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Effects of fine-scale genetic structure on male mating success in gynodioecious *Beta vulgaris* ssp. *maritima*

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Abstract

Plant mating systems are known to influence population genetic structure because pollen and seed dispersal are often spatially restricted. However, the reciprocal outcomes of population structure on the dynamics of polymorphic mating systems have received little attention. In gynodioecious sea beet (*Beta vulgaris* ssp. *maritima*), three sexual types co-occur: females carrying a cytoplasmic male sterility (CMS) gene, hermaphrodites carrying a non-CMS cytoplasm and restored hermaphrodites that carry CMS genes and nuclear restorer alleles. This study investigated the effects of fine-scale genetic structure on male reproductive success of the two hermaphroditic forms. Our study population was strongly structured and characterized by contrasting local sex-ratios. Pollen flow was constrained over short distances and depended on local plant density. Interestingly, restored hermaphrodites sired significantly more seedlings than non-CMS hermaphrodites, despite the previous observation that the former produce pollen of lower quality than the latter. This result was explained by the higher frequency of females in the local vicinity of restored (CMS) hermaphrodites as compared to non-CMS hermaphrodites. Population structure thus strongly influences individual fitness and may locally counteract the expected effects of selection, suggesting that understanding fine scale population processes is central to predicting the evolution of gender polymorphism in angiosperms.

Keywords: gynodioecy, male reproductive success, mating system, paternity analysis, sea beet, sex ratio

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Introduction

Dispersal events play a fundamental role in the evolutionary dynamics of populations by connecting distant demes in a metapopulation network and thereby have a strong impact on the partitioning of genetic diversity within and among structured demes (Loveless & Hamrick 1984; Hamrick & Nason 1996; Ennos 2001; Pannell & Dorken 2006; Sork & Smouse 2006). Beyond the magnitude of gene flow, the strength of the spatial genetic structure also varies with local adaptation processes and with the intrinsic characteristics of the species, including the mating system (e.g. unequal reproductive success). In return, fine-scale population subdivision may affect key evolutionary parameters such as the effective population size, which in turn determines the intensity of genetic drift (Whitlock & Barton 1997). In addition, when the reproductive success of a given genotype depends on its local frequency, population structure, by clustering similar genotypes, may also have a profound effect on individual fitness (Olson *et al.* 2006). In this respect, a better understanding of the interactions between individual fitness and fine-scale population structure requires the study of traits that are under frequency-dependent selection. Different examples of reproductive traits being under frequency dependent selection have been studied in plants, including flower morphology in heterostylous and enantiostylous plants (Jesson & Barrett 2002; Van Rossum *et al.* 2006), color

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polymorphism in rewardless orchids (Gigord *et al.* 2001), S-locus alleles in self-incompatible species (Wagenius *et al.* 2007) or sex-expression in dioecious and gynodioecious species (Fisher 1930; Lewis 1941).

Gynodioecy is a gender polymorphism that refers to plant species in which females and hermaphrodites coexist in natural populations, making this breeding system particularly relevant for addressing questions about the long-lasting evolutionary consequences of population structuring through local variation in sex ratio (Frank & Barr 2001; Olson *et al.* 2005). The genetics underlying sex expression in gynodioecious species commonly involves interactions between cytoplasmic male sterility (CMS) genes located in mitochondria, and nuclear male fertility restorers (Dommée *et al.* 1987; Boutin-Stadler *et al.* 1989; Saumitou-Laprade *et al.* 1994; Koelewijn & Van-Damme 1995; Ronfort *et al.* 1995; McCauley *et al.* 2000b; Dudle *et al.* 2001; Delph *et al.* 2007). The existence of such a cytonuclear polymorphism has attracted interest for several decades and a number of empirical and theoretical studies have attempted to determine the conditions for the stable maintenance of gynodioecy. Typically, CMS genes prevent production of fertile pollen in hermaphrodites, inducing a male sterile (female) phenotype. Theory predicts that a CMS gene should spread in a population as soon as fitness through the female function is higher in females than in hermaphrodites (Gouyon *et al.* 1991; Bailey *et al.* 2003; Dufay *et al.* 2007). This female advantage, due to the reallocation of resources from pollen to ovules or to the avoidance of inbreeding depression, has been measured in several gynodioecious species (e.g. Couvet *et al.* 1986; Asikainen & Mutikainen 2003; Olson *et al.* 2006). As soon as CMS becomes frequent within a population, nuclear (biparentally inherited) alleles which are able to restore male function are likely to be selected for. This is because, when CMS is frequent, genotypes that carry nuclear restorer alleles are the only hermaphrodites that are able to sire hermaphroditic offspring that will keep efficiently transmitting their genes, through both pollen and ovules. Theoretical models, based on the effect of selection only, postulate that the maintenance of cytonuclear polymorphism also requires a cost of restoration of moderate magnitude, so that restorers can increase in frequency when CMS is frequent but cannot reach fixation (Gouyon *et al.* 1991; Bailey *et al.* 2003; Dufay *et al.* 2007). Overall, sex-ratio should evolve according to negative frequency-dependent selection, with CMS genes being selected for when restorers are rare and nuclear restorers being selected for when CMS occurs at high frequency. Although the effect of frequency-dependent selection on gynodioecy is clearly acknowledged, most of the theoretical studies model infinite panmictic populations and do not take into account the fact that population structure and metapopulation dynamics could strongly modify the expected results of frequency-dependent selection (Couvet *et al.* 1998; Dufay & Pannell 2010).

In gynodioecious species sex ratio often varies considerably among populations as well as at a local scale within populations (McCauley *et al.* 2000a; Laporte *et al.* 2001; Olson *et al.* 2005, 2006; Dufay *et al.* 2009). When the spatial distribution of genders is not uniform, the fitness of a given sexual phenotype may depend on the mate availability in the subset of the population with which it interacts, rather than the composition of the entire population (Graff 1999; McCauley *et al.* 2000a; Alonso 2005; Olson *et al.* 2005; Oddou-Muratorio *et al.* 2006; Isagi *et al.* 2007). The interaction between female fitness and local sex-ratio has been investigated in a number of studies and it is now well established that population structure can reduce the fitness of females through pollen limitation when females are spatially clustered, compared with the case of panmixia (e.g. McCauley *et al.* 2000a). However, little is known about the effect of spatial structure of genders on male reproductive success. Intuitively, male reproductive output is expected to increase in female biased patches, when only a few pollen donors are available to sire all the neighbouring females. Once a restorer allele is established in a population, through mutation or migration, the maintenance of gynodioecy in a population will depend on the fate of this restorer, which is influenced by the fitness of individuals carrying the restorer and the intensity of the cost of restoration, but probably also by the intensity of drift and the identity of surrounding individuals in the case of fine scale spatial gender variation. Therefore, studying fine-scale spatial genetic structure and male reproductive success together should provide a more comprehensive insight into the key evolutionary processes that maintain gynodioecy in natural populations.

Here, we study how fine-scale spatial structure and differential male reproductive success among individuals interact to shape the evolution of gender polymorphism in the gynodioecious sea beet, *Beta vulgaris* ssp. *maritima*. Gender expression in this plant species is determined by interactions between maternally inherited CMS genes and biparentally inherited nuclear male fertility restorers. In contrast to some other gynodioecious species, a large part of cytoplasmic diversity in *Beta vulgaris* is not associated with sterilizing factors. Indeed, only four out of the 20 mitochondrial haplotypes described in wild populations are associated with gender polymorphism, meaning that two types of hermaphrodites coexist: restored hermaphrodites (carrying both CMS genes and nuclear restorer alleles) and non-CMS hermaphrodites (Cuguen *et al.* 1994; Forcioli *et al.* 1998; Laporte *et al.* 2001; Fénart *et al.* 2006). A recent

study showed that both pollen quantity and quality vary quantitatively among restored hermaphrodites, suggesting a complex genetic determination of nuclear restoration (Dufay *et al.* 2008). This study also showed that pollen viability was significantly lower in restored (CMS) hermaphrodites than in non-CMS hermaphrodite. These results suggest that restoration of male fertility might be incomplete in some of the restored hermaphrodites. An overall lower pollen quality in restored hermaphrodites may have long-lasting consequences in terms of spread of restoration genes: if restored hermaphrodites do not efficiently compete with non-CMS hermaphrodites, selection for restoration could be slower than predicted by classical models (Gouyon *et al.* 1991; Bailey *et al.* 2003; Dufay *et al.* 2007). Therefore, the sea beet provides a unique opportunity to study the fate of restorer genes by comparing the effective male fitness between restored and non-CMS hermaphrodites.

Additionally, because populations of *B. vulgaris* are known to be highly structured for genes involved in gender polymorphism (Laporte *et al.* 2001), we wanted to assess whether population structure and variation of local sex ratio could affect male reproductive success. In other words, is the fate of restorer alleles only influenced by the poor pollen quality produced by hermaphrodites that carry them, or is it also influenced by the spatial distribution of genders within populations? One way to answer this question is to compare the male reproductive success of the two hermaphroditic forms when (i) restored hermaphrodites are known to produce pollen of lower quality and (ii) fine-scale variation of gender polymorphism is sufficiently pronounced to allow the experimental detection of any effect of population structure on male reproductive success.

