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SEX RATIO VARIATION AMONG GYNODIOECIOUS POPULATIONS OF SEA BEET: CAN IT BE EXPLAINED BY NEGATIVE FREQUENCY-DEPENDENT SELECTION?

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This study is devoted to assess sex ratio variation among 33 populations of the gynodioecious *Beta vulgaris* ssp. *maritima* in Brittany (France) and to explore the causes of this variation. We showed that three different CMS (cytoplasmic male sterility) cytotypes occurred in populations, but strongly differed for their frequencies and the frequency of their associated nuclear restorer alleles (which counteract the effect of CMS and restore male fertility). No correlation was found between CMS and restorer frequencies within populations, which has been previously interpreted as a result of stochasticity. However, neutral genetic variation did not indicate recent population bottlenecks in studied populations. Moreover, no significant correlation was found between female frequency or variance and current population size. Consequently, stochastic processes could not be the major cause of sex ratio variation. Alternatively, empirical estimations of the variation of females, CMS genes and nuclear restorer allele's frequencies were compared to theoretical predictions based on a frequency-dependent selection model of gynodioecy. In particular, we showed that an absence of correlation between CMS and restorer frequencies could also occur without stochasticity. The large variation of sex ratio in *Beta vulgaris* could thus be explained by frequency-dependent selection acting on CMS genes and restorer alleles.

KEY WORDS: ***Beta vulgaris* spp. *maritima*, cytoplasmic male sterility, gynodioecy, restoration of male fertility.**

Sexually polymorphic plants, that is, species composed of two or more sexual phenotypes, are key systems for investigating the effects of selection in natural populations. In such species, different sexual phenotypes typically have contrasted strategies as to how genes are transmitted to the next generation: through pollen only in male individuals, through seeds only in female individuals, or through a combination of both in hermaphrodite and monoecious individuals. As a consequence, the fitness of an individual depends on its own sexual strategy, as well as on the frequency of the different sexual phenotypes in the population (reviewed in Barrett [2002]).

Although the genetic basis of sexual polymorphism remains unclear in most cases (e.g., Charlesworth 2002; Ming et al. 2007 for dioecious species), gynodioecy is one of the plant mating systems for which the genetic determination is the best understood. Gynodioecy is defined as the co-occurrence of females and hermaphrodites in a species, and is the most common sexual polymorphism in plants (Richards 1997). It generally results from the interaction between cytoplasmic male sterility (CMS) genes, which prevent pollen production, entailing a female (male sterile) phenotype on one hand, and nuclear restorer genes that are able to counteract the effects of CMS genes and restore male function (Budar et al. 2003; Chase 2007; Delph et al. 2007) on the other hand.

Because cytoplasmic genes are only transmitted through seeds, theory predicts that a CMS gene should spread in a

population as soon as fitness through the female function becomes higher in females than in hermaphrodites. This fitness effect has been coined "female compensation" by Charlesworth and Ganders (1979, see also Frank 1989 and Gouyon et al. 1991). However, because CMS fixation in a population would entail the loss of pollen production overall, this creates a strong selective pressure in favor of nuclear alleles able to restore pollen production. Hence, restorer genes should rise to fixation following invasion by CMS genes. Empirical studies have shown that restorer genes typically do not reach fixation in natural populations, leading theoretical models based on selection to postulate that a cost of restorer alleles prevents their fixation and allows the maintenance of sexual polymorphism (Gouyon et al. 1991; Bailey et al. 2003; Dufay et al. 2007). Overall, sex ratio in a population should therefore evolve according to negative frequency-dependent selection (thereafter called FDS): CMS genes are selected for when nuclear restorers are rare (i.e., when CMS are mainly carried by female individuals), whereas nuclear restorers are in turn selected for when CMS occurs at high frequencies. Indeed, if FDS drives the evolution of gynodioecy, theory therefore predicts in most cases strong deterministic oscillations of CMS and restorer gene frequencies.

Sex ratio in gynodioecious species generally shows large variation among populations, and this variation has sometimes been considered as a consequence of FDS. According to Gouyon et al. (1991), different populations could be at a different stage of their oscillation dynamics of CMS and restorer gene frequencies, which would explain the observed variation. But so far, this remains a very hypothetical view of gynodioecy dynamics, which is difficult to test experimentally. Moreover, other factors have been proposed to explain variation in sex ratios. First, several studies have shown that female frequencies varied with a specific environmental component, such as herbivory pressure or drought, suggesting that female advantage could be environment dependent (e.g., Gouyon et al. 1983; Thompson and Tarayre 2000; Asikainen and Mutikainen 2003; Ashman et al. 2004; Barr 2004; Caruso and Case 2007). In this case, sex ratio variation would not be due to deterministic oscillations but rather to a variation of the strength of selection for females among populations. Second, other studies showed that population size could also have an effect on sex ratios (Nilsson and Ågren 2006; Caruso and Case 2007), as suggested by theoretical studies (Laporte et al. 2000). Small populations are more prone to genetic drift, and consequently deviations from deterministic models are expected. Hence, these authors stressed the impact of stochastic processes on the dynamics of gynodioecy, suggesting that selective process could be of minor importance compared to the effect of genetic drift in natural populations. Overall, there is currently no clear and synthetic view of the factors underlying sex ratio variation among populations in gynodioecious species (Bailey and Delph 2007). Moreover, theoretical predictions on how selection maintains gynodioecy and affects population sex ratio have obtained minor empirical support so far.

