



Reduced genetic variation mainly affects early rather than late life-cycle stages

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ABSTRACT

A large threat to biological diversity worldwide is habitat fragmentation, with negative effects on organisms arising as a result of changes in the abiotic or biotic environment and via changes in genetic processes. We examined genetic diversity, population structure and gene flow as well as the importance of genetic diversity for plant and population performance in 17 differently-sized populations of the locally rare, self-incompatible, perennial forest herb *Phyteuma spicatum*. Genetic variation, characterized by AFLPs, was low (mean gene diversity, H_e : 0.126) and generally increased with increasing population size. Bayesian analysis of population structure showed that populations located in the same forest patch or in close vicinity formed genetic clusters. Population differentiation conformed to a pattern of isolation by distance (IBD). Gene dispersal distances were higher when estimated within than among populations, suggesting that gene flow is more restricted on a landscape scale. Two seedling vital rates were influenced by population genetic parameters, while most late life-cycle stages and population growth rate were not affected. These results suggest that genetic load is purged early in the life cycle by selective mortality, which may buffer populations against the negative genetic effects of small population size. The overall low levels of genetic diversity, however, imply that populations are poorly equipped to respond to future changes in the environment. This is of special concern as short-distance gene dispersal indicates that colonization of new sites in response to putative changes in the environment is unlikely.

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1. Introduction

One of the largest threats to population viability, species survival and biological diversity worldwide is land-use change including habitat loss and fragmentation (Primack, 2006). Negative effects on plant populations may arise as a result of changes in abiotic and biotic environmental conditions (Kolb, 2008; Olesen and Jain, 1994; Saunders et al., 1991), but also via changes in within- and among-population genetic processes such as genetic drift, inbreeding and gene flow (Ellstrand and Elam, 1993; Keller and Waller, 2002; Young et al., 1996).

Effects of genetic drift, i.e. the random change in allele frequency from one generation to the next, are most pronounced in small populations, in which alleles are either driven to fixation or extinction within relatively short periods of time. As a consequence, genetic diversity within populations will typically decrease, while the differentiation among populations will increase. In populations with low genetic diversity, levels of inbreeding, i.e. the mating between closely related individuals, will be higher,

increasing homozygosity and the expression of recessive deleterious alleles. These processes may in the short term lead to a reduction in fitness of individuals, i.e. inbreeding depression (Fischer and Matthies, 1998; Keller and Waller, 2002; Oostermeijer et al., 1994), and in the long term limit the potential of populations to respond and adapt to changes in the environment (Booy et al., 2000; Jump et al., 2009). The magnitude of such effects, however, may depend on plant mating system and other life-history traits, overall rarity and time elapsed in fragmentation conditions (Aguilar et al., 2008; Leimu et al., 2006), making fragmentation effects on population genetic structure and fitness sometimes difficult to predict (Aparicio et al., 2012).

While a number of studies have examined relationships between population size and genetic diversity (Leimu et al., 2006; Tomimatsu and Ohara, 2003; Van Rossum et al., 2002) or fitness (Kolb, 2005; Leimu et al., 2006), relatively few have examined the importance of genetic variation for fitness (de Vere et al., 2009; Fischer and Matthies, 1998; Lauterbach et al., 2011; Leimu et al., 2006; Schmidt and Jensen, 2000) and we largely still lack studies that incorporate the entire life cycle of species (but see Fischer et al., 2003; Menges and Dolan, 1998; Oostermeijer et al., 1994). More such comprehensive studies are needed, as effects of inbreeding and inbreeding depression may differ between different

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plant traits or life-cycle stages (Angeloni et al., 2011; Husband and Schemske, 1996). Thus, given that population performance responds differently to changes in single fitness components like seed production, growth or survival (Silvertown et al., 1993), the magnitude of genetic effects on particular life-cycle stages may not be predictive of the effects on population viability. Kolb et al. (2010), for example, showed that effects of small population size on fitness components need not translate into effects on population growth rate, suggesting that predictions based on one or few fitness components must be treated with caution.

The erosion of genetic diversity within populations and its effects on plant fitness may be compensated by gene flow between populations. In highly fragmented landscapes, however, immigration of seeds or pollen may be reduced (Kwak et al., 1998; Olesen and Jain, 1994; Sebbenn et al., 2011), preventing the amelioration of the negative effects of genetic drift and inbreeding. Restricted gene flow may enhance population differentiation at the landscape scale (Lauterbach et al., 2011; Schmidt and Jensen, 2000) and create genetic structure on more local scales within populations (Ishihama et al., 2005; Sebbenn et al., 2011; Van Rossum and Triest, 2006; Zeng et al., 2012). Generally, population isolation may also be positive, allowing for the build-up of local adaptations that are not swamped by incoming, poorly adapted genes.

