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GENETIC POPULATION STRUCTURE AND REPRODUCTIVE FITNESS IN THE PLANT *SANGUISORBA OFFICINALIS* IN POPULATIONS SUPPORTING COLONIES OF AN ENDANGERED *MACULINEA* BUTTERFLY

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The loss of genetic variation in small populations through drift and inbreeding is thought to decrease fitness and population viability. In order to evaluate the suitability of small *Sanguisorba officinalis* populations for the long-term conservation of an endangered *Maculinea* butterfly species, we investigated the plant's genetic population structure using amplified fragment length polymorphism (AFLP) and measured life-history traits related to reproduction. Genetic distances between populations were low (mean $F_{ST} = 0.008$) and not correlated with geographic distances, indicating that substantial gene flow compensates for the effects of genetic drift. Analysis of molecular variance indicated the absence of genetic differentiation among different habitat types and low differentiation among populations. High outcrossing rates ($t_m = 0.856$ and $t_m = 0.972$) obtained in two populations suggest that gene flow is promoted by the mating system. Populations differed in the level of intrapopulation genetic variation. These differences were not related to habitat type, population size, or plant density. Mean seed mass and the percentage of germination decreased in small and low-density populations. However, reduced fitness was not related to lower levels of genetic variation. Thus, the observed fitness decline was presumably due to lower habitat quality associated with small populations and low plant densities. The relevance of the results for the conservation of *Maculinea* butterflies is discussed.

Keywords: conservation, fitness, habitat fragmentation, large blue butterflies, *Sanguisorba officinalis*.

Introduction

Habitat fragmentation induced by human activity has become a serious threat to natural populations of many plant species. There is well-founded concern about the impact of small population sizes and increasing isolation on the level of intrapopulation genetic variation. Disruption of gene flow, accompanied by a relative increase of genetic drift, and elevated inbreeding are expected to reduce genetic variation and therefore lower the evolutionary potential of populations to cope with changing environmental conditions (Ellstrand and Elam 1993; Young et al. 1996). The accumulation of genetic load may lead to inbreeding depression acting on reproductive success and individual fitness (Ellstrand and Elam 1993; Reed and Frankham 2003). Hence, the loss of genetic variation may increase the risk of extinction (Frankham et al. 2002). A substantial body of literature provides empirical evidence that plants growing in small populations suffer from severe fitness declines such as reduced seed set (Ågren 1996; Morgan 1999; Kéry et al. 2000), diminished fertility (Menges 1991; Soons and Heil 2002; Vergeer et al. 2003), increased seedling mortality (Oostermeijer et al. 1994; Vergeer et al. 2003), or lowered competitive ability (Pluess and Stöcklin 2004). Moreover, fitness declines have been shown to accompany decreasing levels of genetic variation (Fischer and Matthies 1998; Schmidt and Jensen 2000; Hensen and Oberprieler 2005).

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The effects of isolation and reduced population size have been studied in particular for rare and declining plant species in a conservation context. However, common plant species have attracted far less consideration even though their populations may be affected by habitat fragmentation in the same manner (Lienert et al. 2002; Lienert and Fischer 2003; Hooftman et al. 2004; Galeuchet et al. 2005*a*, 2005*b*). Common and dominant species contribute disproportionately to ecosystem biomass production (Grime 1998; Smith and Knapp 2003) and may provide resources to a large number of organisms. For instance, widely distributed and abundant plant species harbor a greater diversity of herbivorous insects compared to geographically restricted and rare species (Strong et al. 1984). Hence, the decline of common species, or their reduced performance caused by genetic erosion, may have serious consequences for the maintenance of biotic interactions, thereby affecting community composition and biodiversity.

Sanguisorba officinalis is a long-lived perennial herb that occurs throughout the Palearctic, from Western Europe to Alaska and Japan (Hegi 1995). In central Europe, it is a characteristic component of wet grassland habitats. The species is insect pollinated, with syrphid flies, muscid flies, bees, and butterflies being the main visitors (M. Musche, personal observations). Inflorescences are arranged in a hierarchical order, and their numbers vary among plants. One inflorescence contains up to 100 flowers, each of them developing into one fruit containing a single seed. Flowering lasts from June to September. Self-pollination may occur spontaneously in the absence of cross-pollination (Nordborg 1963). Apart from sexual reproduction, plants are able to spread vegetatively by short rhizomes.

Sanguisorba officinalis is an indispensable resource for two endangered large blue butterfly species, *Maculinea nausithous* and *Maculinea teleius*, because it represents their only food plant. Caterpillars of the genus *Maculinea* initially feed on the inflorescences of their food plants until they reach the fourth instar. At this stage they leave the plant to be adopted by workers of specific *Myrmica* host ants. Within ant nests, caterpillars complete their life cycle as social parasites, either being fed by worker ants or by preying on the ant brood (Thomas and Settele 2004). By secreting hydrocarbons that are similar to those of the ant brood, caterpillars prevent predation by the ants during adoption and nest integration (Schonrogge et al. 2004).

