Quantitative genetic variation in declining plant populations

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QUANTITATIVE GENETIC VARIATION IN DECLINING PLANT POPULATIONS

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Contents

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I Ellmer M, Andersson S. 2004. Inbreeding depression in *Nigella degenii* (Ranunculaceae): fitness components compared with morphological and phenological characters. International Journal of Plant Sciences, 165: 1055-1061. (reprinted with permission from the publisher)

II Andersson S, Ellmer M, Jorgensen TH, Palmé A. Quantitative genetic effects of bottlenecks: experimental evidence from a wild plant species, *Nigella degenii*. – Submitted.

III Ellmer M, Johansson LJ, Prentice HC, Andersson S. The structuring of quantitative genetic variation in a fragmented population of *Briza media* (Poaceae). – Manuscript.

IV Ellmer M. Phenotypic variation in *Saxifraga granulata*: past and future effects of habitat fragmentation. – Manuscript.

My contributions to the papers:

Paper I: I had the main responsibility for the experiment design, the collection and analyses of data, and the manuscript preparation.
Paper II: I was responsible for the collection and analyses of data (except for the CPC runs), and was also involved in the manuscript preparation.
Paper III: I was responsible for the collection and analyses of phenotypic data, and the manuscript preparation.
Paper IV: I was responsible for the sampling design, the collection and analyses of data, and the manuscript preparation.

Quantitative genetic variation in declining plant populations

Introduction

Anthropogenic activities have altered natural environments since industrialization and the conditions to which plants and animals are exposed are still changing as a consequence of, for example, overexploitation, habitat destruction, species translocations, pollution and climate change. Habitat destruction is considered to be the most important factor in many areas. When formerly continuous habitats become divided into small and isolated patches of habitat, many species experience a decrease in population size, a reduction in the ability to disperse and repopulate unoccupied patches of habitat and, in many cases, a deterioration of habitat quality owing to, for example edge effects (Saunders et al. 1991; Harrison & Bruna 1999). As well as having direct effects on demographic parameters, these changes may be accompanied by erosion of genetic variation, inbreeding and increased divergence between populations, as a result of genetic drift, reduced gene flow and altered selection regimes. Understanding the genetic consequences of small or declining population size, and more generally, population fragmentation, remains a primary challenge for conservation biologists (e.g., Ellstrand & Elam 1993; Storfer 1996; Young *et al*. 1996; Frankham 1999; Willi *et al*. 2006).

The existence of genetic variation within a population is crucial for its ability to evolve in response to novel environmental challenges. Provided that this variation can be expressed at the phenotypic level and that some phenotypes survive and reproduce better than others, there will be an increase in the proportion of advantageous alleles and genotypes — and a change in the mean phenotype — relative to the previous generation. Therefore, genetically variable populations are expected to evolve morphological, physiological or behavioural mechanisms to cope with the novel conditions (Falconer & Mackay 1996). This sorting process not only results in populations that are better adapted to their local environments, but may also, at least in theory, cause a reduction in the genetic variation — and the potential for further adaptive change — by eliminating those genotypes that deviate from the optimum phenotype. The ability to adapt to future environmental changes will also be reduced if the population has lost variation through stochastic processes

such as genetic drift, inbreeding or bottleneck effects (Falconer & Mackay 1996). Based on empirical data, however, natural populations seem to have a great capacity to maintain, or regenerate, genetic variation by mutation and other mechanisms, especially in phenotypic characters with a polygenic basis (Johnson & Barton 2005). Thus, it is still unclear whether the genetic effects of population fragmentation can reduce the adaptive potential of wild species over timescales considered by most conservation biologists (Willi *et al.* 2006).

The major aim of this thesis was to evaluate the quantitative genetic consequences of small population size and population fragmentation in three wild, nonmodel plant species, using data from a inbreeding/bottleneck experiment or samples of genotypes from a landscape known to have undergone a massive loss of grassland habitat. Particular attention was given to (i) the short- and long-term effects of inbreeding on primary fitness components vs. morphology and phenology, (ii) the genetic (co)variance structure and evolutionary potential of bottlenecked populations, and (iii) the structuring of quantitative variation following human-induced habitat fragmentation. Before addressing these studies, I provide a short introduction to the research field and a background to the main issues raised in this thesis.

Quantitative genetic variation and its estimation

Characters describing life-history, morphology and physiology are generally continuously distributed and controlled by tens to hundreds of loci, each having a small influence on the phenotype. Given the polygenic basis and the additional effects of multiple environmental factors, it is not possible to identify the phenotypic effects of particular alleles nor is it possible to observe Mendelian segregation at the underlying loci. Thus, when inferring patterns and amounts of genetic variation in polygenic characters, it is necessary to use quantitative genetic models that translate allele and genotype frequencies at a large number of unidentified loci into statistical parameters that can be estimated at the phenotypic level (Falconer & Mackay 1996).

The variation in normally distributed characters is most conveniently expressed as the variance around the mean. A primary advantage of the variance is that it can be partitioned into independent components corresponding to identifiable sources of variation. Thus, the total phenotypic variance of a population (V_P) can be interpreted as the sum of two main components: the variance caused by differences between genotypes (V_G) and

the variance arising from environmentally-induced differences between individuals (V_E). The genetic variance represents the inherited part of the variation and can be divided into three components: the additive genetic variance (V_A), the genetic variance caused by dominance effects within loci (V_D) and the genetic variance arising from interactive (epistatic) effects between different loci (V_I). The sum of the latter two components represents the nonadditive genetic variance (Falconer & Mackay 1996).

Additive genetic variance (V_A) results from the segregation of alleles with independent (additive) effects on the phenotype. If allelic effects are additive, the contribution of each allele to the phenotypic value of an individual can be summed across all the loci that influence the character, and the expected phenotypic value of the progeny will approach the mean value of its parents. Such parent-offspring resemblance allows the phenotypic effects of selection to be passed on to the next generation. The additive variance (or a standardized version thereof, see below) can therefore be used as a measure of the future adaptive potential of a population. If alleles interact nonadditively, i.e. if dominance or epistatic variance (V_D , V_I) makes a substantial contribution to the phenotypic variation in the character, then the parent-offspring resemblance — and the genetic response to selection operating at the phenotypic level — will be weak or nonexistent. Although nonadditive variance components make little or no contribution to future adaptive potential, they can play major roles in determining the response to inbreeding or drastic reductions in population size (Falconer & Mackay 1996; Willi *et al.* 2006). For example, there is extensive evidence that inbreeding depression — the decline in fitness with increasing homozygosity — is caused by the increased expression of recessive or partially recessive deleterious alleles in homozygotes (Charlesworth & Charlesworth 1987).

In addition to the purely genetic or environmental determinants of variation, there can be a contribution of variance arising from genotype-by-environment interactions (V_{GxE}), reflecting differences between genotypes in their response to the same environmental difference, and an added variance component called genotype-environment covariance ($COV_{G,E}$), which is caused by the tendency for individuals to experience environments that are correlated with their genotype. Thus, the total phenotypic variance in a polygenic character can be represented as:

$$V_P = V_A + V_D + V_I + V_E + V_{GxE} + COV_{G,E}$$

This expression shows that the variance available for selection (V_A) represents a potentially small proportion of the variation in a character. Judging from empirical data, however, the additive genetic variance usually accounts for a substantial portion of the variation, especially in characters describing life-history, morphology and physiology (Falconer & Mackay 1996; Johnson & Barton 2005).

Different characters may be genetically correlated, a factor that can prevent or facilitate genetic responses to selection, depending on the sign and magnitude of the correlations and the relationship between each character and fitness. For example, a correlation may lead to a nonadaptive change in one character as a result of selection on another character, or facilitate changes in combinations of characters that are correlated and selected in the same direction. To infer the potential for adaptive change in such suites of correlated characters, it is necessary to consider the magnitude and direction of selection operating on each character as well as the additive genetic (co)variance matrix (**G**-matrix), whose diagonal elements are the additive variances for the individual characters and the off-diagonal elements represent the additive covariance for each pair of characters (Falconer & Mackay 1996).

It is relatively straightforward to estimate the genetic, and preferably the additive genetic, component of the (co)variance for phenotypic characters when measurement data from a large number of cloned or pedigreed individuals are available (for different methods, see Falconer & Mackay 1996). Estimates of genetic (co)variance are usually standardized to yield scale-free quantities that can be compared between characters with different scales and ranges. For example, the broad-sense heritability (H^2) is the ratio of the total genetic variance to the total phenotypic variance (V_G / V_P), whereas the narrow-sense heritability (h^2) represents the portion of variance attributable to additive gene effects (V_A / V_P). It is often possible to use H^2 (a more easily estimated parameter) as a proxy for h^2 when inferring the adaptive potential of a natural population. When the heritability of a character is known or inferred to be very high, it may even be possible to approximate the genetic variance from the phenotypic variance observed among field-collected, nonpedigreed individuals: phenotype-based estimates of variation often agree (at least in relative terms) with their quantitative-genetic counterparts (e.g., Leinonen *et al.* 2008). Purely phenotypic approaches have the distinctive advantage of allowing extensive sampling of multiple populations — a necessary feature if the objective of a study is to infer the genetic effects of landscape-scale processes such as habitat fragmentation.

Given the large effort normally required to obtain accurate (co)variance or heritability estimates for quantitative characters, it is not surprising that genetic studies of wild species often focus on allozymes or DNA markers. Molecular polymorphisms have an unambigous genetic basis and behave in an essentially neutral fashion, making them particularly useful for studies of genetic erosion and inbreeding (Ellstrand & Elam 1993; Schemske *et al.* 1994). In some cases, researchers take the data a step further, using them to infer the adaptive potential of populations, presumably under the assumption that patterns of variation at putatively neutral marker loci also provide insights into patterns of variation in ecologically important polygenic characters (Schemske *et al.* 1994). The confidence that can be attached to such extrapolation is limited and empirical data provide no support for a consistent relationship between marker-gene diversity and quantitative genetic variation (e.g., Reed & Frankham 2001). Before more powerful marker systems become available, there is no substitute to phenotype-level analyses in studies that aim to provide information on adaptation and long-term survival of populations (Storfer 1996; Frankham 1999).

Inbreeding depression in fitness components vs. morphology and phenology

Inbreeding depression has received considerable attention in conservation biology, based on the elevated extinction risks observed for small, inbred populations (Newman & Pilson 1997; Frankham 1999; Hedrick & Kalinowski 2000). Although much effort has focused on inbreeding depression in primary components of fitness such as viability and seed production (Husband & Schemske 1996), only a few authors have considered measures of male fertility or evolutionarily important characters (e.g., morphology and phenology) with more subtle or habitat-specific effects on fitness. For characters describing morphology or phenology, it is possible to imagine situations in which the phenotypic response to inbreeding may be advantageous and thus conducive to adaptive or nonadaptive change in the mean phenotype (Rao *et al.* 2002). To evaluate the short- and long-term effects of inbreeding in small or declining populations, it is necessary to estimate inbreeding responses in a broad variety of characters.

Quantitative genetic variation in small populations

Many populations are so small that genetic drift has a predominant effect on the number of gene copies that are passed on to the next generation. As a result of these chance fluctuations, the populations will lose variation and become increasingly differentiated from each other. Regarding quantitative characters, the adaptive potential of a population, measured by the additive genetic variance (V_A), is expected to decrease in direct proportion to the inbreeding coefficient of the population, i.e. at a rate of $1/2N_e$ per generation, where N_e is the effective population size (Falconer & Mackay 1996). Eventually an equilibrium level of variance will be reached, at which point the loss of variance is approximately balanced by mutational input. Under these conditions, the standing levels of additive variance will be directly proportional to the effective population size, the exact relationship depending on the type of selection that operate on the character (Willi *et al.* 2006).

Despite evidence for a link between small population size, reduced gene diversity and low population viability (Newman & Pilson 1997; Leimu *et al.* 2006), there is no conclusive data on the relationship between population size and the evolutionary potential to adapt to novel environmental challenges, as determined by the additive genetic (co)variances for suites of phenotypic characters (Willi *et al.* 2006). A number of authors have documented bottleneck-induced increase of additive variance, and invoked complex genetic mechanisms, for example the "conversion" of epistatic to additive variance, to explain these observations (e.g., Bryant *et al.* 1986). Based on these observations, it has been suggested that bottlenecks have the potential to enhance the adaptive potential of natural populations. However, if an increase in the additive variance is accompanied by severe inbreeding depression in primary components of fitness, then it seems unlikely that a bottlenecked population will have a greater evolutionary potential than the ancestral population (Willi *et al.* 2006). Moreover, as drift is a stochastic process, there should be wide variation around the additive expectations, so that some populations (or characters) show an increase in the additive variance, even if on average the variance decreases (Lynch 1988). So far, only a few bottleneck experiments have been performed on a sufficient scale to account for the detrimental effects of inbreeding depression and the variability in response among replicate lines derived from the same base population (e.g., Whitlock & Fowler 1999).

Quantitative genetic variation in fragmented landscapes

Genetic erosion and inbreeding not only present a threat to rare, threatened species, but can also — and sometimes to a greater degree — have a significant influence on common species, especially those subjected to anthropogenic habitat fragmentation (Young *et al.* 1996). Habitat fragmentation is a pluralistic process, involving not only small-population effects, but also changes in metapopulation dynamics and selection regimes. First, if the original continuous populations are spatially structured into patches of related or locally adapted individuals, as in many plant species (Linhart & Grant 1996), then a local loss of habitat area is expected to cause an immediate reduction of genetic variation as a consequence of bottleneck effects (Nei *et al.* 1975). Second, if the surviving population fragments remain small and isolated for many generations, they will continue to lose variation and become increasingly differentiated as a result of drift and inbreeding (Ellstrand & Elam 1993). Third, there will be an added influence of bottlenecks if the fragmented landscape contains a fraction of unoccupied habitat patches that can be recolonized and if each recolonization involves one or a few founders of local origin (Wade & McCauley 1988). Finally, if the population fragments experience less maladaptive gene flow or more diverse ecological conditions than larger ones, one might expect increased divergent selection after habitat fragmentation (Willi *et al.* 2007). Clearly, it is necessary to consider a broad variety of factors to examine how random and selective processes interact to determine the structuring of quantitative genetic variation — and the adaptive potential of individual populations — within species subjected to habitat fragmentation.

Outlines and objectives of this thesis

The studies in this thesis evaluated the quantitative genetic effects of small population size and population fragmentation, with particular emphasis on the variation in evolutionarily relevant phenotypic characters.

The study presented in **Paper I** compared selfed and outbred progenies of the annual plant *Nigella degenii* (Ranunculaceae) to estimate and compare patterns and amounts of inbreeding depression in different types of characters. The study in **Paper II** was performed to assess the quantitative genetic effects

of a single-founder bottleneck, based on replicate lines established from the same base population as was used in Paper I.

Papers III and **IV** examined the structuring of variation in phenotypic characters — and its possible determinants — in fragmented populations of two common grassland species, *Briza media* (Poaceae) and *Saxifraga granulata* (Saxifragaceae). These studies were based on plant material from the Jordtorp area on the Baltic island of Öland (SE Sweden).

Study systems

Nigella degenii

Nigella degenii Vierh., the species used in Papers I and II, is a diploid, self-compatible plant which occupies more or less disturbed habitats (roadsides, abandoned fields, etc) on the Cyclades, the Greek archipelago. Plants of *Nigella* are erect to ascending, with a branched stem, pinnately dissected leaves and 15-25 mm wide, hermaphroditic flowers adapted for insect pollination. Each flower consists of five white, petal-like sepals, eight stalked bilabiate nectaries and a variable number of stamens. Protandry together with spatial separation of anthers and stigmas (herkogamy) prevents selfing and makes it easy to emasculate *Nigella* flowers for experimental studies. This and related species in the *N. arvensis* complex have diverged for several phenotypic characters. Apart from a large-scale ecogeographical pattern in plant stature, flower size and mating system, there is a mosaic of variation reflected in sharp (random) differentiation among local populations (Strid 1970).

The plants in Papers I and II represented different generations in an extensively replicated inbreeding/bottleneck experiment (Figure 1; see also Methods) and were descendants from seeds collected in a natural population of *N. degenii* subsp. *barbro* Strid on the island of Mikonos. Before initiating the experiment, we used four generations of random outcrossing, involving a minimum of 150 plants per generation, to establish a completely outbred base population in (near) linkage equilibrium.

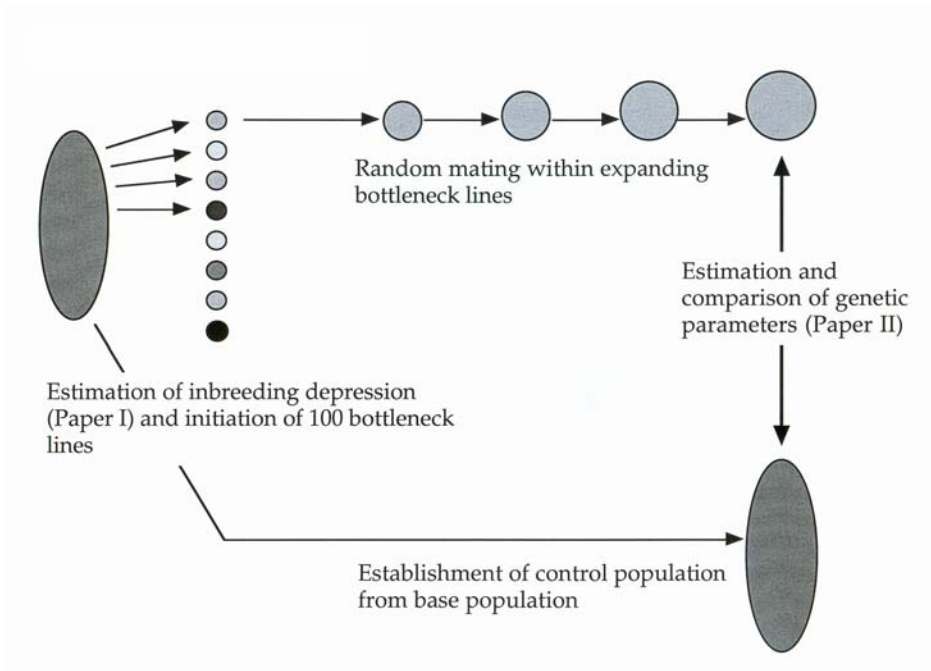

Figure 1. General outline of the inbreeding/bottleneck experiment.

Briza media

Briza media L., the species used in Paper III, is a widely distributed species (western Eurasia) of dry and calcareous, but also moist and acidic grassland. It is an indicator of old, species-rich grasslands as well as a colonizer of young grasslands developing on previously forested or arable sites (Prentice *et al.* 2007). Flowering occurs in June-July when the tussocks produce stems (culms) with roughly pyramidal panicles having a large number of distinctly shaped, flattened spikelets with large protruding anthers and large feathery stigmas typical of wind-pollinated grasses. The one-seeded fruits (caryopses) mostly disperse locally but long-distance dispersal by grazing animals and hay transfer is also possible (Fischer *et al.* 1996; Dixon 2002).

The plants of *Briza* used in Paper III represented clonally-replicated genotypes from 48 grassland patches in the Jordtorp area, grown for several years at randomized positions in a semi-natural (garden) environment before the measurements.

Saxifraga granulata

The study species of Paper IV, *Saxifraga granulata* L., is a self-compatible, herbaceous perennial which occurs in dry, base-rich grasslands (western Eurasia, northern Africa). The self-compatible, protandrous flowers are open in May-June and have five white petals, 10 stamens and a pistil that develops into a capsule. Protandy coupled with herkogamy enhances the opportunity for cross-pollination. Flower visitors include a wide variety of generalist pollinators, mainly members of Diptera and Hymenoptera. A previous study of a *Saxifraga* population in SW Sweden (Skåne) documented moderate to high narrow-sense heritabilities (h^2) for vegetative and floral characters under semi-natural (garden) conditions (Andersson 1996).

The plant material used in Paper IV represented field-collected phenotypes (flower stalks), sampled along 10 to 100 m long transects within 42 grassland patches in the Jordtorp area.

The Jordtorp area

The Jordtorp area includes the village of Jordtorp and four adjacent villages in the parish of Algutsrum on the Baltic island of Öland (SE Sweden).

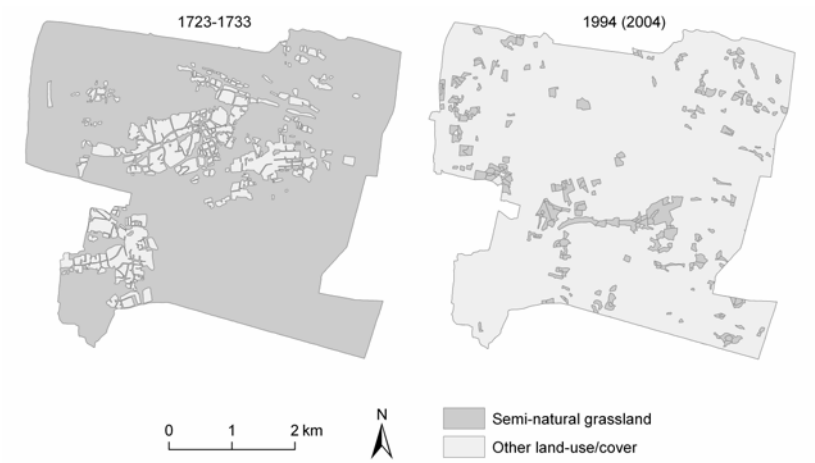

Figure 2. Maps showing the past and present distribution of grasslands within the Jordtorp area on Öland (redrawn using data from Johansson *et al.* 2008).

The landscape in this area consists of a small-scale mosaic of agricultural fields, deciduous forests and various types of grassland, including unimproved, species-rich, semi-natural grasslands. Analyses of historical land-cover maps have revealed a progressive reduction in the area of grassland over the last 300 years and the remaining grasslands have become increasingly fragmented (Figure 2).

The previous studies in this area have resulted in an extensive data set for the remaining grassland fragments, including land-use history, spatial extent, degree of connectivity with other grassland fragments, and ecological attributes such as soil moisture and grazing pressure (Prentice *et al.* 2007; Johansson *et al.* 2008). Several of these parameters have been found to influence, or at least correlate with, other aspects of diversity, for example, allozyme variation within *Briza* (Prentice *et al.* 2006) and various aspects of fine-scale plant diversity (Prentice *et al.* 2007; Johansson *et al.* 2008; Reitalu *et al.* 2009). This background information makes the Jordtorp area a useful study system in which to assess and compare the ecological and genetic effects of past, ongoing and future habitat fragmentation.

Methods

In this section, I provide a short summary of the methods used in this thesis (for detailed information, see the individual papers).

Papers I and II

The main purpose of these investigations was to assess patterns of inbreeding depression and the quantitative genetic effects of a severe bottleneck. To this end, we performed a replicated inbreeding/bottleneck experiment (Figure 1) using *Nigella* as a model system. Following the establishment of the outbred base population, we subjected flowers on each of 100 randomly selected plants to either self- or cross-pollination. A subset of the selfed and outcrossed seeds from each mother plant were sown to provide plants for the study of inbreeding depression (Paper I). The remaining selfed seeds were used to initiate 100 bottleneck lines, each founded from a separate maternal individual. After the selfing event, we expanded the lines using three generations of random outcrossing within lines. Plants in the third outcrossing generation were crossed in many pairwise combinations to produce a large number of

fullsib seed families for each line. These seeds were sown together with a sample of fullsib seed families representing the ancestral base population, to produce plants that could provide measurement data for the quantitative genetic analysis (Paper II).

The plants used in the inbreeding-depression study and the quantitative genetic analysis were scored for a number of characters, including primary fitness components (e.g., survival rate, pollen viability and total flower number) and a suite of morphological or phenological characters that have diverged within the *N. arvensis* complex (e.g., plant height, flowering date and flower size; Strid 1970).

The phenotypic effects of one-generation selfing (Paper I) were quantified as coefficients of inbreeding depression (scaled by the mean or standard deviation of the outcrossed progeny) and tested for significance using chi-square procedures or factorial ANOVAs (controlling for the influence of the mother individual). Maternal estimates of inbreeding depression were also subjected to correlation analyses and principal component analyses to determine the correlated response to inbreeding for combinations of characters.

The quantitative genetic effects of the bottleneck (Paper II) were evaluated by comparing the **G** matrix for the control (base) population with the **G** matrix for each of the surviving lines in the bottleneck category. These comparisons were carried out with the common-principal-component (CPC) technique (Flury 1988; Phillips & Arnold 1999). This method tests whether two or more **G** matrices have completely unrelated structure, whether they share one or more principal components, whether they differ by a simple constant of proportionality, or whether the matrices are equal. We also contrasted the observed within- and among-line (co)variance after the bottleneck to their additive predictions for this experiment.

Papers III and IV

These studies were designed to reveal the quantitative genetic effects of human-induced habitat fragmentation, using samples of *Briza* and *Saxifraga* from the fragmented grassland landscape in the Jordtorp area.

The data set for *Briza* (Paper III) involved nine vegetative and reproductive characters, ranging from the number of culms to the size of the individual spikelets, and represented 740 clonally-replicated genotypes that had been scored for electrophoretically detectable variation at 11 allozyme loci in a previous study (Prentice *et al.* 2006).

The spatially-structured data set for *Saxifraga* (Paper IV) involved seven mostly floral characters that possessed moderate to very high narrow-sense heritability in a previous study of this species (Andersson 1996). It was assumed that genetic factors also made a large contribution to the variation observed in the Jordtorp area.

The measurement data were subjected to canonical variates analyses, nested ANOVSs and multiple regression analyses, to search for signals of genetic drift (reflected by associations with measures of population size and/or habitat connectivity) and local selection (reflected by associations with indicators of local ecological conditions) — processes that might have been enhanced by past and ongoing fragmentation in the Jordtorp area. In the study of *Briza* (Paper III), it was also possible to test for the effects of selection by comparing estimates of between-population variation for the quantitative characters (Q_{st}) with the corresponding estimate for the allozyme loci (F_{st} = 0.006, Prentice *et al*. 2006). In the study of *Saxifraga* (Paper IV), I also performed a series of habitat loss simulations, to assess how the observed variation might respond to further losses of grassland area, both at the regional level and at the level of individual populations.

