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Fine-scale genetic structure of life history stages in the food-deceptive orchid *Orchis purpurea*

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Abstract

In natural plant populations, fine-scale spatial genetic structure can result from limited gene flow, selection pressures or historical events, but the role of each factor is in general hard to discern. One way to investigate the origination of spatial genetic structure within a plant population consists of comparing spatial genetic structure among different life history stages. In this study, spatial genetic structure of the food-deceptive orchid *Orchis purpurea* was determined across life history stages in two populations that were regenerating after many years of population decline. Based on demographic analyses (2001–2004), we distinguished between recruits and adult plants. For both sites, there was no difference in the proportion of polymorphic loci and expected heterozygosity between life history stages. However, spatial autocorrelation analyses showed that spatial genetic structure increased in magnitude with life history stage. Weak or no spatial genetic structure was observed for recruits, whereas adult plants showed a pattern that is consistent with that found in other species with a predominantly outcrossing mating system. The observed differences between seedlings and adults are probably a consequence of changes in management of the two study sites and associated demographic changes in both populations. Our results illustrate that recurrent population crashes and recovery may strongly affect genetic diversity and fine-scale spatial genetic structure of plant populations.

Keywords: deception, gene flow, Orchidaceae, spatial autocorrelation, spatial genetic structure

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Introduction

In natural plant populations, fine-scale genetic structure can be the result of several factors, such as limited seed and pollen dispersal (reviewed in Loveless & Hamrick 1984; Vekemans & Hardy 2004), selection (reviewed in Linhart & Grant 1996) or historical events (e.g. Ingvarsson & Giles 1999; Tero *et al*. 2005), although the relative importance of each factor is hard to discern (Kalisz *et al*. 2001). Vekemans & Hardy (2004) stated that 'on a fine spatial scale the formation of local pedigree structures as a result of limited gene flow is probably the most prevalent cause of spatial genetic structure.' However, Ennos (2001) concluded that where genetic structure attributable to isolation-by-distance processes is present, it is weak and limited to very small scales. In cases where more substantial genetic structure was found, this was often attributable to sampling events during population foundation and regeneration. These conclusions underscore the importance of stochastic events in determining spatial genetic structure and urge to incorporate demographic data and knowledge about the long-term dynamics in population genetic research (Ennos 2001).

One way to investigate the origination of spatial genetic structure within plant populations consists of comparing the spatial genetic structure of different life history stages (Kalisz *et al*. 2001). However, our knowledge in this field is limited at present and the few available studies indicate that spatial genetic structure may increase (Tonsor *et al*. 1993; Kalisz *et al*. 2001) or decrease (Hamrick *et al*. 1993; Epperson & Alvarez-Buylla 1997; Parker *et al*. 2001; Chung

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et al. 2003) in magnitude with life history stage. Reductions in fine-scale genetic structure form seedlings to adults are mostly attributed to loss of individuals when seedling cohorts thin, whereas historical factors or local selection are the most frequent explanations for the increase in spatial genetic structure towards older life history stages.

In orchids, gene flow among populations appears to be variable (Tremblay *et al*. 2005), although some general patterns emerge. First, population differentiation is higher in rewarding species than in deceptive orchid species, suggesting that pollinator-mediated gene flow among populations is higher in deceptive orchids (Cozzolino & Widmer 2005; Tremblay *et al*. 2005). Because there is no reward in deceptive orchids, pollinator visits will mostly be few and brief, and pollinators will quickly leave a floral patch once they have learned that there is no reward (Johnson 2000; Peakall & Schiestl 2004). Therefore, based on pollen dispersal patterns it can be hypothesized that spatial genetic structure within populations of deceptive orchid species is weak and neighbourhood sizes large, whereas in rewarding species within-population genetic structure should be stronger due to higher amounts of within-plant pollinations (geitonogamy) and due to mating among close relatives. Second, despite their tiny size, orchid seed dispersal seems to be limited (e.g. Murren & Ellison 1998; Machon *et al*. 2003), a factor than can also lead to the appearance of significant spatial genetic structure. Indeed, most studies investigating spatial genetic structure in orchid species found significant spatial structure, which was explained by limited seed dispersal distances (e.g. Peakall & Beattie 1996; Machon *et al*. 2003; Chung *et al*. 2004, 2005; Trapnell *et al*. 2004; Trapnell & Hamrick 2005). However, to our knowledge, no study has compared fine-scale spatial genetic structure across life history stages in orchids.