In this study, we used new genetic data together with existing data on plant fitness and population growth rate to examine genetic diversity, population structure and gene flow as well as the importance of genetic diversity for plant and population performance in fragmented populations of the self-incompatible, perennial forest herb *Phyteuma spicatum*. Previous studies with this species have shown that survival and growth of young individuals, flowering rates and seed production were lower in small than in large populations (Kolb, 2005; Kolb et al., 2010). Supplemental hand pollinations and pollinator observations suggested that reductions in seed output were the result of pollen limitation (Kolb, 2008, 2005). Fitness reductions may also have had genetic causes, as suggested by an experimental study in which offspring survival was positively related to the size of the population of origin under common environmental conditions (Kolb, 2005). However, we still lack molecular genetic data which would provide more conclusive evidence for such effects, and we generally lack information about within- and among-population genetic variation in the study system.

Specifically, the major aims of this study were (1) to examine effects of population size on genetic diversity, (2) to examine population differentiation as well as population- and small-scale spatial genetic structure, (3) to estimate gene flow by seed and pollen dispersal, and (4) to test for relationships between population genetic parameters and plant fitness in terms of vital rates, i.e. fitness components, and population growth rate.

2. Materials and methods

2.1. Study species

P. spicatum L. (Campanulaceae) occurs in Central and Atlantic Europe (Hultén and Fries, 1986), and is an iteroparous hemicryptophyte that produces annual rosettes of 2–6 basal leaves and, when flowering, one to several inflorescences on upright stems that carry several cauline leaves (Kolb et al., 2010; Wheeler and Hutchings, 2002). The hermaphroditic flowers are protandrous and pollinated by bumblebees (Kolb, 2008). Spontaneous autogamy and geitonogamy result in no or very few seeds, presumably due to gametophytic self-incompatibility (Huber, 1988); the species can thus be regarded as an obligate outcrosser. Seeds are small and lack special dispersal devices, and most dispersed seeds land close to the par-

ent plant (Wheeler and Hutchings, 2002). The species is diploid (Huber, 1988).

2.2. Study area and populations

The study was conducted in an area of ca. 425 km² in NW-Germany (Fig. 1). The landscape is mostly covered by Pleistocene deposits of the Saale glaciation and is flat to weakly undulating, with elevations varying between 10 and 40 m a.s.l. Forests have been highly fragmented for >250 years (Kelm, 1994) and today cover ca. 13% of the landscape, of which 25% are deciduous hardwood forest (Kolb and Diekmann, 2004). In this area, *P. spicatum* is restricted to mesic or moist, base-rich deciduous hardwood forests and is relatively rare, with about 20 known populations (Fig. 1). The species is included in the red list of vascular plant species in the study area (category “endangered” in the lowlands of Lower Saxony; Garve, 2004). Larger forests support larger populations ($r = 0.58$, $P = 0.016$, $n = 17$), while fragment size is not related to population density ($r = -0.391$, $P = 0.120$, $n = 17$).

We collected genetic data in 17 populations (Fig. 1). These populations were selected because they covered a wide range of population sizes (8–2350 flowering individuals, values are means based on counts from 2008 and 2009; Table 1) and because they all were part of earlier studies, in which effects of population size on fitness and population growth rate were examined (Kolb, 2005; Kolb et al., 2010; Weber and Kolb, 2011; Table 1). Populations were defined as groups of plants being separated from other groups of plants by at least 100 m. Two populations (1 + 2, Fig. 1) were closer (62 m), but still formed very distinct patches of plants, while mean minimum distance to the next population was 1568 m.

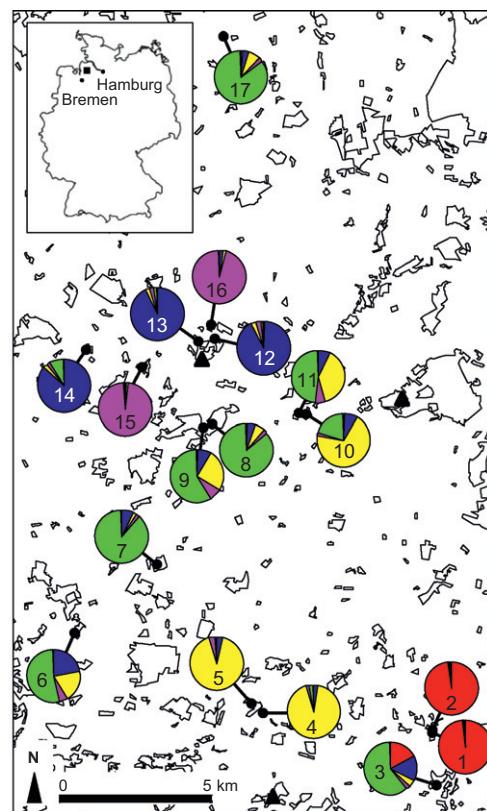


Fig. 1. Forest patches (only those >1 ha) and populations of *Phyteuma spicatum* in the study area, situated between Bremen and Hamburg in NW-Germany. Pie charts show results of a STRUCTURE analysis at $K = 5$ for the 17 study populations (see Fig. B3 in Appendix B). Numbers correspond to those given in Table 1. Populations ($n = 3$) not used in this study are indicated by black triangles.

