doi: 10.1111/j.1420-9101.2007.01454.x

# Variation in pollen production and pollen viability in natural populations of gynodioecious *Beta vulgaris* ssp. *maritima*: evidence for a cost of restoration of male function?

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*Keywords:*

cost of restoration;
cytoplasmic male sterility;
gynodioecy;
partial male sterile;
pollen quantity;
pollen viability.

## Abstract

Gynodioecious species are defined by the co-occurrence of two clearly separated categories of plants: females and hermaphrodites. The hermaphroditic category may, however, not be homogeneous, as male fitness may vary among hermaphrodites as a result of many biological factors. In this study, we analysed estimates of pollen quantity and viability in the gynodioecious *Beta vulgaris* ssp. *maritima*, comparing hermaphrodites bearing a male-fertile cytotype and hermaphrodites bearing cytoplasmic male sterility (CMS) genes, which are counteracted by nuclear restoration factors. We show that: (i) pollen quantity continuously varies among restored hermaphrodites, suggesting a complex genetic determination of nuclear restoration; (ii) pollen viability was lower in restored (CMS) hermaphrodites than in non-CMS hermaphrodites, probably because of incomplete restoration in some of these plants; and (iii) pollen quantity and viability also varied among hermaphrodites with male-fertile cytotypes, possibly a result of a silent cost of restoration. Finally, we discuss the consequences of these results for pollen flow and the dynamics of gynodioecy.

## Introduction

Gynodioecy is a plant breeding system where females and hermaphrodites coexist in populations. This breeding system is the second most common in the European Flora (Richards, 1997). For decades, this system has been a topic of interest for evolutionary biologists for several reasons. First, it can be considered as a possible evolutionary step from hermaphroditism to dioecy and may then constitute a key system to understand the evolution of plant reproductive systems (Charlesworth & Charlesworth, 1978; Desfeux *et al.*, 1996; Barrett, 2002). Second, sex expression in gynodioecious species often results from complex genetic interactions between cytoplasmic male sterility (CMS) genes and nuclear restorer genes (Chase, 2007); this provides the basis for a genetic conflict between cytoplasmic and nuclear genes due to the effect of opposite selective pressures on the sexual phenotype of plants (Cosmides & Tooby, 1981; Saumitou-Laprade *et al.*, 1994; Hurst *et al.*, 1996; Werren & Beukeboom, 1998; Jacobs & Wade, 2003; Delph *et al.*, 2007).

According to evolutionary theory, CMS can spread in a population as soon as fitness through female function is higher in females than in hermaphrodites (female advantage; Charlesworth & Ganders, 1979; Frank, 1989; Gouyon *et al.*, 1991). As cytoplasmic genes are only transmitted through seeds, the loss of the male function (i.e. pollen production) in females is not associated with a cost for CMS genes. Indeed, either only a moderately larger number of seeds or a slightly better quality of seeds is likely to provide CMS with a selective advantage. On the other hand, the loss of pollen production entails a loss of transmission for nuclear genes. As soon as CMS becomes frequent in a population, nuclear alleles that are able to restore pollen production may be selected. Two main factors have been proposed to explain why restorer genes are not driven to fixation in gynodioecious species: (i) gene flow and metapopulation processes that regularly introduce new CMSs in populations (Frank, 1989; Couvet

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*et al.*, 1998); or (ii) a cost induced by restorer alleles: when the corresponding CMS becomes less frequent, restoring male fertility provides no or a small selective advantage, so that the cost causes a decrease in restorer frequency (Charlesworth & Ganders, 1979; Frank, 1989; Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufaÿ *et al.*, 2007). In the latter case, frequency-dependent selection can lead either to limit-cycle dynamics (i.e. undamped oscillations) or to stable equilibrium, that can be reached after a prolonged period of dampened oscillations (Dufaÿ *et al.*, 2007). Thus, both these dynamics may explain the high variations in female frequencies that are sometimes observed among populations in gynodioecious species (Frank, 1989; Gouyon *et al.*, 1991).

Whereas several empirical studies have found a female fitness advantage in gynodioecious species (Alonso & Herrera, 2001; Shykoff *et al.*, 2003 for reviews), little is known about restorer alleles. In particular, although the cost of restoration is a central theme in theoretical studies, only two studies have empirically suggested that restorer genes could negatively affect plant fitness, either through female (de Haan *et al.*, 1997a) or male function (Bailey, 2002). Moreover, although models often assume that restoration of male fertility is achieved through one allele (but see Frank, 1989), empirical work suggests that genetic determination of restoration may be more complex (Charlesworth & Laporte, 1998; Koelewijn, 2003; Touzet *et al.*, 2004). Indeed, a recent study, based on three of the best studied gynodioecious species, pointed out that restoration may be often polygenic (Ehlers *et al.*, 2005). If more than one gene of restoration is involved, male fitness is then expected to quantitatively vary among plants, which could slow down the selection of restorers and thus strongly influence the dynamics of gynodioecy (Charlesworth & Laporte, 1998). To our knowledge, however, few studies have quantified variation in pollen production and quality among gynodioecious populations (but see Glaettli & Goudet, 2006).

In some cases, variation in male fitness among plants is further increased by the presence of cytoplasmic polymorphism in populations. Indeed, although only the most well-known gynodioecious species contain CMS cytoplasms (*Silene vulgaris*: Olson & McCauley, 2002; *Silene acaulis*: Städler & Delph, 2002; *Thymus vulgaris*: Belhassen *et al.*, 1991), this does not seem to be the rule for all species. In particular, non-CMS cytotypes have been reported in at least two gynodioecious species (*Plantago lanceolata*: de Haan *et al.*, 1997b; *Beta vulgaris* ssp. *maritima*: Cuguen *et al.*, 1994). It should be noted that male-fertile cytoplasm in populations includes 'normal' cytoplasm that never produced any sterilizing factors or sterilizing cytoplasm for which the nuclear restorer genes are currently fixed. Because of the lack of CMS markers and data from reciprocal crosses (e.g. Van Damme *et al.*, 2004), the real occurrence of male-fertile cytotypes is usually unknown. Thus, for many gynodioecious species, we do not know whether all hermaphrodites are in fact restored hermaphrodites (CMS) or whether both restored CMS and non-CMS hermaphrodites co-occur in populations. In the latter case, these hermaphrodites may also differ in their pollen production and male fitness abilities.

The cost of restoration, complexity of genetic determination of restoration and co-occurrence of hermaphrodites having different cytoplasms are all possible sources of variation in male fitness and may strongly affect pollen flow, selection of restorer genes and ultimately, the dynamics of gynodioecy. The aim of this study was to assess variation in hermaphrodite potential male fitness in terms of pollen quantity and quality estimated in two natural populations of the gynodioecious wild beet, *B. vulgaris* ssp. *maritima*, including normal hermaphrodites and hermaphrodites bearing one of the two most frequent CMS cytotypes found in wild beet populations (CMS *E*, see Fénart *et al.*, 2006). First, we will examine how pollen production varies among CMS plants, which will give us some clues about the genetic determination of restoration. Second, we will compare pollen quantity and quality in non-CMS and CMS hermaphrodites and discuss the possible consequences on selection of restorer alleles. Third, because frequency-dependent selection is supposed to play an important role in the maintenance of the system, we will compare pollen quantity and quality between two populations that are at two difference stages of gynodioecy dynamics, in terms of CMS and restorer frequencies.