Despite the commonness of *S. officinalis*, the occurrence of the butterflies is often restricted to small sites attributed to secondary succession, where the food plant occurs at low numbers and at low densities (Thomas 1984; Geißler-Strobel 1999). Frequent cutting of meadows does not negatively affect *S. officinalis*; however, it is thought that it prevents the establishment of butterfly populations by causing high mortalities among first-instar caterpillars (Johst et al. 2006) and by lowering habitat quality for the specific *Myrmica* host ants (Thomas 1984). Regarding the importance of small and sparse plant populations for the conservation of the butterfly species, the question arises whether these populations are genetically structured and whether genetic variation is maintained at a sufficient level to ensure high reproductive fitness and to conserve populations in the long term. To answer this question, we investigated 24 *S. officinalis* populations of different sizes and densities, all of them supporting colonies of the butterfly *M. nausithous*. We considered plant populations located in mown and abandoned habitats because agricultural management not only affects the persistence of butterfly populations but also has the potential to create genetic differentiation between plant populations, for example, by altering flowering phenology (Silvertown et al. 2005). In this article, we address the following questions: (1) How is the genotypic variation partitioned between habitats, among populations, and within populations? (2) Do populations located in managed and abandoned habitats show different levels of genetic variation? (3) Are smaller and sparser populations genetically less variable than large and dense populations? (4) What is the mating system of *S. officinalis*, and can it explain the pattern of genetic structure? (5) Is there variation in fitness characters that may be explained by population size, plant density, genetic variation, or habitat type?

Material and Methods

Populations of *Sanguisorba officinalis* were studied in the Upper Rhine Valley (Germany) around the city of Landau (lat. 49°11′56″N, long. 8°8′34″E). The study area is located in the center of the plant's European distribution. The degree of habitat fragmentation is low, and the species occurs frequently in a close network of meadows along small rivers. Plants and additional information were sampled in 24 populations, half of them located in managed meadows and half in fallows (table 1). Sites were selected according to the occurrence of *Maculinea nausithous*; only sites supporting populations of this butterfly were included in this study. Meadows and fallows were equally scattered across the study region. Plant populations were defined as the number of individuals inhabiting a site characterized by a uniform land use and distinct boundaries. The mean distance between study populations was 18 km, ranging from 0.6 to 46.4 km. At each location, population size and plant density were estimated (table 1). Population size was assessed by counting the number of flowering shoots. Plant density was estimated by calculating the mean number of flowering shoots per square meter based on counts within 50 2×2-m squares. Plants for molecular genetic analysis and measurements of reproductive fitness were sampled at maturity in late August 2003. In each population, 20 plants, if available, were chosen randomly, with a minimum distance of 5 m between them to avoid the collection of identical genotypes. Leaf and DNA samples were deposited at the Centre for Environmental Research in Halle (Germany) and are available for further analyses.

As estimates of reproductive fitness, we measured mean seed mass and the percentage of germination on a subsample of 12 plants per population. For this purpose, only the terminal inflorescence of each plant was used. Because *S. officinalis* shows sequential flowering, there is considerable variation in seed development within plants over time. Therefore, lateral inflorescences that generally flower later were excluded from the analysis to minimize the risk of incorporating immature seeds. Seeds were divided into three classes: developed seeds, nondeveloped seeds, and seeds damaged by caterpillars of the butterfly *M. nausithous*. Only developed seeds were considered for further analysis. Mean seed mass was calculated for each plant by dividing the total seed mass of developed seeds by their number.

Seeds were germinated after storing at 4°C for 3 mo to break dormancy. For the germination trials, petri dishes 6 cm in diameter were filled with a 1 : 2 mixture of sand and potting compost (COMPOSANA Anzuchterde, COMPO GmbH, Münster). From the 12 mother plants per population, all developed and undamaged seeds of the terminal flower head (between nine and 83) were used. Germination took place in a common environment (12L : 12D, 25°C). After 3 wk, the number of emerging seedlings did not increase. At this time, seedlings were counted, and the percentage of germination was calculated per seed family and averaged for each population.

