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Reproductive fitness, population size and genetic variation in *Muscari tenuiflorum* (Hyacinthaceae): The role of temporal variation

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ABSTRACT

In plant populations a positive correlation between population size, genetic variation and fitness components is often found, due to increased pollen limitation or reduced genetic variation and inbreeding depression in smaller populations. However, components of fitness also depend on environmental factors which can vary strongly between years. The dry grassland species *Muscari tenuiflorum* experiences long term habitat isolation and small population sizes. We analyzed seed production of *M. tenuiflorum* in four years and its dependence on population size and genetic variation. Genetic diversity within populations was high (AFLP: $H_e = 0.245$; allozymes: $H_e = 0.348$). An analysis of molecular variance revealed considerable population differentiation (AFLP: 26%; allozyme: 17%). An overall pattern of isolation by distance was found, which, however was not present at distances below 20 km, indicating stronger effects of genetic drift. Genetic diversity was positively correlated to population size. Self pollination reduced seed set by 24%, indicating inbreeding depression. Reproductive fitness was not correlated to genetic diversity and a positive correlation with population size was present in two of four study years. The absence of a general pattern stresses the importance for multi-year studies. Overall, the results show that despite long term habitat isolation *M. tenuiflorum* maintains seed production in many years independent of population size. The long term persistence of populations is thus expected to depend less on intrinsic genetic or demographic properties affecting seed production but on successful plant establishment and persistence, which latter are based on conservation and protection of suitable habitats.

Introduction

Many plant species do not exist as large continuous populations but rather as isolated patches that are embedded in a landscape matrix and separated by unsuitable types of vegetation. Patchy habitats often are small in size and are spatially isolated, which is ecologically important because it affects, e.g. population sizes, pollinator movement or seed and pollen dispersal (Drury, 1974; Kearns et al., 1998; Steffan-Dewenter and Tscharntke, 1999) and thus may affect seed production and long term population viability.

Small and isolated populations are particularly vulnerable to the effects of genetic drift (Barrett and Kohn, 1991; Ellstrand and Elam, 1993). In the long term genetic drift will reduce genetic diversity which is essential for adaptation to changing environmental conditions (Hughes et al., 2008). In the short term populations may experience higher levels of inbreeding and subsequentlyreduced individual fitness because inbred plants can have lower seed set, poorer seed quality or reduced offspring survival (Kéry et al., 2000; Mix et al., 2006; Oostermeijer et al., 1995). The effects of genetic drift are even stronger if population sizes fluctuate over time and genetic variation is reduced by bottlenecks (Amos and Harwood, 1998; Ellstrand and Elam, 1993). Isolation additionally leads to increased genetic differentiation of plant populations as effects of drift are not counteracted by gene flow among populations (Aegisdottir et al., 2009; Hensen et al., 2010).

Genetic variation in isolated plant populations has intensively been studied for many plant species (Aguilar et al., 2008; Leimu et al., 2006; Young et al., 1996). In general, habitat isolation and small population size are linked to low genetic diversity with potential negative effects on fitness. However, several species traits like breeding system or life span and local or regional characteristics such as rarity, local population history, but also the type of rarity can affect these relationships (Mix et al., 2006). Negative effects of isolation and reduced population size are expected in species that became isolated or rare only recently, but were more common formerly with larger and well connected populations (Aguilar et al., 2008; Ellstrand and Elam, 1993). In contrast, old rare species that are naturally restricted to small and isolated habitats may be

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less threatened because they are adapted to spatial isolation and small population size (Lutz et al., 2000; Rabinowitz, 1981). Some evidence exists that historically rare and isolated species show no reduction in genetic diversity or fitness with small population size (Aguilar et al., 2008; Brigham, 2003). Mechanisms to counteract negative consequences of isolation and small population size may include long distance dispersal strategies, permanent seed banks or strategies to cope with genetic threats, e.g. by purging genetic load. Together these factors can buffer species from local extinction (Ozinga et al., 2009).

Seed production in plants is of fundamental importance for local and regional persistence. Seed production is affected by both, environmental factors and population characteristics. However, these effects may differ strongly between years (Ågren et al., 2008; Hensen and Wesche, 2006). Factors that contribute to temporal variation include not only weather conditions and limited resources but also biotic interactions with mutualistic pollinators (e.g. Bustamante and Búrquez, 2008; Kenta et al., 2004; Tsaliki and Diekmann, 2011) or pathogens. Because of this temporal variation, single observations may lead to biased results, and observations from multiple years may give more insight into underlying processes (Pfeifer et al., 2006).

Xerothermic dry grasslands represent a type of azonal vegetation in Central Europe that is restricted to spatially isolated regions characterized by low rainfall and therein to limited habitat patches on dry shallow soils and southern exposure. *Muscari tenuiflorum* (Hyacinthaceae) is restricted to these grasslands and thus has a long history of habitat isolation and small population sizes in Central Europe (Herrmann et al., 2006). *M. tenuiflorum* exhibits certain characteristics, like long life span and putatively a high outcrossing rate, which could act as a buffer against loss of genetic variation. Nevertheless, in a one-year study a strong reduction of reproductive fitness in small populations was observed and it was hypothesized to be a consequence of both, pollen limitation and increased inbreeding due to high selfing rates (Weiss and Mahn, 1996). To further elucidate the observed effects we use repeated assessments of seed production, population genetic approaches, and pollination experiments and asked: (i) What is the level of genetic variation within and among long-term isolated populations of *M. tenuiflorum*? (ii) Is reproductive fitness related to population size and genetic variation and how consistent are these relationships across years? (iii) Do pollinator limitation and inbreeding depression lead to reduced seed set?

