 1). The populations of *S. sponhemica* occur in regions that were not covered by glaciers during the last glaciation (Ehlers and Gibbard 2004), except for the populations in the French Jura. In most parts of its distribution area *S. sponhemica* is considered to be extremely rare or critically endangered and is legally protected (Korneck et al. 1996, Holub and Prochazka 2000, Colling 2005, Mirek et al. 2006).

Sampling design

We studied 30 populations from six regions across the whole distributional range of *S. sponhemica*: the Ardennes, the Luxembourg Oesling, the German Mid-Rhine region, the French Jura, the České středohoří and the Českŷ kras (Fig. 1, Table 1). The geographic distances between the sampled populations within the six regions ranged from 0.1 to 14.9 km. To assess whether the studied populations formed a monophyletic group, we used ITS sequence data (ITS-1, 5.8 s and ITS-2) of three plants from each study population (Elvinger, pers. com.). We also included the sequence data from one specimen of the closely related subspecies *S. rosacea* subsp. *rosacea*. As an outgroup, we chose *Saxifraga granulata*. The results of a maximum likelihood tree analysis using version 3 of PhyML (Guindon and Gascuel 2003) with a GTR model of nucleotides substitution clearly indicated that all studied *S. sponhemica* populations formed a monophyletic group (Elvinger, pers. com.). Flow cytometry analysis of the DNA content indicated that all *S. sponhemica* populations had the same ploidy level (Elvinger, pers. com.).

In summer 2002 or 2003, we estimated the size of each population as the number of cushions (Table 1) and sampled 14 cushions along transects of 10-15 m length. Within each transect, we recorded the distances among the sampled plants. The minimum distance between two sampled plants was 100 cm to reduce the chance of sampling the same genetic individual repeatedly, but in populations consisting of less than 14 plants all accessible plants were sampled. In the six largest populations, we placed two transects to test for

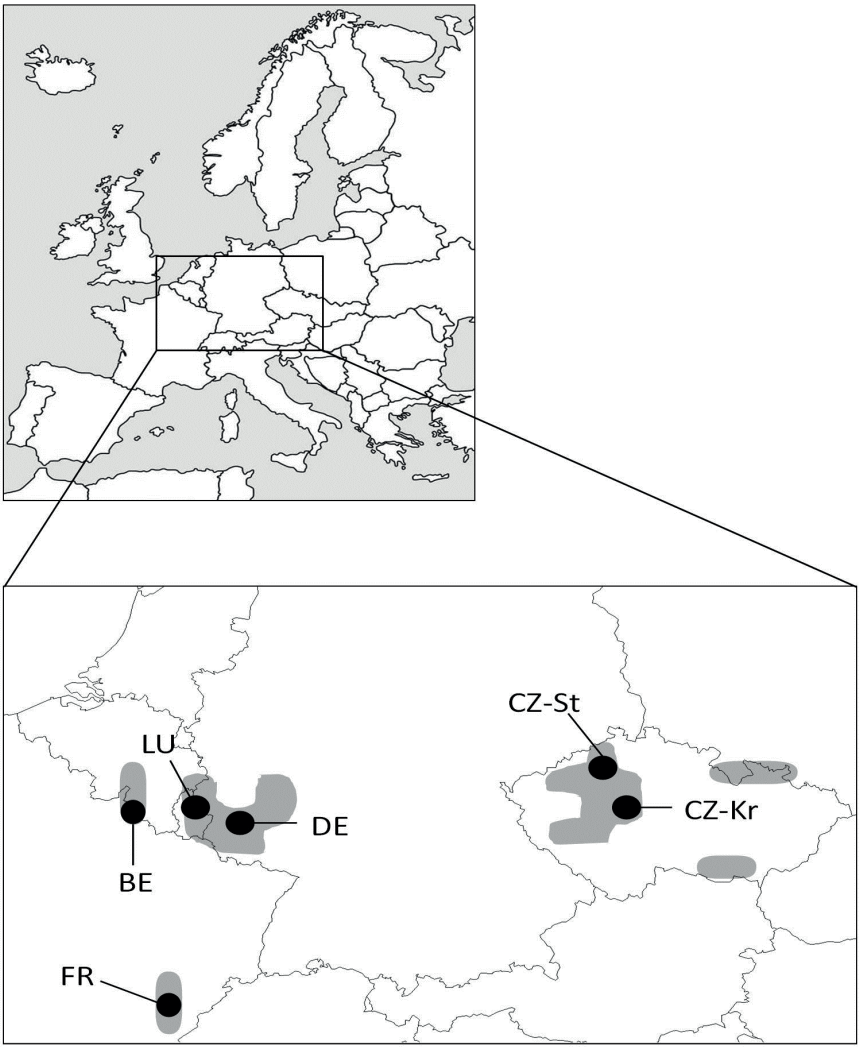

Figure 1. Distribution (grey areas) of *Saxifraga sponhemica* (modified from Jalas and Suominen 1976). The sampling regions are marked as black dots on the map. In Luxembourg (LU) 13 populations were sampled, in Germany (DE) four, in Belgium (BE) five, in France (FR) two, and six in two regions of the Czech Republic: four in Českéstředohoří (CZ-St), and two in Český kras (CZ-Kr)

genetic structuring within the populations. Overall, 459 plants were sampled. Two fresh leaves were collected from each plant, placed in a small paper bag, and immediately frozen in liquid nitrogen. The samples were then stored at -80 °C.

RAPD-PCR

After grinding the frozen leaf material (Retsch MM200, Retsch, Haan, Germany), DNA was extracted using the DNeasy® Plant Mini Kit (QIAGEN, Germany). The DNA concentration of extracted DNA samples was determined by measuring their absorbance at 260 nm with a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany). Amplifications were carried out in 25 µl volumes containing 5 µl of template DNA (5 ng DNA/µl), 8.575 µl ddH2O, 3 µl

Table 1. Genetic diversity of 30 populations of *Saxifraga sponhemica*. *N* number of plants, N_S number of plants sampled, *PPL* proportion of polymorphic loci at the 5 % level, H_{eN} Nei's gene diversity based on allele frequencies calculated with the Bayesian method with non-uniform prior distribution of allele frequencies in a population (Zhivotovsky 1999) assuming that the inbreeding coefficient $F_{IS} = 0.314$, F_{ST} population-specific F_{ST} value.

Geographical Region	Population and habitat	Popcode	N	N_S	*PPL* (%)	H_{eN}	F_{ST}	Location (latitude/longitude)
Oesling (LU)	Bettel, rock	LU1	465	14	86.9	0.261	0.308	N 49.923/E 6.218
	Bettel-Vianden, rock	LU2	536	14	88.5	0.299	0.274	N 49.923/E 6.219
	Kautenbach, rock	LU4	300	14	86.9	0.250	0.373	N 49.952/E 6.016
	Kautenbach, Hockslé, rock	LU5	4	4	83.6	0.329	0.207	N 49.945/E 6.027
	Michelau-Erpeldange, scree	LU6	10	9	75.4	0.222	0.390	N 49.894/E 6.115
	Michelau-Erpeldange, wall	LU7	250	14	95.1	0.310	0.244	N 49.893/E 6.115
	Michelau-Erpeldange, quarry	LU8	188	12	93.4	0.310	0.273	N 49.892/E 6.112
	Unterschlinder, wall	LU10	9600	14	88.5	0.306	0.282	N 49.926/E 6.076
	Unterschlinder, rock	LU11	326	14	90.2	0.286	0.267	N 49.922/E 6.072
	Vianden parking, rock	LU13	100	14	91.8	0.280	0.312	N 49.935/E 6.198
	Vianden-Roth, rock	LU14	157	14	82.0	0.232	0.367	N 49.929/E 6.225
	Vianden tower, wall	LU15	66	14	96.7	0.299	0.261	N 49.933/E 6.208
	Vianden castle, wall	LU16	1100	14	95.1	0.319	0.232	N 49.936/E 6.202
Mid-Rhine (DE)	Frauenburg, rock	DE17	454	13	90.2	0.251	0.398	N 49.667/E 7.282
	Loreleifels, rock	DE19	14	10	90.2	0.301	0.306	N 49.680/E 7.288
	Idar-Oberstein-Hammerstein, rock	DE20	300	13	86.9	0.263	0.317	N 49.687/E 7.299
	Hammerstein crossroads, rock	DE21	58	14	86.9	0.278	0.379	N 49.690/E 7.289
Jura (FR)	Salin-Cernans, rock	FR29	≥10	2	62.3	0.278	0.240	N 46.927/E 5.921
	Planches-sur-Arbois, scree	FR30	50	7	72.1	0.169	0.467	N 46.879/E 5.813
Ardennes (BE)	Bouillon below castle, rock	BE22	199	14	47.5	0.224	0.370	N 49.793/E 5.064
	Bouillon castle, rock	BE23	300	14	88.5	0.244	0.332	N 49.793/E 5.066
	Bouillon Castle Hotel, wall	BE24	43	9	82.0	0.224	0.384	N 49.795/E 5.067
	Bouillon roadsign, rock	BE25	2	2	78.7	0.278	0.160	N 49.791/E 5.062
	Bouillon Bastion Bretagne, wall	BE26	27	12	88.5	0.238	0.317	N 49.797/E 5.069
České středohoří (CZ-St)	Děkovka, rock	CZ31	16	7	73.8	0.220	0.413	N 50.490/E 13.924
	Ostrý, scree	CZ32	405	14	85.2	0.244	0.377	N 50.532/E 13.951
	Boreč, scree	CZ33	90	13	86.9	0.276	0.377	N 50.515/E 13.990
	Blešno, scree	CZ38	125	14	82.0	0.253	0.412	N 50.482/E 13.906
Český kras (CZ-Kr)	Voškov, rock and scree	CZ35	150	13	75.4	0.243	0.430	N 49.918/E 14.197
	Tetínské Skály, rock	CZ37	600	14	83.6	0.260	0.368	N 49.950/E 14.107

MgCl2 (25 mM), 0.5 µl dNTP's (10 mM), 2.5 µl PCR buffer with (NH4)2SO4 (109, Fermentas), 5 µl primer (5 µM), 0.3 µl Taq DNA polymerase (5 units/µl; Fermentas), and 0.125 µl BSA (20 mg/ml). The volumes were held in polycarbonate microtitre plates and covered by adhesive sealing sheets. The plates were then incubated in a thermocycler (iCycler®, Bio-Rad Laboratories) programmed with the following settings: denaturation of the DNA at 94 °C for 2 min, followed by 44 repetitive cycles consisting of denaturation for 45 s at 94 °C, annealing for 2 min 30 s at 36 °C, and extension for 2 min at 72 °C followed by a final extension phase of 5 min at 72 °C. The samples were kept at 4 °C until analysis. Amplified DNA fragments were separated by electrophoresis on precast ReadyAgaroseTM 1.0 % agarose gels with ethidium bromide in 19 TBE buffer (Bio-Rad Laboratories) in an electrical field (85 V, c. 100 min). Gels were visualised under UV light and photographed using the Bio Doc system (Bio-Rad Laboratories).

In a first series of amplifications 40 10mer primers (Kits A, B from Operon Technologies, Alameda, CA) were screened in a random sequence and tested for reproducibility of the amplified fragment profile using four replicates of a single DNA extract. The first eight primers yielding good quality reproducible patterns (primers A5, A7, A9, A19, B7, B10, B17, and B18) were selected for the RAPD analysis of all 459 sampled plants (Table 2). Amplification products were scored visually for presence or absence of reliable bands using the program Quantity/One (Bio-Rad Laboratories) and were treated as phenotypes, with each band position representing a character either present or absent. The final presence-absence matrix contained scores at 61 polymorphic band positions for all samples in the study. We estimated the error rate of the RAPD genotyping by replicating 577 combinations of DNA samples and markers after DNA extraction resulting in 3,686 repeated banding scores (corresponding to 13.2 % of the total dataset). The second scoring was done by the same technician as the first one and the error rate was estimated to be 4.3 %.

For all genetic analyses, except for the analysis of between transect variation, we used 30 populations with only one transect per population. The RAPD fragment presence/absence matrix contained a total of 61 polymorphic loci and 380 plant samples. Because of the error rate of 4.3 %,

Table 2. RAPD primers used

Primer	Sequence
A5	50 -AGGGGTCTTG-30
A7	50 -GAAACGGGTG-30
A9	50 -GGGTAACGCC-30
A19	50 -CAAACGTCGG-30
B7	50 -GGTGACGCAG-30
B10	50 -CTGCTGGGAC-30
B17	50 -AGGGAACGAG-30
B18	50 -CCACAGCAGT-30

we considered plants differing by less than four bands as putative clones belonging to the same genotype (Ehrich et al. 2008). Only one randomly chosen clone per genotype was kept in the RAPD fragment/absence matrix resulting in 352 samples used for further analysis.

Analysis of genetic diversity within populations

To estimate allele frequencies we used the Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999) as implemented in AFLP-SURV version 1.0 (Vekemans 2002) with an estimate of Wright's inbreeding coefficient over all populations (F_{IS}). F_{IS} was calculated using the Bayesian method implemented in HICKORY version 1.0.4 (Holsinger et al. 2002). Genetic diversity within populations was calculated as (1) the percentage of polymorphic loci (*PPL*) at the 5 % level, (2) Nei's gene diversity (expected heterozygosity H_{eN}) according to the method of Lynch and Milligan (1994) which uses the average expected heterozygosity of the marker loci.

Analysis of population structure

To infer population structure at the landscape level and assign individuals to the geographical regions we used the software STRUCTURE v. 2.3.4. which allows the use of dominant markers, such as RAPDs (Pritchard et al. 2000; Falush et al. 2003, 2007). We used the model of no admixture for the ancestry of the individuals without prior information of the regional membership of the populations and assumed that the allele frequencies are correlated among populations. We carried out a total of 300 runs (10 runs each for one to 30 clusters, i.e., K = 1-30) to quantify the amount of variation of the likelihood of each K. We found that a burn-in and Markov Chain Monte Carlo (MCMC) length of 105 each was sufficient as longer burn-ins or MCMC lengths did not change significantly the results. In STRUCTURE, the model choice criterion to detect the K most appropriate to describe the data is given as 'Ln P(D)' which is an estimate of the posterior probability of the data given K. The maximum value of Ln P(D) returned by STRUCTURE, to which we refer as $L(K)$ afterwards, is often taken as the true value of K. However, the distribution of $L(K)$ does often not show a clear mode for the number of groups. We used an ad hoc quantity based on the second-order rate of change of the likelihood function (DK) with respect to K (Evanno et al. 2005) as implemented in STRUCTURE HARVESTER (Earl and von Holdt 2012). It is calculated as $\mathrm{D}K = \mathrm{m}(|L(K+1) - 2L(\mathrm{K}) + \mathrm{L}(K-1)|)/\mathrm{sd}[L(K)]$ where m is the mean and sd the standard deviation. The height of this modal value was used as an indicator of the signal detected by STRUCTURE to find the highest modal value. Finally, the ten runs of the simulation with the highest modal value of DK were aligned using the FullSearch option in CLUMPP (cluster matching and permutation program, Jakobsson and Rosenberg 2007). Convergence of the 10 replicate runs for K = 3 was high as they produced very similar

clustering results as shown by the pairwise *G'* (similarity function) values (> 0.99) for each pair of permutated runs in CLUMPP. The mean membership coefficients were represented as a bar graph using DISTRUCT (Rosenberg 2004).

The genetic structure within and among populations was first analysed using the Bayesian method suggested by Holsinger et al. (2002) as implemented in HICKORY (version 1.0.4, Holsinger and Lewis 2006). This method allows a direct estimate of the overall F_{ST} from dominant markers without assuming previous knowledge of the inbreeding coefficient within populations and Hardy-Weinberg equilibrium. We used HICKORY with a full model and using non-informative priors for f (estimate of F_{IS}) and h_B (estimate of F_{ST}). To ensure that the results were consistent we conducted several runs with default sampling parameters (burn-in = 50,000; sample = 250,000; thin = 50). The method also allowed inference of the within-population inbreeding coefficient F_{IS}.

The genetic structure within and among populations was also analysed on the basis of RAPD allele frequencies with AFLP-SURV assuming the inbreeding coefficient calculated by HICKORY. We used 1,000 permutations to assess the significance of the calculated F_{ST}. A pairwise genetic distance matrix with F_{ST} values was calculated in AFLP-SURV assuming the inbreeding coefficient estimated by HICKORY and used as input for a principal coordinate analysis (PCoA). The partitioning of genetic variation among the clusters identified by STRUCTURE, geographical regions within these clusters, populations within regions, and among individuals within populations was investigated by analysis of molecular variance (AMOVA) using the R-package ade4 (Dray and Dufour 2007).

To test for isolation by distance, we applied Mantel test statistics correlating the pairwise F_{ST} values and the geographic distance matrix using GenAlex 6.501 (Peakall and Smouse 2006, 2012). Significance levels were obtained after performing 999 random permutations for the Mantel test.

High genetic differentiation is not always a consequence of low gene flow, but can also result from a migration-drift disequilibrium when drift plays an important role, such as in small populations (Whitlock and McCauley 1999). We estimated the population-specific F_{ST} values using BAYESCAN 2.01 (Foll and Gaggiotti 2008) with the default settings and used linear regressions to test if there was a relationship between the population-specific F_{ST} values and measures of genetic diversity (PPL and H_{eN}) of populations. We expect a strong relationship if genetic differentiation is strongly affected by genetic drift (both current and historical), reflecting a migration-drift disequilibrium. If on the other hand populations are in migration-drift equilibrium, no such relationship is expected (Cox et al. 2011).

Not all molecular markers are necessarily selectively neutral. We identified markers un-

der divergent or balancing selection with the program BAYESCAN 2.01 with the false discovery rate set to 0.05 (see Foll and Gaggiotti 2008). Several methods of detecting markers under selection have recently been tested by De Mita et al. (2013). The method used by BAYESCAN 2.01 was found to be robust against deviations from the island model and yielded very few false positives in all simulations. To analyse if there is a relationship between putative non-neutral markers and climatic conditions, we obtained the following bioclimatic variables for each study site for the current conditions (interpolations of observed climate data, representative of 1950-2000) in a grid size of about one square kilometre (30 arc seconds) from the WORLDCLIM database version 1.4. (Hijmans et al. 2005): mean annual temperature, temperature seasonality, maximum temperature, minimum temperature, and annual precipitation. We reduced the climatic variables to two principle components using principle component analysis with varimax rotation. We then studied the relationship between the identified non-neutral markers and the two principal components by multiple logistic regressions, using the GLM package of R (version 3.0.1, R Core team 2013). Finally, we removed the loci identified as non-neutral from the dataset and ran a second AMOVA to compare it with the AMOVA based on the complete dataset.

Genetic structure within populations and clonal structure

The genetic structure within populations was studied by autocorrelation analyses using an estimator of the kinship coefficient for dominant markers, F_{ij} (Hardy 2003) as implemented in SPAGeDI version 1.2. (Hardy and Vekemans 2002). This method does not assume Hardy-Weinberg genotypic proportions, but requires an estimate of the departure from these conditions (i.e., that the inbreeding coefficient is known). We used the HICKORY estimate of F_{IS}. The kinship coefficient, F_{ij} is defined as the probability that a random gene from individual *i* is identical to a random gene from individual *j*. To visualize and describe the spatial genetic structure (SGS) within populations of *S. sponhemica*, mean F_{ij} estimates over pairs of individuals at a given distance interval *r*, *F*(*r*), were plotted against distance in a spatial autocorrelogram. If *F*(*r*) tends to decrease linearly with r or ln (*r*), the extent of SGS can be quantified by the slope (*b*) of a regression of mean F_{ij} estimates on r_{ij} or ln (r_{ij}). As (*b*) can depend on the sampling scheme used, we calculated the ratio $-b/(1 - F_{(1)})$ where $F_{(1)}$ is the mean F_{ij} between individuals belonging to the first distance class. $F_{(1)}$ can be considered as an approximation of the kinship coefficient between neighbouring individuals if the first distance class contains enough pairs of individuals. The ratio $-b/(1 - F_{(1)})$ is referred to as the *Sp* statistic (Vekemans and Hardy 2004) and can be used to compare the extent of SGS among populations or species. Standard errors for the mean F_{ij} estimates over pairs of individuals at a given distance interval and the regression slope

(*b*) were assessed by a jack knifing procedure over loci. The significance level of the regression slope (*b*) was evaluated by comparing the observed value with the distribution of (*b*) obtained by 1,000 random permutations.

RESULTS

Genetic diversity within populations

The eight RAPD primers used for analysis generated a total of 61 polymorphic bands. No private (population-specific) bands were observed. Taking into account an error rate of 4.3 %, individuals differing by up to 2.6 (rounded to 3) loci were considered as possible ramets belonging to the same clonal lineage. This resulted in 23 putative clones and 352 unique genotypes. The mean proportion of polymorphic loci (*PPL*) in the 30 populations was 83.8 % and varied among the populations from 47.5 to 96.7 % (Table 1). *PPL* was much lower in the French Jura populations than in those from Luxembourg or Germany (67 vs. 89 %, $P < 0.05$, Tukey's test). Overall, mean Nei's gene diversity (H_{eN}) within populations using the F_{IS} estimated by HICKORY was 0.265, and like *PPL* it was particularly low in the populations from the French Jura and high in those from Luxembourg and Germany, but this difference was only marginally significant ($P = 0.058$). None of the gene diversity measures increased significantly with population size ($r < 0.29$, $P > 0.12$).

Population structure

Using the modal value of D*K* rather than the maximum value of L(*K*) allowed us to identify with STRUCTURE several groups corresponding to the uppermost hierarchical level of partitioning among populations (Fig. 2). The highest modal value of D*K* was at $K = 3$. The first cluster identified by STRUCTURE consisted of three regions (LU, DE and FR), while the other two clusters corresponded to the Belgian (BE) and Czech regions (CZ-St and CZ-Kr) (Fig. 3).

The PCoA analysis based on pairwise F_{ST} distances revealed a clustering pattern very similar to the clusters identified by STRUCTURE (Fig. 4). Populations from the Czech Republic and Belgium formed two distinct clusters whereas the remaining populations formed one large cluster. The PCoA analyses thus confirmed that populations from Luxembourg, Germany and France are closely related.

The posterior mean Bayesian estimate in the HICKORY analysis for F_{IS} (f) was 0.314 ± 0.124 (95 % credible interval 0.085-0.562) suggesting a moderate amount of inbreeding within populations. Estimates of F_{IS} based on HICKORY analysis of dominant markers have to be regarded with caution, but are plausible if consistent with estimates based on other information (Holsinger and Lewis 2006). Our HICKORY estimate of F_{IS} (*f*) was very

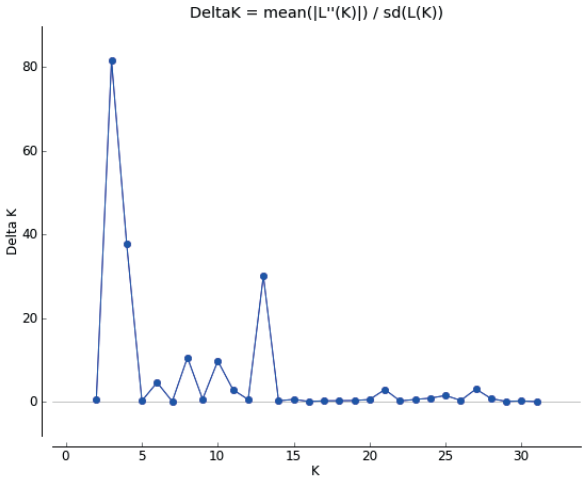

Figure 2. Results of a STRUCTURE analysis (10 runs each for K = 1-30) to infer the population structure of *Saxifraga sponhemica* at the landscape level; ΛK is plotted as a function of K. The highest modal value of ΛK is at K = 3 corresponding to the number of geographical regions (see text for details)

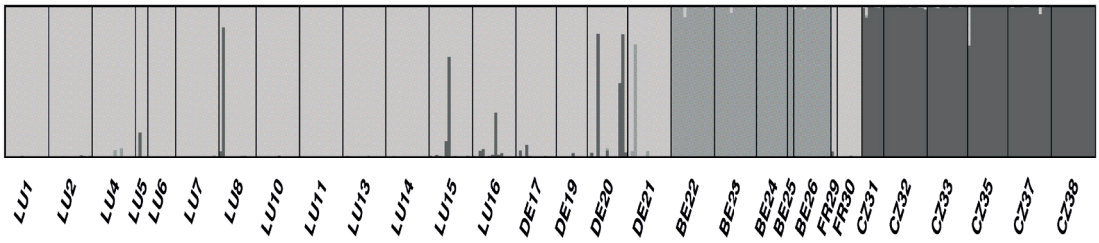

Figure 3. Estimated population structure of *Saxifraga sponhemica* inferred by a Markov chain Monte Carlo Bayesian clustering method (STRUCTURE version 2.2) of RAPD data. Each individual is represented by a vertical line, which is partitioned into a maximum of K = 3 differently shaded segments that represent the individual's estimated membership fractions in three clusters. Vertical black lines separate the 32 populations. Runs of ten simulations were aligned using CLUMPP (see text for details). For population codes, see Table 1

similar to an estimate of F_{IS} = 0.305 computed as $F_{IS} = s/(2 - s)$ (Hartl and Clark 1997) with a self-fertilisation rate (s) of 0.468. The self-fertilisation rate was estimated in a pollination experiment in a large population in Luxembourg (Walisch et al. unpublished). Furthermore, our study was based on a relatively large number of populations and loci suggesting that our inferences about F_{IS} with HICKORY are plausible (Holsinger et al. 2002).

In the Bayesian analysis of population structure with HICKORY, the posterior mean estimate of F_{ST} (h_B) was slightly higher than the traditional estimate of F_{ST} estimated by AFLP-SURV assuming the HICKORY estimate of F_{IS} = 0.314 (F_{ST} = 0.3836 ± 0.0032 and F_{ST} = 0.3369 ± 0.0075, respectively). The AMOVA estimate (Φ_{ST} = 0.377) was similar to the HICKORY estimate. The AMOVA analysis showed that there was significant

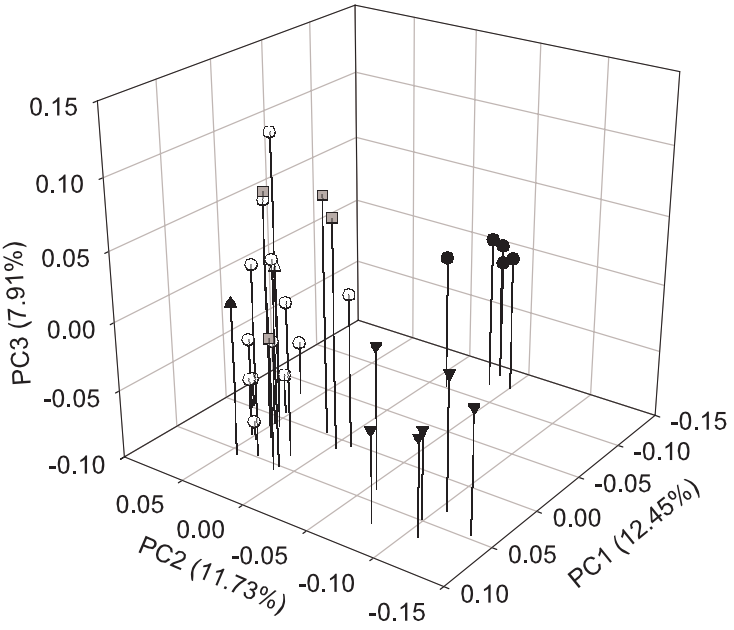

Figure 4. PCoA analysis based on F_{ST} genetic distances derived from RAPD markers of 30 populations of *Saxifraga sponhemica*. Ovals correspond to the three clusters identified by a STRUCTURE analysis. The symbols denote six geographical regions: filled circles Ardennes (BE), unfilled circles Oesling (LU), filled squares Mid-Rhine (DE), filled triangles Jura (FR), dark filled inverted triangles České středohorí (CZ), light filled inverted triangles Český kras (CZ)

Table 3. Partitioning of the genetic variation of *Saxifraga sponhemica* by AMOVA among three genetic clusters as identified by STRUCTURE, six geographical regions within these clusters [Oesling (LU), Mid-Rhine (DE), Jura (FR), Ardennes (BE), České středohoří (CZ), and Český kras (CZ)], populations within regions, and individuals within populations

Source	*df*	Sum of squares	Variance components	Proportion of variation (%)	*P*
Among clusters	2	386.4	1.11	9.4	<0.001
Among regions within clusters	3	211.2	0.84	7.2	<0.001
Among populations within regions	24	869.5	2.47	21.1	<0.001
Within populations	322	2,350.8	7.30	62.3	<0.001

genetic differentiation among the three clusters identified by STRUCTURE, the six geographical regions within the clusters, and among populations within regions (Table 3). Overall, more than 16 % of the variation was among regions. An AMOVA based only on the populations with two transects showed that the variation among transects within populations accounted for 12 % of the total genetic variation, while variation among individuals within transects accounted for another 57 %.

Genetic differentiation among the populations (pairwise F_{ST}) increased with geographic

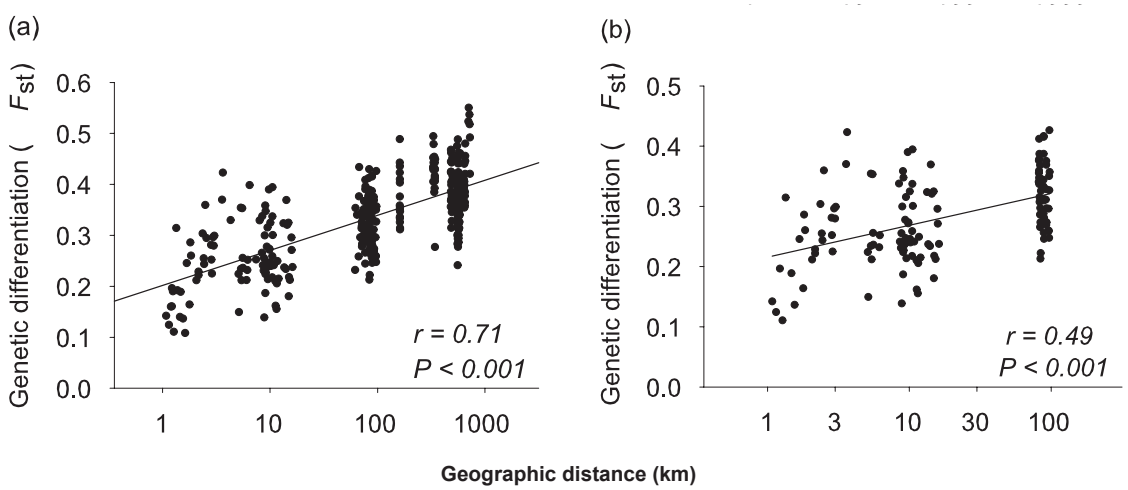

Figure 5. The relationship between genetic distances (pairwise F_{ST}) and geographic distances for a 28 sampled populations of *Saxifraga sponhemica* (all populations except for BE25 and FR29 with only two samples), and for b a subset consisting of the populations from Luxembourg and Germany. *P* values were derived from Mantel tests. Note log scales for geographic distances.

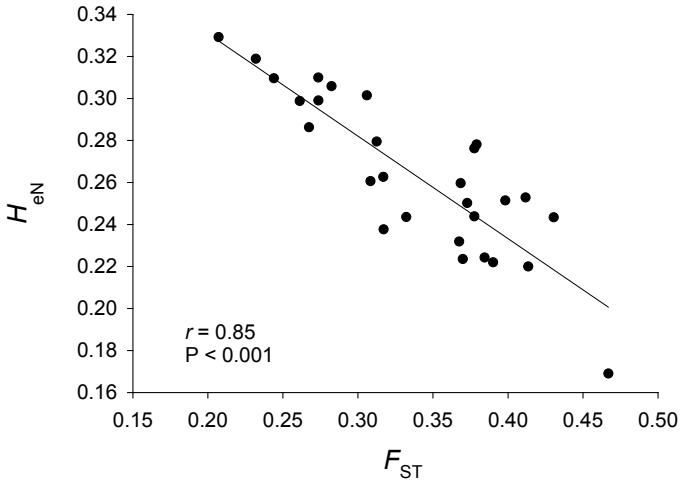

Figure 6. Relation between gene diversity (H_{eN}) and the population specific F_{ST} values in *Saxifraga sponhemica*. The populations (BE25, FR29) with only two samples were excluded from the analysis

distance (Fig. 5a), indicating an isolation by distance (IBD) pattern among the populations. At the regional level, we detected IBD in the western cluster, among the Luxembourg and German populations ($r = 0.49$, $P < 0.001$, Fig. 5b), but not in the eastern cluster, among the Czech populations ($r = 0.22$, $P = 0.20$). However, because of the lower number of populations in the east, the statistical power to detect IBD in the east was much lower than in the west.