We focused on a natural population of *Beta vulgaris* which provided such opportunity. First, we showed that local sex ratio largely varies within our study site because of a pronounced fine-scale cytonuclear structure. Second, a detailed paternity analysis allowed us to characterize the distribution of pollen dispersal events within the studied population. We showed that (i) besides the occurrence of long-distance pollen flow, as usually expected for wind pollinated species, a large part of mating events occur at a very restricted geographical scale, and that (ii) neighbourhood features, such as local conspecific density, affect the patterns of pollen flow. Finally, because quantity and quality of pollen produced by the two hermaphroditic forms were recorded in this particular population, the same year, by a study in tandem with ours (Dufay *et al.* 2008), we used the results of our paternity analysis to answer the following questions: (i) does the lower quality of pollen of restored hermaphrodites cause a reduction of male reproductive success and (ii) does local sex ratio affect the reproductive success of hermaphrodites? This is, to the best of our knowledge, the first study dealing with the effect of gender polymorphism and population structuring on the effective male reproductive success in a gynodioecious species.

Materials and methods

The species

Wild sea beet, *Beta vulgaris* ssp. *maritima* is a diploid species ($2n = 18$) widely distributed along the western coast of Europe and around the Mediterranean basin. It is a short lived perennial and wind-pollinated species (Letschert 1993). *Beta vulgaris* is largely self incompatible, with up to four gametophytic S loci (Owen 1942; Larsen 1977), but up to now, there is no clear knowledge on the levels of outcrossing in natural conditions. There is no vegetative reproduction, and thus dispersal can only occur through seeds and/or pollen movement. Seeds are aggregated in an irregular dry body that contains 1–8 seeds. This seedball has no particular dispersal mechanism and is primarily dispersed by gravity or by water movements during high tide (Fievet *et al.* 2007). This study was carried out in northern France, where sea beets colonize areas located along estuaries, just at the upper level of the tide, cliffs overhanging the sea and other coastal habitats (Letschert 1993; Laporte *et al.* 2001; Arnaud *et al.* 2003b; Viard *et al.* 2004).

Male sterility in *Beta vulgaris* ssp. *maritima* is associated with four particular mitochondrial types, called CMS *E*, *G*, *Svulg* and *H*, the other mitotypes being associated with male fertile phenotypes (Cuguen *et al.* 1994; Forcioli *et al.* 1998; Desplanque *et al.* 2000; Fénart *et al.* 2006). In our study species, historical relationships between CMS and non-CMS haplotypes suggest independent apparitions of CMS haplotypes with sterilizing cytoplasms belonging to different lineages derived from an ancestral non-sterilizing cytoplasm (Fénart *et al.* 2006).

Study site, phenotyping and sampling

We focused on a population located near Audresselles (N 50°49.101, E 1°35.676) in Northern France. The focal population extended over 500 m and consisted of approximately 400 flowering individuals. Within this population, a total of 280 flowering plants were uniformly sampled in order to cover the whole area of the population (Fig. 1). The locations of each sampled plant was mapped, sexual phenotype was determined (female or hermaphrodite), and leaves were collected for molecular studies (Fig. 1a). Among the 206 individuals identified as hermaphrodites, 195 were classified into three

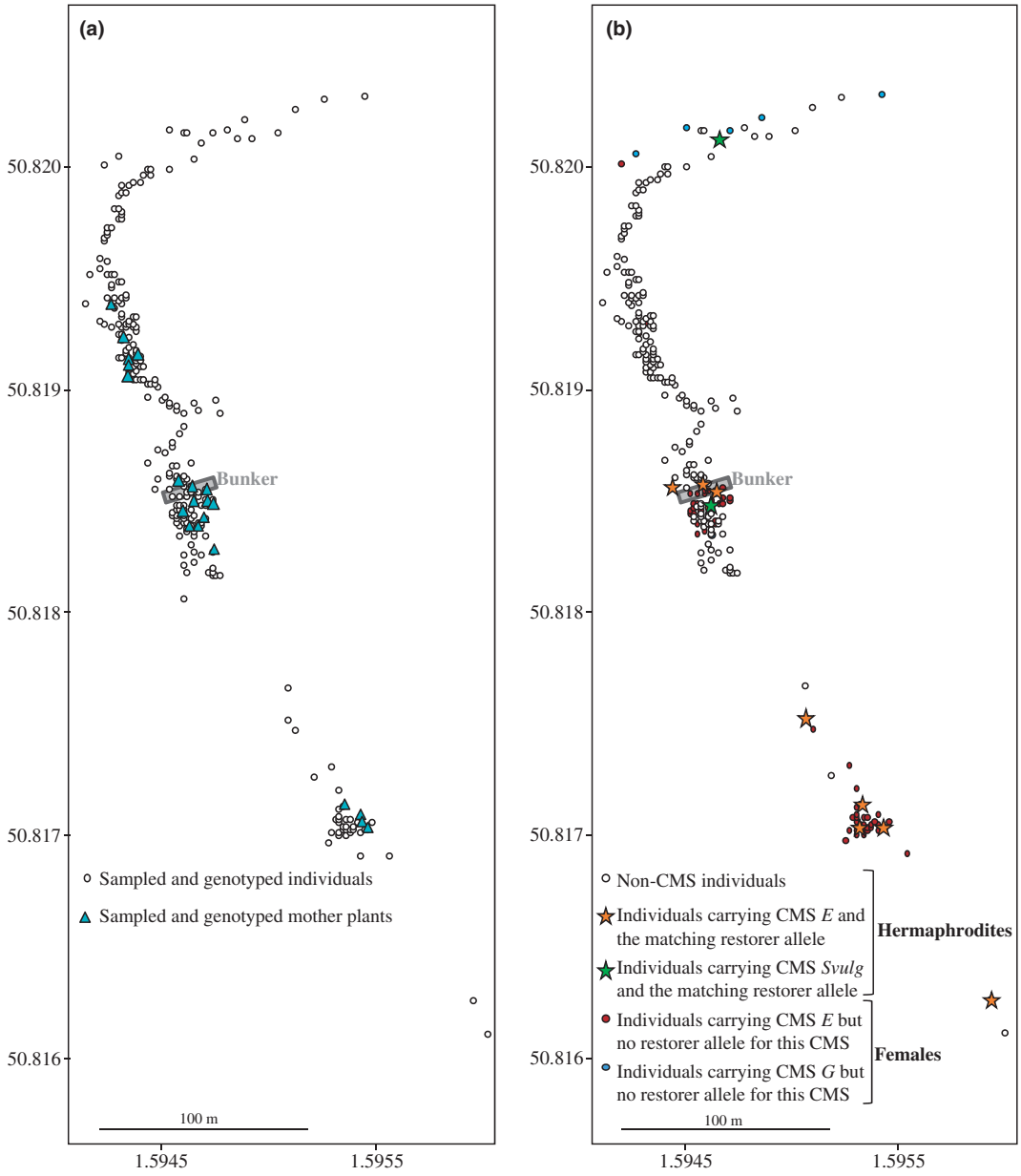

Fig. 1 Spatial locations for sampled individual sea beet (*Beta vulgaris* ssp. *maritima*) plants. (a) Individuals sampled for genotyping and mother plants used for paternity analysis; (b) associated sexual phenotypes and CMSs within the study population.

groups based on relative size, measured by the number of floral stems: group 1 (1 to 15 floral stems, $N = 59$ individuals), group 2 (15 to 30 floral stems, $N = 87$ individuals) and group 3 (more than 30 floral stems, $N = 49$ individuals). We also described the local neighbourhood around each sampled individual by counting the number of flowering hermaphroditic and female individuals within 15 m.

Following cytoplasmic genotyping (see below), we were able to discriminate between normal (carrying a male-fertile cytoplasm) and restored (carrying a CMS-associated cytoplasm) hermaphrodites, and then selected eight females, eleven normal hermaphrodites and two restored hermaphrodites as maternal plants for the paternity analysis (see Fig. 1b). These mother plants were chosen in order to mirror the frequencies of the different genders in the study site. Seeds were randomly collected on five stems for each maternal plant in mid-august. Seeds were then germinated in a greenhouse and grown until each seedling had several leaves. A total of 1019 seedlings were collected for molecular studies (48 ± 12 per seed parent).

In wind-pollinated species, pollen flow often includes substantial amounts of long distance dispersal (Dow &

Ashley 1998; Streiff *et al.* 1999; Burczyk *et al.* 2004; Robledo-Arnuncio & Gil 2005; Fénart *et al.* 2007). Accounting for the paternal contribution of plants growing outside the studied area is important for assessing the connectivity between populations and to understand processes shaping population structure at a larger spatial scale (Sork & Smouse 2006; Slavov *et al.* 2009). In order to study the possibility of external gene flow, the two closest neighbouring populations, situated on both sides of Audresselles, were additionally sampled (20 individuals per population) and mapped. These populations were located 1.2 and 3.5 km from the study site and comprised very few individuals (approximately 30 and 50 individuals respectively).

Genetic data collection

We used a NucleoSpin®96 Plant Kit (Macherey-Nagel) to extract and purify total DNA from dried leaf tissue as described in Fénart *et al.* (2007). This procedure yielded a total of 320 sampled adults and 1019 offspring.