Main results

Paper I

The comparison of experimentally selfed and outbred offspring of *Nigella* revealed significant inbreeding depression in primary components of fitness (flower number and pollen viability) but also demonstrated inbreeding responses in morphological and phenological characters for which the optimal phenotype may be population- or habitat-specific, e.g., plant height and flower size. The data showed extensive between-family variation in the response to inbreeding, but there was no consistent difference in the magnitude of inbreeding depression between fitness components and characters reflecting morphology or phenology, regardless of whether the inbreeding responses were scaled by the mean or the standard deviation of the outcross progeny. Adjusting from general plant vigour (measured by flower number) reduced estimates of inbreeding depression by 13-25%, but the difference between selfed and outbred progeny generally remained significant. Thus, most inbreeding responses probably involved loci with both general and specific effects on the phenotype. These and other observations suggest that

"unconventional" fitness variables, such as male fertility, morphology and phenology, could make significant and partly independent contributions to lifetime inbreeding depression. The observed inbreeding response in flower size could also contribute to the floral reduction accompanying the evolution of selfing in the *N. arvensis* complex.

Paper II

The inbreeding/bottleneck experiment started with 100 lines (each founded from a separate mother plant) but after three generations only 23 lines remained viable. The extinction of lines followed a temporally decreasing trend with 56, 21 and 0 lost lines in the first, second and third generation, respectively. This pattern is consistent with the selective loss or "purging" of those lines that suffered most from inbreeding depression and extends the finding from Paper I that the initial maternal families varied in their sensitivity to inbreeding.

Analyses of the "surviving" bottleneck lines showed stochastic, nonproportional changes in the genetic (co)variance (**G**) matrix: different lines had high or low (co)variance estimates for different characters and there was no support for the equality or proportionality model in CPC analyses that compared each bottleneck line with the control population. Nevertheless, we found a few consistent patterns; for example, leaf and flower size were positively correlated in all cases, indicating a potential for correlated evolution in these characters, even after a severe bottleneck.

The general estimates of the within- and among-line (co)variance for sepal length were in good agreement with the additive predictions for this experiment, whereas the other characters showed an excess of within-line (co)variance and a deficiency of among-line (co)variance; nevertheless, the within-line (co)variances were always lower than the corresponding estimates for the control group.

The results of paper II highlight the idiosyncratic nature of bottleneck effects but also indicate that bottlenecked populations generally have a lower adaptive potential than the ancestral population, given the small proportion of lines that remained viable until the end of the experiment.

Paper III

The *Briza* data were analyzed with a combination of univariate and multivariate methods to provide complementary views of the structuring of phenotypic variation — and its possible determinants — in the Jordtorp area.

A principal component analysis, based on the product-moment correlations among the original characters, indicated the existence of two major axes of variation, one describing overall plant size and another dominated by the number and size of spikelets. Based on these composite variables, almost all the phenotypic variation (> 98%) could be attributed to differences between individual plants within demes (the term for local populations used in Paper III), the remainder occurring between demes from different grassland fragments. A substantial portion of the within-deme variation was explained by differences between genotypes, resulting in relatively high broad-sense heritabilities (H^2 = 0.29-0.34). Although differences between demes accounted for less than 2% of the variation in the composite variables, they explained 37% of the total variation when all the original characters were considered simultaneously in a canonical variates analysis.

The deme-specific means and broad-sense heritabilities for the composite variables were not significantly influenced by descriptors of landscape structure, land-use history and local environmental conditions, regardless of how the data were analysed. Similarly, there was no difference in the multivariate phenotype between categories of plants or demes differing in the various deme/patch descriptors used in this study, the only exception being a weak tendency for the group of spatially restricted demes to have a higher portion of their variation between demes than the group of spatially extensive demes. Although estimates of population structure (Q_{st} = 0-0.03) were similar to the corresponding estimate from the previous allozyme analysis (F_{st} = 0.006; Prentice *et al.* 2006), no significant association was found between quantitative and allozyme-based measures of within-population diversity.

The results from paper III provide no clear evidence that drift, founder events and diversifying selection have played major roles in structuring the quantitative genetic variation within *Briza* in the Jordtorp area, nor do they support the assumption of a consistent relationship between marker diversity and quantitative genetic variation.

Paper IV

As well as searching for signals of local genetic processes (as in Paper III), this study of *Saxifraga* also explored how a further reduction of grassland area might affect the variation present in the Jordtorp area. Nested ANOVAs, based on data from field-collected phenotypes, generally revealed significant variation between populations and between plots in the same population, although the relative magnitude of these components varied between characters. Judging

from a canonical variates analysis, more than 80% of the variation could be attributed to between-population differences.

Consistent with the results for *Briza*, there was no support for genetic drift and founder events being major determinants of the structuring of phenotypic variation within *Saxifraga*: populations differing in size, connectivity or habitat age showed similar divergence in the multivariate phenotype and had similar levels of within-population variation according to the multiple regression analyses. In contrast to the previous study, however, several characters were significantly affected by current grazing pressure, the percent cover of trees or shrubs, or the position along the S-N axis, pointing to the influence of deterministic factors such as selection and/or phenotypic plasticity.

Despite evidence for spatial variation in all characters, there was only a slight or moderate reduction in the regional estimate of the between-population variance following a hypothetical loss of populations, regardless of whether the order of loss was determined by population size, grassland area, habitat connectivity or management intensity: the regional variance remained close to the starting value until only about 10 populations remained in the sample. Similarly, a successive reduction in transect length, simulating a localized loss of habitat, generally had a minor impact on the within-population variance: the variance estimate remained close to the initial value until only one or two sampling plots (corresponding to a transect length of 10-20 m) contributed to the sample.

General discussion

There is ample evidence that reductions in population size have negative effects on short-term population viability and that these changes can be preceeded by, or be correlated with, a reduction in gene diversity at marker loci (Newman & Pilson 1997; Leimu *et al.* 2006). The long-term (evolutionary) consequences have received far less attention, and existing studies provide little support for a consistent relationship between quantitative genetic variation and population size, connectivity or other landscape parameters in wild plant species (Willi *et al.* 2006). In this thesis, I use a combination of approaches to evaluate (i) the short- and long-term effects of inbreeding on different types of characters, (ii) the adaptive potential of bottlenecked populations, and (iii) the structuring of quantitative variation following habitat fragmentation.

The study of inbreeding depression (Paper I) revealed significant levels of inbreeding depression, not only in direct components of fitness such as survival rate, flower number and pollen viability, but also in morphological or phenological characters with presumably more subtle or habitat-correlated effects on fitness. The latter characters showed the same magnitude of inbreeding depression as the primary fitness components; thus, there was no support for the notion that fitness components are more sensitive to inbreeding depression than characters with weaker effects on fitness (DeRose & Roff 1999). As well as influencing the mean phenotype of selfed and outbred progeny, inbreeding depression also seems to be responsible for the large number of lines that went extinct in the bottleneck experiment (Paper II). Taken together, these findings highlight the importance of considering a broad range of fitness consequences before any broad generalizations are made regarding the magnitude of total inbreeding depression and its possible effect on the short- and long-term viability of small or declining populations.

The results from the bottleneck study (Paper II) provide no support for rejecting the widely held view that reductions in population size are detrimental in terms of adaptation and long-term population viability (Willi *et al*. 2006), given the small fraction of lines that survived until the end of the experiment. On the other hand, we observed extensive heterogeneity in the genetic (co)variance structure for those bottleneck lines that remained viable; in fact, several lines showed an increase in the (co)variance for one or several characters, even if on average the (co)variance decreased during the bottleneck. These and other observations illustrate the idiosyncratic nature of bottleneck effects but also raise the possibility that (severe) reductions in population size could enhance, or at least not severely reduce, the evolutionary potential of surviving populations. If so, there is no a priori reason to consider bottlenecked populations as evolutionary ”dead ends”, unworthy of further conservation effort.

In contrast to the large effects of drift and inbreeding seen in the inbreeding/bottleneck experiment, there was little support for stochastic factors being major determinants of the structuring of variation in anthropogenically fragmented populations of *Briza* and *Saxifraga* (Papers III and IV). Contrary to theoretical predictions (Ellstrand & Elam 1993; Young *et al.* 1996) and marker-gene data from fragmented plant populations (Aguilar *et al*. 2008), there was little or no tendency for small, spatially isolated or newly established populations of the two study species to be less variable or more divergent in their mean phenotype than populations with the opposite features. In fact, the

consistent associations observed in *Saxifraga* (Paper IV) pointed to an influence of deterministic rather than stochastic factors. Whatever the reasons for the apparent lack of small-population effects (extensive gene flow, high mutation rates, slow population dynamics, low statistical power, etc), it seems that the structuring of quantitative variation has been relatively insensitive to local stochastic influences, at least over the 300-year time frame considered in these study systems.

The spatially-structured data available for *Saxifraga* (Paper IV) enabled me to determine how the variation between and within populations might be affected under a continued scenario of habitat degradation. Somewhat surprisingly, there were only slight reductions in the estimate of variation following a simulated loss of grassland area, regardless of the "loss scenario" used in the analyses. Thus, substantial losses of grassland area will be required to significantly reduce the phenotypic variation represented in the study species. It would be interesting to perform more extensive habitat loss simulations to determine how different types of genetic and ecological diversity will be influenced by further reductions in the number or size of grassland areas in the Jordtorp area.

The results for *Briza* and *Saxifraga*, together with those for other wild plant species (e.g., Widén & Andersson 1993), indicate that the quantitative genetic effects of habitat fragmentation may be of lesser concern than the more immediate — and more easily studied — effects on species diversity or short-term population viability (e.g., Saunders et al. 1991; Harrison & Bruna 1999; Leimu *et al.* 2006). If this interpretation can be supported by future studies, it seems justified to make conservation-related decisions on the basis of more easily obtained information, for example, species or marker-gene data — rather than focusing directly on adaptive potential and long-term population viability (Schemske *et al.* 1994).

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INBREEDING DEPRESSION IN *NIGELLA DEGENII* (RANUNCULACEAE): FITNESS COMPONENTS COMPARED WITH MORPHOLOGICAL AND PHENOLOGICAL CHARACTERS

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We have compared selfed and outbred offspring from individual plants of the annual plant *Nigella degenii* to examine patterns of inbreeding depression in two direct components of fitness (flower number and pollen viability) and a number of morphological or phenological characters for which the optimal phenotype may be habitat specific. Selfing lowered flower number, plant height, flower size, and pollen viability and caused a shift toward later germination and flowering dates. There was no significant difference in inbreeding depression between fitness components and characters reflecting morphology or phenology regardless of how inbreeding response was estimated. Family-level analyses revealed moderately strong correlated responses involving flower number and each of the nonfitness characters, whereas pollen viability showed an independent response to inbreeding. On the basis of these observations, we hypothesize that morphology and phenology could make a significant contribution to lifetime inbreeding depression in *N. degenii*, that inbreeding responses in different types of characters involve loci with both general and specific effects on the phenotype, and that morphological inbreeding depression has contributed to the evolutionary reduction of floral structures so prevalent in the *Nigella arvensis* complex.

Keywords: Nigella degenii, floral evolution, inbreeding depression, mating system, morphology, phenology.

Introduction

Inbreeding depression, i.e., the decline in mean phenotype with increasing homozygosity (Falconer 1989), has received considerable attention in conservation biology (Hedrick and Kalinowski 2000; Keller and Waller 2002) and evolutionary studies of plant mating systems (Lloyd 1979; Lande and Schemske 1985; Charlesworth and Charlesworth 1987). According to the dominance hypothesis, inbreeding depression is caused by the expression of recessive or partially recessive deleterious alleles in homozygotes, whereas the overdominance hypothesis predicts a link between heterozygosity and gene interactions that cause increased performance relative to both homozygotes (Charlesworth and Charlesworth 1987; Falconer 1989). Although recessive deleterious alleles seem to be the most important source of inbreeding depression (Charlesworth and Charlesworth 1999), there are still too few empirical data to draw general conclusions about the genetic control of inbreeding depression. For example, relatively few attempts have been made to determine whether the heritable basis of inbreeding depression varies across the life cycle (Carr and Dudash 1995; Husband and Schemske 1996; Rao et al. 2002; Andersson and Waldmann 2003).

Much effort has been devoted to the estimation of inbreeding depression in primary components of fitness, particularly those related to vegetative vigor and female function (Charlesworth and Charlesworth 1987; Falconer 1989; Husband and Schemske 1996; Keller and Waller 2002). Only a few studies have considered measures of male fertility (e.g., Carr and Dudash 1995; Jóhannsson et al. 1998; Willis 1999; Stephenson et al. 2001), and there is still a paucity of information on patterns of inbreeding depression in characters that might be weakly related to fitness or under stabilizing selection for a spatially varying optimum, e.g., characters describing morphology or phenology (Willis 1996; Andersson 1997; Shaw et al. 1998; DeRose and Roff 1999; Rao et al. 2002; Andersson and Waldmann 2003). For such characters, one would expect the relative fitnesses of selfed and outcrossed progeny to be habitat specific, a complication in studies that attempt to estimate total inbreeding depression on the basis of data from plants grown in standardized (nonselective) environments (see also Dudash 1990).

Inbreeding depression in morphology or phenology not only affects the relative fitnesses of inbred and outbred progeny but has also been shown to bias measurements of phenotypic selection (Lande and Arnold 1983) toward the detection of strong directional or stabilizing selection in partially inbred populations (Willis 1996). It is also possible to imagine situations in which certain inbreeding responses are advantageous—or at least not deleterious—and thus conducive to evolutionary change in the mean phenotype (Lynch et al. 1999; Rao et al. 2002; see also Charlesworth 1992). For example, there is growing evidence for inbreeding depression in floral size characters (Karoly 1994; Andersson 1996, 1997; Willis 1996; Shaw et al. 1998), a pattern that could facilitate the reduction of floral structures so prevalent in selfing plant lineages (Ornduff 1969). However, there should be little potential for inbreeding-mediated evolution when the phenotypic response to inbreeding represents a side effect of low vigor, given the potentially strong selection

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against those alleles that cause a decline in fitness under inbreeding (Lande and Schemske 1985). To evaluate the potential role of inbreeding depression in phenotypic evolution, it is therefore important to determine whether observed inbreeding responses in morphology or phenology involve loci with general or specific effects on the phenotype (Rao et al. 2002).

Species in the *Nigella arvensis* complex (Ranunculaceae) have undergone considerable phenotypic divergence, especially in the Aegean, where a number of morphologically distinct taxa have been recognized (Strid 1970). For example, flowers of two selfing species, *Nigella doerfleri* Vierh. and *Nigella stricta* Strid, are 50%–60% smaller than the insect-pollinated flowers of *N. arvensis* L. subsp. *arvensis*, the largest-flowered taxon in this species complex. The reduction in flower size and other characters correlated with flower size (plant height, leaf size; Andersson 1997) may be an adaptive response to drought or low pollinator abundance (Strid 1969; Andersson 1997, 2000), but it is also meaningful to consider the direct effects of inbreeding: floral and vegetative size characters showed a negative response to inbreeding in a hybrid population from a cross between two nonautogamous subspecies of *Nigella degenii* Vierh. (Andersson 1997). In this study, we have obtained family-structured data from one of these subspecies to verify the existence of such inbreeding responses in a natural (nonhybrid) population and to provide more detailed information on the relationship between inbreeding depression in different characters. Specifically, we asked, Do measures of morphology and phenology respond to inbreeding, and are these responses of the same magnitude as those recorded for direct components of male or female fitness? Do maternal families respond differently to inbreeding, and are the family-level estimates of inbreeding depression correlated across characters? Is the pattern of inbreeding depression conducive to evolutionary change in the mean phenotype?

Material and Methods

Plant Material

Nigella degenii is an annual, diploid (2n=12), self-compatible, insect-pollinated plant that occurs in more or less disturbed habitats, e.g., roadsides, abandoned terraced fields, seashores, and phrygana vegetation, in Cyclades, the Greek archipelago. The species belongs to the *Nigella arvensis* complex and has been divided into four morphologically distinct subspecies, including subsp. *barbro* Strid, which is endemic to Mykonos and a few other islands in northwest Cyclades (Strid 1970). Plants of *N. degenii* are erect to ascending with pinnatisect leaves and a stem with determinate flowering, starting with the flower terminating the central stem. Each flower consists of five white, petal-like sepals serving as attracting organs; eight stalked, bilabiate nectaries; a variable number of stamens; and a central gynoecium consisting of 3–10 partially united carpels. Protandry coupled with spatial separation of anthers and stigmas prevents within-flower selfing. Fertilized flowers develop into capsules, each consisting of 20–40 seeds (Strid 1969, 1970; Andersson 1997).

The plants used in this study were derived from seeds collected in a population of *N. degenii* subsp. *barbro* on the island of Mykonos (along a stone fence ca. 2.5 km NNW of the town) in the summer of 1993.

Experimental Procedures

Four generations of random outcrossing involving ca. 150 plants per generation were used to establish a completely outbred population in (near) linkage equilibrium for a quantitative genetic analysis (S. Andersson, unpublished data). In this study, we used this plant material as a base population for an inbreeding-depression experiment even though only one generation of outcrossing would have been sufficient for this purpose. In 2000, ca. 150 seeds from the base population were sown in separate pots and arranged in a random pattern on three benches in an insect-free greenhouse. Two randomly chosen flowers on each plant were marked and subjected to one of two pollination treatments: self-pollination or emasculation followed by cross-pollination with pollen from a randomly chosen plant in the same population. In April 2002, five outbred and five selfed seeds from each of 89 plants in the base population ($n = 890$ seeds) were sown individually into 25-cm^2 cells in a series of plastic flats on the same greenhouse bench. Seeds from a given full-sib family were randomized across the whole planting area. Water was supplied as needed, but no fertilizer was applied. Given the lack of inbreeding in the base population, the coefficient of inbreeding was assumed to be 0 and 0.5 for outbred and selfed seeds, respectively (Falconer 1989).

Measurements

The following variables were scored on each selfed or outbred individual: the number of days from sowing to germination, the number of days from sowing to first flowering, plant height, leaf length, leaf-flower distance, flower size, pollen viability, and the total number of flowers initiated. Data on leaf length, leaf-flower distance, and flower size were obtained by preserving the first flower and the uppermost leaf in a microcentrifuge tube filled with 60% ethanol and measuring each character under a dissecting microscope equipped with an ocular micrometer. Leaf length refers to the uppermost leaf on the central stem, while leaf-flower distance refers to the distance between this leaf and the terminal flower (referred to as "peduncle length" in Andersson 1997). Flower size was quantified as the length of one randomly selected sepal per flower. This character is strongly positively correlated with the lengths of the nectaries and stamens (Andersson 1997) and therefore represents an overall measure of flower size. Pollen viability was determined by mixing a small amount of pollen from the first flower in a droplet of aniline blue lactophenol (cotton blue) on a glass slide and using a light microscope to determine the proportion of stained pollen grains (on the basis of 200 counted grains per sample). Cotton blue stains pollen grains that contain starch and is a fairly reliable measure of pollen viability (Stanley and Linskens 1974). The fate of nonflowering plants (germination failure, juvenile mortality) and the presence or absence of pollen in the anthers were also recorded.

Measures of vegetative or reproductive performance, such as germinability, survivorship, pollen viability, and flower number, were regarded as fitness components, while the

number of days to germination or flowering, plant height, leaf length, leaf-flower distance, and flower size represent characters describing morphology or phenology. The fitness components should be positively correlated with fitness irrespective of habitat, whereas the morphological and phenological characters might be subjected to genetic drift or spatially varying selection, as indicated by the extensive among-population variation recorded for most of these variables (Strid 1970; Andersson 1997). Comparative data from Strid (1969) strongly imply that the optimum plant height differs between populations, with more arid island sites selecting for plants with a short stature and more mesic island sites favoring tall individuals. For this reason, we consider plant height as a morphological character rather than as a fitness component.

Flower number is determined relatively late in the ontogeny and represents the most sensitive response variable in resource-manipulation experiments (Andersson 1997); hence, flower number also serves as an integrated measure of general plant vigor.

Statistical Analyses

Frequency data were pooled across families and analyzed with χ^2 procedures to test for differences between selfed and outcrossed progeny. Differences between pollination treatments and maternal families in quantitative variables were tested for significance using mixed-model two-way ANOVA (type III sums of squares), with pollination treatment as "fixed" and family as "random." To test for morphological and phenological inbreeding depression unconfounded by differences in overall plant vigor, we repeated the relevant analyses following the inclusion of total flower number as a covariate (ANCOVA). To normalize the residuals, germination date and leaf-flower distance were log transformed, and pollen viability was arcsine–square root transformed.

To quantify inbreeding responses, we first calculated the difference in the mean between selfed and outbred progeny (using least square means from two-way ANOVA or ANCOVA) and then standardized this quantity by the mean or standard deviation (SD) of the outcross progeny. The resulting parameters will be referred to as δ_{mean} and δ_{SD}, respectively. The first measure represents the conventional coefficient of inbreeding depression (Charlesworth and Charlesworth 1987) and allows direct comparison with estimates from previous inbreeding-depression experiments (Husband and Schemske 1996), whereas the second measure provides a scale-independent estimate of inbreeding depression (DeRose and Roff 1999), allowing comparison of characters for which the choice of scale is arbitrary. For example, the value of δ_{SD} for a phenological character such as flowering date is the same regardless of whether day 0 is chosen to represent the emergence date or the start of the flowering period (Rao et al. 2002). Values of inbreeding depression for phenological parameters were presented as positive, even though selfed offspring had higher means for these variables (i.e., a larger number of days to germination and flowering; see "Results") than outcrossed progeny.

Maternal estimates of inbreeding response—quantified as the difference in the mean of the selfed and outcrossed progeny from each mother plant—were subjected to a product-moment correlation analysis to determine the correlated response to inbreeding for each pair of characters, followed by a principal components analysis (PCA) to summarize the pattern of variation in inbreeding response. Given the (apparent) lack of treatment effects for qualitative variables (see "Results"), we made no attempt to include them in the correlation analyses.

All analyses were performed with SPSS for Windows (release 11.0.0), except for the bivariate and multivariate procedures, which were carried out with SYSTAT on a Macintosh computer.

Results

Population-Level Analyses

Selfing had nonsignificant or marginally significant effects on the frequency of seeds that failed to germinate (10.6% vs. 7.9% for outcrossed progeny; $\chi^2_1 = 1.93$, $P = 0.164$), the frequency of plants that died before flowering (5.0% vs. 2.4% for outcrossed progeny; $\chi^2_1 = 3.78$, $P = 0.052$), and the frequency of flowering plants that produced sterile anthers (2.4% vs. 1.0% for outcrossed progeny; $\chi^2_1 = 2.30$, $P = 0.129$). The magnitude of inbreeding depression for these measures of fitness (measured by δ_{mean}) was 0.029, 0.027, and 0.014, respectively, and all changes were in the predicted direction (i.e., outcrossed progeny outperformed selfed).

Selfed progeny germinated later and had later flowering dates, fewer flowers, shorter stems, lower pollen viabilities, and smaller flowers than plants derived from outcrossing, whereas differences in leaf length and leaf-flower distance failed to reach significance (table 1). Judging from the conventional (mean standardized) estimate of inbreeding depression (δ_{mean}; table 2), there was a stronger effect of inbreeding on pollen viability (0.091), flower number (0.075), and plant height (0.074) than the other characters (<0.050). Scaling by the standard deviation increased the difference between the estimate for pollen viability ($\delta_{SD} = 0.708$) and the corresponding estimates for flower number (0.175) and all the remaining characters (0.105–0.305).

There was no significant difference in inbreeding depression between fitness components and characters reflecting morphology or phenology, regardless of whether the inbreeding response was quantified as δ_{mean} (Mann-Whitney $U_1 = 11.50$, $P = 0.522$) or δ_{SD} (Mann-Whitney $U_1 = 4.00$, $P = 0.505$). Classification of plant height as a fitness component had negligible effects on the results of these comparisons (data not shown).

Differences in pollen viability, germination and flowering date, plant height, and flower size remained significant in analyses accounting for variation in flower number ($P < 0.001$–0.05, ANCOVA; data not shown). Adjusting for flower number had little effect on estimates of inbreeding depression for pollen viability but resulted in lower estimates for all the other characters (table 2). The reduction in δ_{mean} and δ_{SD} was 23%–25% for germination date, plant height, and leaf length; 17%–18% for flowering date and leaf-flower distance; and 13% for flower size.

Table 1

Effects of Pollination Treatment (Outcrossing vs. Selfing) and Maternal Parent on Each of the Quantitative Characters in the Inbreeding-Depression Experiment

	F values			Means			
	Treatment (*T*)	Mother (*M*)	$T \times M$	Outcrossing	*n*	Selfing	*n*
Fitness components:							
Flower number	4.77*	1.39 ns	1.41*	3.80	409	3.52	397
Proportion of viable pollen[a]	44.30***	1.08 ns	1.50**	0.965[b]	389	0.925[b]	358
Morphology/phenology:							
Days to germination[c]	8.05**	1.52*	1.70***	7.82[b]	410	8.13[b]	398
Days to flowering	10.73**	1.43*	1.60***	82.61	399	84.96	374
Plant height (mm)	11.50***	1.70**	1.58**	204.0	406	188.8	390
Leaf length (mm)	1.32 ns	1.97***	1.75***	12.41	390	12.20	368
Leaf-flower distance[c]	3.30 ns	2.94***	1.40*	3.74[b]	390	3.39[b]	368
Flower size (mm)	9.28**	2.12***	1.60***	12.11	390	11.78	368

Note. Data determined by using *F* values and least square means from two-way ANOVA; ns = not significant ($P > 0.05$).

[a] Analyses based on arcsine–square root transformed data.

[b] Mean back transformed to original scale.

[c] Analyses based on log-transformed data.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

Family-Level Analyses

The maternal parent had a significant influence on all but two variables—flower number and pollen viability—and there was a significant interaction between cross type and mother for all variables (table 1). Hence, our data indicate extensive between-family variation, both in the overall mean and in the response to inbreeding.

