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Disentangling the causes of heterogeneity in male fecundity in gynodioecious *Beta vulgaris* ssp. *maritima*

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Summary

- Variation among individuals in reproductive success is advocated as a major process driving evolution of sexual polymorphisms in plants, such as gynodioecy where females and hermaphrodites coexist. In gynodioecious *Beta vulgaris* ssp. *maritima*, sex determination involves cytoplasmic male sterility (CMS) genes and nuclear restorers of male fertility. Both restored CMS and non-CMS hermaphrodites co-occur. Genotype-specific differences in male fitness are theoretically expected to explain the maintenance of cytonuclear polymorphism.
- Using genotypic information on seedlings and flowering plants within two metapopulations, we investigated whether male fecundity was influenced by ecological, phenotypic and genetic factors, while taking into account the shape and scale of pollen dispersal.
- Along with spatially restricted pollen flow, we showed that male fecundity was affected by flowering synchrony, investment in reproduction, pollen production and cytoplasmic identity of potential fathers. Siring success of non-CMS hermaphrodites was higher than that of restored CMS hermaphrodites. However, the magnitude of the difference in fecundity depended on the likelihood of carrying restorer alleles for non-CMS hermaphrodites.
- Our results suggest the occurrence of a cost of silent restorers, a condition supported by scarce empirical evidence, but theoretically required to maintain a stable sexual polymorphism in gynodioecious species.

Introduction

Sexually polymorphic plant species are appealing systems in which to study how evolutionary forces interact to allow the maintenance of distinct phenotypes in natural populations. This issue is particularly intriguing in gynodioecious angiosperms, where strictly female plants are maintained in populations with hermaphroditic plants that gain fitness through both male and female functions (Darwin, 1877). The maintenance of this sexual polymorphism usually involves interactions between cytoplasmic male sterility (CMS) genes and nuclear genes that restore male function (Saumitou-Laprade *et al.*, 1994; Delph *et al.*, 2007). Individuals carrying a non-restored CMS cytoplasm are female, while individuals carrying a non-CMS cytoplasm or carrying a CMS cytoplasm in combination with the matching restorer allele produce functional hermaphroditic flowers.

Theoretical studies have shown that frequency-dependent selection can maintain cytonuclear polymorphism under certain conditions. First, because cytoplasmic genes are only transmitted through ovules, a CMS gene should spread in a population if there is a seed fertility advantage for females compared with hermaphrodites (Charlesworth, 1981; Gouyon *et al.*, 1991; Dufay *et al.*, 2007). Conversely, because nuclear genes are biparentally transmitted, the loss of pollen production directly decreases their transmission. Therefore, nuclear restorers of male fertility are selectively advantageous when CMS genes are frequent in a population. Theory suggests that, to maintain a cytonuclear polymorphism, there must be some forces opposing the fixation of restorer alleles. The only way for frequency-dependent selection to maintain a polymorphism at a nuclear restorer locus is if a fitness cost (i.e. a negative pleiotropic effect) associated with restorer alleles exists, leading to a decrease of either seed or pollen production (Charlesworth, 1981; Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). Theoretical models have explored a number of possible scenarios: a cost of silent restorer alleles (i.e. when restorers are present in a cytoplasm other than the one they restore, either another CMS or a non-CMS cytoplasm) is necessary to maintain the polymorphism, while a cost only associated with expressed restorer alleles (i.e. when restorers are associated with the matching CMS cytotype) usually leads to the loss of cytonuclear polymorphism. As a result of frequency-dependent selection, restorer alleles are favoured when CMS genes are frequent (because they are associated with the rare sex), but counter-selected when CMS genes are rare (because they

are mainly – silently – carried by another cytoplasm and thus costly). However, empirical evidence of such a cost of silent restorers in gynodioecious species remains scarce: only two studies have directly demonstrated its occurrence (involving either a decrease in seed biomass (see de Haan *et al.*, 1997) or a decrease in pollen quality (see Bailey, 2002)); other studies have yielded indirect evidence consistent with a cost of silent restorers (with a decrease in pollen quality; see Dufay *et al.*, 2008 and del Castillo & Trujillo, 2009).

Theoretical predictions are also available for structured metapopulations, whose dynamics affect the maintenance of gynodioecy. For instance, Dufay & Pannell (2010) showed that the combination of selection and drift causes the loss of gynodioecy under scenarios allowing its maintenance under selection alone in panmictic populations, and that seed or pollen dispersal could maintain cytonuclear polymorphism in a subdivided population. Understanding the dynamics of this polymorphism therefore requires detailed estimates of fecundity within natural populations, while taking gene dispersal into account. Here, we report the results of a study that describes how restricted gene flow and male fecundity differences among genotypes interact and affect the evolution of sexual polymorphism in the gynodioecious sea beet, *Beta vulgaris* ssp. *maritima*. Previous studies in this species suggested the existence of a marginal female advantage (Boutin *et al.*, 1988; De Cauwer *et al.*, 2011) but only found indirect evidence for a cost of silent restorers, which may decrease pollen quality (Dufay *et al.*, 2008). In addition, restored CMS hermaphrodites produce low-quality pollen compared with non-CMS hermaphrodites, with a high inter-individual variance among CMS restored hermaphrodites (Dufay *et al.*, 2008; De Cauwer *et al.*, 2011). This may be because more than one dominant restorer allele is necessary to fully restore male function (e.g. Ehlers *et al.*, 2005). If restored hermaphrodites do not compete well with non-CMS hermaphrodites, selection of restorers could be weaker than predicted by classical selection models (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). However, the latter expectation depends on two crucial parameters: the functional link between pollen quality and effective male fecundity of hermaphrodites and the level of population structure, which is known to be particularly pronounced in *B. vulgaris* (e.g. Laporte *et al.*, 2001; De Cauwer *et al.*, 2012). In the current study, we used an approach related to the neighbourhood model, initially developed by Adams & Birkes (1991) and modified by Burczyk *et al.* (2002) and Oddou-Muratorio *et al.* (2005), to investigate the following issues.

- By characterizing the shape and scale of pollen dispersal using molecular analyses of progeny arrays, we gained information about how gene flow occurs among individuals.
- Using these progeny analyses, we investigated the effect of several potentially important phenotypic factors on male fecundity of hermaphrodites (i.e. their ability to produce offspring), including the quality of pollen produced by potential fathers.
- We compared the relative male fecundities of non-CMS and restored CMS hermaphrodites. Because CMS hermaphrodites are known to produce pollen of lower quality, we predict that they will exhibit a reduced male fecundity.
- We predict that the probability of carrying restorer alleles decreases the fecundity of non-CMS hermaphrodites, as expected if silent restorer alleles are costly.
- Finally, we predict that the probability of being fully restored might increase the male fecundity of CMS hermaphrodites, as expected if restoration is polygenic.