## Material and methods

### The species

*Beta vulgaris* ssp. *maritima* is a gynodioecious short-lived perennial species, which is widely distributed along the western coasts of Europe and around the Mediterranean Basin. In northern Europe, wild beets mainly colonize areas located along estuaries, just at the upper level of the tide and, more rarely, cliffs overhanging the sea (Letschert, 1993; Laporte *et al.*, 2001; Fievet *et al.*, 2007). When plants bolt to flowering, stems develop with a main axis bearing flowering branches and sometimes secondary flowering axes. Consequently, each plant can produce up to several thousands of flowers. Plants are self-incompatible and pollinated by wind. In wild beet populations, hermaphrodites often coexist with male-sterile plants that do not produce pollen. Among the 20 mitochondrial haplotypes described in beet, four haplotypes are clearly associated with male sterility: *E*, *G*, *H* and *Svulg* (Saumitou-Laprade *et al.*, 1993; Cuguen *et al.*, 1994; Laporte *et al.*, 1998; Desplanque *et al.*, 2000; Touzet *et al.*, 2004). In contrast with other gynodioecious species, nonsterilizing cytotypes constitute a large part of the mitochondrial diversity in wild beet (Cuguen *et al.*, 1994; Forcioli *et al.*, 1998; Laporte *et al.*, 2001). Moreover, the most frequent cytoplasm is male fertile and because the four

different CMS are derived from it, this male-fertile cytotype is likely to be ancestral (Fénart *et al.*, 2006).

### Study populations and sampling procedures

This work was carried out in two natural gynodioecious populations of wild beet, located on the northern coast of France, 30 km apart. Both populations were linear and comprised approximately 400 plants. One population (Audresselles, 50°49.101′N; 1°35.676′E) was located on a seaside resort; the other population (Canche, 50°32.231′N; 1°35.597′E) was located along the estuary of the river Canche. In April 2005, a total of 398 plants were continuously sampled along the populations: 280 plants were collected in Audresselles and 118 plants were collected in Canche. Sampling consisted in leaf collection and plant labelling and was not affected by plant sex as it took place before flowering. During flowering in June 2005, we recorded the sex ratio by assigning a sexual phenotype to each labelled flowering plant. Plants with brown or white and much reduced anthers were considered to be male sterile. Plants with yellow anthers that produced pollen were considered to be hermaphrodites. Some of the plants had an intermediate phenotype that produced more light-coloured yellow anthers with no obvious pollen grains and, as such, these plants were categorized as intermediate phenotypes. In total, 273 and 96 plants were assigned to a sexual phenotype in Audresselles and Canche respectively; not all labelled plants flowered and some plant could not be retrieved during the second survey in June.

### Molecular analyses

In wild beet, among the four CMSs that have been described, the three most widely distributed are the Owen CMS (also called *Svulg*), which is widely used in plant breeding of sugar beet (Owen, 1945; Arnaud *et al.*, 2003), and two additional ones called *E*, and *G*, which are exclusively found in wild beet populations (Cuguen *et al.*, 1994; Desplanque *et al.*, 2000; Laporte *et al.*, 2001). Extraction and purification of total DNA from the 398 sampled plants were performed using a NucleoSpin®96 Plant Kit, and following the standard protocol for isolation of DNA from dried plant leaf tissue outlined in the NucleoSpin®96 Plant Kit protocol handbook (Macherey-Nagel, Düren, Germany). To characterize the three main CMSs, we used diagnostic cytoplasmic PCR markers. The PCR step for each CMS was conducted using standard procedures. The primers used to amplify the chloroplastic marker associated with CMS *Svulg* are described in Ran & Michaelis (1995). To detect CMS *G*, one unpublished pair of primers was used to amplify a specific fragment (forward primer: TTTCTCTTTATGGATAACCAATTCA – reverse primer: AGGATTCCTTTTGTAACCCAAT). CMS *E* was detected by PCR-RFLP (forward primer: GTTCCC ACTCACGACCCATA – Reverse primer: CCGACTAGTT CCGGGTTC) using the following procedure: 5 μL of the PCR product was digested in a 10-μL reaction with 0.2 mM of spermidin and 1.5 U of *Alu*I, conducted at 37 °C overnight.

### Pollen sample and analysis

Pollen was sampled on plants at the beginning of the flowering period during June 2005. In both populations, we selected genotyped plants, such that both CMS and non-CMS plants were sampled. We focused on CMS type *E*, which was the most frequent CMS cytotype in Audresselles and the only CMS cytotype in Canche (see results below). Moreover, we selected plants that were at the beginning of their flowering phase to obtain results for pollen production that could be compared among plants. In total, pollen was sampled on 115 plants: 43 non-CMS and 23 CMS plants in Audresselles, and 13 non-CMS and 36 CMS plants in Canche. For each plant, two floral buds localized on two different stems of equal size were chosen and two anthers per bud were collected and stored separately in ethanol at 95 °C. Ethanol was then evaporated and samples were placed in oven at 56 °C for 24–48 h to force anther dehiscence. One millilitre of distilled water was then added to each pollen sample and sonicated to separate pollen grains from the anther and each other. Tubes were then vortexed and the number of pollen grains was estimated in 200 μL of solution (this number multiplied by five gives the total pollen count). A particle counter CASY® model TT (Innovatis, Bielefeld, Germany) was used to estimate the number of pollen grains in a solution of 5 mL of pure water CASY® ton for cell counter, in which the 200 μL of distilled water and pollen were diluted. Each sample was shaken to equally distribute pollen in the solution immediately prior to counting. The particle counter then sampled three volumes of 400 μL from the solution and provided the result for the total 1200 μL analysed. The number of detected particles was determined for 400 size classes ranging from 0.125 to 50 μm using the software CASY® excel 2.1. Prior observations had shown that nonviable pollen grains in *B. vulgaris* ssp. *maritima* were of smaller size than viable pollen grains (Boutin *et al.*, 1987). These counts were then used to estimate both total pollen production and fraction of viable pollen grains. Every 20 tubes a blank solution was analysed to estimate the size classes corresponding to pollen grains, by comparing pollen samples with empty solutions.