Seedlings were taken from the germination trials and stored in a deep freezer at −80°C. For the analysis of the genetic population structure, between five and 12 (mean = 10.5) seedlings per population (total number 287) originating from separate seed families were used. Sample size was not correlated with population size ($r = 0.25$, $P = 0.22$), plant density ($r = 0.25$, $P = 0.21$), and gene diversity ($r = -0.21$, $P = 0.31$). Due to the low germination success in some populations, additional seed families from the total sample had to be germinated. Thus, seedlings used for the molecular genetic analysis did not completely descend from those seed families used for the measurement of fitness traits. Outcrossing rates were determined using offspring from two populations (nos. 12 and 16). These populations were selected because they provided the sufficient number of families and siblings necessary for a reliable calculation. From both populations, nine and

Table 1

Summary Data for the 24 Studied Populations of *Sanguisorba officinalis*

Population	Habitat	Population size[a]	Plant density[b]	No. plants analyzed	Gene diversity (H_e)	Mean seed mass (mg)	Germination (%)
1. Landau Reiterwiesen	Fallow	5	.06 (.06)	5	.289 (.020)	1.12 (.14)	5.8 (3.1)
2. Knittelsheim	Fallow	40	.64 (.34)	12	.298 (.021)	.81 (.09)	7.7 (3.7)
3. Zeiskam Rennbahn	Fallow	40	.60 (.24)	9	.289 (.022)	.90 (.13)	11.8 (4.8)
4. Annweiler	Fallow	52	1.29 (.63)	6	.329 (.019)	1.09 (.07)	19.7 (6.7)
5. Schweighofen	Fallow	120	.88 (.52)	10	.301 (.021)	1.41 (.01)	30.1 (7.5)
6. Steinfeld	Fallow	126	2.53 (.90)	9	.303 (.022)	1.32 (.15)	12.1 (4.0)
7. Landau Kläranlage	Fallow	185	2.95 (1.00)	12	.307 (.021)	1.24 (.14)	26.0 (9.2)
8. Schifferstadt	Fallow	550	2.80 (1.00)	8	.308 (.021)	1.35 (.12)	18.7 (4.9)
9. Lustadt Ludwigsmühle	Fallow	550	.62 (.13)	12	.306 (.020)	.98 (.08)	6.5 (3.6)
10. Landau Queichheim	Fallow	600	1.44 (.78)	12	.314 (.019)	1.41 (.13)	26.0 (6.4)
11. Hassloch Pfalzmühle	Meadow	700	.85 (.19)	9	.313 (.020)	1.71 (.16)	21.5 (6.4)
12. Offenbach	Fallow	800	2.38 (.35)	11	.277 (.021)	1.54 (.19)	19.7 (5.6)
13. Bienwaldmühle	Meadow	1000	4.57 (1.04)	11	.288 (.021)	1.14 (.07)	19.6 (5.1)
14. Dernbachtal	Meadow	1000	3.05 (.77)	10	.290 (.021)	1.21 (.11)	17.2 (6.4)
15. Eußerthal	Fallow	1100	3.12 (.69)	9	.287 (.022)	1.44 (.22)	28.8 (6.5)
16. Zeiskam Gärtnerei	Meadow	2100	2.62 (.53)	11	.296 (.021)	1.50 (.11)	27.2 (4.9)
17. Herxheim	Meadow	2200	2.00 (.37)	12	.311 (.020)	1.56 (.14)	26.5 (5.8)
18. Lustadt Lachenmühle	Meadow	2600	3.20 (.45)	12	.297 (.020)	1.35 (.15)	23.7 (6.1)
19. Oberotterbach West	Meadow	2800	10.44 (1.85)	12	.305 (.020)	2.04 (.18)	45.4 (7.6)
20. Freckenfeld	Meadow	3100	1.70 (.45)	11	.281 (.021)	1.73 (.17)	26.7 (5.7)
21. Zeiskam Mühle	Meadow	4500	3.62 (.64)	12	.299 (.020)	1.21 (.10)	12.6 (4.4)
22. Gräfenhausen	Meadow	4900	19.14 (2.79)	12	.315 (.021)	1.58 (.15)	31.3 (8.2)
23. Oberotterbach Ost	Meadow	5300	7.82 (1.57)	10	.317 (.020)	1.23 (.16)	26.6 (7.1)
24. Neuburg Rheindamm	Meadow	8200	7.57 (1.08)	10	.303 (.021)	1.77 (.17)	30.8 (6.1)

Note. Numbers in parentheses are standard deviations.

[a] Number of flowering shoots.

[b] Number of flowering shoots per square meter.

10 seedling families, respectively, containing between seven and 10 siblings per family were analyzed.

We applied amplified fragment length polymorphism (AFLP) as a neutral genetic marker system (Vos and Kuiper 1997). DNA was extracted from the cotyledons and stems of seedlings using the DNeasy 96 plant kit (Qiagen, Hilden, Germany) and quantified by a spectrophotometer (ND 1000, NanoDrop Technologies, Wilmington, DE). AFLPs were obtained according to the AFLP plant mapping kit protocol (Applied Biosystems, Foster City, CA) with the following small changes. For the initial restriction/ligation reaction, 100 ng of genomic DNA was incubated at 37°C for 2 h. DNA fragments were diluted 1 : 5, and preamplification products were diluted 1 : 10 in purified H_2O. The following primer combinations were applied: fluorescent (FAM) *Eco*RI-ACT/*Mse*I-CTA and fluorescent (JOE) *Eco*RI-AGG/*Mse*I-CTG. Outcrossing rates were determined using one selective primer combination (*Eco*RI-ACT/*Mse*I-CTA). AFLP profiles were obtained using an ABI PRISM 310 genetic analyzer. Only fragments ranging from 60 to 500 bp in size and exhibiting a sufficient intensity were considered in the further analysis.