Nuclear diversity

Sampled individuals, including mother plants and seedlings, were genotyped at 10 microsatellite loci: *GAA1*, *GTT1*, *GCC1*, *BVM3*, *CAA1* (Mörchen *et al.* 1996; Viard *et al.* 2002); *SB04*, *SB06*, *SB07*, *SB15* (Richards *et al.* 2004); and *FDSB1027* (McGrath *et al.* 2007). Loci were amplified in two multiplexed PCR. The first multiplex was performed in a 10.5 μL reactions consisting of: 25 ng of DNA template, 1 μL of Buffer 10 × (Perkin-Elmer, Norwalk, CT, USA), 2.9 mM $MgCl_2$, 0.2 μg/μL of BSA, 2% of DMSO, either 0.1 μM (for loci *GTT1*, *BVM3*, *CAA1* and *FDSB1027*) or 0.05 μM of each primer (for locus *GCC1*), 290 μM of each dNTP and 0.9 U/μL of hot start *Taq* polymerase (*AmpliTaq* Gold, Perkin-Elmer, Norwalk, CT, USA). The second multiplex was performed in the same conditions, with 0.1 μM (for loci *GAA1* and *SB04*) or 0.05 μM of each primer (for loci *SB06*, *SB07* and *SB15*). PCR was conducted on a 9700 thermal cycler (Perkin-Elmer, Norwalk, CT, USA) under the following conditions: 5 min denaturing at 94 °C, 45 s denaturing at 94 °C, 45 s annealing at 54 °C and 45 s extension at 72 °C and a final extension step at 72 °C for 10 min, after 40 cycles.

Cytoplasmic diversity

Several cytoplasmic male sterilities (CMSs) have been described in sea beets, of which three are most common: *Owen* CMS (also called *Svulg*), which has been widely used in plant breeding of the sugar beet (Owen 1945; Arnaud *et al.* 2003b; Viard *et al.* 2004), and two others, *E* and *G*, found exclusively in wild beet populations (Cuguen *et al.* 1994; Desplanque *et al.* 2000; Laporte *et al.* 2001; Fénart *et al.* 2006; Dufay *et al.* 2009). Diagnostic cytoplasmic PCR-RFLP markers were used to distinguish among the three main CMSs: two mitochondrial markers that enable to identify the CMSs *Svulg* and *G*, and a chloroplast marker for CMS *E*. Primers for detection of CMS *Svulg* are described in Ran & Michaelis (1995), and those for CMS *G* and CMS *E* in Dufay *et al.* (2008).

We conducted standard PCR procedures in 15 μL reactions consisting of: 25 ng of DNA, 3.5 mM $MgCl_2$, 200 μg/mL of BSA, 200 μM of each dNTP, 0.2 μM of forward and reverse primers, 0.375 U of *Taq* polymerase (Perkin-Elmer). Amplifications were performed with the following method: 3 min denaturing at 95 °C, followed by 35 cycles of 45 s denaturing at 94 °C, 45 s annealing at 55 °C and 1 min extension at 72 °C, with a final extension step of 72 °C for 10 min. Allele sizing could be directly performed for CMS *G* by separating DNA products using a 2% agarose gel and visualizing after ethidium bromide staining under UV light. Polymorphism detection for both CMS *Svulg* and *E* needed a supplementary restriction step before visualization:

- **(i)** for CMS *Svulg*: 5 μL of the PCR product digested in a 10 μL reaction with 0.2 mM of spermidin and 2 U of *Taq*I, conducted at 65 °C overnight, as described in Arnaud *et al.* (2003b).
- **(ii)** for CMS *E*: 5 μL of the PCR product digested in a 10-μL reaction with 0.2 mM of spermidin and 1.5 U of *Alu*I, conducted at 37 °C overnight.

We characterized mitochondrial polymorphism by genotyping individuals at four mitochondrial minisatellite loci: *TR1*, *TR2*, *TR3* and *TR4* (Nishizawa *et al.* 2000). PCRs were carried out in 10.5 μL volumes containing 3 mM $MgCl_2$, 200 μM of each dNTP, 0.2 mg/mL of BSA, 120 μM of each forward and reverse primer, 0.625 U of *Taq* polymerase (Perkin Elmer) and ≈50 ng of template DNA. Cycling conditions consisted of an initial denaturation step of 5 min at 94 °C followed by 30 cycles of 30 s at 94 °C, 60 s annealing at 62 °C, 30 s at 72 °C, and ending with a final extension for 10 min at 72 °C. As the entire mitochondrial genome is generally inherited as a single linkage unit, each genotype combination for these four minisatellite loci was analysed as a single haplotype, as in Fievet *et al.* (2007).

Detection and analysis of PCR products

Allele sizing of both minisatellite and microsatellite multiplex amplified products was performed using an ABI Prism® 3100 Genetic Analyzer 16-capillary array system (Applied Biosystems) as described in Fénart

et al. (2007) for nuclear data and Fénart *et al.* (2008) for cytoplasmic data.

Statistical analyses

Nuclear and cytoplasmic diversity

Genotypic disequilibrium was tested for all locus pairs in GENEPOP version 3.4 (Raymond & Rousset 1995). Exact tests used the Markov-chain method based on the contingency tables for all pairs of loci in each population (Raymond & Rousset 1995). Tests were conducted with the dememorization number set to 10 000, for 1000 batches and 10 000 iterations. Significance of *P*-values were assessed after Bonferroni correction, as suggested by Rice (1989).

Standard population genetic indexes were calculated (number of alleles A_n, observed heterozygosity H_o, gene diversity H_e and unbiased fixation index F_{IS}) in FSTAT version 2.9.3 (Goudet 1995). Significance of single locus F_{IS} as well as mean overall F_{IS} estimate were tested using 10 000 random permutations of alleles among individuals. We also used FSTAT to estimate pairwise population differentiation (F_{ST}) among distinct patches (see below) with 10 000 permutations of the data, using a G test for significance of results (Goudet *et al.* (1996). F_{IS} and F_{IT} estimates were also estimated, and significance was tested by permuting alleles within patches and over the total population with 10 000 permutations.

We estimated inbreeding coefficients to determine whether inbreeding could be responsible for variation in male reproductive success. We estimated individual inbreeding coefficients using a multilocus estimator for each adult individual as well as for progenies, following the procedure described in Ritland & Travis (2004).

Bayesian analysis of population structure

Recent studies indicate that Bayesian clustering methods can accurately describe genetic population clusters, and even in cases of weak spatial genetic structure, for example in newly founded populations likely to be far from migration-drift equilibrium (e.g. Coulon *et al.* 2006; Rowe & Beebee 2007). To assess the number of genetic populations within our study site, we used GENELAND version 1.0.5 (Guillot *et al.* 2005) to visualize genetically distinct groups and to detect genetic discontinuities across the focal area. We analyzed the data first by allowing *K* to vary from one to 10, with 10 independent runs under the following settings: 200 000 MCMC iterations, maximum rate of Poisson process fixed to 100, maximum number of nuclei in the Poisson–Voronoi tessellation fixed to 250, and the Dirichlet model for allelic frequencies. We then inferred the number of groups in our sample from the modal *K* of these 10 runs and ran the MCMC with *K* fixed to this number. Other parameters remained similar to those of the runs with variable *K*. The posterior probability of group membership for each pixel of the spatial domain was then computed (using a burn-in of 10 000 iterations).

Spatial autocorrelation

Once group membership was established for each individual of the studied population, the second step was to determine whether spatially restricted gene flow may promote spatial genetic structure, even within groups of genetically related individuals. We used spatial autocorrelation analyses to examine the spatial arrangement of genetic variability across subpopulations and to compare the strength of spatial structuring from nuclear and cytoplasmic data. These analyses make no assumptions about the scale of spatial patterns or population genetic parameters (Sokal & Oden 1978; Barbujani 1987) and provide a detailed description of gene frequency variation in space that allows inference of micro-evolutionary processes shaping the distribution of gene frequencies (Arnaud *et al.* 2003a; Storfer *et al.* 2007). In this study, we used the kinship coefficient ρ_{ij} developed by Loiselle *et al.* (1995) as autocorrelation index. This kinship coefficient allows the integration of multi-allelic data, or even multiple loci for nuclear data, and is relatively unbiased in the presence of low frequency alleles (Loiselle *et al.* 1995; Aspi *et al.* 2006; Born *et al.* 2008). Ten distance classes were defined in order to obtain approximately the same number of individual pairs within each distance class. We calculated confidence intervals over sampling coordinates (under the null hypothesis of no spatial genetic structure) with 10 000 permutations of haplotypes (mitochondrial minisatellites) and multilocus nuclear microsatellite genotypes using SPAGeDI version 1.2 (Hardy & Vekemans 2002). To compare the strength of spatial genetic structure between nuclear and cytoplasmic data, as well as between genetically differentiated groups of individuals, we used the statistic S_p introduced by Vekemans & Hardy (2004), which is independent on the sampling scheme used, i.e. independent of arbitrarily set distance intervals. S_p statistics were calculated using linear distances and the slope *b* of regressions analyses was tested for significance by randomly permuting individuals over spatial locations, as described above.

