

Fine-scale genetic structure in the orchid Gymnadenia conopsea is not associated with local density of flowering plants

Nina Sletvold, Nina Joffard, Linus Söderquist

▶ **To cite this version:**

Nina Sletvold, Nina Joffard, Linus Söderquist. Fine-scale genetic structure in the orchid Gymnadenia conopsea is not associated with local density of flowering plants. American Journal of Botany, In press, ⟨10.1002/ajb2.16273⟩. ⟨hal-04441652⟩

HAL Id: hal-04441652

https://hal.science/hal-04441652v1

Submitted on 6 Feb 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



HAL Authorization

Check for updates

Received: 3 July 2023 | Accepted: 16 November 2023

DOI: 10.1002/ajb2.16273

RESEARCH ARTICLE

American Journal of Botany

Fine-scale genetic structure in the orchid *Gymnadenia conopsea* is not associated with local density of flowering plants

Nina Sletvold[1] | **Nina Joffard**[1,2] | **Linus Söderquist**[1]

[1]Plant Ecology and Evolution, Department of Ecology and Genetics, EBC, Uppsala University, SE-752 36 Uppsala, Sweden

[2]UMR 8198 –Evo-Eco-Paleo, University of Lille, Lille, France

Correspondence

Nina Sletvold, Plant Ecology and Evolution, Department of Ecology and Genetics, EBC, Uppsala University, SE-752 36 Uppsala, Sweden.
Email: nina.sletvold@ebc.uu.se

Abstract

Premise: Density-dependent pollinator visitation can lead to density-dependent mating patterns and within-population genetic structure. In *Gymnadenia conopsea*, individuals in low-density patches receive more self pollen than individuals in high-density patches, suggesting higher relatedness at low density. Ongoing fragmentation is also expected to cause more local matings, potentially leading to biparental inbreeding depression.

Methods: To evaluate whether relatedness decreases with local density, we analyzed 1315 SNP loci in 113 individuals within two large populations. We quantified within-population genetic structure in one of the populations, recorded potential habitat barriers, and visualized gene flow using estimated effective migration surfaces (EEMS). We further estimated the magnitude of biparental inbreeding depression that would result from matings restricted to within 5 m.

Results: There was no significant relationship between local density and relatedness in any population. We detected significant fine-scale genetic structure consistent with isolation by distance, with positive kinship coefficients at distances below 10 m. Kinship coefficients were low, and predicted biparental inbreeding depression resulting from matings within the closest 5 m was a modest 1–3%. The EEMS suggested that rocks and bushes may act as barriers to gene flow within a population.

Conclusions: The results suggest that increased self-pollen deposition in sparse patches does not necessarily cause higher selfing rates or that inbreeding depression results in low establishment success of inbred individuals. The modest relatedness suggests that biparental inbreeding depression is unlikely to be an immediate problem following fragmentation of large populations. The results further indicate that habitat structure may contribute to governing fine-scale genetic structure in *G. conopsea*.

KEYWORDS

coancestry, density-dependence, fine-scale genetic structure, gene flow, kinship, local density, Orchidaceae, pollen dispersal, relatedness, seed dispersal

Spatial genetic structure is frequent within natural plant populations, reflecting spatially restricted pollen and seed dispersal (Loveless and Hamrick, 1984; Epperson, 2000; Vekemans and Hardy, 2004), historical demographic effects (Ennos, 1994; Epperson and Chung, 2001), or potentially, local selection (Kalisz et al., 2001). In general, within-population genetic structure follows an isolation-by-distance (IBD) model (reviewed by Vekemans and Hardy, 2004), where relatedness among individuals decreases gradually with geographic distance (Rousset, 2000), parallel to the IBD pattern expected among populations at larger spatial scales (Wright, 1943). In addition, habitat heterogeneity such as rocks, trees and shrubs can act as barriers to gene flow, as can mountains and lakes at the landscape level (i.e., isolation by resistance, McRae, 2006). Observed patterns of isolation by distance or resistance suggest that spatially restricted gene flow

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2024 The Authors. *American Journal of Botany* published by Wiley Periodicals LLC on behalf of Botanical Society of America.

is the most important governing process of spatial genetic structure in most systems.

Within species, stronger fine-scale genetic structure has been documented in sparse compared to dense populations (Vekemans and Hardy, 2004), and for a given population density, theoretical studies predict that the strength of spatial genetic structure should increase with spatial aggregation of adult plants (Leblois et al., 2004; Robledo-Aruncio and Rousset, 2010). This prediction has rarely been tested empirically, but was supported by a study that found a positive association between fine-scale genetic structure and individual spatial aggregation across five populations of *Silene ciliata* (Lara-Romero et al., 2016). The stronger structure in patchy populations implies that adjacent individuals on average are more closely related than similarly separated individuals in evenly spaced populations. However, it is presently unclear how this higher average relates to density variation within the population, i.e., whether individuals in dense areas of the population are less related than individuals in sparse areas of a similar size.

One mechanism by which local density can influence gene flow within populations is via density-dependent pollen dispersal, which can result if pollinator attraction or behavior changes with plant density. Several studies have found that pollinators prefer dense patches, but visit a higher proportion of the open flowers on individuals located in low-density patches, leading to more geitonogamous pollination (Klinkhamer and de Jong, 1990; Grindeland et al., 2005; Jing et al., 2013) and higher selfing rates (Karron et al., 1995; Christopher et al., 2021). Direct tracking of pollen movement within populations has shown that more self pollen is deposited on individuals located in sparse patches (Söderquist et al., 2020), indicating less outcrossing and lower mate diversity compared to dense patches, and paternity analyses have documented reduced mate diversity in isolated individuals (Rhodes et al., 2017). At the same time, maximum pollen dispersal distances may decrease with density. Pollinators need to fly longer distances between plants in sparse populations (e.g., Levin and Kerster, 1969; Tero et al., 2005), and paternity analyses have shown significantly longer pollen-dispersal distances for individuals in sparse patches (Tambarussi et al., 2015). While geitonogamous self pollination is expected to be more important in sparse patches, biparental inbreeding is likely to be more important in dense ones (Levin and Kerster, 1969). Similarly, seed dispersal can be density-dependent if animal vectors are involved, but even when the dispersal process is passive, individuals located in sparse patches should have less overlap of seed shadows and less mixing of seeds compared to individuals in dense patches (Nathan and Muller-Landau, 2000). In sum, these processes should cause low-density patches to contain seeds from fewer maternal plants, each mated with a lower number of paternal plants, compared to high-density patches.