While variation in some traits potentially related to male fitness has already been documented in this species (e.g. plant size and pollen quality; see Dufay *et al.*, 2008; De Cauwer *et al.*, 2011), this paternity analysis is the first attempt to bridge the gap between phenotypic variation and male fecundity, allowing us to discuss the consequences of the observed differences in terms of maintenance and dynamics of sexual polymorphism.

Materials and Methods

Study organism

Sea beet, *Beta vulgaris* ssp. *maritima* (L.) Arcangeli, is a diploid species ($2n = 18$) widely distributed along the western coast of Europe and around the Mediterranean basin. It is a wind-pollinated, short-lived perennial (Letschert, 1993). *Beta vulgaris* is generally considered self-incompatible in the wild (Owen, 1942; Larsen, 1977). However, paternity analysis in natural conditions showed very low levels of selfing (De Cauwer *et al.*, 2010b), which suggests the marginal occurrence of a self-fertility factor (Owen, 1942). Each individual bears one to several hundred indeterminate floral stems carrying a long racemose inflorescence at its apex and commonly several secondary flowering axes. Flowers along these stems open successively from bottom to top: a restricted number of the flowers are opened simultaneously within an individual plant. Overall, an individual plant can bear up to several thousand flowers. In *B. vulgaris*, male sterility is associated with four mitochondrial cytotypes, called CMS *E*, *G*, *Svulg* and *H*, which can be identified with molecular markers. These sterilizing cytoplasms coexist with several male fertile cytoplasms, which are never associated with a female phenotype (Cuguen *et al.*, 1994; Desplanque *et al.*, 2000).

Study sites

Our study was carried out in 2007 in Brittany (western France), where sea beets colonize coastal areas (Fievet *et al.*, 2007). Exhaustive sampling of all flowering plants was carried out at two study sites within two isolated coves separated by 30 km. The first site, MOR, extends over *c.* 300 m (N 48°34'168''; E –2°34'831'') and the second one, PAL, over *c.* 600 m (N 48°40'497''; E –2°52'911''). At both sites, individuals appeared to be strongly clustered in space (Fig. 1). In MOR, there were four geographical patches (MA, MB, MC and MD), totalling 1094 sampled individuals, plus four isolated plants growing outside these patches (see Fig. 1 and Table 1). In PAL, 592 individuals were clustered within five patches (PA, PB, PC, PD and PE), and 23 plants were geographically isolated (Fig. 1 and Table 1). No *B. vulgaris* individuals were found for at least 1000 m on either side of these sites.

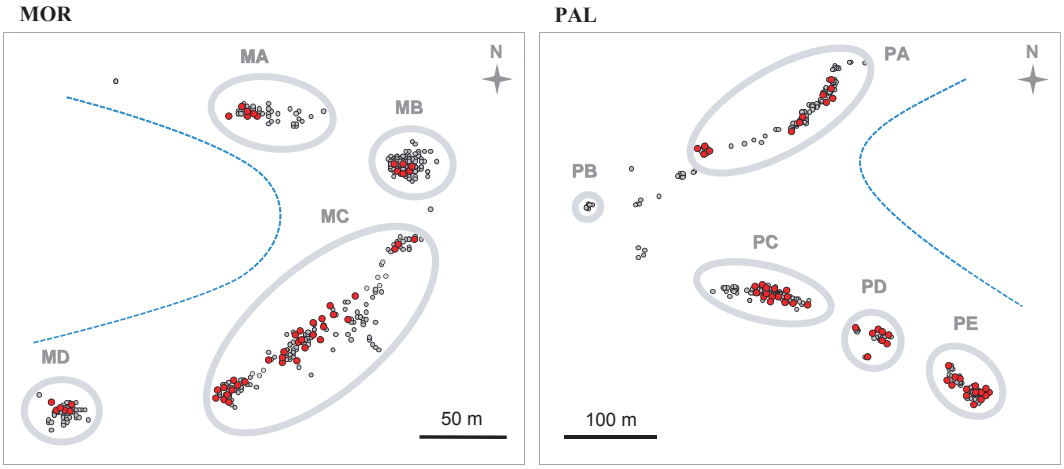

Fig. 1 Spatial distribution of *Beta vulgaris* ssp. *maritima* individuals (grey dots for potential fathers and red dots for the mother plants used for progeny analyses) and delimitation of geographical patches (grey circles) for the two study sites, MOR and PAL. The blue dashed lines show the position of the shore line.

Table 1 Major characteristics of the different geographical patches of *Beta vulgaris* ssp. *maritima* within the two study sites MOR (a) and PAL (b)

(a)

	MA	MB	MC	MD	Overall
N_{TOTAL}	69	635	281	109	1094 (4)
$N_{PHENOLOGY}$	16	45	191	14	266
N_{POLLEN}	16	45	191	14	266
$N_{INVESTMENT}$	0	0	191	0	191
$N_F/N_{HCMS}/N_{HNonCMS}$	0/3/62 (4)	0/0/633 (2)	17/60/195 (9)	0/0/106 (3)	17/63/996 (18)
Sex ratio	0	0	0.063	0	0.016
$N_{MOTHER\ PLANTS}$	6	6	32	6	50
$N_{OFFSPRING}$	143	133	773	150	1199
Mean $_{PROGENY\ SIZE}$ (± SD)	23.83 (± 1.33)	22.17 (± 6.94)	24.16 (± 2.85)	25 (± 0)	23.98 (± 2.29)

(b)

	PA	PB	PC	PD	PE	Overall
N_{TOTAL}	146	18	300	33	95	592 (23)
$N_{PHENOLOGY}$	12	0	120	8	78	218
N_{POLLEN}	12	0	120	8	78	218
$N_{INVESTMENT}$	0	0	221	0	92	313
$N_F/N_{HCMS}/N_{HNonCMS}$	0/0/142 (4)	6/6/2 (4)	61/45/185 (9)	0/0/32 (1)	3/18/71 (3)	70/69/432 (21)
Sex ratio	0	0.429	0.21	0	0.033	0.123
$N_{MOTHER\ PLANTS}$	12	0	15	8	15	50
$N_{OFFSPRING}$	297	0	357	197	368	1219
Mean $_{PROGENY\ SIZE}$ (±SD)	24.75 (± 0.87)	–	23.80 (± 4.13)	24.63 (± 1.06)	24.53 (± 1.81)	24.38 (± 2.51)

N_{TOTAL}, the total number of flowering individuals (with the number between brackets corresponding to isolated individuals, growing outside the geographical patches); $N_{PHENOLOGY}$, the number of plants for which the first day of flowering was recorded; N_{POLLEN}, the number of individuals characterized for pollen production; $N_{INVESTMENT}$, the number of plants for which the total investment in reproduction was estimated; $N_F/N_{HCMS}/N_{HNonCMS}$, the number of females, restored CMS hermaphrodites and non-CMS hermaphrodites, respectively, with the number between brackets corresponding to non-phenotyped individuals; sex ratio, the proportion of females; $N_{MOTHER\ PLANTS}$, the number of mother plants used in the study; $N_{OFFSPRING}$, the number of offspring used in the study.