One main difficulty in assessing what processes are responsible for sex ratio variation is that many studies are based on phenotypic information only (female vs. hermaphrodite) and lack information on identity and frequency of CMS-related cytoplasm (cytotypes) and associated nuclear gene restorers within populations (but see Belhassen et al. 1993; De Haan et al. 1997; Van Damme et al. 2004). When the genetic factors that determine sexual phenotypes are unknown, it is difficult to assess the evolutionary forces that act on male sterility genes and nuclear restorer alleles. On the other hand, when such information is available, it may improve our understanding of the evolutionary dynamics of sex ratios. For example, in a recent study on the gynodioecious *Raphanus sativus*, Murayama et al. (2004) showed that the frequency of CMS genes in populations was not correlated with the frequency of restorer alleles. Because such a correlation is usually expected under the effects of natural selection, the authors interpreted their result as an effect of stochasticity, that would hide the effects of selection.

The current study is devoted to investigate the evolutionary dynamics of gynodioecy by assessing the geographical variation of sex ratio among 33 populations of the gynodioecious wild sea beet, *Beta vulgaris* ssp. *maritima*. Wild beet constitutes a relevant model for understanding the processes responsible for sex ratio variation among populations. In this species, four different CMS cytoplasmic types (cytotypes) co-occur with male fertile (non-CMS) cytotypes, and these various cytotypes can be distinguished by molecular markers. This allows a direct measurement of three parameters of crucial importance: (1) female frequencies (2) CMS frequencies, and (3) the rate of restoration per CMS-related cytoplasm in each population, by recording the frequency of hermaphroditic plants carrying a CMS cytotype. In this study, we focused on a sample of populations that were located in a relatively homogeneous geographical area with regard to climatic and topographic conditions; they occurred in relatively similar coastal habitats and no obvious environmental gradient has been recorded among the 33 sites. Thus, assuming negligible environmental variation for female compensation, this set of populations allowed us to study sex ratio variation, which could be explained by genetic drift and/or FDS. More precisely, we addressed the following questions: (1) How does sex ratio vary among populations in *Beta vulgaris* ssp. *maritima*? (2) How does genetic determination of sex explain sex ratio variation? Do several CMSs co-occur within populations? How do CMS frequencies vary among these populations? How does restoration of male fertility vary among populations, between CMSs, and with time? (3) Could sex ratio variation be due to stochastic effects, that is, do sex ratio, CMS and restorer frequencies, and their variance depend on population size? Do patterns of neutral genetic diversity suggest any signature

of genetic drift? Is there, or not, a statistical correlation between the frequencies of CMS genes and restorer alleles (given that an absence of correlation can be interpreted as the result of stochasticity)? (4) If not, is sex ratio variation consistent with FDS? To do so, we compared our empirical data with expected results from the deterministic effect of FDS using a recent model (Dufay et al. 2007). In particular, because there is currently no clear expectations about the effects of FDS on this issue, we analyzed how the frequencies of CMS genes and restorers were correlated in simulations generated by the FDS model.

Material and Methods

THE SPECIES

Beta vulgaris ssp. *maritima* is a short-lived perennial species, gynodioecious, self-incompatible, wind pollinated, which is widely distributed along the western coasts of Europe and around the Mediterranean Basin. In the North of 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). Among the 20 mitochondrial haplotypes described in beet, four haplotypes are clearly associated with male sterility: *E*, *G*, *H*, and *Svulg* (Cuguen et al. 1994; Laporte et al. 1998; Desplanque et al. 2000; Touzet et al. 2004). In contrast with other gynodioecious species, nonsterilizing mitotypes constitute a large part of the mitochondrial diversity in wild beet (Cuguen et al. 1994; Forcioli et al. 1998; Laporte et al. 2001). Hermaphrodites carrying one of these nonsterilizing mitotypes will be referred to as "normal" hermaphrodites.

STUDIED POPULATIONS AND SAMPLING PROCEDURES

To study the occurrence of gynodioecy in *Beta vulgaris* ssp. *maritima*, we chose to focus our sampling in Brittany, a region with frequent, large populations of sea beet as well as including many gynodioecious populations (Cuguen et al. 1994; Forcioli et al. 1998). Thirty-three populations were sampled to examine sex ratio variation within the Anglo-Normand gulf. These populations were precisely described for neutral cytoplasmic and nuclear genetic variation by Fievet et al. (2007) who described substantial genetic differentiation among all pairwise populations, and no significant departures from Hardy–Weinberg equilibrium for most of them. Among these 33 populations, 10 were insular, distributed in Jersey (5), Guernsey (3) and Chausey Islands (2), and 23 were continentally distributed along the coastline from St Vaast la Hougue (Vaa) in the East to Roscoff (Ros) in the West (Table 1, Fig. 1). The female frequency in population was estimated by counting the number of females (with white or brown empty anthers) over the total of phenotyped plants in the population. For each population, a large sample of flowering individuals was phenotyped, the size of the sample being proportional to the total size of the population. On average, during the first bloom of flowering 185 plants were phenotyped per population (median = 139) for a total of 6008 phenotyped plants, in mid-June 2000, 2002, or 2003 (1 year observation per population).