Paternity analysis, outcrossing rate, pollen dispersal and male mating success

Paternity exclusion probabilities (EP) were computed following Jamieson & Taylor (1997), using the program

CERVUS version 2.0 (Marshall *et al.* 1998). For the 1019 seedlings, paternity assignment was performed using the maximum likelihood-based method described in Marshall *et al.* (1998) and implemented in the program CERVUS version 2.0. All parental individuals (except females) were considered as potential fathers for each offspring (N = 206 hermaphrodites). Paternity likelihood was estimated using the ratio of probabilities (the LOD–score) defined in Meagher (1986). To determine whether the paternity of offspring can be assigned to the hermaphrodite with the highest paternity likelihood, we used the difference in likelihood score between the most likely parental hermaphrodites (ΔLOD). The critical value (Δ_C) of Δ below which paternity cannot be attributed at 80% was determined using a distribution of Δ obtained from 20 000 simulated mating events. This distribution was generated assuming the following parameters: (i) observed allele frequencies at each locus (calculated for the entire data set including the genotypes of all the reproductive adults and offspring) (ii) estimated male breeding population size (number of candidate parents) and the proportion of candidate parents sampled, respectively set to 400 and 0.7, (iii) the proportion of loci mistyped, set to 0. As advised by Oddou-Muratorio *et al.* (2003), it is better to introduce a null scoring error into CERVUS, because a non-null error rate always results in an augmentation of type I errors (i.e. assignment of a wrong father to a given offspring, while the true father is either another sampled individual or a non sampled individual) and type II errors (i.e. non-assignment of paternity to the father of the offspring while this father is among the sampled individuals). For each offspring, the likelihood-based parentage analysis produced three possible alternative outcomes. (i) Paternity could not be significantly assigned to one of the sampled adults, either because the male parent was outside the study area or was one of the non-sampled adults within the population. Also included within this group are the cases where two or more adults were compatible with the offspring but with a difference (Δ) in LOD-score too low to attribute paternity to the most likely parent. (ii) Paternity was attributed to the mother, allowing us to estimate the selfing rate of each mother. (iii) Paternity was significantly attributed to another sampled adult and it was then possible, knowing the position of both mother and assigned father, to calculate their pairwise geographical distance. Deviation of the observed dispersal pattern from the distribution of pollen dispersal events expected under panmixia (i.e. distribution of mate pairs sampled in each distance class) were tested using a chi-square test.

We also investigated the effect of several phenotypical and ecological parameters on male reproductive success. For this we analysed the variance in the number of seedlings sired by each potential father with a logistic regression (Poisson distribution, log-link function, PROC GENMOD, SAS) and corrected for overdispersion (dscale option, PROC GENMOD, SAS). Four factors, describing individual characteristics of the potential fathers were tested: (i) cytoplasmic identity (restored hermaphrodite versus non-CMS hermaphrodites), (ii) size (three levels, according to the number of flowering stems, as above), (iii) geographical position (two levels, defined from the probability of population membership, see results) and (iv) inbreeding coefficient. In addition, four factors describing the local neighbourhood of each potential father within a radius of 15 m were tested: (i) number of flowering plants, (ii) number of flowering females, (iii) number of flowering hermaphrodites, and (iv) the local sex ratio (determined by the frequency of females in the focal area). Finally, we tested whether the factors listed above statistically depended on each other, by comparing all four variables that described local neighbourhoods between (i) restored hermaphrodites and non-CMS hermaphrodites and (ii) plants located in the southern and the northern subpopulation, using non-parametric Wilcoxon Mann–Whitney tests.

As supplementary information, correlation of outcrossed paternity (i.e. the proportion of full sibs within an outcrossed progeny array) was estimated using the maximum-likelihood approach under a mixed-mating system model implemented in the MLTR v3.2 software package (Ritland 2002).

Results

Cytonuclear diversity and sex polymorphism

Male sterilizing cytoplasms were detected at relatively high frequency, particularly the CMS *E*, with 84 individuals out of 280 sampled plants (30%). Two other CMS types were also present but at lower frequencies: six individuals (2.14%) for *G*, and two individuals (0.71%) for *Svulg* (Fig. 1b). The proportion of females was high (29%), suggesting very low frequencies of restorer alleles in this population. We observed only eight hermaphrodites carrying the haplotype associated with CMS *E* (restoration rate of 9.52%). Almost all females and all individuals carrying CMS *E* clustered within patches located in the southern part of the population (see Fig. 1b). We did not observe restored hermaphrodites for CMS *G*, whereas the two individuals carrying CMS *Svulg* were restored for male fertility and were thus expressing a hermaphroditic phenotype. Given the observed rarity of *Svulg* male sterility, we excluded these two hermaphrodites from statistical comparisons of male reproductive success.

Mitochondrial minisatellite loci displayed moderate levels of polymorphism, with one to seven alleles per locus, for a total of 12 alleles giving eight different haplotypes (cf. Table 1). There was a strong linkage disequilibrium between these haplotypes and the CMS genes (all at $P < 0.05$), each of the three CMSs being exclusively associated with a unique minisatellite haplotypes. The levels of diversity exhibited by nuclear microsatellites and cytoplasmic minisatellites were of the same order of magnitude than those that are generally observed in natural sea beet populations (e.g. Fievet *et al.* 2007; Fénart *et al.* 2008). Nuclear microsatellite loci were highly polymorphic with an overall total of 83 alleles, the number of alleles ranging from 2 (*Gcc1*) to 19 (*Caa1*). The number of sampled alleles (A_e), expected heterozygosity (H_e) and estimated intra-population fixation index (F_{IS}) are presented in Table 1. The overall average F_{IS} was non-significant ($F_{IS} = 0.009$). A significant departure from HW genotypic proportions was observed for only two loci (*SB06* and *SB15*). Expected heterozygosity (H_e) was relatively high across all loci (0.157 to 0.846), except for *Gtt1* and *Gaa1*, which showed the lowest values for diversity and expected heterozygosity, as already reported in previous studies (Arnaud *et al.* 2003b; Fievet *et al.* 2007). Significant linkage disequilibrium was detected in 15 of the 45 pairwise comparisons for microsatellite loci (after Bonferroni correction, $P < 0.05$). We also investigated cytonuclear associations between the minisatellite haplotypes and the microsatellite loci: 8 of the 10 pairs showed significant linkage disequilibrium (*1027*, *Bvm3*, *Caa1*, *Gtt1*, *Gaa1*, *SB04*, *SB06* and *SB07*, after Bonferroni correction, $P < 0.05$).

Within the population, individual inbreeding coefficient values varied widely from 0 to 0.966 for the adults, the mean value being 0.0753. Estimates of inbreeding coefficient varied between 0 and 1 for studied seedlings, with a mean value of 0.0832, which was significantly higher than what was observed for adults (Mann–Whitney, $N_{seedlings} = 1019$ $N_{adults} = 280$, $Z = -2.43$, P[two-tailed] = 0.014). The calculation of individual inbreeding coefficient is characterized by a large variance (Ritland & Travis 2004) and there is no direct relationship between the inbreeding coefficient and the intrapopulation fixation index as measured by multilocus level of heterozygosity (Slate *et al.* 2004), which may explain why mean values of inbreeding coefficient differed from mean F_{IS} values.

Within population structure

Individual population membership

First, we ran the MCMC 10 times, allowing K to vary, in order to verify the consistency of the results. All these trial runs in GENELAND identified $K = 2$ as the modal value for the number of genetic populations, which we then fixed to compute the posterior probability of individual membership to one of the clusters, and the population membership for each pixel of the spatial domain (Fig. 2). The transition between the two genetically distinct clusters occurred over a few metres. The southern part of the population appeared to be strikingly separated from the northern part by a thin boundary. The location of this boundary corresponds to that of an ancient bunker, which may be a physical barrier to pollen and seed dispersal. Three groups of plants (19 individuals) located in the northern part of the study area were assigned to the southern group with a very high probability ($P > 0.9$), suggesting seed dispersal over several hundred metres (see Fig. 2). Cytoplasmic identity of these 19 individuals was either non-sterilizing or sterilizing.

Table 1 Genetic diversity estimated for each locus and over all loci in a wild population of sea beet (*Beta vulgaris* ssp. *maritima*). Listed for each locus are the number of sampled alleles per locus (A_n), expected heterozygosities (H_E), intrapopulation fixation indexes (F_{IS}), and the observed allele size ranges

Microsatellite nuclear loci					Minisatellite cytoplasmic loci		
	A_n	H_e	F_{IS}	Allele size range		A_n	Allele size range
1027	7	0.556	0.069^{NS}	175–201	*TR*1	7	439–794
Bvm3	14	0.846	−0.012 ^{NS}	96–123	*TR*2	1	404–404
Caa1	19	0.82	0.032 ^{NS}	139–194	*TR*3	2	420–482
Gcc1	2	0.474	−0.107 ^{NS}	96–99	*TR*4	2	410–438
Gtt1	3	0.23	0.047 ^{NS}	112–118			
Gaa1	3	0.157	0.025 ^{NS}	182–191			
SB04	10	0.332	−0.03 ^{NS}	170–193			
SB06	5	0.682	0.078**	145–165			
SB07	11	0.778	0.044 ^{NS}	245–272			
SB15	9	0.69	−0.066***	140–176			
Mean	8.3	0.557	0.009 ^{NS}				