At the two study sites, all flowering plants were genotyped for RFLP markers diagnostic for CMS cytoplasms (see De Cauwer *et al.*, 2012) and gynodioecy appeared to be based on a simple cytonuclear determination, with fertile cytoplasms and (except for two plants) only one CMS type (CMS *E*, which is the most common source of male sterility in this region; see Dufay *et al.*, 2009). Finally, strong spatial genetic structure was detected within both sites (see De Cauwer *et al.*, 2012).

Characterization of adult individuals

The aim of the study was to explore the effect of various potential sources of variation in male fecundity in *B. vulgaris*. For this purpose, potential fathers were characterized for their spatial localization, for several phenotypic variables, for their cytoplasmic identity and for their probability of carrying a restorer allele.

Spatial localization In the two study sites, global positioning system coordinates of all flowering plants were recorded using a GPS map60CS (GARMIN, Olathe, Kansas, United States) (accuracy of 2–5 m), allowing us to map the location of all individuals within the different geographical patches and then to calculate pairwise distances between individuals chosen as mother plants (see the section on Offspring genotyping) and all potential fathers within each study site.

Flowering phenology Within both study sites, the day of flowering onset was recorded for a random subsample of individuals in the different geographical patches (see Table 1), except the patch named PB, where plants were in bad condition (i.e. a fungal pathogen attacked the aerial parts of the plants). We then calculated the difference in flowering onset (number of days) between individuals chosen as mother plants and all potential fathers.

Pollen production The same random subsample of plants (i.e. plants for which the day of flowering onset was recorded) was used to determine pollen production (Table 1). On the day of flowering onset, two nearly opened buds localized on one of the main floral stems were collected and dissected to obtain two anthers per bud. For each individual, these four anthers were stored separately in 95% ethanol. Details of the counting procedure and the use of the particle counter (CASY® model TT; Innovatis, Bielefeld, Germany) are described in Dufay *et al.* (2008). The number of particles detected was determined for 400 size classes ranging from 0.125 to 50 µm. Pollen size has been shown to be a reliable estimator of pollen viability in *B. vulgaris* (Dufay *et al.*, 2008), with pollen grains typically distributed in two different size categories: small (10.0–13.5 µm), corresponding to nonviable pollen grains, and large (13.6–24.0 µm), corresponding to viable pollen grains (De Cauwer *et al.*, 2011). The mean number of large pollen grains was calculated over the four anthers for each individual.

Investment in reproduction At the end of the flowering season, when plants had reached their full size, the investment in reproduction was estimated by counting the total number of floral stems and measuring the inflorescence length on three randomly chosen floral stems. These measurements were performed on a random subsample of plants in a subsample of patches only (MC in MOR; PC and PE in PAL). The other patches were located on cliffs overhanging the sea, making access too difficult for such measurements. An estimator of overall individual investment in reproduction was then obtained by multiplying the mean inflorescence length (over the three measures) by the total number of floral stems.

Sexual phenotypes and genotypes at sex-determining genes At the end of the flowering season, sexual phenotype was also determined (female or hermaphrodite) for almost all adult individuals within each study site (98.5% in MOR and 97.4% in PAL) by examining several flowers on different parts of each surveyed individual. The cytotype of each plant was known (i.e. genotyped for diagnostic cytoplasmic RFLP markers; see De Cauwer *et al.*, 2012), and it was thus possible to discriminate restored CMS from non-CMS hermaphrodites (Table 1). As no genetic markers are available for restoration genes, one cannot directly predict whether non-CMS hermaphrodites carry (silent) restorer alleles and only indirect methods can be used to estimate their occurrence (e.g. Case & Caruso, 2010). In our study, we used the local restoration rate in CMS individuals as a proxy to estimate this crucial parameter (see the following paragraph).

The local neighbourhood around each individual was characterized by counting the number of restored CMS hermaphrodites, non-CMS hermaphrodites and females within a radius of 10 m. This radius corresponds to the size of a genetic neighbourhood, based on spatial autocorrelation analyses exploring the relationship between pairwise genetic relatedness and pairwise spatial distances among individuals within the same study sites (see De Cauwer *et al.*, 2012). The local restoration rate (i.e. number of restored CMS hermaphrodites divided by the total number of individuals carrying CMS cytoplasms within a radius of 10 m) was used as an estimate of the probability, for any given plant, of carrying restorer alleles. If this proxy is appropriate, the local restoration rate around female plants should be lower than around restored hermaphrodites. Statistical regressions, performed using PROC GENMOD (binomial distribution, using a log-link function) in SAS (version 9.1; SAS Institute, Cary, NC, USA) with a correction for over-dispersion, confirmed this expectation ($\chi^2 = 3.85$; $P = 0.0498$). The average restoration rate around females was 0.48 (± 0.21), significantly lower than around restored CMS hermaphrodites (0.67 ± 0.24). The local restoration rate is thus partly correlated with the probability of carrying restorer alleles. This proxy will therefore be used in subsequent analyses to estimate the genotype at restorer loci for both non-CMS hermaphrodite and restored CMS hermaphrodites.

Biological expectations that can be drawn from this proxy are as follows: in the case of a non-CMS hermaphrodite, the proxy should reflect the probability of carrying a silent restorer allele. If silent restorers are costly, as expected (see the Introduction section), we predict a negative correlation between local restoration rates and male fecundity. In the case of CMS individuals, the fact that they produce pollen indicates that they carry at least one nuclear restorer allele. Previous studies based on pollen quality in *B. vulgaris* suggested that restoration is complex, involving more than just one dominant restorer allele (Dufay *et al.*, 2008; De Cauwer *et al.*, 2011). Restorer alleles could, for instance, be codominant, and/or several partial restorer loci could coexist. In such a situation, we predict a positive correlation between the proxy and male fecundity, because the local restoration rate should reflect the number of restorer alleles in the focal plant.

Offspring genotyping

Offspring genotyping was performed in order to estimate the male fecundity of the different types of hermaphrodites at the two study sites. A total of 50 maternal plants were randomly selected at each study site (six females, five restored CMS hermaphrodites and 39 non-CMS hermaphrodites in MOR; three females, seven restored CMS hermaphrodites and 40 non-CMS hermaphrodites in PAL; see Fig. 1). Seeds were collected in all patches except PB (where fruit set was very low as a result of the action of fungal pathogens attacking the aerial parts of the plants). The distance between mother plants ranged from < 1 to 278 m with a mean of 111 m in MOR, and from < 1 to 542 m with a mean of 232 m in PAL. Seeds were randomly collected from each mother plant in mid-August 2007 and grown in a glasshouse for 2 months. DNA was extracted from dried leaf tissue for 2418 seedlings (Table 1) and purified using the NucleoSpin®96 Plant Kit (Macherey-Nagel, Düren, Germany). Nine nuclear microsatellite loci, previously used to genotype all adult individuals in both study sites, were studied (loci named: *Bmb6*, *Bvm3*, *GTT1*, *CAA1*, *SB04*, *SB06*, *SB07*, *SB15* and *FDSB1027*). Amplification procedures and detection of polymorphism are described in Fénart *et al.* (2008) and De Cauwer *et al.* (2010b). As a result of failures in PCR amplification, 3.67% of the seedling genotypes had missing data.