Table 1

Population size (mean number of flowering individuals, based on counts from 2008 and 2009) and genetic variation of the 17 sampled populations of *Phyteuma spicatum*. Populations are located in the same forest fragment when designated by a similar letter. n is the number of samples included in the AFLP analysis (in populations 5 and 13 additional plants were sampled, see Section 2.3), H_e is gene diversity (expected heterozygosity), PLP is the percentage of polymorphic loci, B_r is band richness, and F_{ij} is the mean kinship coefficient. All populations were part of 1–3 previous studies, as indicated in the last three columns. Note that populations 1 and 2 were excluded from the analyses of the effects of genetic variation on fitness (see Section 2.6).

ID	Population name	Pop. size	n	H_e	PLP	B_r	F_{ij}	Kolb (2005)	Kolb et al. (2010)	Weber and Kolb (2011)
1	Ippensen South (A)	37	19	0.081	19.5	1.205	0.418			(\times)
2	Ippensen North (A)	302	18	0.085	19.5	1.207	0.355	(\times)	(\times)	(\times)
3	Nüttel	701	17	0.126	38.3	1.430	0.110	\times	\times	\times
4	Boitzenbostel East	304	19	0.152	43.0	1.469	0.073	\times		
5	Boitzenbostel West	2350	20 (+16)	0.145	43.0	1.485	0.088	\times	\times	\times
6	Sassenholz	128	18	0.110	30.9	1.342	0.114			\times
7	Wense	37	18	0.132	40.3	1.429	0.110	\times		\times
8	Hahnenhorst East (B)	298	16	0.147	40.9	1.470	0.101			\times
9	Hahnenhorst West (B)	714	16	0.153	45.6	1.511	0.070	\times	\times	\times
10	Wieh East (C)	67	13	0.139	33.6	1.409	0.118	\times	\times	\times
11	Wieh West (C)	16	18	0.119	35.6	1.377	0.115	\times	\times	\times
12	Tadel East (D)	93	17	0.123	32.9	1.350	0.093	\times	\times	\times
13	Tadel West (D)	1219	20 (+34)	0.129	36.9	1.423	0.066	\times	\times	\times
14	Farven	8	13	0.124	29.5	1.362	0.181		\times	\times
15	Reith	27	18	0.124	32.2	1.342	0.152	\times	\times	\times
16	Tadel North	189	16	0.129	31.5	1.363	0.145	\times	\times	\times
17	Fredenbeck	99	17	0.125	39.6	1.423	0.102			\times

2.3. Sampling and genetic analysis

Leaf samples for genetic analysis were taken from 20 adult individuals per population, if available, during 2009. In the two largest populations we sampled 40 additional plants; these were only included in the analysis of small-scale spatial genetic structure and in the STRUCTURE analysis (see Section 2.5). Individuals were sampled randomly from the entire area occupied by each population. The leaves were dried at 50 °C for 48 h and then kept dry in silica gel. We determined the geographic coordinates for each sampled individual, using a high-sensitivity GPS receiver (GARMIN eTrex® H) and measuring tape readings.

Genetic variation was analyzed using amplified fragment length polymorphisms (AFLP) as described in Lachmuth et al. (2010). After initial primer screening, we selected the four primer combinations ACT/CAG, ACG/CTA, ACC/CAG, AGG/CAG (EcoRI/MseI). Some individuals failed the AFLP analysis, resulting in 343 samples across populations (Table 1). We obtained a total of 170 AFLP bands, 149 of which (88%) were polymorphic and used for further analysis. Mean genotyping error rate per locus was 0.481% based on replicate analysis of 104 samples.

2.4. Genetic variation

We determined several estimators of within-population genetic variation. Gene diversity (expected heterozygosity, H_e) and the percentage of polymorphic loci (PLP) were calculated with AFLPSurv 1.0 (<http://ebe.ulb.ac.be/ebe/Software.html>, last accessed October 12, 2012) following Lynch and Milligan (1994). We used the square root method to calculate allele frequencies and assumed an inbreeding coefficient of 0.0 due to self-incompatibility. As the number of samples per population differed, we also estimated band richness (B_r) using AFLPDiv 1.0 (Coart et al., 2005), which accounts for differences in sample size. Data were rarefied to the minimal sample size of $n = 13$. As a measure of population-level relatedness we calculated the mean pairwise kinship coefficient (F_{ij} ; Hardy, 2003).

As populations are likely derived from an ancestral population and are not equally related to one another (see also Section 3.2), they are statistically not independent. We therefore applied phylogenetic generalized least squares (PGLS) regression based on a neighbor-joining tree of Neis genetic distance to test for effects

of log-transformed population size on genetic diversity (H_e , PLP and B_r) and mean relatedness (F_{ij}), using the function “pgls” of the “caper” package in R 2.15.1 (Orme et al., 2012; R Development Core Team, 2012).