MOLECULAR INVESTIGATIONS

For further molecular investigations implying the genotyping of CMS-related cytoplasm, leaf tissue from 1309 individuals was collected. Number of individuals sampled ranged from 16 to 54 per population (median = 39, see Table 1). Extraction and purification of total DNA was performed using a DNeasy® 96 Plant Kit following the standard protocol for isolation of DNA from plant leaf tissue outlined in the DNeasy® 96 Plant protocol handbook (QIAGEN Inc., Hilden, Germany). To estimate the occurrence of the three main CMSs, *E*, *G*, and *Svulg*, we used diagnostic cytoplasmic PCR markers. The primers used as well as the detected polymorphisms are summarized in Table 2. The PCR step for each CMS was conducted using standard procedures. The reaction solution (15 μl) contained 20 ng of DNA, 3.5 mM $MgCl_2$, 200 μg/mL of BSA, 200 μM of each dNTP, 0.2 μM of forward and reverse primers, 0.375 U of *Taq* polymerase (Amplitaq, Applied Biosystems Inc., Foster City, CA). Amplifications were performed using a Applied Biosystems Gene-Amp system 9700 thermocycler, with the following method: 3 min denaturing at 95°C, followed by 35 cycles of 45 sec denaturing at 94°C, 45 sec annealing at 55°C (43°C for *Svulg*) and 1 min extension at 72°C, with a final extension step of 72°C for 10 min. For CMS *Svulg* and *E*, a subsequent restriction step on the PCR product was performed as described in Arnaud et al. (2003) and Dufay et al. (2008). For the present study, we did not attempt to detect the occurrence of CMS *H* because this cytotype is known to be absent from Brittany (Cuguen et al. 1994).

To gain further insight into the demographic and genetic equilibrium and the level of nuclear-cytoplasmic diversity of the 33 studied populations, we used additional molecular information provided by the dataset of seven nuclear microsatellite loci and four mitochondrial minisatellite loci previously described in Fievet et al. (2007) in a spatial genetic structure framework.

RATE OF RESTORATION WITHIN FEMALE PROGENIES

Maternal progenies were collected from female plants from 8 populations out of the 33 studied populations (Table 3). Twenty-one progenies (13 from plants carrying a CMS *G*, 8 from plants carrying a CMS *E*) were sown then vernalized 2 months later in a cold chamber to induce flowering the first year. Vernalized plants were subsequently transplanted into an experimental field for sex determination using the same sexual phenotype classification as in natural populations. For a given CMS, the number of restored hermaphrodites and the total number of plants that were

Table 1. Description of the 33 studied populations of *Beta vulgaris* ssp. *maritima*. For each population are given: acronym, name and geographical coordinates; the data from in situ observations: the number of hermaprodites (H), females (F), and the total of phenotyped plants (N), the data from CMS genotyping: the size of the sample (Ns), frequencies of CMS *E*, CMS *G*, and CMS *Sv*, the overall frequency of CMSs, the estimation of restoration rate for *E*, *G*, and *Sv*.