Materials and methods

Study species and study sites

Muscari tenuiflorum Tausch (Hyacinthaceae, $2n = 2x = 18$) is a perennial, bulbous herb with exclusively sexual reproduction. The biology of the species has been reviewed previously (Herrmann et al., 2006). Its inflorescences consist of lower fertile and upper sterile flowers. The fertile flowers provide pollen and nectar, and the sterile flowers serve as showy structure to attract pollinators, mainly bumblebees. The species is self-compatible, and seems to be mainly outcrossed, although a formal estimate of outcrossing rate was not available. The fruit is a capsule with up to six seeds with no adaptations to long distance dispersal. *M. tenuiflorum* reproduces exclusively by seed and does not propagate vegetatively, as the bulbs do not produce offset bulbs (Herrmann et al., 2006). It takes at least eight to ten years from seedling stage to maturity and adult plants reach ages far beyond. A seed bank does not exist (Herrmann et al., 2006).

The species' distribution ranges in the submeridional and south-temperate zone (Meusel et al., 1965) from south-eastern Europe to Anatolia, Transcaucasia and the Iranian highlands. The species reaches the north-western edge of its distribution in Germany and occurs at open xerothermic dry grasslands and rarely in dry oak-woodlands on south-facing rocky slopes. In Germany, *M. tenuiflorum* is a red list species and categorized as vulnerable. Here, the species is known to occur at 43 sites (Herrmann et al., 2006), 31 of which were included in the current study (Fig. 1). Sites are clustered in two adjacent regions near Halle and Naumburg. Distances between populations range between 100 m and 71 km (23.5 ± 22.0 km, mean $\pm$ SD).

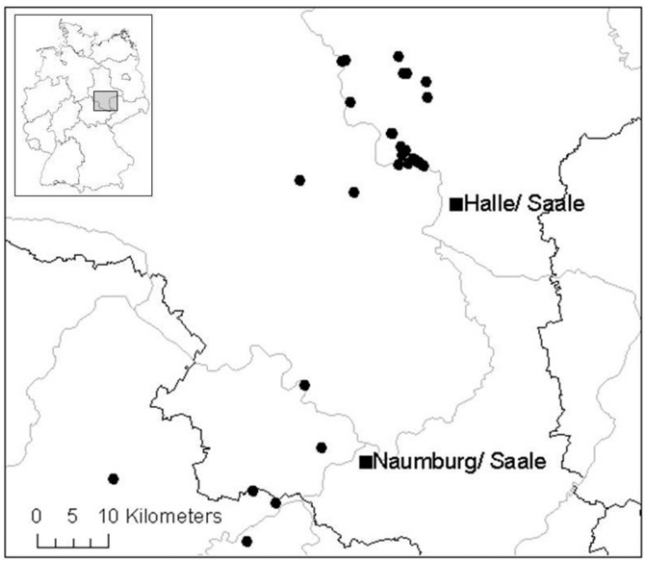

Fig. 1. Geographic location of the study sites of *Muscari tenuiflorum* in Central Germany.

Sampling

As a measure of population size the number of flowering plants was recorded in 1995, 1998, 2000 and yearly from 2004 to 2008, either by individual counts or by estimations. However, not all populations were included in all analyses. For calculations with mean population size we used the arithmetic mean of all available count data (Table 1). In particularly dry years plants may stay dormant. We are therefore aware, that the number of flowering plants may underestimate the population size. However, such effects are expected to occur simultaneously in all populations as they are located in the same region. Additionally, the populations studied range from a few to many thousand individuals. Thus the differences in census sizes among populations are likely representative for differences in total population sizes.

We used both, dominant amplified fragment length polymorphisms (AFLPs) and codominant allozyme markers to characterize genetic variation. For AFLP analyses we randomly collected leaf samples of up to 12 individuals at 30 populations in 2008. Samples were freeze dried immediately after collection. Leaf samples for allozyme analyses were collected from 9 to 39 individuals at 29 populations in 1999. Samples were kept cool and were immediately frozen in the laboratory until analysis.

To quantify components of reproductive fitness we collected, if possible, 20 infructescences per population in July to August of the years 1995 (Weiss and Mahn, 1996), 2000, 2004 and 2007 in 10–18 populations. We determined plant height, the number of fruits, number of intact seeds per capsule and the total number of seeds per plant.

As the study was conducted over several years, weather may have had an effect. The 100 year averages for

Table 1
Summary of study sites and genetic diversity parameters in 31 populations of *M. tenuiflorum*.