2.5. Population genetic structure and gene dispersal

Genetic differentiation among populations was quantified with F -statistics following Lynch and Milligan (1994), calculating overall and pairwise F_{ST} values between populations using AFLPSurv. Population differentiation was further quantified by analysis of molecular variance (AMOVA) using GenAlEx 6.4 (Peakall and Smouse, 2006). We tested for a pattern of isolation by distance (IBD) by correlating pairwise population differentiation, $F_{ST}/(1 - F_{ST})$, with log geographic distance (Rousset, 1997). Significance was assessed by a Mantel test with 9999 permutations using the function “mantel” of the “vegan” package (Oksanen et al., 2011) in R.

Spatial genetic structure was assessed on two different scales. First, to explore overall genetic structure, we used a Bayesian cluster approach adapted to dominant markers, using STRUCTURE 2.3.3 (Falush et al., 2007). Individuals are grouped into genetic clusters representing homogeneous gene pools without a priori information about individual origin. We ran 10 replicate runs, with the number of clusters, K , ranging from 1 to 15, of an admixture model with correlated allele frequencies and used 50,000 for both the length of burn-in period and subsequent number of MCMC (Markov-Chain-Monte-Carlo) repeats. The most probable number of clusters, K , was determined following Evanno et al. (2005). Replicate runs were averaged using CLUMPP 1.1.1 (Jakobsson and Rosenberg, 2007). As STRUCTURE detects only the upper hierarchical structure, a second analysis was performed with one of the two clusters identified in the first analysis. For graphical representation, the two analyses were combined and results of the overall analysis at $K = 5$ are shown.

Second, we assessed small-scale spatial genetic structure using SPAGeDi 1.3 (Hardy and Vekemans, 2002) to estimate individual pairwise kinship coefficients (F_{ij} ; Hardy, 2003) and spatial genetic autocorrelation. Significance of mean pairwise kinship coefficients was tested by permutation ($n = 1000$). We calculated the Sp statistic as a measure of spatial genetic structure independent of sampling scale (Vekemans and Hardy, 2004). A restricted spatial range of 700 m was used in the Sp analysis, which spans distances

within or between nearby forest patches (Fig. 1). We assessed the relative role of seed vs. pollen dispersal from the shape parameter k of the curvature of pairwise kinship coefficients $F(r)$ (Heuertz et al., 2003; Vekemans and Hardy, 2004). When seed dispersal is more restricted than pollen dispersal ($\sigma_s \ll \sigma_p$), a concave curvature ($k > 0$) is expected.

We assessed gene dispersal distance, σ , defined as half of the average parent-offspring distance, on two spatial scales, namely on the population level, based on population differentiation and IBD patterns, and at the individual level, based on small-scale spatial genetic structure, similar to Zeng et al. (2012). In short, we first used the slope (b_{\log}) of the population-level IBD analysis to estimate gene dispersal from $\sigma^2 = (1/b_{\log})/4\pi D$ (Rousset, 1997), where D is the effective population density. Second, we used spatial genetic autocorrelation at the individual level to estimate gene dispersal, as the S_p statistic is expected to equal $1/4\pi D\sigma^2$ in a restricted range of σ to 20σ (Heuertz et al., 2003; Vekemans and Hardy, 2004). We used SPAGeDi which iteratively estimates gene dispersal, and restricted the spatial range to 700 m. For both approaches of gene dispersal estimation, effective density was set to both $0.1\times$ and $0.5\times$ observed median population density (0.21 flowering individuals m^{-2}), as effective density typically ranges between 0.1 and 0.5 of census density (Frankham, 1995).

2.6. Plant fitness and population growth rate

To assess the importance of genetic diversity and mean relatedness for plant and population performance, we used data from three previous studies which had covered between 10 and 14 of the 17 populations included in this study (Table 1). Most of the field data were extracted from a demographic study, in which effects of population size on vital rates and population growth rate were examined during 2006–2008 (see Kolb et al., 2010 for details). The vital rates examined included growth and probability of survival of seedlings, juveniles and adults (2006–2007, 2007–2008); probability of flowering (2006, 2007, 2008); seed production (2006, 2007); and seedling establishment (2006–2007, 2007–2008). Population growth rates (λ) were estimated using integral projection models (2006–2007, 2007–2008). In addition, we used field data on seed production from 2008 and 2009 (Weber and Kolb, 2011). Finally, we used data on offspring fitness, in terms of seedling survival after 32 weeks of growth under common environmental conditions in a greenhouse (Kolb, 2005). We again used PGLS regression to test for effects of genetic variation on fitness. To reduce the number of tests, we restricted our analyses to one of the genetic diversity parameters (H_e) and mean relatedness (F_{ij}), since H_e , PLP and B_r were strongly correlated among each other ($r > 0.9$ and $P < 0.001$), but less to F_{ij} ($r < -0.8$, $P < 0.001$). Furthermore, to avoid spurious correlations, we excluded populations 1 (only one fitness parameter available, Tables 1 and A2 in Appendix A) and 2 (complete fitness data available); both were characterized by particularly low levels of genetic variation and high kinship coefficients (see Sections 3.1 and 3.2).