### Pollen viability

Pollen viability was also estimated with Alexander stain on a subsample of plants to assess the correlation between pollen grain size and viability. To cover the largest variance in pollen viability, we sampled both non-CMS hermaphrodites and plants bearing CMS *E* cytotype, including (restored) hermaphrodites and

intermediate phenotypes. On these plants, an additional freshly opened flower was collected the same day than the floral buds used for particle counter analysis. Within 3 h of collection, pollen was removed from the flower and placed on a glass slide. One drop of Alexander solution (10 mL of 95% ethanol, 1 mL of 1% malachite green in 95% ethanol, 5 g of phenol, 5 mL of 1% acid fuschin in $H_2O$, 0.5 mL of 1% orange G in $H_2O$, 2 mL of glacial acetic acid, 25 mL of glycerol and 50 mL of $H_2O$; Alexander, 1969), which stains viable pollen purple, was added to each pollen sample. A coverslip was used to mix and cover the pollen and Alexander mixture, after which the coverslip was sealed using clear nail varnish. The pollen samples were then examined under a light microscope at ×100 magnification. More than 200 pollen grains per sample, when available, were scored as either purple or green, and the viable proportion of pollen grains was calculated as the ratio of purple-stained pollen grains to the total number of pollen grains. We estimated pollen viability for, respectively, eight and 17 plants in Audresselles and Canche, among which 12 carried a CMS *E* cytotype. It should be noted that Alexander stain is only an estimation and not an absolute measure of viability. Keeping this in mind, we will consider thereafter that it will be a convenient estimate of viability.

### Data analyses

Typical peaks of particles with size ranging from 10 to 24 $\mu$m were observed on samples collected from hermaphrodites, which clearly did not appear on blank samples; thus, we only considered this 10–24 $\mu$m zone for pollen counting. Two subpeaks were recognizable within this size range: 10–13 and 16–24 $\mu$m, with some individuals specifically producing one of the two peaks and some others producing both (Fig. 1). We categorized particles within these two size classes, and considered three variables for subsequent analyses: total pollen quantity, quantity of large pollen grains (16–24 $\mu$m) and ratio of large pollen grains. For each of these three variables, the average value over the four collected anthers per plant was calculated and assigned to each studied plant. The total number of pollen grains and the number of large pollen grains were obtained from the values provided by the particle counter after correcting for the dilution ratio, i.e. by multiplying all values by $5 \times 5200/1200$. The factor 5200/1200 allows to estimate the quantity of pollen grains in the 5-mL solution in which the particle counter sampled, and the five factor allows to estimate the quantity of pollen in the whole anther, as only 200 $\mu$L over a total of 1 mL was used.

Because the quantity of pollen grains appeared to continuously vary among CMS plants (see below), we used the total number of pollen grains (small and large) produced per anther to objectively distinguish between the females and restored hermaphrodites: individuals that produced a small number of pollen grains were considered as females (see below). On this basis, the effects of sexual phenotype (three levels: females (CMS), restored hermaphrodites (CMS) and non-CMS hermaphrodites) and of population (two levels) were tested on

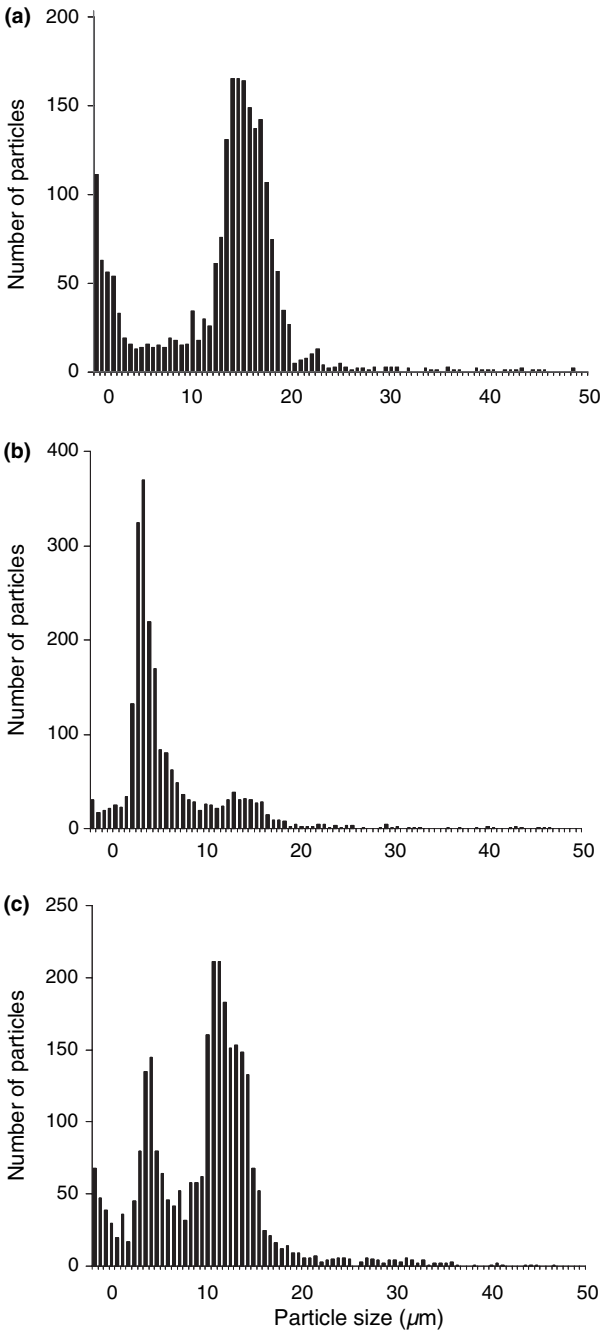


**Fig. 1** Examples of particle counting: number of particles as a function of particle size ($\mu$m). These depict the pollen zone (10–24 $\mu$m) for three different hermaphroditic plants. Plant (a) had one major peak of large pollen grains (16–24 $\mu$m), plant (b) produced mainly small pollen grains (10–13 $\mu$m) and plant (c) produced both.

the frequency and the number of large pollen grains, using an ANOVA (proc GLM, SAS, SAS Institute Inc., Cary, NC, USA). The effects of population and sexual phenotype were also tested on the total number of pollen grains per anther; however, because the number of pollen grains was used to distinguish between females and restored hermaphrodites, females were not included in the data set and this variable was compared only between restored hermaphrodites and non-CMS hermaphrodites. Data were either log transformed (number of pollen grains and number of large pollen grains) or arcsine-square root transformed (frequency of large pollen grains) to obtain normally distributed residuals.

## Results

### CMS cytoplasm frequency, sex ratio and restoration rate in populations

Three PCR markers specific to CMSs enabled us to identify all females. Number of CMS plants, sex ratios and restoration rate (i.e. for a given CMS, the ratio of the number of restored hermaphrodites to the total number of individuals bearing the CMS) in each population are given in Table 1. Whilst sex ratios were similar in both populations (29% females in Audresselles and 31% in Canche), CMS frequencies and restoration rates differed among the study populations. Three different CMSs (*E*, *G* and *Svulg*) were found in Audresselles, with CMS *E* being the most frequent (30% of sampled plants vs. 2% and 0.7% for CMSs *G* and *Svulg* respectively). The rates of restoration for the different CMSs were 0.12, 0 and 1, for CMSs E, G and *Svulg* respectively. These different rates of restoration among the three CMSs are consistent with another study conducted on a larger geographical scale (P. Touzet, unpublished data). In contrast to the Audresselles population, only the CMS *E* was found in Canche. This CMS occurred at a higher proportion than in Audresselles: 79.6% of the 118 sampled plants had the CMS *E*. In this population, the rate of restoration was much higher than in Audresselles: 60% or 42%, depending on whether intermediate phenotypes were included in the estimation or not.