Site	Name	Latitude/longitude	Region	Population size		AFLP				Allozymes			
				Mean (SD)	Years	*N*	H_e	*PLP*	B_r	*N*	H_e	A_r	F_{is}
M01	Dobis	51°36′31.55″/11°46′18.82″	1	1046 (631)	6	12	0.239	52.1	1.42	30	0.349	2.24	0.284**
M02	Gottgau	51°36′31.55″/11°46′18.82″	1	18 (15)	8	4	0.211	35.6	1.36	30	0.199	1.57	0.561***
M03	Schiedsberg	51°38′4.16″/11°55′34.78″	1	8066 (3429)	8	12	0.309	69.2	1.52	31	0.403	2.62	0.09
M04	Schlettau	51°39′59.52″/11°52′13.72″	1	271 (227)	5	12	0.252	56.2	1.43	29	0.438	2.63	−0.004
M05	Krosigk	51°36′53.95″/11°55′47.89″	1	2720 (1545)	6	12	0.287	67.1	1.5	39	0.31	2.29	0.141
M07	Lauchengrund	51°34′5.76″/11°51′20.21″	1	481 (234)	9	12	0.332	74	1.56	30	0.386	2.58	0.184*
M08	Ginsterkuppe	51°33′6.94″/11°52′35.66″	1	884 (1217)	9	11	0.303	71.2	1.48	32	0.375	2.42	0.251**
M09	Küsterberg	51°32′48.54″/11°53′13.08″	1	2462 (1995)	10	12	0.262	58.9	1.46	31	0.384	2.72	0.108
M10	Schulberg	51°32′29.09″/11°52′46.14″	1	247 (172)	10	12	0.272	65.8	1.52	30	0.459	2.32	0.245**
M11	Kerbe	51°31′47.32″/11°52′18.24″	1	86 (73)	10	12	0.258	62.3	1.43	30	0.205	1.66	0.435**
M12	Lunzberg 3	51°31′50.95″/11°53′30.86″	1	259 (122)	9	12	0.225	48.6	1.36	26	0.3	1.66	0.093
M13	Lunzberg 2	51°31′48.68″/11°53′27.24″	1	523 (272)	9	12	0.237	52.1	1.41	31	0.464	2	0.224*
M15	FM Fels	51°31′51.99″/11°54′50.76″	1	38 (32)	7	12	0.22	52.1	1.37	29	0.326	2.09	−0.047
M16	FM Station	51°31′53.93″/11°54′44.53″	1	74 (103)	7	12	0.173	37.7	1.28	30	0.273	2.12	0.175
M17	FM Hang	51°31′57.80″/11°54′37.26″	1	494 (275)	9	12	0.221	52.1	1.37	24	0.17	1.72	0.14
M18	FM Mitte	51°32′6.53″/11°54′19.60″	1	595 (543)	8	12	0.27	63.7	1.46	37	0.43	2.56	−0.041
M19	Langenbogen	51°29′40.31″/11°46′55.61″	1	800 (469)	4	12	0.301	70.5	1.52	29	0.392	2.29	0.08
M20	Gimritz	51°34′9.65″/11°51′32.66″	1	2183 (1853)	6	12	0.302	72.6	1.55	29	0.456	2.66	0.168*
M21	FM Pfad	51°32′11.69″/11°54′4.54″	1	3800 (2334)	4	12	0.254	59.6	1.44	14	0.348	2.46	0.077
M22	FM Ost	51°31′40.03″/11°55′17.24″	1	498 (323)	5	12	0.216	46.6	1.35	30	0.429	2.3	0.184*
M24	Lämmerberg	51°30′34.72″/11°40′16.02″	1	1800 (1424)	4	12	0.272	62.3	1.46	35	0.415	2.15	0.285***
M25	Pfaffengrund	51°39′34.59″/11°45′23.27″	1	23 (23)	3	8	0.248	59.6	1.44	9	0.252	1.67	0.406
M26	Gerbstedt	51°37′54.45″/11°38′10.13″	1	150	2	–	–	–	–	37	0.333	2.52	0.240**
M29	Rothenburg	51°39′44.35″/11°45′46.63″	1	100	1	12	0.163	34.2	1.25	25	0.338	1.67	0.460**
M32	FM Weide	51°32′9.11″/11°54′8.18″	1	250	1	11	0.272	69.2	1.48	–	–	–	–
M33	Reisdorf	51°6′55.53″/11°34′41.11″	2	200	1	12	0.265	60.3	1.47	29	0.441	2	0.128
M34	Bad Sulza	51°6′3.37″/11°37′33.27″	2	200	1	11	0.231	55.5	1.38	10	0.404	2	−0.073
M35	Flurstedt	51°3′6.59″/11°34′4.17″	2	300	1	12	0.209	45.9	1.35	29	0.077	1.44	−0.059
M36	Buttstädt	51°7′46.36″/11°17′54.60″	2	1500	1	11	0.238	54.1	1.37	34	0.491	2.33	0.261**
M37	Tote Täler	51°10′13.82″/11°43′4.83″	2	30	1	12	0.11	21.9	1.16	13	0.244	1.65	0.053
M38	Gleina	51°14′58.61″/11°40′58.30″	2	200	1	12	0.189	43.2	1.28	–	–		–
Mean							0.245	55.81	1.41		0.348	2.15	0.079
SD							0.048	12.68	0.09		0.1	0.38	0.118

Region: north (1), south (2); *Population size*: number of flowering individuals; *Years*: number of years with census data; *N*: sample size; *PLP*: percentage of polymorphic loci; H_e: expected heterozygosity; B_r: band richness; A_r: Allelic richness; F_{IS}: inbreeding coefficient.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

temperature and precipitation during the flowering time (May and June) in the study region are 14.8 °C and 53.4 mm. The respective values in the years of this study, 1995, 2000, 2004 and 2007, were 13.9 °C/55.7 mm, 17.0 °C/34.6 mm, 13.9 °C/85.2 mm and 16.9 °C/90.1 mm, respectively (weather station Leipzig-Schkeuditz, German Weather Service).