Correlation analyses demonstrated significant and moderately strong correlated responses involving flower number and each of the morphological and phenological characters ($|r| = 0.265$–0.642; table 3), whereas estimates of correlated response involving pollen viability failed to reach significance ($|r| < 0.09$). Consistent with these patterns, we found high loadings for all characters except pollen viability on the first principal component, which extracted 46% of the between-family variability in inbreeding response (table 4). The second axis accounted for a supplementary 13% of the variation, contrasting families with high versus low inbreeding depression in pollen viability.

Discussion

Although much attention has focused on the negative effects of inbreeding on direct components of fitness (Charlesworth and Charlesworth 1987; Husband and Schemske 1996; Keller and Waller 2002), there is still a paucity of studies that document patterns and amounts of inbreeding depression in characters reflecting morphology or phenology. In this study, we have obtained family-structured data from an extensive crossing experiment with a self-compatible, nonautogamous population of *Nigella degenii* to compare the phenotypic effects of selfing or outcrossing. Next, we evaluate the results with particular emphasis on the magnitude of inbreeding depression in fitness components versus morphology and phenology, the pattern of correlated response to inbreeding in the different types of characters, and the hypothesis that inbreeding effects have promoted the evolutionary reduction of flower size accompanying the evolution of selfing in the *Nigella arvensis* complex.

Consistent with the results from other wild plant species (Andersson 1996; Willis 1996; Shaw et al. 1998; Rao et al. 2002; Andersson and Waldmann 2003), inbred and outbred offspring of *N. degenii* subsp. *barbro* were found to differ in a wide variety of characters. Selfing depressed measures of

Table 2

Estimates of Inbreeding Depression for Different Characters, Quantified as δ_{mean} and δ_{SD}

	δ_{mean}		δ_{SD}	
	Non-adjusted	Adjusted[a]	Non-adjusted	Adjusted[a]
Fitness components:				
Flower number	0.075	...	0.175	...
Proportion of viable pollen[b]	0.091	0.091	0.708	0.704
Morphology/phenology:				
Days to germination[c]	0.019	0.014	0.252	0.193
Days to flowering	0.028	0.023	0.305	0.251
Plant height	0.074	0.056	0.288	0.216
Leaf length	0.017	0.013	0.105	0.081
Leaf-flower distance[c]	0.049	0.041	0.139	0.115
Flower size	0.027	0.023	0.273	0.237

[a] Values are based on least square means from analyses using flower number as a covariate.

[b] Analyses and means based on arcsine–square root transformed data.

[c] Analyses and means based on log-transformed data.

Table 3

Product-Moment Correlations of Family-Level Estimates of Inbreeding Depression among Characters

Variable	1	2	3	4	5	6	7	8
Fitness components:								
1. Flower number	...							
2. Proportion of viable pollen	0.011	...						
Morphology/phenology:								
3. Days to germination	-0.372^{***}	-0.046	...					
4. Days to flowering	-0.424^{***}	-0.059	0.601^{***}	...				
5. Plant height	0.384^{***}	0.063	-0.493^{***}	-0.597^{***}	...			
6. Leaf length	0.356^{***}	0.083	-0.423^{***}	-0.442^{***}	0.527^{***}	...		
7. Leaf-flower distance	0.352^{***}	-0.059	-0.326^{**}	-0.326^{**}	0.350^{***}	0.413^{***}	...	
8. Flower size	0.265^{*}	0.078	-0.556^{***}	-0.453^{***}	0.642^{***}	0.522^{***}	0.305^{**}	...

Note. Analyses based on 89 families.

* $P < 0.05$.

** $P < 0.01$.

*** $P < 0.001$.

male and female fertility (pollen viability, flower number) and caused a shift toward later germination and flowering dates, shorter stems, and reduced flower size. Most of these responses were also observed in a previous comparison of F1 hybrids and inbred F3 genotypes from a cross between *N. degenii* subsp. *barbro* and *N. degenii* subsp. *jenny* Strid, the most notable exception being flowering date, which showed the opposite response in the hybrid population (Andersson 1997). We have no explanation for the latter discrepancy; however, it is possible that the results from the previous study were confounded by factors related to the use of hybrid genotypes (heterosis effects, breakup of coadapted gene complexes, etc.).

Judging from the absolute values of the conventional inbreeding-depression coefficient (δ_{mean}), flower number and pollen viability showed greater responses to selfing (0.075–0.091) than characters related to morphology or to phenology (<0.075). However, the difference in inbreeding depression between the two types of characters was too small to reach significance in a comparison that also included the three qualitative fitness components (germinability, survivorship, anther fertility; $\delta_{mean} < 0.03$). Furthermore, there was no consistent difference in the level of inbreeding depression between the two quantitative fitness components and the remaining group of morphological and phenological characters when all inbreeding responses were expressed in standard deviation units to account for differences in measurement scale (DeRose and Roff 1999; Rao et al. 2002). Thus, our results for *N. degenii* provide no support for the notion that direct components of fitness are more sensitive to inbreeding depression than characters that are less directly related to fitness (Falconer 1989; DeRose and Roff 1999).

Our results for pollen viability—a component of male fertility—extend similar findings of other recent studies. Carr and Dudash (1995) found inbred offspring of *Mimulus guttatus* (Scrophulariaceae) to produce 28%–33% fewer pollen grains than plants derived from outcrossing. In another study of this species, recessive male-sterility alleles were found to account for 26% of the inbreeding depression in lifetime fitness (Willis 1999). In *Cucurbita texana* (Cucurbitaceae), outcrossed individuals not only initiated more male flowers but also produced pollen that sired more seeds than pollen from selfed plants under conditions of pollen competition (Jóhannsson et al. 1998; see also Stephenson et al. 2001). On the basis of these findings, it seems that male fertility could make a significant contribution to lifetime inbreeding depression, stressing the necessity of considering both sex functions before any broad generalizations are made regarding the negative consequences of inbreeding.

Lifetime inbreeding depression represents the multiplicative effects of all variables that influence fitness. In this investigation, it is possible to obtain a rough measure of total inbreeding depression on the basis of the frequency of seeds that germinated, the frequency of plants that survived to flowering, the frequency of flowering plants producing fertile anthers, the proportion of viable pollen, and the total flower production. The resulting estimate, $\delta_{mean} = 0.217$, is lower than the level of inbreeding depression needed to prevent the invasion of a selfing mutant, according to traditional models ($\delta_{mean} > 0.5$; Lloyd 1979; Charlesworth and Charlesworth 1987). However, our estimate is almost certainly lower than the true level of inbreeding depression: all progenies were raised under favorable growth conditions, a factor that has been shown to reduce the detectable effect of inbreeding in other studies (Dudash 1990), and no attempt was made to account for inbreeding responses

Table 4

Character Loadings for the First Two Components in a PCA of Family-Specific Estimates of Response to Inbreeding

	PC1	PC2
Fitness components:		
Flower number	0.599	−0.206
Proportion of viable pollen	0.085	0.915
Morphology/phenology:		
Days to germination	−0.760	−0.038
Days to flowering	−0.776	−0.029
Plant height	0.811	0.077
Leaf length	0.734	0.034
Leaf-flower distance	0.575	−0.364
Flower size	0.761	0.154
Variance explained (%)	45.6	13.1

in morphology and phenology, whose fitness effects may be habitat specific. It would be interesting to obtain data on the relationship between each character and fitness in the natural habitat to determine whether inbreeding depression in morphology and phenology affects the relative fitnesses of selfed and outcrossed progeny and thus contributes to lifetime inbreeding depression in *N. degenii* subsp. *barbro*.

Results of this study confirm previous observations from other plant species (Carr and Dudash 1995; Husband and Schemske 1996; Rao et al. 2002; Andersson and Waldmann 2003) that families within natural populations vary in their response to inbreeding. Such among-family variation can be attributed to differences in the occurrence of newly risen deleterious mutations and/or variation in past inbreeding history (Schultz and Willis 1995) and has been proposed to facilitate the evolution of selfing or the maintenance of a mixed mating system, involving both selfing and outcrossing (Uyenoyama and Waller 1991). In fact, some authors consider genetic variation in inbreeding response to be a more important factor in mating-system evolution than the average inbreeding depression of a population (Holsinger 1988; Schultz and Willis 1995). To evaluate this hypothesis in *N. degenii*, it is necessary to determine whether the heritable differences in inbreeding depression can be expressed in the field with normal levels of environmental variation.

Our family-level analyses also provided evidence for correlated responses to inbreeding for groups of characters: the magnitude of inbreeding depression in total flower number showed a significant relationship with the magnitude of inbreeding depression in each of the morphological and phenological characters, whereas correlated responses involving pollen viability were too weak to reach significance. Taken together, these results suggest that inbreeding depression in *N. degenii* involves loci with both general and specific effects on the phenotype, contrasting with the more strictly character-specific expression of inbreeding depression seen in previous studies (Carr and Dudash 1995; Husband and Schemske 1996; Rao et al. 2002; Andersson and Waldmann 2003). It is interesting that the matrix of correlated response was strongly correlated ($r = 0.90$) with the family-mean correlation matrix for outbred progeny (data not shown), indicating a close similarity between the pleiotropic effects of those alleles that cause inbreeding depression and those that influence the phenotype of noninbred individuals.

Despite evidence for correlated responses involving flower number, there is no reason to invoke differences in general plant vigor as the principal cause of the observed inbreeding responses in morphology and phenology. The estimates of correlated response were moderate ($|r| < 0.65$), and differences in resource status (measured by flower number) contributed little to the treatment effects in the other characters. For example, estimates of inbreeding depression for flower size, adjusted for flower number, were only 13% lower than those based on "nonadjusted" data. On the basis of this observation, we attribute most of the inbreeding depression in flower size to loci expressed during flower development and not solely to loci influencing overall vigor, an interpretation that also applies to other plant systems (Rao et al. 2002; Andersson and Waldmann 2003).

Comparative and genetic studies of the *N. arvensis* complex suggest that differences in flower size evolved in response to drought and/or low pollinator abundance (Strid 1969), either as a result of direct selection for small flower size as plants became less dependent on pollinators or as a correlated response to selection for reduced plant size at sites occupied by the selfing taxa (Andersson 1997). In addition, experimental data from *N. degenii* indicate that the attracting structures (sepals, nectaries) represent an important sink for assimilates, a factor that should enhance the selective advantage of small flowers under conditions in which large, conspicuous flowers are unnecessary (Andersson 2000). When combined with the negative effects of inbreeding on flower size observed in this investigation, available evidence leads to the hypothesis that the direction of selection and the direction of inbreeding response sometimes converge for this character.

Observed inbreeding responses in this and previous studies of *N. degenii* (Andersson 1997) indicate that alleles for small flowers are recessive (Falconer 1989). The increased expression of recessive alleles under inbreeding not only causes an immediate (nonadaptive) decline in flower size as the proportion of recessive homozygotes increases in the population but also enhances the potential for further adaptive reduction in flower size by exposing these alleles to selection (Charlesworth 1992; Andersson 1997). However, the 2%–3% reduction in flower size after one generation selfing is small relative to the more than 50% decline accompanying the evolution of autogamy within the *N. arvensis* complex (Strid 1970). Thus, it is necessary to invoke a continuous supply of (recessive) mutations suppressing flower size—and a long history of inbreeding—to propose floral inbreeding depression as a persistent force in the evolution of small, autogamous flowers (see also Rao et al. 2002). To provide further insights into the role of inbreeding in facilitating floral evolution, it is necessary to know more about mutation rates and newly risen mutations (magnitude of effects, degree of dominance, pleiotropic side effects, etc.) at loci affecting flower development.

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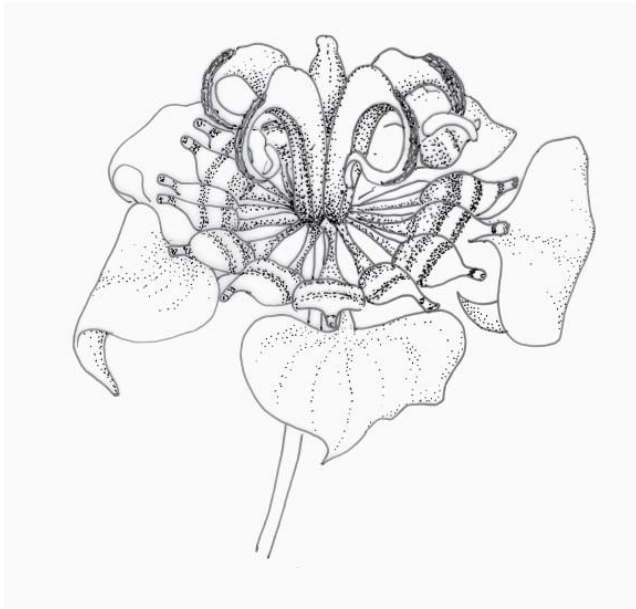

Quantitative genetic effects of bottlenecks: experimental evidence from a wild plant species, *Nigella degenii*

Stefan Andersson, Maarten Ellmer, Tove H. Jorgensen and Anna Palmé

Abstract

Understanding the genetic consequences of changes in population size is fundamental in a variety of contexts, such as adaptation and conservation biology. In the study presented here, we have performed a replicated experiment with the plant *Nigella degenii* to explore the quantitative genetic effects of a single-founder bottleneck. In agreement with theory, the bottleneck caused stochastic, line-specific changes in the **G** matrix. Nevertheless, a significant portion of the (co)variance structure was conserved, and two characters — leaf and flower (sepal) size — turned out to be positively correlated in all data sets, indicating a potential for correlated evolution in these characters, even after a severe bottleneck. The hierarchical partitioning of genetic variance for flower size was in good agreement with predictions from additive theory, whereas the remaining characters showed an excess of within-line variance and a deficiency of among-line variance. The latter discrepancies were most likely a result of selection, given the small fraction of lines (23%) that remained viable until the end of the experiment. Our results highlight the idiosyncratic nature of bottleneck effects but give little support for rejecting the view that bottlenecked populations usually have a lower adaptive potential than the ancestral population.

Introduction

Many populations have undergone severe, temporary reductions in size as a consequence of habitat loss, domestication, environmental catastrophes or founder events (Carson and Templeton 1984; Hewitt 1999; Friar *et al*. 2000; Lee 2002; Briggs and Goldman 2006). Such "population bottlenecks" can have pronounced effects on the genetic constitution of populations and lead to immediate loss of genetic variation (e.g., Nei *et al*. 1975). Yet, despite evidence for bottleneck-induced losses of marker gene diversity (Leberg 1992; Friar *et al*. 2000) and short-term population viability (Newman and Pilson 1997; Saccheri *et al*. 1998), it is still unclear how reductions in population size alter an organisms evolutionary potential to adapt to novel ecological conditions, as determined by the genetic variances and covariances for suites of phenotypic characters (Willi *et al*. 2006).

Stochastic processes such as genetic drift are expected to convert genetic (co)variance within populations into genetic differences between populations. Under a strictly additive model of gene action, the within-population (co)variance after a bottleneck is expected to be 1-F times the genetic (co)variance in the base population, where F is the inbreeding generated during the bottleneck (Wright 1951). This quantity, and the corresponding prediction for the between-population (co)variance (2F times the original (co)variance; Wright 1951), serve as natural baselines against which to compare the observed within- and between-line (co)variance following a bottleneck.

When there are high levels of nonadditive genetic (co)variance in a character, the additive (co)variances behave differently and can even increase, as observed in some bottleneck experiments (e.g., Bryant *et al*. 1986; López-Fanjul and Villaverde 1989; Fernández *et al*. 1995; Cheverud *et al*. 1999; Saccheri *et al*. 2001; Briggs and Goldman 2006; van Heerwaarden *et al*. 2008). Such effects may be attributable to chance increases in the frequencies of recessive or partially recessive deleterious alleles (Robertson 1952; Willis and Orr 1993; Wang *et al*. 1998), or to the release of additive (co)variance as the number of polymorphic loci — and possible interlocus interactions — declines during the bottleneck (Goodnight 1988; Barton and Turelli 2004; López-Fanjul *et al*. 2004). In this context, it must be emphasized that drift can cause considerable random variation around the additive expectations, so that some populations might experience an increase in the additive (co)variance, even if on average the genetic (co)variance decreases (Avery and Hill 1977; Lynch 1988; Zeng and Cockerham 1991). As yet, only a few experiments have been

carried out on a sufficient scale to account for the variability in quantitative genetic parameters among replicate lines derived from the same base population (Cheverud *et al*. 1999; Whitlock and Fowler 1999; Phillips *et al*. 2001; Saccheri *et al*. 2001; Swindell and Bouzat 2005; van Heerwaarden *et al*. 2008).

Although the additive (co)variances in finite populations decline to zero, it probably takes many generations before stochastic processes will cause a reduction in the entire genetic (co)variance (**G**) matrix, especially if there is sufficient independent genetic control of different characters for genetic drift to operate independently on them. Thus, a sudden bottleneck is expected to cause idiosyncratic, element-specific changes in the **G** matrix — and a resultant change in principal component structure — rather than a proportional reduction in all (co)variances (Phillips and Arnold 1999; Phillips *et al*. 2001; Jones *et al*. 2003). Given the strong influence of the **G** matrix on the short-term trajectory of evolution by natural selection (Lande 1979), bottlenecks therefore have the potential to alter the persistence of genetic constraints and the evolutionary potential of natural populations (Wright 1978; Carson and Templeton 1984; Whitlock 1995; Lee 2002).

Populations in the *Nigella arvensis* complex (Ranunculaceae) — a group of six diploid (2n = 12) annual plant species with allopatric distributions in Greece and western Turkey — have diverged for a number of phenotypic characters, especially in the central Aegean (Cyclades). A substantial portion of the large-scale pattern seems to be of an adaptive nature, with taxa occupying the most arid islands having earlier flowering dates, shorter stems, and fewer, smaller and more autogamous flowers than those on more mesic islands (Strid 1969, 1970). In regards to within-species variation, the available data for certain species, e.g. *N. degenii* Vierh., indicate a mosaic of variation unrelated to local habitat conditions (Strid 1970). This and other considerations, for example the disturbed nature of typical *Nigella* habitats and the capacity for some selfing even in the insect-pollinated species, led Strid (1970) to invoke random genetic drift due to bottlenecks as a major evolutionary force in this species complex. Indirect support for this scenario is provided by surveys of putatively neutral molecular markers in Aegean *Nigella* (Bittkau and Comes 2005; Comes *et al*. 2008) and the small difference in estimates of population divergence between phenotypic characters (Q_{ST}) and AFLP markers (F_{ST}) within two subspecies of *N. degenii* (Jorgensen *et al*. 2006).

In the present study on *N. degenii*, we performed a replicated experiment to explore the quantitative genetic effects of a single-founder bottleneck, with

special emphasis on morphological and phenological characters that have diverged within the *N. arvensis* complex. As well as comparing **G** matrices, we contrasted the within- and among-line (co)variances with their additive expectations. Specifically, we asked: Is the bottleneck effect sufficiently strong to alter the persistence of genetic constraints and the adaptive potential of *N. degenii* populations? And, does the quantitative genetic partitioning of (co)variance following the bottleneck conform to additive theory?

Material and methods

Plant material

Nigella degenii occurs in dry, disturbed habitats (mainly in phrygana communities and abandoned fields) on the Cyclades (Greece), where four subspecies have been recognized (Strid 1970). The plants are erect to ascending, with a branched stem, pinnately dissected leaves and 15–25 mm wide, bisexual flowers visited by bees. Each flower has a double perianth with an outer whorl of five whitish, petaloid sepals and an inner whorl of eight stalked, bilabiate nectaries. Protandry, coupled with spatial separation of anthers and stigmas, enhances outcrossing, although the receptive styles sometimes become twisted around the dehiscing anthers, which results in some self-fertilization. Fertilized flowers develop into capsules with up to 100 seeds that lack any special dispersal mechanism. Seed viability declines significantly after 2–3 years (Strid 1969, 1970).

The plants used in this investigation originate from seeds representing 80 maternal plants, scattered throughout a large population ca. 2.5 km NNW of the town on the island of Mikonos. Plants from this site belong to *N. degenii* Vier. ssp. *barbro* Strid, which is endemic to Mikonos and a few neighboring islands (Strid 1970). All crosses and cultivations were carried out under pollinator-free conditions in a greenhouse at the University of Lund, Sweden.

Experimental procedures

Before initiating the bottleneck experiment, we used four generations of random outcrossing, involving a minimum of 150 plants per generation, to establish an outbred base population in (near) linkage equilibrium. In 2000, we sowed ca. 150 seeds from the base population in separate pots, filled with peat soil and placed in a random pattern on three adjacent greenhouse benches. When the majority of the plants had reached anthesis, we subjected a number

of flowers to one of two treatments: (i) self-pollination (ca. 10 flowers per plant) or, (ii) emasculation followed by cross-pollination with pollen from a randomly chosen plant in the same population (one or two flowers per plant). We saved five selfed and five outcrossed seeds from each maternal plant for a separate analysis of inbreeding depression (Ellmer and Andersson 2004) and used the remaining seeds for the bottleneck experiment.

Our experiment simulates a brief bottleneck involving a single founder that set seed by autonomous or insect-mediated selfing, followed by an expansion of the population. Given the potentially low fitness after bottlenecks (Newman and Pilson 1997; Saccheri *et al*. 1998), we initiated as many as 100 bottleneck lines (each founded from a separate individual) to account for possible loss of lines during the expansion phase. After the founder (selfing) event, we expanded the bottleneck lines using three generations of random outcrossing within lines (referred to as G_1–G_3). Each random-mating generation was initiated by sowing up to 45 (G_1) or 108 (G_2–G_3) seeds per line into 25-cm^2 cells (one seed per cell) in one or two plastic trays per line, placed in a random pattern in one or two adjacent greenhouse chambers. Water was supplied as needed, but no extra fertilizer was applied. Once plants began to flower, we assigned half the plants in each line as "males" and the remaining plants as "females", and mated each male to a distinct female within the same line (one flower per cross). In a few cases, it was necessary to use the same plant as a male in one cross and as a female in another cross. Selfing and between-line pollinations were minimized by covering all females with fine-mesh nets before flowering and emasculating all recipient flowers before outcrossing. After flowering, we recorded the number of successful cross-pollinations (fruit set) for each line and mixed an equal number of seeds from every successful cross to form a bulk sample for the next random-mating generation.

Given the use of a single founder for each bottleneck line and the inferred lack of inbreeding in the base population, the coefficient of inbreeding (F) was assumed to be 0.5 immediately after the founder event (Falconer and Mackay 1996). To prevent further inbreeding during the expansion phase we discarded lines with the lowest survival rate (< 50%) and/or the lowest fruit set (< 20 successful crosses) in each random-mating generation. Based on the number of parents involved in successful crosses the final F value of the remaining lines, estimated by adding the new inbreeding in each generation to the F value of the previous generation (Falconer and Mackay 1996), was slightly higher (mean 0.535, range 0.526–0.545) than the inbreeding attributed to the initial founder event.

The establishment of the control population was based on bulked seed samples from the original founders. To detect confounding effects of selection during the expansion phase, we established two control lines, one representing all 100 founders (C_{total}) and the other representing "successful" founders, i.e. founders of bottleneck lines that remained viable two generations after the founder event (C_{subset}). Each founder contributed 10 to 15 outcrossed seeds to a given seed sample. Any difference between the two control lines would indicate that the surviving lines represented a nonrandom (selected) subset of the base population, a potential source of error when inferring the quantitative genetic effects of small population size (Lynch 1988).

To minimize bias arising from differences in growth conditions and mating patterns we subjected the control lines to one additional generation of within-line outcrossing, using the same cultivation and pollination designs as were used for the bottleneck group. The within-line outcrosses involved ca. 250 plants per control line, planted at the same time and in the same greenhouse chamber as the G_3 plants in the bottleneck group.

In 2003, we established a large number of fullsib progenies from the last outcrossing generation (G_3) to obtain phenotypic data for the quantitative genetic analyses. Seeds for this G_4 generation were derived from 110 families in the C_{total} line, 89 families in the C_{subset} line, and 19–21 families per bottleneck line. These were planted individually into 25-cm^2 cells in a series of plastic trays distributed across five adjacent benches in the same greenhouse chamber. Each family contributed two seeds to each bench (a total of 10 seeds per family), randomized across the whole planting area. The resulting plants were given supplementary light (12 h/day) and watered two or three times a week depending on weather conditions.

Measurements

We recorded whether or not a plant had died before flowering (survival status) and scored each flowering plant for five quantitative characters: first flowering date, flower number, plant height, leaf length and sepal length. Data on leaf and sepal length were obtained by preserving the first flowering (terminal) flower and the uppermost leaf on the main stem in a microcentrifuge tube filled with 60% ethanol and then measuring each variable under a stereomicroscope. Sepal length is strongly positively correlated with the length of the other flower parts (Andersson 1997) and therefore provides a general measure of flower size. The quantitative characters exhibit both additive and nonadditive genetic variance within *N. degenii*, as evidenced by parent-

offspring comparisons (Andersson 1997) and data on inbreeding depression (Ellmer and Andersson 2004). They have also been found to define a major axis of differentiation in the *N. arvensis* complex, distinguishing early-flowering taxa with short, few-flowered stems, short leaves and small (selfing) flowers from those with the opposite features (Strid 1969, 1970). Ecological data strongly imply that the optimum phenotype differs between taxa, with more arid sites selecting for small-sized plants and more mesic sites favouring large-sized individuals (Strid 1969).

Phenotypic data were obtained for a maximum of 1837 plants in the control lines and 4542 plants in the 23 bottleneck lines that survived until the end of the experiment (mean 197.5 plants per line).

Initial analyses

The survival data were pooled across families and analyzed with chi-square procedures to test for differences between control lines (C_{total} vs. C_{subset}) and between different lines in the bottlenecked population. The quantitative data were subjected to one-way analyses of variance (ANOVA, type III sums of squares) using "bench" as a categorical variable, to provide residuals adjusted for spatial variation in the greenhouse chamber. Preliminary analyses of these data revealed approximately normal distributions; consequently, we used block-adjusted residuals in all analyses. Differences between the two control lines were tested for significance by univariate and multivariate ANOVAs with line as a fixed factor and family (nested within line) as a random factor. Data for the bottleneck group were subjected to random-effects ANOVAs with line and family (nested within line) as group variables, but also collapsed into line means to provide descriptive statistics on the among-line variation.