### Pollen size vs. pollen viability

The frequency of viable pollen, estimated as the frequency of purple grains with Alexander stain, ranged from 0.09 to 0.99 among the 25 studied plants. For each of these plants, the value of pollen viability was analysed together with the average frequency of large pollen grains obtained with the particle counter: the Pearson's correlation coefficient between the two variables was 0.9 (Fig. 2). In agreement with the results obtained by Kelly *et al.* (2002) on *Mimulus guttatus* and *Collinsia verna*, estimating pollen size is robust method to estimate pollen viability in *B. vulgaris* spp. *maritima*. We will thus use the frequency of large pollen grains as a measure of pollen viability.

### Pollen production in CMS *E* plants

As CMS *E* was the only CMS found in Canche, we focused on this cytotype. The average quantity of pollen per anther (including both large and small grains) was highly variable among CMS *E* plants, ranging from 781 to

**Table 1** Number of plants described as females, intermediates and hermaphrodites for each cytotype (CMSs *E*, *G* and *Svulg* and male fertile cytotype *Nvulg*) in both populations.

| Cytotype | Females | Intermediates | Hermaphrodites | Unknown | Total | Restoration rate |
|---|---|---|---|---|---|---|
| Audresselles | | | | | | |
| CMS *E* | 73 | 0 | 10 | 1 | 84 | 0.12 |
| CMS *G* | 6 | 0 | 0 | 0 | 6 | 0 |
| CMS *Svulg* | 0 | 0 | 2 | 0 | 2 | 1 |
| Non-CMS | 0 | 0 | 182 | 6 | 188 | – |
| Total | 79 | 0 | 194 | 7 | 280 | – |
| Sex ratio (%) | 29 | 0 | 71 | – | – | – |
| Canche | | | | | | |
| CMS *E* | 30 | 24 | 22 | 18 | 94 | 0.6 (0.42) |
| CMS *G* | 0 | 0 | 0 | 0 | 0 | – |
| CMS *Svulg* | 0 | 0 | 0 | 0 | 0 | – |
| Non-CMS | 0 | 0 | 20 | 4 | 24 | – |
| Total | 30 | 24 | 42 | 22 | 118 | – |
| Sex ratio (%) | 31 | 25 | 44 | – | – | – |

Cases for which sex was unknown were plants that had been sampled before flowering, and which either were not flowering or were misled in June. The rate of restoration for a given CMS is the frequency of restored hermaphrodites divided by the total number of individuals, for which a sexual phenotype had been assigned and carrying the CMS under consideration. In the Canche population, two rates of restoration were calculated, taking into account either only restored hermaphrodites or both restored hermaphrodites and intermediates (within brackets).

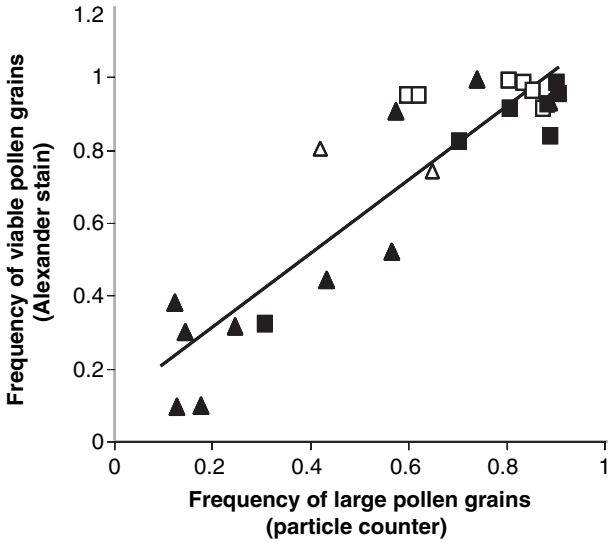


**Fig. 2** Correlation between pollen size and pollen viability in *Beta vulgaris*, measured on 25 plants from two wild populations of Canche (black symbols) and Audresselles (open symbols), comprising both CMS (triangles) and male-fertile (squares) hermaphrodites.

45 520 pollen grains per anther (Fig. 3). Plants that were categorized as females during flowering (i.e. having brown or white and reduced anthers) produced less than 7000 pollen grains. All plants categorized as hermaphrodites (i.e. having yellow anthers and obvious pollen production), thus being restored hermaphrodites, produced more than 13 000 pollen grains. Finally, plants categorized as intermediates in the field, i.e. with more light-coloured yellow anthers and no obvious pollen grains (labelled with an asterisk in Fig. 3) were also intermediates in terms of pollen quantity, and slightly overlapped with females. This suggests that the quantity of pollen varies continuously among CMS *E* plants.

For following analyses, we needed to distinguish among CMS plants, females from plants that express one of several nuclear restoration alleles. CMS plants were thus categorized in two groups, based on their average pollen quantity per anther. The first group (within boxes in Fig. 3) included all plants that had been categorized as females and two plants that had been categorized as intermediates but that produced average quantities of pollen comparable with females. Thereafter, we will consider these plants as females, with little or no pollen production. The second group (outside boxes in Fig. 3) consisted of plants that had been considered as potential pollen producers (categorized in the field as intermediates or hermaphrodites). For the rest of the study, we will consider them as CMS plants bearing one or several restoration alleles of male fertility and we will refer to them as restored hermaphrodites.

The proportion of viable pollen was also highly variable among CMS *E* plants, ranging from 12% to 90%, but was not correlated with the number of pollen

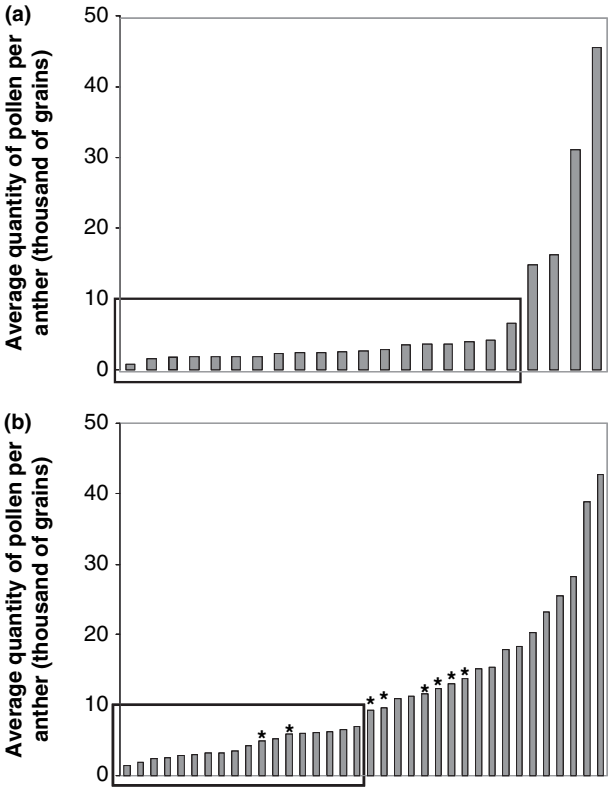


**Fig. 3** Plants carrying the CMS *E* cytotype, from Audresselles (a) and Canche (b) populations, ranked for their quantity of pollen per anther (average value over the four measured anthers). Symbols (*) indicate the plants that had been categorized as intermediates in the field, with lightly coloured yellow anthers and no obvious pollen production. The boxes contain all plants that will be considered as females in further analyses.

grains, irrespective of whether females were included in the data set or not [effect of the ratio of viable pollen on the total number of pollen grains: $P > 0.05$, Proc GLM; Pearson's coefficients $r = 0.13$ ($n = 59$) and $-0.31$ ($n = 22$) for analyses on data set with and without females respectively].