Pollination experiment

To examine the effect of spontaneous self-pollination on seed set and the dependence on population size we conducted a pollinator exclusion experiment in 2004 in 15 populations with up to 60 individuals each (in total 530 plants). Before the start of flowering half of the individuals were bagged with gauze (1 mm mesh size). The other half was marked, left untreated as control with open pollination, but bagged after anthesis to allow collection of all seeds. No manual selfing was conducted, as preliminary experiments had shown that hand pollination treatments damaged the flowers. Moreover, because the stigma is placed in between the anthers, spontaneous self pollination does occur in bagged flowers and no hand self pollination is needed. In August we determined the number of intact seeds per capsule and per plant.

Population genetic analyses

We generated AFLPs following Lachmuth et al. (2010). In short, DNA was extracted from leaf tissue using the DNeasy 96 plant kit (Qiagen, Hilden, Germany) and digested with two restriction enzymes (EcoRI, MseI). After preamplification, we used three selective primer combinations (FAM EcoRI – ACT/MseI-CAG, VIC EcoRI – ACG/MseI – CAC, NED EcoRI – ACC/MseI – CTG). Fragment analysis was performed on an ABI 3130 genetic analyzer with POP7 polymer (Applied Biosystems) and GeneScan 500 LIZ as internal size standard. Only unambiguously scorable polymorphic AFLP bands were manually scored for presence (1) or absence (0) using GeneMapper (version 3.7). An error rate of 3.1% was estimated from 34 samples replicated from the same DNA extract. Analysis of 345 individuals with three primer combinations provided 146 polymorphic loci in the range of 38–493 bp.

We used standard horizontal starch gel electrophoresis to assess genetic variation at allozyme loci. Frozen leaves where extracted with buffer 1 according to Soltis et al. (1983). Four loci that had proven to be polymorphic after screening of 14 enzyme systems were analyzed and genotyped according to Wendel and Weeden (1989): ADH (TBE buffer system), GPI (S4 buffer), MDH and PGM (histidin–citrate pH 7 buffer).

For analysis of the outcrossing rate we germinated 168 seeds from 50 open pollinated plants originating from 11 populations. We genotyped them with AFLP and used the MLTR program (Ritland, 2002) to determine single locus and multilocus outcrossing rates.

For AFLP we calculated within population genetic variation as gene diversity (H_e) and percentage of polymorphic loci (*PLP*) based on allele frequencies which were estimated by the square root method using the inbreeding coefficient derived from the allozyme analysis ($F_{IS} = 0.174$), using the software AFLP-Surv v. 1.0 (Vekemans, 2002). We computed band richness (B_r), a rarefaction

Table 2
Summary of analysis of molecular variance (AMOVA) for the AFLP and allozyme data sets grouped into two regions (north (M01–M32) and south (M33–M38)).

Source of variation	AFLP				Allozymes			
	df	Sum of squares	Variance components	Variation [%]	df	Sum of squares	Variance components	Variation [%]
Among groups	1	182	0.81	3.45***	1	24	0.04	5.47***
Among populations within groups	28	2516	6.15	26.01***	27	213	0.13	17.37***
Within populations	327	5455	16.68	70.55***	1595	928	0.58	77.16**
Total	356	8153	23.65		1623	1165	0.75	

*$p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

measure of genetic variation independent of sample size standardized to the smallest sample size with AFLP-Div 1.0 (Coart et al., 2005). Population differentiation was analyzed with *F*-statistics following Lynch and Milligan (1994) with AFLP-Surv.

For allozymes we calculated within population genetic variation as expected heterozygosity (H_e) and inbreeding coefficient (F_{IS}) averaging over loci. Significance of F_{IS} within samples was estimated by 500 permutations. Allelic richness (A_r) was computed using the rarefaction method based on minimum sample size (El Mousadik and Petit, 1996). Population differentiation (F_{ST}) and descriptive parameters were analyzed in FSTAT v. 2.9.3.2 (Goudet, 1995).

As a composite measure of genetic variation we used the scores of the first axis of a PCA of $H_{e\,(AFLP+allozymes)}$, *PLP*, B_r, A_r and F_{IS} which accounted for 64% of variation (function pca from package pcaMethods, Stacklies et al., 2007).

For both AFLP and allozyme data population structure was assessed with an analysis of molecular variance (AMOVA) with Arlequin (version 3.1.1.1). In order to test for isolation by distance we correlated pairwise geographic distance against pairwise genetic differentiation and checked for significance using a Mantel test based on 1000 permutations with the EcoDist package (Goslee and Urban, 2007). We used pairwise estimates of Slatkins linearized $F_{ST}/(1-F_{ST})$ and log transformed geographic distances (Rousset, 1997).

Statistical analysis

To analyze if seed set (number of seeds per capsule and seeds per plant) in a given year is dependent on population size of that year, we used linear mixed effect models. To account for the temporal pseudoreplication, the sampling unit (year) was included in the models as a random effect. Simple linear regression was used to assess relationships between log transformed population sizes, genetic variation and components of reproductive fitness.

Multiple linear regressions were used to test the combined effects of population size and genetic variation on components of fitness for each study year separately. As independent variables we used log transformed population size and genetic variation. Dependent variables were seeds per capsule and seeds per plant.