Matrix analyses

To assess how the **G** matrix responded to the bottleneck event, we estimated covariance component matrices (based on one-way analyses of covariance among fullsib families) and used the common-principal-components (CPC) technique (Flury 1988; Phillips and Arnold 1999) to evaluate the type of differences between matrices.

As well as estimating the **G** matrix of the control population and each bottleneck line, we calculated the mean **G** matrix following the bottleneck, based on data pooled across lines. To reduce bias arising from differences in the mean phenotype, we normalized the data by subtracting the line mean from the observed value of each individual (Whitlock and Fowler 1999). These

analyses were performed using the program H2boot (Phillips 1998a), which uses a bootstrapping approach to estimate each parameter in the **G** matrix (5000 resamples).

The CPC method allows the (co)variance structure of two or more populations to be compared in a hierarchical fashion, starting from unrelated structure and progressing upwards through partial common principal components (PCPC), common principal components (CPC), proportionality and equality (Flury 1988). Simultaneous analyses of all lines were not computationally feasible; instead, we contrasted the control **G** matrix with the **G** matrix of each bottleneck line and the mean **G** across all bottleneck lines. We used the jump-up approach of Phillips and Arnold (1999) to determine the highest point in the hierarchy at which accumulated differences in the matrices became statistically significant ($P < 0.05$) and considered the model immediately below as the best-fitting model for the observed differences. These analyses were carried out with the program CPCrand (Phillips 1998b), which uses a resampling approach to test the Flury hierarchy (5000 resamples).

Differences in principal components (PC) associated with large eigenvalues often cause the CPC technique to underestimate the degree of shared structure lower in the Flury hierarchy (Houle *et al*. 2002). To address this problem, we explored the consequence of switching the order of major and minor components in the partial models. Based on initial analyses of eigenvalues obtained from the CPCrand output, we considered PC1 and PC2 as ”major” (mean eigenvalue = 347.7) and the remaining ones as ”minor” (mean eigenvalue = 3.4). The major PCs represented variation in plant height and flowering date, whereas the minor PCs had high loadings of the remaining characters (data not shown). As each group of major or minor components involved more than one PC, we repeated the analyses for all possible permutations of PCs within each category and recorded the greatest similarity, i.e. the highest best-fitting model, observed for each matrix comparison.

In most cases, it was necessary to employ the bending option in CPCrand to eliminate negative eigenvalues, i.e. to make the matrices positive definite. Although the validity of this approach remains uncertain (Phillips and Arnold 1999), we found no relation between the number of components shared between **G** matrices and the amount of bending required (Pearson $r = 0.02$, $P > 0.05$). Thus, we assume little or no consistent bias as a result of the bending procedure.

Comparison with additive predictions

As a final step, we contrasted the observed within- and among-line genetic (co)variance (V_g, V_{line}) after the bottleneck to the additive predictions for these parameters, estimated as $E(V_g) = (1\text{-}F)V_{g0}$ and $E(V_{line}) = 2FV_{g0}$, respectively, where F is the final inbreeding coefficient (F = 0.535) and V_{g0} is the genetic (co)variance in the control (base) population (Wright 1951). Following the analyses of each (co)variance in the mean **G** matrix, we extended the analyses to the hierarchical partitioning of variance, as determined by both the within- and among-line genetic variance. The latter analyses were based on variance estimates obtained with restricted maximum likelihood (REML) procedures (Lynch and Walsh 1998), because of the nested experimental design. To assess the significance of differences between observed and predicted values, we computed the approximate 95% confidence interval (CI) of each estimate and its expected value based on the sampling variance obtained for each parameter.

All chi-square tests, ANOVAs and REML analyses were carried out with SPSS for Windows (release 11.0.0).

Results

Patterns of (co)variation before the bottleneck

Plants in the C_{total} and C_{subset} lines had statistically indistinguishable survival rates ($\chi^2 = 0.74$, d.f. = 1, P = 0.39), means (ANOVA: $F < 2.7$, d.f. = 1, 208-228, $P > 0.10$; MANOVA: F = 0.62, d.f. = 5, 193, P = 0.68) and **G** matrices ($P_{EQUALITY} = 0.60$; CPC analysis), despite large sample sizes for both data sets (> 790 individuals). For this reason we pooled data over control lines where appropriate, to provide a single data set against which to compare the lines in the bottleneck category.

Estimates of the genetic variance (V_g) for the (pooled) control line were significantly greater than zero in all cases (Table 1). When scaled by the total phenotypic variance, the genetic variances were higher for first flowering date and sepal length ($H^2 = 0.52$–0.56) than for flower number (0.17), with plant height and leaf length being intermediate (0.31–0.34). The **G** matrix for the control population revealed a major trend distinguishing early-flowering genotypes with many flowers, a tall stem and long leaves, from those with the opposite features, and significantly positive associations among some of the size variables (flower number vs. plant height, leaf vs. sepal length, Table 1).

Table 1. Means, broad-sense heritabilities (H^2) and the **G** matrix for the control population

			G matrix				
Character	Mean	H^2	1	2	3	4	5
1. Flowering date (May)	19.03	0.56*	29.52*				
2. Flower number	6.65	0.17*	-5.52*	1.23*			
3. Plant height (mm)	242.98	0.31*	-102.94*	15.64*	965.82*		
4. Leaf length (mm)	15.03	0.34*	-2.30*	0.06	9.06	1.93*	
5. Sepal length (mm)	11.84	0.52*	-0.31	0.02	2.22	0.55*	0.46*

Values followed by an asterisk (*) are significantly different from zero ($P < 0.05$) as determined by 95% CIs. Estimates of genetic parameters were obtained with the program H2boot (PHILLIPS 1998a).

Table 2. Means, ranges and line-mean correlations for the bottlenecked *N. degenii* population

			Line-mean correlations[a]				
Character	Mean	Range	1	2	3	4	5
1. Flowering date (May)	17.90	9.86-24.11	.				
2. Flower number	6.65	5.13-7.99	-0.75***	.			
3. Plant height (mm)	250.96	202.44-315.28	-0.30	0.33	.		
4. Leaf length (mm)	15.24	12.24-17.53	-0.11	-0.09	-0.02	.	
5. Sepal length (mm)	11.77	9.74-13.10	0.12	-0.13	0.28	0.66***	.

All statistics are based on line-means. *** $P < 0.001$

[a] Entries are Pearson product-moment correlation coefficients.

Means and survival rates after the bottleneck

More than three quarters of the bottleneck lines went extinct during the expansion phase, leaving a total of 23 lines that survived until the end of the experiment and provided data for the quantitative genetic analyses. Most of the losses were caused by low survival rates (40 lines) or low fruit set (16 lines) in the G_1 generation, the remainder reflecting low survival rates (3 lines) or low fruit set (18 lines) in the G_2 generation. No further losses occurred in the G_3 generation.

The surviving lines varied greatly in the fraction of plants that survived to flowering in the G_4 generation (range 63.6–98.2%; $\chi^2 = 243.7$, d.f. = 22, $P < 0.001$). Sixteen lines had survival rates greater than 90%, resulting in an across-line mean (89.9%) similar to the survival rate of the control population (93.7%). For the five quantitative characters, there was extensive among-line variation in the overall mean ($F > 9.2$, d.f. = 22, 483-490, $P < 0.001$ in all cases; nested ANOVAs), with across-line means close to the pooled control population (Tables 1–2). Correlation analyses on the line means revealed a significantly negative association between first flowering date and flower number and a significantly positive association between leaf length and sepal length (Table 2).

*Bottleneck-induced changes in the **G** matrix*

We found three major patterns in the line-specific **G** matrices (Supplementary material) and the mean **G** following the bottleneck (Table 3): (i) negative covariances generally involved flowering date, (ii) flower number, plant height and the lengths of the leaves and sepals showed positive covariance in almost all cases, and (iii) different bottleneck lines had high or low (co)variance estimates for different characters.

There was no support for the hypothesis of equal or proportional **G** matrices in the CPC analyses that compared each bottleneck line with the control group (Table 4). When the partial models were tested with PCs ordered according to the size of their eigenvalues (PC_{size}), a majority of the analyses also rejected the hypothesis of shared structure ($P_{CPC1} < 0.05$). Switching the order of major and minor components ($PC_{reordered}$) increased the level of similarity between matrices: the best-fitting model generally changed

Table 3. The mean **G** matrix (upper values) and its additive prediction (lower values) for the bottlenecked *N. degenii* population

Character	1	2	3	4	5
1. Flowering date (May)	19.62*	.			
	13.73				
2. Flower number	-3.56*	0.84*	.		
	-2.57	*0.57*			
3. Plant height (mm)	-59.62	6.65	602.76*	.	
	-47.87	*7.27*	*449.11*		
4. Leaf length (mm)	-3.18*	0.57*	5.09	1.38*	.
	-1.07	*0.03*	*4.21*	*0.90*	
5. Sepal length (mm)	-0.41	0.08	-3.30	0.26	0.18*
	-0.15	*0.01*	*1.03*	*0.25*	*0.22*

Values followed by an asterisk (*) are significantly different from zero ($P < 0.05$) as determined by 95% CIs. Comparison of CIs (not shown) revealed no significant difference between observed and predicted values. Estimates of genetic parameters were obtained with the program H2boot (PHILLIPS 1998a).

Table 4. The number of comparisons between the **G** matrix of the control population and the **G** matrices of the 23 surviving bottleneck lines that fit different models in the Flury hierarchy. PC_{size} and $PC_{reordered}$ denote whether the partial models were based on size-ordered or reordered components, respectively (for details, see text)

	Number of pair-wise comparisons	
Model	PC_{size}	$PC_{reordered}$
Equality	0	0
Proportionality	0	0
CPC	0	0
CPC3	0	0
CPC2	1	8
CPC1	2	13
Unequal	20	2

CPC indicates a full model with four common components, whereas CPC1, CPC2, etc. indicate partial models involving one, two or more common components.

from no shared structure to a model involving a maximum of one or two common components. In two comparisons, the **G** matrices remained too different to support any of the models in the Flury hierarchy, regardless of how the PCs were ordered in the partial models (Table 4).

Contrary to the large changes observed in the pair-wise CPC analyses, the bottleneck did not significantly influence the mean **G** matrix ($P_{EQUALITY} = 0.36$; CPC analysis based on data pooled over lines), despite large sample sizes for both the control line (1837 plants in 199 families) and the bottlenecked population (4542 plants in 500 families).

Observed vs. predicted (co)variances

Judging from the mean **G** matrix following the bottleneck (Table 3), the magnitude of the (co)variance almost always exceeded the additive expectation,

Table 5. Comparison of REML-based estimates of the within- and among-line genetic variance (V_g, V_{line}) with their additive predictions ($E(V_g)$, $E(V_{line})$) for the bottlenecked *N. degenii* population. CI denotes confidence interval

	Genetic variance within lines				Genetic variance among lines			
Character	V_g	CI	$E(V_g)$	CI	V_{line}	CI	$E(V_{line})$	CI
Flowering date	21.36	17.69, 25.03	14.39	10.75, 18.02	14.36	5.48, 23.24	33.11	24.75, 41.47
Flower number	0.93	0.65, 1.22	0.57	0.31, 0.82	0.43	0.14, 0.71	1.30	0.72, 1.88
Plant height (mm)	653.99	498.41, 809.57	435.15	295.30, 575.01	707.19	272.69, 1141.69	1001.3	679.5, 1323.1
Leaf length (mm)	1.49	1.16, 1.82	0.91	0.63, 1.19	1.51	0.58, 2.44	2.09	1.46, 2.73
Sepal length (mm)	0.20	0.15, 0.24	0.22	0.16, 0.28	0.53	0.21, 0.85	0.50	0.37, 0.63

the only exception being the variance for sepal length, which showed a slight deviation in the opposite direction. None of these differences reached significance (overlapping CI in all cases).

According to the REML-based variance estimates, the within- and among-line genetic variances (V_g, V_{line}) for sepal length were in good agreement with the values predicted under additive theory (Table 5). Although within-line variances for the other characters were lower than the corresponding estimates for the control group (Table 1), they always exceeded the expected values, albeit with overlapping CIs in all cases (Table 5). These characters also showed a deficiency of among-line genetic variance, with CIs excluding the CIs of the additive expectations for two V_{line} estimates (flowering date, flower number) (Table 5).

Discussion

Although much attention has focused on the negative effects of bottlenecks on marker gene diversity and short-term population viability (e.g., Nei *et al*. 1975, Leberg 1992; Newman and Pilson 1997; Saccheri *et al*. 1998; Friar *et al*. 2000), there is still a paucity of experiments in which investigators have manipulated population size to determine the quantitative genetic effects of bottlenecks and so far few studies have used a wild plant species as their model system. Our results for the annual plant *Nigella degenii* highlight the idiosyncratic nature of bottleneck effects but provide little support for rejecting the common notion that bottlenecked populations generally have a lower adaptive potential than the ancestral population, given the small fraction of lines that remained viable after the founder event.

Changes in (co)variance structure

In agreement with population genetic theory of small, isolated populations (Avery and Hill 1977; Lynch 1988; Zeng and Cockerham 1991), we observed considerable variation in the response to the bottleneck, with different lines showing high, or low, genetic (co)variance for different characters. Although this heterogeneity could be a reflection of the large error associated with the estimation of quantitative genetic parameters (Lynch and Walsh 1998), we emphasize that the pair-wise CPC analyses — which compared the **G** matrix for each bottleneck line with the **G** matrix for the control (base) population —

always rejected the hypotheses of equality and proportionality, a result consistent with the generation of wide drift-induced variation in the orientation and magnitude of genetic variance and covariance (Phillips *et al*. 2001; Widén *et al*. 2002).

Differences in one or a few principal components often prevent detection of shared structure lower in the Flury hierarchy (Houle *et al*. 2002). In the case of *N. degenii*, we found greater similarity between the **G** matrices of the control and bottleneck lines after switching the order of major and minor components in the partial models. In fact, the proportion of pair-wise comparisons with similar components (ca. 90%) was somewhat higher than normally found in comparisons of natural or experimental populations of the same species (< 80%; Arnold *et al*. 2008). This pattern indicates (i) that differences in the (co)variances for plant height and flowering time — the main determinants of the major components — had a disproportionately large influence on differences in the principal component structure, and (ii) that the bottlenecked **G** matrices retained a nonnegligible portion of their (co)variance structure. Interestingly, there was no significant difference in **G** between the control line and the pooled bottleneck population, presumably because the line-specific responses cancelled each other out in the pooled data set.

As expected from the presence of shared components, we observed a few consistent associations in the **G** matrices. Apart from the negative associations between flowering time and vegetative size characters, there was a consistent genetic correlation between leaf and sepal length in the control population and within and among different lines in the bottleneck group. This leaf-sepal size association has also been detected in a segregating hybrid population from a cross between our base population (*N. degenii* ssp. *barbro*) and a population of *N. degenii* ssp. *jenny*, and in a comparison of different taxa in the *N. arvensis* complex (Strid 1969, 1970; Andersson 1997). Moreover, family-level analyses of inbreeding depression have revealed highly significant correlated responses involving both leaf and sepal size: families that show high inbreeding depression in leaf length also show high inbreeding depression in sepal length (Ellmer and Andersson 2004). Taken together, these findings indicate that some genes control the development of both leaves and flowers (Andersson 1997) and that it may be difficult for *Nigella* populations to escape this constraint, even after a severe bottleneck.

Our data provide no direct evidence as to the relative importance of different random factors that could contribute to the large, line-specific changes observed in this bottleneck experiment (linkage disequilibrium, simple

fluctuations in allele frequencies, sampling variability, etc). However, it seems that the initial genetic variance among the founders could account for a substantial portion of the variation. The coefficient of variation (CV) in the within-line genetic variance caused by this factor alone (estimated as $[2/N_eL]^{1/2}$, where N_e is the effective population size and L is the number of lines, Lynch 1988) is expected to be 0.29 for this experiment. This quantity represents a sizeable proportion (31-58%) of the observed CV for the five characters measured in this study (CV = 0.50-0.93, calculated from data in supplementary material).

Comparison with additive predictions

The results from the matrix analyses and the hierarchical partitioning of genetic variance generally conformed to the predictions from additive theory: the bottleneck reduced the within-line genetic (co)variance and had a diverging effect on the mean phenotype for all the characters. In the case of sepal length, the partitioning of (co)variance following the bottleneck event was in good quantitative agreement with the additive predictions for this experiment. The remaining characters showed a deficiency of among-line (co)variance and an excess of within-line (co)variance when compared with the predicted values. These patterns imply that "too little" within-line (co)variance was converted into among-line (co)variance for some variables, presumably contributing to the close similarity between the control **G** matrix and the mean **G** matrix of the bottlenecked population.

Comparisons of observed and predicted (co)variances must be interpreted with care when many lines go extinct before the measurements (Lynch 1988) as was the case in the present investigation. For example, the deviating variance estimates for characters other than sepal length could be a manifestation of environmentally-induced selection against lines with extreme means or unusually low genetic (co)variance for these characters. This possibility was evaluated by comparing two control lines, one representing all the initial founders and the other representing founders of lines that remained viable until the end of the experiment. Neither the means nor the **G** matrices significantly differed between the two control lines, as would be expected if the surviving lines represented genotypes better able to survive and reproduce in the greenhouse environment. Nevertheless, we note that the loss of lines followed a temporally decreasing trend during the expansion phase, with 56 lost lines in the first generation, 21 in the second and none in the third. Such patterns are consistent with the selective removal, or "purging", of lines that

suffer from severe inbreeding depression (Lynch 1988) and have the potential to attenuate the quantitative genetic effects of bottlenecks if the characters considered are genetically correlated with fitness.

Quantitative genetic data indicate that both additive and nonadditive genetic effects were segregating in the base population. As expected with a strong additive component of variance, there were no consistent differences between the broad-sense heritabilities for plant stature, flowering time, leaf length and sepal length in the control population (H^2 = 0.31–0.56) and previously estimated narrow-sense heritabilities from a factorial crossing experiment with the same base population (h^2 = 0.27–0.64, A. Palmé and S. Andersson, unpubl. data). As for the nonadditive component, the base population contained sufficient dominance variance for inbreeding to cause significant inbreeding depression in almost all the characters considered in this study (Ellmer and Andersson 2004). However, given the low estimates of inbreeding depression for these characters (< 2% decrease in the mean phenotype per 10% increase in F, Ellmer and Andersson 2004), it seems reasonable to assume that the genetic (co)variances in the base population were mainly due to segregation at nearly additive loci. Thus, there is no reason to invoke bottleneck-induced conversion of nonadditive (co)variance into additive (co)variance (Robertson 1952; Goodnight 1988) to explain why there was an excess of within-line (co)variance for a majority of the characters after the bottleneck.

Although each parent contributed a similar number of seeds to the next random-mating generation, it is conceivable that the final inbreeding coefficient — estimated from the number of parents involved in successful crosses — was underestimated. Many plants failed to produce seeds after outcrossing and there is no guarantee that these losses were randomly distributed across families in the progeny generation. Therefore, the effective number of parents contributing to the next generation was probably lower than the number of parents contributing to the seed samples. The "extra" inbreeding resulting from these differences would reduce the expected within-line genetic variance and increase the expected among-line variance, leading to even larger differences between observed and expected values in the comparative analyses.

The adaptive potential of bottlenecked populations

Our results for *N. degenii* provide no support for rejecting the conventional view (Willis and Orr 1993; Barton and Turelli 2004; Willi *et al*. 2006) that

bottlenecks generally have negative effects on future evolutionary adaptation: most lines were lost during the first or second generation after the founding event. On the other hand, we also note the relatively high survival rate and extensive between-line heterogeneity in the (co)variance structure for those lines that remained viable. Thus, it is premature to rule out the possibility that extreme bottlenecks have the potential to enhance the evolutionary lability of particular populations (e.g., Wright 1978; Carson and Templeton 1984; Cohan 1984; Whitlock 1995). Obviously, the relevance of this idea depends on the strengths and directions of selection in the natural habitat (Lande 1979) and whether the perturbed (co)variance structure could persist into future generations, as found in a large bottleneck experiment with fruit fly *Drosophila melanogaster* (Whitlock *et al*. 2002), or conversely, whether mutation, selection and recombination would return the **G** matrices to their original state before the bottleneck.

Previous studies have shown conflicting results regarding the evolutionary potential of bottlenecked populations. A number of authors have reported bottleneck-induced release of additive variance for a broad variety of characters, including morphology in housefly *Musca domestica* (Bryant *et al*. 1986), viability in fruit fly (López-Fanjul and Villaverde 1989) and flour beetle *Tribolium castaneum* (Fernández *et al*. 1995), desiccation resistance in the fly *Drosophila bunnanda* (van Heerwaarden *et al*. 2008), egg hatching rate in the butterfly *Bicyclus anynana* (Saccheri *et al*. 2001), body weight in mouse *Mus musculus* (Cheverud *et al*. 1999) and cotyledon size in a rapid-cycling population of *Brassica rapa* (Briggs and Goldman 2006). For other study systems, the change in genetic architecture was in good agreement with neutral additive theory (e.g., Wade *et al*. 1996; Whitlock and Fowler 1999; Saccheri *et al*. 2001; Swindell and Bouzat 2005). Based on these findings and the results of this study, more large-scale experiments — accounting for among-line variability in both genetic and demographic parameters — will be needed before any broad generalizations are made regarding the evolutionary potential of bottlenecked populations (Lynch 1988).

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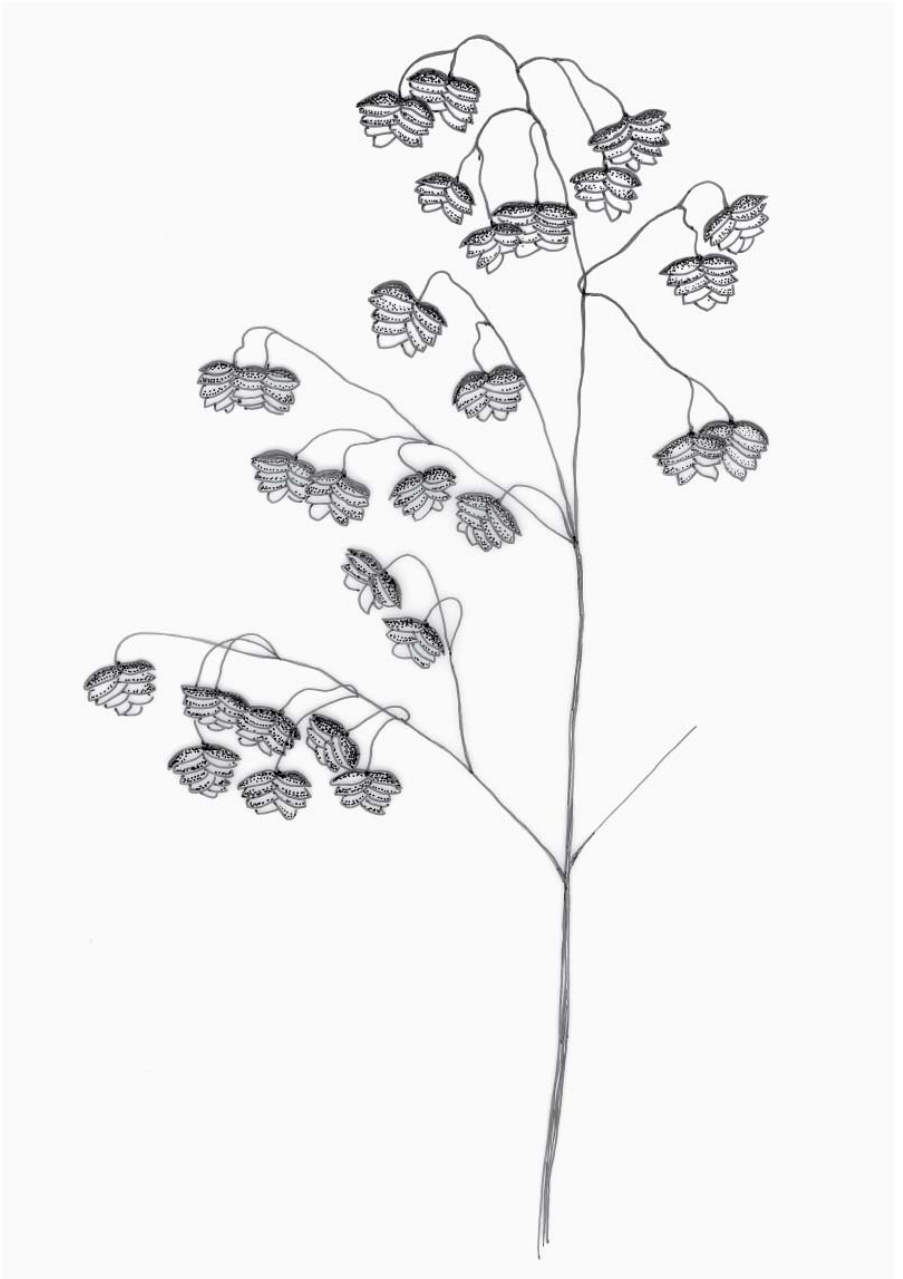
Supplementary material. Genetic variances and covariances for each bottleneck line.