In this study, the fine-scale genetic structure in two populations of the food-deceptive orchid *Orchis purpurea* was investigated. Historical data on long-term population dynamics and regeneration were combined with short-term demographic monitoring and genetic analyses to determine the causes of spatial genetic structure. Historical field books and local reserve managers pointed out that around the half of the previous century, both populations consisted of more than 1000 individuals. Due to changing management practices (lack of coppicing and conversion of the forest to high wood in the first site and artificial fertilizer application in the second), population sizes had seriously decreased to reach a very low number of individuals (less than 50) in the late 1990s, when restoration of the traditional management began. Restoration of the traditional management resulted in rapid population expansion from 1999 onwards. Therefore, these populations represent ideal objects to study how spatial genetic structures evolve during population expansion. Detailed demographic monitoring during 4 years (2001–2004) allowed us to determine seedling recruitment and mortality rates, the spatial pattern of seedling recruitment and to analyse the fine-scale genetic structure of different life history stages in the two studied populations. In addition, data on fruit production rates allowed estimating pollen-mediated gene flow. Amplified fragment length polymorphism (AFLP) markers and spatial autocorrelation analyses using a kinship coefficient for dominant markers (Hardy 2003) were used to answer the following four questions: (i) How is genetic diversity structured within populations of the food-deceptive orchid *O. purpurea*? (ii) What is the relative contribution of pollen and seed dispersal to the overall level of gene flow? (iii) Does fine-scale genetic structure differ among life history stages and which stage shows the strongest spatial genetic structure? (iv) Which factors lie at the basis of possible differences in spatial genetic structure among different life history stages?

Materials and methods

Study species

Orchis purpurea Huds. (Lady Orchid) is a tall, tuberous, perennial orchid with a mainly Mediterranean distribution. It is scattered sparsely through France and Central Europe and extends to Corsica and the mountains of central Italy where it is one of the most common woodland orchid species (Rose 1948). In the UK, the northern part of Belgium (Flanders) and the Netherlands, and Denmark, the species reaches the limits of its northwestern distribution (Rose 1948). In Flanders, Lady Orchid is rare and threatened (Jacquemyn *et al*. 2005a). The natural habitats of *O. purpurea* consist of calcareous grasslands or forests on limestone. In calcareous grasslands, the species can mostly be found in the immediate vicinity of trees and shrubs, whereas in forest habitat it usually occurs in light gaps or along the forest edge.

Orchis purpurea perennates during the winter and its leaves appear above the ground in February. Plants have one to four (sometimes up to seven) basal leaves of elliptic-ovate to lanceolate shape, 2–5 cm wide and 6–20 cm long (Rose 1948). Flowering takes place at the end of May. The height of the flowering stalks varies between 25 and 60 cm, reaching sometimes 80 cm (Jacquemyn *et al*. 2002). Flowering stalks carry 10–50 bright white to purple-brown, self-compatible flowers. The flowers are nectar-less, but they produce a sweet odour and are pollinated by bumblebees or butterflies. Seed capsules ripen by the end of June, followed by dehiscence and seed dispersal in August. Fruit production is generally low (less than 10%) (Darwin 1877; Jacquemyn *et al*. 2002). From mid-August onwards, no living green parts are found above ground.