Nei's gene diversity of a population strongly decreased with its specific F_{ST} value (Fig. 6, $r = -0.85$, $P < 0.0001$), and the proportion of polymorphic loci less strongly ($r_s = -0.39$,

$P < 0.05$), indicating that populations were not in migration-drift equilibrium. The populations BE25 and FR29 (Table 1) which consisted of only two individuals were omitted from this analysis. Using the program BAYESCAN 2.01 (Foll and Gaggiotti 2008), nine loci (15 % of all loci) were identified as outliers and were considered to be putatively under selection or linked to loci under selection. Divergence of five loci (8 %) was higher and that of four (7 %) significantly lower than under a neutral expectation indicating that directional selection may be occurring at similar frequency as balancing selection. We identified two principal components (PCs) by PCA with varimax rotation on climate variables. PC1 explained 6 7 % of the variation and was mainly correlated with temperature seasonality ($r = -0.94$), minimum temperature ($r = 0.83$) and annual precipitation ($r = 0.82$), indicating that PC1 represented decreasing continentality. PC2 explained a further 22 % of the variation and was highly correlated with maximum temperature ($r = 0.98$). Multiple logistic regressions indicated that the frequency of four of the five putative loci under diversifying selection were related to either one or both of the continentality and temperature gradients (Table 4). An AMOVA of a reduced data set without the putatively non-neutral molecular markers resulted in a slightly lower Φ_{ST} value than the AMOVA based on the whole dataset ($\Phi_{ST} = 0.355$ vs. 0.377, respectively).

Table 4 Intercepts and regression coefficients from multiple logistic regression analyses of the relationship between the frequency of four loci putatively under diversifying selection in populations of *Saxifraga sponhemica* and two principal components describing bioclimatic gradients (continentality and maximum temperature) among the sites. Probabilities from Wald tests: * $P < 0.1$, ** $P < 0.01$, *** $P < 0.001$

Locus	*df*	Intercept	$b_{(PC\_CONTIN)}$	$b_{(PC\_MAXTEMP)}$
A09E	29	-0.5177	0.2382 *	0.3999 ***
B07A	29	-0.0360	-0.3946 **	-0.7673 ***
B17F	29	-0.5705	2.0834 ***	-0.3277
B18F	29	0.0588	1.1727 ***	0.8686 ***

Spatial genetic structure within populations

Spatial autocorrelation analysis within populations based on observations across all populations revealed a significant spatial genetic structure within populations of *S. sponhemica* in agreement with an isolation by distance model (Fig. 7). Mean kinship coefficients decreased with distance between plants in the populations ($b = -0.039$, $P < 0.001$), indicating that individual plants growing close together had a higher probability to be genetically related than plants separated by larger distances. Positive values of the mean kinship coefficient were obtained at small geographical distances (<1.5 m), suggesting that neighbouring individuals are genetically more closely related than random pairs of individuals within the populations (Fig. 7). The value for the *Sp* statistic was 0.041 with $F_{(1)} = 0.030$.

The analysis of RAPD phenotypes differing by up to three bands (4.3 % error rate) revealed that 13.7 % of samples were part of 23 putative clonal lineages. Each putative clone was restricted to a single transect. The distance between members of the same putative clone ranged from 0.15 to 6.95 m.

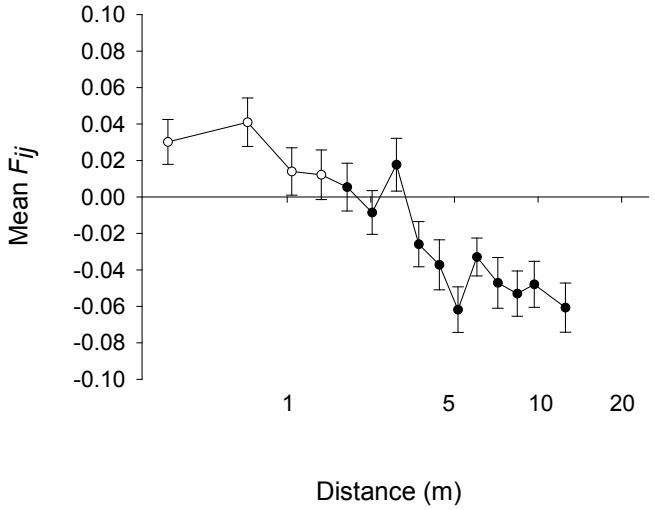

Figure 7. Mean kinship coefficient between pairs of individuals in 30 populations of *Saxifraga sponhemica* that grow at different distances from each other assessed using 61 RAPD markers. Each of the 15 distance classes involves 202-205 pairs of individuals and the total sample consisted of 352 individuals. Means ± 1 SE. The *open symbols* represent significant mean kinship coefficients ($P < 0.05$)

DISCUSSION

Population genetic structure

We found in *S. sponhemica* a strong correlation between genetic and geographical distance, indicating an isolation by distance (IBD) pattern due to gene flow among geographically close populations. However, the current gene flow between most of the populations is very low or absent, because the current distribution is highly disjunct, most extant populations are strongly isolated from each other, and the seeds have no special adaptations for long-range dispersal. The observed pattern of IBD may thus reflect historical gene flow, probably dating back to the last glaciation when suitable open habitats for *S. sponhemica* like screes and rock cliffs were much more abundant (’t Mannetje 2007) and the species was likely to be more common. After the immigration and spread of trees, the populations of *S. sponhemica* would have been restricted to the few remaining open, treeless habitats and been strongly fragmented. A likely explanation for the preservation of the historical genetic pattern is the longevity and clonality of *S. sponhemica*, which slows down genetic drift (Aguilar et al. 2008). Other studies of long-lived ice age relicts,

such as *S. paniculata* (Reisch et al. 2003) and *Dodecatheon amethystinum* (Oberle and Schaal 2011) have also found IBD among populations that are today strongly isolated.

The results of the STRUCTURE and PCoA analyses suggest that the genetic population structure of *S. sponhemica* is hierarchical and consists of three clusters substructured into several populations. The identified genetic groups were, however, not completely concordant with the geographic regions. The distinctness of the populations in the Belgian Ardennes, despite their close proximity to the populations in Luxembourg and Germany suggests that the Belgian populations have been separated for a longer time. The Φ_{ST} value of 0.377 found for *S. sponhemica* is comparable to the average Φ_{ST} values found in a review of a large number of studies that have used dominant molecular markers (RAPDs and AFLPs) to study genetic differentiation in plants (0.34 and 0.35, respectively) and to the mean for other plants with a mixed mating system (0.40, Nybom 2004). The strong genetic differentiation even between *S. sponhemica* populations that are only a few kilometre from each other indicate low gene flow due to restricted pollinator movement and very limited seed dispersal (Ægisdóttir et al. 2009, Colling et al. 2010) between the cliff and scree habitat patches although the seeds of *S. sponhemica* are very small. This is supported by the significant differentiation between subpopulations (transects) within populations.

Loci under natural selection

Most studies on population genetic structure using molecular genetic markers have assumed that these markers are selectively neutral. However, recent genome scan studies showed that a significant amount of among population variation can be due to selection (Strasburg et al. 2012, Manel et al. 2012). In *S. sponhemica*, we found that 15 % of the studied RAPD markers could be considered to be non-neutral outliers, which may be linked to loci under directional or balancing selection, a proportion similar to that found in other genome scan studies (0.4-35.5 %, Strasburg et al. 2012, 10 %, Manel et al. 2012). Variability in four of the loci putatively under selection showed a strong association with climatic gradients, suggesting adaptive genetic variation in response to climate. Climatic factors are strong selective forces and a number of studies have found that genetic variation among natural populations was related to climatic gradients (e.g., Jump et al. 2006, Richardson et al. 2009, Poncet et al. 2010, Cox et al. 2011, Manel et al. 2012). Adaptive genetic variation is important for the potential of a species to adapt to environmental changes, such as ongoing climate change (Hoffmann and Willi 2008, Manel et al. 2012), provided that favourable alleles can spread. However, gene flow is very unlikely among the widely disjunct regions in which *S. sponhemica* occurs.

Within population genetic structure

We found a significant spatial genetic structure within subpopulations at small distances, indicating restricted gene flow in agreement with an isolation by distance model. The *Sp* value of 0.041 (with $F_{(1)} = 0.030$) over all loci was similar to the mean for species with a mixed mating system (0.037, Vekemans and Hardy 2004). Positive mean kinship values indicated that neighbouring individuals (<1.5 m) were genetically more closely related than random pairs of individuals. This could be due to the mixed mating system of *S. sponhemica* that allows geitonogamy, in combination with restricted pollen and seed dispersal. In the sampled *S. sponhemica* populations, putative clones occurred at a low proportion (13.7 %), but over considerable distances (up to 7 m) through the detachment of rosettes.

Genetic diversity within populations

Many studies have found that the genetic variability of populations of rare plants is lower than that of common species (see Gitzendanner and Soltis 2000, Cole 2003, Nybom 2004, references therein), a pattern that was also found in a comparison of 14 rare and common species of the Saxifragaceae (Soltis and Soltis 1991). This has been attributed to genetic drift in the often small and isolated populations of rare plants. In contrast, despite their long isolation, the overall genetic diversity of populations of *S. sponhemica* was similar to that found in other species with a mixed mating system (Nybom 2004). Similarly, other central European ice age relicts, such as *S. paniculata* (Reisch et al. 2003), and *S. aizoides* (Lutz et al. 2000), that occur on rocks have also maintained high genetic diversity despite a fragmented distribution. The maintenance of genetic diversity of these plants is probably due to their longevity and the long-term stability of their habitats (Young et al. 1996, Tang et al. 2010). High gene diversity, combined with moderate to high genetic differentiation and IBD has been found in a number of other cliff species, such as *Centaurea wiedemanniana* (Sözen and Özaydin 2010), *D. amethystinum* (Oberle and Schaal 2011), *Dracocephalum austriacum* (Dostálek et al. 2010), and *Taihingia rupestris* (Tang et al. 2010).

Many studies have found a relationship between the size of the populations and their genetic diversity (Leimu et al. 2006), because small populations have lost variation through genetic drift. In contrast, the genetic diversity of small *S. sponhemica* populations was comparable to that of larger populations. Strong reductions in genetic variability have been found in small populations of plants with a shorter generation time (Fischer and Matthies 1997, Aguilar et al. 2008). The longevity and the long-term stability of habitats of *S. sponhemica* might have buffered the effects of drift on genetic diversity. However, the strong relationship between genetic diversity and population specific F_{ST} values (Fig. 5) indicating a strong gene flow drift disequilibrium suggests that genetic drift has modified the population genetic structure of *S. sponhemica* since the postglacial isolation

of the remnant populations due to forest recolonisation. The particularly low proportion of polymorphic loci in the French Jura populations could be due to founder effects during postglacial warming, because these populations are situated in valleys that had beencovered by ice during the last glaciation (Bichet and Campy 2008).

Conclusions

Our results suggest that long-lived plant species like *S. sponhemica* can maintain historic genetic patterns despite mostly small population sizes and strong isolation. Today, the distribution of *S. sponhemica* is disjunct, consisting of groups of populations that have been strongly isolated from each other for a long time by the spread of trees after the ice age. The extant populations are even at a small geographical scale genetically differentiated, indicating low current gene flow. However, populations still show an isolation by distance pattern, suggesting that the underlying population genetic patterns in *S. sponhemica* were shaped by historical gene flow among interconnected populations during the last ice age.

Fragmentation of populations can result in genetic erosion, i.e., loss of genetic diversity and increased inbreeding (Young et al. 1996, Ouborg et al. 2006) which in turn may result in reduced fitness of plants (Ouborg et al. 1991, Fischer and Matthies 1998, Reed and Frankham 2003). In *S. sponhemica*, considerable genetic variability has been preserved in most populations. We identified several non-neutral markers whose occurrence correlated with climatic gradients, indicating that there is genetic differentiation among populations in traits under selection.

Saxifraga sponhemica is a rare Central European endemic species with few extant populations. Although the habitats of the species are stable and most populations do not appear to be threatened in the short term, extinction of populations due to habitat destruction has been observed (Walisch unpubl.). The small number of populations thus presents a threat to the overall survival of the species. The creation of new populations in suitable habitats within the different regions might thus be considered. Because of the significant clines in non-neutral markers, seeds from the same region should be used to avoid potential maladaptation to local conditions (Becker et al. 2006, 2008).

ACKNOWLEDGMENTS

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Chapter 3

Divergent selection along climatic gradients in a rare central European endemic species, *Saxifraga sponhemica*

published in *Annals of Botany* 115(7), 1177-1190 (2015)
with Guy Colling, Melanie Bodenseh and Diethart Matthies

ABSTRACT

Background and Aims: The effects of habitat fragmentation on quantitative genetic variation in plant populations are still poorly known. *Saxifraga sponhemica* is a rare endemic of Central Europe with a disjunct distribution, and a stable and specialized habitat of treeless screes and cliffs. This study therefore used *S. sponhemica* as a model species to compare quantitative and molecular variation in order to explore (1) the relative importance of drift and selection in shaping the distribution of quantitative genetic variation along climatic gradients; (2) the relationship between plant fitness, quantitative genetic variation, molecular genetic variation and population size; and (3) the relationship between the differentiation of a trait among populations and its evolvability.

Methods: Genetic variation within and among 22 populations from the whole distribution area of *S. sponhemica* was studied using RAPD (random amplified polymorphic DNA) markers, and climatic variables were obtained for each site. Seeds were collected from each population and germinated, and seedlings were transplanted into a common garden for determination of variation in plant traits

Key Results: In contrast to previous results from rare plant species, strong evidence was found for divergent selection. Most population trait means of *S. sponhemica* were significantly related to climate gradients indicating adaptation. Quantitative genetic differentiation increased with geographical distance even when neutral molecular divergence was controlled for, and Q_{ST} exceeded F_{ST} for some traits. The evolvability of traits was negatively correlated with the degree of differentiation among populations (Q_{ST}), i.e. traits under strong selection showed little genetic variation within populations. The evolutionary potential of a population was not related to its size, the performance of the population or its neutral genetic diversity. However, performance in the common garden was lower for plants from populations with reduced molecular genetic variation, suggesting inbreeding depression due to genetic erosion.

Conclusions: The findings suggest that studies of molecular and quantitative genetic variation may provide complementary insights important for the conservation of rare species. The strong differentiation of quantitative traits among populations shows that selection can be an important force for structuring variation in evolutionary important traits even for rare endemic species restricted to very specific habitats.

INTRODUCTION

Selection, drift and gene flow shape genetic variation within and among natural populations and their study is important for conservation and evolutionary biology (Merilä and Crnokrak 2001, Leinonen et al. 2008, 2013). Habitat destruction and fragmentation affect these processes by reducing the size and increasing the isolation of populations (Ellstrand and Elam 1993, Schemske et al. 1994). Plants in small and isolated populations often have a lower performance than those in larger, interconnected populations due to increased inbreeding (Ellstrand and Elam 1993, Fischer and Matthies 1998, Kéry and Matthies 2004). They have lower levels of genetic variation, limited evolutionary potential (Schemske et al. 1994, Young et al. 1996, Willi et al. 2006, Aguilar et al. 2008, Weber and Kolb 2014) and they are more strongly threatened by random environmental fluctuations (Matthies et al. 2004). As a consequence, fragmented populations have a higher risk of becoming extinct (Young et al. 1996).

A recent meta-analysis confirmed a generally positive relationship between the size, the molecular genetic variation and the fitness of plants in populations (Leimu et al. 2006). Neutral molecular genetic variation has been studied extensively, but it is often not or only weakly correlated with adaptive variation and does not inform about the evolutionary potential of populations (Reed and Frankham 2001, Vitt and Havens 2004, Volis et al. 2005, Leinonen et al. 2008). Knowledge of the potential for adaptation to changing environmental conditions is particularly important for rare and threatened species to plan appropriate conservation management (Ye et al. 2013, Weber and Kolb 2014), but there are very few studies of quantitative genetic variation in rare plants (Kramer and Havens 2009).

Genetic variation in quantitative traits of plants can be studied by growing plants from different seed families and populations in a common garden (Vitt and Havens 2004). Moreover, combining studies of adaptive quantitative plant traits (Q_{ST}) and of non-adaptive molecular markers (F_{ST}) makes it possible to estimate the relative contributions of drift and selection to the overall genetic differentiation among populations (Spitze, 1993, Merilä and Crnokrak, 2001). When Q_{ST} and F_{ST} are similar, genetic drift alone can account for the observed genetic differentiation, whereas if Q_{ST} and F_{ST} differ, selection has also shaped differentiation among populations. If Q_{ST} is larger than F_{ST}, the quantitative genetic differentiation is larger than expected by drift alone, and the difference could be assigned to divergent selection and adaptation to local environments, but, if Q_{ST} is smaller, convergent spatially uniform selection could have favoured the same genotypes at different sites (Volis et al. 2005). Although Q_{ST} - F_{ST} comparisons have been criticized (eg. Goudet and Martin 2007, Whitlock 2008, Edelaar et al. 2011), studies based on Q_{ST} - F_{ST} comparisons have provided valuable insights into the causes of spatial genetic divergence

among populations, and the number of studies is growing (Leinonen et al. 2008). Recent meta-analyses have shown that Q_{ST} values are on average higher than F_{ST} values (Leinonen et al. 2008, De Kort et al. 2013) suggesting an important role for directional selection in natural populations.

Evidence for unifying selection ($Q_{ST} < F_{ST}$) is scarce, but has been found in the rare plant species *Clarkia dudleyana* (Podolsky and Holtsford, 1995), *Brassica insularis* (Petit et al. 2001), *Scabiosa columbaria* (Scheepens et al. 2010a) and *Psilopeganum sinense* (Ye et al. 2013). Quantitative variation may be similar to molecular genetic variation if selection has not had enough time to drive divergence (Whitlock and McCauley 1999). In small and isolated populations, Q_{ST} tends to equal F_{ST} because drift is enhanced and the effectiveness of selection is reduced (Gravuer et al. 2005, Johansson et al. 2007). In the rare *Liatris scariosa* var. *novae-angliaea* Q_{ST}-values similar to F_{ST} were found for most traits, possibly due to increased drift and a lower responsiveness to selection (Gravuer et al. 2005). In contrast, a recent study of *Ranunculus reptans* found enhanced directional selection and drift in small populations (Willi et al. 2007). In spite of their usefulness for detecting variation among populations in traits important for evolutionary responses to environmental changes, Q_{ST} - F_{ST} comparisons have only rarely been used in a conservation context (Gravuer et al. 2005, Leamy et al. 2014). Furthermore, to assess the potential of rare species to respond to global change, it will be necessary to know whether traits that are strongly differentiated among populations and are important for adaptation to local conditions maintain sufficient variation (evolvability) within populations. However, little is known about the relationship between the evolvability and among-population differentiation of traits in rare species.

Our model species *Saxifraga rosacea* Moench subsp. *sponhemica* (C.C. Gmel.) D.A. Webb (hereafter referred by its synonym *S. sponhemica* C.C. Gmel) is a rare endemic of Central Europe. Because of its disjunct distribution and its stable and specialized habitat type (treeless screes and cliffs), the species is considered to be an ice age relict (Thorn 1960, Walter and Straka 1970). Because populations of *S. sponhemica* have evolved in long-term fragmented habitats they provide a suitable study system to investigate the joint effects of drift, gene flow and selection in naturally fragmented populations. Moreover, due to its narrow habitat requirements, *S. sponhemica* is a good model species for studying the effect of climatic gradients on genetic differentiation in rare plants, because there is likely to be little variation in habitat conditions apart from climate. A previous study using random amplified polymorphic DNA (RAPD) markers has shown that isolated *S. sponhemica* populations have preserved considerable levels of molecular genetic diversity, presumably because the taxon is longlived (Walisch et al. 2015a). Levels of among-population variation of molecular marker loci were also high. Here, we present

the results of a study of the quantitative genetic variation within and among *S. sponhemica* populations and the performance of plants in a common garden to address the following questions. (1) What has been the relative importance of drift and selection in shaping the distribution of quantitative genetic variation? In particular, is there evidence for adaptive differentiation along climatic gradients for the rare *S. sponhemica*? (2) Do the fitness of plants, quantitative genetic variation, molecular genetic variation and population size correlate positively? (3) Is the differentiation of a trait among populations and its evolvability positively correlated?

MATERIALS AND METHODS

Study species

Saxifraga sponhemica is a perennial cushion plant of rocks and screes whose habitat is naturally fragmented. Flowers of *S. sponhemica* are strongly protandrous. Selfpollination within a flower is possible and geitonogamy is probably quite common (Webb and Gornall 1989). According to a recent study in a large population in Luxembourg, *S. sponhemica* has a mixed mating system with a moderate selfing rate (Walisch et al. unpublished). *S. sponhemica* has a disjunct distribution in Europe. It occurs in three separate areas (1) In the Belgian Ardennes, the Luxembourg Oesling and the German Rhineland, (2) in some isolated locations in the French Jura, and (3) in the Czech Bohemian low mountains (České

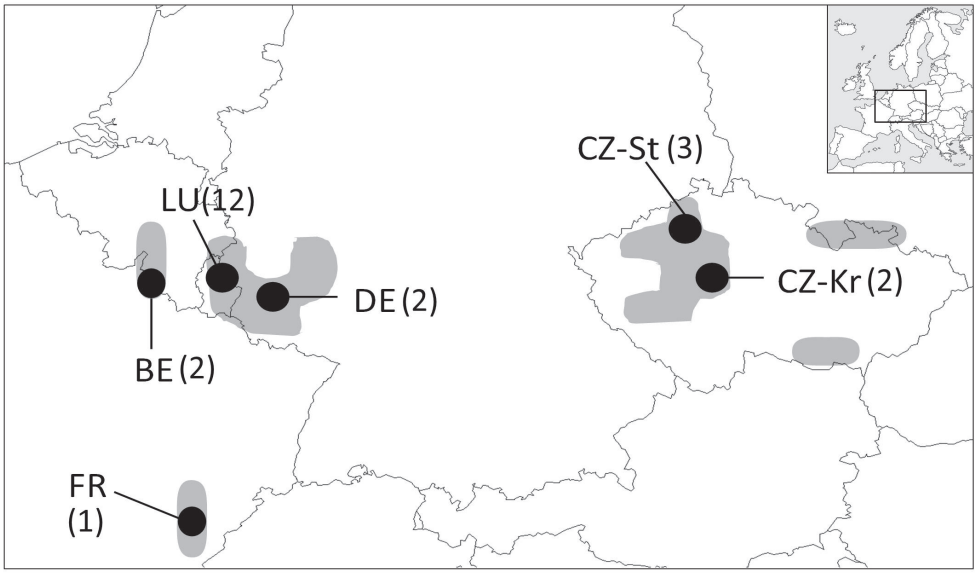

Figure 1. Distribution area (in grey) of *Saxifraga sponhemica* (modified from Jalas and Suominen, 1976). The sampling regions are marked as black dots on the map and the number of study populations are given in parentheses. LU, Luxembourg; DE, Germany; BE, Belgium; FR, France; and five populations in two regions of the Czech Republic: three in České středohoří (CZ-St), two in Český kras (CZ-Kr).

středohoří) and the Czech Bohemian Karst region (Českŷ kras) with isolated populations in the south of Moravia and in the Polish Sudetes (Fig. 1, Jalas et al. 1999, Webb and Gornall 1989). In most parts of its distribution, *S. sponhemica* is considered to be extremely rare or critically endangered, and is legally protected (Korneck et al. 1996, Holub and Procházka 2000, Colling 2005, Mirek et al. 2006). *S. sponhemica* is listed as a species of conservation priority in Central Europe (Schnittler and Günther 1999).

Study populations and bioclimatic variables

In summer 2002 and 2003 we visited 22 populations of *S. sponhemica*, 12 in Luxembourg, two in Belgium, two in Germany, one in France and five in the Czech Republic (Fig. 1, Table 1). We determined the position of each population with a GPS, counted the number of cushions, noted whether it occurred on a rock face, a scree or a stone wall and estimated its main exposition as the absolute deviation from north in degrees. In small

Table 1. Populations of *Saxifraga sponhemica* sampled in Belgium (BE), Germany (DE), Luxembourg (LU) and the Czech Republic (CZ). *N*, number of plants in population; N_{fam}, number of seed families in the garden, No. of plants per family, mean number of plant per seed family grown in the garden, orientation, absolute deviation from north.

Geographical Region	Population and habitat	*N*	N_{fam}	No. of plants per family	Orientation (°)	Location (latitude/ longitude)
Oesling (LU)	Bettel, rock	465	12	6.1	25	N 49.923/E 6.218
	Bettel-Vianden, rock	536	10	6.4	15	N 49.923/E 6.219
	Kautenbach, rock	300	11	5.0	10	N 49.952/E 6.016
	Michelau-Erpeldange, scree	10	11	7.3	46	N 49.945/E 6.027
	Michelau-Erpeldange, scree and wall	250	12	7.4	9	N 49.894/E 6.115
	Michelau-Erpeldange, rock	188	12	6.1	30	N 49.893/E 6.115
	Unterschlinder, wall	96000	12	7.7	63	N 49.892/E 6.112
	Unterschlinder, rock	326	13	7.5	34	N 49.926/E 6.076
	Vianden parking, rock	100	13	7.3	20	N 49.922/E 6.072
	Vianden - Roth, rock	157	14	6.6	68	N 49.935/E 6.198
	Vianden tower, wall	66	12	6.3	10	N 49.929/E 6.225
	Vianden castle, wall	1100	7	7.4	57	N 49.933/E 6.208
Mid-Rhine (DE)	Loreleifels, rock	14	6	6.7	30	N 49.680/E 7.288
	Hammerstein crossroads, rock	58	10	5.8	40	N 49.690/E 7.289
Jura (FR)	Planches-sur-Arbois, scree	50	9	5.8	30	N 46.879/E 5.813
Ardennes (BE)	Bouillon below castle, rock	199	6	6.0	68	N 49.793/E 5.064
	Bouillon Bastion Bretagne, wall	27	7	4.3	5	N 49.797/E 5.069
České středohoří (CZ)	Ostrý, scree	405	9	3.7	20	N 50.532/E 13.951
(CZ-St)	Boreč, scree	90	6	6.5	35	N 50.515/E 13.990
	Blešno, scree	125	7	4.7	10	N 50.482/E 13.906
Český kras (CZ-Kr)	Voškov, rock and scree	150	6	6.3	17	N 49.918/E 14.197
	Tetínské Skály, rock	600	5	5.0	37	N 49.950/E 14.107

populations we determined the number of plants by counting all cushions, while in large populations the total number of plants was estimated by extrapolating counts from parts of the total population area. In subsequent analyses, we combined walls and scree habitats, because in some sites walls had been built at the bottom of screes to hold them, and in these sites plants occurred in both habitat types (Table 1).

We obtained the following bioclimatic variables for each study site (interpolations of observed climate data, representative of 1950-2000) in a grid size of about 1 km^2 (30 arc s) from the Worldclim database version 1.4. (Hijmans et al. 2005): mean annual temperature, minimum temperature of coldest month, maximum temperature of the warmest month, mean diurnal temperature range, temperature annual range, temperature seasonality (SD), annual precipitation, precipitation of the wettest month, precipitation of the driest month and precipitation seasonality (CV). Because these variables were intercorrelated, we identified three principal components (PCs) by principal component analysis (PCA) with varimax rotation (Table 2). The correlations of the PCs with the climatic variables indicated that the first PC (PC CONTIN, 57.6% of the variation) represented continentality, the second (PC SUMMWARM, 24.7% of variation) summerwarmth and the third PC (PC PRECIP, 15.6% of variation) a gradient of precipitation. Continentality was related to the longitude east of the sites of origin ($r = 0.91$, $P < 0.001$) whereas summerwarmth ($r = -0.49$, $P < 0.05$) and precipitation decreased with increasing latitude ($r = -0.61$, $P < 0.01$).

Collection of seeds and cultivation of plants

In 2002-2003 we sampled 5-14 plants (cushions) depending on the number of accessible plants along a transect of 10-15 m length in each of the 22 populations (Table 1). Within

Table 2. Correlations between climatic variables and three principle components extracted from these variables (varimax rotation). The three principle components accounted for 97.6 % of the variation. The strongest correlations ($|r| > 0.7$) are in bold face.

Climate variable	PC1 CONTIN	PC2 SUMMWARM	PC3 PRECIP
Temperature seasonality (SD)	**0.97**	0.04	-0.21
Min. temperature of coldest month	**-0.96**	0.24	0.08
Temperature annual range	**0.92**	0.35	-0.14
Precipitation seasonality (CV)	**0.91**	0.07	-0.35
Mean annual temperature	**-0.72**	0.68	0.09
Max. temperature of warmest month	-0.11	**0.98**	-0.10
Mean diurnal temperature range	0.28	**0.92**	0.07
Precipitation of wettest month	0.02	-0.03	**0.99**
Annual precipitation	-0.56	0.01	**0.82**
Precipitation of driest month	-0.65	0.03	**0.75**

each transect, we recorded the distances among the sampled plants in each transect. If possible, samples were taken from plants that were at least 100 cm apart to ensure that each sample came from a different individual. For comparisons between trait values in the field and in the common garden, we determined for each plant its diameter, the total number of flowering stems and the mean length of up to four randomly selected flowering stems from their base to the uppermost flower. One ripe capsule, if available, and the top part of one rosette were collected from each plant. The rosette was placed in a small paper bag and immediately frozen in liquid nitrogen. The samples were then stored at -80 °C for molecular genetic analysis. The capsules were stored in paper bags in a plastic box with silica gel at 6 °C. At the end of February 2007, two batches of 25 seeds per capsule were placed in Petri dishes on moist filter paper and stored in a growth chamber at 20 °C under a 12 h day/12 h night light regime. Petri dishes were randomized every 3-4 days. Seeds were checked for germination every 2 weeks, and up to 15 seedlings per seed family (hereafter referred to as family) of about 1 cm length were selected at random and transplanted into soaked peat pellets ("Jiffy pots"). The plants were randomly placed into trays and received light from fluorescent tubes (Gro-Lux ®, 28 Watt, Osram Sylvania, USA). In June 2007, the plants were transplanted into square pots of 8 x 8 cm filled with low-nutrient soil (138 mg/L N, 108 mg/L P_2O_2, 158 mg/L K_2O) and 1322 plants were cultivated outdoors in the common garden of the Musée national d'histoire naturelle, Luxembourg.

Measurement of quantitative traits

In May 2008, several traits were measured on plants cultivated in the common garden: diameter of the plants and of the largest rosette, number of rosettes, and the reproductive traits number of flowers, number of flowering stems and length of the longest stem. From mid-April 2009 onwards, the onset of flowering of the plants was recorded every 3 d and one flower in the female stage on a secondary branch was collected per flowering plant. The flowers were put into holes in PCR micro-plates without overlapping of the petals and photographed with a digital camera (Nikon Coolpix 995, 3.34 Megapixels). At least two petals per flower were attached to black cardboard sheets and were scanned at a resolution of 600 dpi. Furthermore, one leaf was collected from the base of the longest stem of each plant. The leaves were fixed to a cardboard sheet with self-adhesive tape and scanned at a resolution of 600 dpi together with a length standard. The leaves were then dried and weighed individually.

Using the scans and photo images, we counted the number of leaf lobes and determined the length, width, perimeter and area of leaves and petals with ImageJ 1.42 (National Institute of Health, USA). In addition, we calculated the variables shape of the leaves and petals as the ratio between their perimeter and area, leaf narrowness as the ratio between

leaf length and width, number of flowers per rosette as the ratio between the number of flowers and the number of rosettes of a plant, and specific leaf area (SLA) as the ratio between leaf area and mass. As several of the variables were correlated, we extracted two PCs each from a PCA with varimax rotation of the reproductive and vegetative traits (Table 3). The PCs were treated as quantitative traits. For certain analyses, we divided both vegetative and reproductive traits into morphological and fitness-related life-history traits (Table 3). Of the traits not listed in Table 3, SLA and leaf narrowness were treated as morphological traits and flowering time as a life-history trait.