Species that are predominantly outcrossing generally have strong inbreeding depression at early life cycle stages (Husband and Schemske, 1996; Sletvold et al., 2013), and if seeds resulting from self-pollination have reduced viability and germination success, the link between mating events and relatedness of established individuals will be weak (Broyles and Wyatt, 1991; Fenster et al., 2003). In such cases, a higher selfing rate may affect numerical dynamics more than genetic structure; i.e., selfing may lead to fewer seeds in sparse than in dense patches, but seeds (and established individuals) will not necessarily be more related. It is thus unclear if density-dependent pollen or seed dispersal will leave a spatial genetic signature in the presence of inbreeding depression, and studies that link local density variation and relatedness are needed to improve our understanding.

The orchid family contains many species that today are of conservation interest due to declines associated with habitat loss and change (Shefferson et al., 2020). Many species are long-lived perennials, and with ongoing fragmentation, the kinship structure of the original population may influence reproductive success in remnant fragments for a long time (Ishihama et al., 2005). Orchid populations frequently have significant within-population genetic structure, typically at very local scales (<5–10 m; Chung et al., 2004, 2005; Jacquemyn et al., 2006, 2009). Such fine-scale genetic structure has been attributed to both restricted pollen and seed dispersal, where especially the latter has been documented to be surprisingly local (Machon et al., 2003; Jacquemyn et al., 2007; Jersákova and Malinová, 2007; Brzosko et al., 2017; Kotilínek et al., 2020), despite minute, wind-dispersed orchid seeds. In addition, orchid germination success has been found to be higher in proximity of established plants (Diez, 2007; Jacquemyn et al., 2012), perhaps because orchids need a mycorrhizal partner to germinate and progress into the seedling stage and sharing the parental fungal strain could potentially favor the establishment of seedlings.

In this study, we focused on the perennial terrestrial orchid *Gymnadenia conopsea* (Orchidaceae), a declining species in many European countries (Meekers et al., 2012). *Gymnadenia conopsea* is an insect-pollinated outcrossing species, but geitonogamous pollination can contribute to seed production, as the species is fully self compatible. In an earlier pollen staining experiment in a large population with substantial variation in flowering plant density, we documented a 193% higher proportion of self pollen deposited in sparse patches compared to dense ones (Söderquist et al., 2020), indicating density-dependent pollinator behavior. If also seed dispersal mainly is short-range, local genetic substructuring should depend on density. However, previous controlled crossings have also documented substantial inbreeding depression at the seed stage in Scandinavian populations (Sletvold et al., 2012; Söderquist et al., 2020), potentially weakening the fine-scale genetic structure. Here, we used SNPs to test the prediction of density-dependent genetic structure in two large populations with extensive small-scale density variation, one of which is the population with the documented density-dependent pollen dispersal mentioned above. Based on previous estimates of inbreeding depression from controlled crosses in the study populations (Söderquist et al., 2020), we also estimated the effect of biparental inbreeding that would result if matings were restricted to within a 5-m

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

neighborhood. We specifically asked whether (1) relatedness between individuals decrease with local flowering density, (2) fine-scale genetic structure is evident on a scale that corresponds to spatial density variation in the population and reflects isolation by distance or habitat structure, and (3) increased biparental inbreeding is likely to severely reduce seed production in case of rapid population fragmentation.

MATERIALS AND METHODS

Study species and sites

Gymnadenia conopsea is a perennial tuberous orchid on a variety of limestone habitats in Eurasia. It flowers from early June to early July, producing a single inflorescence with 10–70 pink flowers. The flowers produce nectar and are strongly scented. It is self compatible, but requires pollinators to produce fruits. In the study populations, pollination is mainly nocturnal, and the most frequent visitors are *Deilephila porcellus* (Sphingidae) and *Autographa gamma* (Noctuidae) (Joffard et al., 2022). Fruit set is usually high (60–100%), and a single fruit typically contains 300–1500 seeds that are dispersed by wind in late summer. Germination depends on association with a compatible fungus providing carbohydrates, and when established, *G. conopsea* can associate with a wide spectrum of mycorrhizal partners (Stark et al., 2009). In vitro experiments suggest that it requires specific Ceratobasidiaceae fungal species to promote protocorm formation and growth to a seedling (Gao et al., 2020). The belowground stage lasts for about 1.5 years before the first aboveground leaves emerge (Meekers et al., 2012), and the plant typically spends several years in the vegetative stage before flowering.

In this study, we focused on two populations on the Baltic island of Öland, where the habitats of *G. conopsea* range from dry calcareous grassland to fen margins. The focal populations, Alböke and Störlinge, are separated by 20 km, and situated in grazed grasslands. Both populations contain several thousand flowering individuals, with considerable variation in local density (1 to >20 plants/m^2). A previous pollen-staining experiment in the Störlinge population documented a 72% increase in the probability of self-pollen deposition in sparse patches (1–3 plants/m^2) compared to dense ones (>8 plants/m^2; Söderquist et al., 2020). Controlled crosses in the two populations have documented substantial inbreeding depression, with self fertilization resulting in 29% (Alböke) and 56% (Störlinge) fewer seeds than produced by outcrossing (Söderquist et al., 2020).