Modelling the dispersal kernel and heterogeneity in male fecundity

The aim of the study was to disentangle the effects of various sources of variation on male fecundity (i.e. ability to produce offspring). A spatially explicit mating model was used to investigate how male reproductive success depended on (1) the physical distance between individuals, (2) the geographical patch, (3) flowering synchrony, (4) the investment in reproduction, (5) pollen production, (6) cytotype (CMS vs non-CMS) and (7) the probability of carrying restorer alleles. The method used here is related to fractional attribution of paternity, where each offspring is partially assigned to each of the possible candidate fathers on the basis of their relative likelihoods of parentage (see Oddou-Muratorio *et al.*, 2005; Jones *et al.*, 2010). Analyses were carried out independently for each study site (MOR and PAL).

Following Burczyk *et al.* (2002) and Oddou-Muratorio *et al.* (2005), we assumed that each offspring could be the result of either self-pollination, pollen coming from outside the study sites or pollen coming from one of the sampled individuals. A seed o sampled on a mother plant j_o with genotype g_{jo} was then expected to have the genotype g_o with probability:

$$P(g_o|g_{j_o}) = sT(g_o|g_{j_o}, g_{j_o}) + (1-s-m)\sum_{k:father}\pi_{j_o k}\,T(g_o|g_{j_o}, g_k) + mT(g_o|g_{j_o}, AF) \quad \text{Eqn 1}$$

(s, the selfing rate; m, the rate of incoming pollen flow; $(1-m-s)$, the probability that the pollen donor is inside the study site; π_{jk}, the composition of the pollen pools (described below; Eqn 3).) The allele frequencies (AF) in the incoming pollen pool were measured independently in MOR and PAL from the estimated contribution of individuals located outside the study sites (i.e. through offspring that have no compatible father inside the study sites). The transition probabilities T (.|.,.) are the Mendelian likelihoods of observing a genotype for a seedling, conditional on the genotype of the parents (Meagher, 1986). The information carried by the genotypes of all seeds sampled in one site was combined into a log-likelihood function, assuming that all fertilization events were independent,

$$\log L = \sum_{o:offspring}\log\left[sT(g_o|g_{j_o}, g_{j_o}) + (1-s-m)\sum_{k:father}\pi_{j_o k}\,T(g_o|g_{j_o}, g_k) + mT(g_o|g_{j_o}, AF)\right]. \quad \text{Eqn 2}$$

The proportion of pollen from each father k in the pollen pool of each mother j originating from all known fathers, π_{jk}, was assumed to follow the mass-action law,

$$\pi_{jk} = \frac{Disp_{jk}\,Pop_k\,Pheno_{jk}\,Fec_k\,Sex_k}{\sum_{l:father} Disp_{jl}\,Pop_l\,Pheno_{jl}\,Fec_l\,Sex_l}. \quad \text{Eqn 3}$$

$Disp_{jk}$ is taking into account the effect of physical distance between individuals. This effect was modelled using a dispersal kernel, describing the probability density that a pollen grain lands at a given position away from the source. As suggested in previous studies (Oddou-Muratorio *et al.*, 2005; Fénart *et al.*, 2007), we investigated several shapes for the dispersal kernel, including the exponential-power function and the logistic function (reviewed in Austerlitz *et al.*, 2004).

The exponential-power kernel used to model the effect of distance on mating probability was given by:

$$Disp_{jk} = \frac{b}{2\pi a^2\Gamma(2/b)}\exp\left[-\frac{d_{jk}^b}{a^b}\right] \quad \text{Eqn 4}$$

(d_{jk}, the distance between mother j and father k; Γ, the Gamma function; a, a scale parameter for distance; b, a shape parameter (Clark, 1998).) The average pollen dispersal distance (δ) is then given by $\delta = [a\,\Gamma(3/b)/\Gamma(2/b)]$.

The logistic function was given by:

$$Disp_{jk} = \frac{b}{2\pi a^2\Gamma(2/b)\Gamma(1-2/b)}\left(1+\frac{d_{jk}^b}{a^b}\right)^{-1}. \quad \text{Eqn 5}$$

Pop_k in $(0,+\infty)$ takes into account the differences in individual male fecundities resulting from potential microenvironmental

variations among the different geographical patches within each study site (see Fig. 1).

$Pheno_{jk}$ takes into account the effect of flowering synchrony and is given by a Gaussian-like curve,

$$Pheno_{jk} = \exp\left[-\frac{\left(\Delta_{jk} - \Delta_{opt}\right)^2}{2\sigma_\Delta^2}\right] \quad \text{Eqn 6}$$

(Δ_{jk}, the difference in phenology between mother j and father k; Δ_{opt}, the optimal difference in phenology; ${\sigma_\Delta}^2$, the variance of the Gaussian curve.) A positive Δ_{opt} means that fathers flowering earlier than a given mother have a higher reproductive success than males flowering synchronously.

Fec_{jk} takes into account the effects of pollen production (PP_k) and investment in reproduction (RI_k) through an exponential relation,

$$Fec_k = \exp\left[b_{RI}\left(RI_k - \overline{RI}\right)\right] \exp\left[b_{PP}\left(PP_k - \overline{PP}\right)\right] \quad \text{Eqn 7}$$

(b_{PP} and b_{RI}, selection gradients describing the effect of pollen production and investment in reproduction on male fecundity.) For the individuals that were not scored for pollen production or investment in reproduction, the corresponding exponential term was replaced by a parameter NS_{PP} (different in the different patches) or NS_{RI} (independent of the patch).

Finally, the effect of the cytotype (CMS vs non-CMS) and the effect of the probability of carrying restorer alleles were also taken into account. To assess the effect of the cytotype, we set $Sex_k = F = 0$ for females, $Sex_k = H_{CMS}$ for restored CMS hermaphrodites, $Sex_k = H_{NonCMS} = 1$ for non-CMS hermaphrodites and $Sex_k = NT$ for non-typed individuals (i.e. individuals for which the sexual phenotype was not scored).

The effect of the probability of carrying restorer alleles was considered through the local CMS restoration frequency around individual k ($P_{Freq,k}$), using the following equation:

$$Neigh_k = \exp\left[b_{Freq,k} P_{Freq,k}\right], \quad \text{Eqn 8}$$

where $b_{Freq,k}$ is b_{HCMS} if individual k is a restored CMS hermaphrodite, $b_{HNonCMS}$ for a non-CMS hermaphrodite, and 0 for females and non-typed individuals.