Sixty-nine polymorphic loci were identified and scored for presence and absence using Genographer software (ver. 1.6.0, J. J. Benham, Montana State University). Genetic diversity and population genetic structure were assessed using the method of Lynch and Milligan (1994), based on allele frequencies determined with a Bayesian method with nonuniform prior distribution (Zhivotovsky 1999), and assuming Hardy-Weinberg genotypic proportions, with the software AFLP-Surv, version 1.0 (Vekemans 2002). We used gene diversity, which is equivalent to expected heterozygosity (H_e) under Hardy-Weinberg conditions (Nei 1987), as a measure of within-population genetic diversity. Assuming that mating patterns did not differ among populations, an analysis of molecular variance (AMOVA; Excoffier et al. 1992) was calculated to estimate the partition of genotypic variance between habitats, among populations, and among individuals within populations. To test whether genetic distances between populations follow a model of isolation by distance, the significance of the correlation between geographic and genetic distances (F_{ST}) was checked by a Mantel test based on 1000 permutations. AMOVA and Mantel tests were calculated using the software Arlequin, version 2.000 (Schneider et al. 2000).

Inbreeding coefficients and outcrossing rates were estimated for two populations (nos. 12 and 16) based on the analysis of 17 polymorphic AFLP loci in a total of 183 individuals. Multilocus locus outcrossing rates (t_m) were calculated using WinMLTR (Ritland 2002). Estimates and standard deviations were derived from 1000 bootstrap replicates. Percentile method was applied to test whether differences between both populations were significant.

A *t*-test was applied to compare mean gene diversity between habitats (meadows vs. fallows). Pearson correlation coefficients were used to analyze the relationship between gene diversity, logarithm of population size, logarithm of plant density, and population means of the two fitness traits seed mass

and percentage germination. The analyses were performed for all populations and separately for the two habitat types. In an ANCOVA, the combined effects of habitat type, population size, density, and H_e on seed mass were determined. We allowed for curvilinear effects of population size and plant density by incorporating their logarithmic terms. In the ANCOVA on the percentage of germination, additionally, mean seed mass was included as an independent variable. The initial models were reduced stepwise backward following Crawley (2002). The least significant terms were removed first. Model simplification aimed at reducing Akaike Information Criterion (AIC; Sakamoto et al. 1986). The residuals of the final models were checked for normality using a Shapiro test. Correlation coefficients and statistical models were calculated using the software package R, version 2.1.0 (R Development Core Team 2004).

Results

Populations were not genetically differentiated, as indicated by an overall F_{ST} value of 0.008 (0.322 SE), which did not differ significantly from 0. The hierarchical partitioning of genetic variation by AMOVA revealed the absence of significant genetic differentiation between the two types of habitat, meadows and fallows (table 2). An AMOVA derived mean φ_{CT} value of 0.026 indicated significant genetic differentiation among populations within habitats, however, which explained only 2.4% of the total molecular variance. More than 97% of the entire genotypic variation was found between individual plants within populations. Mantel statistics revealed that genetic population structure did not follow a pattern of isolation by distance ($r = 0.12$, $P = 0.10$).

Intrapopulation genetic variation measured as gene diversity (H_e) was variable, ranging from 0.277 to 0.329. This variability was not due to habitat type ($t = 0.03$, $P > 0.05$). A relationship between population size and gene diversity could not be demonstrated ($r = 0.11$, $P > 0.05$), and there was no correlation between plant density and gene diversity ($r = 0.25$, $P > 0.05$).

Parental inbreeding coefficients derived for two populations were −0.122 (population 12) and −0.199 (population 16; mean = −0.16). The multilocus (t_m) outcrossing rate of population 12 and 16 was 0.856 (±0.078 SD) and 0.972 (±0.075 SD), respectively, and did not differ significantly between populations ($P > 0.05$, percentile method).