For the analysis of the pollinator exclusion experiment, generalized linear models were used to analyze treatment effects on the number of seeds per capsule and per plant. Treatment effects on the number of seeds per capsule were tested using an *F* test. The effect on the number of seeds per plant was tested with quasi-Poisson errors and a log-link function. We adjusted the χ^2 statistics to account for overdispersion employing a corrected *F* test (Crawley, 1993). The factor site was included into the models as interaction term. To analyze if treatment effects were dependent on population size we calculated the log response ratio of seed set for each population, $\ln RR_S = \ln(S_{PE}/S_{OP})$, with S_{PE} and S_{OP} being mean seed set under pollinator exclusion and open pollination, respectively (Hedges et al., 1999). The $\ln RR_S$ was then regressed against log transformed population size.

If no other program is mentioned, statistical tests were performed in R v. 2.7.2. (R Development Core Team, 2008).

Results

Genetic variation within and among populations

Singlelocus and multilocus estimates of outcrossing rate were $t_s = 0.61$ (SD 0.05) and $t_m = 0.81$ (SD 0.05), indicating a mixed mating system with a high proportion of outcrossing and a considerable level of biparental inbreeding ($t_m - t_s = 0.2$). At the population level, estimates of gene diversity of AFLP differed widely among populations and H_e ranged from 0.110 to 0.332, with a mean of 0.245 (Table 1). Percentage polymorphic loci (*PLP*) ranged from 21.9% to 74.0%, with a mean of 55.8%. Genetic differentiation between populations was high (overall $F_{ST} = 0.252 \pm 0.081$ SE). Values for pairwise F_{ST} ranged from 0.016 to 0.561. Hierarchical partitioning by AMOVA showed that 3.5% of variation was due to differences between the northern and southern region, 26% resided among populations and 70.5% within populations (Table 2). Mantel tests revealed a significant positive relationship between genetic differentiation and geographic distance in the whole dataset ($r = 0.336$, $p = 0.005$; Fig. 2). However, no pattern of isolation by distance was found when the two regions were analyzed separately ($p > 0.3$), indicating a predominant role of genetic drift relative to gene flow.

Allozyme analysis revealed widely differing levels of variation, with allelic richness (A_r) ranging from 1.44 to 2.72 and H_e ranging from 0.077 to 0.491 (Table 1). The inbreeding coefficient (F_{IS}) ranged between −0.077 and 0.561 (mean 0.174). Out of 29 populations, F_{IS}

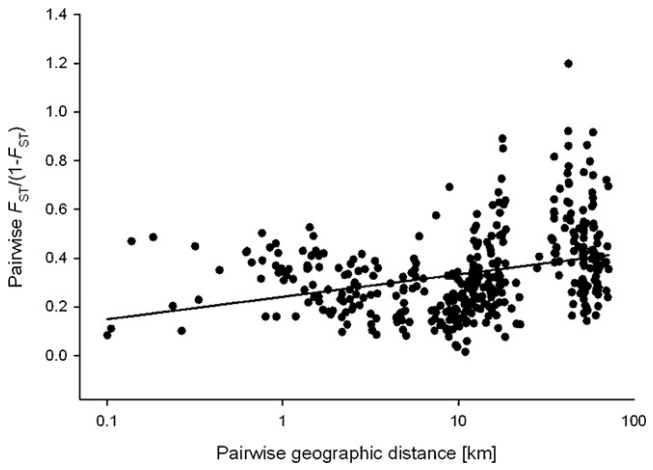

Fig. 2. Relationship between pairwise genetic and geographical distances for 30 *M. tenuiflorum* populations (AFLP data); ($r = 0.336$, Mantel $p = 0.005$).