By maximizing the log-likelihood log$L$ (Eqn 2), we jointly estimated the following parameters: levels of selfing (s) and of incoming pollen flow (m), the dispersal parameters (a and b), the effect of geographical patches (Pop_1, Pop_2… Pop_{max}), the effect of flowering phenology (Δ_{opt} and σ_Δ), the effect of investment in reproduction (b_{RI} and NS_{RI}), the effect of pollen production (b_{PP}, and NS_{PP1}, …NS_{PPmax}), the effect of the cytotype (H_{CMS} and NT) and the effect of the probability of carrying restorer alleles (b_{HCMS} and $b_{HNonCMS}$). The log-likelihood function was maximized numerically using the quasi-Newton algorithm in MATHEMATICA 7.1 (Wolfram Research, Champaign, Illinois, United States). Several contrasted initial values for the maximization of the parameters were used to confirm that we reached a global maximum (when the initial values actually led to the different maxima, we kept the one that reached the higher log-likelihood). Parameters describing the relative male reproductive success of non-scored individuals (NS_{RI} and NS_{PP1}, …NS_{PPmax}) and of non-genotyped individuals (NT) were incorporated in the models to improve the fit to data, but these results will not be described further in the paper.

The significance of each effect was tested with a Type III likelihood-ratio test (LRT). For each test, the log-likelihood of a model without the tested effect was computed. The deviance (i.e. twice the difference between the log-likelihood obtained for the complete model and the log-likelihood obtained for the model without the tested effect) was then compared to a χ^2 distribution, with the number of degrees of freedom equal to the difference in the number of parameters between the two models.

The confidence intervals for the parameters were obtained through a bootstrap procedure using mother plants as sampling units. For each replication, we successively sampled mother plants with replacement and equal probabilities and kept all their seedlings' genotypes, until we reached the same total number of seedling genotypes in the bootstrapped data set as in the real data set. To reach exactly the same total number of seedlings, we sampled without replacement the correct number of seedlings among those of the last mother drawn. We then estimated the parameters on the bootstrapped data set by maximizing the log-likelihood (Eqn 2). For each study site, we derived 95% symmetric confidence intervals for all parameters from 250 bootstrapped data sets.

Results

Variation in phenotypic traits among hermaphrodite types

One of the aims of this study was to compare CMS and non-CMS hermaphrodites for their male fecundity, while taking into account the possible effect of several phenotypic factors. The first step was to analyse how the different phenotypic factors varied between the two hermaphrodite categories in order to assess whether the explanatory variables used in the spatially explicit models are statistically independent.

The effect of cytotype on the date of flowering onset differed in the two study sites: the two cytotypes were statistically indistinguishable in MOR, while restored CMS hermaphrodites started to flower significantly earlier than non-CMS hermaphrodites in PAL (Supporting Information Table S1). In MOR, non-CMS hermaphrodites started to flower on average on the 8th day of the survey and the restored CMS hermaphrodites on the 9th day. In PAL, non-CMS hermaphrodites started to flower on average on the 11th day of the survey and the restored CMS hermaphrodites on the 8th day.

The cytotype had no significant effect on the overall investment in reproduction in either site (Table S1). In MOR, the cumulated within-individual inflorescence length was on average 197 cm (SE = 23) for non-CMS hermaphrodites and 281 cm (SE = 81) for restored CMS hermaphrodites. In PAL, the

average was 355 cm (SE = 31) for non-CMS hermaphrodites and 452 cm (SE = 78) for restored CMS hermaphrodites. The date of flowering onset had a strong and significant effect on the overall investment in reproduction, with small individuals flowering, on average, later than individuals showing a higher investment in reproduction.

Our results confirmed that restored CMS hermaphrodites are poor pollen producers compared with non-CMS hermaphrodites (Table S1). In MOR, the average number of viable pollen grains was 910 (SE = 34) for non-CMS hermaphrodites and 629 (SE = 80) for restored CMS hermaphrodites. In PAL, the average was 1096 (SE = 47) for non-CMS hermaphrodites and 823 for restored CMS hermaphrodites (SE = 121). In both sites, date of flowering onset also had a significant effect on pollen production, with early individuals being better pollen producers on average, whatever their cytotype.

Spatially explicit mating models

Spatially explicit mating models were used to estimate the overall levels of selfing and incoming pollen flow, to characterize the shape and scale of pollen dispersal and to explore the impact of various factors on male fecundity (i.e. on the ability to produce offspring). In particular, the effects of several phenotypic traits on male fecundity were assessed, the male fecundities of restored-CMS and non-CMS hermaphrodites were compared and the effect of the probability of carrying restorer alleles was explored for the two hermaphroditic categories.

Immigration and selfing The estimated proportion of seeds fathered by foreign pollen was similar in both study sites (m = 0.12 and 0.08 in MOR and in PAL, respectively; see Table 2), suggesting low levels of incoming pollen flow at both sites. The

Table 2 Values of all the parameters estimated using the spatially explicit mating model for the two study sites, MOR (a) and PAL (b), along with their confidence intervals (CIs) obtained through a bootstrap procedure using *Beta vulgaris* ssp. *maritima* mother plants as sampling units

(a)

Parameter	Value	CI
Migration rate (m)	0.12	(0.10, 0.16)
Selfing rate (s)	0.02	(0.00, 0.03)
Average pollen dispersal distance (δ)	23.17	(17.0, 31.7)
Shape parameter (b)	0.60	(0.49, 0.76)
Estimated relative male fecundity for individuals in MA (Pop_{MA})	0.40	(0.02, 2.2)
Estimated relative male fecundity for individuals in MB (Pop_{MB})	0.07	(0.00, 0.40)
Estimated relative male fecundity for individuals in MC (Pop_{MC})	1[a]	–
Estimated relative male fecundity for individuals in MD (Pop_{MD})	0.02	(0.00, 0.44)
Optimal difference in phenology (Δ_{opt})	0.55	(-3.8, > 20)
Variance of the Gaussian distribution (σ_Δ)	9.85	(6.0, > 20)
Selection gradient for pollen production (b_{PP})	0.0009	(0.0006, 0.0012)
Selection gradient for investment in reproduction (b_{RI})	0.0010	(0.0007, 0.0013)
Estimated relative male fecundity for restored CMS hermaphrodites (H_{CMS})	0.34	(0.09, 0.88)
Estimated relative male fecundity for non-CMS hermaphrodites (H_{NonCMS})	1[a]	–
Estimated relative male fecundity for females (F)	0[a]	–
Selection gradient for restoration rate in the neighbourhood for restored CMS hermaphrodites (b_{HCMS})	0.80	(– 0.23, 2.63)
Selection gradient for restoration rate in the neighbourhood for non-CMS hermaphrodites ($b_{HNonCMS}$)	– 0.75	(– 1.64, 0.13)
Selection gradient for restoration rate in the neighbourhood for females (b_F)	0[a]	–