Table 2

Analysis of Molecular Variance for Plants from Different Populations within Two Habitats

Source of variation	df	Sum of squares	Variance components	Variation (%)
Among habitats	1	17.1	.039	.39
Among populations within habitats	22	269.3	.241***	2.41
Within populations	228	2214.3	9.712***	97.20
Total	251	2500.7	9.992	

*** $P < 0.001$.

Mean seed mass across populations averaged 1.36 mg (±0.29 SD). There was a significant correlation between the logarithm of population size and mean seed mass ($r = 0.62$, $P < 0.001$; fig. 1). A similar relationship was found between the logarithm of plant density and seed mass ($r = 0.49$, $P < 0.05$; fig. 1), whereas gene diversity was not related to seed mass ($r = 0.05$, $P > 0.05$; fig. 1). Within habitat types, a significant positive association could only be found between the logarithm of population size and seed mass in fallows (table 3). Including all variables and the factor habitat into one ANCOVA showed that variation in mean seed mass was explained best singly by the logarithm of population size rather than habitat differences, gene diversity, or plant density. The final model explained 39% of the entire variance in seed mass (table 4). The population mean of percentage of germination was 21.7 (±9.3 SD). The logarithm of both population size ($r = 0.68$, $P < 0.001$) and plant density ($r = 0.49$, $P < 0.05$) showed a positive correlation with the percentage of germination (fig. 1). Gene diversity was not correlated with the percentage of germination ($r = 0.21$, $P < 0.05$; fig. 1). The incorporation of population size, plant density, and gene diversity into one ANCOVA that considered the effects of habitat and seed mass showed that variation in the percentage of germination was explained by seed mass and to a minor extent by the logarithm of plant density (table 5). Increasing seed mass resulted in a larger germination success (fig. 2). Gene diversity, population size, and habitat had no significant effect. About 75% of the entire variance in germination was explained by the final model (table 5).

Discussion

AMOVA indicated that most of the genetic variation of *Sanguisorba officinalis* in the sampling area occurred within populations, whereas genetic differentiation was low among populations and absent among habitats. The overall F_{ST} value (0.008) was low, suggesting a high level of gene flow. This assumption is supported by the fact that genetic distances between populations did not correspond to geographic distances. The absence of isolation by distance combined with low population differentiation indicates high gene flow compensating the effects of genetic drift (Hutchison and Templeton 1999). Although this pattern of genetic structure may be ascribed to historical gene flow to a certain extent, there are good reasons for assuming the current maintenance of considerable gene flow among populations. First, *S. officinalis* is still common in the area surrounding the populations under study. The high connectivity among habitats should facilitate genetic exchange. Second, flowers are pollinated by highly mobile insect taxa such as syrphid flies, which are known to migrate over long distances within short time frames (Aubert et al. 1969), allowing large-scale pollen transfer within one flowering season. Because seeds lack apparent mechanisms for long-distance dispersal, it is likely that a large fraction of gene flow is maintained by the exchange of pollen. Third, the mating system has been shown to influence the level of gene flow and thus the partition of genetic variation. Generally, it is expected that selfing enforces population differentiation whereas outcrossing promotes gene flow (Loveless and Hamrick 1984).