RAPD-PCR

Molecular genetic data for plants from all study populations were available from a genetic study (Walisch et al. 2015a). For the current study, we used only the data from the sub-set of populations for which quantitative genetic data were available (Table 1). After removing nine band positions that had been identified as putative non-neutral loci (Walisch et al. 2015a), we obtained a final matrix of 287 samples and 52 neutral loci for our 22 study populations. Genetic diversity within populations was estimated with AFLP-SURV as Nei's gene diversity (expected heterozygosity H_{eN}) according to the method of Lynch and Milligan (1994). We calculated F_{ST}, its standard error and genetic distances as pairwise F_{ST} values in AFLP-SURV, and calculated 95% confidence intervals from the standard error of F_{ST}.

Statistical analysis

We studied the effects of population size, habitat type, orientation and of the three principal bioclimatic PCs CONTIN, PRECIP and SUMMWARM on mean vegetative and reproductive traits in populations of *S. sponhemica*. For each plant trait, we calculated the Bayesian information criterion (BIC) of all possible models using the leaps package (version 2.9., Lumley 2009) in R (version 3.0, R core team 2014). We selected the model for which the BIC was minimal and studied the relationship between each plant trait and explanatory variables by multiple regression. We performed a correlation analysis between the following traits of plants that had been measured in a population in the field and in the common garden: plant diameter and the number and mean length of flower stems.

Analyses of variance (ANOVAs) with population and family as factors were conducted for all traits. According to the hierarchical design, the effect of population was tested against the variation between families. Before the analyses, all traits were checked for normally distributed residuals. Leaf area, plant diameter, number of rosettes, number of flowers per plant, number of flowering stalks, and the number of flowers per rosette were square-root transformed, and leaf shape and petal shape were log-transformed. To estimate between-population genetic

Table 3. Loadings of reproductive and vegetative traits of *S. sponhemica* on principal components derived from (a) vegetative (73.7% of variation) and (b) reproductive traits (80.0% of variation) after varimax rotation. The strongest correlations ($|r| > 0.6$) are in bold face. For some analyses, traits were also divided into morphological (M) and life-history traits (Lh).

(a) Vegetative traits	PC LEAF-SIZE (M)	PC PLANT SIZE (Lh)
(M) Leaf area (sqrt)	**0.97**	-0.01
(M) Leaf perimeter	**0.93**	0.01
(M) Leaf length	**0.87**	0.05
(M) Leaf width	**0.86**	-0.01
(M) Number of leaf lobes	**0.61**	0.17
(M) Leaf shape (log)	**-0.82**	-0.02
(Lh) Plant diameter (sqrt)	0.04	**0.93**
(Lh) Number of rosettes (sqrt)	-0.15	**0.86**
(M) Diameter of largest rosette	0.28	**0.74**
(b) Reproductive traits	PC PETAL-SIZE (M)	PC REPRO (Lh)
(M) Petal perimeter	**0.99**	0.05
(M) Petal area	**0.99**	0.04
(M) Petal shape (log)	**-0.97**	-0.05
(M) Petal length	**0.89**	0.07
(M) Petal width	**0.84**	0.02
(M) Diameter of flower	**0.76**	0.05
(Lh) Number of flowers per plant (sqrt)	-0.05	**0.97**
(Lh) Number of flowering stems (sqrt)	-0.07	**0.90**
(Lh) Number of flowers per rosette (sqrt)	0.08	**0.84**
(Lh) Length of the longest flowering stem	0.21	**0.73**

variation (Q_{ST}), heritability (h_2) and evolvability (genetic coefficient of variation, $CV_{genetic}$; Houle 1992), we calculated variance components between populations (V_{pop}), between families within populations (V_{fam}) and between individuals within families (V_{error}) for each trait by restricted maximum likelihood with the varcomp function of the R-package ape version 3.1-4 (Paradis et al. 2004). Heritability (h_2) was calculated as $h_2 = (V_{fam}/2*\theta) / (V_{fam} + V_{error})$, and the evolvability (genetic coefficient of variation) as $CV_{genetic} = \sqrt{(V_{fam}/2* \theta)}/\text{mean}$, where θ is a measure of the kinship of the plants. We used untransformed values to calculate mean evolvability as suggested by Hansen et al. (2011). For selfed plants θ is 0.5, for full-sibs 0.25 and for half-sibs 0.125 (Jimenez-Ambriz et al. 2007). We estimated a selfing rate of 47% after Charlesworth (1988) from the multiplicative fitness function number of flowers per seed calculated for offspring from selfed, open-pollinated, and outcrossed flowers in a large population of *S. sponhemica* in Luxembourg (Walisch et al. unpublished). Knowing that about 50% of our plants originated from selfings we assumed that the other half were full-sibs and used a value of 0.375 for θ. The assumption of full-sibs in the case of unknown relationships between offspring from a family provides conservative estimates of quantitative genetic parameters (Podolsky and Holtsford, 1995). Q_{ST} was thus computed as $V_{pop} / (2* [V_{fam}/2*\theta] + V_{pop}) = V_{pop} / (2.67*V_{fam} + V_{pop})$.

We estimated 95% confidence intervals for Q_{ST} by the jackknife technique following O'Hara and Merilä (2005). We calculated the mean Q_{ST} of the reproductive and vegetative traits as the sum of the numerators divided by the sum of the denominators of the individual Q_{ST} values, after standardizing the sums of the variance components for each trait to 1 as suggested by Chapuis et al. (2007) to avoid that some traits had an undue influence on the overall average. We used regressions to study the relationship between mean evolvability and mean heritability of each trait over all populations, as well as the relationship between mean evolvability and mean heritability per population over all traits.

We estimated the genetic variability of quantitative traits as mean evolvability ($CV_{genetic}$) over vegetative and over reproductive traits, and studied the relationship between quantitative genetic variability and molecular genetic variability by regressions. We also studied the effects of population size, rock as a habitat, orientation and the three bioclimatic PCs CONTIN, PRECIP and SUMMWARM on the mean evolvability of vegetative and reproductive traits and their PCs. For each plant trait, we calculated the BIC for all possible models using the leaps package in R (Lumley 2009, R core team 2014). We selected the model for which the BIC was minimal and studied the relationship between each plant trait and the explanatory variables by multiple regressions.

As a measure of quantitative genetic distances, Mahalanobis distances were calculated for morphological and for life-history traits averaged over families. Mahanalobis distances measure distance in multivariate space taking into account correlations among traits and are independent of the scale of the traits (Legendre and Legendre 1998). We compared the pairwise quantitative genetic distance matrix with the geographic distance matrix while controlling for the effect of neutral genetic drift as measured by pairwise F_{ST} (partial Mantel tests). Significance levels were obtained after performing 1000 random permutations for the Mantel test. Significant partial Mantel correlations suggest that clinal variation in quantitative traits cannot be explained by non-adaptive (i.e., isolation-by-distance) mechanisms alone. We also analysed if there was a relationship between quantitative genetic distances and climatic distances calculated as pairwise euclidian distances based on the three bioclimatic PCs CONTIN, PRECIP and SUMMWARM. All Mantel tests were calculated using the program zt version 1.1 (Bonnet and Van de Peer 2002) with 1000 permutations. All statistical analyses, if not stated otherwise, were carried out with SPSS 19.0 (IBM Corp. 2010)

RESULTS

Principal component analysis of vegetative traits identified two main components. The first component (PC LEAFSIZE) explained 49.8% of the total variation and correlated closely with leaf traits (Table 3a). The second component (PC PLANTSIZE) accounted for 23.9% of the variation and was closely related to variables that measured the size of the plants. The first principal component (PC PETALSIZE) from the PCA of reproductive traits accounted

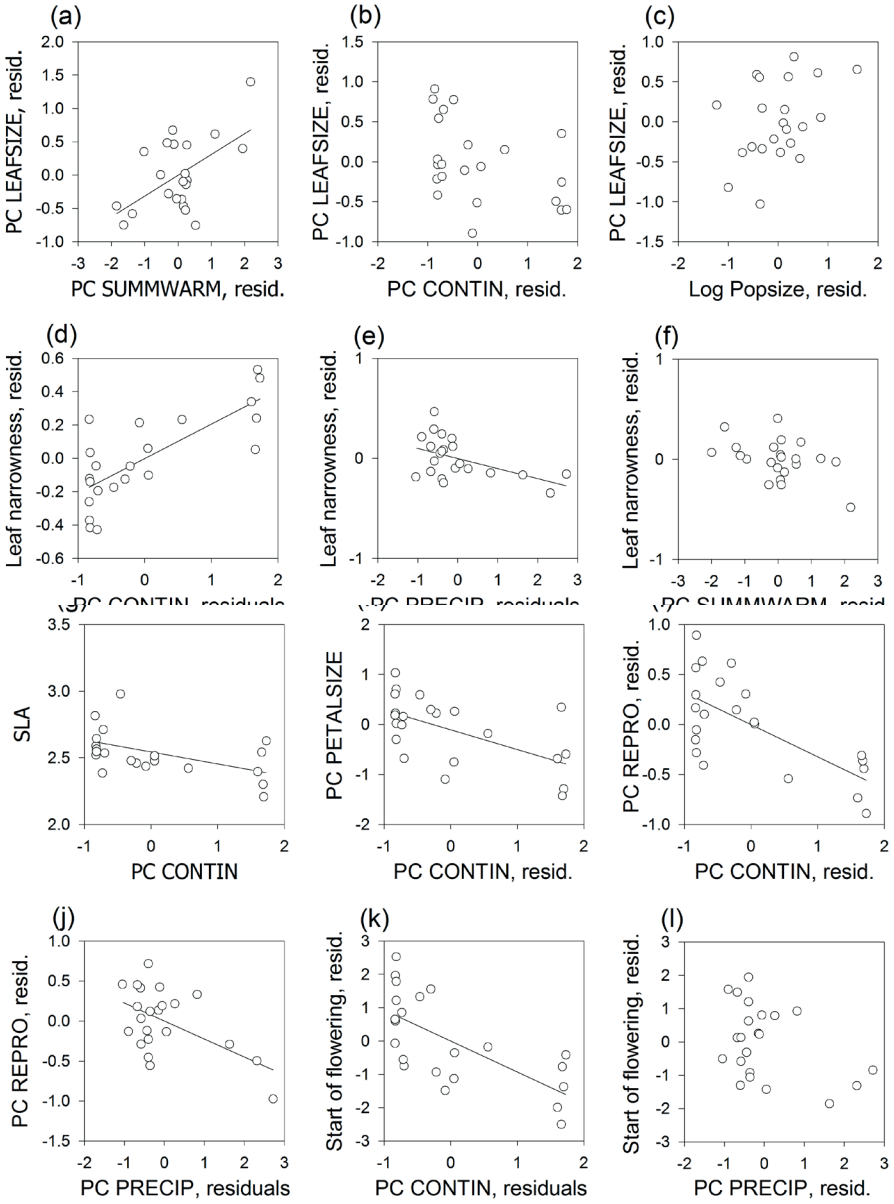

Figure 2. Relationships between (a, b, c) PC LEAFSIZE, (d, e, f) leaf narrowness, (g) SLA, (h) PC PETALSIZE, (i, j) PC REPRO, (k, l) start of flowering of *Saxifraga sponhemica* and significant explanatory variables. Shown are regression plots (g, h) in the case of single explanatory variables and partial regression plots (a, b, c, d, e, f, i, j, k, l) in the case of several explanatory variables. For statistical analysis see Table 4.

for 50.7 % of the variance and was closely related to petal traits such as petal area and perimeter (Table 3b). The second component (PC REPRO) explained 29.3% of the variation and correlated strongly with variables such as the number of flowers and the length of the flowering stalks.

Variation among trait means

The variation among populations and among families within populations of *S. sponhemica* grown was highly significant for all vegetative and reproductive traits, and for the four components extracted from these traits (all $P < 0.01$). To study possible causes of the differences among populations, regression analyses of the influence of habitat characteristics of the populations of origin on the four trait PCs and on three traits that were not related to these components (leaf narrowness, SLA and start of flowering) were carried out.

The size of leaves increased with summer warmth (Fig. 2a) and decreased with continentality of a site (Fig. 2b, Table 4), and PC LEAFSIZE was the only trait for which the best set of predictors included the size of the populations of origin (Fig. 2c). However, in single linear regressions, only plant diameter in the common garden was related to population size ($r = 0.45$, $P < 0.05$). In contrast to leaf size, leaf narrowness decreased with summer

Table 4. The effects of habitat characteristics of the population of origin and its size on mean trait values of plants of *Saxifraga sponhemica* grown in a common garden. (a) Vegetative traits, (b) reproductive traits. We present the models for which the Bayesian information criterion is minimal. Possible explanatory variables were the principal components PC SUMMWARM, PC CONTIN, PC PRECIP, and rock habitat (rock faces = 1, walls and screes = 0), exposition (absolute deviation from north), and population size. No significant relationship with any of the variables was found for PC PLANTSIZE. Only PCs and dependent variables not strongly correlated with the PCs (s. Table 2) were studied. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Dependent variable	r^2	df	F	Explanatory variable	β	*t-value*
(a) Vegetative traits						
PC LEAFSIZE	0.44	18	4.68 *	PC SUMMWARM	0.52	2.84 *
				PC CONTIN	-0.36	-2.04
				Log Popsize	0.33	1.83
Leaf narrowness	0.66	18	11.75 ***	PC CONTIN	0.68	5.00 ***
				PC PRECIP	-0.33	-2.43 *
				PC SUMMWARM	-0.29	-2.09
SLA (sqrt)	0.26	20	6.94 *	PC CONTIN	-0.51	-2.63 *
(b) Reproductive traits						
PC PETALSIZE	0.35	20	10.67 **	PC CONTIN	-0.59	-3.27 **
PC REPRO	0.57	19	12.49 ***	PC CONTIN	-0.62	-4.10 ***
				PC PRECIP	-0.43	-2.86 **
Start of flowering	0.51	19	10.06 **	PC CONTIN	-0.65	-4.06 ***
				PC PRECIP	-0.31	-1.91

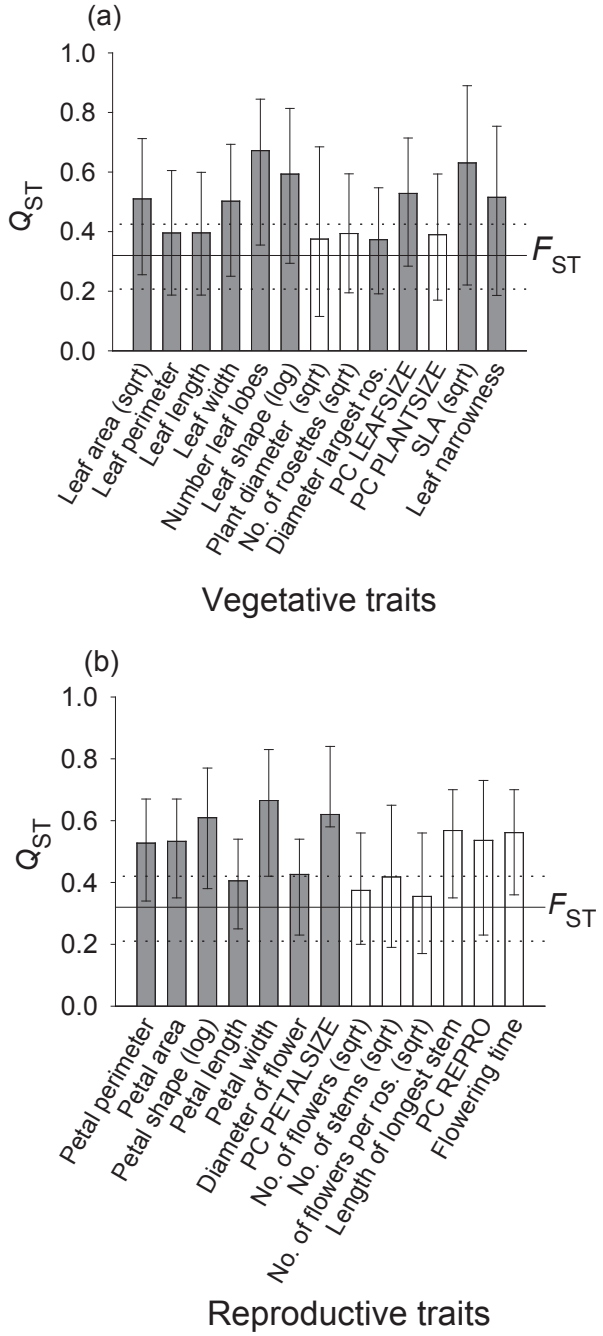

Figure 3. Mean genetic differentiation in quantitative traits between populations (Q_{ST}) for (a) vegetative and (b) reproductive traits of *Saxifraga sponhemica*. Vertical error bars indicate 95% confidence limits of Q_{ST}. White bars indicate life history traits and grey bars morphological traits. Horizontal lines show the 95% confidence limits of F_{ST}.

warmth (Fig. 2f) and increased with continentality (Fig. 2d), but was also related negatively to precipitation at a site (Fig. 2e). SLA (Fig. 2g), petal size (Fig. 2h), reproduction (Fig. 2i), and the starting date of flowering (Fig. 2k) were all related negatively to continentality. In addition, reproduction (Fig. 2j) and the starting date of flowering (Fig. 2l)

decreased with precipitation. Both the mean length of the longest stem in a population in the field and in the common garden ($r = 0.53$, $P < 0.05$) and the number of flower stems per rosette ($r = 0.54$, $P < 0.01$) in a population in the field and the common garden were correlated (see Supplementary Data Fig. S1).

Genetic differentiation among populations

We divided both vegetative and reproductive traits into morphological and life-history traits (Table 2, Fig. 3). Quantitative genetic differentiation among populations (Q_{ST}) was mostly higher for morphological than for life-history traits, both for vegetative (Fig. 3a) and for reproductive traits (Fig. 3b). The Q_{ST} values of all individual traits and the mean Q_{ST} value (0.49) were higher than the F_{ST} value (0.32), suggesting diversifying selection. The mean difference between Q_{ST} and F_{ST} was more pronounced for morphological traits (0.20) than for life-history traits (0.13). However, for only two of the reproductive morphological traits (petal width and PC PETALSIZE) the 95% confidence intervals of the Q_{ST} and the F_{ST} values did not overlap. The coefficients of determination for the most supported regression models relating trait means and explanatory habitat characteristics were positively correlated with the genetic differentiation among populations (Q_{ST}) ($r = 0.39$, Fig. 4), indicating that for traits with a high Q_{ST} habitat characteristics were a better predictor of trait means than for traits with a lower Q_{ST}.

The pairwise quantitative genetic distance (Mahalanobis distance) and the molecular genetic

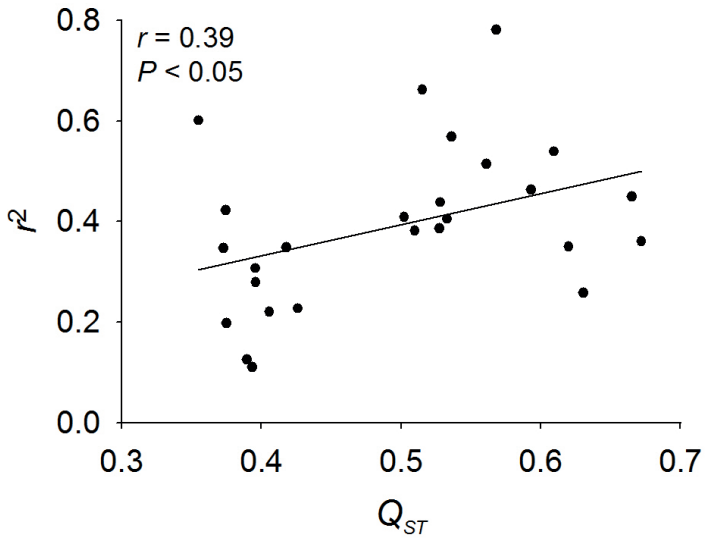

Figure 4. The relationship between the amount of variation (r^2) in a trait that could be explained by environmental differences between sites and the quantitative genetic differentiation (Q_{ST}) between populations for that trait. r^2-values were taken from regression analyses for which the Bayesian information criterion was minimal.

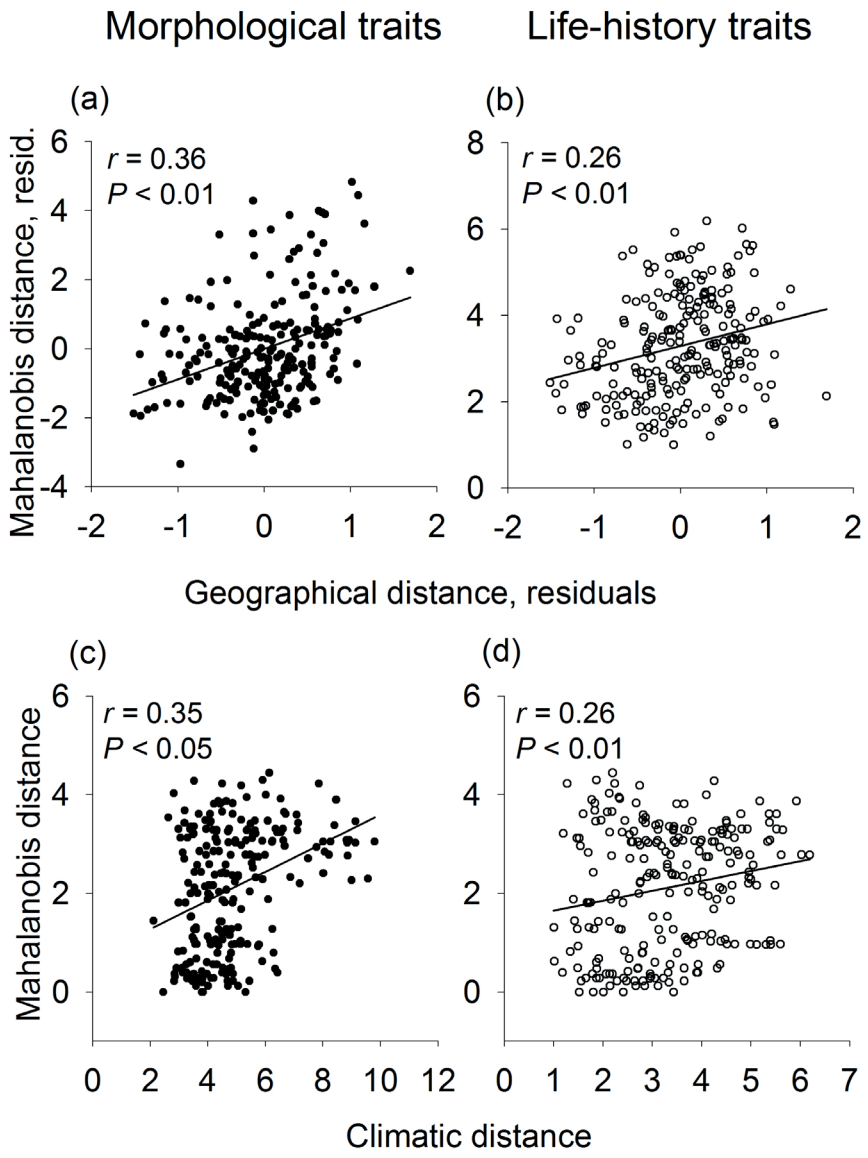

Figure 5. Relationships between pairwise quantitative-genetic distances between populations of *S. sponhemica* based on (a, c) morphological traits (filled symbols) and (b, d) life-history traits (open symbols) and (a, b) pairwise geographical distances and (c, d) pairwise climatic distances. In (a) and (b) partial regression plots are presented, in which the effects of geographical distance are adjusted for those of molecular genetic distance (pairwise F_{ST}).

distance (pairwise F_{ST} value) were correlated for both life-history traits ($r = 0.30$, $P < 0.01$, Mantel test) and morphological traits ($r = 0.28$, $P = 0.06$, Mantel test). In a partial Mantel test the pairwise quantitative genetic distance increased with geographical distance both for morphological and for life-history traits, controlled for the effect of neutral molecular divergence (Fig. 5a, b). This indicates that the quantitative genetic differentiation among populations cannot be explained by non-adaptive (i.e., isolation-by-distance) mechanisms alone but that local adaptation due to diversifying selection also plays a role. Climatic distance

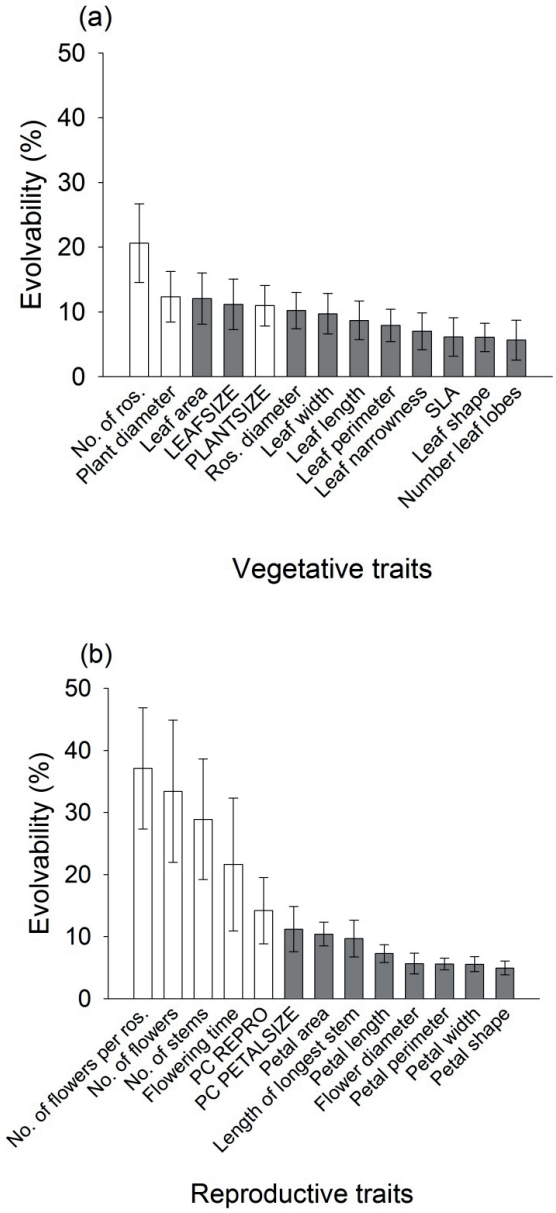

Figure 6. Mean evolvability ± 95% confidence limits of (a) vegetative and (b) reproductive traits of *S. sponhemica*. White bars indicate life-history traits and grey bars morphological traits.

between populations also increased with geographical distance ($r = 0.83$, $P < 0.001$) indicating a clinal variation in climate. Furthermore, quantitative genetic distance was correlated with climatic distance, suggesting that climate is a diversifying selective force (Fig. 5c, d).

Quantitative genetic variation within populations

Quantitative genetic diversity, estimated as evolvability ($CV_{genetic}$), was significantly larger than zero in all traits and was on average higher for reproductive traits (15.0%) than for vegetative traits (9.9%), and particularly high for reproductive traits related to the life-history of the plants, such as the number of flowers and flowering stems per plant and

start of flowering (Fig. 6). In contrast, the evolvability of morphological traits like leaf and petal shape was particularly low. Heritabilities of vegetative traits ranged from 0.095 (SLA) to 0.204 (leaf width) and those of reproductive traits from 0.154 (length of longest stem) to 0.308 (flowering time), and were significantly larger than zero in all traits but did not differ among life-history and morphological traits.

In multiple regressions, the main influences on the genetic diversity of quantitative traits within populations were the summer warmth in the populations of origin and whether populations were growing on rocks or on screes and walls (Table 5). Genetic variability of leaf narrowness, of petal perimeter and of petal area increased with summer warmth, while that of start of flowering decreased. Genetic variability of populations from rock habitats was higher for several traits related to petal size and shape than that of populations from screes and walls. Leaf width was the only trait whose evolvability was related (negatively) to population size. The evolvability of all other traits was not significantly related to population size (all $r < 0.21$, all $P > 0.12$) in simple linear regressions.

Table 5. The effects of habitat characteristics of the population of origin and its size on the evolvability of quantitative traits (genetic coefficient of variation) of *Saxifraga sponhemica* grown in a common garden. (a) Vegetative traits, (b) reproductive traits. We present the models for which the Bayesian information criterion is minimal. Possible explanatory variables were the principal components PC SUMMWARM, PC CONTIN, PC PRECIP, and rock habitat (rock faces = 1, walls and = 0), exposition (absolute deviation from north), and population size. No significant relationship with any of the variables was found for the vegetative traits PC LEAFSIZE, PC PLANTSIZE, leaf area, leaf perimeter, leaf length, number of leaf lobes, leaf shape, plant diameter, number of rosettes, SLA, and rosette diameter; and the reproductive traits PC PETALSIZE, petal length, petal width, number of flowers, number of stems, length of longest stem. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Dependent variable	r^2	df	F	Explanatory variable	β	t-value
(a) Vegetative traits						
Leaf width	0.19	19	4.54 *	Log Popsize	-0.43	-2.13 *
Leaf narrowness	0.45	19	7.90 **	PC SUMMWARM	0.72	3.93 ***
				Rock habitat	-0.38	-2.06
(b) Reproductive traits						
Petal perimeter	0.46	19	8.13 **	Rock habitat	0.44	2.43 *
				PC SUMMWARM	0.37	2.06
Petal area	0.44	19	7.54 **	Rock habitat	0.46	2.51 *
				PC SUMMWARM	0.33	1.79
Petal shape (log)	0.28	20	7.84 *	Rock habitat	0.53	2.80 *
Diameter of flower	0.32	20	9.45 **	Rock habitat	0.57	3.07 **
No. of flowers per rosette	0.25	20	6.64 *	PC CONTIN	0.50	2.58 *
Start of flowering	0.24	19	5.84 *	PC SUMMWARM	-0.48	-2.42 *

Heritability (h^2) and evolvability of each trait in the populations were strongly correlated (all $r > 0.84$, all $P < 0.001$). Averaged over all traits per population, mean evolvability of traits in the populations varied from 7% - 35%. There was a strong positive relationship between mean evolvability and mean heritability of a trait (averaged over all populations) ($r = 0.93$, $P < 0.001$), but no relationship between the mean evolvability and heritability of all traits per population ($r = 0.20$, $P = 0.33$). Mean genetic variation of quantitative traits in a population (mean evolvability) did not increase with molecular genetic variability (Nei's gene diversity) per population, either for vegetative traits ($r = -0.16$, $P = 0.48$) or for reproductive traits ($r = -0.21$, $P = 0.36$). Molecular genetic variation in the populations was also not positively correlated with evolvability of the individual vegetative (r from -0.39 to 0.10, $P > 0.075$) or reproductive traits (r from -0.33 to 0.03, $P > 0.13$). Evolvability and heritability were both negatively related to among-population differentiation (Q_{ST}) of a trait, but the relationship was significant for evolvability only ($r = -0.52$, Fig. 7).

Relationships between fitness-related traits and estimates of genetic diversity

All fitness-related traits increased in the common garden with the molecular genetic diversity of the population of origin (see Supplementary Data Fig. S2): PC PLANTSIZE ($r = 0.53$, $P < 0.05$), plant diameter ($r = 0.44$, $P < 0.05$), number of rosettes ($r = 0.52$), number of flowers ($r = 0.56$) and PC REPRO ($r = 0.52$, all $P < 0.05$). In contrast, traits that were not related to fitness did not correlate with the molecular genetic diversity of the population. Moreover, there was no significant correlation between the mean of any

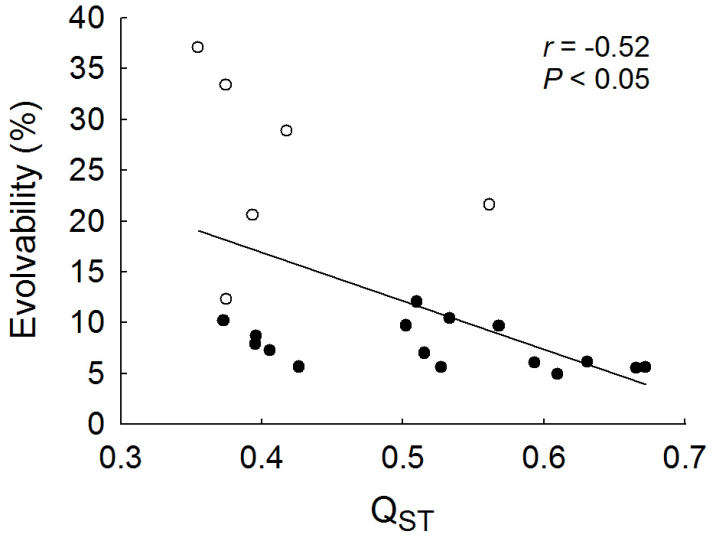

Figure 7. The relationship between mean evolvability of a trait and its differentiation between populations (Q_{ST}) for life-history traits (open symbols) and morphological traits (filled symbols) of *S. sponhemica*. The fitted line is based on both types of traits.

trait and its mean evolvability or heritability in a population (all $|r| < 0.3$, all $P > 0.30$), indicating that performance and morphology were not related to quantitative genetic variation. Fitness traits did not correlate with the mean of the evolvabilities of all measured traits per population (all $|r| < 0.3$, all $P > 0.30$).