(b)

Migration rate (m)	0.08	(0.05, 0.12)
Selfing rate (s)	0.03	(0.01, 0.07)
Average pollen dispersal distance (δ)	6.53	(5.1, 8.8)
Shape parameter (b)	2.65	(2.34, 3.35)
Estimated relative male fecundity for individuals in PA (Pop_{PA})	3.50	(0.34, 21)
Estimated relative male fecundity for individuals in PB (Pop_{PB})	0[a]	–
Estimated relative male fecundity for individuals in PC (Pop_{PC})	0.66	(0.02, 5.5)
Estimated relative male fecundity for individuals in PD (Pop_{PD})	0.08	(0.012, 0.86)
Estimated relative male fecundity for individuals in PE (Pop_{PE})	1[a]	–
Optimal difference in phenology (Δ_{opt})	8.00	(4.7, > 20)
Variance of the Gaussian distribution (σ_Δ)	7.20	(5.0, > 20)
Selection gradient for pollen production (b_{PP})	0.0005	(0.0002, 0.0008)
Selection gradient for investment in reproduction (b_{RI})	0.0010	(0.0007, 0.0013)
Estimated relative male fecundity for restored CMS hermaphrodites (H_{CMS})	0.17	(0.05, 0.43)
Estimated relative male fecundity for non-CMS hermaphrodites (H_{NonCMS})	1[a]	–
Estimated relative male fecundity for females (F)	0[a]	–
Selection gradient for restoration rate in the neighbourhood for restored CMS hermaphrodites (b_{HCMS})	0.93	(– 0.50, 3.10)
Selection gradient for restoration rate in the neighbourhood for non-CMS hermaphrodites ($b_{HNonCMS}$)	– 0.78	(– 1.59, 0.03)
Selection gradient for restoration rate in the neighbourhood for females (b_F)	0[a]	–

[a]Parameters fixed in the models.

estimated self-pollination rates were very low in both sites (s = 0.02 in MOR and 0.03 in PAL; see Table 2).

Effect of distance on mating probability The effect of physical distance between individuals on the mating probability was strong and highly significant in both study sites (see Table 3). Several dispersal kernels were tested. The best fit was obtained with the exponential power function in MOR and with a logistic function in PAL, leading to differing probabilities of long-distance dispersal with a rather thin-tailed kernel in PAL and a fat-tailed kernel in MOR (see Fig. 2a). Both models yielded low estimates of mean pollen dispersal distances (δ = 23.17 and 6.53 m in MOR and in PAL, respectively; see Table 2).

Table 3 Significance of the different effects taken into account in the spatially explicit mating models on male fecundity, for *Beta vulgaris* ssp. *maritima* plants in the two study sites, MOR (a) and PAL (b)

(a)

	df	− log*L*	ΔAIC	*P*
Complete model	20	13 900		
Dispersal	18	15 534	3264	0
Geographical patch	17	13 907	8	0.0029
Flowering phenology	18	13 903	2	0.0498
Pollen production	15	13 966	122	0
Investment in reproduction	18	13 954	104	0
Cytotype * restoration rate in the neighbourhood	16	13 907	6	0.0073
Restoration rate in the neighbourhood (restored CMS hermaphrodites)	19	13 901	0	0.1573
Restoration rate in the neighbourhood (non-CMS hermaphrodites)	19	13 906	10	0.0005
Restoration rate in the neighbourhood: slope difference between the two hermaphroditic types	19	13 901	0	0.1573

(b)

	df	− log*L*	ΔAIC	*P*
Complete model	20	12 769		
Dispersal	18	14 700	3858	0
Geographical patch	17	12 782	20	0
Flowering phenology	18	12 800	58	0
Pollen production	15	12 792	36	0
Investment in reproduction	18	12 847	152	0
Cytotype * restoration rate in the neighbourhood	16	12 793	40	0
Restoration rate in the neighbourhood (restored CMS hermaphrodites)	19	12 770	0	0.1573
Restoration rate in the neighbourhood (non-CMS hermaphrodites)	19	12 777	14	0.0001
Restoration rate in the neighbourhood: slope difference between the two hermaphroditic types	19	12 773	6	0.0047

log*L* values are the log-likelihoods obtained for the complete model and for the models without each tested effect. ΔAIC (Akaike information criterion) describe the relative strength of the different tested effects. *P*-values were obtained with Type III likelihood-ratio tests.

Effects of ecological, phenotypic and genetic factors on male reproductive success Geographical patch of origin had a significant effect on male mating probabilities in both study sites (Table 3), suggesting that micro-environmental variation explains some of the heterogeneity in male fecundity. We also found a significant effect of flowering synchrony on male fecundity (Table 3). In both populations, our results suggest that it is advantageous for potential fathers to start flowering before mother plants. However, the optimal difference in phenology differed between the sites: in MOR, male fecundity was maximal for fathers starting to flower 0.55 d before mother plants, whereas in PAL, the estimated optimal asynchrony was 8 d (Fig. 2b; Table 2). As expected, male fecundity increased significantly with pollen production (Fig. 2c; Table 3). Investment in reproduction also had a strong and significant effect on male fecundity (Fig. 2d; Table 3). With regard to the cytotype of hermaphrodites, our results suggest that non-CMS hermaphrodites have a higher fecundity than restored CMS hermaphrodites in both study sites (Table 2). The overall difference in male fitness between the two cytoplasmic categories includes both the cytoplasmic effect detected here and a contribution of the effect of pollen production. Because pollen production was significantly lower in restored CMS hermaphrodites, restored CMS hermaphrodites should suffer from an even stronger disadvantage in male fitness than suggested by the results summarized in Table 2. Finally, male fecundity was affected by the probability of carrying restorer alleles (the restoration rate in the neighbourhood; see Table 3). The effect depended on the cytotype (see Fig. 2e): for non-CMS hermaphrodites, the local restoration rate (i.e. the probability of carrying silent restorer alleles) had a significantly negative effect on fecundity; and for restored CMS hermaphrodites, there was a nonsignificant trend for an increase in fecundity with local restoration rate (i.e. fitting this slope did not significantly improve the models; see Table 3). When further comparing the slopes between the two categories of hermaphrodites within each study site, the difference was significant in PAL (P = 0.0047; see Table 3) but not in MOR.