Plant sampling and genotyping

To examine whether kinship is influenced by local flowering plant density, we sampled pairs of plants situated in patches with varying density. We randomly selected 45 and 18 focal plants in the Alböke and Störlinge populations, respectively. To quantify flowering plant density, we centered a 1-m^2 quadrat on the focal plant and counted all flowering individuals within this square. We also sampled the closest neighboring plant to all focal plants and measured the distance between the two individuals. This resulted in 45 pairs separated by 1 to 110 cm in patches containing 2 to 20 flowering individuals per square meter in the Alböke population, and 18 pairs separated by 12 to 95 cm in patches containing 2 to 15 flowering individuals per square meter in the Störlinge population. Density and pair distance were weakly negatively correlated in both populations (Alböke: $r = -0.45$, Störlinge: $r = -0.31$). Across populations, estimates of flowering density are highly correlated between consecutive years ($r > 0.7$, $N = 18$), and although local flowering density will fluctuate with the overall flowering frequency in any specific year, we expect the relative ranking of dense and sparse patches to be robust.

In the Alböke population, we also recorded the position of all 90 individuals to allow for a more general assessment of the fine-scale genetic structure. The 90 individuals were all located within a 45 × 30 m area, and we marked the outline of the area and positioned each individual with an X and Y coordinate. We also recorded the position of large rocks and *Juniperus communis* bushes within the area (Figure 1). To illustrate density variation, we fitted a thin plate spline surface to the values of all pairs using the Tps function from the R package fields (Nychka et al., 2021), interpolated density across the area using the interpolate function from the terra package (Hijmans, 2022), and plotted the resulting values as a contour plot using the ggplot2 package (Wickham, 2016).

From each focal plant, we collected fresh leaf material on silica gel for DNA extraction with the Qiagen Plant DNeasy kit (Qiagen, Germantown, MD, USA). We genotyped all DNA samples ($N = 126$) using a custom-designed Illumina iSelect chip with 2803 SNP assays (hereafter referred to as the Orchid3K chip). To construct the Orchid3K chip, we initially used double-digest RAD sequencing (ddRAD-seq) to generate genome-wide polymorphism data, as described in Söderquist et al. (unpublished manuscript). Genotyping was performed using the Illumina Infinium assay, and the results were analyzed using the software GenomeStudio 2.0.3 (Illumina, San Diego, CA, USA) at the SNP&SEQ Technology Platform of SciLifeLab in Uppsala, Sweden. The SNP conversion rate was 75.2%, resulting in 2109 SNPs with an average call rate of 95.3%. Reproducibility was 100% (0 conflicts in 14050 duplicate tests). We removed duplicates ($N = 3$ individuals with complete similarity, indicating clonality), loci typed in less than 1/3 of the individuals, invariant sites, and singletons. After filtering, 1437 and 1315 loci were left in Alböke ($N = 87$ individuals) and Störlinge ($N = 36$ individuals), respectively.

Data analyses

We calculated expected heterozygosity (H_E), observed heterozygosity (H_O), and the inbreeding coefficient (F_{IS}), using the

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

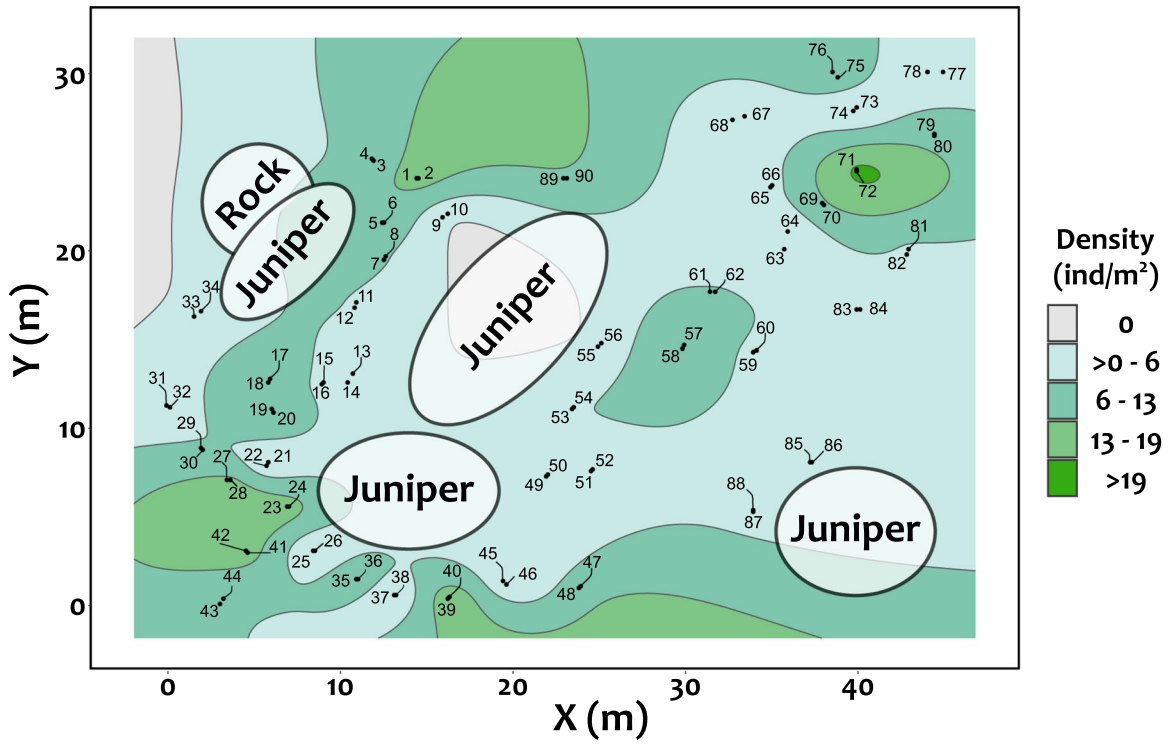


FIGURE 1 Pairs of individuals of *Gymnadenia conopsea* included in the analysis of fine-scale genetic structure in the Alböke population ($N = 45$). Flowering density was interpolated across the map from the values measured at each pair.

basic.stats function in the hierfstat package in R version 3.6.0 (R Core Team, 2019). Values for H_E, H_O, and F_{IS} were averaged across all loci.