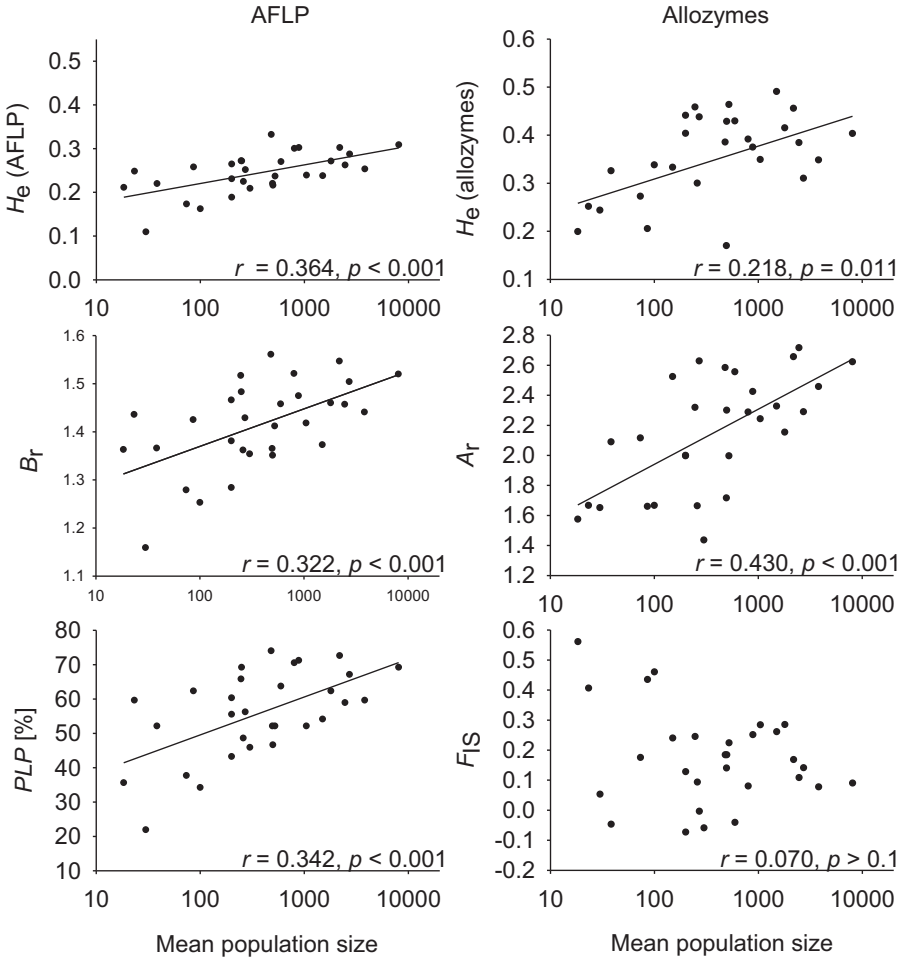

Fig. 3. Correlations between mean population size (number of flowering individuals) and measures of genetic variation of AFLP and allozymes.

was significantly positive for 13 populations, indicating departure from Hardy–Weinberg equilibrium and a lack of heterozygotes. Genetic differentiation was high (overall $F_{ST} = 0.211 \pm 0.036$ SE), with pairwise F_{ST} values ranging from −0.031 to 0.784. Similarly, AMOVA revealed 5.47%, 17.37% and 77.16% of variation among regions, among populations within regions and within populations, respectively (Table 2). No pattern of isolation by distance was detected for allozymes ($p > 0.145$).

Patterns of genetic variation were largely consistent between allozyme and AFLP marker systems as revealed by significant correlations (H_e: $r = 0.2$, $p = 0.017$; F_{ST}: $r = 0.443$, $p = 0.003$, Mantel test). All measures of genetic diversity for both genetic marker systems except for F_{IS} were positively correlated to mean population size ($p < 0.05$; Fig. 3).

Components of reproductive fitness, population size and genetic variation

The pollinator exclusion experiment revealed that even without pollinator visit, considerable seed set was found, indicating self compatibility. At the population level the number of seeds per capsule and seeds per plant differed significantly between treatments ($p < 0.001$) and sites ($p < 0.001$) and was reduced under pollinator exclusion. Pollinator exclusion reduced the number of seeds per capsule by 24.6% (±13.1 SD) and seeds per plant by 23.7% (±14.5 SD) (Fig. 4). The reduction of seed set after pollinator exclusion was not significantly correlated to population size or any measure of genetic variation ($p > 0.051$).

Estimates of components of reproductive fitness varied strongly among years and among populations. Overall, population size had a significant positive effect on the number of seeds per plant ($p < 0.001$) and on the number of seeds per capsule ($p < 0.01$), but such effects differed between the four study years ($p < 0.04$). Separate linear regressions for each of the study years showed that for the number of seeds per plant the relationship was significantly positive in two of four years (1995 and 2000; $p < 0.05$). The number of seeds per capsule was correlated to population size only in 1995 ($p < 0.01$; Fig. 5).

A correlation analysis of the population means of the fitness components with the measures of genetic diversity revealed a significant correlation only for plant height with $H_{e\_allozymes}$ ($r = 0.336$, $p = 0.011$) and with F_{IS} ($r = -0.290$, $p = 0.021$).

Multiple regressions on the combined effects of population size and genetic variation on components of fitness for each study year separately revealed no effect of genetic variation. However, population size positively affected the number of seeds per plant in 1995 ($p < 0.01$).

Discussion

Our study revealed three main findings. First, populations of *M. tenuiflorum* harbor high levels of genetic variation but are affected by genetic drift resulting in population differentiation and reduction of genetic variation in smaller populations. Second, pollinator exclusion leads to a moderate reduction of seed set. Third, seed set was positively correlated to population size in some years, but