Discussion

Power and limitation of the modelling approach

Like any other approach, maximum likelihood models are subject to drawbacks. A recent paper suggested unexpectedly high Type 1 error rates for likelihood ratio tests (Klein *et al.*, 2011). This is because these models do not take into account the entire variance in fecundity, but only the limited part explained by the covariates studied. In this case, if some individuals have high mating probabilities, the numerous offspring sired on some mother plants will appear as correlated matings, while classical maximum likelihood models assume independent events. This results in over-dispersion and incorrect inferences (underestimated *P*-values; see Klein *et al.*, 2011). Therefore, the confidence intervals computed through a bootstrap procedure using mother plants as sampling units might better illustrate the uncertainty of the estimated parameters. We obtained quite wide confidence intervals, especially

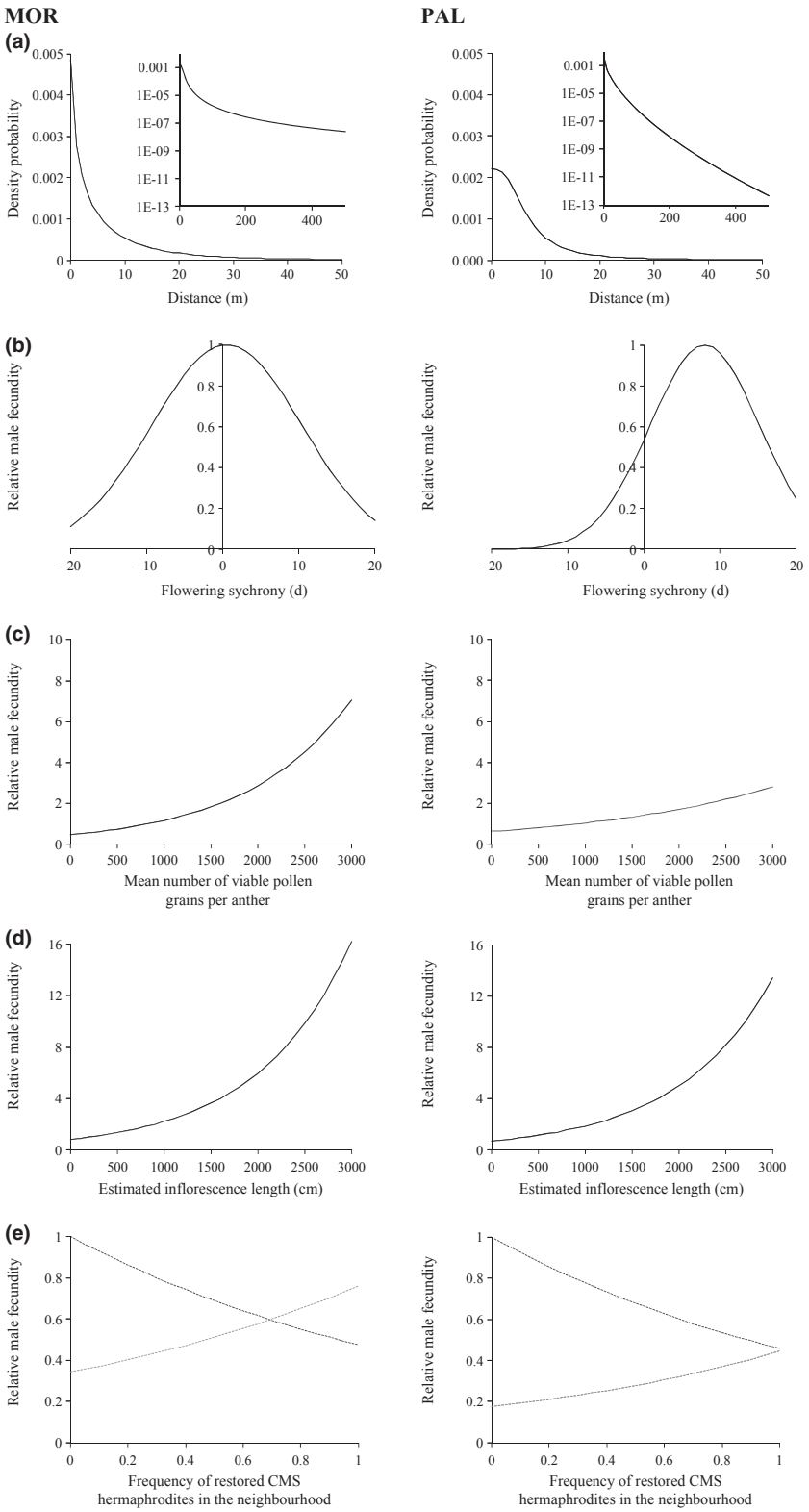

Fig. 2 Effects of (a) physical distance between potential fathers and mother plants in the *Beta vulgaris* ssp. *maritima* populations, (b) flowering synchrony (difference in the date of flowering onset between potential fathers and mother plants), (c) pollen production (mean number of viable pollen grains per anther), (d) investment in reproduction (estimated inflorescence length), and (e) local cytoplasmic male sterility (CMS) restoration rate, for non-CMS hermaphrodites (black dashed line) and restored CMS hermaphrodites (grey dashed line), on the relative male fecundity. All these effects were estimated using spatially explicit mating models in the two study sites (MOR and PAL). In the top-right corner of the curves showing the effect of physical distance on relative male fecundity (a), a log-plot version of the same curves providing a better representation of the tails is shown.

for the parameters describing the effect of phenology, as well as for the parameters describing the effect of the genotype for sex-determining genes. Keeping this in mind, the fact that the analyses yielded comparable trends over the range of estimated parameters in both study sites suggests that our results might be biologically relevant.

Dispersal capabilities and mating system

Using the genotypes of all potential fathers and of a set of progeny arrays, we assessed the effect of physical distance on mating probabilities by estimating the shape and scale of the pollen-dispersal function in *B. vulgaris*. Our results confirmed the previous observation that mating distances are highly variable in natural populations of *B. vulgaris*, with a majority of pollination events occurring at restricted geographical distances and a small proportion of long-distance pollen flow (Fénart *et al.*, 2007; De Cauwer *et al.*, 2010b). Consistently with this predominance of short-distance siring events, the models yielded low estimated mean pollen dispersal distances and suggested limited levels of pollen flow coming from outside the study sites. This result was quite unexpected in a wind-pollinated species where viable pollen grains can be found at high altitude (Meier & Artschwager, 1938).

Our results also suggest different patterns of long-distance pollen flow within the study sites, implying fewer long-distance dispersal events in PAL than in MOR. This difference between sites is not attributable to the different spatial configurations of individuals because it refers to differences between dispersal kernels (and not differences between the distributions of realized dispersal distances), whose estimations are known to be robust to the spatial configuration of sources and recipients (Robledo-Arnuncio & García, 2007). There were no obvious physical barriers to pollen flow within both study sites, so these differences may arise from contrasted levels of exposure to the pollen dispersal vector, the wind. Although fat-tailed dispersal kernels are increasingly considered as a common feature in natural plant populations (e.g. Hardy *et al.*, 2004; Devaux *et al.*, 2005; Oddou-Muratorio *et al.*, 2005; Gérard *et al.*, 2006; Goto *et al.*, 2006), this study illustrates that, for a given species, the shape of pollen dispersal can vary among localities.