3. Results

3.1. Genetic variation within populations and relationships with population size

Among populations, gene diversity (H_e), the percentage of polymorphic loci (PLP) and band richness (B_r) varied between 0.081 and 0.153 (mean \pm SD, 0.126 ± 0.020), 19.5% and 45.6% (34.9 ± 7.5) and 1.205 and 1.511 (1.388 ± 0.086), respectively (Table 1). Kinship coefficients (F_{ij}) varied between 0.066 and 0.418 (0.142 ± 0.097). Populations 1 and 2, which were located in the same forest frag-

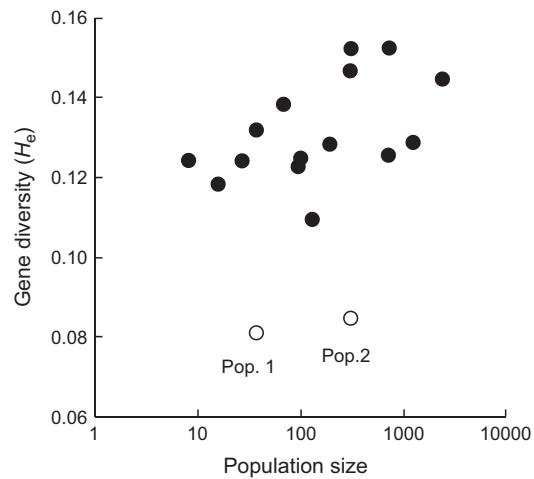


Fig. 2. Relationship between gene diversity (H_e) and log-transformed population size in 17 populations of *Phyteuma spicatum*. Two putatively bottlenecked populations (populations 1 and 2, $H_e < 0.1$) are indicated by open circles.

ment and separated by only 62 m (Fig. 1), had high kinship coefficients and particularly low levels of genetic variation (Table 1).

Across all populations, population size had a positive effect on B_r (PGLS regression, $P = 0.040$, see Table A1 for full statistical results) and a negative one on F_{ij} ($P = 0.041$). When excluding the two seemingly bottlenecked populations 1 and 2, we also detected a significant positive effect of population size on PLP ($P = 0.028$). While H_e was not significantly affected in either analysis ($P = 0.110$ across all populations, and $P = 0.129$ when populations 1 + 2 were excluded) the overall relationship between H_e and population size was still positive (Fig. 2), as expected from the strong positive correlation between PLP , B_r and H_e (see Section 2.6).

3.2. Population genetic structure

Populations were significantly differentiated from each other (overall $F_{ST} = 0.193$, $P < 0.001$). However, populations 1 and 2 were more strongly differentiated compared to the remaining 15 populations (mean pairwise F_{ST} of populations 1 and 2 against all others = 0.372). This suggests that these two populations have a

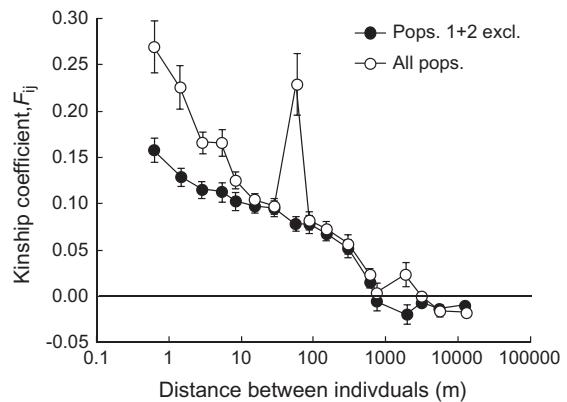


Fig. 3. Small-scale spatial genetic autocorrelation (mean pairwise kinship coefficients per distance class \pm SE) of *Phyteuma spicatum* individuals, using all individuals (open circles) and all excluding those of populations 1 and 2 (solid circles). All mean kinship coefficients were significant ($P < 0.05$). Positive and negative kinship coefficients indicate that individuals were more or less related than random individuals, respectively.

common origin and have experienced a population bottleneck. This is corroborated by the Bayesian cluster analysis with STRUCTURE, which revealed two gene pools, with populations 1 and 2 representing one pool separated from all other populations (see Fig. B1 in Appendix B). Population 3, which is located spatially closest to populations 1 and 2 (Fig. 1), showed some admixture from their gene pool indicating closer relationships among these three populations. A second STRUCTURE analysis of the remaining 15 populations, which had an overall F_{ST} of 0.134 ($P < 0.001$), revealed some evidence for further structure at $K = 4$ (Fig. B2), resulting in a total of five clusters ($K = 5$, Figs. 1 and B3). Populations that were located within the same forest patch (e.g. populations 1 + 2, 12 + 13) or in close vicinity (e.g. populations 4 + 5) mostly grouped into the same cluster. AMOVA analyses revealed that 26% and 74% of the variation resided among and within populations, respectively. Taking the STRUCTURE clusters into account, 14% of the variation resided among clusters, 14% among populations within clusters and 72% within populations. Population differentiation conformed to a pattern of isolation by distance (correlation between $F_{ST}/(1-F_{ST})$ and log geographic distance: Mantel $P = 0.001$, $b_{\log} = 0.154$), indicating that more distant population pairs are genetically more differentiated than closer pairs and that populations are in regional equilibrium between gene flow and drift.