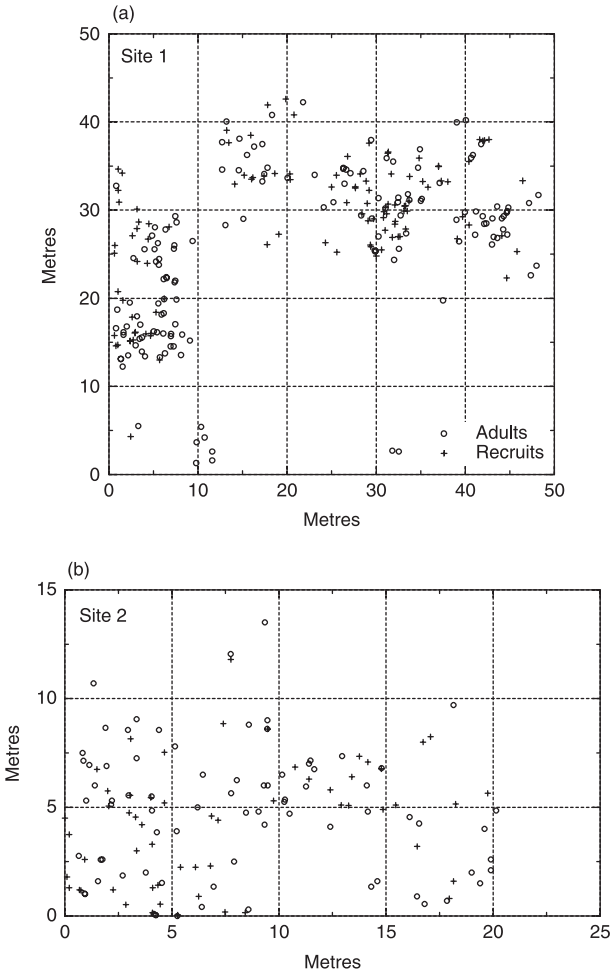

Fig. 1 Spatial distribution of genotyped adults and recruits of *Orchis purpurea* in two sites.

Study sites and demographic monitoring

Two sites were selected to study fine-scale population genetic structure of *O. purpurea* (Fig. 1a, b). During the last decades, both populations had seriously decreased in size due to a lack of management (Site 1) and application of artificial fertilizer (Site 2). Restoration of the original management began in 1999. At that time, both populations consisted solely of adult, mostly vegetative plants and no seedlings were observed. From 2001 to 2004, both sites were subject to intensive demographic monitoring. During this period, all individuals were mapped and their life history stage determined. At the same time, the number of flowering and nonflowering adult plants was determined. At the moment of sampling (2004), the first site consisted of 340 individuals (193 adults and 147 recruits) and was located in a large forest gap that comprised an area of *c.* 2500 m² (density = 0.136 individuals/m²) (Fig. 1a). The second site was situated in a species-rich calcareous grassland immediately bordering forest habitat and comprised an area of 375 m². The second site consisted of 172 individuals (density = 0.459 individuals/m²) (Fig. 1b), of which 99 individuals (58%) were adults. The maximum distance between individuals was 57.69 m and 20.45 m for the first and second site, respectively (Fig. 1a,b). Demographic analyses further showed that on average, 58% and 51% of all adult plants flowered in a given year in Sites 1 and 2, respectively, and only a small proportion of individuals flowered for three consecutive years (27% and 37%, respectively). Further, it was shown that once seedlings appeared above the ground, mortality rates were very low (mean mortality rate: 8% and 3%, respectively). In addition, clonal growth was only observed in a very few (less than 10) individuals, indicating that vegetative spread is almost completely absent in this species.

Fruit production

In order to get an idea of fruit production and overall pollen transfer rates, for each flowering individual the number of flowers was also counted at the time of leaf sampling. At the end of June, the number of fruits was counted and fruiting success was calculated as the number of fruits divided by the number of flowers.

Sampling design and AFLP protocol

In May 2004, a total of 389 individuals (adults and seedlings) were sampled from both populations (Table 1). Young leaf material was collected and immediately frozen in liquid nitrogen. Before DNA extraction, plant materials were freeze-dried for 48 h and homogenized with a mill (Retsch MM 200) to fine powder. Total DNA was

Table 1 The proportion of polymorphic loci (*P*) and expected heterozygosity (H_E), slope *b* of the regression of pairwise kinship coefficients on the logarithm of geographical distance with significance level, the kinship coefficient between close relatives ($F_{(1)}$) and the *Sp* statistic estimated in two *Orchis purpurea* populations. Results for all individuals and for adults and recruits separately are shown

	n	*P*	H_E	*b*	$F_{(1)}$	*Sp*
Site 1						
All individuals	254	58.3	0.2064	−0.0140***	0.0260	0.0144
Adults	144	61.1	0.2069	−0.0195***	0.0391	0.0195
Recruits	110	58.3	0.2053	−0.0093***	0.0073	0.0094
Site 2						
All individuals	135	61.1	0.2114	−0.0145***	0.0212	0.0148
Adults	78	61.1	0.2117	−0.0239***	0.0405	0.0249
Recruits	57	61.1	0.2166	−0.0045 NS	0.0046	0.0045

***$P < 0.0001$; NS, not significant.

extracted from 30 mg of freeze-dried leaf material using methods described in Dendauw *et al*. (2002). After extraction, DNA concentrations were estimated on 1.5% (w/v) agarose gels.