To examine within-population genetic structure in the Alböke population, we used the spatial autocorrelation approach implemented in the software SPAGeDI version 1.4 (Hardy and Vekemans, 2002). Under the isolation-by-distance model, theory predicts that the kinship coefficient, F_{ij}, decreases approximately linearly with the natural logarithm of the physical distance between individuals (Loiselle et al., 1995). In the case of two individuals, i and j, F_{ij} can be defined as $F_{ij} = (Q_{ij} - Q_m)/(1 - Q_m)$, where Q_{ij} is the probability of identity by state between two randomly chosen genes from i and j, and Q_m is the mean probability of identity by state between two randomly chosen individuals from the reference population. Note that the estimated kinship coefficient will be negative whenever individuals are sharing fewer alleles than the population average. To examine fine-scale genetic structure, we computed average F_{ij} values for each of the five following distance classes: 0–5 m, >5–10 m, >10–20 m, >20–30 m, and >30 m. Intervals were chosen to ensure sufficient number of pairs (>100) over each distance class. We then assessed the significance of average F_{ij} values using permutation tests, in which spatial distances were permuted randomly among pairs of individuals ($n_{perm} = 10000$). To test the hypothesis that there was significant spatial genetic structure, the observed regression slope of F_{ij} on ln (Distance), b, was compared with those obtained after 10,000 random permutations.

We further investigated the effect of patch density on the kinship coefficient of sampled pairs in Alböke and Störlinge using linear models. The log-transformed kinship coefficient was used as the response variable, and local density, distance between the focal individual and its closest neighbor, and their interaction were used as explanatory variables. Linear models were run in R version 3.6.0 with the function lm, and the significance of fixed effects was assessed using type III sum of squares with the function Anova from the car package.

To identify potential habitat barriers to gene flow, we used the software EEMS (Estimated Effective Migration Surfaces, v 20160605; Petkova et al., 2016), which uses a stepping stone model in which migration is only possible between neighboring demes, distributed in a grid, at a user-specified density. The estimated migration rates are interpolated across the whole extent of the demes specified to create a visual summary of the observed genetic diversity in relation to geographic location. Areas where observed genetic similarities decline faster than expected are considered barriers to gene flow. We determined a deme density of 400 and 500 to be suitable for our data set, by first running RUNEEMS_SNPS with a range of different deme densities. We ran three runs per deme density with 6 million Markov chain Monte Carlo iterations, 2.5 million burn-in iterations, and 99,999 thinning iterations. The final plot was averaged over all runs and both deme densities, using the rEEMSplots package v0.0.0.9000, as suggested by Petkova et al. (2016). We also used conStruct v 1.0.4

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Sample size (N), proportion of polymorphic loci (Pp), expected heterozygosity (H_E), observed heterozygosity (H_O), and the inbreeding coefficient (F_{IS}) in the two *Gymnadenia conopsea* populations (Pop.).

Pop.	N	P_p	H_E	H_O	F_{IS}
Alböke	87	0.731	0.226	0.217	0.040
Störlinge	36	0.707	0.247	0.241	0.023

(Bradburd, 2019) to check for distinct genetic clusters, but found none (results not shown).

To estimate biparental inbreeding depression of progeny resulting from matings restricted to within 5 m, we assumed that the magnitude of inbreeding depression is proportional to the exponential of the kinship coefficient (Morton et al., 1956): inbreeding depression = exp[−B(Kinship)], where −B is the inbreeding load (Charlesworth and Charlesworth, 1987). This approach is equivalent to assuming that inbreeding depression is caused by the multiplicative effect of a large number of deleterious alleles of small effect. When data are available for mean trait values (W) of known outbred and inbred individuals with a single known level of relatedness (F) (for example, offspring resulting from selfing versus outcrossing), the inbreeding load B can be estimated as twice the ratio $-\log_e(W_S/W_O)/F$, where W_S and W_O are trait values of offspring resulting from selfing and outcrossing, respectively, and F is the coefficient of relatedness of the inbred group (Lynch and Walsh, 1998, p. 278). We used inbreeding depression quantified at the seed stage (number of seeds produced) by earlier controlled crosses to quantify the inbreeding load in the two study populations (Söderquist et al., 2020), which resulted in $B = 2 \times -\log_e(618/865) = 0.675$ in Alböke, and $B = 2 \times -\log_e(576/1309) = 1.641$ in Störlinge. In the previous experiment, the minimum distance between plants used for outcrossings was 5 m, but in large populations such as Alböke and Störlinge, distances were usually considerably longer. Here, we estimated the mean biparental inbreeding depression that would result from matings between plants separated by less than 5 m, by combining the average kinship coefficient for that distance class with the inbreeding load quantified at the seed stage.

RESULTS

The populations had similar genetic diversity (Table 1). Pairwise kinship coefficients (F_{ij} estimates) ranged from −0.074 to 0.33 in Alböke and from −0.050 to 0.25 in Störlinge. The kinship coefficient of pairs of nearby individuals did not vary with local density or distance between individuals in any of the two populations (Table 2, Figure 2). In Alböke, kinship coefficients were positive and significantly higher than expected at random for distances shorter than 10 m, with the highest values found for the smallest distance class (0–5 m, $F_{ij} = 0.02$; Figure 3). In contrast, F_{ij} estimates were negative for distances longer than 10 m, and significantly lower than expected at random for distances longer than 20 m. The slope of the regression line between the kinship coefficient and the natural logarithm of the distance between individuals was statistically significant ($b = -0.008$, $P < 0.0001$). The EEMS suggested that juniper bushes and rocks may act as barriers to gene flow within the Alböke population, but also pointed to reduced gene flow in an area where there were no close barriers (Figure 4).

TABLE 2 The effect of local flowering density, distance between individuals, and their interaction, on relatedness (kinship coefficient) in the Alböke and Störlinge populations, analyzed with ANOVA.