Acronym	Population	Latitude N	Longitude W	In situ observations				Data from CMS genotyping							
				H	*F*	*N*	Female freq.	Ns	Freq. CMS *E*	Freq. CMS *G*	Freq. CMS *Sv*	Freq. all CMSs	Resto. rate on CMS *E*	Resto. rate on CMS *G*	Resto. rate on CMS *Sv*
VAA	St Vaast la Hougue	49.587300	1.262366	50	0	50	0.00	44	0.00	0.00	0.00	0.00	–	–	–
CAP	Cap lévi	49.688383	1.473283	133	0	133	0.00	45	0.00	0.00	0.00	0.00	–	–	–
DI	Port de Dielette	49.549938	1.864895	91	36	127	0.28	38	0.26	0.05	0.00	0.32	0.30	0.00	–
PB	Portbail	49.330391	1.709271	300	52	352	0.15	32	0.19	0.09	0.28	0.56	0.67	0.00	0.89
ORM	Le gué de l'Orme	49.218540	1.608931	75	11	86	0.13	54	0.20	0.02	0.00	0.22	0.45	0.00	–
AGO	Pointe d'Agon	49.001552	1.574942	17	1	18	0.06	16	0.06	0.00	0.06	0.13	0.00	–	1.00
BRI	Briqueville sur mer	48.933679	1.543426	50	3	53	0.06	51	0.12	0.00	0.00	0.12	0.67	–	–
EP	Gué de l'Epine	48.650683	1.392766	34	9	43	0.21	42	0.17	0.17	0.05	0.38	0.86	0.00	0.50
M	Mont St Michel	48.633171	1.509457	165	122	287	0.43	34	0.18	0.35	0.18	0.71	0.67	0.17	0.67
VI	Le Vivier (Cancale)	48.633171	1.781600	317	68	385	0.18	30	0.57	0.00	0.00	0.57	0.65	–	–
GRO	Pointe du Grouin	48.711166	1.844400	69	20	89	0.22	37	0.35	0.00	0.00	0.35	0.38	–	–
V	Plage du Verger	48.696383	1.879883	89	39	128	0.30	33	0.27	0.12	0.00	0.39	0.33	0.00	–
LU	Iles Besnard	48.690816	1.950566	225	20	245	0.08	39	0.38	0.00	0.00	0.38	0.80	–	–
PO	Port St Jean	48.536508	1.961664	125	46	171	0.27	36	0.47	0.03	0.00	0.50	0.65	0.00	–
BO	Beaussay	48.577038	2.175217	236	82	318	0.26	35	0.17	0.20	0.00	0.37	0.33	0.29	–
PLE	Phéhérel plage	48.654383	2.365216	32	1	33	0.03	32	0.03	0.00	0.00	0.03	0.00	–	–
MOR	Plage du Morvan	48.570250	2.579102	79	3	84	0.04	50	0.08	0.00	0.00	0.08	0.75	–	–
PAL	Plage de Palus	48.676033	2.883883	111	16	127	0.13	48	0.25	0.04	0.00	0.29	0.67	0.00	–
ARC	Pointe de l'Arcouest	48.820466	3.021600	382	28	410	0.07	43	0.16	0.02	0.00	0.19	0.71	0.00	–
ROY	Trévou Tréguignec	48.824166	3.350100	172	25	197	0.13	47	0.06	0.06	0.00	0.13	0.00	0.00	–
TRE	Trébeurden	48.774033	3.582500	197	11	208	0.05	48	0.02	0.10	0.00	0.13	0.00	0.60	–
PRI	Primel Trégastel	48.713033	3.817850	182	34	216	0.16	44	0.25	0.00	0.00	0.25	0.36	–	–
ROS	Roscoff	48.720995	4.008234	588	37	625	0.06	48	0.04	0.06	0.00	0.10	0.50	0.33	–
CHOA	Grande grève (Chausey)	48.876691	1.837569	309	64	373	0.17	41	0.27	0.00	0.00	0.27	0.45	–	–
CHOB	La Cale (Chausey)	48.872551	1.826068	44	3	47	0.06	39	0.10	0.05	0.00	0.15	0.75	0.50	–
PE	Penbrook (Guernsey)	49.507483	2.532266	215	49	264	0.19	34	0.00	0.18	0.00	0.18	–	0.00	–

Continued

Table 1. Continued.

Code	Population	Latitude N	Longitude W	In situ observations				Data from CMS genotyping							
				H	*F*	*N*	Female freq.	Ns	Freq. CMS *E*	Freq. CMS *G*	Freq. CMS *Sv*	Freq. all CMSs	Resto. rate on CMS *E*	Resto. rate on CMS *G*	Resto. rate on CMS *Sv*
RB	Lihou (Guernsey)	49.454966	2.653266	117	23	140	0.16	33	0.15	0.09	0.00	0.24	0.40	0.00	–
MH	Moulin Huey (Guernsey)	49.426216	2.548950	40	0	40	0.00	34	0.00	0.00	0.00	0.00	–	–	–
ET	Etacq (Jersey)	48.240850	2.246383	157	41	198	0.21	39	0.10	0.03	0.23	0.36	0.75	0.00	0.44
B	Bonne Nuit Bay (Jersey)	49.251333	2.115350	125	10	135	0.07	38	0.11	0.00	0.00	0.11	0.25	–	–
SC	St Catherine's bay (Jersey)	49.220550	2.025750	171	4	175	0.02	49	0.12	0.00	0.00	0.12	0.83	–	–
CL	St Clement Bay (Jersey)	49.167116	2.061800	108	17	125	0.14	36	0.06	0.61	0.00	0.67	0.50	0.82	–
BP	Port bay (Jersey)	49.178600	2.208733	124	2	126	0.02	40	0.18	0.00	0.00	0.18	0.86	–	–
			Total			6008		1309							

phenotyped within the progeny were summed over the different progenies within each population. Rate of restoration within female offspring (named *RFo* hereafter) was calculated as the ratio of restored hermaphrodites over the total number of plants that were phenotyped.