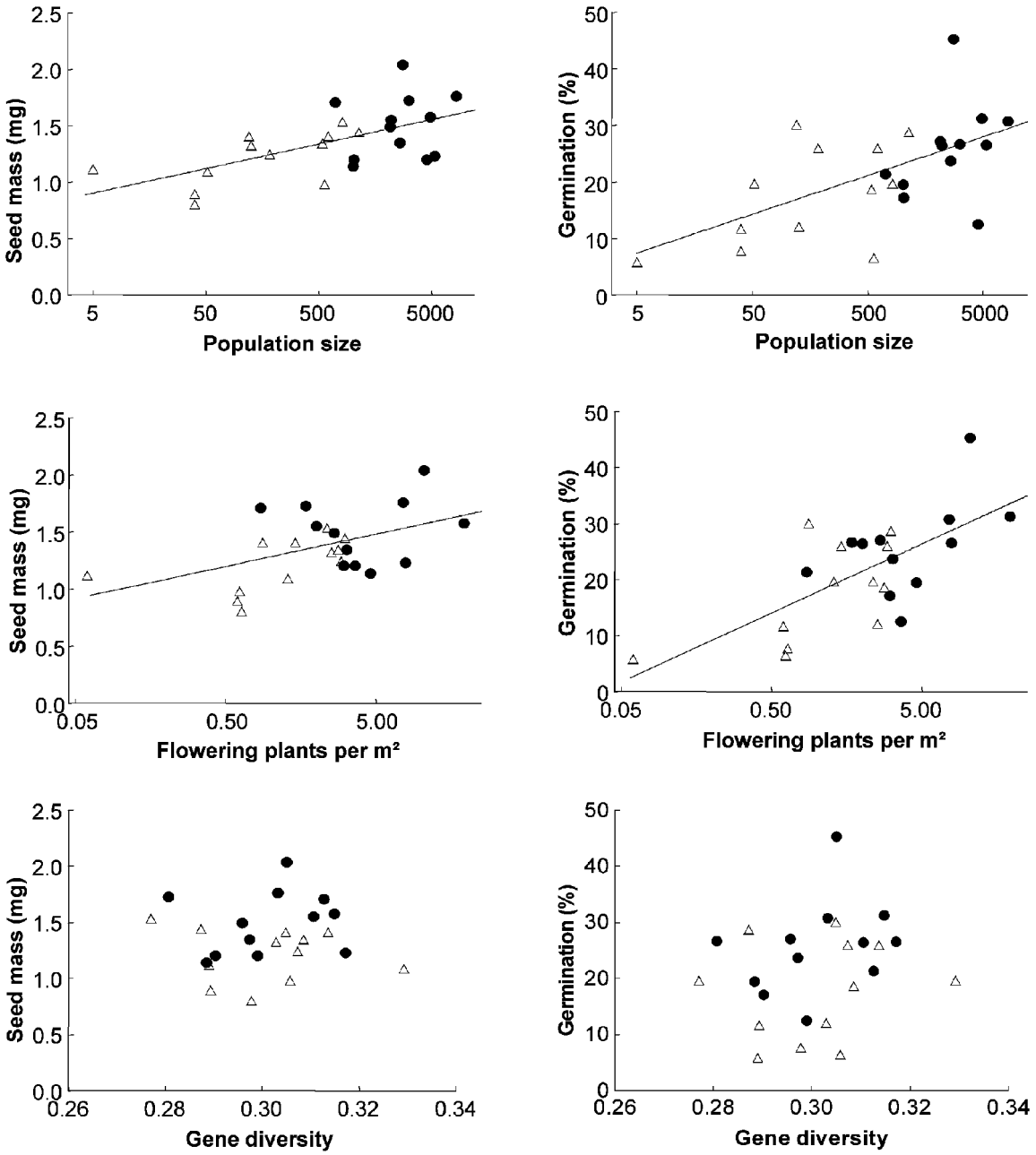

Fig. 1 Mean seed mass (left column) and mean percentage of germination (right column) in relation to population size, density of flowering plants, and gene diversity. Regression lines indicate significant correlations across all populations (table 5). Triangles represent populations from fallows, and circles represent populations from meadows.

Although *S. officinalis* has been described as partially selfing (Nordborg 1963), high outcrossing rates were found in two selected populations, suggesting a strong impact of the mating system on the distribution of genetic variability. The pattern obtained from this study fits well into the result of a meta-analysis by Nybom and Bartish (2000), which shows that long-lived and outcrossing species maintain the largest amount of genetic variation within populations.

There was no genetic differentiation between meadows and fallows. Despite the delay of flower development on managed meadows caused by frequent cutting (M. Musche, personal observation), gene flow between habitat types does not seem to be restricted. One reason for this result may be the long flowering period of *S. officinalis*, which may enable the exchange of pollen in spite of phenological differences of peak flowering. Due to the above-mentioned seed dispersal limitations, it seems unlikely that plants growing in fallows represent offspring of meadow populations located nearby. However, the lack of differentiation may reflect patterns of ancient population structure. Genetic drift should act slowly in perennial

Table 3

Pearson Correlation Coefficients Examining the Relationship between Population Size, Plant Density, Gene Diversity, and Fitness Traits

	Mean seed mass			Germination (%)		
	Within fallows	Within meadows	All populations	Within fallows	Within meadows	All populations
Log population size	.60*	.18	.62***	.53	.34	.68***
Log plant density	.53	.06	.49*	.61*	.50	.49*
Gene diversity	−.13	.19	.05	.18	.30	.21

* $P < 0.05$.
*** $P < 0.001$.

species with low demographic turnover (Loveless and Hamrick 1984). The fallows investigated in this study have developed from former meadows due to secondary succession. Although the exact time of abandonment is unknown, most fallows are out of regular use for at least 10 yr (J. Settele, personal observation). It might be possible that plants growing in fallows represent the survivors of the succession and have preserved the genetic composition of the initial meadow habitats. Studies reporting molecular genetic differentiation between habitats varying in agricultural practice were carried out either on annual plant species with high demographic turnover (Steinger et al. 2002) or in habitats characterized by a long and constant management regime (Kölliker et al. 1998; Silvertown et al. 2005).

The level of intrapopulation genetic variation differed among populations. These differences could not be explained by the type of habitat. Similar results were found in an allozyme study on the perennial plants *Carex davalliana* and *Succisia pratensis* (Billeter et al. 2002). In these species, similar levels of genetic variation within mown and abandoned meadows could be demonstrated in spite of considerable differentiation between populations. Genetic variation may be influenced by differential selection pressures associated with habitat heterogeneity (Linhart and Grant 1996). Selective forces related to agricultural practice, e.g., defoliation and fertilization frequency (Kölliker et al. 1998), or species composition of plant communities (Odat et al. 2004) have been shown to affect the molecular genetic variation within plant populations. However, selection primarily acts on quantitative traits rather than neutral molecular markers that are largely affected by drift (Reed and Frankham 2001). This may explain the similar levels of genetic variation found in meadows and fallows.