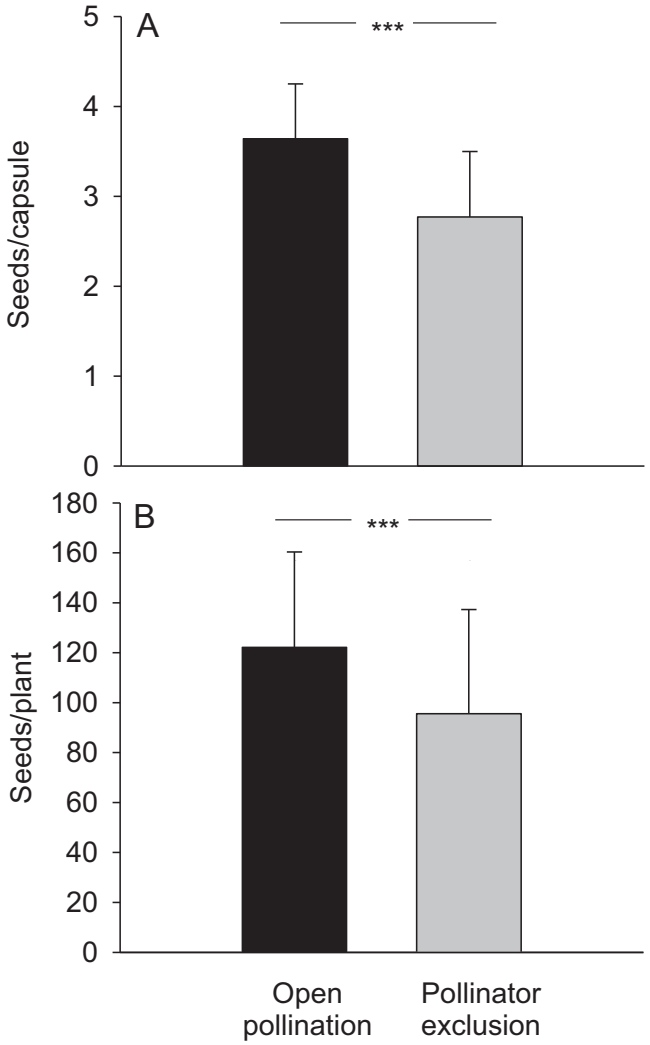

Fig. 4. Pollinator exclusion experiment: effects of pollination treatments on the mean number of seeds/capsule (A) and seeds/plant (B) (mean ± SD).

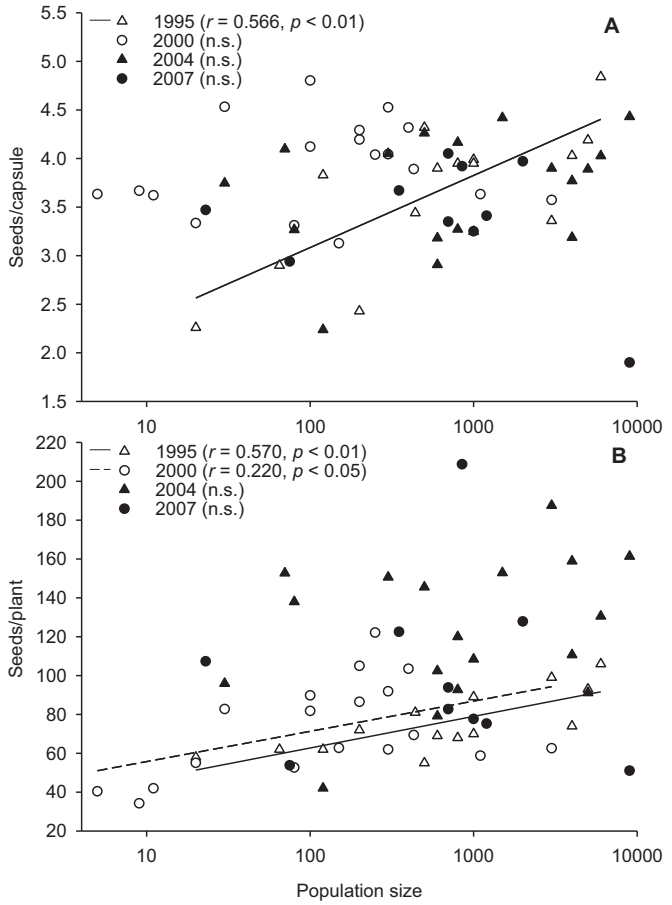

Fig. 5. The relationship between population size (number of flowering individuals) and the population mean of the number of seeds per capsule (A) and seeds per plant (B) in four different years. Regression lines are displayed for the significant correlations in 1995 (solid line) and 2000 (dashed line).

in two of four years there was no significant relationship. Thus, *M. tenuiflorum* does not generally match the pattern of higher fitness and genetic diversity in large populations that was observed in many species (Leimu et al., 2006). Two not mutually exclusive processes may drive a positive correlation between population size and components of reproductive fitness. These processes are pollen limitation because of lower pollinator abundances and inbreeding depression because of higher selfing rates in small populations, both of which may vary temporally.

Seed set

The exclusion experiment revealed that components of reproductive fitness in *M. tenuiflorum* were positively influenced by pollinator visits, although self pollination also results in considerable seed set. Principally, lowered seed set under pollinator exclusion could be attributed to a reduced quantity of pollen reaching the stigmas. Pollen quantity can itself be influenced by species density, pollinator visitation rate or pollinator efficiency (Ghazoul, 2006; Johnson et al., 2003; Wilcock and Neiland, 2002). In large populations higher pollinator activity due to a higher number of flowering plants or larger patch size is expected (Wilcock and Neiland, 2002) and can therefore result in higher seed set in these populations. However, we did not find a general positive relationship between population size and components of reproductive fitness. Also, the abundance of co-flowering species was not related to seed set (unpubl. data). Besides pollen quantity pollen quality has also been shown to affect seed set. Lower reproductive fitness after self pollination, as we found it in this study, has often been attributed to inbreeding depression (Husband and Schemske, 1996). To some extent our results suggest inbreeding effects on plant performance since plant height was positively connected to heterozygosity and negatively to inbreeding coefficients. Nevertheless, our results provide no clear evidence for inbreeding depression in small populations of *M. tenuiflorum*. First, there is no significant relationship between inbreeding coefficients and population sizes despite lower genetic diversity in small populations. Although the highest F_{IS} values were mainly represented in the smallest populations, which may be the result of the long history of inbreeding, there were also very small populations at Hardy–Weinberg equilibrium. The latter may have experienced recent declines and individuals are still representatives of larger and more outcrossing populations. The absence of a connection between population size and F_{IS} has frequently been reported and attributed to a possible selection against homozygotes and an absence of homozygous rare alleles in small populations or Wahlund effects in large populations (Honnay and Jacquemyn, 2007; Leimu et al., 2006; Raijmann et al., 1994; Young et al., 1999). Second, assuming that inbreeding is responsible for lower seed set in small populations we would expect to find stronger effects of the pollination treatment in small populations than in large ones because of a possible higher relatedness of individuals.