Restoration rate within female progenies provides an estimation of the frequency of restorer allele in the pollen cloud. This frequency should directly depend on the frequency within the population of restored hermaphrodites carrying the considered CMS cytotype, as well as on the frequency of silent restorers (i.e., restorer alleles carried by hermaphrodites that do not bear the corresponding CMS), which was unknown in our study. We aimed to compare our obtained restoration rates to expected rates under two extreme scenarios, regarding to the frequency of silent restorers: (1) if restorer alleles only occur in hermaphrodites carrying the considered CMS (i.e., no silent restorers) and (2) if restorers occur at the same frequency in all hermaphrodites, whatever their cytotype are. Considering the simple case of a dominant restorer allele, these expected restoration rates were calculated as:

$$\mathrm{E}(RFo) = \alpha * freq(\mathrm{RH})_{\mathrm{i}}/[1 - freq(\mathrm{fem})] \quad (1)$$

$$\mathrm{E}(RFo) = \alpha * freq(\mathrm{RH})_{\mathrm{i}}/freq(\mathrm{CMS}i), \quad (2)$$

where E(*RFo*) is the expected rate of restoration in female's offspring (on CMS *i*); $freq(\mathrm{RH})_{\mathrm{i}}$ is the frequency of restored hermaphrodites bearing the CMS *i* in the population; *freq*(fem) is the frequency of females (of any CMS type), *freq*(CMS *i*) is the frequency of CMS *i* in the population and α is a factor varying from 0.5 to 1, according to the frequency of homozygotes for restorer allele ($\alpha = 0.5$ means that all restored hermaphrodites are heterozygotes; $\alpha = 1$, that they all are homozygotes). In scenario (1), females can only receive a restorer allele (with a probability equal to α) when pollen comes from a restored hermaphrodite carrying the same CMS. In all other cases, when pollen comes from hermaphrodites with normal or another CMS cytotype, no restorer allele can be transmitted. In scenario (2), the restorer allele occurs at the same frequency in the whole population, irrespective of the cytotype. We thus used the frequency of restored hermaphrodites among CMS *i* plants, as an estimation of the frequency of the restorer allele within the whole population. For each population and for each considered CMS, we then compared observed restoration rates within female progenies to these two ranges of expected values.

STATISTICAL ANALYSES

To estimate restoration rates within populations, we focused on individuals that carried a CMS-related cytotype after molecular screening. Based on field phenotyping, we scored the number of hermaphrodites for each of the three CMS cytotypes. The restoration rate was subsequently calculated as this number of (restored)

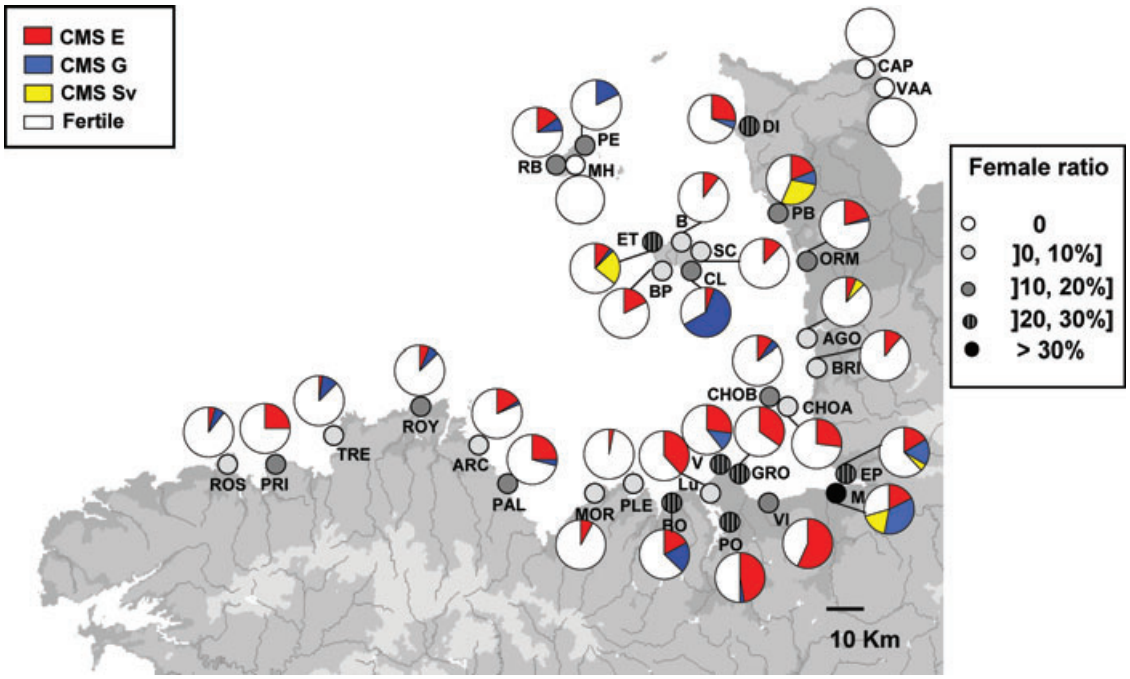

Figure 1. Map of the Anglo-Normand gulf and spatial location of the 33 studied populations of *Beta vulgaris* ssp. *maritima*. For each population, the estimated in situ female frequency is shown, as well as the frequency of CMS-cytotype *E* (red), CMS-cytotype *G* (blue), CMS-cytotype *Svulg* (yellow) and male-fertile cytoplasms (white).

hermaphrodites divided by the number of plants carrying the considered CMS cytotype.