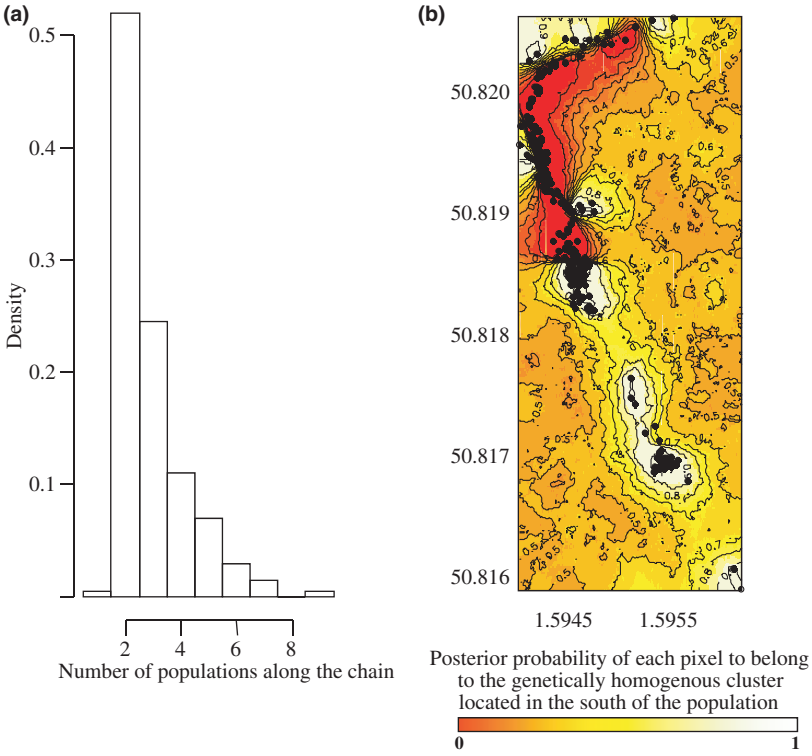

Fig. 2 (a) Posterior distribution of probability density of the number *K* of populations (b) Map of the posterior probability of population membership of each pixel of the spatial domain to belong to one of the two genetically homogenous clusters, using the Bayesian analyses of population structure described in Guillot *et al.* (2005). Scale of X and Y axis designed geographical coordinates in decimal degrees.

The two subpopulations did not differ in gene diversity, allelic richness ($P > 0.05$ in both cases, *t*-test using loci as resampling units), or inbreeding levels estimated following Ritland and Travis method (Mann-Whitney using individuals as resampling units, $N_{north} = 160$, $N_{south} = 120$, $Z = -0.1739$, P[two-tailed] = 0.431). F_{IS} fixation indexes were also estimated within each of the two genetically distinct clusters: no significant departure from Hardy–Weinberg expectations within these subpopulations was observed ($F_{IS} = 0.007$ and -0.017 for the southern and the northern part of the population, respectively). Genetic differentiation between the two groups was highly significant, based on both nuclear ($F_{ST} = 0.03$, $P < 0.001$) and cytoplasmic data ($F_{ST} = 0.36$, $P < 0.001$). Furthermore, we investigated the cytonuclear associations at the subpopulation level for the eight loci that displayed significant linkage disequilibrium over the whole population. Only three comparisons remained significant: *Bvm3* in both subpopulations, and *Caa1* and *Gaa1* in the northern subpopulation only. As the disequilibrium at *Bvm3* implies different alleles and haplotypes, depending on the subpopulation, it is most probably due to a genetic structuring effect within the study site.

Frequencies of male sterile cytoplasms and females differed between the two genetically distinct clusters. In the southern cluster, 65% of the individuals had a sterilizing cytoplasm and 60% had a female phenotype. In the northern cluster, only 5% of individuals possessed a sterilizing cytoplasm and 4% a female phenotype. Additionally, 75% of restored hermaphrodites were located in the southern cluster.

Spatial genetic structure

Results of the spatial autocorrelation analysis showed that the southern cluster was highly structured relative to the northern cluster (Fig. 3). As shown by correlograms, a continuous decline in genetic similarity with physical distance was only observed for the southern cluster (Fig. 3a, c). In this cluster, there was also a considerable difference in the extent of spatial genetic structure between cytoplasmic and nuclear genetic variation: the S_P statistic for nuclear markers was 18-fold lower than for cytoplasmic haplotypes, whereas in the northern cluster there was very low spatial genetic structure for either marker type ($S_P = 0.0168$ and 0.005 respectively, Fig. 3b, d). These observations suggested contrasted patterns of spatial genetic structure between the two genetically differentiated clusters of individuals as well as a general trend for isolation by distance processes for both cytoplasmic and nuclear data over the whole population.

Contemporary pollen flow

The cumulative exclusion probability was high ($EP = 0.993$), suggesting that the loci were highly

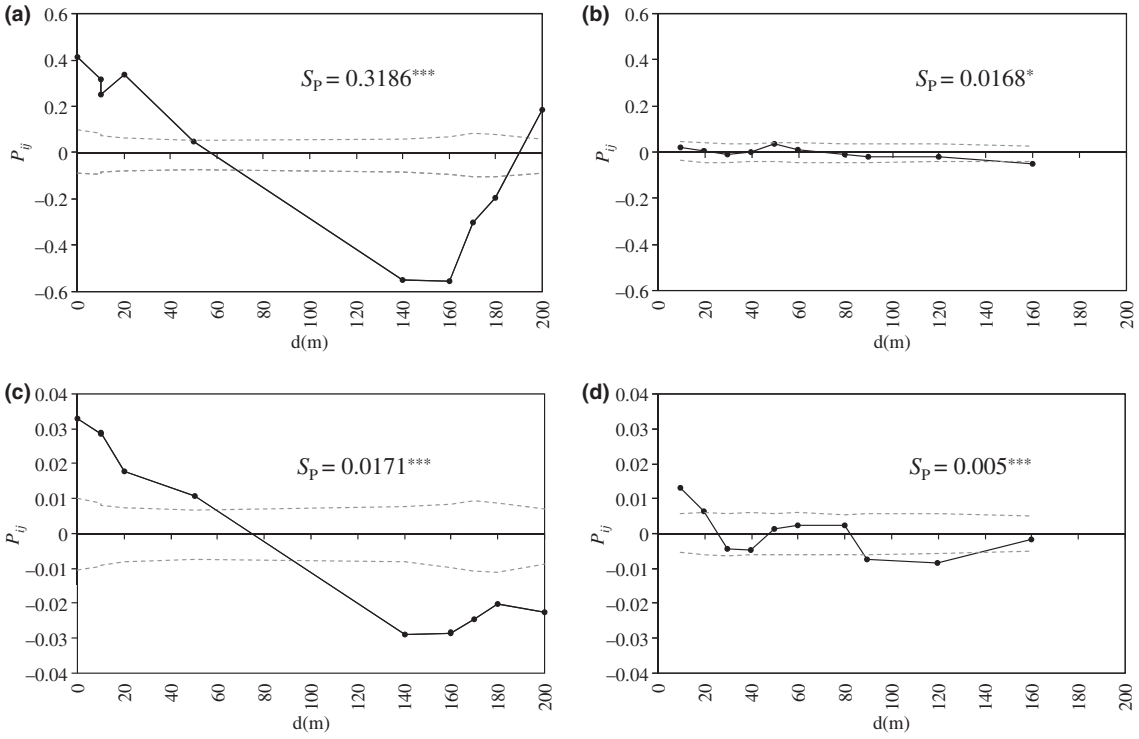

Fig. 3 Average pairwise kinship coefficient (*p*ij, Loiselle *et al*. 1995) between individuals as a function of the distance (meters). Patterns of relatedness based on cytoplasmic haplotypes for individuals in the southern cluster (a) and the northern cluster (b), and patterns of relatedness based on nuclear data for individuals in the southern cluster (c) and the northern cluster (d). The dashed lines represent upper and lower 95% confidence intervals for the null hypothesis of no relatedness between individuals. *: $P < 0.05$; ***: $P < 0.001$.

suitable for paternity analysis. Out of 1019 seedlings, 492 (48%) had no compatible father among the reproducing individuals collected (either because the male parent was outside the study area or was among the non-sampled adults within the population), 526 (51%) had only one compatible father (confidence level of 80% or more), and one showed a mismatch with the genotype of the mother at one locus (this seedling was excluded in further analyses). None of the studied seedlings showed equal probability to be assigned to two or more fathers.

Among the 526 seedlings with paternity assignments, 13 (3%) were sired with high probability by a father situated in one of the two additional neighbouring populations sampled to gauge rate of external pollen flow. This suggests that pollen flow can occur over several kilometres (at least up to 3.5 km) along the coastline. Within the studied population, paternity was assigned to the mother of 14 seedlings, providing an overall selfing rate estimate of 3%. Among the 13 hermaphroditic mother plants, only five showed partial selfing, often at very low rates (1 to 3 seedlings per mother) with the exception of one individual (7 out of 19 seedlings assigned to the mother plant).

Based on the outcrossed mating events within the population, pollen dispersal distances ranged up to 355 m, with an average of 96 m and median of 24 m. The resulting frequency distributions of pollen dispersal distances departed from the expected distribution under random mating and clearly indicated a pollen flow constrained over relatively short distances (Fig. 4a). Twenty-nine percent of the pollination events occurred between the two clusters defined above. By accounting for densities of conspecifics near the assessed fathers, we found that the pattern of local density significantly affected pollen dispersal distance. Pollen flow was limited to short distances for fathers situated in patchy areas (Fig. 4c), with an average of 27 m and a median of 9 m, whereas the pollen dispersal was significantly higher for spatially isolated fathers (Fig. 4b), with an average of 104 m and median of 80 m (Mann–Whitney, $N_{\text{isolated}} = 96$, $N_{\text{non-isolated}} = 96$, $Z = 9.46$, P[two-tailed] < 10^{-4}).