Determinants of male fecundity heterogeneity

While the physical distance between potential fathers and mother plants was clearly the most important determinant of mating-success, the spatially explicit mating models also enabled us to identify additional factors influencing male fecundity in gynodioecious *B. vulgaris*.

First of all, male fecundity was significantly affected by the geographical patch of origin of potential fathers. This factor potentially encompasses various abiotic and biotic factors that are difficult to disentangle in our study, such as sun exposure, soil type and composition, water availability, individual density, competition with other plant species, the presence and nature of herbivores or the relative age of the patch. Additional experiments are needed to identify more precisely the sources of micro-environmental variations in male fecundities.

Beyond the effect of micro-environmental variation, we also investigated the impact of various phenotypic factors on male fecundity, including flowering phenology, investment in reproduction and pollen production. Flowering phenology is a characteristic that is likely to contribute importantly to individual male fecundity (e.g. Burczyk & Prat, 1997; Gérard *et al.*, 2006), especially in species showing short individual flowering periods and large variability for flowering dates among individuals within populations. In *B. vulgaris*, individual flowering duration is known to be rather long (> 1 month on average; Archimowitsch, 1949; De Cauwer *et al.*, 2011) and flowering is largely synchronous within populations (De Cauwer *et al.*, 2010a), potentially limiting the effect of flowering phenology on male fecundity. However, our results suggest that flowering synchrony had a significant effect on male fecundity in *B. vulgaris* populations, with male fecundity being maximal for potential fathers starting to flower before mother plants in both sites. It remains difficult to generalize this result, as the optimal delays between male and female flowering were different between the two study sites. Finally, we found that restored CMS hermaphrodites tended to start flowering significantly earlier than non-CMS hermaphrodites in PAL only. While comparing the reproductive abilities among sex categories is the basis for understanding the evolutionary dynamics of sexual polymorphism, this suggests that all reproductive traits should be taken into account.

The results of the spatially explicit mating models also suggested that investment in reproduction had a significant effect on male fecundity. As expected, hermaphrodites with the highest levels of investment had a higher male fecundity than hermaphrodites exhibiting more moderate flowering. Several studies have already suggested that flowering intensity is an important determinant of male fecundity in plant populations (Burczyk & Prat, 1997; Oddou-Muratorio *et al.*, 2005). However, while our measures of investment in reproduction probably reflect quite accurately the total number of flowers per individual (see De Cauwer *et al.*, 2011), it was also necessary to take into account the intrinsic quality of flowers, through measures of pollen production. Pollen production is likely to have an important effect on male reproductive success (e.g. Torimaru *et al.*, 2012). In *B. vulgaris*, the number of viable pollen grains per anther is known to vary extensively among individuals (Dufay *et al.*, 2008; De Cauwer *et al.*, 2011) and had to be taken into account in the spatially explicit mating models to give a comprehensive picture of the phenotypic determinants of male fecundity. The current study clearly illustrates the functional link between pollen production and male fecundity: individuals producing a limited amount of viable pollen suffer from a decrease in their male fecundity.

Finally, spatially explicit mating models also allowed us to test for genotype effects. First, we found a strong effect of the cytotype, with non-CMS hermaphrodites having a higher male fecundity than CMS ones. One likely explanation for this difference is incomplete restoration of male fertility at least in some CMS-hermaphrodites, although this was not confirmed by other analyses (i.e. effect of the probability of carrying restorer alleles in

CMS hermaphrodites; see the end of this paragraph). Because both pollen production and cytotype simultaneously affected male fecundity in the models, the lower fecundity of CMS hermaphrodites is not only attributable to their low pollen quality. Other phenotypic differences may thus exist between the two categories of hermaphrodites, such as the germination ability of viable pollen grains. Secondly, male fecundity was also affected by the probability of carrying restorer alleles, with the effect depending on the cytotype. On one hand, our study suggests that the probability of carrying restorer alleles reduces male fecundity of non-CMS hermaphrodites in both study sites, suggesting the occurrence of a cost of silent restorers which is a theoretical condition for the maintenance of a stable sexual polymorphism (Delph *et al.*, 2007). On the other hand, the probability of carrying restorer alleles had no effect on restored CMS hermaphrodites. We were expecting a positive effect, which would have confirmed our hypothesis that CMS hermaphrodites have a higher male fecundity when they are more likely be fully restored. Further studies are needed to understand why CMS hermaphrodites exhibit a lower male fecundity than non-CMS ones.

Variations in male fecundity: potential effects on the dynamics of gynodioecy

A large number of empirical studies have attempted to test theoretical predictions about how selection can maintain cytonuclear gynodioecy. The first prediction is that females should produce more seeds and/or seeds of better quality than hermaphrodites, which has been confirmed repeatedly (reviewed in Shykoff *et al.*, 2003). The second prediction is the occurrence of a cost of silent restorers which prevents the fixation of restorer alleles and thus the loss of cytonuclear gynodioecy, whatever the magnitude of that cost (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). This cost has been observed in only very few species (see McCauley & Bailey, 2009 and references therein). The current study suggests that the probability of carrying silent restorer alleles decreases the male fecundity of non-CMS hermaphrodites. While previous studies described possible effects of silent restorers on phenotypic traits (e.g. pollen quality or seed biomass; see de Haan *et al.*, 1997; del Castillo & Trujillo, 2009), this study is the first to deal with direct measurements of male fitness in natural conditions.

The second salient result of this study is that CMS hermaphrodites showed an overall lower male fecundity compared with non-CMS hermaphrodites. This effect has not received attention in theoretical studies so far, but one can hypothesize that it could lower the frequency and/or the probability of maintenance of restorer alleles in wild populations. Consequently, gynodioecy should be more difficult to maintain, compared with classical theoretical predictions (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007). However, it is important to note that male performance of restored CMS hermaphrodites (and thus, the dynamics of restorer alleles) can be affected by other factors. For instance, male fitness of hermaphrodites also depends on the patterns of sex structure of the population in which they grow. *Beta vulgaris* populations classically display strong spatial structure for sex-determining genes (e.g. Laporte *et al.*, 2001). In extreme cases, this strong genetic structuring can lead to the existence of demes in which nearly all individuals carry a CMS gene and in which restored CMS hermaphrodites are the only local pollen producers (De Cauwer *et al.*, 2010b). Extinction and recolonization dynamics, in disturbed coastal habitats, could thus persistently create situations where restored hermaphrodites benefit from a strong transient advantage, allowing rapid selection and local maintenance of restorer alleles. The fitness associated with restorer alleles will then depend on the relative proportion of CMS and non-CMS cytotypes, and thus on the age and/or the level of isolation of the population.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 Results of the general linear models testing for the effect of cytotype (restored cytoplasmic male sterility (CMS) hermaphrodites or non-CMS hermaphrodites) on the three phenotypic traits used in this study (date of flowering onset, investment in reproduction and pollen production) in the two study sites, MOR and PAL

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