We then used logistic regression (binomial distribution, Logit function, using proc GENMOD, SAS, version 9, SAS Institute Inc., Cary, NC) to analyze the variation of female frequencies, CMS frequencies, and restoration rates in populations. Depending on the analysis, the effects of population size (using as an estimation the number of plants that were phenotyped), CMS cytotype identity and number of CMS cytotypes occurring in the population were tested. Data were corrected for overdispersion (dscale option, proc GENMOD). To determine whether the variance in sex ratio depended on population size, we used the same method as Nilsson and Ågren (2006): we calculated the deviation of each population from the average sex ratio and we then regressed the absolute mean of this deviation with population size, using a proc GLM, SAS. To determine whether restoration rate increased with CMS frequency, we performed for each recorded CMS separately a linear regression on arcsine-square root transformed frequencies and recorded the coefficient of determination R^2. For all analyses using GLM models, we checked for the normality of residuals distribution; the normality hypothesis was never rejected (Kolmogorov–Smirnov test; $P > 0.15$ for all of these analyses presented below).

The study carried out on two types of populations, 10 insular and 23 continental populations. If insular populations are more isolated than those from the continent, one might expect strong founder effects to generate a larger variance in genetic and phenotypic characteristics. For this reason, for all of the variables that will be analyzed in this study (female frequency, CMS frequency, restoration rate), we tested for a difference between insular and continental populations. As no significant difference was found for any variable, we will present throughout this study island and continental populations within the same dataset, with no distinction between these two types of populations.

In an attempt to test whether the 33 sampled populations exhibited genetic evidence for departure from mutation–drift equilibrium as the result of recent population bottlenecks, we used methods based on loss of alleles and heterozygosity from selectively neutral loci (reviewed in Cornuet and Luikart [1996]). When a population experiences a reduction in effective size, allelic diversity is reduced faster than is heterozygosity because rare alleles tend to be eliminated more easily. As a result, there is a transient deficiency in the number of alleles found in a sample of individuals, that is, the observed number of alleles is less than that expected from the observed heterozygosity until genetic drift and new mutations begin to reestablish mutation–drift equilibrium (Cornuet and Luikart 1996). The relationship between heterozygosity and allele number differs according to the mutational process. The seven microsatellite loci used in Fievet et al. (2007) may not follow a simple stepwise mutation model (SMM) because they displayed pairs of alleles that differ by less than

Table 2. PCR primers and polymorphism for target CMSs in *Beta vulgaris* ssp. *maritima*.

CMS[1]	Target	DNA[2]	Forward primer sequence: 5′ to 3′	Reverse primer sequence: 5′ to 3′	Ta[3] (°C)	Fragment[4] size (bp)	Restriction	Polym.[4] (bp)	Reference
Svulg	petG-psbE	Cp	CAGTTACAAATAATCCAG	CACGATATGTGTAGATG	43	563	*Hind*III	454/563	Ran and Michaelis (1995)
G	*nad9*	Mt	TTTCTCTTTATGGATAACCAATTCA	AGGATTCCTTTTGTAACCCAAT	55	639/1028	–	639/1028	Ducos et al. (2001)
E	*psbA/trnK*	Cp	GTTCcCACTCAcGACCCATA	CCGACTAGTTCCGGGTTC	55	672	*Alu*I	(229+111)/340	Dufay et al. (2008)

[1]CMS for which the PCR marker has been developed.
[2]Mitochondrial (Mt) or chloroplastic (Cp).
[3]Temperature of annealing.
[4]In the x/y expression. x corresponds to the size of to the fragment(s) of the target CMS, y the size of the other cytoplasms.

one repeat unit, suggesting imperfect or compound microsatellite fragments. Therefore we used statistical tests for detecting heterozygosity excess under either the classical infinite allele model of mutation (IAM) or the two-phase model (TPM) that allow for a small proportion of multistep mutation changes. Using the software BOTTLENECK (Cornuet and Luikart 1996), simulations with 100,000 replicates were generated for each sampled populations to obtain the distribution of the gene diversity expected from the observed number of alleles under the assumption of mutation–drift equilibrium, given the sample size. Wilcoxon tests were applied to assess the statistical significance for departure from equilibrium. Bonferroni corrections for multiple tests were applied following Rice (1989).

Finally, to assess whether genetic diversity was a function of population size, linear regressions were performed (using proc GLM, SAS) between population size in one hand, and several measures of genetic diversity on the other hand, including mean nuclear allelic richness (*Ar*), mean nuclear gene diversity (*He*) and mean F_{IS}. All of these variables were calculated over the seven nuclear microsatellite loci. The same analysis was performed for the mean cytoplasmic allelic richness (*Ar*) calculated over the four mitochondrial minisatellite loci.