DISCUSSION

Evidence for selection

Several lines of evidence suggest that selection shaped the quantitative genetic structure of populations of the rare and highly fragmented *S. sponhemica*. Most population trait means of *S. sponhemica* were significantly related to climate gradients, suggesting adaptive genetic differentiation in quantitative traits among populations (see Whitlock 2008). Such patterns could also result from non-selective processes such as genetic drift (Vasemägi 2006, Kawakami et al. 2011), which is considered to be the primary evolutionary force leading to population divergence among fragmented populations (Lynch 1986, Ye et al. 2013). However, we found significant relationships between overall quantitative genetic distances between populations as measured by Mahalanobis distances and geographical distances, even after controlling for differentiation in neutral molecular genetic variation in partial Mantel tests. This shows that the quantitative genetic differentiation between populations was higher than could be explained by neutral processes. In line with this, we also found for some traits that Q_{ST} was significantly higher than F_{ST}.

In contrast to our results, previous studies of quantitative genetic variation in rare species either found that non-selective processes were sufficient to explain the observed differentiation in quantitative traits among populations, or even found evidence for uniform selection (*Scabiosa canescens*, Waldmann and Andersson, 1998, *Centaurea corymbosa* and *Brassica insularis*, Petit et al. 2001, *Liatris scariosa*, Gravuer et al. 2005, *Primula sieboldii*, Yoshida et al. 2008, *Psilopeganum sinense*, Ye et al. 2013). In small fragmented populations, the effectiveness of selection can be reduced and, because rare species often have a narrow ecological niche, their populations may be exposed to homogeneous selective forces that result in low population differentiation in quantitative traits (Petit et al. 2001, Ye et al. 2013). *S. sponhemica* also has a very restricted range of habitats, but nevertheless showed strong population differentiation related to climate gradients. This could be due to the fact that the maximum distance between the populations of *S. sponhemica* was much larger than in previous studies of rare species. Our study populations were thus exposed to different climatic conditions exerting divergent selective pressures.

Plants from sites with a harsher, more continental climate had smaller, narrower and thicker leaves and smaller petals, flowered earlier and reproduced less than those from more Atlantic

sites. Smaller and thicker leaves could be an adaptation to reduce water loss during warm and dry spells (Scheepens et al. 2010b) at the continental sites. Early flowering could be an adaptation to avoid heat or drought periods (Latta and Gardner 2009, Franks 2011), which may be exacerbated by the dark rock faces or screes in *S. sponhemica* habitats that strongly absorb solar radiation. The earlier timing of reproduction and the harsher climate may have led to the observed lower reproduction and reduced petal size (Obeso 2002) at the more continental sites. A further indication that climatic differences among sites drive local adaptation is given by the positive correlation between quantitative genetic and climatic distances.

Evolvability of traits and its relationship to differentiation among populations

The genetic variability (evolvability) of quantitative traits varied among populations. The larger evolvability of petal size traits of plants which originated from rock faces was possibly due to more spatially variable selection (e.g. Kelly 1992, McLeod et al. 2012) and restricted gene flow within *S. sponhemica* populations (Walisch et al. 2015a), resulting in small-scale local adaptation (Knight and Miller 2004, Paccard et al. 2013). Likewise, the larger evolvability of petal size and leaf narrowness could be caused by a spatially or temporally varying selection due to environmental heterogeneity (eg., Schemske and Horvitz 1989, Kelly 1992, Siepielsky et al. 2009) at sites with warmer summers. Conversely, the evolvability of the start of flowering decreased with warmer summers, and the evolvability of leaf narrowness was lower in rock habitats, suggesting that less adapted genotypes have been filtered out by selection. The loss of genetic variability within populations due to selection is in agreement with quantitative genetic theory (Bulmer 1971, Visscher et al. 2008).

The degree of differentiation among *S. sponhemica* populations (mean Q_{ST}value) decreased with the evolvability of a trait within populations. Heritability showed the same trend as evolvability. In contrast, previous studies of the relationship between heritability and Q_{ST} of traits found non-significant negative (Steinger et al. 2002), non-significant positive (Podolsky and Holtsford 1995, Bonnin et al. 1996) or significant positive correlations (Andersson 1991, Yang et al. 1996, Lynch et al. 1999). A positive relationship has been interpreted as support for the hypothesis that traits with a high evolvability respond more strongly to divergent selective forces (Lynch et al. 1999, Hansen et al. 2011). In contrast, the negative relationship between evolvability and Q_{ST} found for *S. sponhemica* could be the result of differences in the strength of selection for individual traits. Traits under strong selection will tend to show little variability within populations (low evolvability), but will vary strongly among populations (high Q_{ST}) due to local adaptation to strongly diverging environmental conditions. Our results thus suggest that selection shaped quantitative genetic variation not only among, but also within populations of *S. sponhemica*.

Variation in morphological traits was more strongly affected by selection than variation in life-history traits, as shown by the lower evolvability and larger mean Q_{ST}-F_{ST} differences for morphological traits. Furthermore, morphological traits were more strongly correlated with geographic and climatic distances than life-history traits, reinforcing the conclusion that life-history traits showed a weaker response to directional selection than morphological traits. Stronger differentiation among populations for morphological than life-history traits has also been found in other empirical studies (Merilä and Crnokrak 2001, Leinonen et al. 2008, 2013, De Kort et al. 2013). It has been suggested that life-history traits respond more slowly to selection because they are influenced by multiple genes of small effect and by interactions among genes that result in non-additive genetic effects (Kruuk et al. 2008, Morrissey et al. 2012).

Mean evolvability and mean heritability of a trait averaged over all populations correlated strongly, whereas the means over all traits per population did not correlate. The first result is in contrast and the second lends support to the general conclusion of reviews by Houlé (1992) and Hansen et al. (2011) that evolvability and heritability are generally not correlated. It has been suggested that this is due to positive relationships between additive genetic variances and other components of variance (Hansen et al. 2011).

Quantitative genetic variation and its relationship to molecular genetic variation and fitness

Quantitative and molecular genetic variation are both subject to drift and are predicted to decline in small populations (Schemske et al. 1994, Lynch 1996, Young et al. 1996, Aguilar et al. 2008). However, neither molecular (Walisch et al. 2015a) nor quantitative genetic variation in *S. sponhemica* was related to population size. There are several non-mutually exclusive explanations for the absence of an effect of population size on quantitative variation. Either stabilizing or divergent selection within populations may have over-ridden the effects of genetic drift (Reed and Frankham 2001), effective population sizes may have been different from census sizes (Lynch and Hill 1986), and there may have been insufficient generations at a small size to reduce genetic diversity in the long-lived *S. sponhemica* (Walisch et al. 2015a). Other field studies have produced varying results. *Salvia pratensis* and *Scabiosa columbaria* (Ouborg et al. 1991), *Ranunculus reptans* (Willi et al. 2007), and *Phyteuma spicatum* (Weber and Kolb 2014) showed an increase in phenotypic variation with increasing population size, whereas for *Senecio integrifolius* quantitative genetic variation was larger in a small subdivided population than in a large interconnected population (Widén and Andersson 1993). In several other species no relation between heritability and population size was detected (Widén and Andersson 1993, Waldmann and Andersson 1998, Podolsky 2001, Widén et al. 2002, Steinger et al. 2002, Gravuer et al. 2005, Ellmer et al. 2011).

The fitness of plants tends to be reduced in small and fragmented populations (Fischer and Matthies 1998, Leimu et al. 2006), because of genetic erosion (Aguilar et al. 2008, Young et al. 1996) and increased inbreeding (Ellstrand and Elam 1993). In *S. sponhemica*, all fitness traits were significantly reduced in populations with low molecular variability, suggesting inbreeding depression. In contrast, plant fitness did not correlate with the evolvability of a trait or with the mean evolvability of all traits per population. Molecular variation appears to be a better predictor of inbreeding effects in populations than variation in quantitative traits.

Neither mean evolvability nor the evolvability of individual traits was related to the molecular genetic diversity of *S. sponhemica*. Because of the strong influence of selection on variation in quantitative traits observed in *S. sponhemica* this is not surprising. Our results contribute to the increasing body of evidence that studies of molecular variation provide little insight into variation of traits important for adaptation to changing conditions (Reed and Frankam 2001, McKay and Latta 2002, Steinger et al. 2002, Vitt and Havens 2004, Gravuer et al. 2005). To assess the amount of adaptive genetic variation present in populations of rare species, conservation studies should thus investigate quantitative traits and not molecular markers.

CONCLUSIONS

We found that selection has strongly influenced the variation in quantitative traits within and among populations of *S. sponhemica*. This is in agreement with the conclusions of general meta-analyses of the role of selection for population differentiation in plants (Merilä and Crnokrak 2001, Leinonen et al. 2008, 2013, De Kort et al. 2013), but in contrast to the results of other studies on rare plant species (Gravuer et al. 2005). Our results indicate that selection can be an important force for structuring variation in evolutionary important traits even for rare endemic species restricted to very specific habitats, if climatic conditions vary. The negative relationship between the differentiation of a trait among populations of *S. sponhemica* and its evolvability indicates that those traits that are important for adaptation to a changing climate have the least evolutionary potential within populations. Adaptation to climate change in *S. sponhemica* may thus require gene flow between populations, which is, however, unlikely due to the strong fragmentation of the populations.

We found no significant relationships between the genetic variability of any of the quantitative traits and molecular genetic variability. Our results thus support the conclusion that molecular genetic variation is not suitable to predict the short term evolutionary potential of populations or population divergence, because of the importance of selection

for the variation in quantitative traits (Reed and Frankham, 2001). Quantitative traits in relation to environmental conditions should therefore be studied to assess the potential for adaptation (Vitt and Havens 2004, Whitlock 2008). In contrast, low molecular genetic variability appears to be a better indicator for the risk of inbreeding depression than low variability in quantitative traits. We conclude that studies of genetic variation of molecular markers and of quantitative traits may provide complementary insights important for the conservation of rare species.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Fig. S1: Relationship between population means of (a) the length of stems and (b) the number of flowerstems per rosette of *S. sponhemica* plants measured in the field and in the common garden. Fig. S2: Relationship between (a) plant diameter, (b) number of rosettes, (c) PC PLANTSIZE, (d) number of flowers, (e) PC REPRO in populations and their molecular diversity H_{eN}.

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Chapter 4

Effects of recent habitat fragmentation on molecular and quantitative genetic variation of the grassland plant *Saxifraga granulata*

in preparation with
Guy Colling, Sylvie Hermant and Diethart Matthies

ABSTRACT

Formerly common species are expected to be particularly susceptible to the recent fragmentation of their habitats but the effects of fragmentation on their population genetics are hardly known. Semi-natural dry mesic grasslands have been recently fragmented over the past decades due to agricultural intensification. We used the grassland plant *Saxifraga granulata* as a model species to study (1) the relative importance of drift and selection in shaping the genetic variation among populations and within populations (2) the clonal diversity, clonal spread and small scale genetic structure within the populations, (3) the relation between plant fitness, quantitative genetic variation, molecular genetic variation and population size. We studied the molecular genetic structure of 19 *S. granulata* populations in a restricted geographic area in Luxembourg and neighbouring Germany using RAPD markers. We grew plants from several families per population in a common garden and determined the variation of quantitative plant traits among and within populations. Differentiation for quantitative traits (Q_{ST}) was slightly lower than differentiation for molecular markers (F_{ST}) suggesting that homogenising selection for optimal trait values has contributed to the variation among populations. Contrary to our expectation, the level of differentiation among fragmented *S. granulata* populations was low and did not increase with the geographical distance among populations. Moreover, molecular genetic diversity of populations was high and not correlated with their size or with plant performance. Gene flow by long distance dispersal as well as longevity, clonality and polyploidy of *S. granulata* may have prevented genetic erosion within and strong genetic differentiation among populations. Although clonality was restricted as shown by high clonal diversity and limited clonal spread it shaped spatial genetic structure at small spatial scales within populations. The spatial genetic structure within populations, indicated an isolation by distance due to reduced gene flow by localised seed dispersal, geitonogamous pollination and biparental inbreeding. In the studied populations of *S. granulata* the effects of genetic drift due to recent habitat fragmentation are not yet perceptible. However, it is important to preserve extant populations and increase the size of small populations to avoid genetic erosion in the future. Management measures should maintain gene flow among populations.

Introduction

Since the late 1950s the intensification of agricultural land use at the expense of traditional land management practices has caused a decline of semi-natural grasslands in Western Europe (Matthies 2000, Poschlod et al. 2005). In Sweden for example, the area of semi-natural grasslands has decreased by 90% since the 1870s (Bernes 1994) and in Western Europe many formerly common grassland species have declined and now occur in smaller and more isolated populations (Saunders et al. 1991, Oostermeijer et al. 1996).

In most plant species, even continuous and large populations are structured into groups of locally adapted and related individuals (e.g. Linhart and Grant 1996) and a decrease of habitat area results in a loss of spatially restricted genotypes and of genetic diversity (Nei et al. 1975). If populations remain small and isolated over successive generations, as a result of genetic drift and inbreeding they are expected to continuously lose genetic variation and become increasingly differentiated (Ellstrand and Elam 1993, Schemske et al. 1994, Young et al 1996). This process has been confirmed both in rare and in common species (e.g. Jacquemyn et al. 2004, Leimu et al. 2006, Aguilar et al. 2008).

Fragmented populations face an increased extinction risk (Young et al. 1996), because as they lose genetic variation, the level of inbreeding increases and inbreeding depression reduces the performance of plants (Ellstrand and Elam 1993, Fischer and Matthies 1998a, Kéry and Matthies 2004). They are also more strongly threatened by environmental and demographic fluctuations (Matthies et al. 2004). Moreover, very small and isolated populations have a lower evolutionary potential (Schemske et al. 1994, Young et al. 1996, Willi et al. 2006, Aguilar et al. 2008, Weber and Kolb 2014). A positive relationship between the size, the genetic diversity and the fitness of plants in populations is commonly observed in plant studies (Leimu et al. 2006, Aguilar et al. 2008). Genetic diversity in these studies is, however, usually measured using neutral molecular markers which is often only weakly related to variation in quantitative traits, which determines the potential of a population to adapt to changing conditions and persist in the long term (Reed and Frankham 2001, Leinonen et al. 2008, Walisch et al. 2015b, Mittell et al. 2015). Despite its importance for the survival and conservation of plant populations, the evolutionary potential of populations has been studied far less often than their molecular genetic diversity (Kramer and Havens 2009, Edwards 2015).

The genetic effects of fragmentation on grassland species are variable. For several formerly common grassland species such as *Gentiana pneumonanthe* (Raijmann et al. 1994), *Gentianella germanica* (Fischer and Matthies 1998a), *Globularia bisnagarica* (Honnay et al. 2007), *Salvia pratensis* and *Scabiosa columbaria* (Van Treuren et al. 1991) a positive relation between genetic diversity and population size has been found. In species like

Succisa pratensis (Vergeer et al. 2003), *Primula vulgaris* (Jacquemyn et al. 2003), *Gentianella germanica* (Fischer and Matthies 1998b), *Gentiana ciliata* (Kéry and Matthies 2004), and *Scorzonera humilis* (Colling and Matthies 2004) the performance of plants was lower in in small than in large populations. Furthermore, fragmented populations of certain species were genetically more strongly differentiated than continuous ones (Van Rossum et al. 2004, Honnay et al. 2007, Lauterbach et al. 2012). However, other studies did not find a relationship between genetic diversity and population size or fitness (Ouborg and Van Treuren 1994, Young et al. 1996, Honnay et al. 2007, Lauterbach 2012, Münzbergova et al. 2013) or did not find population differentiation as a result of fragmentation (e.g. Münzbergova et al. 2013, James and Jordan 2014, van der Meer and Jacquemyn 2015).

The sensitivity of plants to the effects of fragmentation is partly determined by their life history traits. A recent meta-analysis found that outbreeding and wind-pollinated species maintain more genetic variation within and differentiate less among fragmented populations than species with other combinations of traits (Aguilar et al. 2008). The response of plants to fragmentation increases with the number of generations since the start of fragmentation. Longevity may thus delay the response because it extends the time between generations and therefore reduces the loss of alleles through genetic drift (Young et al. 1996, Jacquemyn et al. 2003, Münzbergova et al. 2013, van der Meer and Jacquemyn 2015). However, a recent meta-analysis did not confirm this (Honnay and Jacquemyn 2007). Other plant traits such as polyploidy or a persistent seed bank may also buffer the negative effects of fragmentation on population genetic diversity and structure (Young et al. 2000, James and Jordan 2014, van der Meer and Jacquemyn 2015). Moreover, many perennial plants propagate clonally which increases the time between generations delaying genetic erosion through fragmentation (Honnay and Bossuyt 2005). The relative importance of clonal versus sexual recruitment affects the genetic diversity and structure within populations and clonal plants are assumed to have lower genotype diversities and genetic variation within populations (Ellstrand and Roose 1987). However, several reviews demonstrated that most clonal plants have genetic diversities similar to non-clonal plants and that even a low rate of seedling recruitment may be sufficient to maintain genetic diversity (Eriksson 1989, 1993, Watkinson and Powell 1993, Hangelbroek 2002). Studying jointly the clonal pattern and the genetic structure at a small spatial scale allows to estimate the amount and extent of clonal growth and to estimate the amount of gene flow through pollen and seeds at small spatial scales. If seed dispersal is limited to the close vicinity around mother plants, and if pollen dispersal is also limited, a strong genetic structure will establish, reflecting a pattern of isolation by distance within populations, where geographically close individuals are more closely related than random pairs of individuals (Vekemans and Hardy 2004). Diverging adaptation to small-scale variation of environmental

conditions may also shape the spatial molecular genetic structure at small spatial scales (eg. Linhart and Grant 1996, Van Rossum et al. 2004).

If large populations become isolated, they may have an enhanced potential to adapt to local conditions because of a reduced inflow of potentially maladapted genes from other populations resulting in a diminished risk of outbreeding depression (Lopez et al. 2009). Populations in fragmented landscapes may therefore be more strongly locally adapted (Jakobsson and Dinnetz 2005) and more differentiated in adaptive pHeNotypic characters than interconnected populations (Willi et al. 2007). Conversely, the effectiveness of selection may be reduced and drift may be enhanced in small and isolated populations (Gravuer et al. 2005, Johansson et al. 2007). Even though the importance of studies on local adaptation for conservation biology is now widely recognised, there are still few quantitative genetic studies on the relative contribution of neutral non-adaptive and of selective processes to the genetic variation among populations (Willi et al. 2006). The variation in adaptive traits within and among populations can be studied by growing plants from different families and populations in a common garden study and measuring quantitative plant traits. Knowledge of both the genetic differentiation in potentially adaptive quantitative traits (Q_{ST}) and in neutral molecular genetic markers (F_{ST}) measured for plants from the same populations allows one to estimate the relative contributions of drift and of selection to the overall genetic variation among populations (Merilä and Crnokrak 2001). If Q_{ST}-F_{ST} is close to zero, drift is the major evolutionary force shaping the overall genetic differentiation among populations. If Q_{ST}-F_{ST} is larger or smaller than zero, tHeN divergent or stabilizing selection are contributing to the overall genetic variation among populations (Volis et al. 2005). In most studies, Q_{ST} was found to be larger than F_{ST} indicating that divergent selection is common in plant populations (e.g. meta-analyses by Leinonen et al. 2008, de Koort et al. 2013). However, there are very few quantitative genetic studies on common or recently fragmented grassland species and they obtained conflicting results. In a study of *Scabiosa columbaria* in calcareous grasslands within a very restricted area of the Swiss Jura (Scheepens et al., 2010a) unifying selection was detected, while in a study in a small geographic area in Sweden, *Scabiosa columbaria* showed signs of divergent selection (Waldmann et al. 1998). In highly fragmented temperate grasslands of Australia *Rutidosis leptorrhynchoides* showed divergent selection along environmental gradients (Pickup et al. 2012). More joint studies of quantitative and molecular genetic diversities on formerly common and recently fragmented species are needed.

Old and naturally rare species such as *S. sponhemica* (Walisch et al. 2015a) and *S. azoides* (Lutz et al. 2000) have been found to withstand the negative effects of fragmentation for thousands of years as a result of their longevity and clonality and they have preserved evolutionary potential (Walisch et al. 2015b). However, formerly common and recently

fragmented species are expected to show a stronger response to fragmentation than naturally rare species, because they have suffered recent and much more rapid declines in the number and size of populations (Huenneke 1991, Aguilar et al. 2008). In a comparative review, formerly common species more frequently showed a decrease of genetic diversity with population size than historically rare species (Brigham 2003). A recent meta-analysis also found that common species face a higher risk of genetic erosion due to recent fragmentation than naturally rare species because they host comparatively higher genetic diversity (Aguilar et al. 2008). They concluded that conservation efforts should be directed towards common and recently fragmented, and mainly outcrossing species because they are more susceptible to the effects of fragmentation (Aguilar et al. 2008). In order to plan appropriate conservation measures, there is a need for integrated studies that investigate the effects of fragmentation on the genetic diversity, the evolutionary potential, as well as the relative contributions of drift and selection to the genetic differentiation among populations.

We studied the molecular and quantitative genetic variation within and among populations of the long-lived and clonal semi-natural grassland species *Saxifraga granulata* in Luxembourg and a neighbouring area of Germany to investigate the effects of the recent fragmentation of dry mesophilous grasslands on populations of the plant. In addition, we also investigated the small-scale genetic structure within populations and clonal patterns. *S. granulata* is a formerly common grassland species that used to occur in dry mesic grasslands all over Western Europe (Walisch et al. 2012, van der Meer and Jacquemyn 2015), but has strongly declined in the last decades and is now threatened in several European regions (Korneck et al. 1996, Niklfeld 1999) as a result of the use of fertilizers and conversion of traditional grassland to silage meadows. In Luxembourg and surrounding regions, remnant populations still exist in semi-natural mesophilous grassland patches, but the fragmentation of their habitat may lead to reduced gene flow, genetic erosion and increased inbreeding, and reduced performance of populations. We expect that *S. granulata* is vulnerable to these effects of fragmentation, life history traits of the species such as longevity, polyploidy and clonality may alleviate these effects. We addressed the following questions. (1) How is genetic variation distributed among populations and individuals and does the genetic distance between populations and individuals increase with their geographical distance? (2) What is the clonal diversity and how far are clones spread in the populations? (3) What is the relative contribution of drift and selection to genetic differentiation in *S. granulata* and is there evidence for adaptive variation along environmental gradients? (4) Are the fitness of plants, quantitative genetic variation, molecular genetic variation and population size positively correlated ?

Materials and methods

Study species

Saxifraga granulata L. is a perennial herb that propagates both by seeds and by small bulbils produced at the base of the plant (Weber 1995, Stroh 2015). The seeds are very small (ca. 0.5 × 0.3 mm, ca. 40 μg) and dispersed by wind. After the aboveground parts have withered over the summer months, a new basal rosette grows in the autumn which overwinters and may flower the next spring. The flowers of *S. granulata* are protandrous, but self-compatible (Walisch et al. 2012, Hansen and Molau 1994). Pollination is assured by a wide range of insect species including flies and solitary bees (Hansen and Molau 1994). Geitonogamous selfing within the same genet is common as a result of asynchronous ripening of flowers on the same genet. A pollination study in a large population of *S. granulata* in Luxembourg revealed a mixed mating system with an estimated selfing rate of 55% (Walisch et al. 2012). In central Belgium *S. granulata* is octoploid (van der Meer et al. 2014). *S. granulata* occurs mainly in mesic to dry grasslands and is widespread across northern, western and central Europe reaching its southern range limit in North-Africa (Stroh 2015). It is considered to be threatened in most parts of its range, and populations have declined and have become increasingly fragmented over the past decades. The main causes for this decline are the conversion of grasslands into arable fields or urban developments, changes in agricultural practices, such as the increased fertilisation of meadows (Walisch et al. 2012) and the use of broad-spectrum herbicides (Stroh 2015).

Study sites and collection of samples

In early June 2003 we visited 19 populations of *S. granulata* in the Gutland and Minette regions of Luxembourg and in the state of Rheinland-Pfalz in Germany (Table 1). The distance between the sampled populations ranged from 0.05 to 60.6 km. The longitude and latitude of the centre of each population were determined with a GPS. To determine the size of the populations we counted the number of flowering plants. In small populations all flowering plants were counted, whereas in large populations we extrapolated population size from counts in parts of the population area. For each site, we obtained the bioclimatic variables mean diurnal temperature range, mean annual temperature, temperature seasonality (SD), minimum temperature of the coldest month, maximum temperature of the warmest month, temperature annual range, annual precipitation, precipitation seasonality (CV), precipitation of the wettest month and precipitation of the driest month in a grid size of about 1 km^2 (30 arc s, interpolations of observed climate data, representative of 1950-2000) from the Worldclim database version 1.4. (Hijmans et al. 2005). A principal component analysis of climate variables identified two principal components

Table 1. Genetic diversity of 19 populations of *Saxifraga granulata.* N, number of flowering plants in the population; N_{mol}, number of plants used for RAPD analysis; N_{fam}, no. of seed families in the garden; No. of plants per family, number of plants per seed family grown in the garden; *PPL*, proportion of polymorphic loci at the 5% level; H_{eN}, Nei's gene diversity based on allele frequencies calculated with the Bayesian method with non-uniform prior distribution of allele frequencies in a population (Zhitvotosky 1999) assuming that the inbreeding coefficient F_{IS} = 0.643, F_{ST}, population-specific F_{ST} value.

Geographical region	Population name	N	N_{mol}	N_{fam}	No. of plants per family	*PPL (%)*	H_{eN}	Location (lat./long.)
Luxemburg	Bertrange 1	67	14	15	6.4	94.2	0.338	N 49.605/E 6.058
	Bertrange 2	8,000	13	15	5.7	92.3	0.378	N 49.605/E 6.060
	Bertrange 3	59	14	12	5.0	90.4	0.356	N 49.609/E 6.026
	Bertrange 4, Léi	1,000	14	15	6.0	96.2	0.361	N 49.597/E 6.067
	Sprinkange, Bitschenheck	22,000	14	15	6.5	98.1	0.348	N 49.572/E 5.951
	Mamer 1	2,400	14	15	5.9	96.2	0.364	N 49.611/E 6.026
	Mamer 2	3,430	12	11	5.9	84.6	0.327	N 49.602/E 6.018
	Mamer 3	11	14	13	6.0	94.2	0.345	N 49.614/E 6.018
	Mamer 4	210	11	15	6.1	96.2	0.331	N 49.621/E 6.018
	Niedercorn 1	14,800	14	14	5.6	94.2	0.342	N 49.548/E 5.903
	Niedercorn 2	8,000	14	14	6.0	100	0.377	N 49.548/E 5.903
	Roedgen 1	200	14	15	6.3	94.2	0.350	N 49.570/ E 6.043
	Roedgen 2	5,100	14	15	6.1	92.3	0.339	N 49.571/E 6.041
	Lallange 1	15	13	11	5.8	71.2	0.287	N 49.490/E 6.001
	Lallange 2	900	13	15	6.5	96.2	0.373	N 49.489/E 6.000
Germany	Geizenburg 1	4,150	10	14	5.4	96.2	0.329	N 49.682/E 6.708
	Geizenburg 2	200	11	14	5.6	96.2	0.347	N 49.682/E 6.710
	Gusterath 1	2,700	14	14	5.6	90.4	0.302	N 49.709/E 6.706
	Gusterath 2	5,000	13	14	6.6	90.4	0.357	N 49.706/E 6.703

(PCs) which explained 93.5% of the total variation. The first PC (PRECIP) explained 76% and correlated strongly with annual precipitation (r = 0.97), precipitation of the driest (r = 0.92) and of the wettest month (r = 0.98), and was negatively correlated with mean diurnal temperature range (r = - 0.81). The second PC (TEMP) explained a further 17.5% of the variance and was highly correlated with the maximum temperature of the warmest month (r = 0.99), annual mean temperature (r = 0.94) and minimum temperature of the coldest month (r = 0.86). In each population we selected 14 plants along a 15 m transect. In most populations, the plants were at least 0.5 m apart in order to minimize the chance of sampling clones. However, in very small populations distances were smaller. To calculate the distances between the plants we recorded their relative spatial position along the transect. From each plant we collected one ripe capsule and 1-2 basal leaves. We counted the number of flowers of each plant for comparisons between the mother

plants in the field and their offspring raised in the common garden. To study the clonal structure within populations, we collected 1-2 leaves from all plants in a randomly selected plot (1 x 1 m) in the population Mamer and in a plot in the population Cents (0.4 x 0.4 m) and recorded their positions within the plots. The leaf samples were immediately frozen at – 80° for molecular genetic analysis.

Cultivation of plants

At the end of August 2009, we placed two batches of 15 seeds per capsule in Petri dishes on moist filter paper and stratified them in a growth chamber at 4°C for four weeks. The temperature was raised to 20 °C at the end of October and the seeds were put under a 12h day/12h night light regime. The position of the Petri dishes was randomised every 3-4 d. Seed germination was recorded every two weeks and 6 to 10 seedlings per capsule (hereafter referred to as 'seed family') of a minimum size of 1 cm were selected at random and planted into soaked peat pellets ('Jiffy pots'). The plants were put into trays and placed under fluorescent tubes (Gro-Lux, 28W, Osram Sylvania, USA). Dead seedlings were recorded and replaced until mid-January 2010. In March we again checked the survival of plants and measured the largest diameter of each plant, the width of the largest leaf and the number of flowers.

RAPD-PCR

The dried leaf material was ground (Retsch MM200, Retsch, Haan, Germany), and DNA extracted using the DNeasy® Plant Mini Kit (QIAGEN, Germany). We carried out amplifications in 25 µl volumes containing 5 µl of template DNA (5 ng DNA/µl), 8.575 µl ddH2O, 3 µl MgCl2 (25 mM), 0.5 µl dNTP's (10 mM), 2.5 µl PCR Buffer with (NH4)-2SO4 (10X, Fermentas), 5 µl Primer (5 µM), 0.3 µl Taq DNA Polymerase (5 units µl, Fermentas), and 0.125 µl BSA (20 mg/ml). The volumes were held in polycarbonate microtitre plates and covered by adhesive sealing sheets. The plates were tHeN incubated in a thermocycler (iCycler®, Bio-Rad Laboratories) programmed with the following settings: Denaturation of the DNA at 94°C for 2 min, followed by 44 repetitive cycles consisting of denaturation for 45 s at 94°C, annealing for 2 min 30 s at 36°C and extension for 2 min at 72 °C followed by a final extension phase of 5 min at 72°C. The samples were kept at 4°C until analysis. Amplified DNA fragments were separated by electrophoresis on precast ReadyAgaroseTM 1.0% Agarose gels with ethidium bromide in 1xTBE buffer (Bio-Rad Laboratories) in an electrical field (85 V, c. 100 min). The gels were put under UV light and photographed using the Bio Doc system (Bio-Rad Laboratories).l of template DNA

Table 2**.** RAPD primers used.