## Variation of pollen quantity and viability among sexual phenotypes and populations

The total number of pollen grains (viable and nonviable pollen grains) did not significantly differ between CMS-restored hermaphrodites and non-CMS hermaphrodites ($F_{1,77} = 0.03$; $P = 0.85$); only the population had an effect, individuals from Audresselles producing significantly more pollen grains than individuals from Canche ($F_{1,77} = 14.5$; $P = 0.003$). The ratio of viable pollen grains differed significantly among populations and sexual phenotypes, with a marginally significant effect of their interaction (Table 2). Overall, plants from Audresselles produced a higher proportion of viable pollen than plants

**Table 2** ANOVA of size of pollen grains, as a measure of pollen viability in *Beta vulgaris* on a subsample of plants of Audresselles and Canche populations ($n$ = 115).

| Source | d.f. | Type III SS | $F$ | $P$ |
|---|---|---|---|---|
| Population | 1 | 0.86 | 17.53 | <0.0001 |
| Sexual phenotype | 2 | 2.10 | 21.38 | <0.0001 |
| Population × sexual phenotype | 2 | 0.26 | 2.61 | 0.08 |
| Error | 109 | 5.36 | | |

The frequency of viable (large) pollen grains per anther (arcsine-square root transformed) depended on the sexual phenotypes (females, restored hermaphrodites and true hermaphrodite individuals), the population and their interaction.

from Canche. Concerning the effect of sexual phenotype, non-CMS hermaphrodites produced a significantly (Tukey test, $P < 0.0005$) higher proportion of viable pollen grains (0.7) than females and restored hermaphrodites (respectively 0.41 and 0.47) with no significant difference between females and restored hermaphrodites (Tukey pairwise comparison, $P > 0.1$). The same sex effect holds when individuals classified as intermediates in the field were removed from the data set: restored hermaphrodites produced a lower proportion of viable pollen grains than non-CMS hermaphrodites (sex effect: $F_{1,107} = 20.30$; $P < 0.0001$). Regarding the marginally significant interaction between both factors, the pairwise comparisons indicated that non-CMS hermaphrodites from Audresselles produced a significantly higher proportion of viable pollen than non-CMS hermaphrodites from Canche (Tukey pairwise comparison, $P < 0.05$). By contrast, restored CMS hermaphrodites of the two populations were not significantly different from each other ($P > 0.1$).

When combining both variables (i.e. number of pollen grains and frequency of viable pollen grains), an estimation of the number of viable pollen grains could be obtained. Using this response variable, a significant effect was found for population, sexual phenotype and their interaction (Table 3). Overall, plants from Audresselles produced a significantly higher quantity of viable pollen grains than plants from Canche. Non-CMS hermaphrodites, restored hermaphrodites and females produced on average, respectively, 25 081 ± 2078 ($n$ = 56), 9069 ± 2358 ($n$ = 22) and 1305 ± 82 ($n$ = 37) viable pollen grains per anther, with all values being significantly different from each other (Tukey pairwise comparisons, $P < 0.05$). The significant effect of the interaction between population and sexual phenotype was due to a difference between non-CMS hermaphrodites of Canche and Audresselles ($P < 0.05$), whereas the restored hermaphrodites of the two populations belonged to the same statistical category.

**Table 3** ANOVA of the number of viable (large) pollen grains per anther (log transformed) in *Beta vulgaris*, on a subsample of plants of Audresselles and Canche populations ($n$ = 115).

| Source | d.f. | Type III SS | $F$ | $P$ |
|---|---|---|---|---|
| Population | 1 | 6.59 | 12.69 | 0.0005 |
| Sexual phenotype | 2 | 118.70 | 114.31 | <0.0001 |
| Population × sexual phenotype | 2 | 10.57 | 10.18 | <0.0001 |
| Error | 109 | 56.59 | | |

The number of viable pollen grains depended on the sexual phenotype (females, restored hermaphrodites and true hermaphrodites individuals), the population and their interaction.

### Focus on non-CMS hermaphrodites

The same statistical analyses were conducted using the data set containing only non-CMS hermaphrodites. For all variables, a population effect was found (quantity of pollen grains: $F_{1,55} = 12.34$, $P = 0.0009$; frequency of viable pollen grains: $F_{1,55} = 10.66$, $P = 0.0019$; quantity of viable pollen grains: $F_{1,55} = 17.96$, $P < 0.0001$), non-CMS hermaphrodites from Audresselles producing higher quantity and quality pollen than non-CMS hermaphrodites from Canche. Regarding the ratio of viable pollen grains, it should be noted that population differences were not due to an overall lower pollen viability of all non-CMS hermaphrodites from Canche, but rather to a high proportion of non-CMS hermaphrodites from Canche that produced extremely low frequencies of viable pollen grains, whereas some others produced viable pollen in frequencies comparable with non-CMS hermaphrodites from Audresselles: 74% of non-CMS hermaphrodites from Audresselles showed a ratio of pollen viability higher than 0.8, against only 38% of non-CMS hermaphrodites from Canche (Fig. 4).

## Discussion

### Variation of pollen among CMS plants: some clues about the genetics of restoration?

The study on pollen quantity revealed a continuous variation of this trait among CMS plants, partly due to the occurrence of intermediate phenotypes (as already noticed by Boutin *et al.*, 1987). These intermediate phenotypes, visually recognizable with anthers being lighter coloured and with no obvious pollen production are also called partial male steriles and have been reported in *B. vulgaris* ssp. *maritima* in other CMS systems (Owen, 1942, 1945; Hjerdin-Panagopoulos *et al.*, 2002; Touzet *et al.*, 2004), as well as in other gynodioecious species (Van Damme & Van Delden, 1982; Koelewijn & Van Damme, 1996; Charlesworth & Laporte, 1998; Glaettli & Goudet, 2006). The presence of intermediate phenotypes usually suggests that at least two different loci or co-dominant alleles at the same locus are involved in the process of restoration.