Significant small-scale spatial genetic structure (SGS) was detected (Fig. 3). Kinship coefficients (F_{ij}) of individuals within and among populations 1 and 2 were extremely high, suggestive of both population bottlenecks and close relationships between the two populations. When these populations were excluded, a monotonous decline of pairwise kinship coefficients was observed. Significantly positive and negative kinship coefficients were detected below and above ca. 1 km, respectively, indicating that these individuals were more ($F_{ij} > 0$) or less ($F_{ij} < 0$) related than random individuals. In the distance range up to 700 m, the S_p statistic was 0.0236 (CI 0.0200–0.0273), and the relationship between kinship coefficient and log distance was almost linear with only minimal concave curvature ($k = 0.018$; cubic regression $r^2 = 0.841$, $P < 0.001$). The latter suggests that seed dispersal was not much more restricted than pollen dispersal at this spatial scale.

3.3. Gene dispersal

At the individual scale, based on SGS, estimates of historical gene dispersal σ were 9.9 m (SE: 1.1 m) and 20.0 m (2.1 m) when effective population density was set to 0.5 and 0.1 of the census density, respectively. At the population scale, based on IBD patterns among populations, estimates of σ were smaller and ranged between 2.3 m and 5.2 m for all populations ($b_{\log} = 0.15$), and between 5.2 m and 11.5 m when populations 1 and 2 were excluded ($b_{\log} = 0.03$). This suggests that gene flow among populations is more restricted than that within populations.

3.4. Genetic variation, plant fitness and population growth rate

Most of the fitness estimates as well as population growth rate were not affected by genetic diversity (H_e) or mean relatedness (F_{ij}) (PGLS regression, in all cases $P > 0.05$, Table A2). However, we detected a significant positive effect of H_e on seedling growth in one of two years in the field ($P = 0.004$; Fig. 4a) as well as a significant negative effect of F_{ij} on seedling survival under common environmental conditions ($P = 0.048$; Fig. 4b). H_e also positively influenced the probability of flowering in one of three years ($P = 0.035$; Fig. 4c). None of the fitness estimates, however, was significantly affected by genetic variation when applying a Bonferroni correction to account for the large number of tests (Table A2).