Primer	Sequence
A4	5'- AATCGGGCTG- 3'
A7	5'- GAAACGGGTG- 3'
A11	5'- CAATCGCCGT- 3'
C1	5'- TTCGAGCCAG - 3'
C2	5'- GTGAGGCGTC - 3'
C6	5'- GAACGGACTC - 3'
C8	5'- TGGACCGGTG - 3'

In a first series of amplifications 60 10-base primers (Kits A, B, C from Operon Technologies, Alameda, California) were screened in a random sequence and tested for reproducibility of the amplified fragment profile using four replicates of a single DNA extract. The first seven primers yielding good quality reproducible patterns (primers A4, A7, A11, C1, C2, C6, C8) were selected for the RAPD analysis of 250 sampled plants (Table 2). Presence or absence of reliable bands on amplification products were scored visually using the program Quantity/One (Bio-Rad Laboratories) and were treated as pHeNotypes, with each band position representing a character either present or absent. The final presence – absence matrix contained scores at 54 polymorphic band positions for all samples in the study. We replicated 356 combinations of DNA samples and markers after DNA extraction to estimate the error rate of the RAPD genotyping resulting in 2771 repeated banding scores (corresponding to 20.5 % of the total dataset). The second scoring was done by the same technician as the first one and the error rate was estimated to be 6.6%. Because of the error rate of 6.6%, we considered plants differing by up to 3.6 (rounded to 4) loci as putative clones belonging to the same genotype (Ehrich et al. 2008). We only kept one randomly chosen putative clone per genotype in the RAPD matrix resulting in 247 samples used for further analysis.

We identified markers under divergent or balancing selection with the program BAYESCAN 2.01 with the false discovery rate set to 0.05 (see Foll and Gaggiotti 2008). Several methods of detecting markers under selection have recently been tested by De Mita et al. (2013). The method used by BAYESCAN 2.01 was found to be robust against deviations from the island model and yielded very few false positives in all simulations. We removed any markers that were putatively non-neutral and used the resulting matrix of neutral loci in subsequent analyses.

DATA ANALYSIS

Molecular genetic diversity within populations and structure among populations

To estimate allele frequencies we used the Bayesian method with non-uniform prior distribution of allele frequencies (Zhivotovsky 1999) as implemented in AFLP-SURV version 1.0 (Vekemans 2002) with an estimate of Wright's inbreeding coefficient over all populations (F_{IS}). F_{IS} was calculated using the approximate bayesian computation for *F*-statistics (ABC4F) for dominant data (Foll et al. 2008). Genetic diversity within populations was calculated as (i) the percentage of polymorphic loci (*PPL*) at the 5% level, and (ii) Nei's gene diversity (expected heterozygosity H_{eN}) according to the method of Lynch and Milligan (1994) which uses the average expected heterozygosity of the marker loci.

To test for genetic clusters of the 19 study populations at the landscape level we used the software STRUCTURE v. 2.2 which allows the use of dominant markers like RAPDs (Pritchard et al. 2000, Falush et al. 2003, 2007). We used a model with correlated allele frequencies and assumed population admixture with population ID's as prior information. ALPHA, the Dirichlet parameter for the degree of admixture was set to 1 for all populations. To quantify the amount of variation of the likelihood of each *K*, we carried out a total of 10 independent runs of *K* = 1- 19. We found that a burn-in and MCMC length (Markov Chain Monte Carlo) of 10^5 each was sufficient as longer burn-ins or MCMC lengths did not change significantly the results.

The genetic structure among populations was analysed on the basis of RAPD allele frequencies using AFLP-SURV assuming the inbreeding coefficient F_{IS} calculated by ABC4F. The significance level of the calculated F_{ST} was assessed by 1000 permutations. A pairwise genetic distance matrix with F_{ST} values was calculated in AFLP-SURV using F_{IS}. The partitioning of genetic variation among populations and among individuals within populations was investigated by analysis of molecular variance (AMOVA) using GenAlex6 version 6 (Peakall and Smouse 2006, see Excoffier et al. 1992, Stewart and Excoffier 1996).

Molecular genetic structure within populations and clonal structure

We used spatial autocorrelation analyses with an estimator of the kinship coefficient for dominant markers, F_{ij} (Hardy 2003) as implemented in SPAGeDI version 1.2. (Hardy and Vekemans 2002). As an estimate of the departure from Hardy-Weinberg genotypic proportions we used F_{IS} calculated by ABC4F. The kinship coefficient, F_{ij} determines the probability that individuals *i* and *j* share an identical gene. Mean F_{ij} estimates over pairs of individuals at a given distance interval *r*, *F(r)* were plotted against distance in a spatial autocorrelogram. In the case of a linear decrease of *F(r)* with *r* or ln (*r*), the extent of the spatial genetic structure (SGS) can be quantified by the slope (*b*) of a regression of mean F_{ij} estimates on r_{ij} or ln (r_{ij}). As (*b*) can depend on the sampling scheme used, we calculated the ratio $-b/(1-F_{(1)})$

where $F_{(1)}$ is the mean F_{ij} between individuals belonging to the first distance class. $F_{(1)}$ can be considered as an approximation of the kinship coefficient between neighbouring individuals if the first distance class contains enough pairs of individuals. The ratio $-b/(1-F_{(1)})$ is referred to as the *Sp* statistic (Vekemans and Hardy 2004) and can be used to compare the extent of SGS among populations or species. Standard errors for the mean F_{ij} estimates over pairs of individuals at a given distance interval and the regression slope (b) were assessed by a jackknifing procedure over loci, and the significance level of the regression slope (b) was calculated by comparing the observed value with the distribution of (b) obtained by 1000 random permutations.

We studied the clonal structure within two populations of *S. granulata* by sampling all ramets in a plot and assuming that ramets which shared the same multilocus RAPD genotype belonged to the same genet. We also determined the probability (P_G) that two random, sexually produced multilocus genotypes are identical under the assumption of random mating. Therefore we first calculated the probability that two unrelated plants shared the same banding pattern at locus i as $1 - 2p_i(1 - p_i)$, where pi is the frequency of the band at locus i in the population (Bizoux and Mahy 2007). The probability that two unrelated plants have the same RAPD pHeNotype just by chance, assuming that all loci are independent (unlinked) was tHeN estimated as $\Pi[1 - 2pi(1 - p_i)]$ with the product taken over all loci. We quantified genotype diversity in two plots (McGlaughlin and Friar 2007) by calculating (1) mean clone size (N/G), where N is the number of sampled individuals and G is the number of genets, (2) the proportion of genotypes detected (PD = G/N) and (3) Simpson's diversity index (D) corrected for finite sample size as $D = 1 - \Sigma n_i(n_i - 1)/N(N - 1)$, where ni is the number of samples of genotype i and N is the total number of samples (Simpson 1949). The D values lie between 0 and 1, where at the low end of the range all sampled individuals belong to one genotype and at the upper end each individual has a unique genotype.

Within and between population quantitative genetic variation

We checked whether the distribution of residuals was normal for each trait and square-root-transformed the number of flowers per plant to obtain normally distributed residuals. We tested whether the trait variables were intercorrelated and performed a principal component analysis (PCA) with varimax rotation to identify principal components. We studied the effects of population size and of bioclimatic principal components on all traits by multiple regression. We determined the Bayesian information criterion (BIC) for all possible models by using the leaps package (version 2.9, Lumley 2009) in R (version 3.1.0, R core team, 2014) and selected the model for which BIC was minimal.

We conducted analyses of variance with population and family as factors for all traits in

a hierarchical design which tested the effect of population against the variation between families. To obtain estimates of between population genetic variation (Q_{ST}), heritability (h_2) and evolvability (genetic coefficient of variation, $CV_{genetic}$, Houle 1992), we calculated variance components between populations (V_{pop}), between families within populations (V_{fam}) and between individuals within families (V_{error}) for each trait by restricted maximum likelihood with the varcomp function of the R-package ape version 3.1-4 (Paradis et al., 2004). Heritability (h^2) was calculated as $h^2 = (V_{fam}/2*\theta) / (V_{fam} + V_{error})$, and the evolvability (genetic coefficient of variation) as $CV_{genetic} = \sqrt{(V_{fam}/2*\theta)}/mean$, where θ is a measure of the kinship of the plants. To calculate mean evolvability we used untransformed variables (Hansen et al., 2011). For θ we used a value of 0.5 for selfed plants and 0.25 for full-sibs (Jimenez-Ambriz et al., 2007). In a previous pollination study conducted in a large population of *S. granulata* in Luxembourg, the estimated selfing rate was 55% (Walisch et al., 2012). We inferred that 55% of offspring originated from selfings in our study populations and assumed that the remaining 45% of offspring were full-sibs to obtain a value of 0.3875 for θ. The assumption of full-sibs wHeN relationships between offspring from a family are unknown provides conservative estimates of quantitative genetic parameters (Podolsky and Holtsford, 1995). Q_{ST} was HeNce calculated as

$$V_{pop} / (2* [V_{fam}/2*\theta] + V_{pop}) = V_{pop} / (2.58*V_{fam} + V_{pop}).$$

We estimated 95% confidence intervals for Q_{ST} by the jackknife technique following O'Hara and Merilä (2005). The mean Q_{ST} of all traits was calculated as the sum of the numerators divided by the sum of the denominators of the individual Q_{ST}-values, after standardizing the sums of the variance components for each trait to 1 as suggested by Chapuis et al. (2007) to avoid that some traits had an undue influence on the overall average. We used regression analyses to explore the relationship between the evolvability ($CV_{genetic}$) and heritability (h_2) of each trait in a population and averaged over all traits per population. We estimated the genetic variability of quantitative traits as mean evolvability over all traits and studied the relation between quantitative genetic variability and molecular genetic variability by regressions. We investigated the effects of population size and of the climate factors PRECIP and TEMP on the evolvabilities of individual traits and on the mean evolvability of all traits in a population. We calculated the Bayesian information criterion (BIC) for all possible models using the leaps package in R (Lumley 2009, R core team 2014), selected the model with the lowest BIC value and performed regression analysis between evolvabilities of traits and their best predicting variables.

We applied Mantel test statistics to analyse whether the pairwise F_{ST} values and the geographic distance matrix were correlated, a sign of isolation-by-distance. Mantel test statistics were calculated using the program zt version 1.1 (Bonnet and Van de Peer, 2002)

and significance levels were obtained after performing 1000 random permutations. All statistical analyses, if not stated otherwise, were carried out with SPSS 19.0 (IBM Corp., 2010).

RESULTS

Variation among traits

According to the Bayesian information criterion leaf width was best explained by the bioclimatic factor PRECIP, whereas plant diameter, and number of flowers were best explained by TEMP. However, none of the plant traits was significantly related to their best explanatory variable in regression analyses ($r^2 < 0.09$, $P > 0.20$), indicating that plant traits were not significantly influenced by the size of their populations or by the climatic conditions at their locations. Population means of plant traits measured in the common garden (number of flowering stems and flowers, length of flowering stems) were only weakly correlated with the same traits measured in the field ($|r| < 0.22$, $P > 0.16$).

Genetic diversity within populations

The seven RAPD primers used for analysis generated a total of 54 polymorphic bands. No private (population-specific) bands were observed. Taking into account an error rate of 6.6%, pHeNotypes which shared less than 5 bands were considered putative clones. We identified three clonal lineages. The distance between members of the same putative clone ranged from 0.01 to 0.93 m. We removed putative clones as well as two band positions that had been identified as putative non-neutral loci (C02F and C02G, 4% of all loci) by the program BAYESCAN 2.1. and obtained a final matrix of 247 unique genotypes and 52 neutral loci for our study populations. The mean proportion of polymorphic loci (*PPL*) in the 19 populations was 92.8% and varied among the populations from 71.2% to 100% (Table 1). Mean Nei's gene diversity (H_{eN}) using the F_{IS} estimated by ABC4F (F_{IS} (*f*) = 0.643 ± 0.04 , 95% credible interval 0.823-0.993) was 0.345 and varied from 0.287 in a very small population of Lallange to 0.378 in a large population in Niedercorn. Quantitative genetic diversity within populations estimated as evolvability (*CV*gen) was significantly larger than zero for all traits (Fig. 1). The mean evolvability averaged over all traits in a population varied from 9 to 31%. Evolvability and heritability (h^2) of the individual traits in the populations were strongly correlated (all $r > 0.68$, all $P < 0.001$), and mean evolvability and mean heritability averaged over all traits in a population were also strongly correlated ($r = 0.89$, $P < 0.001$).

Mean evolvability of leaf width increased significantly with the two molecular genetic diversity measures H_{eN} ($r = 0.58$, $P < 0.01$) and *PPL* ($r = 0.55$, $P < 0.05$), mean evolvability

of plant diameter increased with H_{eN} ($r = 0.55$, $P < 0.05$) and mean evolvability of flower number increased with *PPL* ($r = 0.53$, $P < 0.05$). There was a strong positive relationship between the mean evolvability over all traits and H_{eN} (Fig. 2) or *PPL* ($r = 0.61$, $P < 0.01$) in a population, but neither measure of molecular genetic diversity increased significantly with population size ($r < 0.37$, $P > 0.12$, Fig. 3).

Population means and evolvabilities of plant diameter, leaf width and number of flowers were negatively correlated (Figs. 4a, b, c), but none of the trait means correlated signif-

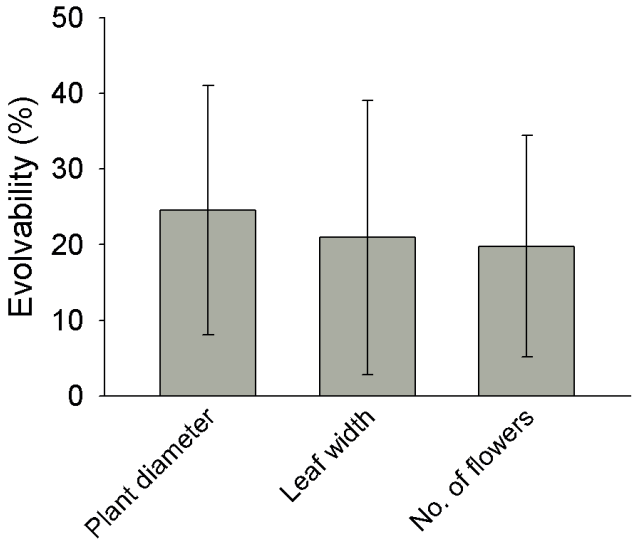

Figure 1. Mean evolvability ± 95 % confidence limits of measured traits of *Saxifraga granulata*.

icantly with molecular genetic diversity ($|r| < 0.28$, $P > 0.24$). The population Lallange 1 (Table 1) was omitted as an outlier from the analyses because it had exceptionally low mean trait values and low evolvabilities. According to the Bayesian information criterion (BIC) the main influence on the evolvability of traits and on the mean evolvability over all traits in a population was the bioclimatic factor TEMP. In regression analyses however, only the evolvability of leaf width was marginally significantly related to TEMP ($r = 0.47$, $P = 0.05$), but none of the evolvabilities of the other traits was related to TEMP ($r < 0.33$, $P > 0.18$). The population Lallange 1 was also omitted from these analyses.

Genetic variation among populations

We did not detect an upper level of partitioning among populations using STRUCTURE. The highest modal value of ΔK was at $K = 2$, but the differences among K-values were not consistent and the inferred clusters were not related to geography, suggesting that

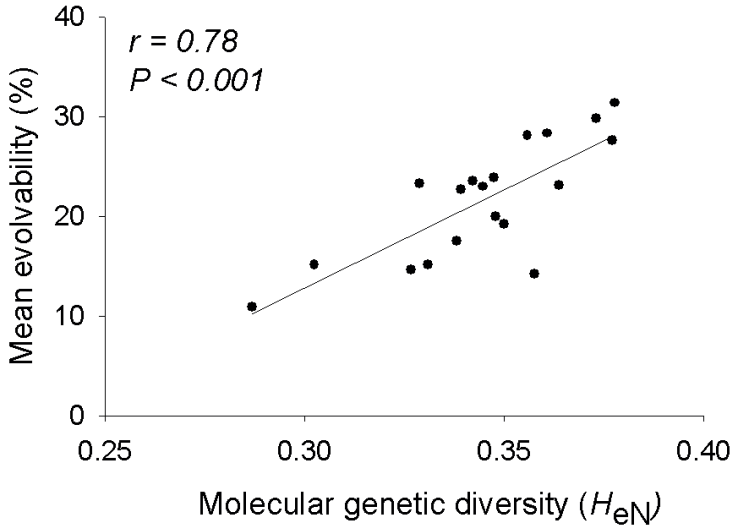

Figure 2. Relationship between mean evolvability of all measured traits in a population and molecular genetic diversity (Nei's gene diversity) of the population.

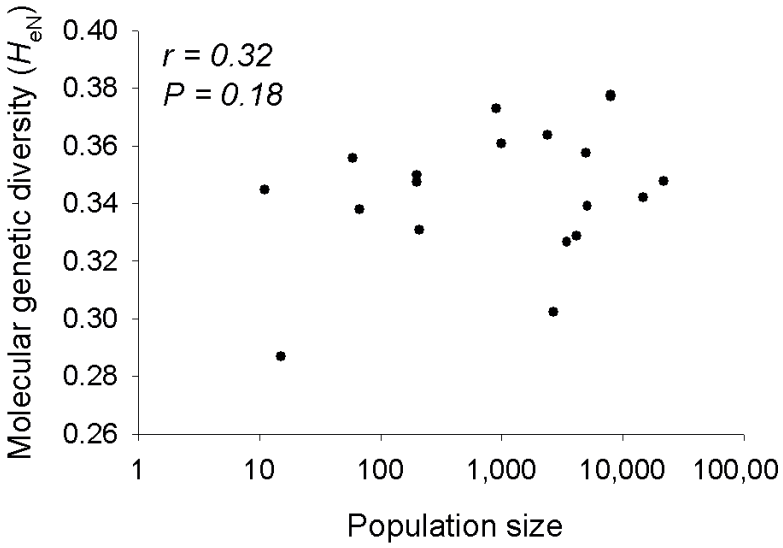

Figure 3. The relationship between Nei's gene diversity, H_{eN}, and the size of a population.

populations are part of a larger regional group. The AMOVA analysis showed that 11% of the variation was among populations ($P < 0.001$), while variation among individuals within populations accounted for 89%. Divergence of the two putatively non-neutral loci was higher than under a neutral expectation suggesting directional selection. The dataset including the putatively non-neutral markers thus yielded a slightly higher Φ_{st} value than the dataset which contained only neutral loci (12% versus 11%). F_{ST} estimated by AFLP-SURV assuming $F_{IS} = 0.643$ was 0.079 ± 0.1348.

The quantitative genetic differentiation among populations estimated as mean overall

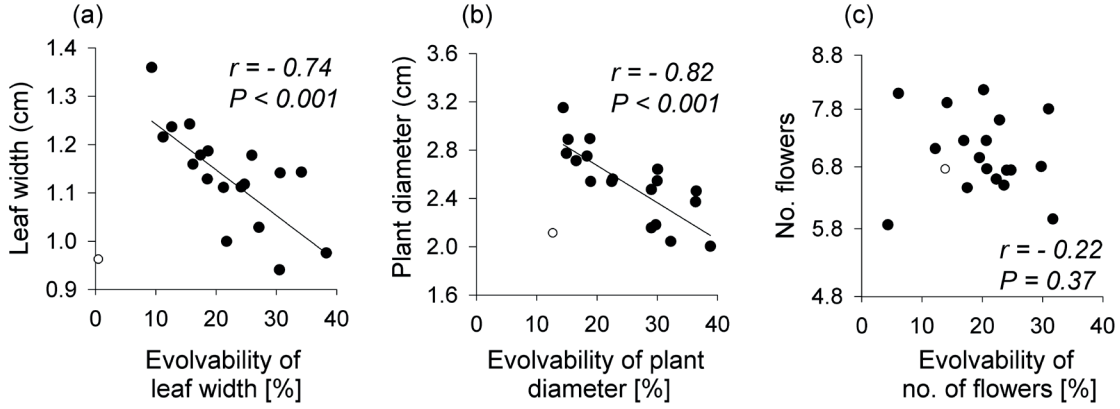

Figure 4. Relationships between trait means and the evolvabilities for (a) leaf width, (b) plant diameter and (c) number of flowers in a population. The open symbol denotes the population Lallange 1 which was excluded from the analyses.

Q_{ST} amounted to 0.044 and the difference between overall Q_{ST} and F_{ST} was 0.039. The Q_{ST} values all traits were slightly lower than the F_{ST} value but for plant diameter the confidence intervals were overlapping indicating that neutral processes, such as drift as well as stabilizing selection shape the quantitative genetic differentiation among populations (Fig. 5). Finally, the pairwise molecular genetic (pairwise F_{ST}) and geographic distances were not correlated ($r = 0.113$, $P = 0.2$), suggesting that an isolation by distance pattern did not exist among our study populations.

Spatial genetic variation within populations and clonal structure

Spatial autocorrelation analysis revealed a significant spatial genetic structure within populations based on observations across all populations. Mean kinship coefficients decreased with distance between plants in the populations ($b = -0.003$, $P < 0.01$), indicating that individual plants growing at less than 50 cm from each other had a higher probability to be genetically related than plants separated by larger distances. Positive values of the mean kinship coefficient were obtained at a small geographical distance (50 cm) suggesting that neighbouring individuals are genetically more closely related than random pairs of individuals within the populations and negative values were obtained at about 5 m (Fig. 6). The value for the *Sp* statistic was 0.003 with $F_{(1)} = 0.007$.

The analysis of the clonal structure of plants of *S. granulata* at a small scale showed that plot 1 contained 37 genotypes and plot 2 11 genotypes (Fig. 7a,b). The mean proportion of genotypes (PD) detected was 0.65 (0.51 in plot 1 and 0.79 in plot 2). The mean size of clones ranged from 2 to 7 rosettes per genotype (mean = 3.3) and the mean distance between rosettes of the same genet was 3.4 cm in plot 1 and 17.1 cm in plot 2, while the maximum distance was 11.2 cm in plot 1 and 25 cm in plot 2 (Figs. 7ab). Simpson's di-

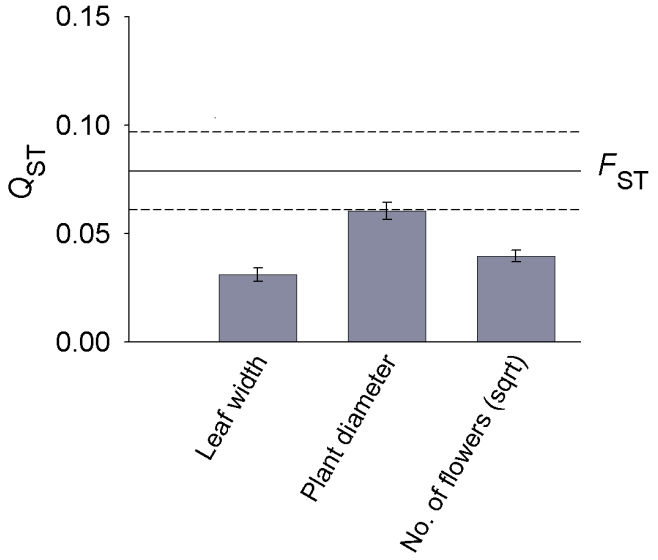

Figure 5. Mean genetic differentiation in quantitative traits between populations (Q_{ST}) of *Saxifraga granulata*. Vertical error bars indicate 95% confidence limits of Q_{ST}. Horizontal dotted lines show the 95% confidence limits of F_{ST}.

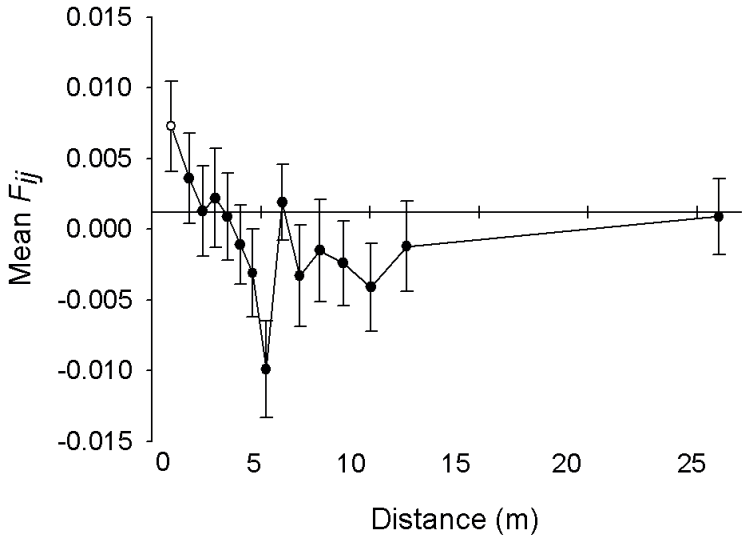

Figure 6. Mean kinship coefficient correlogram between pairs of individuals that grow at different distances from each other in 19 populations of *Saxifraga granulata* assessed using 52 RAPD markers. Each of the 15 distance classes involves 1910-1914 pairs of individuals and the total sample consisted of 247 individuals. Means ± 1 SE. The open symbol represents a significant mean kinship coefficient. ($P < 0.05$)

versity index (D) was 0.97 for the two plots combined. The probability that two ramets share the same genotype by chance was $2.7 * 10^{-7}$ in plot 1 and $9.1 * 10^{-13}$ in plot 2, indicating that plants sharing the same RAPD pHeNotype were most probably clones.

The spatial autocorrelation analysis at the plot level showed a significant spatial genetic structure and the slope of the linear regression between the mean kinship coefficients and the

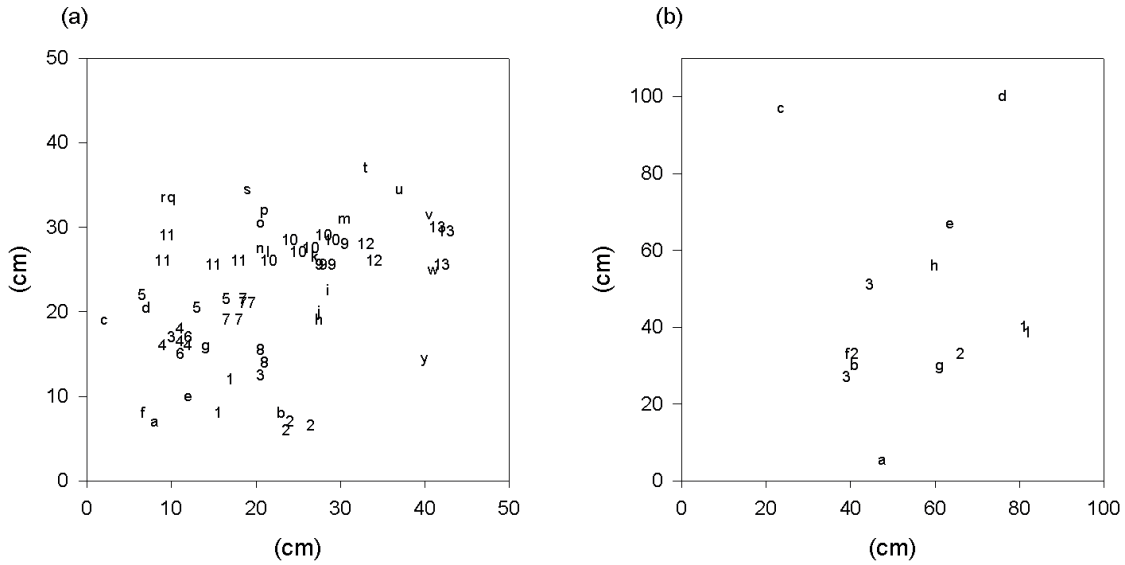

Figure 7. Maps showing the position of the sampled ramets of *Saxifraga granulata,* each represented by a number or a letter. (a) Plot 1, (b) plot 2. Ramets of the same genotype share the same number while unique ramets are designated by letters. *Letters* start with *a* and *numbers* start with 1 separately in each plot.

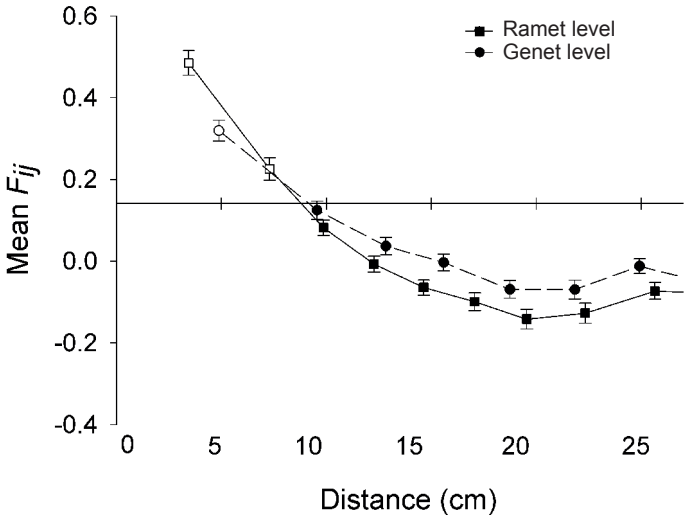

Figure 8. Mean kinship coefficient correlogram between pairs of ramets and genets that grow at different distances from each other in two plots (100 x 100 cm and 40 x 40 cm) in two populations of *Saxifraga granulata* as assessed using 69 RAPD markers. Error bars represent ± 1 SE. The open symbols represent significant mean kinship coefficients ($P < 0.05$).

geographical distances were significantly negative ($b = -0.129$ at the ramet level, $b = -0.091$ at the genet level, $P < 0.0001$) indicating that plants growing in close proximity have a higher probability to be related than those separated by larger distances. The mean kinship coefficient was positive at very small geographical distances (ramets < 7.3 cm, genets < 4.9 cm,

Fig. 8) and the value of the *Sp* statistic was 0.250 at the ramet level and 0.134 at the genet level. The slope was steeper and the *Sp* statistic larger at the ramet than at the genet level, suggesting that clones enhance the spatial structure of the populations at small spatial scales.

DISCUSSION

Genetic variation among populations

Contrary to our expectation, the level of differentiation among fragmented *S. granulata* populations was low and did not increase with the distance between populations. The Φ_{ST} value (0.11) of our *S. granulata* study populations was much lower than the mean overall Φ_{ST} found in compilations of studies using dominant markers (0.34-0.35, mixed mating species Φ_{ST} = 0.27-0.40, long-lived species Φ_{ST} = 0.25, Nybom and Bartish 2000, Nybom 2004). Our Φ_{ST} value was also lower than the Φ_{ST} values obtained in other studies at a similar geographical scale (e.g., Tollefsrud et al. 1998, Allnutt et al. 1999, Colling et al. 2010, Müller et al. 2012, Walisch et al. 2015a). Similarly low levels of genetic variation among populations have been found in another study on *S. granulata* along two river systems in Belgium (G_{ST} = 0.093 – 0.042), probably reflecting past gene flow due to higher connectivity among populations (van der Meer and Jacquemyn 2015). Likewise, the weak genetic structure in our *S. granulata* study populations may reflect the past connectivity of populations and this structure may have been maintained since the beginning of fragmentation a few decades ago, by the longevity, the clonality and the polyploid nature of the species. Our F_{ST} value (0.079) was similarly low as that of *Pimelea spinescens* in fragmented temperate grasslands in Southeast Australia (F_{ST} = 0.07, James and Jordan 2014) which has also been explained by a combination of past connectivity of populations, plant longevity and a seed bank preserving the similarity among populations.

In *S. granulata* we did not find an isolation-by-distance pattern (IBD), indicating that the genetic variation among populations does not increase with distance among populations, which is surprising in spatially isolated populations where a reduction of natural gene flow with increasing distance between populations would be expected. The lack of IBD could be due to the effect of strong genetic bottlenecks or founder events. Two of our populations are known to have been founded in the 1970s in the mining sites of Lallange and some of the other populations may have experienced drastic bottlenecks due to the recent and rapid fragmentation and deterioration of their grassland habitats. Another factor that may have weakened the IBD pattern is long-distance dispersal of seeds (van der Meer and Jacquemyn, 2015). The seeds of *S. granulata* are very small and light and may therefore be carried across long distances by strong winds, or they may be dispersed between

non-adjacent populations by mowing machines (Bonn and Poschlod 1998) carrying remains of hay between meadows. Long distance dispersal has been detected in Svalbard populations of the congener *Saxifraga oppositifolia* which has seeds of similar size and shape (Müller et al. 2012). Finally, the low maximum distance between the sampled populations may also have affected the variation among populations (e.g. Garnier et al. 2004, Crispo and HeNdry 2005).