AFLP analysis was carried out according to Vos *et al*. (1995), using commercial kits and following the protocol of Roldán-Ruiz *et al*. (2000). The enzymes *Eco*RI and *Mse*I were used for DNA digestion. Each individual plant was fingerprinted with three primer combinations: *Eco*RI-AGG/*Mse*I-CTGC, *Eco*RI-AGG/*Mse*I-CTGG and *Eco*RI-AGG/*Mse*I-CTAG. Fragment separation and detection took place on an ABI PRISM 377 DNA sequencer on 36 cm denaturing gels using 4.25% polyacrylamide (4.25% acrylamide/bisacrylamide 19/1, 6 M Urea in 1× TBE). GeneScan 500 ROX-labelled size standard (Applied Biosystems) was loaded in each lane. The fluorescent AFLP patterns were scored using GENEMAPPER version 3.7 (Applied Biosystems). We scored the presence or absence of each marker in each individual plant. Each individual displayed a unique banding pattern.

Data analysis

The proportion of polymorphic loci and expected gene diversity were calculated for each site using AFLP-SURV (Vekemans *et al*. 2002), assuming Hardy–Weinberg equilibrium and following methods outlined in Lynch & Milligan (1994). Allele frequencies were estimated using the Bayesian approach of Zhivotovsky (1999). In addition, for each site both measures were also calculated for adults and seedlings separately.

In order to identify differences in fine-scale spatial genetic structure between life-history stages spatial autocorrelation analyses were used. Under isolation-by-distance processes, theory predicts that the multilocus kinship coefficient F decreases approximately linearly with the natural logarithm of the physical distance between individuals. Hardy (2003) recently developed a multilocus estimator of the pairwise relatedness coefficient between individuals specifically adapted to dominant genetic markers (AFLP and RAPD). We used the estimator of the kinship coefficient for dominant markers:

$$Fd_{ij} = \frac{1 + F_I}{2} \frac{\sum_k [(X_{ki} - \hat{D}_k)(X_{kj} - \hat{D}_k) + \hat{D}_k(1 - \hat{D}_k)\hat{h}_k^2/(n-1)]}{\sum_k \hat{D}_k(1 - \hat{D}_k)\hat{h}_k^2}$$