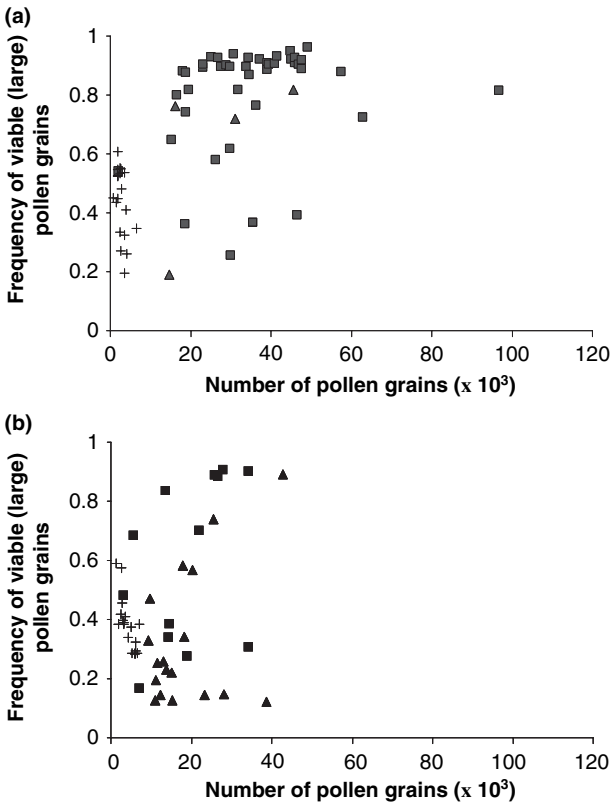


**Fig. 4** Pollen viability and quantity in all plants in Audresselles (a) and Canche (b). Crosses picture females, triangles picture restored hermaphrodites and squares picture male fertile (non-CMS) hermaphrodites.

Moreover, in our study, the quantity of pollen per anther was highly variable, even among fully restored hermaphrodites (excluding intermediate phenotypes) (Fig. 3). This is probably at least partly due to environment: even though pollen production was measured for all plants at the beginning of flowering and in the same geographical region, one cannot exclude micro-environmental variation among plants. Future studies conducted in controlled conditions will help to understand whether the variation in pollen quantity among restored hermaphrodites is only due to environmental variations or whether polygenic determination of restoration can also be invoked in *B. vulgaris* ssp. *maritima*, as recently hypothesized for several gynodioecious species (Ehlers *et al.*, 2005).

We estimated variation in pollen viability among plants, using pollen size, a good estimator of pollen grains viability. Some plants had high pollen production but suffered from very low quality. As a matter of fact, pollen viability did not correlate with pollen quantity in restored CMS hermaphrodites, suggesting that another factor, independent of the process controlling pollen quantity, may act on viability. Viability of pollen also exhibited large variation among restored CMS hermaphrodites, but was not continuously distributed (Fig. 4). This suggests that pollen viability may have a simpler mechanism of genetic determination than the number of pollen grains or may be less sensitive to environmental variation. Interestingly, the same phenomenon was observed in an experiment in controlled conditions on a maternal progeny segregating for male fertility on another CMS (M. Dufaÿ, unpublished results).

## Are restored hermaphrodites good pollen producers?

In most studied gynodioecious species, only two types of plants are considered: females and restored hermaphrodites, both bearing CMS genes. In such case, dynamics and maintenance of gynodioecy rely on differences in fitness between these two types of plants. In the case of *B. vulgaris* ssp. *maritima*, an additional question must be raised: are restored CMS hermaphrodites as fit as non-CMS ones? In other words, does the presence of male sterility genes, although counteracted by restorers, affect the performance of these plants, compared with genotypes that do not bear any active male sterility gene? Restorers have been shown in several crop species to modify the transcripts of the CMS genes (Hanson & Bentolila, 2004; Chase, 2007). However, if restorers cannot efficiently modify the transcripts or if this modification drives some negative effects on the expression of other genes, one might expect a lower fitness for restored hermaphrodites (discussed in Delph *et al.*, 2007).

In this study, we found that restored CMS hermaphrodites did not produce lower quantity of pollen but did produce pollen of lower viability than non-CMS hermaphrodites. This lower quality of pollen may be partly due to an incomplete restoration of male fertility: indeed, a group of restored CMS hermaphrodites, possibly lacking one allele of restoration, exhibited very low levels of pollen viability and consequently strongly decreased the average quality of pollen in restored hermaphrodites (Fig. 4). Some other restored hermaphrodites were demonstrated to reach very high frequencies of viable pollen, comparable with the non-CMS hermaphrodites that produced the highest proportion of viable pollen (Fig. 4). This suggests that a complete restoration of CMS is possible to achieve.

Because pollen viability and quantity were not correlated, the difference between non-CMS and restored CMS hermaphrodites in pollen viability remained even when excluding intermediate phenotypes from the analyses. Consequently, even CMS plants that appeared to be well restored for their quantity of pollen can have low male performance. We also compared the number of viable pollen grains between non-CMS and CMS hermaphrodites, using the combined measures of pollen quantity and viability and thus providing an estimate of potential male fitness. Restored CMS hermaphrodites that produced a very high number of pollen grains but of

low viability had consequently a lower potential male fitness than non-CMS hermaphrodites.

### Consequences for selection of restorer alleles

Our results suggest that some of the restored CMS hermaphrodites potentially contribute poorly to pollination in natural populations. Because restoration alleles can at least be transmitted through seeds, incompletely restored hermaphrodites may constitute a reservoir of restorers in the population until the appearance of fully restored hermaphrodites, which would efficiently produce pollen and change the population sex ratio. Such complex determination may then slow down the positive selection of restorer factors and help to maintain females in populations, as recently showed by Bailey & Delph (2007). Moreover, restorer alleles can also be eliminated by drift before the apparition of an efficient combination of restorer factors in the population (Charlesworth & Laporte, 1998). This would consequently increase the frequency of females, especially in isolated and/or small populations. A complex determination of the restoration of male fertility may thus constitute an alternative explanation to the correlation between female frequencies and population size that have been observed in some gynodioecious species (e.g. Caruso & Case, 2007).

### Differences among populations: the result of a silent cost of restoration?

The observed differences in pollen viability between populations could be due to environmental variation between these populations. However, an effect of the interaction between population and sexual phenotype suggested that the various sexual phenotypes were not equally affected by this population effect. When restored hermaphrodites were compared between populations, no statistical difference was found. By contrast, a significant difference was shown among non-CMS hermaphrodites, plants from Canche having lower pollen viability than plants from Audresselles. Besides the global population effect, non-CMS hermaphrodites from Canche seem to have a lower potential male fitness than those from Audresselles.

Interestingly enough, these two populations had strongly contrasting ratios of CMS and of restoration of male fertility. The Audresselles population exhibited low frequencies of both CMS and restorers, whereas we found high frequencies of CMS and restorers in the Canche population, which suggests that they are at two different stages of the gynodioecy dynamics. In Canche, restored hermaphrodites represented half of the hermaphrodites of the population. Thus, as a result of pollen flow within the population, many non-CMS hermaphrodites were likely to carry the restorer alleles as well. Consequently, a cost of restoration could explain why non-CMS hermaphrodites in Canche had lower pollen viability, and ultimately a lower potential male fitness than non-CMS hermaphrodites from Audresselles. Interestingly, the lower performance by hermaphrodites in Canche was due to a high frequency of individuals suffering from very low pollen viability, whereas the other non-CMS hermaphrodites produced good levels of pollen viability (Fig. 4). Conversely, the frequency of non-CMS hermaphrodites in Audresselles, exhibiting this low pollen quality was lower than 10%. This shows that low pollen viability possibly occurred in non-CMS hermaphrodites in Audresselles, but at a frequency that is consistent with a lower restorer frequency in this population.