However, there was no such effect of population size or genetic variation on the response to the pollination treatments. Probably genetic diversity, which is clearly reduced in small populations, is still high enough to prevent negative effects of inbreeding depression.

Temporal variation

In the repeated seed set measurements we found a positive relationship between population size and seed set. However, both, seed set values and also the relationship between population size and seed set differed greatly between years. Although successful reproduction is essential for population persistence or long term population growth, inter-annual variation in seed production might be of minor importance in such long lived species as *M. tenuiflorum*. Generally, inter-annual variation in seed set is very common in long lived plants and single years of low seed production do not critically weaken the long term performance of a population. In contrast, high survival rates of adult plants are more important for population survival in long lived species (Bossuyt and Honnay, 2006). It is known that individuals of *M. tenuiflorum* can get very old (Herrmann et al., 2006), which presents an effective buffer against varying reproductive output among years.

Overall, population size positively affected seed set but still, in two of four study years seed set was independent of population size. Thus, a positive connection between population size and seed set seems to depend on annually changing environmental conditions, which are most likely weather conditions during flowering or fruiting. Both flower development and reproduction in *M. tenuiflorum* are sensitive to weather conditions as the whole inflorescence may wither in bud stage in very dry years (Herrmann et al., 2006). Seed and fruit set in those plants that still flower in dry years are likely to be most strongly affected by water stress. It is well known that drought during seed and fruit maturation leads to seed or fruit abortion (e.g. Aragon et al., 2008; Lloyd, 1980). Fluctuating abiotic conditions are known to cause interannual variation in fruit and/or seed set. In *Rubus chamaemorus*, for example, detrimental late frosts affected flower and fruit development (Ågren, 1988). Delayed flower development after unfavorable spring conditions can influence fruit set indirectly by an asynchronization of flowering with pollinator activity (Ågren, 1988; Ågren et al., 2008). Thus, possible effects on seed set that are related to population size, such as pollinator activity or herbivory may be concealed by environmental effects. Indeed, precipitation during the flowering period in those study years with no relationship between seed set and population size was very high compared to the long term mean, thus releasing plants from soil water stress. This is consistent with the finding that inbreeding depression is greater under stressful conditions compared to benign environments (Armbruster and Reed, 2005).

The sensitivity of seed set to abiotic conditions also stresses the importance of multi-year studies to elucidate patterns that influence seed set, particularly in systems like these xerothermic dry grasslands that are sensitive to variability of environmental conditions.

Our estimates of components of reproductive fitness were measured in the field, where other environmental conditions like, e.g. soil characteristics, management history or interspecific interactions can influence observed patterns. Growing individuals from different sites in the same environment or measuring environmental parameters in situ would surely help to identify environmental effects and to disentangle them from genetic impacts on reproductive fitness.

Genetic diversity within populations

Genetic variation was high in most populations, which is typical for mainly outcrossing, sexually reproducing and long lived species (Hamrick and Godt, 1989; Loveless and Hamrick, 1984; Nybom, 2004). Such species generally show high genetic variability within and low differentiation among populations (Hamrick and Godt, 1996; Šmídová et al., 2011). However, outcrossing species are especially sensitive to fragmentation and show stronger reductions in genetic diversity and the number of alleles compared to selfing species (Aguilar et al., 2008). Recent population declines and population extinctions in *M. tenuiflorum* may cause such genetic consequences of fragmentation (Dannemann et al., 1999; Frank and Neumann, 1999). In fact, genetic variation was related to population size, but strong reductions were only found at census sizes of less than 200 flowering individuals. Probably, the predominantly outcrossing mating system of the species could maintain high diversity within populations (Loveless and Hamrick, 1984; Nybom, 2004). Additionally, the long individual life span in this geophyte may act as a buffer against the loss of genetic variation (Ellstrand and Elam, 1993). Also, due to cessation of flowering in very dry years, the variation of census size is not necessarily affecting total population size. This is consistent with the prediction that the genetic response to fragmentation is delayed in long lived species (Ewers and Didham, 2006; Lowe et al., 2005).

We found high genetic differentiation among populations although most genetic variation lies within populations. The absence of isolation by distance at the local scale confirms the low gene flow among populations and a strong influence of genetic drift. Similar patterns have been reported from other naturally isolated and rare species (e.g. Aegisdottir et al., 2009; Kuss et al., 2008; Šmídová et al., 2011). Additionally, colonization of new habitats is unlikely although suitable but unoccupied sites do exist. The heavy seeds can only be dispersed in the fur or hooves of grazers (Herrmann et al., 2006). However, sheep pasturing, the traditional land use practice in former times, strongly declined since 1989 (Herrmann et al., 2006), and the current conservation management focuses on removal of biomass but less on functional connection of habitats through movement of sheep between sites.

In conclusion, our findings show that despite long term habitat isolation and recent declines, *M. tenuiflorum* populations mostly maintain high levels of genetic variation and seed production, the latter being affected by population size only in single years. Slow growth, long lifespan and high outcrossing rate may partly buffer effects of population size. Thus, long term persistence of populations is expected to depend less on intrinsic genetic or demographic properties affecting seed production but on successful plant establishment and persistence which is based on conservation and protection of suitable habitat.

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