where F_I is the inbreeding coefficient, $\hat{D}_k$ is the observed frequency of the dominant phenotype at locus k in the sample, n is the sample size and $\hat{h}_k^2$ is the estimated heritability for locus k (Hardy 2003). Average multilocus kinship coefficients per distance interval were then computed for the following distance classes: 3, 6, 11, 16, 23, 29, 35 and 44 (Site 1), and 3, 5, 7, 9, 11 and 14 m (Site 2) and were plotted against distance. Standard errors for the multilocus estimates of the kinship coefficients per distance class were estimated using a jackknife procedure over the loci. To test the hypothesis that there was significant spatial genetic structure, the observed regression slope of Fd_{ij} on $\ln(r_{ij})$, $\hat{b}_F$, was compared with those obtained after 9999 random permutations of individuals among positions. In this formula, $\ln(r_{ij})$ is the natural logarithm of the physical distance between samples i and j. This procedure has the advantage that all the information is contained in one single test statistic, and the results are independent of arbitrarily set distance intervals (Vekemans & Hardy 2004). Spatial genetic structure was also quantified by the '*Sp*' statistic, which is calculated as $-\hat{b}_F/(1 - \hat{F}_{(1)})$, where $\hat{F}_{(1)}$ is the mean $\hat{F}_{(ij)}$ between individuals belonging to the first distance interval and $\hat{b}_F$ is the regression slope of $\hat{F}_{(ij)}$ on r_{ij} (Vekemans & Hardy 2004). $\hat{F}_{(1)}$ can be considered a good estimate of the kinship between pairs of neighbours, on the condition that the first distance interval contains enough pairs of individuals to obtain reasonably precise $\hat{F}_{(1)}$ values, even if these pairs are not necessarily neighbours (Vekemans & Hardy 2004). The reciprocal of *Sp*, $-(1-\hat{F}_{(1)})/\hat{b}_F$, is an estimate of the neighbourhood size (Vekemans & Hardy 2004). In order to understand the relative contributions of pollen (σ_p) and seed (σ_s) dispersal to the overall level of gene flow (σ), we followed the method outlined in Heuertz *et al*. (2003). Using the average kinship coefficients $F(r)$ per distance interval, data were fitted to a polynomial regression of the third power: $f(r) = a + b\ln(r) + c[\ln(r)]^2 + d[\ln(r)]^3$. The curvature of $f(r)$ is given by the second derivative: $k = 2c + 6d\ln(r)$, where r_1 is the middle of the first distance interval. k values larger than 0 indicate concavity, suggesting leptokurtic gene flow and more restricted seed dispersal than pollen dispersal ($\sigma_s << \sigma_p$), whereas k values smaller than 0 indicate convexity ($\sigma_s \geq \sigma_p$) (Vekemans & Hardy 2004).

Analyses were performed for the whole population and for seedlings and adults, separately. All analyses were performed using SPAGEDI version 1.1b (Hardy & Vekemans 2002).

Results

Gene diversity

Using three primer pairs, a total of 36 polymorphic AFLP bands between 50 bp and 500 bp were scored. The percentage of polymorphic markers at the 5% level and gene diversity within populations were similar for both populations: 58.3 and 61.1, and 0.2064 (SD = 0.0309) and 0.2114 (SD = 0.0304), for Sites 1 and 2, respectively. There was also no difference in the number of polymorphic loci and gene diversity between seedlings and adults (Table 1).

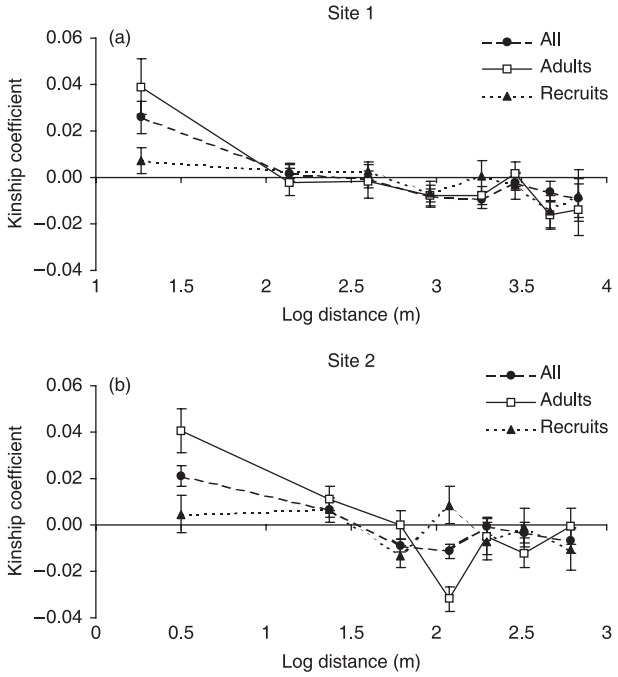

Fig. 2 Correlograms of kinship coefficients for all individuals, adults and recruits, respectively. Correlograms were calculated for two populations of *Orchis purpurea*.

Spatial genetic structure

The spatial autocorrelation analysis revealed significant spatial genetic structure in both sites (Table 1, Fig. 2). The slope of the regression line between the kinship coefficient and the natural logarithm of the distance between individuals, *b*, was highly significant ($P < 0.0001$) (Table 1) and both sites showed similar *b* values. Kinship coefficients between neighbours ($F_{(1)}$) were also very similar between sites (Fig. 2, Table 1). There were, however, large differences between the adults and seedlings in both populations. Adults showed a much stronger genetic structure than seedlings, and this pattern was consistent for both sites as *b* values were three and four times larger for adults than for seedlings (Table 1). In addition, *Sp* statistics were also higher for adults than for seedlings (Table 1). In both sites, the shape of the regression between the kinship coefficients and the logarithm of the distance was concave (i.e. *k* values were larger than 0), suggesting that seed dispersal was more restricted than pollen dispersal ($\sigma_s << \sigma_p$).