This study may provide a third case of a cost of restoration in a gynodioecious species, after having been suggested in *P lanceolata*, (de Haan *et al.*, 1997a) and *Lobelia siphilitica* (Bailey, 2002). The restoration cost suggested by our study may imply a 'silent' (also called 'alien') cost of restoration, as they occur in the presence of the 'other' cytoplasm (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Delph *et al.*, 2007). In a species containing both CMS and non-CMS cytotypes, such an alien cost has been shown to be necessary to maintain gynodioecy (Dufaÿ *et al.*, 2007). Ideally, to measure a cost associated with restoration alleles, controlled crosses assisted with genetically linked molecular markers could be conducted.

## Conclusion

We have demonstrated that pollen quantity and viability can exhibit large variation within and among gynodioecious populations, which could be partly attributed to the action of the restorer genes and their possible associated cost. Therefore, we would expect highly variable contributions to pollination among diverse types of hermaphrodites in gynodioecious species. Future studies should attempt to understand the consequences of this variation in pollen on the effective male fitness of the various types of hermaphrodites and on selection of restorer alleles. In other word, how do the pollen traits we measured effectively relate to male fitness and real gender functionality? This question is currently addressed through a paternity analysis conducted within the Audresselles population by comparing restored and non-CMS hermaphrodites in their success to sire a seedling.

## Acknowledgments

We thank Y. Michalakis for letting M.D. use his laboratory particle counter during preliminary measures of pollen production and P. Agnew for his nice help in the use of the machine and in the results analysis. We thank E. Hesse and J. Pannell and two reviewers for very helpful comments on this manuscript as well as J. Shykoff for her time and her help with discussion of ideas and statistics. This work was funded by a grant from

the 'Contrat de Plan Etat/Région Nord-Pas-de-Calais' and by a 'BQR' from the University of Lille 1.

## References


Alexander, M.P. 1969. Differential staining of aborted and non-aborted pollen. *Stain Technol.* **44**: 117–122.

Alonso, C. & Herrera, C.M. 2001. Neither vegetative nor reproductive advantages account for high frequency of male-steriles in southern Spanish gynodioecious *Daphne laureola* (Thymelaeaceae). *Am. J. Bot.* **88**: 1016–1024.

Arnaud, J.-F., Viard, F., Delescluse, M. & Cuguen, J. 2003. Evidence for gene flow via seed dispersal from crop to wild relatives in *Beta vulgaris* (Chenopodiaceae): consequences for the release of genetically modified crop species with weedy lineages. *Proc. R. Soc. Lond. B* **270**: 1565–1571.

Bailey, M.F. 2002. A cost of restoration of male fertility in a gynodioecious species, *Lobelia siphilitica*. *Evolution* **56**: 2178–2186.

Bailey, M.F. & Delph, L.F. 2007. Sex-ratio evolution in nuclear-cytoplasmic gynodioecy when restoration is a threshold trait. *Genetics* **176**: 2465–2476.

Bailey, M.F., Delph, L.F. & Lively, C.M. 2003. Modeling gynodioecy: novel scenarios for maintaining polymorphism. *Am. Nat.* **161**: 762–776.

Barrett, S.C.H. 2002. The evolution of plant sexual diversity. *Nat. Rev. Genet.* **3**: 274–284.

Belhassen, E., Dommée, B., Atlan, A., Gouyon, P.H., Pomente, D., Assouad, M.W. & Couvet, D. 1991. Complex determination of male sterility in *Thymus vulgaris* L.: genetic and molecular analysis. *Theor. Appl. Genet.* **82**: 137–143.

Boutin, V., Pannenbecker, G., Ecke, W., Schewe, G., Saumitou-Laprade, P., Jean, R., Vernet, P. & Michaelis, G. 1987. Cytoplasmic male sterility and nuclear restorer genes in a natural population of *Beta maritima*: genetical and molecular aspects. *Theor. Appl. Genet.* **73**: 625–629.

Caruso, C.M. & Case, A.L. 2007. Sex ratio variation in gynodioecious *Lobelia siphilitica*: effects of population size and geographic location. *J. Evol. Biol.* **20**: 1396–1405.

Charlesworth, B. & Charlesworth, D. 1978. A model for the evolution of dioecy and gynodioecy. *Am. Nat.* **112**: 975–997.

Charlesworth, D. & Ganders, F.R. 1979. The population genetics with cytoplasmic–genic male-sterility. *Heredity* **43**: 213–218.

Charlesworth, D. & Laporte, V. 1998. The male sterility polymorphism of *Silene vulgaris*: analysis of genetic data from two populations, and comparison with *Thymus vulgaris*. *Genetics* **150**: 1267–1282.

Chase, C.D. 2007. Cytoplasmic male sterility: a window to the world of plant mitochondrial–nuclear interactions. *Trends Genet.* **23**: 81–90.

Cosmides, L.M. & Tooby, J. 1981. Cytoplasmic inheritance and intragenomic conflict. *J. Theor. Biol.* **89**: 83–129.

Couvet, D., Ronce, O. & Gliddon, C. 1998. The maintenance of nucleocytoplasmic polymorphism in a metapopulation: the case of gynodioecy. *Am. Nat.* **152**: 59–70.

Cuguen, J., Saumitou-Laprade, P., Forcioli, D., Mörchen, M., Van-Dijk, H. & Vernet, P. 1994. Gynodioecy and mitochondrial DNA polymorphism in natural populations of *Beta vulgaris* ssp. *maritima*. *Genet. Sel. Evol.* **26**: S87–S101.

Delph, L.F., Touzet, P. & Bailey, M.F. 2007. Merging theory and mechanism in studies of gynodioecy. *Trends Ecol. Evol.* **22**: 17–24.

Desfeux, C., Maurice, S., Henry, J.P., Lejeune, B. & Gouyon, P.H. 1996. Evolution of reproductive systems in the genus *Silene*. *Proc. R. Soc. Lond. B* **263**: 409–414.

Desplanque, B., Viard, F., Bernard, J., Forcioli, D., Saumitou-Laprade, P., Cuguen, J. & Van Dijk, H. 2000. The linkage disequilibrium between chloroplast DNA and mitochondrial DNA haplotypes in *Beta vulgaris* ssp. *maritima* (L.): the usefulness of both genomes for population genetic studies. *Mol. Ecol.* **9**: 141–154.

Dufaÿ, M., Touzet, P., Maurice, S. & Cuguen, J. 2007. Modelling the maintenance of male-fertile cytoplasm in a gynodioecious population. *Heredity* **99**: 349–356.

Ehlers, B., Maurice, S. & Bataillon, T. 2005. Sex inheritance in gynodioecious species: a polygenic view. *Proc. R. Soc. Lond. B* **272**: 1795–1802.