Male reproductive success

For each sampled hermaphrodite, we analyzed the number of assigned seedlings as a function of several

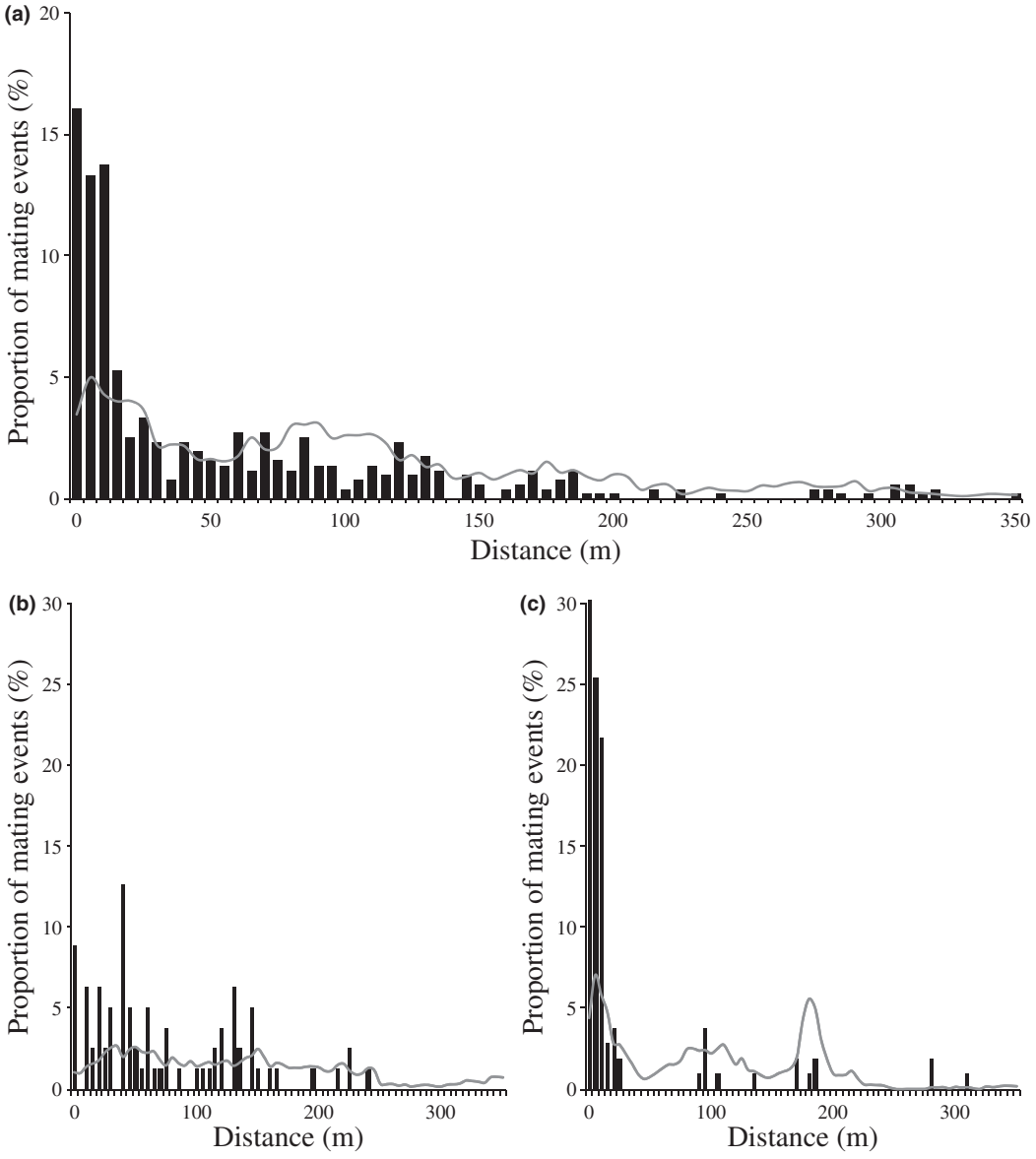

Fig. 4 Proportion of mating events as a function of geographical distance between mates within the studied population for (a) all the outcrossing pollination events (N = 526 seedlings), (b) spatially isolated fathers (< 15 individuals in a 15 m neighborhood, N = 96 seedlings), and (c) fathers situated in patchy areas (> 45 individuals in a 15 neighborhood, N = 96 seedlings). The grey line represents the expected numbers of mating events under the null hypothesis of random mating (*i.e.* the proportion of mate pairs sampled in each distance class).

parameters: cytoplasmic identity (CMS versus non-CMS hermaphrodites), inbreeding coefficient, individual size, geographical location (south versus north), as well as quantitative descriptors of the neighbourhood of each focal plant (number of flowering individuals, number of females, number of hermaphrodites and local sex ratio, i.e. female frequency around each focal plant). Because some of the factors listed above were dependent on each other (see below), we chose to test each factor separately in a first step. The results are summarized in Table 2.

Neither individual size nor inbreeding coefficient significantly affected the number of sired seedlings. However, we did record an effect of cytoplasmic identity, with restored hermaphrodites that appeared to sire a higher number of seedlings than non-CMS hermaphrodites. There was also a geographical effect, with hermaphrodites located in the southern cluster being associated with a higher number of seedlings than plants located in the northern cluster. We also found a significant effect of the neighbourhood of each potential father: the number of flowering plants, the number of

Table 2 Results of eight log linear models carried out on the number of assigned seedlings (following a Poisson distribution) for all potential fathers in the study population of sea beet (*Beta vulgaris* ssp. *maritima*)

Source of variation	df	χ^2	*P*	Effect
Individual characteristics				
Cytoplasmic identity	1	5.88	0.0153	CMS > Non-CMS
Geographical location	1	10.13	0.0015	South > North
Size of the individual	2	0.47	0.7907	—
Inbreeding coefficient	1	0.25	0.6198	—
Neighbourhood characteristics (within a radius of 15 m around each focal plant)				
Number of plants	1	17.32	$<10^{-4}$	Positive
Number of females	1	20.98	$<10^{-4}$	Positive
Number of hermaphrodites	1	2	0.1589	—
Female frequency	1	15.30	$<10^{-4}$	Positive

In each of the eight models, the effect of one factor was tested (either related to the individual characteristics or to the neighbourhood of each plant). These analyses were performed with 195 potential fathers, except for the effect of local female frequency, which could not be calculated for four potential fathers that had no other plants in their vicinity. North and south refer to the two clusters inferred with Bayesian clustering methods. CMS and non-CMS refer to sterilizing and non-sterilizing cytoplasms respectively.

females and the local female ratio within a radius of 15 m all had a strong positive effect on their estimated number of seedlings; only the number of hermaphrodites around plants had no significant effect (Table 2). One must notice that we obtained similar results with descriptors of local neighbourhood based on other values of radiuses around each hermaphrodite (data not shown).

In all, three major types of factors seemed to affect male fitness of the hermaphrodites: cytoplasmic identity, geographical location and local neighbourhood. These factors appeared to be strongly inter-dependent; plants in the southern cluster had more locally available mates (number of flowering plants, number and frequency of females; Table 3). More importantly, number and frequency of females in neighbourhoods was significantly higher for restored (CMS) hermaphrodites (20.30 ± 14.68 females, i.e. 62.47% ± 27.20% of females) than non-CMS hermaphrodites (3.02 ± 6.33 females, i.e. 8.59% ± 15.93% of females). Thus, restored hermaphrodites probably experienced less pollen competition than hermaphrodites carrying a non-sterilizing cytoplasm. For example, for each of the four mother plants situated in the extreme south, more than 50% of the assigned seedlings were sired by one of the three directly neighbouring restored hermaphrodites (Fig. 5). As a result of the reduced number of potential fathers in the areas were the sex ratio was female biased, the estimated paternity correlation (i.e. fraction of seedlings that share the same father) is significantly higher in the southern part of the population (0.161 ± 0.036), compared to what was observed in the northern cluster (0.084 ± 0.016).