Effect	SS	df	F	P
Alböke ($N = 42$)				
Intercept	29.90	1	46.9	<0.0001
Density	0.0426	1	0.0669	0.797
Distance	0.103	1	0.162	0.690
Density × Distance	0.0238	1	0.0374	0.848
Residual	24.20	38		
Störlinge ($N = 18$)				
Intercept	22.99	1	10.69	0.00559
Density	1.462	1	0.679	0.424
Distance	0.364	1	0.169	0.687
Density × Distance	0.229	1	0.107	0.749
Residual	30.11	14		

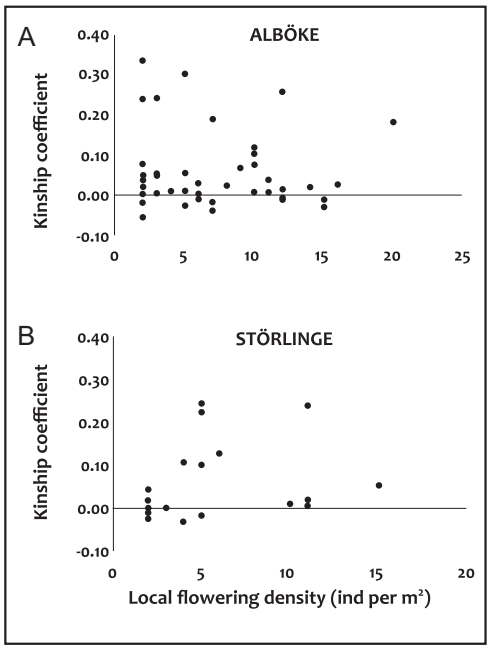


FIGURE 2 Pairwise relatedness in relation to the density of flowering individuals (ind.) of *Gymnadenia conopsea* in the (A) Alböke ($N = 42$) and (B) Störlinge ($N = 18$) populations.

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

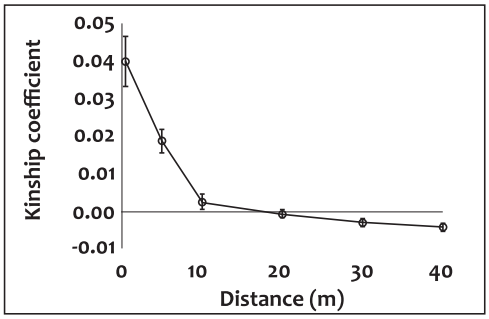


FIGURE 3 Correlogram of kinship coefficients in relation to distance for all individuals in the Alböke population of *Gymnadenia conopsea*. Error bars indicate 95% CI.

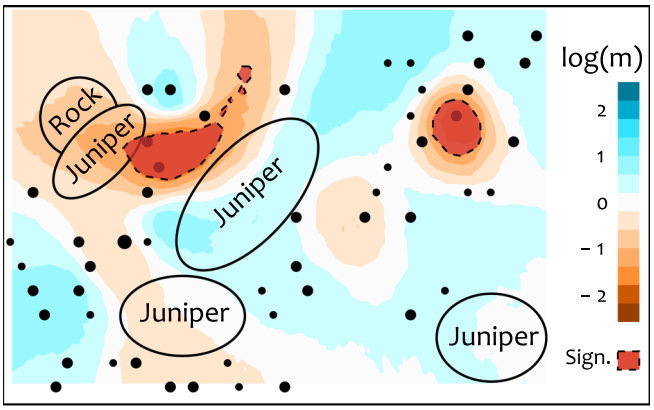


FIGURE 4 Effective migration surfaces modelled using EEMS, indicating areas of reduced (orange) or increased (blue) gene flow as compared to average. Areas within dashed lines have significantly reduced migration ("sign", posterior probability >95%). Results were averaged over deme densities 400 and 500 for three runs each. Black dots indicate locations of sampling, and dot size indicates number of samples in each deme.

The estimated inbreeding depression at the seed stage resulting from mating distances below 5 m was 1–3% in the Alböke population and 3–6% in the Störlinge population (assuming identical fine-scale genetic structure as in Alböke).

DISCUSSION

The spatial autocorrelation analysis documented significant within-population genetic structure in the Alböke population of *Gymnadenia conopsea*, but relatedness between pairs of plants did not vary with local density of flowering individuals in any of the two populations. Below, we discuss possible reasons for this pattern.

Based on an earlier pollen staining experiment (Söderquist et al., 2020), we expected individual pairs in sparse patches to be more closely related than individuals in dense patches, but this was not the case. Clearly, the 72% increase in the probability of self-pollen deposition and the 193% higher proportion of self-pollen deposited in sparse patches compared to dense ones, did not leave a spatial genetic signature. Several processes between mating and the formation of a new generation of flowering plants could explain the lack of a density-effect on relatedness. First, the self pollen deposited onto the stigmas may not necessarily achieve ovule fertilization if a surplus of outcross pollen is available. Although little is known about pollen competition in orchids, an advantage of outcross over self pollen has been documented in many mixed maters (reviewed by Goodwillie and Weber, 2018). Second, abortion of inbred seeds may severely limit the number of seeds that actually are added to the population. The magnitude of inbreeding depression at the seed stage has earlier been estimated to 29% and 56% in the two populations, and if inbreeding depression accumulates across the life cycle in line with findings in other outcrossing species (reviewed by Husband and Schemske, 1996, and Sletvold et al., 2013), very few plants resulting from selfing may reach the flowering stage. If so, the close to zero inbreeding coefficient in both populations (Alböke $F_{IS} = 0.040$, Störlinge $F_{IS} = 0.023$) does not necessarily indicate random mating, but rather that very few inbred plants remain at the flowering stage. A comparison of inbreeding coefficients or fine-scale genetic structure at different life stages could clarify whether inbred plants fail to establish or die at a later stage (Jacquemyn et al., 2006). It could be that the main effect of higher self-pollination in sparse patches or populations is seed discounting and that selfing predominantly affects numerical dynamics. In line with this possibility, open-pollinated plants in dense patches on average produced 20.4% more viable seeds than plants in sparse patches in the previous pollen staining experiment (Rosenberg, 2019). Supplemental hand-pollinations in dense and sparse patches could clarify the role of pollen quality limitation.