Signs of stabilizing selection among populations

We found weak signs of stabilizing selection in *S. granulata* populations.The Q_{ST} values for leaf length and the number of flowers were lower than F_{ST}, and Q_{ST} for plant diameter was also lower, but its confidence interval overlapped that of the F_{ST} value, suggesting that homogenizing selection for optimal trait values as well as drift have shaped the variation among populations. The studied populations occur in a restricted area in similar environments, and thus have likely experienced similar selection pressures on fitness-related traits. In contrast, most quantitative genetic studies have reported divergent selection (Merilä and Crnokrak 2001, Leinonen et al. 2008). In *Scabiosa columbaria* populations from a restricted area (37 x 11 km) in the Jura mountains the Q_{ST} values for aboveground biomass and relative growth rate were also very low (0.000-0.004) and were smaller than G_{ST} (CI 0.08-0.16) suggesting unifying selection (Scheepens et al 2010a) and in *Psilopeganum sinense,* populations sampled at a maximum distance of 287 km along the Yangtse river, the traits height of the tallest stem, area of the largest leaf and total number of flowers of populations also showed much lower Q_{ST} (0.067-0.13) than F_{ST} (0.47), indicating unifying selection (Ye et al. 2013). Furthermore, in the rare plants *Brassica insularis* and *Centaurea corymbosa*, Q_{ST} was lower than F_{ST} for juvenile traits (Petit et al. 2001). The fact that Q_{ST} is similar or slightly lower than F_{ST} does, however, not necessarily exclude directional selection, but merely indicates that the hypothesis that the present structure has been shaped by drift and homogenizing selection cannot be rejected. We may not have detected an effect of divergent selection because the maximum spatial extent of our study was too small (< 61 km). Indeed, Q_{ST}-F_{ST} differences increase with increasing geographical distances among populations, possibly due to the joint actions of decreasing gene flow and increasing environmental differences between populations (De Koort et al. 2013). However, populations of *Scabiosa columbaria* also showed divergent selection within a small spatial area (Waldmann et al. 1998). Similarly, populations of *Saxifraga sponhemica* sampled within a small area of Luxemburg and Germany (< 96 km maximum distance), had a larger overall Q_{ST} than F_{ST} (0.39 vs. 0.27, Walisch unpublished results) indicating divergent selection. Populations of *S. sponhemica* may be more strongly differentiated than those of *S. granulata* because they have been isolated for a longer time with limited or no gene flow among populations (Walisch et al 2015b).

Clonality and genetic structure at a small spatial scale

Clonal diversity (proportion of distinguishable genotypes PD and the Simpson's diversity index *D* found in two populations of *S. granulata* were high compared to the mean values for mainly vegetatively reproducing species (PD = 0.27, *D* = 0.75) (Widén et al. 1994) and to those found in a compilation of RAPD studies on clonal species (mean *D* = 0.74, range 0.35–1.00 and mean PD = 0.44, range 0.00–0.94, Hangelbroek et al. 2002). Similar results have been found in other clonal species such as *Narcissus pseudonarcissus* (PD = 0.57, Colling et al., 2010), *Potamogeton pectinatus* (0.76, Hangelbroek et al. 2002), or the grassland populations of *Ranunculus ficaria* (0.80, Reisch and Scheitler 2009) and *Viola calaminaria* (0.76-0.90, Bizoux and Mahy 2007). It has been suggested that in these species clonality is less important and repeated seedling recruitment is occurring (Eriksson 1993). In a demographic study of *V. riviniana*, an even higher genotypic diversity (0.93-0.99) resulted from high seedling recruitment in the populations and high mortality of clonal ramets (Auge et al. 2001). A simulation study demonstrated that rare events of seedling recruitment may be sufficient to maintain high diversity in populations of clonal species (Watkinson and Powell 1993) and an empirical study of the widespread arctic *Saxifraga cernua* found that rare sexual events were sufficient to maintain genetic diversity in populations, also at small spatial scales (Kjølner et al. 2004). We conclude that in populations of *S. granulata*, the propagation through bulbils is less important than sexual reproduction through seedling recruitment. Low levels of seedling establishment combined with weak clonal propagation may maintain clonal diversity.

Most clones formed clusters with a low spatial extent (3.5 cm). This was comparable to the clonal extent of clones of *Narcissus pseudonarcissus* formed by bulbils (4.5 cm). Only a small proportion of *S. granulata* clones had a larger spread of up to 25 cm, comparable to the clonal spread of *Viola calaminaria* by rhizomes (Bizoux and Mahy 2007), but smaller than the spread by adventitious buds and rhizomes in *Viola riviniana* (0.1-1.7 m, Auge et al. 2001). As insects tend to preferentially pollinate neighbouring flowers (De Jong et al. 1992, Rademaker and de Jong 1998), the risk of biparental inbreeding between clonal ramets is larger if they grow in clusters. Because the negative effects of inbreeding may be extremely high in populations of *S. granulata* (Walisch et al. 2012) mortality of inbred offspring is likely to be high and this may lower the reproductive success of a population. We found that genetically distinct individuals can grow in close proximity to each other, suggesting that outbreeding probably occurs quite frequently.

There was a significant spatial genetic structure within plots at the ramet level (including clones) and at the genet level suggesting that there is isolation by distance at very small distances (< 7.5 cm). Isolation by distance is a sign of localized drift from limited gene

flow via pollen or seeds (Hardy and Vekemans 1999). The strong small-scale genetic structure at distances below 10 cm may be due to very localised seed dispersal geitonogamous pollination or biparental inbreeding. Clonality increased the kinship coefficient, indicating that it strengtHeNed the spatial genetic structure. A similar importance of clonal growth through bulbils at small distances has been found in a study of *Narcissus pseudonarcissus* (Colling et al. 2010). At the transect level, where plants had been sampled at a minimum distance of 50 cm, there was a weaker spatial genetic structure, confirming an isolation by distance pattern at distances of up to 80 cm. The *Sp* statistics at the transect level, expressing the level of relatedness and scale of genetic structure, were about ten times smaller than those of its congener *S. sponhemica* (*Sp* = 0.041, $F_{(1)}$ = 0.03) and those of species with a mixed mating system (mean *Sp* = 0.04, mean $F_{(1)}$ = 0.098, Vekemans and Hardy 2004), and were at the lower end of values for mainly outcrossing or self-incompatible species. They were similar to the mean values of wind-pollinated or of animal dispersed species (*Sp* = 0.006-0.009, Vekemans and Hardy 2004). The weaker structure at the transect level probably mainly reflected pollination of genetically close individuals resulting in biparental inbreeding (Walisch et al. 2012). Furthermore, the dispersal of the small seeds over large distances by wind or mowing activities may have weakened the genetic structure found at the transect level in *S. granulata*. Moreover, extreme inbreeding depression may have led to selection against selfed individuals, favouring outbred individuals (Walisch et al. 2012).

No effects of fragmentation on the genetic diversity of populations and on plant performance

Genetic diversity is sensitive to drift and predicted to decline in small and isolated populations. The recent fragmentation of *S. granulata* populations had, however, not yet any negative impact on within-population genetic diversity or plant performance, given the high overall molecular genetic diversities H_{eN} and *PPL* and the absence of a correlation between population size, genetic diversity and plant performance.

The overall molecular genetic diversity H_{eN} of populations was high in comparison to the mean H_{eN} found in RAPD studies (0.214, Nybom and Bartish 2004), for long lived perennials (H_{eN} = 0.21, Nybom 2004), or for widespread species (H_{eN} = 0.22). A microsatellite marker study on riparian *S. granulata* populations in Belgium estimated a within-population genetic diversity H_s = 0.68 (van der Meer and Jacquemyn 2015). This diversity level is comparable to the genetic diversity H_{eN} = 0.314 of our study, because the genetic diversities estimated by microsatellite marker studies are on average almost three times higher than the diversity estimates from dominant marker studies (Nybom 2004). The high genetic diversity could be due to the polyploid nature of *S. granulata*

(van der Meer and Jacquemyn 2015). Because they contain more copies of the genome, populations of polyploid species have a higher potential for mutations and they are buffered against the loss of alleles through drift (Meirmanns and van Tienderen 2013). The genetic diversity of *S. granulata* populations was also slightly larger than the genetic diversity of populations of the long-term fragmented *S. sponhemica* in Luxembourg and Germany (H_{eN} = 0.28, Walisch et al. 2015a). Formerly common species often have higher genetic diversities than historically rare species (Aguilar et al. 2008) as a result of past habitat connectivity and gene flow counteracting genetic erosion in the species (Levin 1995, Münzbergova et al. 2013). Because common species generally host comparatively higher genetic diversity, they are also more susceptible to genetic erosion (Aguilar et al. 2008).

Small *S. granulata* populations had not yet lost genetic variation as a result of drift. Only one small population in an open cast mine (Lallange) had a lower genetic diversity, probably due to a founder effect a few decades ago. This is not in line with the commonly found positive correlation between population size and genetic diversity in plant species (Leimu et al. 2006). However, a lack of a correlation between population size and genetic diversity has been found in studies of *Scabiosa columbaria* populations in Sweden (Waldmann et al. 1998), and of the long-lived and clonal glacial relicts *S. sponhemica* (Walisch et al. 2015a) and *S. azoides* (Lutz et al. 2000) which have maintained genetic diversity over thousands of years despite high levels of fragmentation. It is likely that the longevity, clonality and polyploidy of *S. granulata* have delayed genetic erosion due to drift and thus preserved genetic diversity in the recently fragmented populations (Nybom et al., 2004, van der Meer and Jacquemyn 2015).

We found no effect of molecular genetic diversity on the performance of populations in contrast to the results of a study of the related *S. sponhemica* (Walisch et al. 2015b). A possible explanation is the restricted range of genetic diversities in our study populations (0.287-0.378). Furthermore, the extremely high inbreeding depression in *S. granulata* (Walisch et al. 2012) may eliminate offspring from selfing and increase the proportion of outcrossed, more genetically variable and fitter individuals in all the populations.

Contrary to our expectations, the performance of populations and quantitative genetic diversity were negatively correlated. Thus, in populations where plants were larger and had larger leaves, the evolutionary potential of these traits was reduced. One explanation could be that in populations the selection of a fitter larger pHeNotype as a result of (balancing) selection, may be accompanied by a loss of quantitative genetic variability within populations, as stated by quantitative genetic theory (Bulmer 1971, Visscher et al. 2008).

Relation between molecular and quantitative genetic diversity

There was a positive relationship between the mean evolvability of each studied trait and the molecular genetic diversity in a population. Overall mean evolvability over all traits also increased with molecular genetic diversity, which suggests that molecular genetic diversity is a good predictor of evolvability in our study, in contrast to the results of many other studies (Reed and Frankham 2001, Leinonen et al. 2008, Mittell et al. 2015). This is probably due to the fact that in our study populations were sampled in similar habitats in a small region, and there was thus no adaptation to local edaphic conditions and differences in climate were small. We also found a positive relation between the evolvability and the heritablility (h^2) of each trait and averaged over all traits in a population, which does not lend support to the general conclusions of reviews by Houlé (1992) and Hansen (2011) that evolvability and heritability are generally not correlated. This may be due to positive relationships between additive genetic variances and other components of variance (Hansen et al. 2011).

CONCLUSIONS

S. granulata is an example of a newly rare species that has not yet suffered from the ongoing fragmentation of its habitat over the past 60 years. High genetic diversity and low differentiation among populations reflect the past interconnection of populations. The polyploid nature and the longevity of *S. granulata* are the most likely factors that have delayed the genetic erosion within and the genetic differentiation among the populations. Although we did not find high levels of clonal propagation in *S. granulata*, clonal growth makes genets potentially immortal and is also a potent buffer against the loss of diversity in populations (Eriksson 1993, Watkinson and White 1986). Moreover, long distance dispersal, by wind or mowing machines may have prevented genetic erosion in the increasingly isolated populations. We did not find evidence for divergent selection in the measured traits, but we detected weak signs of stabilizing selection suggesting that homogeneous selection pressures favour an optimal pHeNotype in the studied populations.

While longevity, clonality and polyploidy have preserved past genetic diversity in populations of *S. granulata*, management is required to ensure the maintenance of this diversity into the future. It is important to preserve the extant populations and increase the size of small populations to avoid genetic erosion because of drift. Gene flow should be maintained among populations by, for example, transport of hay.

Chapter 5

Effects of inbreeding and interpopulation crosses on performance and plasticity of two generations of offspring of a declining grassland plant

published in *American Journal of Botany* 99(8), 1300–1313. (2012)
with Guy Colling, Myriam Poncelet and Diethart Matthies

ABSTRACT

Premise of the study: Inbreeding depression is a major evolutionary force and an important topic in conservation genetics because habitat fragmentation leads to increased inbreeding in the populations of many species. Crosses between populations may restore heterozygosity resulting in increased performance (heterosis), but may also lead to the disruption of coadapted gene complexes and to decreased performance (outbreeding depression).

Methods: We investigated the effects of selfing, and of within and between population crosses on reproduction and the performance of two generations of offspring of the declining grassland plant *Saxifraga granulata*. We also subjected the first generation of offspring to a fertilisation and two stress treatments (competition and defoliation) to investigate whether the effects of inbreeding and interpopulation gene flow depend on environmental conditions.

Key results: Inbreeding depression affected all traits in the F_1 ($\delta = 0.07 - 0.55$), but was stronger for traits expressed late during development and varied among families. The adaptive plasticity of offspring from selfing and from interpopulation crosses in response to nutrient addition was reduced. Outbreeding depression was also observed in response to stress. Multiplicative fitness of the F_2-generation after serial inbreeding was extremely low ($\delta > 0.99$), but there was heterosis after crossing inbred lines. Outbreeding depression was not observed in the F_2.

Conclusions: Continuous inbreeding may drastically reduce the fitness of plants, but effects may be environment-dependent. When assessing the genetic effects of fragmentation and interpopulation crosses, both the possible effects on the mean performance of offspring and on its adaptive plasticity should be considered.

INTRODUCTION

Inbreeding depression, the decrease in fitness as a result of inbreeding, is a major topic of evolutionary and ecological research, because of its importance for the evolution of mating systems and life history traits (Crnokrak and Barrett 2002, Charlesworth and Willis 2009, Cheptou and Donohue 2011). Understanding inbreeding depression has become particularly important in conservation biology, because the ongoing fragmentation of habitats has increased inbreeding levels in the populations of many species (Keller and Waller 2002, Mustajärvi et al. 2005, Honnay and Jaquemyn 2007, Angeloni et al. 2011). In fragmented populations, changes in pollinator behavior and reduced availability of mates may increase pollinations between closely related individuals and self-pollination, resulting in increased inbreeding (Sih & Baltus 1987, Mustajärvi et al. 2001, Honnay et al. 2005). Mating between close relatives in fragmented populations may result in inbreeding depression negatively affecting various components of plant fitness like reproduction, survival and growth, as well as population growth rate (Husband & Schemske 1996, Fischer and Matthies 1998, Keller and Waller 2002, Kolb 2005, Leimu et al. 2006, Ramula et al. 2007, Wagenius 2010, Angeloni et al. 2011). Inbreeding may also reduce the adaptive plasticity of plants in response to changes in the environment (Fischer et al. 2000, Kéry et al. 2000). Because of its negative effects on plant fitness, increased inbreeding in fragmented populations is a major concern for conservation. Inbreeding is also of great interest to evolutionary plant biologists, because variation among genotypes in inbreeding depression is thought to be a major factor in the evolution of plant breeding systems (Holsinger 1988, Uyenoyama et al. 1993, Schultz and Willis 1995, Picó et al. 2004).

The magnitude of inbreeding depression may depend on environmental conditions. The environmental dependency of inbreeding depression can manifest itself in two ways (Cheptou and Donohue 2011): (1) The fitness of inbred progeny may be reduced more strongly under stressful conditions than that of outbred progeny. Many studies have found inbreeding depression to be stronger under stressful conditions (Armbruster and Reed 2005, Fox and Reed 2010, Cheptou and Donohue 2011). However, this pattern is not universal and a recent metaanalysis found no evidence for a general interaction between environment and the strength of inbreeding depression and called for more detailed studies of the interaction between environment and the dynamics of inbreeding depression over time (Schlichting and Levin 1986, Angeloni et al. 2011). (2) Inbred progeny may be less capable of capitalizing on favourable environments (Cheptou and Donohue 2011), i.e. show less adaptive plasticity (Kéry et al. 2000, Pluess and Stöcklin 2004, but see Berg et al. 2005).

The magnitude of the negative effects of inbreeding depends on the breeding system and may vary among life stages (Husband & Schemske 1996, Angeloni et al. 2011). Inbreeding depression is in general higher in predominantly outcrossing than in selfing species, but this difference decreases from early to late life stages (Husband and Schemske 1996, Mustajärvi et al. 2005, Angeloni et al. 2011). While inbreeding depression at early stages may be caused by strongly deleterious alleles that may be effectively purged in self-fertilizing species, negative effects of inbreeding on late traits (i.e. expressed at later life stages) may be due to the accumulated effects of many mildly deleterious alleles, which are more difficult to purge (Husband and Schemske 1996). Little is known, however, about the timing of inbreeding depression in plants with a mixed mating system (Husband and Schemske 1996, but see Mustajärvi et al. 2005).

For counteracting the negative genetic effects of fragmentation such as increased drift and inbreeding, it has been suggested that gene flow needs to be retained or artificially increased among the remnant plant populations (Storfer 1999, Keller and Waller 2002, Hufford and Mazer 2003). Increased fitness of the hybrids (heterosis) from interpopulation crosses and a genetic rescue effect has been demonstrated for several plant species (Vergeer et al. 2004, Erickson and Fenster 2006, Willi et al. 2007). However, the fitness of offspring from interpopulation crosses may also be reduced because of outbreeding depression (see Montalvo et al. 1997, Hufford and Mazer 2003). Two mechanisms, an ecological and a genetic one, may cause outbreeding depression. If the environmental conditions of the two parent populations differ and the populations are adapted to these conditions, the hybrids may be maladapted to both parental environments. Another mechanism is the breakup of coadapted gene complexes by recombination (Price and Waser 1979, Lynch 1991, Hufford and Mazer 2003). Outbreeding depression has been observed in a number of species (Waser and Price 1989, 1994, Fischer and Matthies 1997, Edmands 1999, Grindeland 2008), but no consensus has emerged on its importance in comparison to heterosis after interpopulation crosses (Edmands 2007, but see Frankham et al. 2011). The negative effects of interpopulation crosses should mainly be expressed from the second generation of offspring (F_2) on, because they may be masked in the F_1 by heterosis (Edmands and Timmermann 2003). However, very few studies have investigated effects of interpopulation crosses on the F_2-generation (Fenster and Galloway 2000, Hufford and Mazer 2003, Willi et al. 2007, Crémieux et al. 2010, Volis and Zhang 2010) and their results have varied. Like the effects of inbreeding, the effects of interpopulation crosses may be environment-dependent, but little is known about the influence of the environment on the strength of heterosis or outbreeding depression (Armbruster et al. 1997, Fenster and Galloway 2000, Edmands and Deimler 2004, Edmands 2007).

The aim of this study was to investigate the potential effects of increased inbreeding re-

sulting from fragmentation and of interpopulation crosses on the self-compatible protandrous plant *Saxifraga granulata* L. *S. granulata* is a typical species of dry grasslands, which has strongly declined in the last decades and is now threatened in several European regions (Korneck et al. 1996, Niklfeld 1999) due to the alteration and fragmentation of its habitats. We investigated the effects of selfing, and within and between population crosses on reproduction and offspring performance of two generations of plants of *S. granulata* from a large remnant population. We grew the first and second-generation offspring in a common garden and recorded seed production, survival, and performance. In addition, we subjected the first generation offspring to competition, defoliation, and fertilization treatments to investigate whether the effects of inbreeding and interpopulation gene flow depend on environmental conditions.

We addressed the following questions: (1) Do selfing and interpopulation crosses affect seed production and offspring performance, and are the effects on offspring performance environment-dependent? (2) Which life stages of the first and second offspring generation are most affected by inbreeding depression, heterosis or outbreeding depression? (3) Is there among family variation in inbreeding and outbreeding depression?

MATERIALS AND METHODS

Study species

Saxifraga granulata L. is a perennial herb that is capable of propagating sexually via seeds and vegetatively via small bulbils at the base of the plant. An individual ramet consists of a basal rosette that may produce a stem with petiolate leaves. Genets are perennial, but individual ramets are annual. Stems of *S. granulata* produce flowers in May and June. The flowers are self-compatible and are visited by a wide range of insect species, including flies and solitary bees (Hansen and Molau 1994). Flowers are protandrous and arranged in a cymose inflorescence with the apical flowers opening first. The differential ripening of the flowers allows geitonogamous selfing within the same genet. Some populations have been found to be gynodioecious (Stevens and Richards 1985). The fruit is a capsule with two locules, each containing ca. 250 seeds. The seeds are very small, (ca. 0.5 x 0.3 mm, ca. 40 μg), brown, and covered with small papillae. *S. granulata* occurs in mesic to dry grasslands and occasionally on rocks and has a West Eurasian and North African distribution. Populations in Europe have been declining in the last decades as a result of changes in agricultural practices, in particular, the increased fertilization of meadows, which results in increased competition by grasses.

Experiment 1: Effects of pollen source on reproduction and offspring performance

In May 2001, we randomly chose 22 individuals in a large population of *S. granulata* (~5000 individuals) in a hay meadow at Huneschwanz, ca. 12 km west of the city of Luxembourg. On each plant, five flowers that were about to open were selected and marked with differently colored cotton threads. The flowers of each plant received randomly one of the following five pollination treatments: (1) One flower was left untreated to investigate the reproductive success of open-pollinated flowers ('open pollination'). (2) A second flower was bagged with a fine-meshed cloth (0.3 mm) to prevent insects from visiting the flowers and to assess spontaneous self-pollination ('autonomously selfed'). The other three flowers were emasculated at the start of the experiment and also bagged. After the stigmata had become receptive, one flower (3) was hand-pollinated with pollen from the same plant ('hand-selfed'), (4) one was pollinated with pollen from a plant of the same population, at least 10 m away ('within population cross'), (5) and one was pollinated with pollen from a different population 10 km away ('between population cross'). For the hand-pollinations we carefully pulled out one to two ripe stamens of two to three randomly chosen donor plants and gently rubbed the anthers against the receptive stigma of a flower. After hand-pollinations, the bags were closed again to prevent natural pollination. In mid June the ripe fruits were collected, put into paper bags, and left to dry at room temperature. Capsules were then opened and ripe seeds counted. The ripe seeds were weighed per capsule and stored in paper bags at 6 °C.

In September 2002, we placed ca. 100 seeds from each fruit (hereafter referred to as a seed family) on moist filter paper in two Petri dishes. Seeds were kept in darkness at 4 °C for 5 weeks to break their dormancy, then kept in a growth chamber at 20 °C under a 12 h day/12 h night light regime. Lighting was provided by fluorescent tubes. Petri dishes were randomised every 3 to 4 days. Germination of the seeds was checked every third day. At the end of October 2002 we randomly selected, if available, 30 seedlings from each seed family and planted them into soaked peat pellets ('Jiffy pots'). The plants were kept in a heated glasshouse and received 16 h/day additional light from high-pressure sodium lamps (SON-T Agro 400 W, Philips). The position of the plants was randomized every two weeks.

The survival of the plants was recorded 60 d after planting, and the width of the largest leaf and the number of leaves were recorded for each plant. The leaves of *S. granulata* are wider than long, and leaf width was strongly correlated with leaf biomass ($r = 0.89$, $n = 29$, $P < 0.001$). In mid February 2003 (day 118), survival of the plants was recorded, and they were then transplanted into pots (11 cm diameter) filled with low-nutrient soil (138 mg/L N, 108 mg/L P_2O_2, 158 mg/L K_2O). At the beginning of March, the pots

were placed into the experimental garden of the National Museum of Natural History of Luxembourg and protected against frost by a foil tunnel until April. Plants were watered if necessary. At the beginning of May, 200 d after planting, plant leaf traits were measured again. In mid June 2004, around day 603, when nearly all the plants had reached maturity, the diameter of each plant and the width of the largest leaf was measured and the number of leaves and flowers per plant counted. The magnitude of inbreeding depression based on the various traits was calculated from the fitness of outcrossed (within population) and selfed flowers as the proportional reduction in performance of inbred relative to that of outcrossed offspring (Johnston and Schoen 1994).

Experiment 2: Plasticity of offspring

At the end of July 2003, we selected, if available, two plants at random from each seed family from the first experiment and transplanted them into larger pots (14 cm diameter) filled with low-nutrient soil. On 17 September 2003, we determined the diameter of each plant as an estimate of initial size. Then we subjected each seed family randomly to one of four treatments: fertilization, competition from grasses, defoliation, or untreated control. Plants to be fertilized received 1.5 ml of liquid fertilizer (Bayfolan® Special, Bayer, Brussels; NPK 8:6:4) mixed with 250 ml of water and 20 g of long-term slow-release fertilizer (Hornoska® Depot, Günther, Erlangen, Germany, NPK: 24 : 8 : 16) to each pot. For the competition treatment, we sowed 200 seeds of the grass species *Dactylis glomerata* per pot. For the defoliation treatment, we removed 90% of the leaves with scissors. The defoliation was repeated on day 51 of the experiment. We assessed the effect of the treatments by determining the number of leaves, the width of the largest leaf, and the number of flowers per plant in spring 2004 after 215 d of growth.

Experiment 3: Performance of the F_2*-generation*

In June 2004, we randomly selected two plants from each of the seed families produced by the handpollinated flowers (WPC, BPC, and hand-selfed) of the first experiment, and in addition the remaining offspring from two hand-selfed flowers. The plants were arranged randomly in a separate plot in the experimental garden. We emasculated one flower per plant and protected it with a fine-meshed cloth to prevent insects from visiting. Six to 10 days after emasculation, we (1) carried out within-family crosses using offspring from selfed flowers (S → WFC) (Fig. 1), (2) we randomly crossed offspring from different families resulting from within-population crosses (WPC → WPC) and (3) crossed offspring from different families resulting from between-population crosses (BPC → WPC). Finally, we (4) crossed different families resulting from selfed flowers (S → BFC). Per treatment, 18-30 replicate crosses were carried out. The treatments were meant to simu-

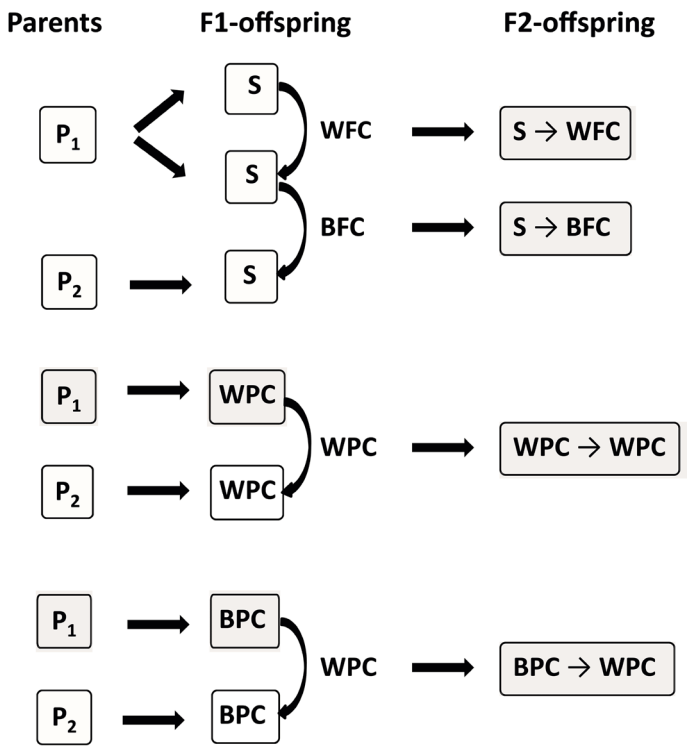

Figure 1. Schematic diagram showing the various combinations of pollination treatments to which plants of *S. granulata* were subjected. S, selfed; WFC, within-family-cross; BFC, between-family-cross; WPC, within-population-cross; BPC, between-population-cross.

late the situation after a population is founded (1) by a single seed, (2) by several individuals from a single population, (3) by several individuals from two populations, and (4) the situation that two seeds from a single population found separate populations that come into contact after one generation of inbreeding. For pollinations, we picked two male phase flowers from a donor plant and gently rubbed them against the ripe stigma of the receiver flower. The ripe fruits were collected and stored in a dry place for two months. The number of fully developed seeds and seed mass were determined as described.

On 6 September 2004, we randomly selected two batches of ca. 30 seeds each from each fruit and placed them on wet filter paper in two Petri dishes. The germination test was conducted as in experiment 1. We counted the number of germinated seeds every 5 d for 6 weeks. Seedlings were cultivated in Jiffy pots as in experiment 1. On 3 March 2005 we recorded survival and transplanted the surviving plants into pots (11 cm diameter) filled with low-nutrient soil and placed them into the experimental garden of the National Museum of Natural History in Luxembourg.

After 17 weeks of growth outside, 182 d after the start of germination, the number of leaves and the width of the largest leaf were determined. The same traits were measured

in the next year on 14 April (546 d). On 13 May 2006 (575 d), in addition the number of stems and flowers and the length of stems and inflorescences of flowering individuals were determined.

Data analysis

Plant size of F_1 and of F_2 progeny was calculated as the number of leaves multiplied by the width of the largest leaf (cumulative leaf width). Several multiplicative fitness functions were calculated to study the effect of pollination treatments on reproduction and performance of the F_1 progeny. We calculated the number of flowers produced by the F_1 descendents of a flower as the number of surviving offspring per flower multiplied by the mean number of flowers produced after 603 d of growth. We also calculated the number of flowers per seed as a measure of the fitness of offspring.

The selfing rate (*s*) was estimated after Charlesworth (1988) from the multiplicative fitness function number of flowers per seed (w) calculated for offspring from selfed, open-pollinated, and outcrossed flowers as:

$$s = (w_{open} - w_{outcrossed})/(w_{selfed} - w_{outcrossed})$$

To study the effect of successive pollination treatments on the performance and reproduction of the F_2 progeny, we calculated the number of flowers produced by the descendants of an F_1 flower 575 d since planting of the F_2. We also calculated a bigenerational fitness function as the cumulative leaf width of the F_2 descendants of one flower of the parent generation pollinated in the field.

To investigate the effects of pollination treatment on reproduction and offspring performance at each measurement time, we used analyses of variance with mother plant as a block factor. We partitioned the effect of pollination treatment into orthogonal contrasts: (1) the two selfing vs. the open and hand-crossed treatments (2) open pollination vs. hand-crossed, (3) within population vs. between population crosses, (4) spontaneously selfed vs. handselfed. All effects were tested against the residual variation among the pollinated flowers. For the analysis of seed production all four contrasts were used, for the analysis of offspring traits data for spontaneously selfed and hand-selfed flowers were pooled.

We performed pairwise correlations between inbreeding depression levels of traits and used the graphically sharpened false discovery rate (FDR) to obtain adjusted *P*-values for multiple comparisons (Benjamini and Hochberg 2000, Pike 2010).

To test for differences in levels of inbreeding depression among families, we compared the effect of inbreeding (hand-selfing), outbreeding (hand-crossing) and mother plant on offspring performance. A significant mother plant-by-pollination interaction

indicates that the levels of inbreeding depression differ among mother plants (Johnston and Schoen 1994). Following Johnston and Schoen (1994), we log-transformed fitness measures to analyse relative effects. We further tested which mother plants had been significantly affected by selfing using multiple simple main effects tests (Pedhazur 1982, Pico et al. 2004) and adjusted the *P* values for multiple comparisons using the graphically sharpened false discovery rate (Benjamini and Hochberg 2000, Pike 2010).

To investigate the effects of the environmental treatments applied to the offspring of the various crosses, we used two-factorial ANOVAs. The effect of environmental treatment was partitioned into three orthogonal contrasts: (1) fertilized vs. non-fertilized, (2) stressed (defoliated or under competition) vs. control, and (3) competition vs. defoliation. To investigate the effects of the successive pollination treatments on F_1 reproduction and F_2 performance, we partitioned the effect of pollination treatments into three orthogonal contrasts: the within-family cross treatment (S→WFC) vs. the three other treatments, (2) crosses between selfed families (S→BFC) vs. crosses between crossed families (WPC→WPC and BPC→WPC), and (3) crosses between offspring from WPC (WPC→WPC) vs. crosses between offspring from BPC (BPC→WPC).