Fruiting success

Of a total of 8505 flowers investigated, only 721 (8.48%) were successfully pollinated [354 (6.53%) and 367 (11.88%) in Sites 1 and 2, respectively]. A total of 56 plants [41 (35.65%) in Site 1 and 15 (23.08%) in Site 2] did not produce any fruit. Fruit production rates per plant are presented in Fig. 3. In the majority of the plants less than 5% of all flowers were successfully pollinated and only a very small fraction of plants had more than 30% of its flowers pollinated.

Discussion

We found no differences in genetic diversity between life-history stages within the two studied populations of *Orchis purpurea*. These results are consistent with previous studies

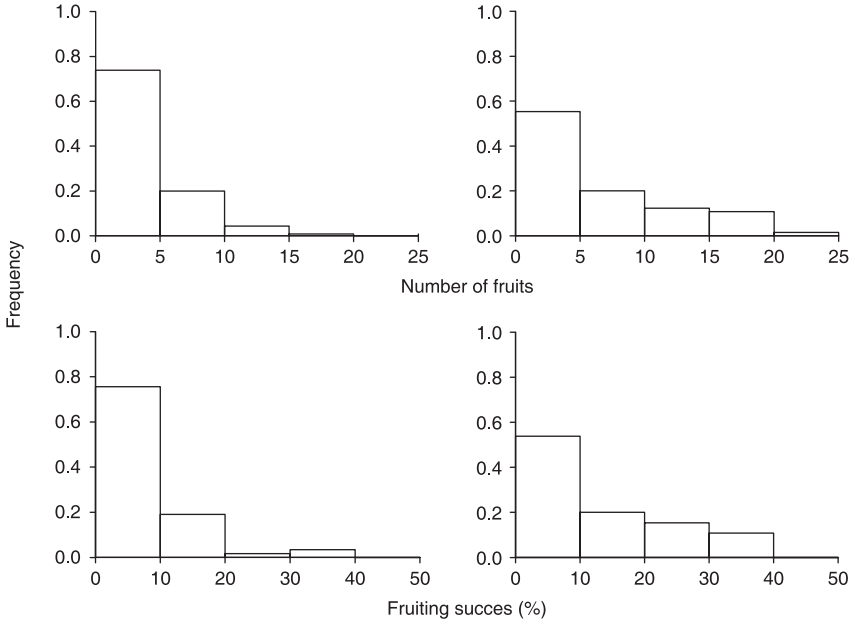

Fig. 3 Histograms of the number of flowers per plant successfully pollinated and fruit set for Site 1 and Site 2, respectively.

that have investigated temporal variation in genetic diversity among cohorts within populations (e.g. Linhart *et al*. 1981; Schnabel & Hamrick 1990; Hossaert-McKey *et al*. 1996; Chung *et al*. 2003). On the other hand, spatial autocorrelation analyses revealed significant spatial genetic structure, which increased in magnitude with life history stage. The observed *Sp* statistics (0.0144 and 0.0148) for the whole population (i.e. seedlings and adults together) are within the range for species with a predominantly outcrossing breeding system (range: 0.0020–0.0393, mean: 0.0126, SD: 0.0101) (Vekemans & Hardy 2004). The results agree with other studies that have investigated spatial genetic structure of deceptive orchids (e.g. *Caladenia tentaculata*, Peakall & Beattie 1996; *Orchis cyclochila*, Chung *et al*. 2005).