In the Alböke population, we found significant genetic structure at a small spatial scale (<5 m), in line with many previous studies on orchids (reviewed by Chung et al., 2011). The genetic neighborhood size, i.e., the distance where a focal individual will be more related to its neighbors than to an average individual in the population, was suggested to be around 15 m, as indicated by the point at which the coancestry correlogram first intersects the *x*-axis (Figure 3). This spatial structure is most likely due to restricted seed dispersal and suggests that the majority of seeds fall within a distance of 15 m from the maternal plant. While the exact distance should be interpreted cautiously because it will vary with scale of sampling (Fenster et al., 2003; Vekemans and Hardy, 2004), the estimate of 15 m is similar to distances inferred from genetic data in other orchids (Chung et al., 2004) and to maximal distances observed in seed dispersal studies (Brzosko et al., 2017). Still, the spatial structure was weak, with a mean kinship coefficient of 0.02 in the closest distance class (<5 m), and with only a few of the included pairs being as closely related as half sibs. The weak structure contrasts with several orchid species where the immediate neighborhood consists of

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

closely related individuals (Chung et al., 2004, 2005). However, because the match between pedigree-based predictions and marker-based estimates of kinship can be poor (Goudet et al., 2018), we encourage careful interpretation of actual relatedness.

Although genetic distance on average declined gradually with distance, the EEMS analysis suggests that juniper bushes and large rocks can act as barriers to gene flow, creating additional small-scale spatial genetic structure. Most likely, bushes and rocks restrict seed dispersal by wind more than pollen dispersal by moths, suggesting that bush encroachment following land-use change could contribute to small-scale population subdivision. However, the EEMS also pointed to reduced gene flow in an area with no nearby rocks or bushes (Figure 4), and it is clear that a more rigorous analysis of potential habitat barriers requires sampling across larger, replicated areas.

The modest kinship coefficients suggest that increased biparental inbreeding following a fragmentation is unlikely to be an immediate problem for these populations. If the Alböke population was subdivided to an extent that restricted all matings to within 5 m of an individual, the average reduction in seed production compared to complete outcrossing would initially only be 1–3%. If we assume that a similar spatial genetic structure exists in Störlinge, the reduction in seed production would be slightly higher, 3–6%. It should be noted that even if we are underestimating inbreeding depression by focusing at the seed stage only, this underestimation will only have minor effects because of the weak relatedness. It further suggests that, unless the selfing rate increases dramatically, seed production will be maintained for a period after fragmentation. While genetic diversity will decline due to a direct effect of reduction in population size (sampling effect), it will likely take time to build up a kinship structure that will reinforce loss of diversity via biparental inbreeding. Further studies that estimate spatial genetic structure within populations of varying size, or track genetic structure during a process of decline, are needed to understand effects of fragmentation.

CONCLUSIONS

This study suggests that large populations of *G. conopsea* have significant but weak genetic structure and high outcrossing rates. The modest relatedness suggests that fragmentation of large populations is unlikely to cause biparental inbreeding depression. This conclusion should hold even if lifetime inbreeding depression is much stronger than our estimate for the seed stage, simply because matings between closely related individuals will continue to be rare. Potentially higher inbreeding in sparse patches or isolated individuals is not evident in the genetic structure, which could be explained by severe inbreeding depression. In sum, habitat structure may be as important as local plant distribution in governing fine-scale genetic structure in *G. conopsea*.

AUTHOR CONTRIBUTIONS

N.S. and L.S. conceived the study questions, and N.S. collected the field data. N.J. and L.S. analyzed the data. N.S. led the writing of the manuscript. All authors contributed critically to revisions and gave final approval for publication.

ACKNOWLEDGMENTS

We thank J. Ågren for valuable comments on the manuscript and L. Vikström and M. Uscka-Perzanowska for DNA extractions. We thank J. Fant, C. Goodwillie, and an anonymous reviewer for constructive comments that improved the presentation of the study. Genotyping was done by the SNP&SEQ Technology Platform in Uppsala (www.genotyping.se). The facility is part of the National Genomics Infrastructure supported by the Swedish Research Council for Infrastructures and Science for Life Laboratory, Sweden. The computations were enabled by resources provided by Uppsala University at UPPMAX. This study was funded by grants 2014-00601 and 2018-01397 from The Swedish Research Council Formas to N.S.

DATA AVAILABILITY STATEMENT

Associated data are available from Dryad: https://doi.org/10.5061/dryad.dbrv15f7m (Sletvold et al., 2023).

ORCID

Nina Sletvold http://orcid.org/0000-0002-9868-3449
Nina Joffard http://orcid.org/0000-0003-3712-6080
Linus Söderquist http://orcid.org/0000-0002-9894-4119

REFERENCES

Bradburd, G. 2019. conStruct: Models spatially continuous and discrete population genetic structure. R package version 1.0.4. Website: https://CRAN.R-project.org/package=conStruct

Broyles, S. B., and R. Wyatt. 1991. Effective pollen dispersal in a natural population of *Asclepias exaltata*: the influence of pollinator behavior, genetic similarity, and mating success. *American Naturalist* 138: 1239–1249.

Brzosko, E., B. Ostrowiecka, J. Kotowicz, M. Bolesta, A. Gromotowicz, M. Gromotowicz, A. Orzechowska, et al. 2017. Seed dispersal in six species of terrestrial orchids in Biebrza National Park (NE Poland). *Acta Societatis Botanicorum Poloniae* 86: 3557.

Charlesworth, D., and B. Charlesworth. 1987. Inbreeding depression and its evolutionary consequences. *Annual Review of Ecology and Systematics* 18: 237–268.

Christopher, D. A., J. D. Karron, W. R. Semski, P. A. Smallwood, D. W. Trapnell, and R. J. Mitchell. 2021. Selfing rates vary with floral display, pollinator visitation and plant density in natural populations of *Mimulus ringens*. *Journal of Evolutionary Biology* 34: 803–815.

Chung, M. Y., J. D. Nason, and M. G. Chung. 2004. Spatial genetic structure in populations of the terrestrial orchid *Cephalanthera longibracteata* (Orchidaceae). *American Journal of Botany* 91: 52–57.