In contrast to the large number of studies demonstrating a positive correlation between population size and the amount of genetic variation (reviewed in Leimu et al. 2006), we did not find a significant relationship. Theory predicts a loss of genetic variation in small populations due to the effects of random genetic drift (reviewed in Frankham 1996; Young et al. 1996). Extensive gene flow may compensate this loss, leading to high levels of genetic variation regardless of population size (Van Rossum et al. 1997). Considering the low differentiation among populations, genetic variation in small populations of *S. officinalis* is likely to be maintained by gene flow originating from larger source populations. Alternatively, small populations may preserve genetic variation if arising from the recent fragmentation of formerly large populations (Schmidt and Jensen 2000). As already stated, the longevity of *S. officinalis* may have contributed to such a pattern. Further, it has been suggested that selection favors the survival of heterozygous individuals, thereby increasing average heterozygosity and conserving genetic variation (Raijmann et al. 1994; Luijten et al. 2000). However, due to the methodological limitations of the dominant marker system, we were not able to analyze heterozygosity at the individual level. Finally, uniform levels of genetic variation may result from recent simultaneous invasions of empty habitat networks (Leimu and Mutikainen 2005). However, such a scenario seems unlikely to explain our results, because *S. officinalis* has been described as common throughout the study region in the past (Hindelang 1900).

We did not find a relationship between plant density and the level of intrapopulation genetic variation. Many studies have examined genetic variation in relation to population size, but plant density has rarely been considered. A negative relationship between plant density and allelic richness was demonstrated in the herb *Primula veris* (Van Rossum et al. 2004), whereas genetic variation in *Primula elatior* was not affected by the mean distances between plants (Van Rossum et al. 2002). Heterozygote deficiency was not associated with density in *Thymus vulgaris* (Tarayre and Thompson 1997). Similarly, Gram and Sork (1999) failed to find a general relationship in a sample of common tree species. Particularly in insect-pollinated species, genetic drift and inbreeding may reduce genetic variation at low plant densities independent of population size. Theoretical (Charnov 1976; Pyke 1984) and empirical (Heinrich 1979; Cibula and Zimmerman 1984; Klinkhamer et al. 1989) studies have demonstrated higher rates of intraplant pollinator flights if travel distances between

Table 4

ANCOVA of Mean Seed Mass

Source of variation	df	Sum of squares	Mean square	F
Log population size	1	7.5937e−07	7.5937e−07	13.91**
Residuals	22	1.2006e−06	5.4570e−08	

Note. Nonsignificant terms (habitat, gene diversity, plant density, log plant density, and population size) that did not increase the explanatory power of the model were eliminated stepwise backward. The model explained approximately 39% of the observed variation ($r^2 = 0.39$).
** $P < 0.01$.

Table 5

ANCOVA of Percentage of Germination

Source of variation	df	Sum of squares	Mean square	*F*
Seed mass	1	628.17	628.17	27.29***
Log plant density	1	220.75	220.75	9.59**
Log population size	1	53.66	53.66	2.33
Residuals	20	460.30	23.01	

Note. Nonsignificant terms (habitat, gene diversity, population size, and plant density) that did not increase the explanatory power of the model were eliminated stepwise backward. The model explained approximately 75% of the observed variation ($r^2 = 0.75$).

** $P < 0.01$.

*** $P < 0.001$.

plants increase. Such a shift in pollinator behavior should result in enhanced self-pollination and geitonogamy. Wider spacing between plants may also cause pollinators to move to the nearest neighbor rather than attending plants randomly, thereby increasing the probability of nonrandom mating (Schaal 1978). Restricted fertilization among few genotypes should, in turn, enhance the exposure of populations to the effects of genetic drift. Moreover, if neighboring plants are genetically related (Schoen and Latta 1989), pollination among them should increase biparental inbreeding. Considering the negligible genetic differentiation among *S. officinalis* populations, it seems likely that any effects that may be caused by the described mechanisms are compensated by gene flow.

Mean seed mass declined in small populations of *S. officinalis*. Such a pattern has already been observed in other plant species (Vergeer et al. 2003; Hensen and Oberprieler 2005). Reduced reproductive fitness in small populations may result from a loss of genetic variation (Fischer and Matthies 1998; Lienert et al. 2002), pollen limitation (Byers 1995; Ågren 1996), or poor habitat quality (Widén 1993; Oostermeijer et al. 1994). As the decline in seed mass was not accompanied by decreasing levels of genetic variation, inbreeding depression appears to be unlikely. Pollen limitation may occur in our study populations, but it should mainly affect fruit and seed set (Knight et al. 2005). We excluded nondeveloped seeds from the calculation of the mean seed mass. Therefore, potential effects of pollen limitation should be small. Generally, seed size variation within many species has a large environmental component, but the heritability of this trait seems to be low (Baskin and Baskin 2001). Factors such as nutrient availability (Lewis and Koide 1990), soil moisture (Stamp 1990), or defoliation (Maun and Cavers 1971) can influence seed development until maturity. Small and sparse populations of *S. officinalis* were mostly located in fallows, whereas managed meadows harbored large and dense populations. Thus, the positive association between population size, plant density, and seed mass may reflect differences in habitat quality. Separately calculated correlation coefficients showed that the effect of population size was particularly strong within successional fallow habitats containing the smallest populations. These habitat fragments often suffer from overgrowing by shrubs and trees that compete with *S. officinalis* for light and nutrients. Generally, interspecific competition in plants can reduce maternal investment into seeds (Platenkamp and Shaw 1993).