	Character or character combination														
Line	FD	FD-FN	FD-PH	FD-LL	FD-SL	FN	FN-PH	FN-LL	FN-SL	PH	PH-LL	PH-SL	LL	LL-SL	SL
1	16.24	-0.49	-116.7	-2.04	-1.483	0.345	34.05	0.690	0.271	1740*	12.74	-2.7	0.726*	0.227	0.160*
2	31.80*	-6.03	-79.0	-6.82	-1.565	1.165	1.37	1.322	0.351	94	9.21	7.0	2.400*	0.817	0.349*
3	33.31*	-4.24	-160.6	-3.30	-1.145	0.956*	16.01	-0.031	0.133	1021*	13.77	-4.9	1.629*	0.192	0.163
4	15.12*	-5.18	-169.8	-0.86	0.541	1.635*	34.12	0.521	-0.167	1167*	-81.15	-82.6	0.722	0.185	0.070
5	27.35*	-4.12	-62.2	-3.28	-0.402	0.323	-7.42	0.661	0.414	62	-34.06	-4.0	0.731	-0.129	0.142*
6	25.54*	-4.74	-59.2	-3.59	-1.680	0.154	13.91	0.464	0.300	252	66.21	6.8	0.940*	0.241	0.343*
7	9.61*	-2.83	-26.2	-3.32	-1.561	0.917	24.91	0.681	0.445	753*	28.31	14.8	1.710*	0.945	0.325*
8	22.89*	-2.11	-30.6	-3.75	-0.379	-0.057	6.43	0.134	-0.024	38	-1.35	-0.7	0.768	0.280	0.090
9	14.90*	-2.31	-63.8	-2.41	-0.721	1.366*	28.60	-0.388	0.119	1055*	29.41	27.5	2.063*	0.335	0.165*
10	9.43*	-0.73	-3.7	0.26	0.445	-0.084	-4.60	-0.267	0.180	572*	-1.38	-1.4	0.251	0.245	0.066*
11	9.73*	-1.69	8.9	-1.57	-0.158	0.910*	8.01	-0.186	-0.111	136	-3.51	4.4	0.342	0.101	0.050
12	32.55*	-5.44	-133.8	-4.77	-0.991	1.042*	13.38	1.063	0.145	880*	-85.20	-101.7	1.702*	0.283	0.090*
13	15.21*	-2.01	44.7	-2.58	0.108	0.567	-10.45	0.245	-0.149	244	14.21	12.7	0.736	0.296	0.255*
14	20.03*	-6.16	-97.4	-5.90	-0.394	2.106*	11.07	1.964	0.435	1840*	66.49	37.8	2.518*	0.430	0.382*
15	3.74*	-1.66	-10.5	0.38	0.362	1.033*	7.53	0.270	-0.209	116	24.50	6.6	-0.011	0.177	0.213*
16	26.76*	-5.93	-88.4	-2.47	0.160	1.452*	30.09	0.612	0.021	908*	38.53	37.6	0.618	0.313	0.181*
17	11.08*	-1.91	-93.9	0.02	-0.964	0.239	20.61	0.157	0.170	997*	-4.03	29.5	2.469*	0.285	0.261*
18	9.42	-0.54	-19.3	-1.33	0.240	0.607	-10.52	0.291	0.106	373	12.39	2.5	1.893*	0.212	0.078
19	11.57*	-0.53	-61.3	-3.84	-0.144	-0.235	-12.63	0.326	-0.110	292	-0.95	-11.0	1.054	0.035	0.237*
20	22.48*	-3.62	-14.9	-2.04	1.289	0.170	-3.76	0.469	-0.195	181	5.93	-0.1	0.443	0.142	0.175*
21	10.58*	-2.29	-74.2	-2.85	0.261	0.340	4.30	0.484	-0.093	904*	-2.31	-9.6	1.017*	-0.080	0.105
22	28.91*	-5.15	-19.7	-3.32	-1.180	0.968	-4.76	0.095	0.232	496*	17.09	8.6	0.650*	0.144	0.170*
23	38.48*	-10.17	-29.4	-12.77	-0.118	3.011*	0.62	3.553	-0.222	-17	-15.06	-9.8	6.050*	0.290	0.078

Character abbreviations: FD, flowering date; FN, flower number; PH, plant height; LL, leaf length; and SL, sepal length. Values followed by an asterisk (*) are significantly different from zero ($P < 0.05$) as determined by 95% CIs. Estimates of genetic parameters were obtained with the program H2boot (PHILLIPS 1998a).



The structuring of quantitative genetic variation in a fragmented population of *Briza media* (Poaceae)

Maarten Ellmer, Lotten J. Johansson, Honor C. Prentice, Stefan Andersson

Abstract

Knowledge of the structuring of quantitative genetic variation and its determinants is useful when inferring the adaptive potential and long-term viability of species subjected to natural or human-induced habitat fragmentation. In this study of *Briza media*, we have related patterns of heritable variation in phenotypic characters to descriptors of landscape structure, land-use history and local ecological conditions, using samples of clonally-replicated genotypes from a landscape known to have undergone a massive loss of semi-natural grassland. Analyses of composite variables, derived from a principal component analysis, attributed 29-34% of the phenotypic variation to differences between genotypes from the same deme. Differences between demes accounted for less than 2% of the variation in each of the derived variables, and for 37% of the multivariate variation when all the nine original characters were considered simultaneously in a canonical variates analysis. Despite evidence for heritable variation, neither the deme-specific means nor the broad-sense heritabilities for the derived variables were consistently associated with any of the descriptor variables. These results, together with previous data on allozyme variation and fine-scale plant species diversity from the same study system, indicate that the structuring of quantitative genetic variation has been relatively insensitive to habitat fragmentation, at least over the 300-year time frame considered in this study.

Introduction

The ecological and genetic effects of habitat fragmentation remain a primary issue in conservation biology, especially in areas where populations have become fragmented through human activities such as forestry, agricultural development and urbanization (Young *et al.* 1996; Saunders *et al.* 1991; Harrison & Bruna 1999). Habitat fragmentation results in smaller habitat areas and therefore smaller population sizes, but also restricts dispersal and reduces the ability of species to (re)colonize unoccupied patches of habitat. Species in fragmented habitats may also suffer from poor habitat quality, especially when the patch boundary is large relative to the amount of interior habitat (edge effects), as in small or linear remnants of habitat (Saunders *et al.* 1991; Harrison & Bruna 1999).

Habitat fragmentation has considerable potential to influence the genetic constitution of species and populations. First, if the original continuous populations are spatially structured into patches of related or locally adapted individuals, as in many plant species (e.g., Linhart & Grant 1996), then a local loss of habitat area is expected to cause an immediate reduction of genetic variation as a result of bottlenecks (Nei *et al.* 1975). Second, if the surviving population fragments remain small and isolated for several generations, they will continue to lose variation and become increasingly differentiated as a result of genetic drift and inbreeding (Ellstrand & Elam 1993; Young *et al.* 1996). Finally, there will be an added influence of bottlenecks if the fragmented landscape contains a fraction of unoccupied habitat patches that can be recolonized and if each recolonization involves one or a few founders of local origin (Wade & McCauley 1988). Studies of marker gene diversity generally support these predictions, both for initially rare and common species (e.g., Jacquemyn *et al.* 2004; Vellend 2004; Leimu *et al.* 2006; Aguilar *et al.* 2008).

Genetic drift as a consequence of habitat fragmentation may present a threat to a plant species by increasing the expression of deleterious recessive mutations (inbreeding depression), by causing the loss of self-incompatibility alleles and by reducing the variation necessary for adaptation and long-term survival in changing environments (Storfer 1996; Frankham 1999; Leimu *et al.* 2006; Willi *et al.* 2006). However, it is also possible to imagine situations in which the long-term viability of populations may increase after habitat fragmentation, if, for example, the effective population sizes remain large and the increased isolation enhances the potential for local genetic adaptation by reducing the inflow of maladapted alleles from other populations. Under these

conditions, one might expect populations in fragmented landscapes to exhibit greater local adaptation (Jakobsson & Dinnetz 2005) and to become more divergent in adaptive phenotypic characters than more continuously distributed populations (Willi *et al.* 2007). Although quantitative genetics and local adaptation are receiving increasing attention in conservation biology (Storfer 1996; Frankham 1999; Carvajal-Rodrígues *et al.* 2005), it is still uncertain how random and selective processes interact to determine the structuring of quantitative genetic variation — and the adaptive potential of local populations — within species subjected to natural or anthropogenic habitat fragmentation.

Cessation of traditional management practices, for example grazing and mowing, has caused a massive loss of unimproved, species-rich, semi-natural grasslands during the last century (Bruun *et al*. 2001). In Sweden, for example, the area of semi-natural grasslands has decreased by more than 90% since the 1870s (Bernes 1994) and many grassland species now exist as small, isolated patches in a surrounding matrix of unsuitable habitat (Cousins 2001; Johansson *et al.* 2008). The loss of grasslands has been accompanied by reductions in species diversity (Kiviniemi & Eriksson 2002) and the local extinction of subspecies or varieties adapted to traditional grazing or mowing practices (e.g., Karlsson 1986). As for the general effects on genetic structure, the available data from grassland species indicate considerable heterogeneity, with some showing clear signs of drift (e.g., *Scabiosa columbaria*, van Treuren *et al*. 1994; *Arnica montana*, Luijten *et al*. 2000; *Primula veris*, van Rossum *et al*. 2004) and others in which the genetic structure remains more-or-less the same as in the previous continuous landscape (e.g., *Filipendula vulgaris*, Weidema *et al.* 2000; *Anthyllis vulneraria*, Honnay *et al*. 2006). There is still a paucity of empirical data, especially on phenotypic characters, that can be used to draw general conclusions about the structuring of heritable variation in fragmented grassland landscapes and how different types of diversity, for example, species richness and genetic variation within species, respond to grassland fragmentation (cf. Antonovics 2003; Vellend 2004).

Prentice *et al.* (2006) used allozyme data from the outcrossing, perennial grass *Briza media* (henceforth *Briza*) to explore the relationship between genetic diversity and various descriptors of landscape structure and land-use history in a fragmented grassland landscape on the Baltic island of Öland (SE Sweden). Their data suggest a weak, though significant, impact of drift, manifested as a slight reduction of gene diversity in demes from weakly connected grasslands, but also point towards a role for selection, manifested as

a convergence of allele frequency profiles as young, secondary grasslands on former arable or forested land mature towards the more uniform, species-rich communities characteristic of the old, relict grasslands. More recent studies have documented significant impacts of grassland age, patch connectivity, management (grazing) intensity and soil moisture on measures of plant species richness for remnant grasslands in the study area (Prentice *et al.* 2007; Johansson *et al.* 2008; Reitalu *et al.* 2009). In view of these observations, it becomes meaningful to examine whether similar patterns exist for quantitative genetic measures of variation.

In this study, we have obtained phenotypic data from clonally replicated plants of *Briza*, derived from the same genotypes as were used in the allozyme analyses, to address the following questions: Is the structuring of quantitative genetic variation consistent with a history of genetic drift in small, isolated demes, and do demes in young, secondary grasslands show signs of recent founder effects? Is the quantitative variation correlated with indicators of local habitat conditions, e.g. soil moisture and grazing intensity, as predicted under a local selection scenario? Finally, does the structuring of quantitative variation follow the same patterns as those previously observed for allozyme variation and plant species diversity?

Material and methods

Study species

The perennial, diploid (2n = 14) grass *Briza media* L. is common in a wide variety of base-rich grasslands and has an extensive distribution in western Eurasia (Dixon 2002). As well as being an indicator of old, species-rich grasslands (Ekstam & Forshed 1992), *Briza* also occurs as an early colonizer of young grasslands developing on previously forested or arable sites (Prentice *et al*. 2007). Flowering occurs in June-July, when the small (<10 cm) tussocks develop up to 60 cm tall stems (culms) with a large number of distinctly shaped, flattened spikelets arranged in loose, roughly pyramidal panicles. The florets have large protruding anthers, copious pollen production and large feathery stigmas typical of wind-pollinated grasses, and they mature into one-seeded, indehiscent fruits, termed caryopses. Experimental studies suggest that most caryopses disperse locally (< 1 m from the mother plant; Dixon 2002), but long-distance dispersal of caryopses and undehisced spikelets is also possible, either by grazing animals (Fischer *et al*. 1996) or hay transfer (Dixon

2002). Plants of *Briza* possess a gametophytic self-incompatibility system (Murray 1974).

Study area

The Jordtorp area includes the village of Jordtorp and four adjacent villages in the parish of Algutsrum on the Baltic island of Öland (SE Sweden). The area covers approximately 22 km^2 and consists of a small-scale mosaic of arable land, deciduous forest and grasslands (Johansson *et al.* 2008). Historical land-cover maps for the Jordtorp area (dating back to the early 18th century) show a progressive reduction in the area covered by semi-natural grassland over the last 300 years and the remaining grasslands have become increasingly fragmented (Fig. 1). Most of the present grasslands are situated in former outfields that lay outside the villages until the late 18th or early 19th century. These grasslands have a continuity of at least 274 years and will be termed "primary" throughout this paper. The remaining "secondary" grasslands developed during the 20th century on previously arable or forested land (Johansson *et al.* 2008). Ungrazed (abandoned) grasslands are undergoing succession towards forest, with an increasing cover of both shrubs and trees (Reitalu *et al.* 2009). Populations of *Briza* can be found in both grazed and ungrazed grassland fragments.

Sampling, cultivation and data collection

The plant material in this study was derived from a sample of 885 vegetative shoots collected in 48 local populations (hereafter "demes") of *Briza* within the Jordtorp area during July-August in 2001-02 (Prentice *et al.* 2006). The chosen demes were spread throughout the study area and represented both primary and secondary grassland fragments (Fig. 1). A maximum of 26 vegetative shoots, separated by not less than 3 m, were collected in each deme. The sampling of shoots proceeded outwards from the centre of each deme until the sample contained 26 shoots or no further *Briza* was encountered. The number of sampled shoots thus provides a rough estimate of the area occupied by the local *Briza* population (hereafter "deme area").

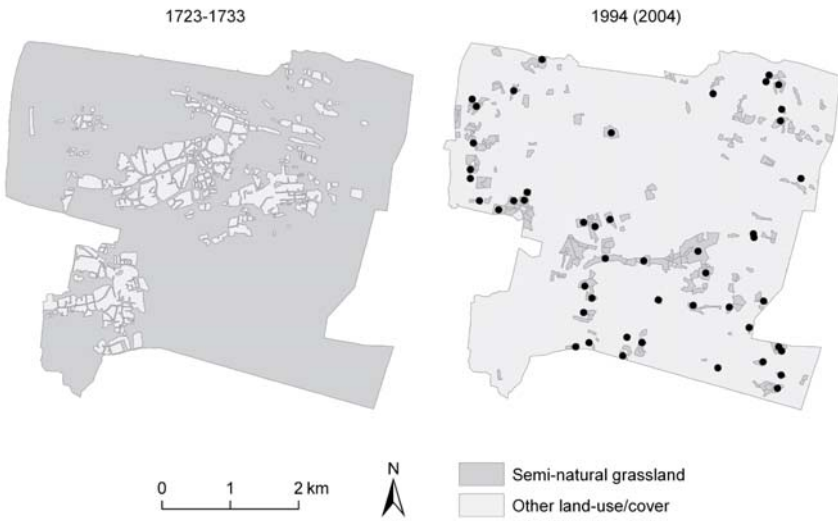


Figure 1. Maps showing the past and present distribution of grasslands within the Jordtorp area (redrawn using data from Johansson *et al.* 2008). Circles denote locations of sampled *Briza media* demes.

The sampled shoots, grown in separate pots in an experimental garden (University of Lund, S Sweden), were scored for variation at 11 polymorphic allozyme loci (Prentice *et al.* 2006). In 2004, each tussock was split into a maximum of three shoots ("ramets"). Each ramet was trimmed to approximately the same size, planted in a separate pot with standard peat-based soil, and assigned to a random position in a sunny part of another garden (University of Lund, S Sweden). Watering was supplied as needed, but no fertilizer was applied. The plants were repotted three times during the cultivation period. The ramets from the same field-collected shoot are regarded as clonal replicates of the same "genet" in the statistical analyses.

In 2005, we recorded the number of culms for all ramets and pressed two culms from each ramet. The following characters were scored on the dried culms: the lengths of the two uppermost leaves, the height of the culm (including the panicle), the total number of spikelets and the length of the lowermost side branch in the panicle. Two spikelets per panicle were studied under a stereo-microscope to obtain data on spikelet size, characterized by length, width and floret number. Data were averaged over culms and spikelets

to provide single values for each ramet. All characters were approximately normally distributed, except for culm number which had to be log-transformed to fulfill the assumption of normality. Phenotypic data were obtained for a maximum of 1662 ramets, representing 736 genets.

The measured characters define a major axis of variation within the genus *Briza*, contrasting *B. major*, a species with a tall culm, long leaves, short panicle-branches and a few large, many-flowered spikelets from *B. media* and other species with short culms, short leaves, long panicle-branches and numerous small, few-flowered spikelets (S. Andersson & M. Ellmer, unpublished data). Based on the extensive between-species variation in all variables and the putatively strong effects of culm, spikelet and floret number on reproductive fitness, we consider the characters as evolutionarily labile and potentially under selection.

A number of population, landscape and historical variables were used to characterize the deme or grassland fragment from which the genets were sampled. Four descriptors — deme area (the number of shoots sampled; range 1-25), fragment area (range 1380-138970 m^2), the percent fragment perimeter bounded by adjacent grassland (range 0-100%), and grassland age (the minimum time from the transition from forest or arable land to grassland; range 0-265 years) — were chosen to indicate differing potential for genetic drift as a consequence of small population size and low inter-fragment connectivity, and the possible influence of recent founder events in demes representing secondary grasslands. The remaining descriptors were chosen to characterize the past and present selection regime: soil moisture (scored as 1 [dry], 2 [moderately moist] or 3 [moist]), current grazing intensity (scored as 1 [ungrazed], 2 [slightly or moderately grazed] or 3 [well-grazed]) and two indicators of longer-term management intensity: the percent cover of trees and bushes (both scored as 1 [0%], 2 [1-10%], 3 [10-25%], 4 [25-50%] or 5 [50-70%]). All these data were obtained from interpretation of field-validated infrared aerial photographs (M. Ihse, M. Kindström and H.C. Prentice, unpublished data).

The eight deme/fragment descriptors (or variants thereof) have been found to correlate with allozyme-based estimates of gene diversity in *Briza* (Prentice *et al.* 2006) or with measures of fine-scale plant species diversity, recorded within 50 x 50 cm plots in remnant grasslands in the study area (Prentice *et al.* 2007; Johansson *et al.* 2008; Reitalu *et al.* 2009).

Univariate analyses

A combination of univariate and multivariate analyses were employed to provide complementary views of the structuring of phenotypic variation and the genetic processes that may be responsible for the between-deme variation. The univariate analyses were carried out to, first, quantify the quantitative genetic variation in a way that allowed comparison with the corresponding data for allozyme markers, and second, to explore whether the mean phenotype and the level of heritable variation within demes covaried with the deme/fragment descriptors. To avoid redundancy in these analyses, we first performed a principal component analysis (PCA) to extract the first two principal components (PCs) of the between-ramet variation (Dunn & Everitt 1982), based on the product-moment correlations among the original characters. The PCs were used to characterize individual phenotypes in all univariate analyses.

After the calculation of deme means, the data were analysed with analyses of variance (type III sum of squares) and then subjected to variance component analyses, based on the restricted maximum likelihood method because of the unbalanced experimental design (Lynch & Walsh 1998). These analyses quantified and tested the significance of the variance between demes (V_{deme}), between genets within demes (V_{genet}) and between ramets within genets (V_{ramet}), using a model in which all factors were considered as random. We quantified the level of population structure for each PC as Q_{st} (Spitze 1993), estimated as $V_{deme} / (V_{deme} + 2V_g)$, where V_g is the additive genetic variance within demes, approximated by V_{genet} under the assumption that the between-genet variation was mostly additive (Falconer 1989; Lynch & Walsh 1998). Q_{st} is the quantitative genetic analogue of the fixation index (F_{st}, Wright 1951) and therefore allows direct comparison with the between-deme variation estimated for the allozyme loci (F_{st} = 0.006, Prentice *et al.* 2006). A close similarity between Q_{st} and F_{st} suggests that the phenotypic characters and the marker loci have been affected by the same evolutionary force(s), whereas a significant difference indicates a stronger impact of diversifying ($Q_{st} > F_{st}$) or unifying ($Q_{st} < F_{st}$) selection (Spitze 1993; Waldmann & Andersson 1998; Leinonen *et al.* 2008).

To quantify the within-deme genetic variation, we first obtained a general estimate of the broad-sense heritability (H^2) for each PC, calculated as $V_{genet} / (V_{genet} + V_{ramet})$ using data from the overall REML analyses, and then carried out separate variance component analyses for each deme (using the genet as the highest group level) to obtain deme-specific H^2 estimates. An estimate of H^2

sets an upper limit to the amount of additive variation available for selection (Falconer 1989; Lynch & Walsh 1998).

Linear regression analyses were performed to explore the association between the deme-specific mean or H^2 estimate for each PC and the eight deme/fragment descriptors considered in this study. As a first step, we regressed the dependent variable on each descriptor using univariate regression, i.e. without separating direct and indirect relationships between variables. Second, we used multiple regression to assess the direct relationship between each descriptor and the response variable, holding all the other descriptor variables constant. Third, we utilized a stepwise selection procedure to search for subsets of descriptors with high predictive value. The stepwise analyses used a forward selection procedure and $P = 0.05$ as a cutoff for entry of other descriptor variables in the regression model. To remove covariation associated with geography, we entered the distance along the W-E and S-N axes of the study area as covariates in the multiple regressions (cf. Prentice *et al.* 2006).

To further compare polygenic and monogenic variation, we used the product-moment correlation coefficient (r) to determine how the H^2 of each deme covaried with allozyme-based estimates of within-deme gene diversity (H, expected heterozygosity) and within-deme allelic richness (A) (for details on how these data were obtained, see Prentice *et al.* 2006).

Regression and correlation analyses based on the nine original characters (rather than PCs) or direct estimates of V_{genet} (rather than H^2) gave quantitatively similar results and provided little additional insight (data not shown).

Multivariate analyses

As a final step, we used canonical variates analysis (CVA) to assess patterns of variation between populations at the multivariate level. CVA is similar to PCA except that the new composite variables (canonical variates, CVs) maximize the differences between groups defined a priori, rather than the overall variation as in a PCA. The CVs are calculated in a way that accounts for covariance between variables: differences between groups that are perpendicular to the major axes of within-group variation make a greater contribution to the separation between groups than those that are in the same direction as the within-group variation (Dunn & Everitt 1982). We thus assume that the multivariate analyses provide a complementary view of the structuring of variation. CVA also provides estimates of Wilks' lambda, which quantifies the proportion of the total variance that is due to within-group variation. In the

present study, we focused on the proportion of the variance that is due to *between-group* variation, calculated as 1-Wilks' lambda.

Individual ramets were used as within-group replicates in all CVAs and the groups were defined as individual demes, or groups of ramets representing demes with potentially differing impact of drift, founder effects or past selection, as reflected by differences in the various deme or fragment descriptors (see above). For deme area and fragment area, we used the median as a cutoff to provide two categories of demes or ramets for each descriptor variable (small vs. large area). For grassland age, we contrasted demes/ramets from primary vs. secondary grasslands. A comparison of ramets from secondary demes on previously arable vs. forested land provided a test for overall differences in selective factors (such as soil fertility) related to past land-use.

The significance of differences between groups of demes, for example, demes in grasslands with differing grazing intensity, was evaluated by contrasting the matrix of pairwise Euclidean distances between deme means (based on all the original characters and referred to as "phenotypic distances") with dissimilarity matrices expressing absolute differences in each descriptor variable. A pairwise difference in a two-state descriptor (e.g. grassland age and past land-use) was assigned a value of 0 if two demes belonged to the same category or 1 if they represented different categories. A Mantel permutation procedure (9999 repetitions) was used to determine whether corresponding elements in two distance matrices were significantly correlated with respect to each other. The geographic distance between demes was entered as a covariate in all matrix comparisons.

All analyses were carried out with SPSS (version 15.0) or NTSYSpc (version 2.20) for Windows.

Results

Univariate analyses

The PCA revealed high positive loadings for all phenotypic variables on the first principal component (PC1), which extracted 54.3% of the total variation

Table 1. Character loadings for the nine vegetative and floral characters on the first principal components (PC1,2) in a PCA based on the product-moment correlations among the original characters.

Character	PC1	PC2
No. of culms (log)	0.514	0.417
Culm height	0.781	0.033
Length of lower leaf	0.764	0.400
Length of upper leaf	0.623	0.349
Length of panicle branch	0.878	0.083
No. of spikelets	0.699	0.553
Spikelet length	0.785	-0.540
Spikelet width	0.790	-0.466
No. flowers per spikelet	0.733	-0.585
Variance explained (%)	54.3	18.0

(Table 1). The second component (PC2), which explained an additional 18.0% of the variation, had high loadings for spikelet number and measures of spikelet size, contrasting individuals with many, small spikelets from those with the opposite features. These two components will be referred to as "plant size" and "spikelet size-number ratio", respectively, even though some of the size variables also contributed to the second axis (Table 1).

According to the ANOVAs and variance component analyses, the variation among replicate ramets within genets (V_{ramet}) accounted for most of the variation in plant size (69.8%) and spikelet size-number ratio (65.8%), in contrast to the moderate variance associated with differences between genets within demes (V_{genet}; plant size: 28.5%; spikelet size-number ratio: 34.2%; $P < 0.001$ in both cases) and the low variance attributed to between-deme variation ($V_{deme} < 2\%$, $P \geq 0.05$). The overall estimate of Q_{st} was 0.030 for plant size and 0 for spikelet size-number ratio.

Table 2. The relationship between different deme/fragment descriptors and deme-specific means and H^2 estimates for plant size (PC1) and spikelet size-number ratio (PC2), as indicated by standardized regression coefficients from univariate regression analyses (N = 48 in all analyses). Each entry corresponds to a separate regression.