Chung, M. Y., J. D. Nason, and M. G. Chung. 2005. Spatial genetic structure in populations of the terrestrial orchid *Orchis cyclochila* (Orchidaceae). *Plant Systematics and Evolution* 254: 209–219.

Chung, M. Y., J. D. Nason, and M. G. Chung. 2011. Significant demographic and fine-scale genetic structure in expanding and senescing populations of the terrestrial orchid *Cymbidium goeringii* (Orchidaceae). *American Journal of Botany* 98: 2027–2039.

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Diez, J. M. 2007. Hierarchical patterns of symbiotic orchid germination linked to adult proximity and environmental gradients. *Journal of Ecology* 95: 159–170.

Ennos, R. A. 1994. Estimating the relative rates of pollen and seed migration among plant populations. *Heredity* 72: 250–259.

Epperson, B. K. 2000. Spatial genetic structure and non-equilibrium demographics within plant populations. *Plant Species Biology* 15: 269–279.

Epperson, B. K., and M. G. Chung. 2001. Spatial genetic structure of allozyme polymorphisms within populations of *Pinus strobus* (Pinaceae). *American Journal of Botany* 88:1006–1010.

Fenster, C. B., O. J. Hardy, and X. Vekemans. 2003. Quantifying gene flow from spatial genetic structure data in a metapopulation of *Chamaecrista fasciculata* (Leguminosae). *Evolution* 57: 995–1007.

Gao, Y., Z. Zhao, J. Li, N. Liu, H. Jacquemyn, S. Guo, and X. Xing. 2020. Do fungal associates of co-occurring orchids promote seed germination of the widespread orchid species *Gymnadenia conopsea*? *Mycorrhiza* 30: 221–228.

Goodwillie, C., and J. J. Weber. 2018. The best of both worlds? A review of delayed selfing in flowering plants. *American Journal of Botany* 105: 641–655.

Goudet, J., T. Kay, and B. S. Weir. 2018. How to estimate kinship. *Molecular Ecology* 27: 4121–4135.

Grindeland, J. M., N. Sletvold, and R. A. Ims. 2005. Effects of floral display size and plant density on pollinator visitation rate in a natural population of *Digitalis purpurea*. *Functional Ecology* 19: 383–390.

Hardy, O. J., and X. Vekemans. 2002. SPAGeDi: a versatile computer program to analyse spatial genetic structure at the individual or population levels. *Molecular Ecology Notes* 2: 618–620.

Hijmans, R. J. 2022. Terra: spatial data analysis. Website: https://rspatial.org/pkg/index.html

Husband, B. C., and D. W. Schemske. 1996. Evolution of the magnitude and timing of inbreeding depression in plants. *Evolution* 50: 54–70.

Ishihama, F., S. Ueno, Y. Tsumura, and I. Washitani. 2005. Gene flow and inbreeding depression inferred from fine-scale genetic structure in an endangered heterostylous perennial, *Primula sieboldii*. *Molecular Ecology* 14: 983–990.

Jacquemyn, H., R. Brys, K. Vandepitte, O. Honnay, and I. Roldán-Ruiz. 2006. Fine-scale genetic structure of life history stages in the food deceptive orchid *Orchis purpurea*. *Molecular Ecology* 15: 2801–2808.

Jacquemyn, H., R. Brys, K. Vandepitte, O. Honnay, I. Roldán-Ruiz, and T. Wiegand. 2007. A spatially explicit analysis of seedling recruitment in the terrestrial orchid *Orchis purpurea*. *New Phytologist* 176: 448-459.

Jacquemyn, H., T. Wiegand, K. Vandepitte, R. Brys, I. Roldán-Ruiz, and O. Honnay. 2009. Multigenerational analysis of spatial structure in the terrestrial, food-deceptive orchid *Orchis mascula*. *Journal of Ecology* 97: 206–216.

Jacquemyn, H., R. Brys, B. Lievens, and T. Wiegand. 2012. Spatial variation in below-ground seed germination and divergent mycorrhizal associations correlate with spatial segregation of three co-occurring orchid species. *Journal of Ecology* 100: 1328–1333.

Jersáková, J., and T. Malinová. 2007. Spatial aspects of seed dispersal and seedling recruitment in orchids. *New Phytologist* 176: 237-241.

Jing, X., L. Hong, and Q. Rui. 2013. Unexpectedly high outcrossing rate in both dense and sparse patches in self-compatible *Pedicularis rex* (Orobanchaceae). *Plant Systematics and Evolution* 299: 49–56.

Joffard, N., C. Olofsson, M. Friberg, N. Sletvold. 2022. Extensive pollinator sharing does not promote character displacement in two orchid congeners. *Evolution* 76: 749–764.

Kalisz, S., J. D. Nason, F. M. Hanzawa, and S. J. Tonsor. 2001. Spatial population genetic structure in *Trillium grandiflorum*: the roles of dispersal, mating, history, and selection. *Evolution* 55: 1560–1568.

Karron, J. D., N. N. Thumser, R. Tucker, and A. J. Hessenauer. 1995. The influence of population density on outcrossing rates in *Mimulus ringens*. *Heredity* 75: 175–180.

Klinkhamer, P. G. L. and T. J. de Jong. 1990. Effects of plant size, plant density and sex differential nectar reward on pollinator visitation in the protandrous *Echium vulgare* (Boraginaceae). *Oikos* 57: 399–405.

Kotilínek, M., T. Tesitelova, J. Konar, P. Fibich, L. Hemrová, P. Koutecký, Z. Munzbergová, and J. Jersáková. 2020. Seed dispersal and realized gene flow of two forest orchids in a fragmented landscape. *Plant Biology* 22: 522–532.

Lara-Romero, C., A. García-Fernández, J. J. Robledo-Arnuncio, M. Roumet, J. Morente-López, A. López-Gil, and J. M. Iriondo. 2016. Individual spatial aggregation correlates with between-population variation in fine-scale genetic structure of *Silene ciliata* (Caryophyllaceae). *Heredity* 116: 417–423.