MODEL SIMULATIONS

If CMS frequency mirrors the likelihood for a nuclear gene to be carried by a CMS plant, and thus to be only transmitted by seeds, restorer alleles are usually thought to be strongly selected for when CMS is frequent. Consequently, one might expect CMS and restorer frequencies to be positively correlated. Hence, one of our aims was to determine whether restoration rate increased with the frequency of CMS-related cytotypes in populations. However, if frequency-dependent selection creates large oscillations of frequencies, as it had been suggested by several models, it is not clear whether this positive correlation remains. To answer this question, we used the predictions of a theoretical model proposed to study the conditions of maintenance of gynodioecy with a CMS and a male fertile cytotype (Dufay et al. 2007). This model was based on a large, panmictic population, and analyzed the stable maintenance of gynodioecy as a function of three fitness parameters: a cost of restorer allele, a female advantage and a CMS cost effect (thus fitness of female plants is the product of CMS effect and female advantage). This model showed that a large set of fitness parameters allowed the stable maintenance of gynodioecy, either producing a limit cycle or a stable equilibrium reached after a long phase of oscillations of CMS and female frequencies. We chose one realistic outcome generated by the model, as an example of oscillating frequencies, based on fitness parameters fitting to wild beet (Boutin et al. 1988), in particular a small female fitness of male sterile plants (cost of restoration = 0.3, female advantage = 1.4 and CMS effect = 0.75; thus, female fitness of

Table 3. Observed and expected values of *RFo* (rate of restoration in female offspring). For each measure, this table provides the population from which female progenies were sampled, the CMS carried by the mother plant, the number of plants within female offspring that were phenotyped (the value within brackets indicates the number of families, i.e., the number of mother plants used for the measure). Predicted values were calculated as indicated in the text. For each scenario, the lowest value was for α=0.5 (i.e., all restored hermaphrodites in the population were heterozygous at the restorer locus) and the highest for α=1 (i.e., all restored hermaphrodites in the population were homozygous).

Acronym	CMS	Sample size		Observed *Rfo*	Expected *Rfo* under scenario (1)	Expected *Rfo* under scenario (2)
DI	*G*	140	(3)	0.021	0	0
PB	*E*	41	(1)	0.219	[0.083–0.154]	[0.333–0.667]
M	*G*	163	(4)	0.092	[0.042–0.100]	[0.083–0.167]
PAL	*E*	64	(3)	0.234	[0.074–0.190]	[0.333–0.667]
PAL	*G*	67	(1)	0	0	0
ARC	*E*	71	(2)	0.309	[0.053–0.125]	[0.357–0.714]
ARC	*G*	50	(1)	0.020	0	0
ROY	*G*	36	(2)	0	0	0
TRE	*G*	112	(2)	0.143	[0.030–0.067]	[0.300–0.600]
PRI	*E*	94	(2)	0.011	[0.042–0.108]	[0.182–0.364]

male sterile = 1.05, i.e., 5% of female advantage compared to normal hermaphrodites). This set of parameters provided dampened oscillations of frequencies, which is more realistic in a population of finite size (see Fig. 3A). We recorded CMS frequency and restoration rate, defined as the ratio of CMS plants carrying a restorer allele, over 500 generations (until the point equilibrium was reached). If gynodioecious populations follow such a dynamics, each observation in a given population could be one of these 500 points. We then randomly sampled 1000 times 30 points among the 500 points (sampling with replacement). This provided 1000 different datasets that could all have been obtained by recording CMS frequency and rate of restoration over a sample of 30 different populations following this type of deterministic oscillations of frequencies over time. The choice of sampling 30 points was made to fit with what we observed in the field in term of number of gynodioecious populations (see results), allowing a comparison between simulated and field data. For each of these 1000 datasets, we assessed whether CMS frequency and restoration rate were correlated by recording the coefficient of determination R^2. The aim of this analysis was to compare this distribution of R^2 values expected under the hypothesis of deterministic oscillations with R^2 values that were obtained empirically on the various CMS cytotypes in this study, as described above.

Results

GYNODIOECY, CMS FREQUENCIES, AND SEX RATIOS

Overall, 14.6% of the 6008 plants that were scored during this study were females. Female plants were found in 30 populations over the 33 sampled populations (Fig. 1). In the 30 gynodioecious populations, female frequencies varied from 0.02 in Port Bay to 0.43 in Mont Saint-Michel (median = 0.135). Neither the value of female frequency ($\chi^2 = 1.03$, df = 1, $P = 0.31$) nor its variance (deviance from the average female frequency: $F_{1,29} = 0.04$, $P = 0.85$) depended on population size.

Three different CMS-related cytotypes, *E*, *G*, and *Svulg*, were found in this study. Among plants that were screened for cytoplasmic markers, all plants that had been scored as females carried one of these three CMS cytotypes, meaning that all possible sources of male sterility were detected in the study area. Over the 1309 individuals screened for cytoplasmic markers, 24% were found carrying one of the three target CMS cytotypes, the other plants being associated with a male fertile cytotype (Fig. 1). It must be noted that male fertile cytotypes were found in all populations. In nongynodioecious populations, they represented the totality of screened individuals, meaning that hermaphroditic populations were not populations in which one or several CMS were completely restored for male fertility. In gynodioecious populations, male fertile cytotypes were carried by from 29% (in Mont Saint Michel) to 97% (in Pléhérel) of screened individuals. The frequency of CMS plants found in each population did not depend on population size ($\chi^2 = 0.46$, df = 1, $P = 0.49$).