Descriptor	Response variable			
	$Mean_{PC1}$	$Mean_{PC2}$	H^2_{PC1}	H^2_{PC2}
Grassland continuity	-0.239 ^{ns}	-0.091 ^{ns}	-0.247 ^{ns}	-0.013 ^{ns}
Deme area	-0.112 ^{ns}	-0.160 ^{ns}	-0.104 ^{ns}	-0.202 ^{ns}
Fragment area	-0.137 ^{ns}	-0.010 ^{ns}	-0.081 ^{ns}	0.117 ^{ns}
% perimeter bounded by grassland	0.088 ^{ns}	0.238 ^{ns}	-0.028 ^{ns}	0.151 ^{ns}
Soil moisture	0.082 ^{ns}	-0.157 ^{ns}	0.035 ^{ns}	-0.072 ^{ns}
Grazing pressure	-0.076 ^{ns}	0.007 ^{ns}	0.281 ^{ns}	0.119 ^{ns}
Bush cover	-0.027 ^{ns}	0.041 ^{ns}	-0.041 ^{ns}	0.161 ^{ns}
Tree cover	-0.181 ^{ns}	-0.145 ^{ns}	0.139 ^{ns}	-0.275 ^{ns}
Position along W-E axis	0.301 *	-0.190 ^{ns}	0.170 ^{ns}	-0.058 ^{ns}
Position along S-N axis	-0.058 ^{ns}	0.034 ^{ns}	0.001 ^{ns}	-0.008 ^{ns}

* $P < 0.05$, ns $P > 0.05$

Table 3. The relationship between different deme/fragment descriptors and deme-specific means and H^2 estimates for plant size (PC1) and spikelet size-number ratio (PC2), as indicated by standardized partial regression coefficients from multivariate regressions (N = 48 in all analyses). Each column corresponds to a separate regression. The proportion of variance explained by each regression model (R^2) is also given.

Descriptor	Response variable			
	$Mean_{PC1}$	$Mean_{PC2}$	H^2_{PC1}	H^2_{PC2}
Grassland continuity	-0.100 ^{ns}	-0.028 ^{ns}	-0.323 ^{ns}	0.050 ^{ns}
Deme area	-0.073 ^{ns}	-0.272 ^{ns}	0.204 ^{ns}	-0.327 ^{ns}
Fragment area	-0.151 ^{ns}	0.030 ^{ns}	-0.014 ^{ns}	0.128 ^{ns}
% perimeter bounded by grassland	0.042 ^{ns}	0.253 ^{ns}	-0.103 ^{ns}	0.035 ^{ns}
Moisture	0.335 ^{ns}	-0.221 ^{ns}	-0.119 ^{ns}	0.078 ^{ns}
Grazing pressure	-0.245 ^{ns}	0.028 ^{ns}	0.351 ^{ns}	0.100 ^{ns}
Bush cover	0.267 ^{ns}	-0.067 ^{ns}	0.043 ^{ns}	0.203 ^{ns}
Tree cover	-0.246 ^{ns}	-0.002 ^{ns}	0.010 ^{ns}	-0.351 ^{ns}
Position along W-E axis	0.367 *	-0.272 ^{ns}	0.126 ^{ns}	-0.122 ^{ns}
Position along S-N axis	-0.035 ^{ns}	0.079 ^{ns}	0.114 ^{ns}	0.024 ^{ns}
R^2	0.059	-0.024	-0.036	0.013

* $P < 0.05$, ns $P > 0.05$

The overall estimate of the broad-sense heritability (H^2) was 0.29 for plant size and 0.34 for spikelet size-number ratio. Values of H^2 for individual demes ranged between 0 and 0.82 for plant size and between 0 and 0.86 for spikelet size-number ratio.

Judging from the results of the regression analyses (Tables 2, 3), the means and H^2 values of individual demes were not significantly influenced by any of the deme/fragment descriptors used in this study, the only exception being a weakly significant increase in mean plant size with increasing distance along the west-east axis ($Mean_{PC1}$). The proportion of variation explained by the multiple regressions was always low (R^2 < 6%, Table 3) and none of the descriptors were retained as significant predictors in the stepwise analyses.

Deme-specific H^2 estimates for plant size or spikelet size-number ratio were not significantly correlated with allozyme-based estimates of within-deme gene diversity (H) ($|r| < 0.113$, $P > 0.05$) or within-deme allelic richness ($|r| < 0.112$, $P > 0.05$).

Multivariate analyses

A CVA based on all phenotypic characters revealed modest (though significant) variation among the 48 demes considered in this study (1-Wilks' lambda = 0.374, $P < 0.001$). No distinct clusters were revealed by the first two canonical axes (CV1-2; Fig. 2), which jointly explained a total of 49.6% of the variation (Table 4). Rather, there was a more-or-less gradual transition from a large, central cluster of demes with intermediate scores (close to 0) to scattered demes with low or high values on either the first or second axis (Fig. 2).

The phenotypic distance between demes increased with increasing geographical distance between the grassland fragments from which the demes originated ($r = 0.179$, $P < 0.05$; Mantel test), especially for demes classified as secondary ($r = 0.403$, $P < 0.05$ vs. $r = 0.041$, $P > 0.05$ for demes in primary patches). Based on these observations, we entered the geographic distance matrix as a covariate in all subsequent matrix comparisons.

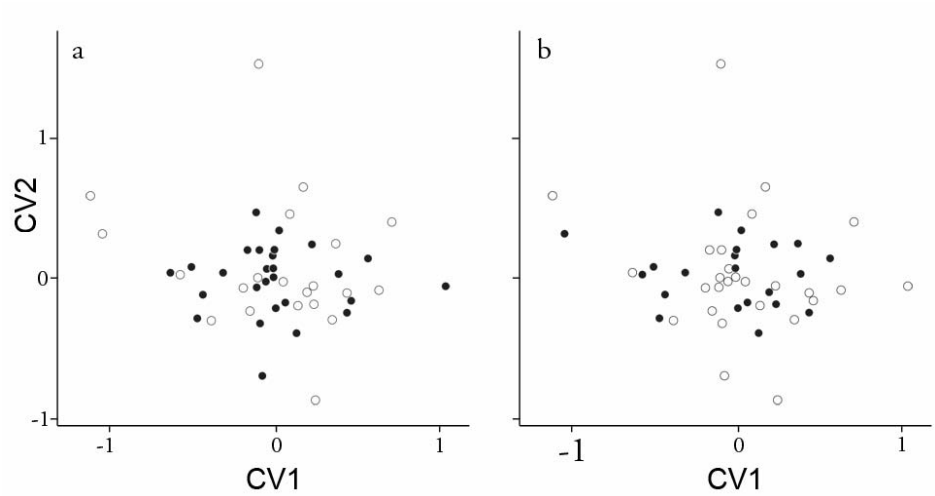


Figure 2. Canonical variates plot of the 48 demes of *Briza media* considered in this study. The demes are represented by their centroids. Different symbols in (a) denote demes classified as small (open circles) or large (closed circles). Different symbols in (b) denote demes from grasslands classified as primary (open circles) or secondary (closed circles).

There was no consistent difference in the mean phenotype between ramets in different deme or fragment size categories: the proportion of variation explained by the between-group difference was low (1-Wilks' lambda ≤ 0.010; CVA) and permutation tests revealed no significant association between the phenotypic distance matrix and the matrix expressing differences in deme or fragment area ($|r| \leq 0.06$, $P > 0.05$; Mantel test) (Table 5). Judging from the results of separate CVAs, demes from small and large grassland fragments showed similar partitioning of variation between demes (1-Wilks' lambda = 0.357-0.383); however, the group of spatially restricted demes tended to have a higher proportion of their variation between demes (1-Wilks' lambda = 0.418) than the group of spatially extensive demes (1-Wilks' lambda = 0.339). This pattern is also apparent in the plot of the first two canonical variates from the overall analysis (Fig. 2a).

Ramets from primary and secondary grassland fragments were similar in their mean phenotype, as shown by low between-group variation in CVA (1-Wilks' lambda = 0.012) and a nonsignificant difference according to the permutation analyses ($r = -0.028$, $P > 0.05$). A separate CVA for each category showed that demes from old, primary grasslands had a somewhat higher between-deme

Table 4. Character loadings for the nine vegetative and floral characters on the first canonical variates (CV1,2) in a CVA using deme as a class variable.

Character	CV1	CV2
No. of culms (log)	-0.289	-0.227
Culm height	0.647	-0.175
Length of lower leaf	0.288	0.216
Length of upper leaf	0.281	0.369
Length of panicle branch	0.302	0.122
No. of spikelets	0.250	-0.358
Spikelet length	0.365	0.221
Spikelet width	0.483	0.154
No. flowers per spikelet	0.329	0.328
Variance explained (%)	33.4	16.2

Table 5. Results of multivariate analyses comparing different categories of ramets or demes based on the nine vegetative and floral characters considered in this study. 1-Wilks' lambda denotes the fraction of variance that is due to between-group variation in CVAs, whereas r denotes the correlation coefficient from a matrix-based comparison (Mantel test). None of the statistics were significantly different from 0 ($P > 0.05$).

Grouping variable	1-Wilks' lambda	r
Grassland age (primary vs secondary fragments)	0.012	-0.0275
Deme area (small vs. large)	0.003	-0.0559
Fragment area (small vs. large)	0.010	-0.0051
Soil moisture (3 categories)	0.037	-0.0608
Grazing pressure (3 categories)	0.024	-0.0393
Bush cover (5 categories)	0.035	-0.0470
Tree cover (5 categories)	0.020	-0.1004
Previous land-use (forest vs arable land)	0.035	-0.1041

component of diversity (1-Wilks' lambda = 0.435) than those from secondary grasslands (1-Wilks' lambda = 0.312), a pattern also found in the ordination

plot from the overall analysis (Fig. 2b). However, the most deviating points in Fig. 2 represented demes that were both small and primary, making it difficult to separate between grassland age and deme size as the primary cause of their divergence.

Ramets from sites with differing soil moisture or management intensity, measured by current grazing intensity or bush/tree cover, had similar phenotypes (1-Wilks' lambda < 0.040), with low, non-significant test statistics in the permutation analyses ($|r| \leq 0.100$, $P > 0.05$). Judging from the separate analysis of demes from young, secondary patches, there was no significant influence of previous land-use (forest vs. arable land) on the multivariate phenotype (1-Wilks' lambda = 0.035; $r = -0.104$, $P > 0.05$).

Discussion

Considerable attention has been devoted to the genetic consequences of habitat fragmentation, especially those that can be detected at putatively neutral marker loci or in direct components of fitness or population viability (for reviews, see Leimu *et al*. 2006; Aguilar *et al.* 2008). Only a few studies have considered measures of long-term adaptive potential and most of these are based on small numbers of populations or samples of populations for which no historical data are available (Willi *et al.* 2006). As a consequence, we still lack a clear understanding of how the widespread fragmentation of semi-natural grasslands impacts the genetic variation in those characters that are likely to be the targets of current or future selection forces, such as those associated with life history, physiology and morphology. In this study of *Briza*, we related patterns and amounts of quantitative genetic variation in phenotypic characters to landscape structure, land-use history and local ecological conditions, using samples of genotypes from an old, agricultural landscape with a known history of fragmentation. We also evaluated whether the observed patterns parallel those seen in previous analyses of marker gene variation and small-scale species diversity (Prentice *et al*. 2006, 2007; Johansson *et al.* 2008; Reitalu *et al.* 2009).

The structuring of quantitative genetic variation

A PCA indicated the existence of two partly independent axes of variation, one describing general plant size (PC1) and another dominated by the number and

size of spikelets (PC2). Based on these variables, almost all the variation could be attributed to differences between ramets from the same deme, the remainder (< 2%) occurring between demes from different grasslands. The overall estimate of the broad-sense heritability was high for both PCs (0.29-0.34), indicating a relatively large genetic contribution to the variation observed at the within-deme level. In contrast to the low between-deme variation found at the level of individual characters, differences between demes accounted for a sizeable fraction of the variation when data were aggregated across all the original characters in a CVA (37%), indicating that the direction of the between-deme differences often deviated from the major axes of the within-deme variation (Dunn & Everitt 1982).

Fragmented populations are expected to lose heritable variation and become increasingly differentiated from each other as a result of genetic drift and bottleneck effects (Ellstrand & Elam 1993), especially under a meta-population scenario in which unoccupied habitat patches become (re)colonized by a few founders of local origin (Wade & McCauley 1988). However, our results provide no clear evidence that drift and/or founder events have played major roles in structuring the quantitative genetic variation within *Briza* in the study area. First, although a few spatially restricted demes proved to be divergent in the multivariate analyses, we found no tendency for the small demes to have the lowest broad-sense heritabilities — regardless of whether deme size was expressed as deme area or the spatial extent of the grassland occupied. Second, no significant association was found between the heritability and the percentage of grassland fragment perimeter bounded by adjacent grassland, despite the potentially large effective population sizes and/or extensive gene flow associated with high inter-patch connectivity. Finally, our data provide no evidence of increased between-deme variation or reduced within-deme variation for demes on young, secondary grasslands relative to those representing old, relict grassland fragments; in fact, the proportion of variation attributable to between-deme variation in the CVAs showed a trend in the opposite direction, i.e. slightly more divergence among demes on old, primary fragments.

Characters under selection are expected to show habitat-correlated variation and to be more strongly differentiated between populations than allele frequencies at neutral marker genes as a consequence of selection for different optimum phenotypes in different environments (Spitze 1993; Linhart & Grant 1996), especially in fragmented landscapes where distances between populations may be sufficiently long to reduce maladaptive gene flow into local

gene pools (Ellstrand & Elam 1993). If selection has been strong and persistent in direction, one would also expect habitat-correlated variation in the heritability, given the expected elimination of inferior genotypes associated with directional or stabilizing selection (Falconer 1989). Although grasses generally have a great capacity to evolve locally adapted populations (e.g., Snaydon & Davies 1982; Prentice *et al.* 1995), we found no support for selection being a major determinant of the structuring of quantitative genetic variation in *Briza*: neither the means nor the deme-specific H^2 values were significantly correlated with soil moisture, measures of management intensity or environmental factors related to past land-use. In addition, the estimates of Q_{st} for plant size and spikelet size-number ratio (0-0.030) were not consistently larger than the allozyme-based value (F_{st} = 0.006; Prentice *et al.* 2006), contrasting with the relatively large difference observed in other Q_{st}-F_{st} comparisons (Leinonen *et al.* 2008).

We cannot exclude the possibility that some unmeasured selective factor is contributing to the variation in the measured characters or that selection is operating on a finer (or coarser) scale than that considered in this investigation. For example, the positive association between phenotypic and geographic distances seen in the multivariate analyses may suggest that spatially adjacent demes have experienced more similar selection forces than those separated by larger distances. Similarly, the eastward increase in general plant size is consistent with a history of spatially varying selection on this character, with eastern sites favouring larger individuals. However, the joint effect of all selective factors (including unmeasured ones) has been too weak to substantially increase Q_{st} above the F_{st} value. Hence, it seems unlikely that diversifying selection pressures have made a substantial contribution to the structuring of genetic variation observed in the univariate analyses.

The apparent lack of strong drift and selection effects may have several possible causes. First, the effects may have been diminished by long-distance gene flow, mediated by wind-pollination or propagules dispersed by grazing cattle or hay transfer (Fischer *et al.* 1996; Dixon 2002), a factor that also explains the low between-deme component of allozyme diversity previously reported for this study system (Prentice *et al.* 2006). Second, based on the high mutation rate inferred for quantitative characters (Lynch & Walsh 1998) and the potentially slow population dynamics of perennial grasses, there may have been insufficient time for genetic drift or selection to influence the structuring of variation in the phenotypic characters, i.e. the genetic variation may still reflect the larger population sizes that existed in the previously more

continuous grassland habitat (Keyghobadi *et al.* 2005; see also Reitalu *et al.* 2009). Third, our analyses may have lacked the statistical power to detect existing relationships between quantitative genetic parameters and deme/patch descriptors, given the possible inclusion of nongenetic or nonadditive variance in the H^2 and Q_{st} estimates. A large nongenetic or nonadditive component would make selection inefficient and thus reduce, or obscure, the signature of past selection forces; furthermore, the loss of quantitative variance caused by drift does not follow the standard predictions when there are high levels of nonadditive variance in the characters (Willi *et al.* 2006). However, the fact that all genotypes were cultivated under uniform garden conditions for 4 or 5 years (and repotted three times during this period) before the measurements is likely to have minimized "carry-over" effects from the original field environment. Regarding the Q_{st}-F_{st} contrast, available evidence from other studies indicates that nonadditive variance is unlikely to mask the effect of selection on the signatures of divergent or unifying selection (Leinonen *et al.* 2008; Santure & Wang 2009).

Although results from experimentally manipulated populations often agree with the expected loss of quantitative genetic variation in small, isolated populations (Willi *et al.* 2006), there is little consensus over the relationship between adaptive potential and population size, connectivity or other landscape parameters in wild plant species. Ouborg *et al.* (1991) demonstrated a positive association between the level of phenotypic variation and population size in *Scabiosa columbaria* and *Salvia pratensis*. Quantitative genetic studies of Swedish populations of *Scabiosa columbaria*, *S. canescens* and *Tephroseris integrifolia* found no consistent link between low heritability and small population size (Widén & Andersson 1993; Waldmann & Andersson 1998) — in agreement with results for the annual plant *Clarkia dudleyana* in North America (Podolsky 2001). Widén *et al.* (2002) detected large, idiosyncratic differences in the genetic (co)variance structure between Cretan populations of *Brassica cretica* and attributed this pattern to a long history of drift and population isolation. A recent study of a fragmented meta-population of *Ranunculus reptans* documented erosion of additive genetic variation as a consequence of small population size, but also demonstrated that Q_{st} between small populations exceeded that expected from drift alone, pointing to an enhanced role for both genetic drift and selection for these populations (Willi *et al.* 2007). A recent transplantation experiment with *Carlina vulgaris* indicate that the strength of local adaptation may be favoured by small population size and/or high population isolation (Jakobsson & Dinnetz 2005), while no such

pattern was observed in a transplant study of *Lychnis flos-cuculi* (Bowman *et al*. 2008). Together with the present results from *Briza*, the available data indicate considerable heterogeneity in the extent to which habitat fragmentation causes a reduction in long-term adaptive potential.

Quantitative genetic variation vs. allozyme and species diversity

Surveys of genetic variation in wild species generally focus on putatively neutral marker genes (Leimu *et al*. 2006; Aguilar *et al.* 2008). The validity of the assumption that this type of variation is representative of the variation in polygenic characters has been questioned (Reed & Frankham 2001; Carvajal-Rodrígues *et al.* 2005; Leinonen *et al.* 2008) and our results for *Briza* provide no support for a consistent relationship between marker diversity and quantitative genetic variation. First, although partitions of diversity were similar ($Q_{st} \approx F_{st}$), there was no obvious association between quantitative and allozyme-based measures of within-deme diversity. Second, in contrast with the allozyme results, which suggest weak, though significant, impacts of both neutral and selective processes (Prentice *et al*. 2006), we observed no clear signs of drift and selection in the present study, even though quantitative genetic estimates of variation generally have the same or higher precision than those based on marker genes (Carvajal-Rodrígues *et al.* 2005). The results of this study reinforce the notion that local genetic processes sometimes operate differently on monogenic and polygenic characters and that conservation strategies based solely on marker gene variation may be misleading (Storfer 1996; Frankham 1999).

Plant species diversity is significantly affected by landscape structure, land-use history and local ecological conditions among remnant grasslands in the study area: the dominant gradient of variation in plant community composition is explained by soil moisture (Prentice *et al*. 2007) and measures of fine-scale species diversity have been found to increase with increasing grassland age, patch connectivity and/or grazing pressure (Johansson *et al.* 2008; Reitalu *et al.* 2009). Although these associations point to a major role for processes that contribute to the environmental sorting of species at the community level, we reiterate that no such patterns were observed for the phenotypic variation considered in the present investigation. Thus, it seems that measures of species diversity and adaptive potential may respond differently — or at different rates — to the same change in landscape structure (cf. Antonovics 2003; Vellend 2004).

In order to be able to make recommendations for management, restoration and maintenance of biological diversity in fragmented landscapes, it is necessary to understand how different types of genetic and species diversity respond to habitat fragmentation. The differences inferred for *Briza* need to be confirmed by more powerful statistical analyses (accounting for differences in sample sizes and in the particular descriptors used to quantify different deme/fragment characteristics, etc), but lead to the suggestion that quantitative genetic parameters can remain relatively stable during habitat fragmentation and that changes in long-term adaptive potential may be of lesser concern than the more immediate effects on population viability or species diversity (Saunders *et al.* 1991; Harrison & Bruna 1999; Kiviniemi & Eriksson 2002; Leimu *et al.* 2006). As more studies begin to integrate data across different types of diversity, it will be possible to make broader generalizations regarding the biological consequences of habitat fragmentation — and the conservation strategies needed to minimize the loss of biodiversity in fragmented grassland landscapes.

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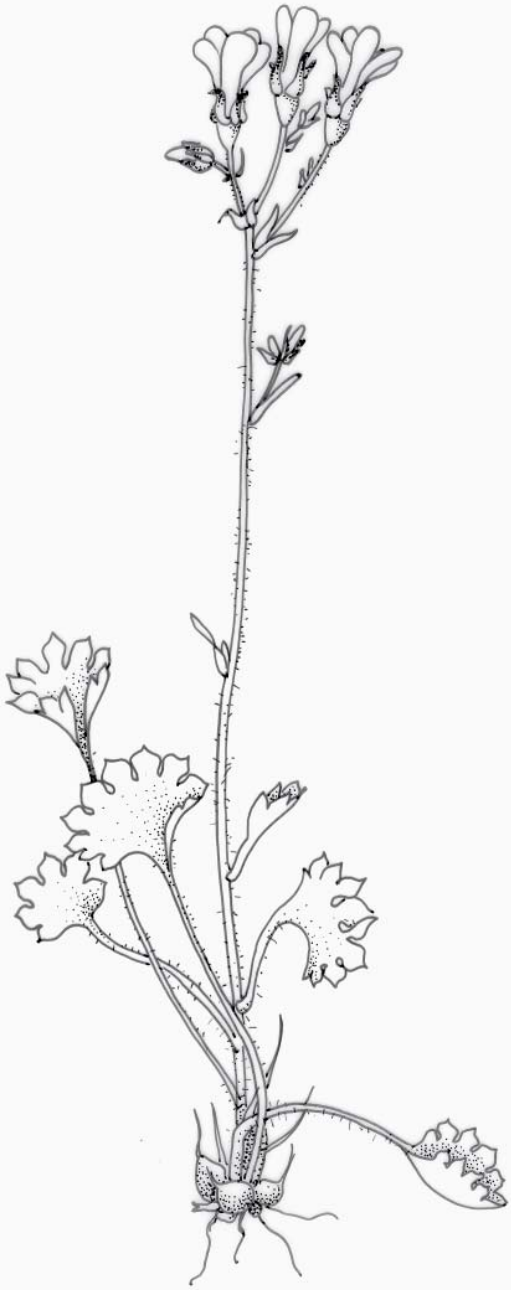

Phenotypic variation in *Saxifraga granulata*: past and future effects of habitat fragmentation

Maarten Ellmer

Abstract

A central question in conservation biology is how different measures of biodiversity respond to human-induced habitat fragmentation. In this study of the grassland herb *Saxifraga granulata*, I related patterns and amounts of phenotypic variation to population size, habitat connectivity and local habitat conditions, using field-collected phenotypes from a grassland landscape with a known fragmentation history and characters known to possess significant heritability under near-natural (garden) conditions. As well as searching for signals of drift, founder events and selection — local genetic processes that might have been enhanced by past habitat fragmentation, I performed a series of habitat loss simulations to determine how the currently observed variation might respond to a further reduction of grassland area. My results provide little support for drift and founder events as major determinants of the structuring of variation, but they agree with an influence of diversifying selection (possibly enhanced by phenotypic plasticity), mediated through ecological factors related to, for example, grazing intensity. Despite evidence for spatial variation in all characters, there was only a slight or moderate reduction in the regional estimate of the between-population variance following a simulated loss of habitat fragments. Similarly, the within-population variation was not structured in a way that markedly affected the estimates of within-population variance following a simulated reduction of the areas occupied by the individual populations. Thus, substantial losses of grassland area will be required to significantly reduce the phenotypic variation represented in the study area.

Introduction

Destruction and fragmentation of natural and seminatural habitats, caused by anthropogenic factors such as urbanization, forestry and agriculture, is a significant threat to the maintenance of biological diversity (Young *et al.* 1996; Saunders *et al.* 1991; Harrison & Bruna 1999). When formerly continuous habitats become divided into small and isolated fragments, many species experience a decrease in population size, a reduction in the ability to disperse and repopulate unoccupied patches of habitat and, in many cases, a deterioration of environmental quality owing to, for example, edge effects. As well as influencing species diversity (Saunders *et al.* 1991; Harrison & Bruna 1999), these changes may also be accompanied by erosion of intraspecific genetic variation and increased divergence between populations as a consequence of increased genetic drift, reduced gene flow and altered metapopulation dynamics (Wade & McCauley 1988; Ellstrand & Elam 1993; Young *et al.* 1996, Leimu *et al*. 2006; Aguilar *et al.* 2008). If small, isolated population fragments experience less maladaptive gene flow and/or more diverse environmental conditions than larger ones, it is also reasonable to expect increased divergent selection and an enhanced potential for local adaptation following habitat fragmentation (Jakobsson & Dinnetz 2005; Willi *et al.* 2007). Clearly, there is a great opportunity for both stochastic and selective processes to shape the structuring of variation within fragmented landscapes, particularly for potentially adaptive phenotypic characters such as those reflecting life history, physiology and morphology (e.g., Storfer 1996; Frankham 1999; Willi *et al.* 2006).

Measures of genetic diversity and population structure, preferably combined with data on the spatial distribution of particular alleles or phenotypes, can provide valuable insights into the random and selective processes operating in fragmented landscapes (Ellstrand & Elam 1993; Young *et al.* 1996; Leimu *et al*. 2006; Leinonen *et al.* 2008; Aguilar *et al.* 2008). Knowledge of how variation is structured is also useful when developing conservation strategies that aim to avoid further losses of genetic diversity (e.g., Neel & Cummings 2003a,b) or, alternatively, when inferring how different components of diversity will be influenced under a continued scenario of habitat degradation. In the latter approach, it is useful to consider a range of conservation strategies or ”loss scenarios”. The expected reduction of genetic variation should, for example, depend on whether the loss of habitat is random or nonrandom with respect to the genetic or phenotypic characteristics of the

affected (sub)populations. While population structure is a central concern when designing sampling strategies for maintaining allelic diversity at marker loci (Bengtsson *et al.* 1995; Neel & Cummings 2003a,b), I am not aware of any attempts to assess how measures of quantitative variation will be affected by further reductions in the number of populations or in the areas available to individual populations, based on the phenotypic variation observed in the present-day landscape.