To compare the magnitude of inbreeding depression observed for different traits, we calculated the family means of inbreeding coefficients for both generations, i.e., the means of the per-family estimates of inbreeding depression, as suggested by Johnston and Schoen (1994).

Data were transformed if necessary prior to analysis to achieve normally distributed residuals and homogeneity of variances. Binomial variables like survival and flowering, seed set and germination were analyzed by analyses of deviance. Mean deviances due to a factor were divided by their appropriate error mean deviances, analogous to the calculation of *F* ratios in ordinary analysis of variance (Francis et al. 1993). All analysis were carried out with SPSS for Windows 11.0 (SPSS 2001).

RESULTS

Experiment 1: Effects of pollinator exclusion and pollen source on fecundity and offspring performance

Seed set, seed mass (Table 1) and germination (*quasi*-$F_{21,62} = 8.96$, $P < 0.001$) were influenced by maternal plant identity. This suggests that there is considerable genetic or maternal variation for early traits. The different pollination treatments influenced the number of seeds (Table 1, Fig. 2a). Seed production by caged flowers that were not hand-pollinated was 74% lower than that by hand-selfed flowers, indicating that spontaneous selfing was low. Seed production of hand-selfed flowers was 23% lower than that by hand-crossed flowers

Table 1. Effects of pollination treatment on seed production (number of seeds per capsule) and seed mass of *S. granulata*. Results of analyses of variance. The effect of pollination treatment was partitioned into orthogonal contrasts: Selfed vs. cross-pollinated (i.e. hand-crossed and open-pollinated) flowers, open-pollinated vs. hand-crossed, within-population crosses (WPC) vs. between-population crosses (BPC) and spontaneously- (auto-) selfed vs. hand-selfed. ***, $P < 0.001$.

		Number of seeds per capsule		Seed mass	
Source of variation	df	MS	*F*	MS	*F*
Mother plant	21	66417	2.84 ***	401.39	5.48 ***
Pollination treatment	3-4	371760	15.90 ***	1889.87	25.80 ***
Selfed vs. crossed	1	487151	20.83 ***	2555.60	34.88 ***
Open vs. hand-crossed	1	527262	22.54 ***	2921.91	39.88 ***
WPC vs. BPC	1	274	0.01	192.11	2.62
Auto vs. hand-selfed	1	472354	20.20 ***		
Residual	60-73	23386		73.26	

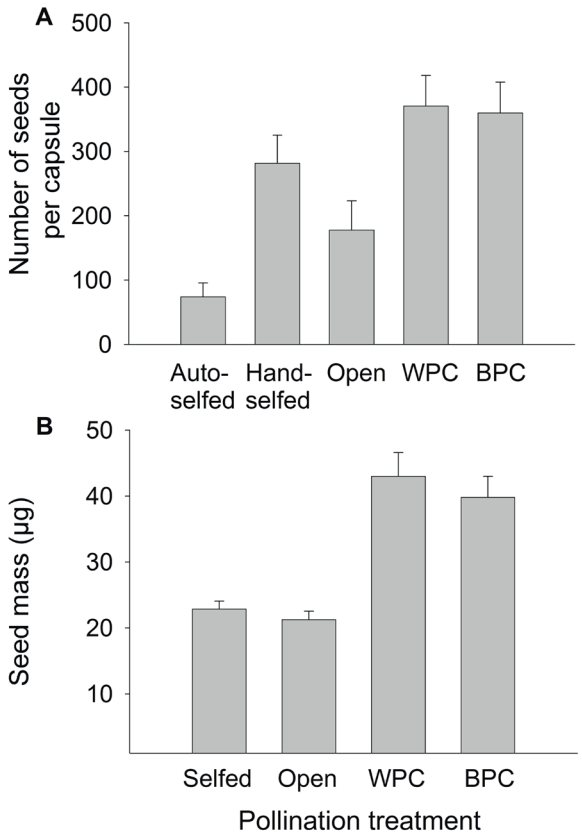

Figure 2. The effect of pollination treatments on (A) number of seeds per capsule, and (B) mean seed mass of *Saxifraga granulata*. Flowers were either bagged and left to auto-self, hand-selfed, open-pollinated or hand-crossed with pollen from the same population (WPC) or from a different population (BPC). Means + 1SE.

indicating inbreeding depression in seed production, but 58% higher than that by open-pollinated flowers, indicating insufficient pollination by insects. Handcrosses increased the number of seeds by 105% compared to open pollination (Table 1, Fig. 2a).

Pollination treatments also influenced seed mass and germination. The seeds of selfed and open-pollinated flowers were of similar size and 48% smaller than seeds from hand-crossed flowers (Fig. 2b). Seeds produced by pollination within and between populations did not differ in size. Germination was generally high (>50%), but the germination of seeds produced by open-pollinated flowers was 25% lower than that of seeds from hand-crossed flowers (*quasi-F* $_{1,62}$ = 12.38, $P < 0.001$).

Survival of the plants was high as long as they were raised indoors (>90%), but already after 118 d of growth, survival of selfed seeds was 9% lower than that of seeds from open pollinations or hand-crosses (91.6% vs. 95%, *quasi*-$F_{1,62}$ = 4.34, $P < 0.05$), indicating inbreeding depression. After 16 months of growth outdoors (day 603), plants resulting from between-population crosses and from open pollinations had similar survival, whereas survival of plants from within-population crosses was 16% lower and similar to that of plants from selfing (Fig. 3a, Table 2).

The pollination treatments also affected the growth and flowering of the offspring. These effects were due to the lower performance of the selfed offspring, while there were no significant differences among the other three treatments. The size of inbred offspring at the end of the experiment in terms of cumulative leaf width was 27% lower (93.0 cm vs. 127.7 cm; $F_{1,53}$ = 13.28, $P < 0.001$), the proportion of plants flowering was 4% lower (93.6% vs. 97.4%; *quasi*-$F_{1,56}$ = 17.27, $P < 0.001$) and the number of flowers per plant 52% lower (Fig. 3b, Table 2).

Table 2. Effects of pollination treatments on survival, reproduction and multiplicative fitness of *S. granulata* after 603 days of growth. Results of analyses of deviance or variance. The effect of pollination treatment was partitioned into three orthogonal contrasts: selfed vs. cross-pollinated (i.e. hand-crossed and open-pollinated) flowers, open-pollinated vs. hand-crossed, and within-population crosses (WPC) vs. between-population crosses (BPC). Number of flowers per flowering plant was log-transformed prior to analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

		Survival		Flowers per flowering plant		Number of flowers per seed	
Source of variation	df	MD	*Quasi-F*	MS	*F*	MS	*F*
Mother plant	21	18.61	1.92 *	0.094	3.48 ***	781.08	2.39 **
Pollination	3	23.93	2.46	0.591	21.85 ***	5014.52	15.36 ***
Selfed vs. crossed	1	20.00	2.06	1.709	63.24 ***	12096.52	37.05 ***
Open vs. hand-crossed	1	12.37	1.27	0.048	1.77	2935.76	8.99 **
WPC vs. BPC	1	39.43	4.06 *	0.014	0.53	11.29	0.04
Residual	62-67	9.71		0.027		326.48	

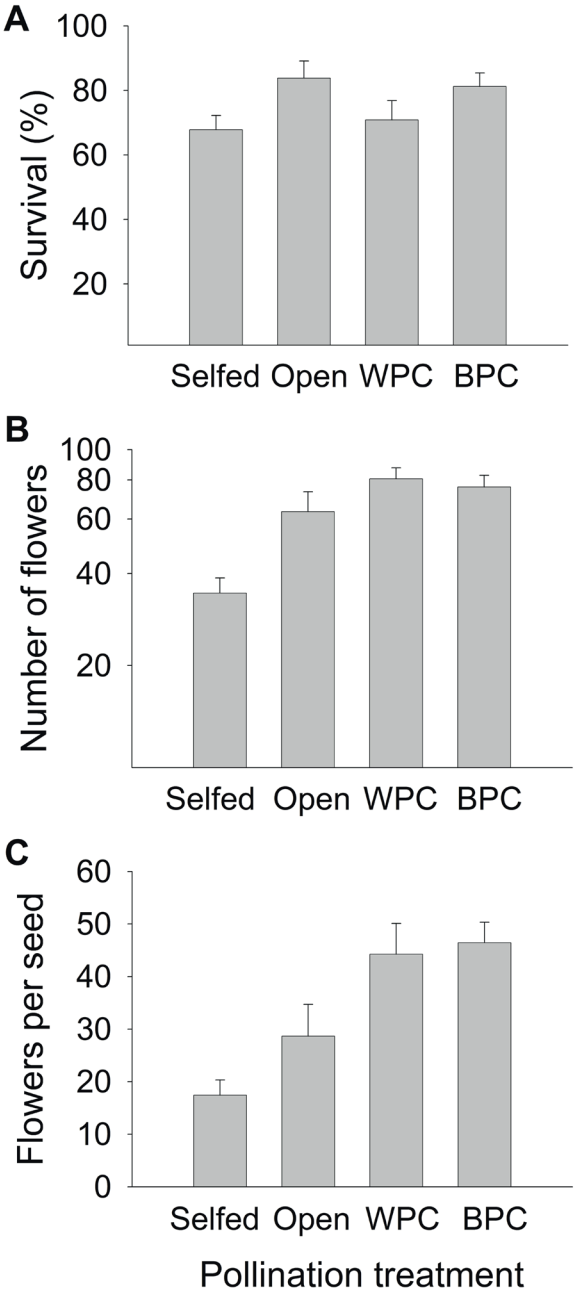

Figure 3. The effect of pollination treatments on the performance of offspring of *S. granulata.* (A) survival over two years, (B) number of flowers per plant, and (C) the multiplicative fitness function number of flowers produced by the offspring of a seed. Flowers were either selfed, open-pollinated or hand-crossed with pollen from the same population (WPC) or from a different population (BPC). Note log-scale for number of flowers. Means + 1SE.

We calculated two multiplicative measures of fitness. The first was the number of flowers produced per seed, which describes the fitness of the offspring. The fitness of offspring from the selfing treatment was much lower (Fig. 3c, Table 2). The fitness of offspring resulting from open pollinations was higher than that of selfed, but 36% lower than that

of offspring from hand-crosses, indicating that part of the seeds resulted from selfing. Using the formula of Charlesworth (1988), we estimated a selfing rate of 55%. The second measure of fitness included in addition the number of seeds produced per pollinated flower, i.e., was equivalent to the number of flowers produced by all the descendants (seeds) of a flower after 2 years of growth. The effect of the pollination treatments on this fitness function was similar to that on the other function. Offspring of selfed flowers produced fewer flowers than both offspring from open-pollinations and hand-crosses (54% and 87% fewer; $F_{1,67} = 51.61$, $P < 0.001$), and offspring from open pollinations produced 72% fewer flowers than offspring from hand-outcrosses ($F_{1,67} = 28.65$, $P < 0.001$). For both multiplicative measures of fitness there were no significant differences between offspring resulting from within- and between-population crosses.

Pairwise correlations between maternal mean inbreeding depression levels of the early traits number of seeds, seed mass, germination, early plant size, and survival, did not show any significant relations after adjusting *P* values for multiple comparisons by the graphically sharpened false discovery rate (Benjamini and Hochberg 2000, Pike 2010) (Table 3). We found, however, significant correlations between levels of inbreeding depression for the late traits plant diameter and number of flowers per plant and between flowering probability and number of flowers per plant (Table 3), and marginally significant correlations between inbreeding depression levels of plant diameter and flowering. Pairwise comparisons between the inbreeding depression levels of early and late traits did not show any

Table 3. Pairwise correlations (Spearman rank correlations) of maternal mean inbreeding depression (δ) among early and late life cycle traits of *S. granulata*. Early fitness traits were the number of seeds per capsule, seed mass, germination, survival until 60 days and cumulative leaf width after 60 days. Late fitness traits were survival until 603 days, plant diameter, flowering probability and the number of flowers at 603 days. Significant correlations are marked in bold face (adjusted *P-value*s < 0.05, obtained by the graphically sharpened false discovery rate; Benjamini and Hochberg, 2000) and marginally significant correlations are marked in italics (adjusted P-values < 0.01).

	Early traits					Late traits		
	Seed mass	Germination	Survival (60 days)	Leaf width (60 days)	Survival (603 days)	Plant diameter (603 days)	Flowering (603 days)	Flowers per plant
Early traits								
Seeds per capsule	-0.24	0.23	0.18	-0.18	-0.15	-0.11	-0.51	-0.50
Seed mass		0.25	0.003	0.36	0.40	0.27	0.17	0.31
Germination			-0.23	0.34	*0.55*	0.21	0.05	0.10
Survival (60 d)				-0.21	-0.18	**-0.66**	-0.36	-0.50
Leaf width (60 d)					0.44	0.22	0.18	0.02
Late traits								
Survival (603 d)						0.53	0.16	0.41
Plant diameter (603 d)							*0.61*	**0.73**
Flowering								**0.73**

positive relations after adjusting for multiple comparisons. There was only one significant negative correlation between the inbreeding depression of early survival and the plant diameter at flowering. We found a marginally significant correlation between inbreeding depression levels of germination and late survival.

Among-family variation in the effects of inbreeding and interpopulation crosses

Our design allowed us to test whether the offspring of individual mother plants differed in their response to inbreeding and outbreeding. We found a significant interaction between mother plant identity and selfing effect for plant size at day 60, day 200, day 603 and for the multiplicate fitness function number of flowers produced per seed (Table 4), indicating that there was among-family variation in the effects of inbreeding. Although the size of selfed and outcrossed offspring after 200 d did not differ for most families, we found four of 18 mother plants (22%) whose outcrossed offspring were significantly larger (22%-57%) than selfed offspring, indicating inbreeding depression. Furthermore, we found 3 mother plants (17%) whose selfed offspring were significantly larger (7%-8%, Fig. 4a) than outcrossed offspring, indicating past purging in these families. For the fitness function flowers per seed, 7 families out of 19 (37%) showed significant inbreeding depression (49%-631%, Fig. 4b).

Individual mother plants also responded differently to the type of outcrossing. We found a significant interaction between the effects of mother plant and cross type for plant size at day 60, day 200, day 603, and for the multiplicate fitness function number of flowers produced per seed (Table 4). For plant size after 200 d, we found four of 20 mother plants

Table 4. Effects of pollination treatments on the performance of offspring of *S. granulata* from different mother plants. Plant size was measured as cumulative leaf width after 60 and 200 days and as plant diameter after 603 days. Significant interaction terms indicate significant among family variation in inbreeding and outbreeding effects for a trait. The multiplicative fitness function flowers produced per seed sown was determined after 603 days of growth. The traits were log transformed prior to analysis. Results of analyses of variance. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Source of variation	df	*F*-values			
		Leaf width 60 days	Leaf width 200 days	Plant diameter 603 days	Flowers per seed sown
Mother plant	20	10.10 ***	5.25 ***	2.78 ***	12.26 ***
Pollination	2	6.14 **	3.49 *	10.34 ***	4.77 *
Selfed vs. crossed	1	15.34 **	2.79	22.62 ***	8.28 *
WPC vs. BPC	1	0.01	4.61 *	1.35	0.23
Mother plant x Pollination	33-37	4.61 ***	3.66 ***	2.37 ***	4.24 ***
Mother plant x (Selfed vs. crossed)	14-18	3.80 ***	4.81 ***	2.01 *	4.77 ***
Mother plant x (WPC vs. BPC)	19	2.06 **	2.63 ***	2.64 ***	3.73 ***

(25%) whose BPC offspring were significantly larger (17%-25%, Fig. 4c) than WPC offspring, indicating heterosis. For the multiplicative fitness function, four (20%) mother plants showed significant and strong heterosis (124%-433%), whereas two families (10%) showed strong outbreeding depression (20%-303%) (Fig. 4d).

While both inbreeding and outbreeding depression varied among families, they were not related. In particular, there was no indication that families that had low inbreeding depression had relatively high outbreeding depression. Instead, the relationship between inbreeding and outbreeding depression in the multiplicative fitness functions was positive, but very weak, both for the number of flowers produced per seed ($r = 0.14$, $P = 0.61$) and the number of flowers produced by the descendants of a flower ($r = 0.07$, $P = 0.80$).

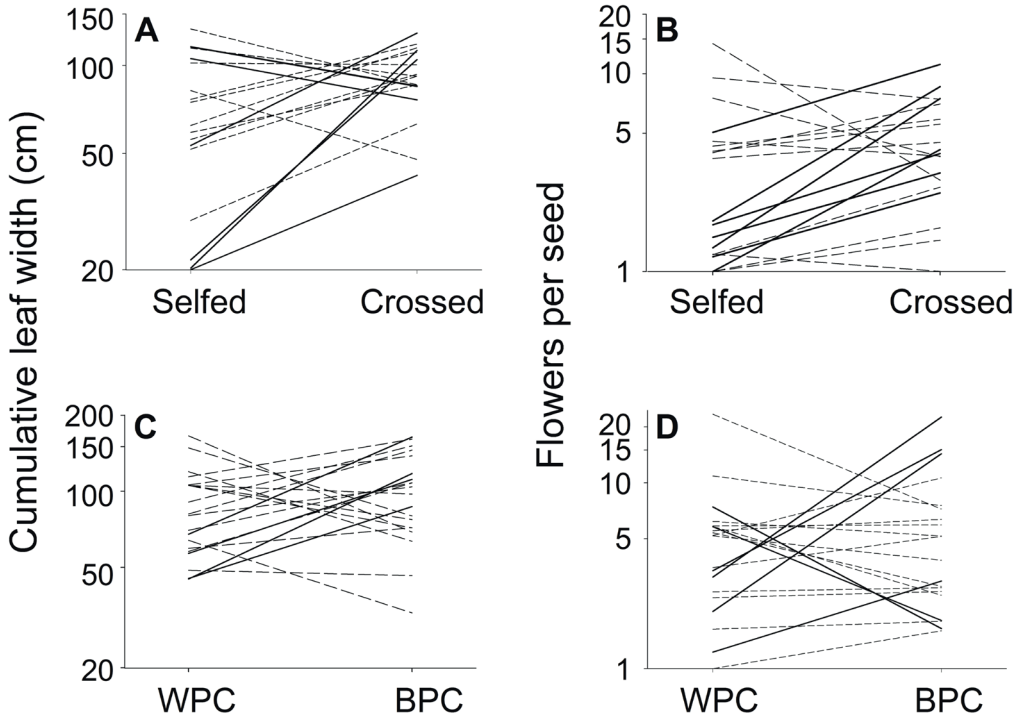

Figure 4. Variation among families of *S. granulata* in the response to selfing and outcrossing (A, B) and to within population (WPC) and between population (BPC) crosses (C, D). The size of offspring was measured at 200 d of growth as cumulative leaf width (A, C); multiplicative fitness was measured as the number of flowers produced by the offspring of a sown seed after 603 d (B, D). Lines connect offspring from the same mother plant. Solid lines indicate that the differences between selfing and outcrossing or between WPC and BPC were significant within a family (simple main effects test, and adjusted *P* values for multiple comparisons using the graphically sharpened false discovery rate (Benjamini and Hochberg, 2000, Pike, 2010, $P < 0.05$)).

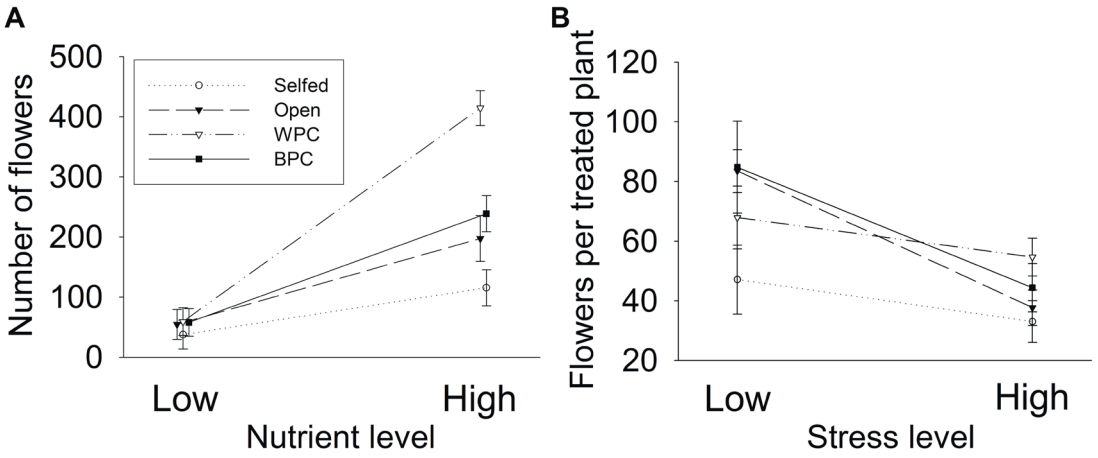

Figure 5. The response of offspring of *S. granulata* resulting from different pollination types to (A) nutrient addition and (B) stress (defoliation or competition). Flowers were either selfed, open-pollinated or hand-crossed with pollen from the same population (WPC) or from a different population (BPC). Vertical bars denote ±1 SE.

Experiment 2: Effect of pollination treatments on the plasticity of offspring under different environmental treatments

The nutrient addition treatment and the two stress treatments, competition by grass and repeated defoliation, affected both the number of flowers per plant and the multiplicative fitness function number of flowers produced per plant initially treated, but the effects depended on the type of cross from which the treated plants had originated (Fig. 5, Table 5). Offspring from selfed flowers increased their flower production in response to nutrient addition less than offspring from the other cross types, indicating inbreeding depression, and offspring from WPC reacted far more strongly than offspring from BPC, indicating lower adaptive plasticity and outbreeding depression for BPC offspring (Fig. 5a). The pattern for multiplicative fitness was very similar, except for an additional difference in the response of WPC and BPC offspring to stress through competition or defoliation. BPC offspring were far more sensitive against stress than WPC offspring (-48% vs. -20% in comparison to the control), again indicating lower adaptive plasticity and outbreeding depression (Fig. 5b). All these effects remained qualitatively the same, if plant size at the start of the experiment was included as a covariate in the analysis; i.e. the effects were independent of initial differences in plant size.

Experiment 3: Effects of repeated inbreeding and outbreeding on reproduction of the F_1 generation and performance of the F_2

The different types of pollination treatments to which the F_1-plants were subjected influenced both their reproduction and the performance of their offspring (Fig. 6, Table 6).

Table 5. Effects of plant treatments on performance of offspring of *S. granulata* resulting from different pollination types. Performance was measured after 215 days of growth as number of flowers per plant and by the multiplicative fitness function flowers produced per plant present at the start of the experiment. Offspring resulting from four pollination treatments (selfed, open-pollinated, crossed within populations [WPC] and between populations [BPC]) were subjected to four treatments: control, high nutrients, and the two stress treatments competition by grass and defoliation. The effect of pollination treatment was partitioned into three orthogonal contrasts: Selfed vs. crossed or open-pollinated, open pollinated vs. hand-crossed, and WPC vs. BPC. The effect of plant treatment was also partitioned into three orthogonal contrasts: High nutrients vs. no nutrient addition, stressed (i.e. with competition or defoliated) vs. control, and stress type (competition vs. defoliated). * $P < 0.05$, *** $P < 0.001$.

	Number of flowers				
Source of variation	df	MS	*F*	MS	*F*
Mother plant identity	21	11233	6.36 ***	11433	5.57 ***
Pollination	3	41016	23.21 ***	42889	20.89 ***
Self vs. Crossed/Open [Selfing contrast]	1	31252	17.69 ***	28804	14.03 ***
Open vs. Hand-crossed [Open contrast]	1	52982	29.98 ***	61048	29.74 ***
WPC vs. BPC	1	38813	21.97 ***	38815	18.91 ***
Plant treatment	3	132058	74.74 ***	123197	60.02 ***
High nutrients vs. Low nutrients	1	393611	222.76 ***	368517	179.52 ***
Stress vs. Control	1	112	0.06	78	0.04
Stress type	1	2449	1.39	996	0.48
Pollination x Plant treatment	9	15519	8.78 ***	17229	8.39 ***
Selfing contrast x Nutrient contrast	1	78911	44.66 ***	74766	36.42 ***
Selfing contrast x Stress contrast	1	2017	1.14	3611	1.76
Selfing contrast x Stress type	1	48	0.03	68	0.03
Open contrast x Nutrient contrast	1	10647	6.03 *	30897	15.05 ***
Open contrast x Stress contrast	1	3750	2.12	4512	2.20
Open contrast x Stress type	1	546	0.31	254	0.12
WPC vs. BPC x Nutrient contrast	1	37774	21.38 ***	31285	15.24 ***
WPC vs. BPC x Stress contrast	1	5646	3.19	9563	4.66 *
WPC vs. BPC x Stress type	1	328	0.19	104	0.05
Residual	35-36	1767		2053	

Flowers of selfed offspring that had been crossed with offspring from the same family produced 70% fewer seeds than those that had been crossed with a plant from another selfed offspring family, and 82% fewer seeds than outcrossed offspring that had been outcrossed again (Fig. 6a). Crosses between different selfed families resulted in 40% fewer seeds than crosses between offspring of outcrossed plants. These differences were partly due to differences in the size of the F_1 plants, but all effects remained significant if plant diameter at flowering time or the number of flowers were included as covariates in the analysis. The low number of seeds produced by the serially inbred plants reflected both the effect of inbreeding on the performance of the mother plants and that of early inbreeding depression in the F_2 generation. By comparing the seed production of serial inbred plants, serial outbred plants, and of inbred offspring that were subsequently outbred,

we could estimate the effects of the first and second inbreeding. The reduction in seed production by early inbreeding depression in the F_2 generation (-70%) was much stronger than that caused by the reduced performance of the inbred mother plants (-40%).

The pollination treatments also affected the mass of the seeds produced by the F_1 and the performance of the F_2 plants. These effects were exclusively due to the very low performance of the strongly inbred offspring, i.e., the offspring of selfed plants that had been crossed with plants from the same family. In comparison to the performance of the offspring from the three types of F_1 between-family crosses, their seed mass was 54% lower ($F_{1,61} = 7.47$, $P < 0.01$), germination 59% lower ($F_{1,65} = 26.62$, $P < 0.001$), survival over two seasons 92% lower ($Quasi\text{-}F_{1,59} = 10.96$, $P < 0.01$), and their cumulative leaf width was 75% lower ($F_{1,29} = 9.15$, $P < 0.01$). None of the strongly inbred plants flowered, but 80% of the others flowered. There were no differences in the performance of the offspring from the three types of between family crosses (all $P > 0.05$); in particular, the performance of offspring from crosses between inbred families was similar to that of offspring from crosses between outcrossed plants, indicating a heterosis effect. The same pattern as for the individual fitness-related traits was found for multiplicative fitness in the F_2, estimated as cumulative leaf width produced by the offspring of a F_1-flower, which was 99.5% lower in the strongly inbred plants than in the offspring from the three other cross

Table 6. The effects of subjecting two generations of plants to different pollination treatments on seed production of F_1 offspring, multiplicative fitness of F_2 offspring, and bigenerational multiplicative fitness. Flowers of *S. granulata* plants in the field were subjected to selfing (S), within population crosses (WPC) or between population crosses (BPC). The F_1 offspring of selfed flowers were then either crossed within families (S→WFC) or crossed between families (S→BFC). The F_1 offspring of both WPC and BPC were subjected to within population crosses (WPC→WPC and BPC→WPC). The effects of pollination treatment combinations were partitioned into three orthogonal contrasts: the within-family cross treatment (S→WFC) vs. the three other treatments, (2) crosses between selfed families (S→BFC) vs. crosses between crossed families (WPC→WPC and BPC→WPC), and (3) crosses between offspring from WPC (WPC→WPC) vs. crosses between offspring from BPC (BPC→WPC). Fitness estimates were log-transformed prior to analysis. ** $P < 0.01$, *** $P < 0.001$.

		Number of seeds F_1		Fitness F_2		Bigenerational fitness	
Source of variation	df	MS	*F*	MS	*F*	MS	*F*
Pollination	3	707512.03	17.47 ***	16.44	10.43 ***	88.20	10.03 ***
(S→WFC) vs. other treatments	1	1768179.40	43.65 ***	47.23	29.95 ***	242.16	27.55 ***
(S→BFC) vs. (WPC→WPC and BPC→WPC)	1	354335.47	8.75 **	1.46	0.93	16.61	1.89
(WPC→WPC) vs. (BPC→WPC)	1	21.24	0.01	0.63	0.40	5.83	0.66
Capsule	83	40507.23		1.58		8.79	

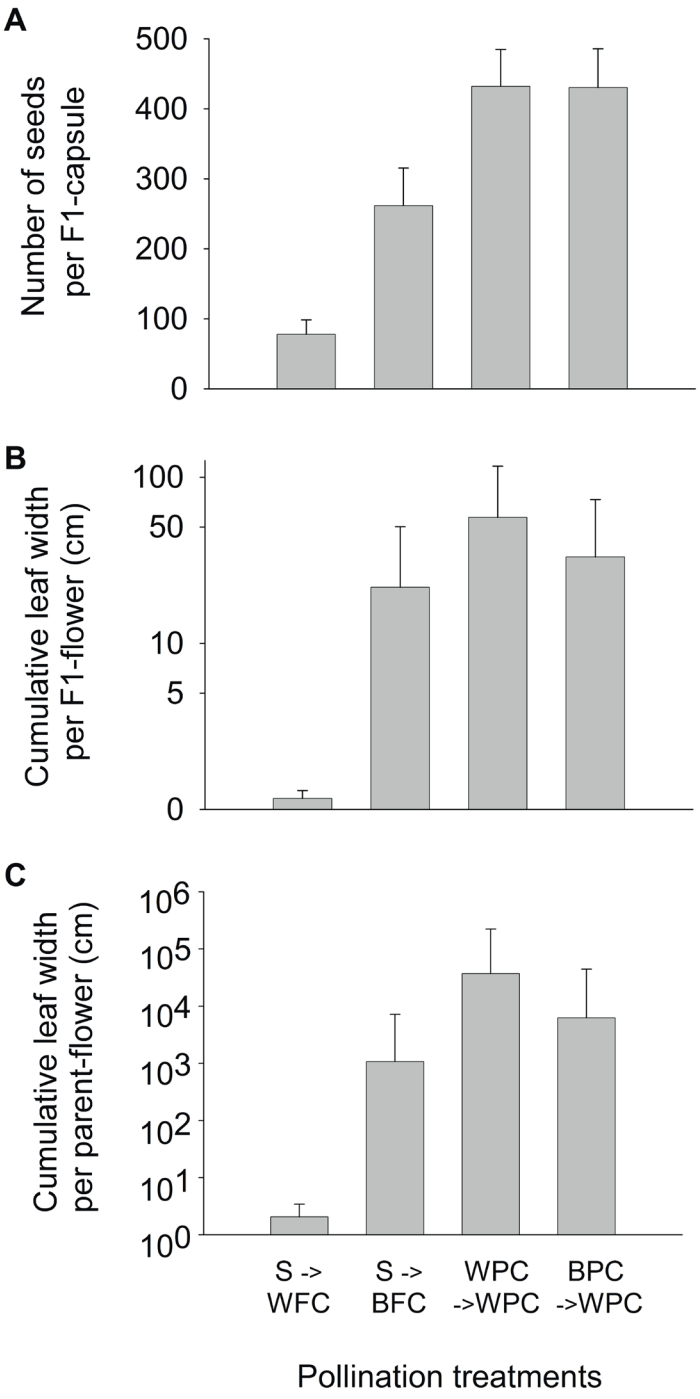

Figure 6. The effects of two successive pollination treatments on the performance of *S. granulata.* (A) Seed production of F_1-offspring, (B) multiplicative fitness of F_2-offspring (cumulative leaf width produced per F_1-flower), (C) bigenerational multiplicative fitness (cumulative leaf width produced per parent-flower). Flowers of *S. granulata* plants in the field were subjected to selfing (S), within population crosses (WPC) or between population crosses (BPC). The F_1-offspring of selfed flowers were then either crossed within families (S→WFC) or crossed between families (S→BFC). The F_1-offspring of both WPC and BPC were subjected to within population crosses (WPC→WPC and BPC→WPC). Note log-scales for fitness. Means +1 SE.