We found a positive relationship between germination success and both population size and plant density, but germination was unrelated to the level of genetic variation. Population size dropped out of the analysis when all parameters were taken into account, but the effect of plant density remained significant. Again, in fallows containing the sparsest populations, the effect of density was strong, whereas in meadows, where plant density is generally higher, the effect was marginal. Several mechanisms may account for the density effect. Like population size, density may represent an indicator for any component of habitat quality that, in turn, can influence reproductive success (Bosch and Waser 2001). Environmental effects are likely because the decline in germination rate was not accompanied by decreasing levels of genetic variation. However, the reduced germinability of seeds originating from low-density populations of *S. officinalis* may also be due to genetic factors. As already mentioned, modified pollinator behavior at low plant density may cause enhanced self-pollination, geitonogamy, and biparental inbreeding. Density-dependent outcrossing rates have been found frequently (Murawski et al. 1990; Watkins and Levin 1990; Van Treuren et al. 1993). Additionally, selfed seeds may feature a lower survival probability, particularly in outcrossing species (Farris and Mitton 1984; Van Treuren et al. 1994). Under these circumstances, the density effect may indicate inbreeding depression. Studies investigating seed germination in relation to population size, plant density, or genetic diversity have produced a variety of results ranging from positive correlations (Menges 1991; Soons and Heil 2002; Vergeer et al. 2003) to inconsistent patterns (Oostermeijer et al. 1994; Ouborg and Van Treuren 1995; Lammi et al. 1999; Morgan 1999; Kéry et al. 2000; Costin et al. 2001) and negative associations (Widén 1993). The repeatedly observed positive correlation between seed mass or seed size and germination characteristics such as germination rate (Gómez 2004), percentage of germination (Weis 1982; Ouborg and Van Treuren 1995; Van Mölken et al. 2005), or timing (Simons and Johnston 2000) shows that there is substantial maternal influence on this trait. In this respect, our result is in line with previous findings. Maternal seed mass also explained the largest amount of variance in the percentage of germination in *S. officinalis* and

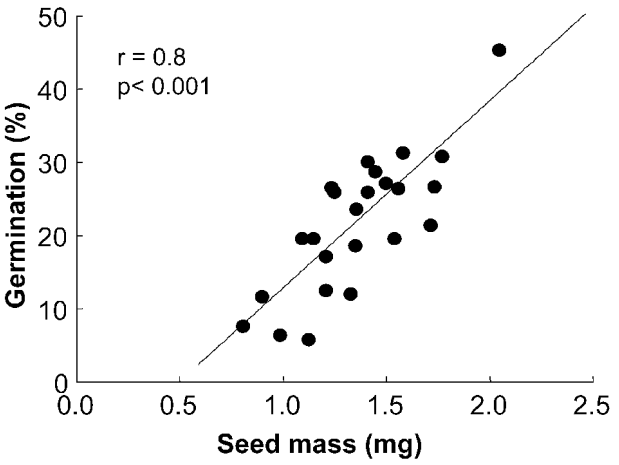

Fig. 2 Relationship between mean seed mass and mean percentage of germination.

should therefore affect recruitment of seedlings much more than the mechanisms underlying the density effect.

The fact that population size, plant density, and habitat were not independent from each other complicates the evaluation of their relative influence on the fitness traits under study. Future experimental manipulations, e.g., pollination experiments, will be required to investigate whether the observed variation in seed mass and the percentage of germination are caused by inbreeding depression or pollen limitation or are based on the environmental background. As genetic variation was not related to seed mass and germination, environmental factors are presumably the main determinants of both fitness traits.

Our results have the following implications for the conservation of the rare *Maculinea* butterfly species that rely on small habitat fragments. First, the diminished reproductive capability of *S. officinalis* in these habitats may increase the extinction risk of plant populations, thus making them less suitable for the long-term conservation of *Maculinea nausithous* and *Maculinea teleius*. Second, as small populations have retained high levels of genetic variation and gene flow from the surrounding areas seems sufficient to prevent future loss, conservation efforts should focus on the improvement of habitat quality. This could be achieved by mowing fallows occasionally to halt secondary succession. However, this recommendation may be true for areas only where *S. officinalis* remains common. Higher isolation and lower levels of gene flow at the range margins of the distribution may expose small plant populations to the effects of genetic drift and inbreeding that might demand alternative conservation strategies.

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