Conventional analyses of phenotypic variation, for example common-garden studies and quantitative-genetic analyses, use measurements from a large number of individuals, derived from known parents and grown in some "standard" environment, to extract the genetic, and preferably the additive-genetic, component of the variation (Lynch & Walsh 1998). This approach can provide "clean" estimates of the phenotypic variation available for selection, but is time- and labour-intensive, and usually precludes the inclusion of more than one or a few populations in the analyses. Besides, common-garden estimates of genetic variation may be misleading if they have been measured in unnatural laboratory or garden environments. Earlier studies have shown that phenotypic estimates of variation generally agree with their quantitative-genetic counterparts (Weigensberg & Roff 1996; Waitt & Levin 1998; Leinonen *et al.* 2008) and that purely phenotypic data often confirm hypotheses about the effects of selection and other genetic processes (e.g., Yeaman & Jarvis 2006; Betti *et al.* 2009). One way of circumventing the problems associated with the conventional approaches may, therefore, be to approximate the genetic variance from the variation observed among naturally-occurring phenotypes. The disadvantage of this field-based method is that the confounding effects of nongenetic and nonadditive genetic factors may be large for particular species or characters. The remainder of this paper will be based on the premise that this price is worth paying, and that the drawbacks of this approach are more than compensated for by the advantage of being able to sample a larger number of populations — which is necessary if the objective of a study is to infer the genetic consequences of landscape-scale processes such as habitat fragmentation.

Unimproved, semi-natural grasslands are among the most species-rich habitats in northern and central Europe (Bruun *et al*. 2001) and represent the main remnants of the old, traditional agricultural landscape in Sweden (Bernes 1994). Cessation of grazing and mowing, caused mainly by the reduction in extensive animal husbandry, has resulted in a massive loss of semi-natural grasslands during the last century. The remaining grassland fragments have

become increasingly fragmented and are often in the process of undergoing succession towards forest (Bernes 1994; Cousins 2001). Although considerable attention has focused on the reduction of species and gene diversity in fragmented grassland landscapes (e.g., van Treuren *et al.* 1994; Luijten *et al.* 2000; Weidema *et al.* 2000; Kiviniemi & Eriksson 2002; Lindborg & Eriksson 2004; van Rossum *et al.* 2004; Honnay *et al.* 2006; Prentice *et al.* 2006, 2007; Johansson *et al.* 2008; Reitalu *et al.* 2009), only a few authors have examined measures of quantitative variation in fragmented populations of grassland plants and the few studies that exist are restricted to direct components of fitness (Leimu *et al.* 2006) or based on small samples of populations (Widén & Andersson, 1993; Waldmann & Andersson 1998; for an exception, see Willi *et al.* 2007). No effort has yet been made to predict how levels of quantitative genetic variation (or proxies thereof) might be affected by a further loss of grassland area.

The present study of the grassland herb *Saxifraga granulata* (Saxifragaceae) aims to infer the quantitative-genetic effects of both past and future habitat fragmentation, using spatially-structured samples of phenotypes from an old, agricultural landscape known to have undergone a massive loss of grassland area (Johansson *et al.* 2008), and characters known to possess significant heritability under semi-natural (garden) conditions (Andersson 1996). I focused on the following questions: Is the structuring of phenotypic variation consistent with a history of genetic drift and recent founder events (reflected by associations with measures of population size and/or habitat connectivity) or divergent selection (reflected by associations with indicators of local ecological conditions)? How much of the current between-population variation will be lost under a continued scenario of habitat degradation? Will a reduction in the spatial extent of individual populations — caused by localized habitat losses — cause a significant decline in the within-population variation?

Material and methods

Plant material

The self-compatible, herbaceous perennial *Saxifraga granulata* L. (2n = 32-60) (henceforth *Saxifraga*) occurs in a variety of dry, base-rich grasslands in western Eurasia and northern Africa. Flowering occurs from late May to early June, when the plants develop one or several flowering stalks from basal rosettes with petiolate leaves and axillary bulbils. Each flower has five white petals, 10

stamens and a central pistil with two partly united carpels, each having its own style. Protandy coupled with herkogamy enhances the opportunity for outcrossing. Fertilized flowers develop into capsules, each with hundreds of small seeds. Flower visitors include a wide variety of generalist pollinators, mainly members of Diptera and Hymenoptera (Lindgaard Hansen & Molau 1994).

The present investigation involves 42 populations of *Saxifraga*, sampled in 2005 and 2006 within an old, agricultural landscape on the Baltic island of Öland (SE Sweden). The study area, which covers about 22 km^2, includes the village of Jordtorp and four adjacent villages in the parish of Algutsrum. As a result of the cessation of traditional management practices, this area has lost more than 80% of its semi-natural grasslands over the last 300 years (Johansson *et al*. 2008) and remaining grasslands exist as isolated patches in a matrix of arable land, deciduous forests and improved (fertilized) grassland (Fig. 1).

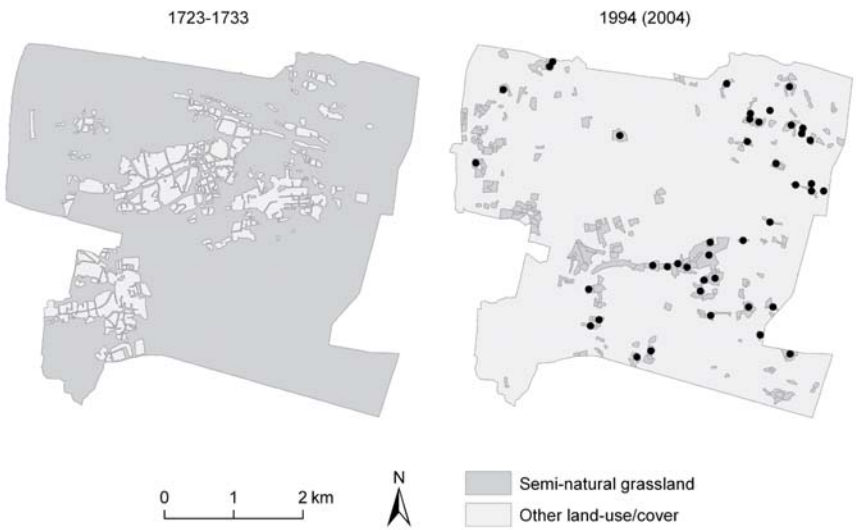

Figure 1. Maps showing the past and present distribution of grasslands within the Jordtorp area (redrawn using data from Johansson *et al.* 2008). Circles denote locations of sampled *Saxifraga granulata* populations.

The grassland fragments vary greatly in their spatial extent, connectivity and current ecological attributes such as soil moisture and grazing pressure (Prentice *et al.* 2007; Johansson *et al.* 2008). Many sites are abandoned (ungrazed) and therefore undergoing succession towards forest, as indicated by increased cover values of trees and shrubs (Reitalu *et al.* 2009). Most of the study populations represent true grassland fragments with a continuity of at least 274 years. A few represent young, secondary grasslands that have developed during the 20th century on previously arable or forested land (Johansson *et al.* 2008).

Phenotypic measurements

Populations were sampled by collecting flowering stalks along one or two transects within each population at peak flowering (early June). The transects were laid out along the longest axis of each population and varied in length between 10 and 110 m, depending on the spatial extent of the population. At every 10 m along the transects, I collected 5-6 stalks (each from a separate leaf rosette) within a sampling plot covering an area of 5 x 5 m. The sampled rosettes were separated by a minimum of 0.5 m to avoid possible duplicate sampling of the same genotype. When the density of flowering plants was low, I established the sampling plots at 5-m intervals and/or sampled fewer stalks per plot. The sampled stalks were placed in plastic bags and transferred to a laboratory, where I measured their height and preserved one fully-developed, female-stage flower on each stalk in a labelled microcentrifuge tube filled with 70% ethanol. The preserved flowers were measured under a stereo-microscope to obtain data on petal length, petal width, stamen length, style length and stigma size (one measurement per flower).

Stalk height and measures of petal size may determine the risk of being grazed and/or the plants' visual display to local pollinators, while the remaining variables may influence the frequency with which visiting insects contact the anthers (stamen length) or stigmas (style length, stigma size), as required for successful outcrossing (Ågren *et al.* 2006; Andersson 1996). Previous work on *Saxifraga* also indicate a potential for indirect selection on some of the characters, mediated through differences in general plant vigour (Andersson 1996). Based on these considerations and the extensive between-patch variation in spatial extent, habitat connectivity and ecological attributes, there should be ample opportunity for both stochastic and selective processes to influence the quantitative characters measured in this study.

All six characters showed significant genetic variation in a southern Swedish population (Skåne), with narrow-sense heritabilities (h^2) ranging from 0.24 for stalk height to more than 0.75 for stylar characters (Andersson 1996). To test the assumption that genetic factors also made a potentially large contribution to the variation observed in the present study, I measured the six characters on pairs of adjacent stalks from a separate sample of spatially separated rosettes (each assumed to represent a single genotype) within each of seven populations and used the resulting data to quantify the between-rosette component of variation (see below).

Phenotypic data were obtained for a maximum of 1863 flowering stalks in the spatially-structured population samples (representing 47 transects and 43 populations) and 272 stalks in the paired-stalks sample (representing seven populations). All variables were approximately normally distributed and no transformation was necessary.

Statistical analyses

Phenotypic data from the spatially-structured population samples were subjected to nested analyses of variance (ANOVA, type III sum of squares) to assess the effects of collection year (2005 vs 2006), transect (nested within year) and sampling plot (nested within transect). As all but two populations were represented by a single transect, the main effect of transect will henceforth be referred to as a "population" effect. Collection year was regarded as a fixed factor, whereas population and plot effects were regarded as random. The variance attributable to each random-effect factor was estimated with restricted maximum likelihood procedures, based on the same model as was used in the ANOVAs, and then divided by the total variance to obtain relative variance components. Similar approaches were used to test and quantify the variance attributed to the effects of population and rosette (nested within population) for the paired-stalks sample. Following the detection of significant year effects in the initial analyses (see Results), a one-way ANOVA was performed for each variable with year as a group variable to provide data (residuals) adjusted for between-year differences. The year-adjusted residuals were used to characterize individual phenotypes in all subsequent analysis.

Canonical variates analyses (CVA) were used to search for patterns that might indicate a history of genetic drift, for example, unusually high levels of divergence among small, isolated or young populations. A CVA simplifies multivariate data by assigning the majority of the variation to a set of new composite variables, referred to as canonical variates, that maximize the

differences between groups defined a priori (Dunn & Everitt 1982). Individual stalks were within-group replicates and the groups were defined as individual populations. I displayed the centroid of each population in a scatter plot of the first two canonical variates and used Wilks' lambda to estimate the proportion of variance attributable to differences between populations (calculated as 1-Wilks' lambda). Following the overall analysis, I performed separate CVAs for groups of populations differing in size or level of habitat connectivity, approximated by the total number of stalks sampled or the percentage of grassland perimeter bounded by adjacent grassland, respectively (see below). For the latter analyses, the median was used as a cutoff to provide two contrasting categories for each variable.

To identify determinants of variation at the level of individual characters, I carried out a series of regression analyses with the phenotypic mean or variance of a population as a response variable and the following descriptors as independent variables: population size (the total number of stalks sampled; (range 6-91), the area of the grassland fragment occupied (range 1817-138973 m^2), the percent of the perimeter of the grassland fragment bounded by adjacent grassland (range 0-74%), soil moisture (scored as 1 [dry], 2 [moderately moist] or 3 [moist]), current grazing intensity (scored as 1 [ungrazed], 2 [slightly or moderately grazed] or 3 [well-grazed]), and two indicators of longer-term management intensity, the percent cover of trees and shrubs, respectively (both scored as 1 [0%], 2 [1-10%], 3 [10-25%], 4 [25-50%] or 5 [50-70%]). Stalk number is largely a function of population (transect) length and (to a lesser extent) the density of flowering rosettes within sampling plots, and therefore provides a relative integrated measure of population size. The remaining descriptors were quantified using GIS analysis and interpretation of field-validated IR aerial photographs (M. Ihse, M. Kindström and H.C. Prentice, unpubl. data). To detect (and remove) covariation associated with geography, the positions along the W-E and S-N axes of the study area were also included in the analyses.

First, I used multiple regressions to assess the direct relationship between each descriptor and the response variable, holding all the other descriptors constant. Second, I used a stepwise selection procedure to search for subsets of descriptors with high predictive value, based on a forward selection procedure and $P = 0.05$ as a cutoff for entry of additional variables in the regression model. Multicollinearity was not considered a serious problem, given the relatively low associations between the descriptors used in this study (Pearson $r = 0.005$-0.530).

Population size, together with fragment area and percent adjacent grassland were assumed to reflect differing potential for genetic drift as a consequence of small population size and/or low habitat connectivity, whereas soil moisture and measures of management (grazing) intensity were assumed to characterize past or current selection regimes (Ellmer *et al.* 2009). A majority of these descriptors (or variants thereof) have been found to affect, or at least correlate with, other measures of diversity in the study area, for example, measures of gene diversity within the grass *Briza media* (Prentice *et al.* 2006) and various aspects of fine-scale plant diversity within remnant grassland fragments (Prentice *et al.* 2007; Johansson *et al.* 2008; Reitalu *et al.* 2009).

All statistical analyses were carried out with SPSS (version 15.0) for Windows.

Simulations

A series of habitat loss simulations were carried out to determine how the currently observed variation might respond to a further loss of grassland area, both at the regional level and at the level of individual populations. In the region-level analyses, I explored how the variance among population means changed during the successive removal of populations from the regional sample of populations. The order of removal was specified by each of three scenarios. In the first, second and third scenarios, the removals followed the rank order of increasing population size, grassland area and percent adjacent grassland, respectively, based on the assumption that demographic or ecological stochasticity make small, spatially restricted or isolated population fragments most sensitive to extinction (Saunders *et al.* 1991). In the fourth scenario, the removals followed the rank order of increasing management intensity, based on the finding that the long-term persistence of *Saxifraga* and other grassland specialists tends to decline with increasing frequencies of more competitive, grazing-sensitive species (Reitalu *et a.* 2009). The rank order for management intensity was based on the product of the grazing score (three levels) and the reversed scores for percent bush or tree cover (five levels) to integrate the effects of both past and current management intensity; simulations based on a single management indicator, for example, the current grazing score, gave similar results and provided little additional insight (data not shown). For comparison, I also examined how the regional variance estimate changed during the random exclusion of 1 to N-1 populations, drawn without replacement from the total sample of N = 47 populations (Neel & Cummings 2003a,b). This procedure was repeated 1000 times. To allow comparison between characters, all variance

estimates were scaled by the variance over the entire sample of populations (the baseline variance).

To assess how a localized reduction of grassland area might affect the variation represented in a population, I determined the relationship between the phenotypic variance and the number of adjacent sampling plots contributing to the sample of phenotypes from that population. Starting from the beginning or the end of the transect, I successively decreased the length of the transect segment that contributed to the overall sample (recalculating the phenotypic variance following each removal) until only the first (or last) sampling plot contributed to the sample. To simplify comparison, the variance estimate for each segment length was divided by the total variance of the population and then averaged across the forward and backward removal sequence for each transect. As no clear pattern emerged at the level of individual transects (data not shown), I also averaged data across transects to obtain a single "variance change curve" for each character.

It is important to stress that all simulations refer to the immediate effects of habitat loss, not on how the phenotypic variation might change through genetic drift or altered selection regimes during subsequent generations.

All habitat loss simulations were carried out with Excel™ for Windows and Resampling Stats™ for Macintosh.

Results

Nested ANOVAs revealed a significant effect of collection year for all variables except stalk height and stigma size (Table 1). Based on yearly means (data not shown), plants sampled in 2006 had 3-22% larger means for all characters than those sampled in 2005, a year when the spring was unusually warm and dry. Differences between populations usually explained a larger portion of the total variance than differences between plots within the same population, the only exception being stigma size which showed the opposite pattern (Table 1).

Analyses on the paired-stalks data (Table 2) confirmed the existence of strong population effects but also revealed significant variation between stalks from different rosettes for all variables. The effect of rosette accounted for 46-71% of the total phenotypic variation (Table 2).

Table 1. Results of nested ANOVAs for stalk height and floral characters, with collection year (2005 vs 2006), population (nested within collection year) and plot (nested within population) as categorical variables. The relative variance components (% variance) for the random-effect factors are also given.

Character	Source	df	F	P	% variance
Stalk height	Collection year	1	0.76	ns	
	Population (year)	45	24.96	***	55
	Plot (population)	321	2.83	***	12
	Residual	1492			33
Petal length	Collection year	1	34.31	***	
	Population (year)	45	16.80	***	39
	Plot (population)	321	1.79	***	8
	Residual	1493			53
Petal width	Collection year	1	30.18	***	
	Population (year)	45	7.63	***	22
	Plot (population)	321	1.93	***	12
	Residual	1493			66
Stamen length	Collection year	1	4.33	*	
	Population (year)	45	9.87	***	26
	Plot (population)	321	1.64	***	8
	Residual	1493			66
Style length	Collection year	1	55.63	***	
	Population (year)	45	6.24	***	20
	Plot (population)	321	2.00	***	13
	Residual	1482			67
Stigma size	Collection year	1	1.01	ns	
	Population (year)	45	2.74	***	7
	Plot (population)	321	1.98	***	15
	Residual	1480			78

Table 2. Results of nested ANOVAs for the paired data on stalk height and floral characters, with population and stalk pair (nested within population/year) as categorical variables. The relative variance components (% variance) for the random-effect factors are also given.

Character	Source	df	F	P	% variance
Stalk height	Population	7	15.59	***	36
	Rosette (population)	128	9.85	***	52
	Residual	133			12
Petal length	Population	7	3.82	***	10
	Rosette (population)	128	8.43	***	71
	Residual	136			19
Petal width	Population	7	4.46	***	11
	Rosette (population)	128	6.33	***	65
	Residual	136			24
Stamen length	Population	7	4.39	***	11
	Rosette (population)	128	3.17	***	46
	Residual	136			43
Style length	Population	7	6.29	***	16
	Rosette (population)	128	4.05	***	51
	Residual	136			33
Stigma size	Population	7	6.29	***	2
	Rosette (population)	128	4.05	***	58
	Residual	136			40

Product-moment correlation analyses of year-adjusted data showed positive associations between characters, ranging from r = 0.08 (stamen length vs. stigma size) to r = 0.60 (petal length vs. petal width) ($P < 0.001$ to 0.01 in all cases). Stalk height was moderately positively correlated with the floral characters (r = 0.32-0.51).

A CVA based on all populations revealed considerable divergence in the multivariate phenotype: as much as 81% of the total variation was due to

differences between populations (1- Wilks' lambda = 0.81). A display of the variation along the first two canonical variates (Fig. 2), which together explained 82% of the variation (Table 3), showed a gradual transition from a loose, central cluster of populations to a few scattered populations with either high or low values on the first or second axis. Replicate transects from the same population (four cases marked with lines in Fig. 2) were separated by relatively short distances in the CVA plot.

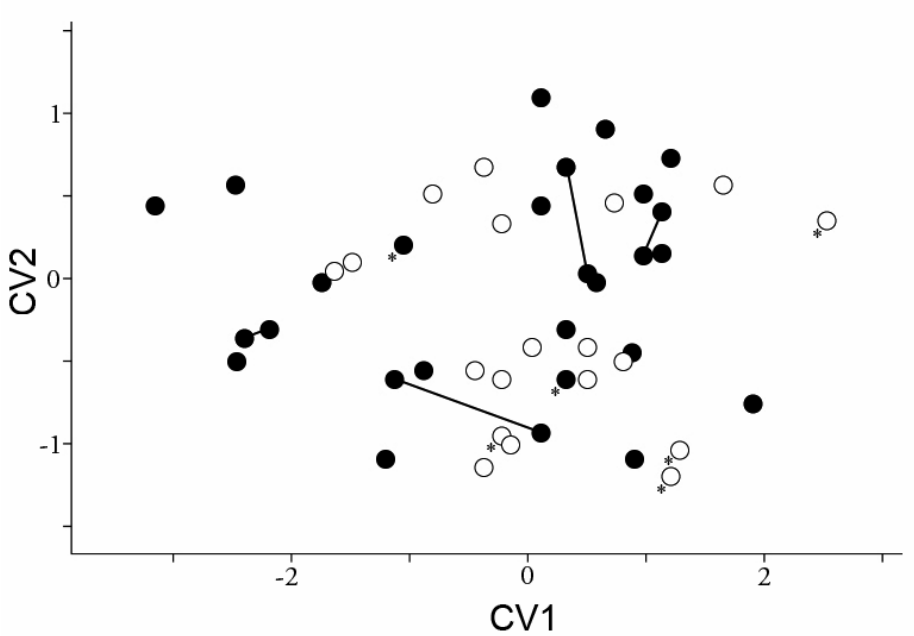

Figure 2. Canonical variates plot of the populations of *Saxifraga granulata* used in this study. The populations are represented by their centroids: small populations = open circles; large populations = closed circles. Asterisks denote populations from young, secondary grassland patches. Continuous lines connect replicate transects from the same population (when available).

Populations classified as small or large overlapped greatly and showed similar levels of divergence in the ordination plot (Fig. 2). A separate CVA for each size category confirmed this pattern: the proportion of variation attributed to differences between populations was similar for the small-size group (1-Wilk's lambda = 0.79) and the large-size group (1-Wilks' lambda = 0.81). As for the contrast between populations from weakly vs. strongly connected grassland fragments, there was no separation in the CVA plot (not shown) nor did separate CVAs reveal elevated levels of between-population variation in the low-connectivity group (1-Wilks' lambda = 0.74) compared with the high-connectivity group (1-Wilks' lambda = 0.86). Populations growing on young, secondary grasslands were intermingled with those representing old, relict grasslands in the CVA plot (Fig. 2).

Table 3. Character loadings for stalk height and floral characters on the first canonical variates (CV1-3) in a CVA using population as a class variable.

Character	CV1	CV2	CV3
Stalk height	0.915	-0.651	0.112
Petal length	0.339	0.538	0.038
Petal width	-0.067	0.414	0.430
Stamen length	0.131	0.084	-0.817
Style length	0.032	0.478	0.019
Stigma size	-0.333	-0.071	0.365
Variance explained (%)	68.5	13.3	10.7

Judging from the regression analyses, plants in spatially extensive grassland fragments had shorter stems and smaller-sized flowers than plants growing in small fragments (Table 4). The means for all characters increased with increasing grazing pressure and increasing cover by shrubs and trees, with regression coefficients significantly greater than zero in many cases. There was a significant northward increase in mean petal length, stamen length and style length, but no significant effect of population size, soil moisture, percent

Table 4. The relationship between different population/fragment descriptors and population-specific means for stalk height (SH), petal length (PL), petal width (PW), stamen length (STA), style length (STY) and stigma size (STI), as indicated by standardized regression coefficients from multivariate regression analyses (N = 47 in all cases). Each column corresponds to a separate regression. An underlined value denotes a case in which the descriptor was a significant predictor in the stepwise analyses. The proportion of variance explained by each regression model (R^2) is also given.

Descriptor	Response variable					
	SH	PL	PW	STA	STY	STI
Population size	-0.079 ns	0.156 ns	0.121 ns	0.110 ns	0.144 ns	0.108 ns
Fragment area	-<u>0.394</u> **	-0.243 *	-0.089 ns	-0.253 ns	-0.196 ns	0.038 ns
% adjacent grassland	-0.128 ns	0.157 ns	0.200 ns	-0.019 ns	0.076 ns	-0.013 ns
Soil moisture	0.328 ns	0.061 ns	-0.044 ns	-0.048 ns	0.139 ns	0.299 ns
Grazing pressure	0.178 ns	0.332 *	0.391 *	0.154 ns	<u>0.485</u> **	<u>0.576</u> **
Bush cover	0.399 *	0.265 ns	0.277 ns	0.212 ns	0.299 ns	0.506 **
Tree cover	0.351 *	0.294 **	0.255 ns	0.221 ns	<u>0.519</u> ***	0.357 *
Position along W-E axis	-0.144 ns	-0.244 ns	-0.165 ns	-0.094 ns	-0.069 ns	-0.093 ns
Position along S-N axis	0.284 ns	<u>0.556</u> ***	0.334 ns	<u>0.586</u> **	<u>0.400</u> **	-0.086 ns
R^2	0.442	0.542	0.284	0.436	0.523	0.325

*** P < 0.001, ** P < 0.01, * P < 0.05, ns P > 0.05

Table 5. The relationship between different population/fragment descriptors and population-specific variances for stalk height (SH), petal length (PL), petal width (PW), stamen length (STA), style length (STY) and stigma size (STI), as indicated by standardized regression coefficients from multivariate regression analyses (N = 47 in all cases). Each column corresponds to a separate regression. The proportion of variance explained by each regression model (R^2) is also given.

Descriptor	Response variable					
	SH	PL	PW	STA	STY	STI
Population size	0.109 ns	-0.008 ns	-0.118 ns	-0.135 ns	0.178 ns	-0.062 ns
Fragment area	-0.198 ns	0.037 ns	-0.016 ns	0.048 ns	-0.283 ns	-0.008 ns
% adjacent grassland	0.153 ns	-0.180 ns	-0.072 ns	-0.020 ns	0.157 ns	-0.189 ns
Soil moisture	0.285 ns	0.211 ns	-0.348 ns	0.268 ns	0.121 ns	0.216 ns
Grazing pressure	-0.251 ns	-0.167 ns	0.208 ns	0.053 ns	-0.099 ns	-0.240 ns
Bush cover	0.234 ns	0.310 ns	-0.201 ns	0.158 ns	0.122 ns	0.252 ns
Tree cover	-0.065 ns	0.085 ns	0.161 ns	0.138 ns	0.429 ns	0.221 ns
Position along W-E axis	0.347 ns	0.078 ns	0.153 ns	0.030 ns	-0.063 ns	-0.107 ns
Position along S-N axis	-0.051 ns	0.157 ns	0.329 ns	-0.283 ns	0.037 ns	-0.181 ns
R^2	0.180	-0.075	0.012	-0.026	-0.006	-0.108

ns $P > 0.05$

adjacent grassland or the position along the W-E axis. Overall, the descriptors jointly explained 28-54% of the variation in the mean phenotype. The influences of fragment area, current grazing intensity, tree cover and S-N position were sufficiently strong for these descriptors to be retained as significant predictors in some of the stepwise analyses (underlined values in Table 4).