Supposing that no distinction had been made between seedling and adult plants, the observed spatial genetic structure could indicate limited pollen flow and seed flow, or limited seed flow and random pollen flow (Kalisz *et al*. 2001). Based on the shape of $F(r)$ estimates at short distances, it was concluded that pollen dispersal was much larger than seed dispersal ($\sigma_s << \sigma_p$). These results are corroborated by field observations that also suggested that pollen flow in the investigated populations is largely random. The few pollinators that we observed (mostly bumblebees) foraged on a few plants before leaving the population or turning to other, rewarding plant species. These observations seem to be in accordance with other deceptive orchid species, for which it was shown that pollen-mediated gene flow was large. Peakall & Beattie (1996), for example, showed that pollen dispersal distances might be as large as 50 m in the deceptive *C. tentaculata*. Similarly, Trapnell & Hamrick (2005) showed pollen dispersal distances of more than 200 m in the tropical orchid *Laelia rubescens*. Moreover, our data on fruit set also showed a low amount of successful pollinations and a large fraction of plants showing no fruit set at all (on average, only 6.5% and 11.9% of the flowers had successfully set seed), indicating that none of the populations are able to attract a large amount of pollinators and that pollination is highly unpredictable.

There were, however, strong differences in the extent of spatial genetic structure between seedling and adult plants. Strong spatial genetic structure was observed in adult plants, whereas the extent of spatial genetic structure was weak or even absent for recruits. Several mechanisms may be brought forward to explain why the spatial genetic structure of seedlings is less pronounced than that of the adult plants. First, the weak spatial genetic structure in recruits may be the result of high mortality rates of seedlings (Epperson & Alvarez-Buylla 1997). Kalisz *et al*. (2001), for example, demonstrated that in *Trillium grandiflorum*, thinning of post-dispersal seed clusters to single juvenile individuals led to decreased spatial genetic structure in juveniles. However, this mortality-driven rarefaction is most unlikely for the two populations studied here, as our 4-year demographic data showed that seedling mortality in both sites was very low. On average, once a seedling was observed above-ground, the probability of disappearing next year was very low (~5%). This suggests that mortality cannot be invoked as an explanation for the weak or absent genetic structure observed in the seedling stage.

A more plausible explanation may be found in the relative contributions of seed and pollen flow to total gene flow before and after disturbance. Before 1999, a dense vegetation and tightly packed humus layer were present, which are not very likely to allow suitable germination conditions and seeds to be dispersed over large distances. Flowering frequencies were also much lower under these conditions, resulting in very low fruit production (Jacquemyn *et al*. 2002) and no seedlings were observed before 2000. Hence, under these unfavourable conditions, no or very few recruitment is to be expected, whereas selection of related individuals or fine–scale genetic interactions of mycorrhizal associations could have favoured the survival of spatial aggregates of relatives (Taylor & Bruns 1999). In plants showing vegetative spread, clonal growth may also have contributed to the strong spatial structure (e.g. Jacquemyn *et al*. 2005b). However, our demographic analyses showed that clonal growth was restricted to a very few individuals. Moreover, none of the investigated plants displayed the same banding pattern, indicating that vegetative spread did not significantly contribute to the strong spatial structure observed in adult plants.

Once the vegetation (especially *Hedera helix*) and the thick humus layer were removed and more individuals started to flower, seeds could be dispersed over larger distances and germinate over a larger area. Moreover, as a limited number of individuals flowered for three consecutive years, gene flow by pollen and seeds may differ substantially from one year to the next, which may also result in overlapping seed shadows and the observed low degree of relatedness between neighbouring recruits. Hence, overlapping seed shadows, random mating and improvement of germination conditions are the most likely explanations for the weak spatial genetic structure in the seedling stage.

In conclusion, our results suggest that the strong differences in spatial genetic structure between seedlings and adult plants are most likely the result of the historical development of both populations. These findings stress the importance of incorporating both short- and long-term demographic data in studies of fine-scale spatial genetic structure. If the differences between life history stages found are due to disturbances related to the altered management of the sites, we can expect that, once the present adult plants die, the populations will probably reach a situation in which no or limited fine-scale spatial genetic

structure is present. It would be therefore extremely interesting to monitor the further development of these populations and repeat the genetic analyses once the plants, which are currently at the seedling stage, reach the adult stage. In addition, estimation of instantaneous gene flow through paternity/parentage analyses or the Two-Gener approach would also be very useful to disentangle historical vs. contemporary gene dispersal in this species and to assess the impact of changing environmental conditions and human disturbances on gene dispersal patterns.

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