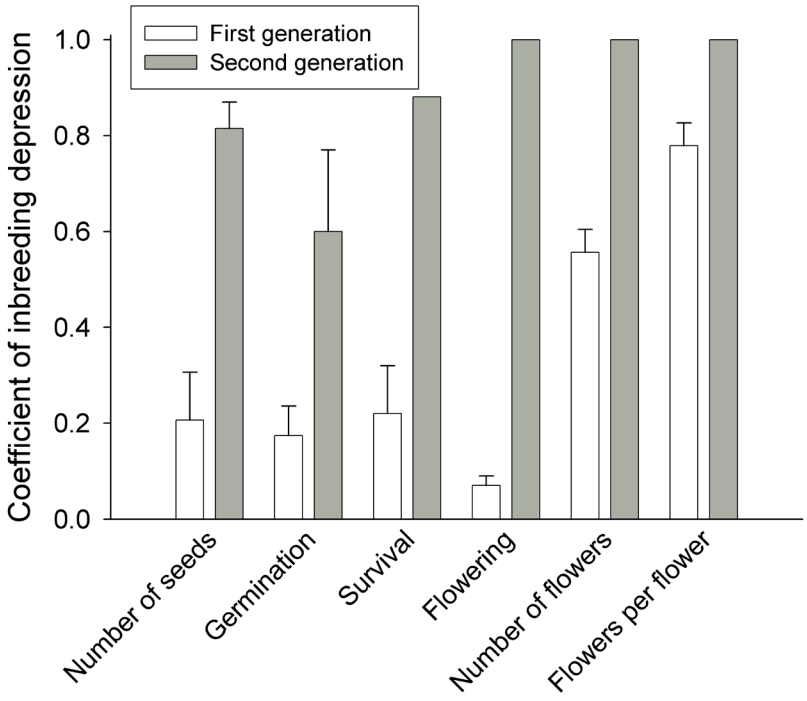

Fig 7: Coefficients of inbreeding depression (δ) for components of fitness and cumulative fitness (flowers per flower) in two generations of inbred offspring of *Saxifraga granulata*. Coefficients were calculated as the means of the per-family estimates of inbreeding depression. Means +1 SE.

types (Fig. 6b, Table 6). Even stronger were the effects of strong inbreeding on bigenerational multiplicative fitness, measured as the cumulative leaf width of the descendents produced by one flower of the parent generation (99.99% reduction, Fig. 6c).

A comparison of family means of inbreeding depression along the life cycle of *S. granulata* showed that during the development of the first inbred generation inbreeding depression was c. 20% for early traits like seed number and germination, but was also 20% for survival and 7% for flowering, whereas inbreeding depression for flower production was much higher (56%) (Fig. 7). In the second inbred generation, inbreeding depression of early traits was high, but flower production was even more affected.

DISCUSSION

Breeding system

The seed set of bagged flowers of *S. granulata* was very low in comparison to that of hand-selfed flowers, indicating that spontaneous selfing is inefficient and that the plant is dependent on pollinators for seed production. This could lead to pollen limitation of reproduction, in particular in small populations, which are frequently less attractive for

pollinators (e.g. Aizen and Feinsinger 1994, Young et al. 1996). Even in the large study population, seed production of open-pollinated (control) flowers was lower than that of handpollinated flowers, indicating pollen limitation.

Hand-selfed flowers of *S. granulata* produced 23% fewer seeds than outcrossed flowers. In a similar study with *S. granulata* by Hansen and Molau (1994), no differences in seed set between selfed and outcrossed flowers were found in a Danish population, whereas a study by Stevens (1988) in an English population recorded 49% fewer seeds after selfing than after outcrossing. The lower seed set of selfed flowers in our study could be either an indication of self-incompatibility or the result of early negative effects of inbreeding on seed development. Several authors have required at least a relative reduction of 80% in the success of selfed vs. outcrossed seed set to indicate self-incompatibility (see Bawa 1974, Igic et al. 2008). The much lower reduction in *S. granulata* suggests that the lower seed set was an effect of inbreeding. All other *Saxifraga* species studied so far have also been found to be self-compatible (Holderegger 1996, Gugerli 1997, Meier and Holderegger 1998, Brochmann and Hapnes 2001).

Based on a multiplicative measure of offspring fitness, we calculated a selfing rate of 55%, indicating that *S. granulata* has a mixed mating system. The high selfing rate in *S. granulata* could be due to within-flower selfing, geitonogamy (De Jong et al. 1993) and biparental inbreeding. Although *S. granulata* is protandrous, bagged flowers produced some seeds indicating that within-flower selfing is possible. Moreover, geitonogamy may be common in *S. granulata,* because the male and female phases of flowers within inflorescences overlap (Hansen and Molau 1994) and *S. granulata* can form large genets by clonal growth through the formation of bulbils.

Effects of inbreeding

Selfing resulted in strong inbreeding depression in all measures of fitness, indicating that *S. granulata* is sensitive against inbreeding. The cumulative inbreeding depression of 0.78 was much higher than the mean of 0.39 calculated for 47 angiosperms by Husband and Schemske (1996) and the mean of 0.49 for outcrossed angiosperms. It is generally thought that inbreeding depression decreases with the selfing rate (Johnston and Schoen 1996), i.e. populations or species with high rates of selfing should show low levels of inbreeding depression. However, in spite of the estimated selfing rate of 55% in *S. granulata*, inbreeding depression was very strong. Several other studies have also found high levels of cumulative inbreeding depression in species with mixed mating systems (e.g. 0.63 in *Lychnis viscaria* [Mustajärvi et al. 2005], 0.85 in *Bulbine bulbosa* [Owen et al. 2007] and 0.74 in *Silene nutans* [Thiele et al. 2010]). A recent meta-analysis found that levels of inbreeding depression increase with population size in plants (Angeloni et al. 2011),

because baseline inbreeding is higher in small populations and purging stronger. The very high level of inbreeding depression found in our large remnant population of *S. granulata* indicates that the species is likely to be negatively affected by the continuing fragmentation of its populations which results in small and more isolated populations with increased levels of inbreeding (Young et al. 1996, Aguilar et al. 2008). Even under the benign conditions in the experimental garden the negative effects of inbreeding were very strong in the current study. Inbreeding depression in the field might be even higher, as several studies have found that the negative effects of inbreeding are exacerbated in the field (Dudash 1990, Koelewijn 1998).

Inbred offspring of *S. granulata* also exhibited reduced plasticity. Both growth and flower production increased more strongly in response to higher nutrient levels in outcrossed than in inbred offspring. The reduced plasticity of inbred *S. granulata* indicates that small remnant populations, in which inbreeding is likely to be high, will be less able to cope with increased competition by grasses due to nutrient enrichment. There are few other studies on the influence of inbreeding on adaptive plasticity (Dudash et al. 2005). Our results are in line with those of Kéry et al. (2000) who found that plasticity in response to fertilizer was reduced in plants from small populations of *Primula veris*, presumably due to inbreeding depression. Similarly, in the hermaphroditic snail *Physa acuta*, inbreeding reduced the expression of adaptive plasticity (Auld & Relyea 2010). In contrast, Schlichting & Levin (1986) found no consistent relationship between plasticity and levels of inbreeding in *Phlox drummondii*.

Variation among traits

By far the highest level of inbreeding depression was found in the late trait number of flowers (0.61, see Fig. 7). Our results thus support the notion that inbreeding affects late more strongly than early traits (Husband & Schemske 1996, Glaettli and Goudet 2006, Thiele et al. 2010). Inbreeding depression in late traits may be more difficult to purge because they are under polygenic control by mildly deleterious alleles which accumulate in the genome (Charlesworth et al. 1991, Lande and Schemske 1985, Byers and Waller 1999, Willis 1999, Thiele et al. 2010). The stronger inbreeding depression in late traits could also be due to early acting genes that have pleiotropic effects on later traits. However, the lack of correlations between levels of inbreeding depression in early and late traits suggests that genes causing inbreeding depression are independent among life stages (Husband and Schemske 1996). In contrast, we found two significant correlations among inbreeding depression levels of late traits, indicating that they may be affected by the same set of genes which are expressed gradually across the life cycle (Koelewijn et al. 1999). Our results stress the importance of screening late stages like flowering to avoid

underestimating inbreeding depression (Glaetti and Goudet 2006, see also Willis 1993, Mayer et al. 1996, Melser et al. 1999, Wagenius et al. 2010).

Among-family variation in the response to pollination treatments

We found considerable variation among families of *S. granulata* in inbreeding depression for plant size and multiplicative fitness, which is in line with the results of other studies on inbreeding depression (Schoen 1983, Kalisz 1989, Agren and Schemske 1993, Dudash et al. 1997, Koelewijn 1998, Pico et al. 2003, 2004). This was true for traits that showed no inbreeding depression at the population level like plant size at 200 d (Fig. 4a) as well as for traits that showed strong population inbreeding depression like cumulative fitness (Fig. 4b). While some families showed strong inbreeding depression, in others inbred individuals actually outperformed outcrossed progeny (negative inbreeding depression). These plants could have a selective advantage in fragmented populations in which there is strong inbreeding (Holsinger 1991, Uyenoyama et al. 1993, Pico et al. 2004).

Studying among family variation in addition to mean trait values may lead to a deeper understanding of the evolutionary processes within populations in response to inbreeding. Variation in the response to inbreeding among families is often considered to indicate the potential of a population to evolve towards increased or even complete selfing or outcrossing (Agren and Schemske 1993, Dudash et al. 1997, Pico et al. 2003, 2004). However, the variation in the response to inbreeding may be due to effects other than differences in inbreeding load among families, such as maternal effects, the inbreeding history of parents and the decrease of genetic diversity in inbred families in comparison to outbred families (Fox 2005, Kelly 2005, Moorad and Wade 2005).

We also found among-family variation in the response to inter-population crosses. Several of the families showed strong heterosis after between-population crosses, while at least two families showed significant and strong outbreeding depression. This indicates that plants in the study population differed in their genetic load and in their susceptibility to outbreeding depression. Although a number of studies have shown effects of interpopulational crosses on mean fitness trait values, very few have investigated among-family differences in the effects of outbreeding on plant performance (see Muola et al. 2011), and those did not find significant among-family variation in the effects of outbreeding on plant fitness traits. Because the plants were grown in a common garden, outbreeding depression could not be due to the dilution of locally adapted genotypes, but must be due to the genetic mechanisms of epistasis and underdominance (Fenster and Galloway 2000, Hufford and Mazer 2003, Galloway and Etterson 2005).

Variation among families in both the strength of inbreeding and outbreeding depression could potentially be due to differences in the breeding value of mother plants, i.e., additive genetic effects. Families with high fitness would be expected to show stronger outbreeding depression after interpopulation crosses than families with below average fitness, because the breeding value of their mates is likely to be lower than their own, and the breeding value of the hybrids is the average of the breeding values of their parents (Escobar et al. 2008). Conversely, high-fitness families would tend to show lower than average inbreeding depression, resulting in a negative correlation between inbreeding depression and outbreeding depression shown by families. However, in our study correlations between inbreeding and outbreeding depression for multiplicative fitness functions were very weak and positive, providing no evidence that additive effects contributed to the estimated levels of inbreeding and outbreeding depression.

Effects of serial inbreeding

Selfing plants of *S. granulata* and subjecting the offspring to full-sib crosses resulted in extreme inbreeding depression ($\delta = 99.5\%$) in the second generation. In contrast, the crossing of inbred offspring from different families of *S. granulata* restored the performance of progeny to a similar level as that of continuously outbred progeny. The extreme inbreeding depression shows that the foundation of a new population by a single seed of *S. granulata* is very unlikely, because the second offspring generation has very low fitness. Moreover, the evolution of complete selfing appears to be highly unlikely. Although in the F_1 some families performed better under selfing than under outcrossing, which is seen as favorable for the evolution of selfing (Uyenoyama et al. 1993, Pico et al. 2004), after a second generation of inbreeding fitness was consistently extremely low.

Similar series of inbreeding events may also occur in spatially structured populations due to limited seed dispersal and restricted pollen transfer. However, while in large populations there is a chance that the fitness of offspring of inbred plants may rebound by outcrossing with unrelated individuals as in our experiment, in small populations such a rebound may not be possible, because of the reduction of genetic diversity and heterozygosity by genetic drift (van Treuren et al. 1991, Ellstrand and Elam 1993, Fischer and Matthies 1998, Aguilar et al. 2008).

The expected consequences of repeated inbreeding on fitness depend on the genetic basis of inbreeding depression (Charlesworth and Willis 2009). Inbreeding increases the frequency of homozygotes. Two mechanisms, overdominance and partial dominance, may result in a reduction of fitness with increasing homozygosity (Charlesworth and Charlesworth 1987). If heterozygotes have higher fitness than both types of homozygotes (overdominance), fitness should decline log-linearly under serial inbreeding (Carr and

Dudash 2003, Charlesworth and Willis 2009). If, however, inbreeding depression is due to recessive deleterious alleles that are more likely to be expressed in inbred individuals (partial dominance), then continuous strong inbreeding may expose these alleles to selection and purge them from a population, leading to a rebound in fitness (Crnokrak and Barrett 2002). Evidence for both mechanisms has been found, but partial dominance is commonly thought to be the most important genetic basis for inbreeding depression (Charlesworth and Willis 2009). The extreme inbreeding depression in the second generation in *Saxifraga granulata* indicates that a single generation of inbreeding did not strongly reduce the genetic load. Similarly to our results, Ozimec and Husband (2011) found that cumulative inbreeding depression in *Chamerion angustifolium* increased over three generations of inbreeding and concluded that the purging of deleterious mutations did not keep pace with the increase in homozygosity. This is in contrast to the results of most serial inbreeding experiments that found some evidence for purging (Crnokrak and Barrett 2002) and could be due to the combined effect of many deleterious alleles which may be difficult to purge (Koelewijn 1998, Dudash et al. 1997, Carr and Dudash 1997). In addition, we cannot exclude that overdominance contributed to inbreeding depression in *S. granulata*, and because fitness decreased stronger than log-linearly with repeated inbreeding, effects of synergistic epistasis may also have contributed (Carr and Dudash 2003, Charlesworth and Willis 2009).

Effects of interpopulation crosses

We found outbreeding depression in *S. granulata* in the F_1 only for survival, but not for overall fitness, as there were no differences in overall fitness between offspring from between and within population crosses in experiment 1. However, in the $F_{1,}$ generation the negative effects of the breakup of coadapted gene complexes may be hidden by heterosis effects. Due to segregation, the masking of outbreeding depression by heterosis will decrease in subsequent generations. It has therefore been suggested that it is necessary to study the F_2-generation to be able to exclude possible negative effects of interpopulation crosses (Keller et al. 2000, Hufford and Mazer 2003). Negative effects of intraspecific hybridization only in the F_2 have, for instance, been found in *Agrostemma githago*, *Papaver rhoeas* and *Silene alba* (Keller et al. 2000), and *Senecio glaucus* (Volis and Zhang 2010). In contrast, in *S. granulata* we found no negative effects of inter-population crosses in the F_2 generation. However, in *S. granulata* the expression of outbreeding depression was strongly dependent on environmental conditions. In the $F_{1,}$ the robustness to stress caused by grass competition and defoliation was lower in offspring resulting from between- than within-population crosses and the increase in fitness in response to nutrient addition was smaller, indicating outbreeding depression and reduced plasticity (Richards et al. 2006) in

the response to stress or nutrients. While a number of studies have found a negative effect of interpopulation crosses on mean reproduction or offspring performance in a common garden (e.g., Waser and Price 1991, Fischer and Matthies 1997, Becker et al. 2006) we are not aware of another study that has shown an effect of interpopulation crosses on plant plasticity.

CONCLUSIONS

The continuing habitat fragmentation is likely to increase inbreeding in remnant populations of many grassland plants. Our findings suggest that the declining grassland plant *S. granulata* is very sensitive against inbreeding. It has been suggested that among-family variation in inbreeding depression might buffer to some extent the effects of inbreeding on population viability (Pico et al. 2004). However, while we found significant among family variation in inbreeding depression, this represented only a weak buffer against the drastic negative effects of inbreeding that occurred after serial inbreeding in the F_2 generation. Such serial inbreeding may frequently occur in small populations where mating partners are closely related. The extremely low fitness of serially inbred offspring makes purging of the genetic load in small populations unlikely and will also prevent the founding of new populations by a single seed, in spite of self-compatibility.

Possible genetic effects of interpopulation crosses such as heterosis and outbreeding depression are also important considerations in conservation biology. There has been a lot of discussion about whether to try to rescue inbred populations by increasing gene flow through management and whether to use mixed seed sources from several populations for the restoration of populations, but no clear consensus has emerged (Waser and Price 1994, Fischer and Matthies 1997, Hufford and Mazer 2003, Becker et al. 2006, Wagenius et al. 2010).

Our results suggest that not only inbreeding depression (Cheptou and Donohue 2011), but also outbreeding depression is environment-dependent, and that in conservation biology the effects of interpopulation crosses on both the mean performance of offspring and on its adaptive plasticity should be considered. Crossbred offspring were more affected by stress (defoliation and competition by grasses) and, like inbred offspring, were less able to profit from high nutrient availability. A reduced adaptive plasticity in the response to nutrients may be relevant for conservation, because eutrophication in grassland habitats is a common problem and plants that are less capable of capitalizing on increased nutrient availability and that are less robust to competition and defoliation may have a lower competitive ability.

In conclusion, in *S. granulata* we found evidence for both inbreeding and outbreeding depression. However, our results support the view that the threats to small inbred populations due to inbreeding depression may be far greater than those posed by interpopulation crosses and that artificially increasing gene flow as a management measure may result in genetic rescue.

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Summary

Zusammenfassung

The aim of this thesis was to study the ecology, genetics and evolution of two congeneric species with different fragmentation histories. *Saxifraga sponhemica* is a glacial relict species of long-term fragmented lowland rock and scree habitats, and has been naturally rare for thousands of years with a disjunct distribution in Central Europe. *Saxifraga granulata* is a formerly common species of species-rich semi-natural grasslands that has become recently fragmented due to the intensification of agricultural practices such as the use of fertilizers and conversion of traditional grassland to silage meadows since the 1950s. Formerly common species that have recently become fragmented are expected to show a stronger response to fragmentation than naturally rare species, because they have suffered recent and much more rapid declines in the number and size of populations, hampering an evolutionary response. The negative effects of fragmentation are reduced genetic diversity due to drift and inbreeding, reduced mean fitness and increased extinction rates of populations. Relatively few studies have so far analysed the effects of fragmentation on formerly common species in comparison to naturally rare species and results are not equivocal.

An analysis of the genetic diversity and the genetic structure of 30 populations of *S. sponhemica* based on RAPD-markers showed that in most populations considerable genetic variability has been preserved due to the longevity of *S. sponhemica*. An isolation by distance pattern of genetic differentiation suggested historical gene flow during the last glaciation when suitable habitats for were much more abundant. Our results suggest that long-lived plant species can maintain historic genetic patterns despite the small size and strong isolation of populations. Several RAPD loci were identified to be non-neutral and their frequencies correlated with climatic gradients, indicating natural selection. Adaptive genetic variation could be important for adaptation of *S. sponhemica* to environmental changes like ongoing climate change.

Strong evidence for divergent selection was found in a quantitative genetic study of *S. sponhemica*. Most population trait means were significantly related to climate gradients, indicating adaptation. Quantitative genetic differentiation increased with climatic distance and with geographical distance, even when neutral molecular divergence was controlled for, and quantitative genetic differentiation (Q_{ST}) exceeded molecular genetic differentiation (F_{ST}) for some traits. The evolvability of traits was negatively correlated with the degree of differentiation among populations (Q_{ST}), i.e. traits under strong selection showed little genetic variation within populations. The evolutionary potential of a population was not related to its size, the performance of the population or its neutral genetic diversity. However, performance in the common garden was lower for plants from populations with reduced molecular genetic variation, suggesting inbreeding depression due to genetic erosion. The findings suggest that studies of molecular and quantitative

genetic variation may provide complementary insights important for the conservation of rare species. The taxon does not appear to be genetically threatened in the short term, but populations are threatened by habitat destruction. A conservation measure could be to create new populations in suitable habitats with seeds from the same region to avoid local maladaptation.

Formerly common species are expected to be particularly susceptible to the recent fragmentation of their habitats but the effects of fragmentation on their population genetics are hardly known. We studied the molecular genetic structure of 19 *S. granulata* populations in a restricted geographic area in Luxembourg and neighbouring Germany using RAPD markers. We grew plants from several families per population in a common garden and determined the variation of quantitative plant traits among and within populations. Differentiation for quantitative traits (Q_{ST}) was slightly lower than differentiation for molecular markers (F_{ST}) suggesting that homogenising selection for optimal trait values has contributed to the variation among populations. Contrary to our expectation, the level of differentiation among fragmented *S. granulata* populations was low and did not increase with the geographical distance among populations. Moreover, molecular genetic diversity of populations was high and not correlated with their size or with plant performance. Gene flow by long distance dispersal as well as longevity, clonality and polyploidy of *S. granulata* may have prevented genetic erosion within and strong genetic differentiation among populations. Although clonality was restricted as shown by high clonal diversity and limited clonal spread it shaped spatial genetic structure at small spatial scales within populations. The spatial genetic structure within populations indicated an isolation by distance due to reduced gene flow by localised seed dispersal, geitonogamous pollination and biparental inbreeding. In the studied populations of *S. granulata* the effects of genetic drift due to recent habitat fragmentation are not yet perceptible. However, it is important to preserve extant populations and increase the size of small populations to avoid genetic erosion in the future. Management measures should maintain gene flow among populations.

Inbreeding depression is a major evolutionary force and an important topic in conservation genetics, because habitat fragmentation has lead to increased inbreeding in the populations of many species. Crosses between populations may restore heterozygosity, resulting in increased performance (heterosis), but may also lead to the disruption of coadapted gene complexes and to decreased performance (outbreeding depression). We investigated the effects of selfing and of within and between-population crosses on reproduction and the performance of two generations of offspring of the declining grassland plant *Saxifraga granulata* (Saxifragaceae). Inbreeding depression affected all traits in the F_1 generation, but was stronger for traits expressed late during development and varied among families. We also subjected the first generation of offspring to a fertilization and

two stress treatments (competition and defoliation) to investigate whether the effects of inbreeding and interpopulation gene flow depend on environmental conditions. The adaptive plasticity of offspring from selfing and from interpopulation crosses in response to nutrient addition was reduced. Outbreeding depression was also observed in response to stress. Multiplicative fitness of the F_2 generation after serial inbreeding was extremely low, but there was heterosis after crossing inbred lines. Outbreeding depression was not observed in the F_2. The results suggest that continuous inbreeding may drastically reduce the fitness of plants, but effects may be environment-dependent.

Overall, the results of this thesis advance knowledge on the role of time since habitat fragmentation, of historic connectivity among populations, and of life history traits such as longevity and clonality on the processes of selection and drift that shape the genetic variation within and among populations. It stresses the importance of using both molecular and quantitative genetic tools to gain complementary insight for the conservation of rare and endangered plant species. It also highlights the importance of knowledge about the susceptibility of populations to increased inbreeding and the potential risks of artificially increasing gene flow between populations of recently fragmented species for their effective conservation.

Das Ziel dieser Arbeit war es, ökologische, genetische und evolutionäre Aspekte zweier Arten der Gattung *Saxifraga* mit unterschiedlicher Fragmentierungsgeschichte zu untersuchen. *S. sponhemica* ist eine Art mit einer disjunkten Verbreitung in Zentraleuropa, die als Eiszeitrelikt auf isolierten Felswänden und Schutthalden wächst. Es handelt sich um eine Art, die seit Tausenden von Jahren selten ist und deren Populationen meist seit langem voneinander isoliert sind. *Saxifraga granulata* hingegen ist eine früher häufige Art artenreicher, halb-natürlicher, mesophiler Mähwiesen, die seit den Ende der 50er-Jahre durch die chemische Düngung und die Umwandlung von traditionellem Grasland in Silowiesen verändert und fragmentiert wurden. Es wird angenommen, dass einst häufige Arten, die erst kürzlich fragmentiert wurden, stärker unter den negativen Effekten der Fragmentierung leiden als Arten, deren Populationen seit langem fragmentiert sind, weil der Rückgang in der Anzahl und Größe ihrer Populationen über einen viel küzeren Zeitraum erfolgte und evolutionäre Anpassungen während dieses kurzen Zeitraumes unwahrscheinlich sind. Negative Effekte der Fragmentierung für Populationen sind verringerte genetische Diversität durch Drift und Inzucht, eine verringerte Fitness der Pflanzen aufgrund von Inzuchtdepression und in der Folge ein erhöhtes Aussterberisko der Populationen. Es gibt jedoch nur wenige Studien, die den Effekt der Fragmentierung auf einst häufige Arten und natürlich seltene Arten derselben Gattung vergleichend untersucht haben.

Eine Studie der genetischen Diversität und der genetischen Struktur von 30 *S. sponhemica* Populationen mit Hilfe von molekularen RAPD-Markern zeigte, dass dank der Langlebigkeit der Art die Populationen eine hohe genetische Diversität bewahrt haben. Die genetische Differenzierung der Populationen nahm mit ihrer Entfernung voneinander zu, was auf historischen Genfluss während der letzten Eiszeit hindeutet, als für *S. sponhemica* geeignete Habitate häufiger waren und es vermutlich viel mehr Vorkommen der Art gab. Unsere Ergebnisse deuten darauf hin, dass langlebige Pflanzen historische genetische Muster trotz ihrer heute kleinen und z.T. extrem isolierten Populationen über lange Zeiträume bewahren können. Die Häufigkeit mehrerer vermutlich nichtneutraler molekularer Loci korrelierte mit Klimavariablen der Wuchsorte, was auf Selektion hindeutet. Diese adaptive genetische Variation könnte für die Anpassung von *S. sponhemica* an zukünftige Umweltveränderungen wie den Klimawandel wichtig sein.

Um die quantitativ-genetische Variation von Merkmalen innerhalb und zwischen Populationen von *S. sponhemica* zu erfassen, wurden Samen verschiedener Individuen in den Populationen gesammelt und Keimlinge unter gleichen Bedingungen angezogen. Wir fanden verschiedene Hinweise auf divergente Selektion in den Populationen. Die meisten Merkmale zeigten klinale Variation in Beziehung zu Klimagradienten. Die genetische Differenzierung zwischen Populationen nahm mit den klimatischen Unterschieden und geographischen Distanzen zwischen Populationen zu, auch wenn für die molekular-gene-

tische Differenzierung kontrolliert wurde. Außerdem war die quantitativ-genetische Variation einiger Merkmale zwischen den Populationen deutlich höher als die der molekular-genetischen Marker. Merkmale die sich stark zwischen Populationen unterschieden, zeigten weniger Variation innerhalb von Populationen. Es gab keine Beziehung zwischen der quantitativ-genetischen Diversität, d.h. dem evolutionären Potential einer Population, und ihrer Populationsgrösse, ihrer Fitness, oder ihrer neutral-genetischen Diversität. Die Fitness von Pflanzen aus Populationen mit geringer molekular-genetischer Diversität war reduziert, was ein Hinweis auf Inzuchtdepression ist. Unsere Ergebnisse weisen darauf hin, dass molekular- und quantitativ-genetische Studien komplementäre Erkenntnisse liefern können, die beide für die Erhaltung von Arten wichtig sind. Die Populationen von *S. sponhemica* sind gegenwärtig eher durch Habitatzerstörung gefährdet als durch genetische Probleme. Eine Naturschutzmaßnahme könnte darin bestehen, neue Populationen in geeigneten Habitaten anzusiedeln. Dabei sollten Samen aus der gleichen Region verwendet werden, um Fehlanpassungen zu vermeiden.

Es wird angenommen, dass einst häufige Arten besonders anfällig sind für die negativen Effekte der rezenten Fragmentierung ihrer Habitate, aber es gibt nur wenige Studien zu den Effekten der Fragmentierung auf die Populationsgenetik dieser Arten. Wir untersuchten deshalb die molekular-genetische Variation innerhalb und zwischen 19 kürzlich fragmentierten Populationen von *Saxifraga granulata* in einem geografisch eng begrenzten Gebiet in Luxemburg und Deutschland anhand von RAPD-Markern. Zusätzlich wurden Nachkommen verschiedener Familien der Art aus unterschiedlichen Populationen im Versuchsgarten angezogen und eine quantitativ-genetische Studie durchgeführt. Die genetischen Unterschiede zwischen den Populationen in quantitativen Merkmalen waren z.T. geringer als diejenigen in neutralen Markern, was auf stabilisierende Selektion hindeutet. Entgegen unseren Erwartungen war die Differenzierung zwischen den fragmentierten Populationen gering und nahm nicht mit der geographischen Distanz zwischen Populationen zu. Außerdem war die genetische Diversität innerhalb von Population hoch und war nicht mit der Populationsgröße oder der mittleren Pflanzengröße korreliert. Gründe dafür könnten eine Ausbreitung der Samen über längere Distanzen durch den Wind oder durch Mähmaschinen, sowie die Langlebigkeit, Klonalität und Polyploidie von *S. granulata* sein, die einen Verlust der genetischen Diversität durch Drift und die genetische Differenzierung zwischen Populationen verhindert haben. Wir fanden eine räumliche genetische Struktur auf geringer Distanz innerhalb von Populationen, was auf eingeschränkten Genfluss durch geringe Ausbreitung der Samen, geitonogame Bestäubung oder biparentale Inzucht hindeutet. Die Effekte der genetischen Drift durch rezente Habitatfragmentierung sind noch nicht sichtbar in den untersuchten *S. granulata* Populationen. Es ist trotzdem wichtig, die bestehenden Populationen zu schützen und die Grösse der Populationen zu

erhöhen, um einen Verlust an genetischer Diversität zu vermeiden. Naturschutzmassnahmen sollten den Genfluss zwischen den Populationen erhalten.

Inzuchtdepression beeinflusst die Evolution von Populationen und ist ein wichtiges Thema der Naturschutzbiologie, weil die Fragmentierung der Habitate zu erhöhter Inzucht in den Populationen vieler Arten geführt hat. Kreuzungen zwischen Populationen könnten einerseits den Grad der Heterozygotie und die Fitness der Nachkommen steigern, andererseits aber auch die Fitness von Pflanzen durch das Aufbrechen koadaptierter Genkomplexe verringern. Wir untersuchten den Einfluss von Inzucht und Auszucht innerhalb und zwischen Populationen auf die Reproduktion und die Vitalität zweier Generationen von Nachkommen der zurückgehenden Grünlandart *S. granulata*. Inzuchtdepression beeinflusste alle Merkmale in der F_1 Generation; dabei variierte der Einfluss zwischen Familien und die Inzucht wirkte sich stärker auf Merkmale aus, die sich spät im Lebenszyklus der Pflanze ausprägen. Pflanzen der F_1 Generation wurden einer Düngung und zwei Stressbehandlungen (Konkurrenz und Entblätterung) unterzogen, um zu untersuchen ob der Einfluss von In- und Auszucht umweltabhängig ist. Ingezüchtete Pflanzen wiesen eine geringere adaptive Plastizität als Reaktion auf Düngung auf und zeigten Auszuchtdepression als Reaktion auf die Stressbehandlungen. Die multiplikative Fitness der nochmals ingezüchteten F_2 Generation war extrem gering. Kreuzungen ingezüchteter Linien zeigten keine Auszuchtdepression, sondern Heterosis. Unsere Ergebnisse deuten darauf hin, dass fortgesetzte Inzucht die Fitness der Pflanzen drastisch reduziert, aber dass die Effekte umweltabhängig sind.

Insgesamt erweitern unsere Untersuchungen das Wissen über den Einfluss der Dauer der Fragmentierung, der historischen Konnektivität der Populationen und von Eigenschaften wie Langlebigkeit oder Klonalität auf die evolutionären Prozesse von Selektion und genetischer Drift, welche die genetische Variation innerhalb und zwischen Populationen bestimmen. Die Studien zeigen dass molekulare und quantitativ genetische Untersuchungen ergänzende Informationen für den Schutz von seltenen und gefährdeten Pflanzen liefern. Darüber hinaus zeigen sie die Bedeutung von Studien zur Empfindlichkeit von Populationen gegenüber Inzucht und zum Potential und den Risiken einer künstlichen Erhöhung des Genflusses zwischen den Populationen kürzlich fragmentierter Arten für einen effektiven Artenschutz.

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Erklärung

Ich versichere, dass ich meine Dissertation

“ The ecology, genetics and evolution of two *Saxifraga* species with different fragmentation histories”

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe.

Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Luxembourg, den 13.08.2015 _____

Tania Walisch