Multiple regressions based on the within-population variance as response variable revealed no significant associations with any of the descriptors considered in this study (Table 5). The fraction of variation explained was always low (R^2 < 18%) and none of the population or patch descriptors were retained as significant predictors in the stepwise analyses.

There was only a slight or moderate reduction in the regional estimate of the between-population variance following a hypothetical loss of populations, regardless of whether the order of extinction was determined by population size, habitat area, connectivity or management intensity (Fig. 3). The regional variance remained relatively close to the baseline until about 10 populations remained in the sample, the only exception being stigma size for which the regional variance started to decline at relatively large sample sizes, especially when the order of removal was determined by population size or habitat area (Fig. 3a,b). As expected, there was no consistent change in the regional variance estimate when populations were ”lost” at random (Fig. 3e). Samples with fewer than 10 populations showed extensive scatter around the baseline, reflecting the large sampling variability associated with the estimation of variances for small samples.

A successive reduction in transect length, simulating a reduction in the areas occupied by the individual populations, had a minor impact on the variance estimated for the populations: the mean variance remained close to the baseline or starting value until only one or two sampling plots (corresponding to a transect length of 10-20 m) contributed to the sample (Fig. 4). Below this point, there was a noticeable reduction in the mean within-population variance; however, spatially restricted samples still captured a substantial portion of the variation present in the populations (> 80%).

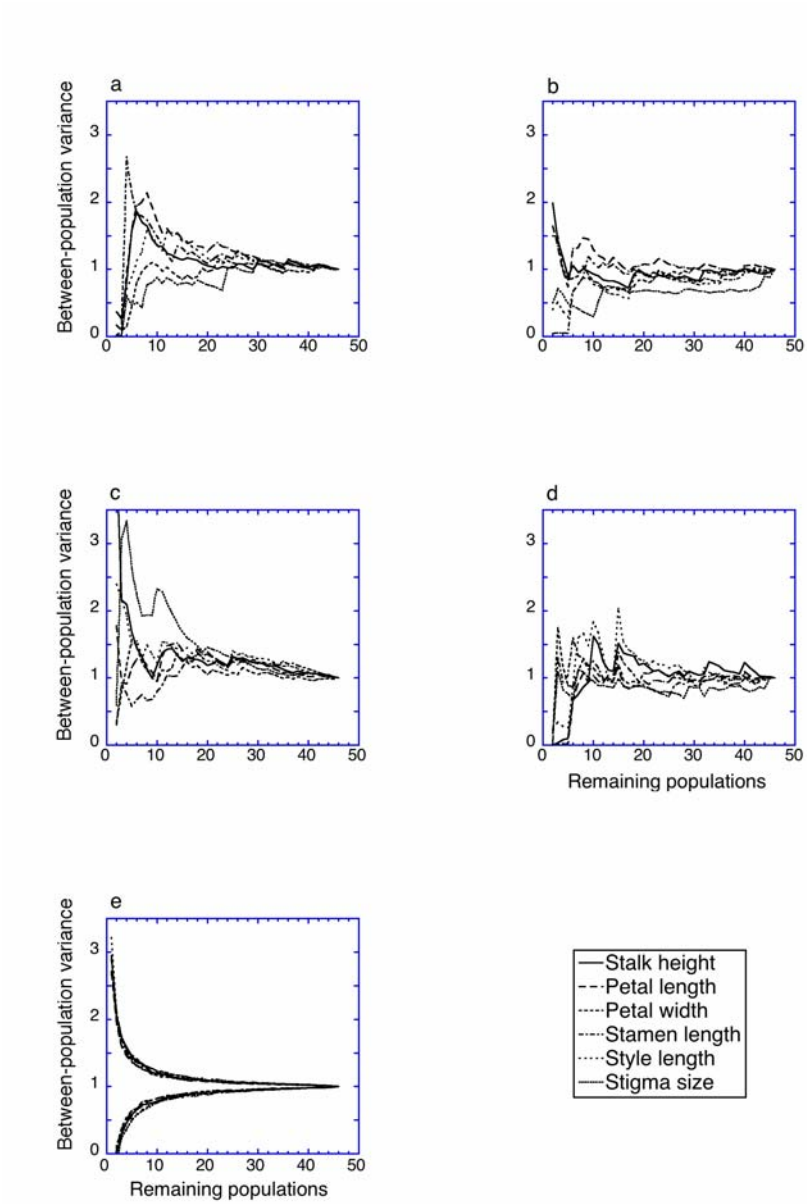

Figure 3. The regional estimates of the between-population variance following a successive removal of populations from the regional sample of populations. The order of removal followed the rank order of (a) population size, (b) habitat area, (c) habitat connectivity (measured by percent adjacent grassland), and (d) management intensity (measured by past and current grazing intensity). The lowermost graph (e) shows the 95% confidence intervals for the regional variance (the mean was very close to one, not shown) when populations were removed at random in a Monte Carlo simulation. All variances have been scaled so that a value of one equals the total variance (over the entire sample of populations). See text for details.

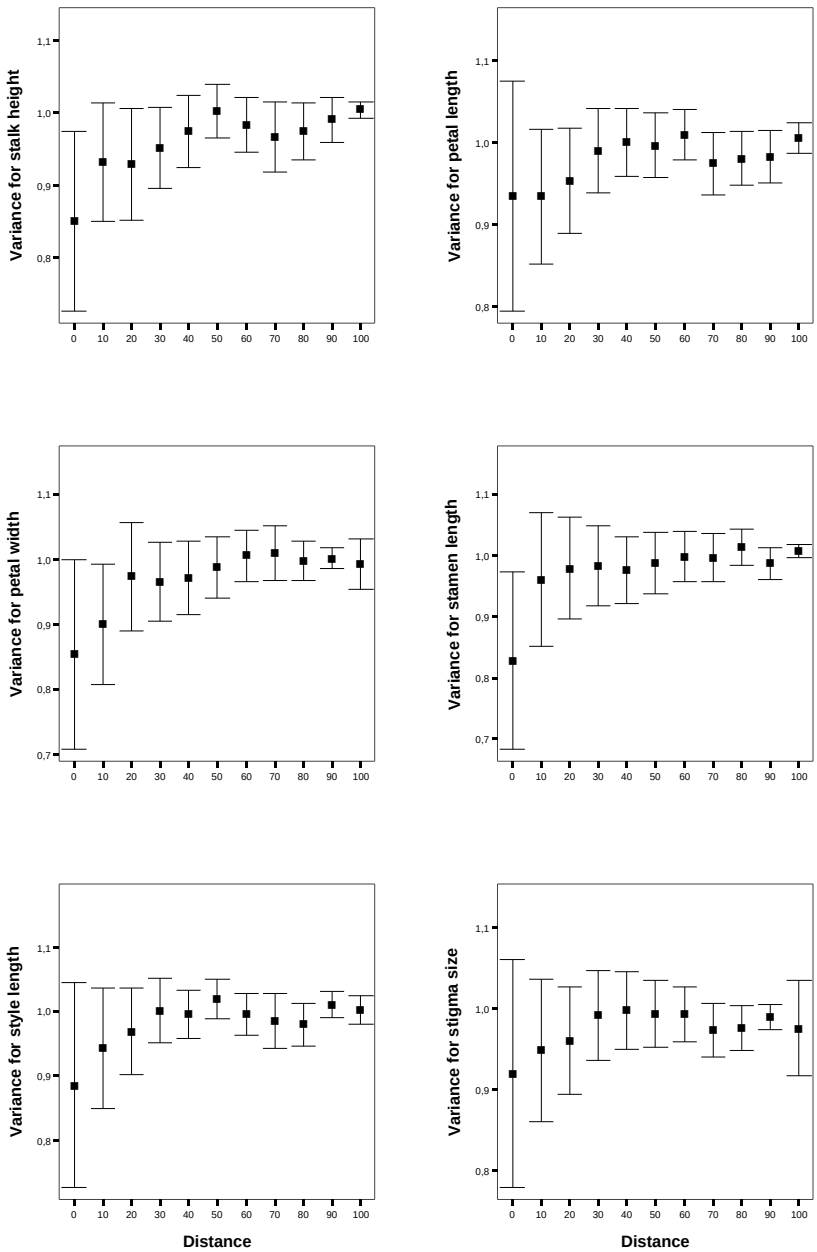

Figure 4. The relationship between the observed within-population variance (measured by the mean and its 95% confidence interval) and the length of the sampling transect contributing to the sample of phenotypes from the population. All variances have been scaled so that a value of one equals the variance over the entire transect. Data averaged across transects. See text for details.

Discussion

Understanding the ecological and genetic consequences of habitat fragmentation is crucial to conservation biology, especially in regions where many populations have become small and isolated as a result of human activities. Despite evidence for a link between small population size, reduced gene diversity and low population viability (Young *et al.* 1996; Leimu *et al.* 2006; Aguilar *et al.* 2008), it is still uncertain whether habitat fragmentation can affect ecologically important polygenic variation over the timescales considered by most conservation biologists (Storfer 1996; Willi *et al.* 2006; Ellmer *et al.* 2009). Further, no attempt has yet been made to predict how the remaining variation will be influenced under a continued scenario of habitat degradation, based on the structuring of variation in the present-day landscape. The aims of the present study of *Saxifraga granulata* were twofold: (i) to search for signals of drift, founder events and selection — processes that might have been enhanced by past and ongoing fragmentation — and (ii) to explore how a further reduction of grassland area will affect the variation present in the study area. The use of phenotypic variation as a proxy for quantitative genetic variation allowed extensive sampling of populations differing in size, connectivity and local habitat conditions.

Genetic effects of past and ongoing habitat fragmentation

Population genetic theory predicts small, isolated populations will be less genetically variable and more divergent than large populations as a result of genetic drift and/or founder events (Wade & McCauley 1988; Ellstrand & Elam 1993; Young *et al.* 1996). Although marker-based analyses of grassland species generally support these predictions (e.g., van Treuren *et al.* 1994; Luijten *et al.* 2000; van Rossum *et al.* 2004; for exceptions, see Weidema *et al.* 2000; Honnay *et al.* 2006), there was no tendency for the smallest or most isolated populations of *Saxifraga* to possess the lowest within-population phenotypic variation in the characters measured. Additionally, there was no evidence suggesting elevated levels of between-population variation for numerically small populations, populations in small or weakly connected grasslands, or populations established in young, secondary grasslands. Leaving aside any obscuring effects of phenotypic plasticity (see below), these observations provide little support for drift and (recent) founder events as major determinants of the structuring of quantitative variation within the study area.

Natural selection can cause extensive habitat-correlated variation in the mean phenotype (Linhart & Grant 1996; Leinonen *et al.* 2008), especially when populations are genetically isolated and/or exposed to unusually diverse environmental conditions, as may be the case in species subjected to habitat fragmentation (Jakobsson & Dinnetz 2005; Willi *et al.* 2007). As for the characters used in this study, stalk height can be expected to influence the risk of being grazed or the plants' visual display to pollinators (Ågren *et al.* 2006), while the floral characters could affect either display or the amount of pollen picked up or deposited per pollinator visit (Andersson 1996). Correlation data from this and a previous investigation of *Saxifraga* (Andersson 1996) also indicate the potential for characters to evolve by indirect selection on some other character. Therefore, one would expect local selection — mediated by edaphic factors, grazing animals and perhaps pollinators — to cause, or at least facilitate, divergence in the mean phenotype. Results from the regression analyses agree with a history of selection, even if the selective agents and the role of phenotypic plasticity (see below) remain to be elucidated. First, the mean for stalk height and floral size characters declined with increasing long-term management intensity (measured by tree or shrub cover), indicating a selective advantage of small plant size at sites with a long and continuous history of cattle grazing. However, there was a positive (rather than negative) association between current grazing intensity and most of the studied characters, a result in direct conflict with the supposition that grazing animals impose negative selection on size characters. Therefore, if grazing pressure plays a role in determining the mean phenotype of *Saxifraga* populations, its effect must be mediated through ecological factors other than size-dependent herbivory. Second, several characters showed negative relationships with the spatial extent of the grassland occupied, pointing to the influence of some ecological factor(s) correlated with habitat area, for example, edge effects. Third, there was a significant northward increase in the mean for some characters, suggesting the influence of large-scale spatial differences in habitat structure or management history (L.J. Johansson, T. Reitalu, M.T. Sykes, K. Hall and H.C. Prentice, unpubl. data).

It must be stressed that stalk height and floral characters were positively correlated and that the signs of most habitat-phenotype associations were consistent across characters: when a particular descriptor had a positive (or negative) effect on the mean stalk height of a population, it also had a positive (or negative) influence on one or several floral variables. Thus, most associations could result from some ecological factor operating on some general

aspect of plant or flower size rather than being a result of multiple selection pressures, each operating on a separate character. Further studies are needed to determine the relationships between the measured characters and individual fitness under field conditions to confirm that the observed habitat-phenotype associations have been caused, or at least strengthened, by diversifying selection forces.

The results for *Saxifraga* can be compared with those documented for the perennial, wind-pollinated grass *Briza media*, another grassland species that have been investigated in the study area. Data from an allozyme study of this species (Prentice *et al.* 2006) suggested weak, though significant, impacts of genetic drift (reflected by an association between gene diversity and percent adjacent grassland), but also indicated a role for convergent selection, manifested as a convergence of allele frequency profiles as young, secondary grasslands mature towards the more uniform communities characteristic of old, permanent grasslands. More recently, Ellmer *et al.* (2009) used clonal replicates of the same *Briza* material to determine whether drift and selection have left any imprint at the phenotypic level. The results from this follow-up study provided little evidence to suggest that the effects of local genetic processes have been sufficiently strong to influence the structuring the quantitative variation within *Briza*: differences between demes accounted for a minor fraction of the variation (less than 2% in variance component analyses) and showed no significant association with differences in current landscape structure, past land-use history and local habitat conditions. The lack of detectable pattern was largely attributed to the high mutation rate inferred for polygenic characters (Lynch & Walsh 1998) and the possible lack of time for drift or selection to influence the structuring of quantitative genetic variation in the study area (Ellmer *et al.* 2009). Whatever the factor(s) underlying the more consistent habitat-phenotype associations found in the present investigation, it seems that measures of phenotypic variation in *Saxifraga* and *Briza* have responded differently, or at different rates, to the same change in landscape structure.

The use of phenotypic variation as a proxy for genetic variance allows extensive sampling of populations but relies on the assumption that the observed variation has a strong heritable basis, i.e. that the effects of genetic drift and selection are sufficiently strong to override the effects of phenotypic plasticity. Indirect evidence in favour of this assumption comes from observations that phenotype-based data often reveal patterns similar to those found using quantitative-genetic approaches (Weigensberg & Roff 1996; Waitt

& Levin 1998; Leinonen *et al.* 2007). As for the present study, I note that most of the within-population variation could be attributed to differences between rather than within rosettes, an observation consistent with — but not proving — the existence of genetically-based differences. Additional support for a strong heritable component is provided by a factorial crossing experiment with a *Saxifraga* population in another region: the variance attributed to additively acting genes was unusually high for stylar characters ($h^2 > 0.75$) and moderate for the other characters ($h^2 = 0.24$-0.54) (Andersson 1996). However, given the significant difference between years detected for several characters in the present study, I cannot dismiss the possibility that some of the observed variation has been caused by environmentally-induced responses to local growth conditions, even though the year effect itself could be removed statistically.

The effects of future habitat fragmentation

Given the slow population dynamics of many species in fragmented landscapes, the current levels of species or genetic diversity may be too high to be maintained in the long term, and future losses of diversity may therefore be expected even if the present-day landscape is maintained (Lindborg & Eriksson 2004; Keyghobadi *et al.* 2005). In addition to considering the existence of such ”extinction debts”, it is also meaningful to ask how measures of diversity will be affected by a continuing loss of habitat area. To address this question in the present investigation, I performed a series of habitat loss simulations, both at the regional level and at the level of individual populations, to determine the number and spatial extent of populations needed to reliably represent the phenotypic variation in the study area.

Although differences between populations accounted for a substantial portion of the phenotypic variation, there was only a slight or moderate reduction in the regional estimate of the between-population variance following a simulated loss of populations, regardless of whether the extinction order was determined by differences in population size, habitat area, connectivity or management intensity. The regional variance decreased for most of the characters but only when the number of populations was small (< 10). For stigma size, the variance started to decline at smaller sample sizes, especially when the removals followed the rank order of population size or grassland area. These declines were, however, often overshadowed by the extensive fluctuations caused by the small sample sizes.

In *Saxifraga*, which lacks special adaptations for long-distance seed dispersal, there is a potential for vegetative reproduction by axillary bulbils to create patches of identical genotypes, and further patchiness may be caused by local drift, micro-scale selection or individual (plastic) responses to microsite heterogeneity. If such patchiness is present, then a localized loss of habitat area might be expected to cause a loss of variation not present in other parts of the population. However, although differences between plots within populations turned out to be significant for all characters, this variation was not structured in a way that markedly affected the variance estimates following a simulated reduction of the area occupied by a population: plants sampled over a distance of just 10 or 20 m still captured more than 80% of the variance present in the population.

Few other studies have used data from natural populations to examine how measures of genetic diversity (or proxies thereof) will be affected under different scenarios of habitat loss or population conservation. Neel & Cummings (2003a) used allozyme data from four rare plant species in Monte Carlo simulations to assess how well allele numbers are represented when different numbers of conspecific populations are sampled for conservation purposes. On average, currently advocated conservation intensities represented 67-83% of all alleles and 85-93% of all common alleles (alleles having a frequency of more than 0.05 in at least one population). Their results further showed that it was necessary to conserve 16-29 (53-93%) of the sampled populations to have a 90-95% probability of capturing all common alleles, a generally accepted standard of genetic conservation. In a parallel study, Neel & Cummings (2003b) assessed the genetic consequences of applying ecological reserve design guidelines (selected by four conservation professionals) to the four study species. Selecting populations according to reserve guidelines generally did not capture more allozyme diversity than selecting populations at random; in fact, two expert strategies performed worse than random when the diversity measure was common alleles. Consequently, the number of populations selected was far more important than how those populations were selected. Whether this conclusion also applies to the conservation of adaptive phenotypic variation remains to be investigated.

The results of the present study suggest that substantial losses of grassland area will be required to significantly reduce the phenotypic variation represented in the study area. This interpretation may also apply to *Briza*, given the large proportion of quantitative variation stored within rather than between populations and the lack of consistent habitat-phenotype associations

in this species (see above). Given that these results can be generalized to other plant species, it seems that changes in quantitative genetic parameters may be of lesser concern than the more immediate effects of fragmentation on population viability and species diversity (Saunders *et al.* 1991; Harrison & Bruna 1999; Leimu *et al.* 2006). In this context, it must be stressed that variance estimates for quantitative characters only account for differences in the realized phenotype, not for the presence/absence of particular alleles as in marker-based analyses. It is quite likely that the same phenotype can result from different combinations of alleles (genetic redundancy sensu Goldstein & Holsinger 1992) and that it would be easier to detect strong fragmentation effects at the individual loci underlying the phenotypic characters. However, because selection operates on phenotypes, not genotypes, it seems highly relevant to focus on variances or variance-related parameters if the aim is to determine how well small or spatially restricted samples capture the variation available to selection at the phenotypic level. To explore how natural sampling processes, such as those occurring during habitat fragmentation, affect the allelic diversity at loci influencing quantitative phenotypic characters, it will be necessary to develop marker systems with sufficient power to distinguish between alternative alleles at a potentially large number of loci — a difficult (but not impossible) task if the alleles have small effects on the phenotype (for possible approaches, see Vasemägi & Primmer 2005; Bonnin *et al.* 2007).

The simulation results only concern the immediate effects of habitat degradation, not on how the progressively smaller and more isolated populations will be affected by genetic drift and selection in subsequent generations. For example, the stochastic effects of drift can be expected to increase under a continued scenario of habitat degradation, adding to the genetic extinction debt that may have been generated during previous generations (Keyghobadi *et al.* 2005). In addition, it is very likely that measures of species diversity will respond differently to a simulated loss of grassland area, given the positive relationships between species richness and habitat area previously observed in the study area (T. Reitalu, O. Purschke, L.J. Johansson, K. Hall, M.T. Sykes and H.C. Prentice, unpubl. data). It would be interesting to perform more extensive habitat loss simulations — involving both genetic and ecological aspects of diversity — to predict how diversity in general will be affected by a further reduction in the number or size of grassland areas. Given the increasing number of datasets available for remnant grasslands in the Jordtorp area (Prentice *et al.* 2006, 2007; Johansson *et al.* 2008; Reitalu *et al.* 2009; Ellmer *et al.* 2009), this area provides a useful study

system in which to evaluate the multiple effects of past, ongoing and future habitat fragmentation and to develop conservation strategies that aim to minimize the loss of biodiversity in fragmented grassland landscapes.

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47. Britt Snogerup (1983) The genus *Odontites* (Scrophulariaceae) in north-west Europe.
48. Lennart Folkeson (1983) Heavy-metal pollution of forest ecosystems: effects on vegetation and mineralization of organic matter.
49. Gunilla Olsson (1984) Old-field forest succession in the Swedish west coast archipelago.
50. Urban Emanuelsson (1984) Ecological effects of grazing and trampling on mountain vegetation in northern Sweden.
51. Bo Wiman (1985) Aerosol dynamics in coniferous forests - empirical and theoretical analyses.
52. Björn Rörslett (1985) Regulation impact on submerged macrophyte communities in some Norwegian lakes.
53. Bo Sundström (1986) The marine diatom genus *Rhizosoleni.*
54. Gösta Regnéll (1986) Fen grassland in southern Sweden - the application of plant ecology to a problem of nature conservancy.
55. Sture Wijk (1986) *Salix herbacea* and the alpine snow-bed environment.
56. Bo Bergkvist (1986) Metal fluxes in spruce and beech forest ecosystems of South Sweden.
57. Hilde Nybom (1987) Apomixis in the genus *Rubus* and its effects on reproduction.
58. Roy Franzén (1987) Biosystematics of the *Achillea clavennae* and *A. ageratifolia* groups (Asteraceae).
59. Annette Carlström (1987) The flora and phytogeography of Rhodos, Simi, Tilos and the Marmaris peninsula (SE Greece, SW Turkey).
60. Jan Thomas Johansson (1987) Taxonomic and morphological studies in the tribe Morindae (Rubiaceae).
61. Göran Svensson (1987) Studies on fossil plant communities, stratigraphy and development of peatlands in South Sweden.
62. Brita Svensson (1987) Studies of the metapopulation dynamics of *Lycopodium annotium* and its microenvironment.
63. Poul Hansen (1988) Statistical modeling of soil-macrofungal realtionships in south Swedish beech forests.
64. Lars Fröberg (1989) The calcicolous lichens on the Great Alvar of Öland, Sweden.
65. Thomas Landström (1989) The species of *Ornithogalum* L. subgenus Ornithogalum (Hyacinthaceae) in Greece.
66. Ingibjörg Svala Jonsdottir (1989) The population dynamics, intraclonal physiology and grazing tolerance of *Carex bigelowii*.
67. Ursula Falkengren-Grerup (1989) Soil acidification and vegetation changes in South Swedish forests.
68. Linus Svensson (1990) Flora morphology and the effects of crossing distance in *Scleranthus*.
69. Stefan Andersson (1990) Population differentiation in *Crepis tectorum* (Asteraceae): past and current patterns of natural selection.

70. Bengt L. Carlsson (1990) Controls on the growth and population dynamics of *Carex bigelowii.*
71. Dan Berggren (1990) Species of Al, Cd, and Cu in forest soil solutions - analytical methods, mobilization mechanisms, and toxicity to plants.
72. Ullmer Norden (1992) Soil acidification and element fluxes as related to tree species in deciduous forests of south Sweden.
73. Jan-Erik Mattsson (1993) The genus *Vulpicida*.
74. Maud E. Andersson (1993) Aluminium and hydrogen ions - limiting factors for growth and distribution of beech forest plants.
75. Mostafa Asadi (1994) The genus *Elymus* L. (Poaceae) in Iran: Biosystematic studies and generic delimitation.
76. Nils Cronberg (1994) Genetic diversity and reproduction in *Sphagnum* (Bryophyta): Isozyme studies in *S. capillifolium* and *S. rubellum*.
77. Torleif Bramryd (1994) Effects on growth and nutrition of sewage sludge application in acid pine forests (*Pinus sylvestris*, L.) in a temperature gradient in Sweden.
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79. Jörg Brunet (1994) Importance of soil solution chemistry and land use to growth and distribution of four woodland grasses in south Sweden.
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82. Martin Ljungström (1994) Beech (*Fagus sylvatica*) seedling growth and nutrition - effects of acid soils and liming.
83. Ulf Arup (1995) Littoral species of the lichen genus *Caloplaca* in North America.
84. Leif Jonsson (1995) Effects of restoration on wooded meadows in southeastern Sweden.
85. Arne Thell (1996) Anatomy and taxonomy of cetarioid lichens.
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87. Stefan Ekman (1996) The corticolous and lignicolous species of *Bacidia* and *Bacidina* in North America.
88. Alex Haxeltine (1996) Modelling the vegetation of the Earth.
89. Christer Albinsson (1996) Vegetation structure and interactions on mires.
90. Helena Runyeon (1997) Variation in *Silene vulgaris* and *S. uniflora* (Caryophyllaceae): genetic diversity, gene flow and habitat selection.
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94. Gudrun Berlin (1998) Semi-natural meadows in southern Sweden – changes over time and the relationship between nitrogen supply and management.
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99. Anna-Carin Linusson (1999) Changes in plant community diversity and management effects in semi-natural meadows in southern Sweden.
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110. Gunnar Thelin (2000) Nutrient imbalance in Norway spruce.
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143. Lotten Jönsson Johansson (2008) Semi-natural grasslands: landscape, history and plant species diversity. (Joint PhD project with the Department of Physical Geography and Ecosystems Analysis.)
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