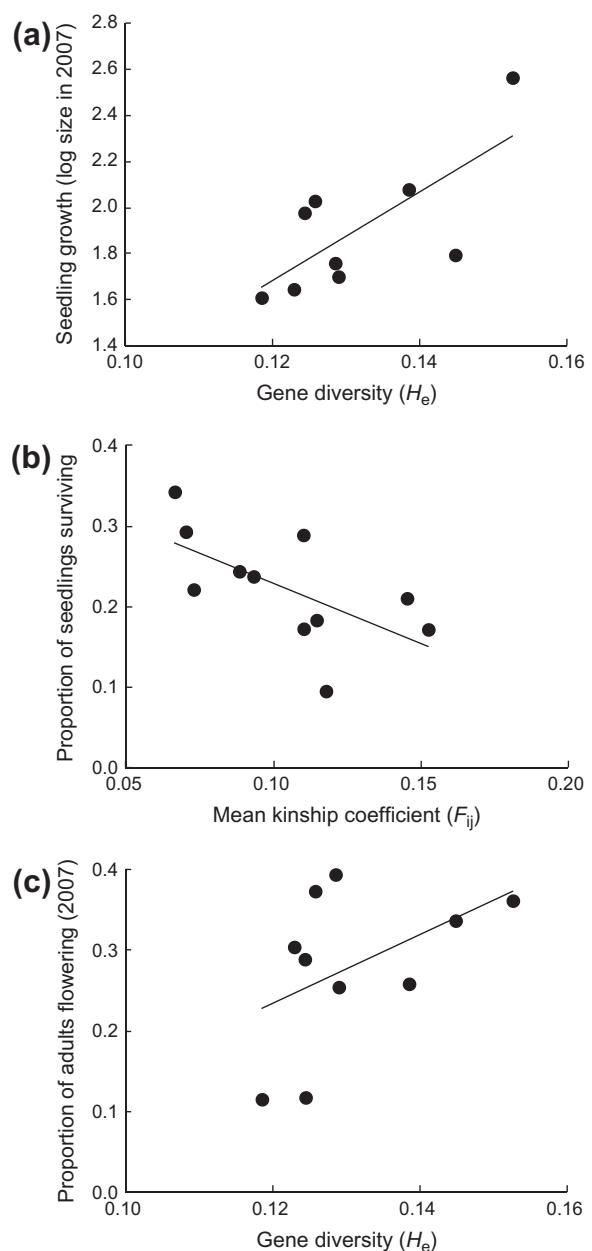


Fig. 4. Relationship between genetic parameters (H_e or F_{ij}) and fitness in populations of *Phyteuma spicatum*. In (a) seedling growth is expressed as the log-transformed size in the year after emergence (data from Kolb et al. (2010)), in (b) seedling survival is expressed as the mean proportion of seedlings surviving after 32 weeks of growth under common environmental conditions in greenhouse (data from Kolb (2005)), and in (c) the probability of flowering is expressed as the proportion of adults that flowered (data from Kolb et al. (2010)). PGLS regression: (a) $P = 0.004$, (b) $P = 0.048$, and (c) $P = 0.035$ (see Table A2 in Appendix A for full statistical results).

4. Discussion

4.1. Genetic variation within populations

Overall, *P. spicatum* populations showed a relatively low genetic diversity (mean $H_e = 0.126$) compared to other outcrossing species (mean AFLP-derived estimates of H_e for outcrossing, mixed-mating and selfing species: 0.183 (CI: 0.168–0.197, $n = 95$), 0.148 (CI: 0.127–0.169, $n = 45$), 0.076 (CI: 0.043–0.109, $n = 23$), Table C1 and Fig. C1 in Appendix C). Three non-mutually exclusive factors may have contributed to the low levels of genetic variation. First,

populations have been fragmented for at least 250 years when forest fragmentation had already reached its current extent (Kelm, 1994). The species' fragmentation history may be even much longer, given that it is confined to base-rich forest sites, which in turn are not very common in the landscape. The species is therefore, at least to some extent, naturally rare. This long fragmentation history implies relatively strong negative effects on genetic diversity. Correspondingly, Aguilar et al. (2008) showed that species subjected for >100 years in fragmentation conditions were more negatively affected compared to species subjected to such conditions for <50 years. Second, past population bottlenecks and founder effects may have reduced genetic variation due to genetic drift. Two of our study populations had particularly low levels of genetic variation compared to other populations of similar or even smaller size. A likely explanation is that these populations experienced a genetic bottleneck at some point in time. Alternatively, the populations could have been founded by only a few founder seeds. At last, our study populations are located close to the margin of the species' distribution range (Hultén and Fries, 1986). Marginal populations are generally thought to be genetically less variable as a consequence of smaller effective population size and greater geographical isolation relative to geographically more central populations (Eckert et al., 2008). Unfortunately, we only have data for one additional, large population located about 100 km south of our study region and thus still close to the species' range margin. The genetic variation of this population was within the range observed in our study populations ($H_e = 0.119$, $PLP = 36.2\%$, $B_r = 1.446$, $F_{ij} = 0.114$). This could suggest that genetic variation may be low across larger parts of the species' range, but still does not preclude that genetic diversity may be higher closer to the center of the species' distribution range.

Consistent with the results obtained for other species (Leimu et al., 2006), population size was positively related to genetic diversity. Effects of population size on PLP and B_r were stronger than on H_e . This is in line with theory, which predicts that predominantly rare alleles get lost due to increased negative effects of genetic drift in small populations (Nei et al., 1975). Relatively high levels of heterozygosity, on the other hand, may be maintained among the remaining alleles through the outcrossing mating system of the species. PLP and B_r may therefore be more sensitive to differences in population size than H_e . Similar results were found for other self-incompatible forest herbs. Van Rossum et al. (2002) found for *Primula elatior* that small populations had fewer alleles per locus than large populations but that they also maintained high levels of heterozygosity. Likewise, Tomimatsu and Ohara (2003) found for *Trillium camschatcense* that population size affected both PLP and the number of alleles per locus, but not H_e .

4.2. Population genetic structure and gene flow

Populations were significantly differentiated from each other, and population differentiation followed an isolation-by-distance pattern (IBD), i.e. close populations were genetically more similar than more distant population pairs. The comparatively high genetic differentiation among populations is likely caused by the spatial isolation of forest fragments within the otherwise agriculturally-used landscape, restricting pollen and seed dispersal and thus gene flow to nearby populations (see also below), in addition to the fact that the species probably was always relatively rare in the area.

The STRUCTURE analyses showed that genetic diversity is hierarchically structured. The first analysis clustered individuals into two groups, with those from the two bottlenecked populations being separated from all others. Still, further sub-structuring was evident when repeating the analysis for the latter group. We suggest a scenario of one original larger gene pool represented by the green colors in Fig. 1 from which some populations diverged

as a result of random genetic drift, enhanced by population bottlenecks and historical land-use differences. This interpretation is supported by the observation that populations located within the same forest patch or in close vicinity mostly grouped into the same cluster. At least two populations (4 and 5), which are located in nearby, but separate forest patches today, were likely one larger population in former times, as evidenced by historical maps ('Kurhannoversche Landesaufnahme') that show a continuous forest cover in this area during 1765–1770.