When all factors were tested simultaneously on the number of seedlings per potential father, only cytoplasmic identity and the number of flowering plants in the neighbourhood remained significant, as well as the interaction between cytoplasmic identity and female frequency in the vicinity of each focal plant (Table 4). Interestingly, in this model, restored (CMS) hermaphrodites had a marginally lower male fitness than non-CMS ones. In other words, when correcting for the variability in the neighbourhood of each potential father (that favours restored hermaphrodites, cf. Table 3), restored (CMS) hermaphrodites apparently lose their reproductive advantage. This suggests that the better male fitness of restored hermaphrodites highlighted by the one factor model (cf. Table 2) is not due to their intrinsic capability of siring seeds, but rather only to their geographical location. Finally, the significant interaction between cytoplasmic identity and female frequency in the neighbourhood was due to a positive effect of female frequency for restored (CMS) hermaphrodites only. Indeed, while local

Table 3 Results of non-parametric tests (Wilcoxon Mann–Whitney) on four variables describing the neighbourhood of each potential father (N = 195) as a function of either cytoplasmic identity of the plant or its geographical location

Variable	Source of variation	*N*	*Z*	*P*	Effect
Number of plants	Cytoplasmic identity	195	2.17	0.03	CMS > Non-CMS
	Geographical location	195	4.09	$<10^{-4}$	South > North
Number of females	Cytoplasmic identity	195	3.82	$<10^{-4}$	CMS > Non–CMS
	Geographical location	195	7.06	$<10^{-4}$	South > North
Number of	Cytoplasmic identity	195	–2.41	0.02	Non-CMS > CMS
hermaphrodites	Geographical location	195	–1.46	0.14	—
Female frequency	Cytoplasmic identity	191	4.99	$<10^{-4}$	CMS > Non-CMS
	Geographical location	191	7.39	$<10^{-4}$	South > North

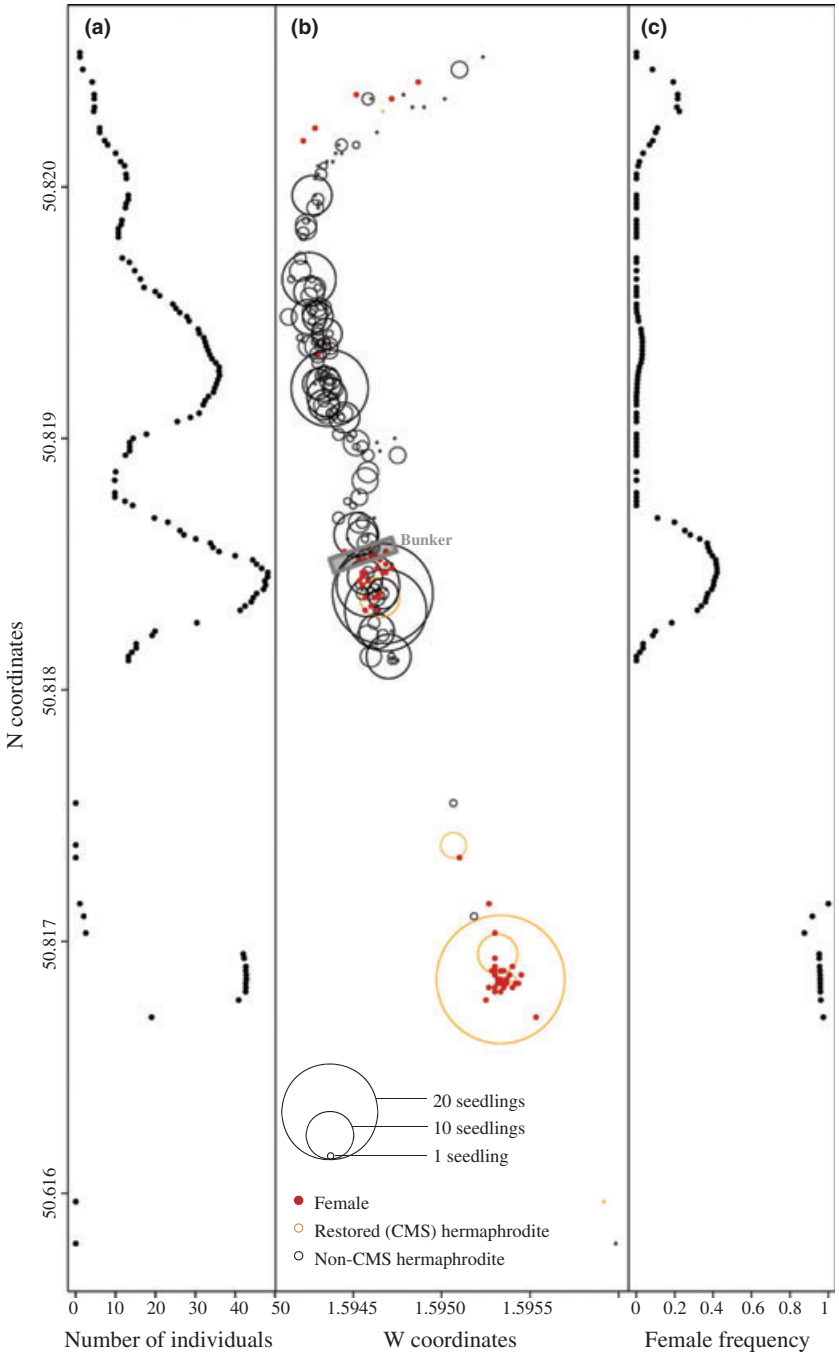

Fig. 5 Spatial variation in male reproductive success within the study site, for non-CMS hermaphrodites and restored hermaphrodites (b), along with spatial variation in the number of individuals calculated within a radius of 15 m (a) and spatial variation in the female frequency within the same radius (c). The bunker marks the boundary between the northern and the southern subpopulations defined by the Bayesian assignment test.

female frequency strongly varied across the population for restored hermaphrodites, with a particularly beneficial neighbourhood in the southern sub-population, local sex-ratio was virtually constant among non-CMS hermaphrodites (see Fig. 5).

Discussion

Many theoretical and empirical studies of gynodioecy have focused on the question of how a joint cytonuclear polymorphism can be maintained at the sex-determining loci. Theory has shown that the maintenance of sexual polymorphism is affected by (i) selection on both male and female fitness components (Gouyon *et al.* 1991; Bailey *et al.* 2003; Dufay *et al.* 2007), and (ii) population structure and gene flow (Frank 1989; Pannell 1997; Couvet *et al.* 1998; McCauley *et al.* 2000a). This study investigates both processes by characterizing fine-scale genetic structure and contemporary gene flow along with male reproductive success, to understand

Table 4 Results of the complete log linear model testing for all the main factors simultaneously, carried out on the number of assigned seedlings (following a Poisson distribution) for all potential fathers in the study population of sea beet (*Beta vulgaris* ssp. *maritima*)

Source of variation	*dF*	χ^2	*P*	Effect
Cytoplasmic identity	1	3.55	0.0597	CMS < Non-CMS
Geographical location	1	2.40	0.1217	—
Size of the individual	2	1.17	0.5572	—
Inbreeding coefficient	1	0.06	0.8012	—
Number of plants	1	8.52	0.0035	Positive
Female frequency	1	1.71	0.1911	—
Cytoplasmic identity * Female frequency	1	6.89	0.0087	Positive effect for CMS plants only

All the interactions between main factors were tested, and only Cytoplasmic identity * Female frequency had a significant effect. CMS and non-CMS refer to sterilizing and non-sterilizing cytoplasms respectively.

the evolutionary dynamics of gynodioecy in the sea beet.

The rise of population genetic structure and variation of local sex ratio

Local genetic structure in plant populations is shaped by a combination of factors involving gene flow, selection and genetic drift, which in turn are influenced by mating system, and ecological factors, such as density or dispersal capabilities of seed and pollen (Loveless & Hamrick 1984). In this study, we observed strong differences in the extent of spatial genetic structure between nuclear and cytoplasmic loci, and found that at a small spatial scale, genetic structure of maternally inherited cytoplasmic markers was stronger than at nuclear loci. This is likely due to a difference in the mode of inheritance: nuclear genes are dispersed via seed and pollen, whereas mitochondrial genes are only dispersed via seeds. This has been demonstrated in other studies, in which maternally inherited cpDNA or mtDNA were more structured than nuclear DNA, both at the local, within-population scale and at the larger metapopulation scale (McCauley 1998; Oddou-Muratorio *et al.* 2001; Olson & McCauley 2002; Fievet *et al.* 2007).

Using Bayesian clustering analyses based on nuclear multilocus genotypes, we observed a strong genetic partition in the study population between two distinct north and south clusters. The partitioning of the clusters corresponds with the location of a potential landscape barrier (a bunker), which may limit pollen and seed dispersal between the south and north. However, we also detected some first generation migration events involving 19 individuals located in the northern cluster that were assigned to the southern. This suggests that seeds may periodically travel several hundred metres, probably following water movements during high tide (e.g. Fievet *et al.* 2007). Similarly, paternity analyses (see below) indicated that a substantial proportion of pollen dispersal occurs between the two clusters. This striking genetic discontinuity of the studied population goes along with marked differences in CMS genes and sex ratios. Ninety per cent of the plants carrying male sterilizing cytoplasms were found in the southern part of the population. Consequently, sex ratios were also markedly different between the two sub populations (60% and 4% of females in the southern and the northern cluster, respectively). This structure in sex ratio is expected to persist into future generations because offspring sex-ratio from females is usually female biased whereas offspring from hermaphrodites is hermaphrodite biased. Local structure of cytoplasmic types and sexual phenotypes seems to be a general trend in many gynodioecious species (e.g. Manicacci *et al.* 1996; Laporte *et al.* 2001; Olson *et al.* 2006) and it has been shown to affect individual fitness, in terms of female fertility (McCauley *et al.* 1996; Graff 1999; Taylor *et al.* 1999), but also in terms of male fertility, as we show in this study. In the particular case of *Beta vulgaris*, two types of hermaphrodites are found in natural populations because of the co-occurrence of male fertile and male sterile cytoplasms. In this regard, we found a similar spatial trend in the geographical occurrence of restorer alleles with most of the restored hermaphrodites located in the southern cluster.