Leblois, R., F. Rousset, and A. Estoup. 2004. Influence of spatial and temporal heterogeneities on the estimation of demographic parameters in a continuous population using individual microsatellite data. *Genetics* 166: 1081–1092.

Levin, D. A. and H. Kerster. 1969. Density-dependent gene dispersal in *Liatris*. *American Naturalist* 103: 61–74.

Loiselle, B. A., V. L. Sork, J. Nason, and C. Graham. 1995. Spatial genetic structure of a tropical understorey shrub, *Psychotria officinalis* (Rubiaceae). *American Journal of Botany* 82: 1420–1425.

Loveless, M. D., and J. L. Hamrick. 1984. Ecological determinants of genetic structure in plant populations. *Annual Review of Ecology and Systematics* 15: 65–95.

Lynch, M., and B. Walsh. 1998. Genetics and analysis of quantitative traits. Sinauer, Sunderland, MA, USA.

Machon, N., P. Bardin, S. J. Mazer, J. Moret, B. Godelle, and F. Austerlitz. 2003. Relationship between genetic structure and seed and pollen dispersal in the endangered orchid *Spiranthes spiralis*. *New Phytologist* 157: 677–687.

McRae, B. H. 2006. Isolation by resistance. *Evolution* 60: 1551–1561.

Meekers, T., M. J. Hutchings, O. Honnay, and H. Jacquemyn. 2012. Biological flora of the British Isles: *Gymnadenia conopsea s.l. Journal of Ecology* 100: 1269–1288.

Morton, N. E., J. F. Crow, and H. J. Muller. 1956. An estimate of the mutational damage in man from data on consanguineous marriages. *Proceedings of the National Academy of Sciences, USA* 42: 855–563.

Nathan, R., and H. C. Muller-Landau. 2000. Spatial patterns of seed dispersal, their determinants and consequences for recruitment. *Trends in Ecology and Evolution* 15: 278–285.

Nychka, D., R. Furrer, J. Paige, and S. Sain. 2021. Fields: tools for spatial data. Website: https://github.com/dnychka/fieldsRPackage

Petkova, D., J. Novembre, and M. Stephens. 2016. Visualizing spatial population structure with estimated effective migration surfaces. *Nature Genetics* 48: 94–100.

R Core Team. 2019. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Website: https://www.R-project.org/

Rhodes, M. K., J. B. Fant, and K. A. Skogen. 2017. Pollinator identity and spatial isolation influence multiple paternity in an annual plant. *Molecular Ecology* 26: 4296–4308.

Robledo-Arnuncio, J. J., and F. Rousset. 2010. Isolation by distance in a continuous population under stochastic demographic fluctuations. *Journal of Evolutionary Biology* 23: 53–71.

Rosenberg, V. 2019. The effects of local density on pollination and pollinator-mediated selfing in the self-compatible orchid *Gymnadenia conopsea*. M.Sc. thesis, Uppsala University, Uppsala, Sweden.

Rousset, F. 2000. Genetic differentiation between individuals. *Journal of Evolutionary Biology* 13: 58–62.

Shefferson, R. P., H. Jacquemyn, T. Kull, M. J. Hutchings. 2020. The demography of terrestrial orchids: life history, population dynamics and conservation. *Botanical Journal of the Linnean Society* 192: 315–332.

Sletvold, N., J. M. Grindeland, P. Zu, J. Ågren. 2012. Strong inbreeding depression and local outbreeding depression in the rewarding orchid *Gymnadenia conopsea*. *Conservation Genetics* 13: 1305–1315.

Sletvold, N., M. Mousset, J. Hagenblad, B. Hansson, and J. Ågren. 2013. Strong cumulative inbreeding depression over two growing seasons

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

in two Scandinavian populations of the self-incompatible perennial herb *Arabidopsis lyrata*. *Evolution* 67: 2876–2888.

Sletvold, N., N. Joffard, and L. Söderquist. 2023. Data from: Fine-scale genetic structure in the orchid Gymnadenia conopsea is not associated with local density of flowering plants (Version 2) [Data set]. Dryad. https://doi.org/10.5061/DRYAD.DBRV15F7M

Stark, C., W. Babik, and W. Durka. 2009. Fungi from the roots of the common terrestrial orchid *Gymnadenia conopsea*. *Mycological Research* 113: 952–959.

Söderquist, L., A. Broberg, V. Rosenberg, and N. Sletvold. 2020. Predicting heterosis and inbreeding depression from population size and density to inform management efforts. *Journal of Applied Ecology* 57: 1459–1468.

Tambarussi, E. V., D. Boshier, R. Vencovsky, M. L. M. Freitas, and A. M. Sebbenn. 2015. Paternity analysis reveals significant isolation and near neighbor pollen dispersal in small *Cariniana legalis* Mart. Kuntze populations in the Brazilian Atlantic Forest. *Ecology and Evolution* 5: 5588–5600.

Tero, N., J. Aspi, P. Siikamäki, and A. Jäkäläniemi. 2005. Local genetic population structure in an endangered plant species, *Silene tatarica* (Caryophyllaceae). *Heredity* 94: 478–487.

Vekemans, X., and O. J. Hardy. 2004. New insights from fine-scale spatial genetic structure analyses in plant populations. *Molecular Ecology* 13: 921–935.

Wickham H. 2016. ggplot2: Elegant graphics for data analysis. Springer-Verlag NY, NY, USA.

Wright, S. 1943. Isolation by distance. *Genetics* 28: 114–138.

How to cite this article: Sletvold, N., N. Joffard, and L. Söderquist. 2024. Fine-scale genetic structure in the orchid *Gymnadenia conopsea* is not associated with local density of flowering plants. *American Journal of Botany*. e16273. https://doi.org/10.1002/ajb2.16273

15372197, 0, Downloaded from https://bsapubs.onlinelibrary.wiley.com/doi/10.1002/ajb2.16273 by Cochrane France, Wiley Online Library on [05/02/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License