Populations were also characterized by a significant small-scale spatial genetic structure (SGS) and its strength ($Sp = 0.0236$) was very similar to that observed in other herbaceous outcrossing plants (mean $Sp = 0.024$; Michalski and Durka, 2012). SGS was more pronounced than, for example, in outcrossing species of managed grasslands (e.g. *Veronica chamaedrys*: $Sp = 0.0023$; Kloss et al., 2011), indicating that comparatively undisturbed forest communities allow for the development of SGS. Individuals growing close to each other were thus more likely to be genetically related than individuals further apart.

Estimates of historical gene flow distances derived from the IBD pattern ranged between 2.3 m and 11.5 m. The gene flow distances estimated from the SGS were about twice as high (9.9–20.0 m), suggesting that pollen and seed dispersal distances were less restricted on local scales (i.e. within populations and thus similar habitat) compared to the landscape scale (i.e. between populations which in turn are often separated by agricultural land). At the landscape scale, gene flow thus appears to be limited to nearby populations, with mean distances between populations of ca. 1.5 km and a matrix of agricultural land not allowing frequent migration of pollinators between populations. Similar gene flow distances were estimated by Ishihama et al. (2005) for the perennial forest herb *Primula sieboldii* (15.7–18.4 m), a species which is also mainly pollinated by bumblebees and lacks apparent morphological adaptations to dispersal.

Analysis of the relative contributions of seed and pollen dispersal to gene flow provided no strong evidence for different dispersal distances of seeds vs. pollen, although *P. spicatum* lacks specific adaptations to long-distance seed dispersal, while its main pollinators, bumblebees, are known to fly relatively long distances (Knight et al., 2005). Van Rossum and Triest (2006), on the other hand, found for the forest herb *Primula elatior* that seed dispersal was more restricted than pollen dispersal. *Primula elatior* and *P. spicatum* differ strongly in seed size and number, and the smaller and more numerous seeds of *P. spicatum* are likely dispersed to farther distances than those of *P. elatior*. Ultimately, however, only direct estimates of pollen and seed dispersal distances (e.g. using molecular-marker based paternity analyses) will give more conclusive results.

4.3. Genetic variation, plant fitness and population growth rate

In our study, two seedling vital rates appeared to be influenced by population genetic parameters, while most late life-cycle stages and population growth rate were not affected. Specifically, we found that seedling growth was higher in genetically more diverse populations and that seedling survival was lower in populations with higher mean kinship coefficients. These results suggest that both genetic drift and biparental inbreeding have lead to the observed fitness reductions. Similarly, de Vere et al. (2009) found for *Cirsium dissectum* that reduced genetic diversity had a negative impact on the survival of seedlings grown under standard conditions. The fact that we found effects mainly at the seedling stage and less so at late life-cycle stages suggests that deleterious alleles are purged in early life-cycle stages by the increased mortality of seedlings with a high inbreeding coefficient, releasing populations from their genetic load. Still, we also detected a positive relation-

ship between H_e and the probability of flowering in one of three years. Despite the effects of purging, populations still differ in their genetic variability, which could translate into effects on fitness if fitness-relevant alleles are rare or missing. Significant effects of genetic variation on fitness components were found only in single years for which fitness data were available. Such temporal variation is expected (Hornemann et al., 2012) as genetic variation is temporally more constant than fitness components which may vary strongly among years. The lack of relationship between genetic parameters and population growth rate (λ) can be explained by the fact that λ is not very sensitive to the changes in the vital rates that appeared to be affected by inbreeding depression (Kolb et al., 2010). Given our finding that population size and genetic diversity are positively related, the results of this study are consistent with those of Kolb et al. (2010), who showed that the survival and growth of young individuals as well as the proportion of adults flowering were lower in small populations and that most adult vital rates and λ were not related to population size.

4.4. Conclusions

From a conservation genetic point of view, *P. spicatum* appears to be negatively affected by habitat fragmentation, with small and isolated populations suffering from lower levels of genetic diversity and increased levels of biparental inbreeding as well as reductions in gene flow, which in turn seems to cause inbreeding depression mainly in early stages of the species' life cycle. The latter, however, may be beneficial at the population level, because populations are released from their genetic load. As a result, population growth rates, which are mostly influenced by differences in the survival and growth of adults and much less so by seedling and juvenile vital rates or reproduction (Kolb et al., 2010), are neither related to genetic diversity (this study) nor to population size (Kolb et al., 2010). Populations may thus be able to buffer the negative genetic effects of small population size. Still, the overall low levels of genetic diversity observed may be a reason for concern, as populations are poorly equipped to respond to future changes in the environment (Booy et al., 2000; Jump et al., 2009). This is especially relevant as patterns of population differentiation and short-distance gene dispersal indicate that colonization of new sites in response to putative changes in the environment is unlikely.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocon.2012.11.019>.

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