Interestingly, within-population structure appeared to be stronger in the southern cluster compared to the northern cluster, especially for cytoplasmic haplotypes. Variation in cytoplasmic diversity within and among populations can occur from either stochastic or selective factors influencing the spread of advantageous alleles (Olson & McCauley 2002). If seed dispersal between populations is a rare event, a newly founded population should have only a few cytoplasmic haplotypes. Hence, this stronger genetic structure could be the consequence of a recent founder event involving genetically related individuals that shared the same sterilizing cytoplasm (see Manicacci *et al.* 1996). This being the case, even pollen flow between subpopulations would not have had enough time to homogenize the family structure that arises after a founder event. This hypothesis is supported by significant genetic differentiation between the north and south clusters, both for nuclear and for cytoplasmic data. Additionally, the fact that the southern cluster was mainly composed of females, that are strictly seed dispersers, could reinforce this observed strong genetic structure. The observed bias in sex ratio in the southern cluster also restricts the number of

neighbouring potential fathers, which reinforces the correlated paternity within a progeny array. As a consequence, a larger part of the seedlings of each progeny are full sibs, which results in local accumulation of related individuals in family clusters (see Torimaru *et al.* 2007).

Pollen flow within and between subpopulations

Beyond historical estimates of gene flow, we used progeny arrays and paternity analysis to gain some insights on the pattern of real-time pollen flow. Pollen dispersal followed a leptokurtic distribution with most dispersal events occurring at short distances and a long tail of low level pollen dispersal over larger distances. Indeed, more than 40% of mating events occurred at spatial scales not exceeding 15 m, which was unexpected for a weedy anemophilous species. Beyond a simple spatial-dependent pattern of pollen dispersal, the density of conspecific individuals is a major determinant of pollination distance and effective number of pollen donors (e.g. Oddou-Muratorio *et al.* 2006; Fénart *et al.* 2007). In this study, we found that the pattern of local plant density strongly affected pollen dispersal distance. Pollen flow was limited to short distances for fathers situated within dense patches of individuals, whereas the distance between mates was significantly higher for spatially isolated fathers. The individuals located within dense patches of flowering beets compete in a pollen cloud saturated by the closest neighbouring conspecifics (see Fénart *et al.* 2007). Therefore, strong clustering of individuals should counteract the long distance pollination events expected in a wind-pollinated species, and may accentuate patterns of isolation-by-distance (e.g. García *et al.* 2005; Ishihama *et al.* 2006; but see Isagi *et al.* 2007; Byrne *et al.* 2007).

To understand the processes occurring at larger scale, pollen flow at longer distances must be taken into account. In our study, 29% of the pollination events occurred between the north and south clusters. This result may seem contradictory with the significant differentiation between the two sub-populations. However, this measurement of gene flow only represents real time events that do not necessarily result immediately in a genetic homogenization over the whole population. In other words, the strong genetic structure observed in our study site, probably resulting from distinct founder events, persists despite the currently high rates of pollen flow because the study species is perennial. Furthermore, 2.5% of the seedlings were apparently sired by fathers located in two neighbouring populations sampled outside the study site, suggesting the possibility of long distance pollen flow in sea beet (over several kilometres). Other studies based on paternity analysis have also reported long distance immigration events (e.g. Goto *et al.* 2006; Bittencourt & Sebbenn 2007; Bacles & Ennos 2008; Slavov *et al.* 2009). It should be noted, however, that the vast majority of studies reporting patterns of contemporary pollen dispersal focused on either entomophilous species or wind-pollinated trees. To the best of our knowledge this study is the first to investigate pollen flow in a weedy wind-pollinated species, meaning that any generalization or comparison is difficult to assess (but see Fénart *et al.* 2007 for a study of the same species in an agronomical context). These results are especially important because rare long distance pollen-mediated gene flow can have dramatic consequences by introducing locally novel alleles into a breeding neighbourhood (Frank & Barr 2001; Sork & Smouse 2006). In a general context, the introduction of alleles in a population has obvious implications in terms of genetic diversity. In the particular case of gynodioecy, long distance pollen dispersal is likely to affect the evolutionary dynamics of sex ratios by introducing restorers of male fertility.

Male reproductive success and evolutionary dynamics of gynodioecy

As already outlined, two types of hermaphrodites coexist in natural populations of gynodioecious sea beets: those carrying a sterilizing cytoplasm (restored hermaphrodites) and those with a non sterilizing cytoplasm. Another study conducted in tandem with ours, the same year, estimated pollen viability on the same individual plants that were used in the current study and showed that non-CMS hermaphrodites produced better pollen than restored hermaphrodites (Dufay *et al.* 2008). The paternity analysis aimed to investigate whether these phenotypic differences actually resulted in differences in male reproductive success. Quite surprisingly our results showed that the opposite may be the case: restored hermaphrodites (CMS hermaphrodites) appeared to sire more seedlings than normal hermaphrodites. This unexpected observation is a likely consequence of the spatial structure, since CMSs and sexes tended to be clustered and restored hermaphrodites experienced a stronger availability of females in their direct vicinity. This particular structure probably causes spatial variation in pollen competition and the restored hermaphrodites were the only available fathers in the areas where the sex ratio was female biased. As a consequence, as soon as it benefits of the advantage of being rare, even a bad pollen producer can efficiently transmit its genes. The interplay between female fitness and local sex ratio has been studied in several gynodioecious species, all of which found that individual fitness cannot be predicted without taking into account

the local sex ratio (Graff 1999; Taylor *et al.* 1999; Alonso 2005). The present study shows that the same is true for male fitness. In this particular case, clustering of females and restored hermaphrodites apparently counteracts the expected disadvantage of restored hermaphrodites in terms of mean number of pollination events. This particular relationship between local structure and fitness is similar to what is observed in self-incompatible species, where reproduction can be negatively affected by identity of neighbouring individuals, in cases of local low diversity of self-incompatibility alleles (Wagenius *et al.* 2007). Such idea could be generalized to any system in which individual fitness is likely to depend on the identity of the neighbouring individuals.

The frequencies of sexual phenotypes and the results of paternity analyses actually fit theoretical predictions of selection models: in the first phase, CMS genes increase in frequency by benefiting from a female advantage. After a few generations, the population is then mainly composed by females (Gouyon *et al.* 1991; Couvet *et al.* 1998; Dufay *et al.* 2007). At this stage, selection is expected to favour restorers of male fertility because of pollen limitation occurring in female biased populations (Gouyon *et al.* 1991; Bailey *et al.* 2003; Dufay *et al.* 2007). In other words, restorers are expected to increase in frequency because of their association with the rare gamete type, pollen. In our empirical study, pollen flow is mainly local, making this fitness advantage inherently dependent on the composition of the direct neighbourhood. Our results thus suggest that frequency-dependent selection occurs at a very restricted spatial scale (i.e. within a genetically distinct cluster).

What remains unclear is what occurs in the following stages of the evolutionary dynamics of gynodioecy. There are probably two advantages of being a hermaphrodite in an area where females are common, in terms of male reproductive success: (i) there are more potential mates per capita to receive pollen and (ii) female are expected to be better mates, due to the female reproductive advantage that is expected in gynodioecious species. Such female advantage has not been found yet in our study species, but it has been clearly demonstrated in many other gynodioecious species (e.g. Avila-Sakar & Domínguez 2000; Thompson & Tarayre 2000; Marshall & Ganders 2001; Lopez-Villavicencio *et al.* 2005). Nonetheless, in the first stages of the selection of restorers, female advantage may be lowered by pollen limitation. These considerations point out that population structure can affect the nuclear and cytoplasmic genomes in a different way, even in the same plant. In terms of more general evolutionary biology, strong spatial genetic structure is likely to amplify the expected effects of frequency dependent selection by clustering together similar genotypes.

Conclusions and perspectives

This study demonstrates that spatially-restricted gene dispersal can enhance fine-scale structuring of both genetic diversity and sexual phenotypes. Because this study was carried out on a population which was characterized by extremely contrasted local sex ratios and genotype frequencies, it clearly illustrates how fine scale population structure strongly affects the individual male fitness, and may even counteract the expected effects of natural selection. This result was quite unexpected over such a geographically restricted scale, especially in a wind-pollinated species. Empirical studies that look at the maintenance of gynodioecy, the evolution of sex-ratio, or more generally the dynamics of any polymorphic trait under frequency-dependent selection should probably not considerer gene or morph frequencies at the scale of the whole population, but rather investigate how these frequencies vary at a fine geographical scale. A better understanding of the interplay between neighbourhood characteristics and individual fitness within populations is indeed required to identify the relevant spatial scale at which frequency dependent selection is likely to drive polymorphism dynamics. This also underlies the need for spatially explicit models, which should integrate the effect of local genotype frequencies together with gene flow among demes on the evolution of polymorphism.

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This work was a part of I.D.C.'s Master thesis in population genetics and dynamics of gynodioecy in wild *Beta vulgaris* populations. M.D. works on the ecology and the evolution of plant reproductive systems using theoretical modelling and field-work in natural populations**.** J.C. and J.-F.A. are interested in the evolution of mating system in plants and in its consequences on patterns of spatial genetic structure. More information about the activities of the ''Laboratoire de Génétique et Evolution des Populations Végétales'' can be found on the following web site: http://gepv.univ-lille1.fr/