Fénart, S., Touzet, P., Arnaud, J.-F.. & Cuguen, J. 2006. Emergence of gynodioecy in wild beet (*Beta vulgaris* spp. *maritima* L.): a genealogical approach using chloroplastic nucleotide sequences. *Proc. R. Soc. Lond. B* **273**: 1391–1398.

Fievet, V., Touzet, P., Arnaud, J.-F. & Cuguen, J. 2007. Spatial analysis of nuclear and cytoplasmic DNA diversity in wild sea beet (*Beta vulgaris* ssp. *maritima*) populations: do marine currents shape the genetic structure? *Mol. Ecol.* **16**: 1847–1864.

Forcioli, D., Saumitou-Laprade, P., Valero, M., Vernet, P. & Cuguen, J. 1998. Distribution of chloroplast DNA diversity within and among populations in gynodioecious *Beta vulgaris* ssp. *maritima* (Chenopodiaceae). *Mol. Ecol.* **7**: 1193–1204.

Frank, S.A. 1989. The evolutionary dynamics of cytoplasmic male sterility. *Am. Nat.* **133**: 345–376.

Glaettli, M. & Goudet, J. 2006. Inbreeding effects on progeny sex ratio and gender variation in the gynodioecious *Silene vulgaris* (Caryophyllaceae). *New Phytol.* **172**: 763–773.

Gouyon, P.H., Vichot, F. & Van Damme, J.M.M. 1991. Nuclear-cytoplasmic male sterility: single point equilibria versus limit cycles. *Am. Nat.* **137**: 498–514.

de Haan, A.A., Hundscheid, M.P.J. & van Hinsberg, A. 1997a. Effects of CMS types and restorer alleles on plant performance in *Plantago lanceolata* L.: an indication for cost of restoration. *J. Evol. Biol.* **10**: 803–820.

de Haan, A.A., Mateman, A.C., Van Dijk, P.J. & Van Damme, J.M.M. 1997b. New CMS types in *Plantago lanceolata* and their relatedness. *Theor. Appl. Genet.* **94**: 539–548.

Hanson, M.R. & Bentolila, S. 2004. Interactions of mitochondrial and nuclear genes that affect male gametophyte development. *Plant Cell* **16**: S154–S169.

Hjerdin-Panagopoulos, A., Kraft, T., Rading, I., Tuvesson, S. & Nilsson, N.-O. 2002. Three QTL regions for restoration of *Owen* CMS in sugar beet. *Crop Sci.* **42**: 540–544.

Hurst, L.D., Atlan, A. & Bengtsson, B.O. 1996. Genetic conflicts. *Q. Rev. Biol.* **71**: 317–364.

Jacobs, M.S. & Wade, M.J. 2003. A synthetic review of the theory of gynodioecy. *Am. Nat.* **161**: 837–851.

Kelly, J.K., Rasch, A. & Kalisz, S. 2002. A method to estimate pollen viability from pollen size variation. *Am. J. Bot.* **89**: 1021–1023.

Koelewijn, H.P. 2003. Variation in restorer genes and primary sexual investment in gynodioecious *Plantago coronopus*: the

trade-off between male and female function. *Proc. R. Soc. Lond. B* **270**: 1939–1945.

Koelewijn, H.P. & Van Damme, J.M.M. 1996. Gender variation, partial male sterility and labile sex expression in gynodioecious *Plantago coronopus*. *New Phytol.* **132**: 67–76.

Laporte, V., Merdinoglu, D., Saumitou-Laprade, P., Butterlin, G., Vernet, P. & Cuguen, J. 1998. Identification and mapping of RAPD and RFLP markers linked to a fertility restorer gene for a new source of cytoplasmic male sterility in *Beta vulgaris* ssp. *maritima*. *Theor. Appl. Genet.* **96**: 989–996.

Laporte, V., Viard, F., Bena, G., Valero, M. & Cuguen, J. 2001. The spatial structure of sexual and cytonuclear polymorphism in the gynodioecious *Beta vulgaris*: I/at a local scale. *Genetics* **157**: 1699–1710.

Letschert, J.P.W. 1993. *Beta* section *beta*: biogeographical patterns of variation and taxonomy. *Wageningen Agric. Univ. Pap.* **93**: 1–137.

Olson, M.S. & McCauley, D.E. 2002. Mitochondrial DNA diversity, population structure, and gender association in the gynodioecious plant *Silene vulgaris*. *Evolution* **56**: 253–262.

Owen, F.V. 1942. inheritance of cross- and self-sterility and self-fertility in *Beta vulgaris*. *J. Agric. Res.* **64**: 679–698.

Owen, F.V. 1945. Cytoplasmically inherited male-sterility in sugar beet. *J. Agric. Res.* **71**: 00.

Ran, Z. & Michaelis, G. 1995. Mapping of a chloroplast RFLP marker associated with the CMS cytoplasm of sugar beet (*Beta vulgaris*). *Theor. Appl. Genet.* **91**: 836–840.

Richards, A.J. 1997. *Plant Breeding Systems*. Chapman & Hall, London.

Saumitou-Laprade, P., Maggouta, F., Cuguen, J., Wattier, R., Van Dijk, H., Vernet, P. & Michaelis, G. 1993. Cytoplasmic male sterility in *Beta vulgaris* ssp. *maritima* and the nucleocytoplasmic conflict. In: *Plant Mitochondria with Emphasis on RNA Editing and Cytoplasmic Male Sterility* (A. Brennicke & U. Kück, ed.), pp. 249–258. VCH, Weinheim.

Saumitou-Laprade, P., Cuguen, J. & Vernet, P. 1994. Cytoplasmic male sterility in plants: molecular evidence and the nucleocytoplasmic conflict. *Trends Ecol. Evol.* **9**: 431–435.

Shykoff, J.A., Kolokotronics, S.-O., Collin, C.L. & Lopez-Villavicencio, M. 2003. Effects of male sterility on reproductive traits in gynodioecious plants: a meta-analysis. *Oecologia* **135**: 1–9.

Städler, T. & Delph, L.F. 2002. Ancient mitochondrial haplotypes and evidence for intragenic recombination in a gynodioecious plant. *Proc. Natl Acad. Sci. U.S.A.* **99**: 11730–11735.

Touzet, P., Hueber, N., Bürkholz, A., Barnes, S. & Cuguen, J. 2004. Genetic analysis of male fertility restoration in wild cytoplasmic male sterility G of beet. *Theor. Appl. Genet.* **109**: 240–247.

Van Damme, J.M.M. & Van Delden, W. 1982. Gynodioecy in *Plantago lanceolata* L. I. Polymorphism for plasmon type. *Heredity* **49**: 303–318.

Van Damme, J.M.M., Hundscheid, M.P.J., Ivanovic, S. & Koelewijn, H.P. 2004. Multiple CMS-restorer gene polymorphism in gynodioecious *Plantago coronopus*. *Heredity* **93**: 175–181.

Werren, J.H. & Beukeboom, L.W. 1998. Sex determination, sex ratios, and genetic conflict. *Annu. Rev. Ecol. Syst.* **29**: 233–261.

*Received 21 May 2007; revised 25 September 2007; accepted 25 September 2007*