Among gynodioecious populations, 11 populations contained one CMS cytotype, 15 contained two different CMSs and four populations contained all three CMS cytotypes. A logistic regression performed on the 30 gynodioecious populations revealed that the frequency of individuals bearing a CMS (irrespective of their cytotype) increased when several different CMSs co-occurred in the population ($\chi^2 = 3.74$, df = 1, $P = 0.05$): the frequency of CMS individuals was significantly

lower in populations containing only one CMS-related cytotype compared with populations containing two or three CMS-related cytotypes.

As expected when male sterility is determined by cytoplasmic genes, the frequency of females in populations significantly increased with the frequency of individuals carrying a CMS-related cytotype ($\chi^2 = 25.51$, df $= 1$, $P < 0.0001$). Indeed, the more CMS cytotypes in a population, the more individuals are expected to express male sterility, provided that they do not bear a restorer gene. In the same way, the frequency of females depended on the number of different CMS cytotypes occurring within populations ($\chi^2 = 6.28$, df $= 2$, $P = 0.04$). More precisely, females were significantly more frequent (26% of females on average) within populations containing three different cytotypes compared to populations containing either one (9% of females) or two (15% of females) different CMSs (contrast analyses in proc GENMOD, $P < 0.05$).

The three CMSs had different frequencies within populations. Overall, CMS *E* was the most frequent one, occurring in 29 out of the 30 gynodioecious populations and was found in 16% of all screened individuals whatever the phenotype they exhibited. In contrast, CMS *G* was found in only 7% of plants overall and in 18 populations; CMS *Svulg* in only 2% of individuals overall across five populations. A logistic regression performed on the number of CMS plants over the number of screened individuals in each population indicated that these differences in frequency were significant among the three CMS-related cytotypes ($\chi^2 = 34.08$, df $= 2$, $P < 0.0001$; all contrast analyses being significant, $P < 0.05$).

TESTS OF GENETIC EQUILIBRIUM

Because populations could be prone to wide oscillations in size, the current population size solely could not be a reliable estimator of stochasticity intensity. Consequently, we used genetic data from a previous study conducted on the same populations (Fievet et al. 2007) to test whether the 33 populations exhibited genetic evidence for departures from mutation–drift equilibrium as the result of recent population bottlenecks. For 28 populations out of 33, no deviations from mutation–drift equilibrium were detected from the expected heterozygosity based on the number of alleles, either under the IAM or the TPM model of mutation. Only the populations M, Vi, Bo, ChoB, and Pe exhibited minor deviations with significant heterozygosity excess at $P < 0.05$ using Wilcoxon tests. Nonetheless, the statistical significance remained for none of these populations after Bonferroni correction. Altogether, these results are suggestive of no recent reduction in population size and suggest a genetic equilibrium for all sampled populations. Moreover, no correlation was found between population size and nuclear genetic diversity for any of the measures of diversity we tested (i.e., allelic richness *Ar*, *He*, and F_{IS}; $P > 0.1$ for all analyses). The same result held for cytoplasmic *Ar* based on mitochondrial minisatellite loci.

RESTORATION OF MALE FERTILITY WITHIN POPULATIONS

Restoration rates (estimated from genotyped plants) were significantly different among CMS-related cytotypes ($\chi^2 = 9.01$, df $= 2$, $P = 0.01$). On average, 50%, 15%, and 66% of CMS plants were restored for male fertility, for CMS *E*, *G*, and *Svulg*, respectively. Contrast analyses performed by proc GENMOD revealed that the rate of restoration was significantly lower for CMS *G* than for the two other CMSs ($P < 0.05$), with no difference between CMS *E* and *Svulg* ($\chi^2 = 0.55$, df $= 1$, $P = 0.46$). By considering each CMS cytotype individually, we found no effect of population size on restoration rate (CMS *E*: $\chi^2 = 0.21$, df $= 1$, $P = 0.65$; CMS *G*: $\chi^2 = 0.1$, df $= 1$, $P = 0.75$; CMS *Svulg*: $\chi^2 = 1.17$, df $= 1$, $P = 0.28$).

To determine whether the rate of restoration increased with CMS frequency, we reduced our analyses to CMS *E* and *G* because CMS *Svulg* was found only in five populations (Fig. 2A,B). A linear regression performed on populations containing CMS *E* revealed that restoration rate (arcsine-square root transformed) only marginally depended on CMS *E* frequency ($F(1,27) = 3.65$; $P = 0.07$; coefficient of determination $R^2 = 0.17$). Considering CMS *G*, the same analysis showed that restoration rate significantly increased with CMS *G* frequency ($F(1,16) = 8.63$; $P = 0.009$), although the part of the model explained by CMS *G* frequency was moderate (coefficient of determination $R^2 = 0.33$). However, in this case, the correlation entirely relied on one particular point (Saint Clement Bay—CL): when this particular point was removed from the dataset, the R^2 value dropped to 0.07, leading to a situation of no correlation between CMS and restorer frequencies. To compare these empirical results with theoretical expectations under FDS, the same regression analyses were performed on the 1000 datasets obtained by randomly sampling 30 points on a theoretical limit cycle (Fig. 3A). Overall, this provided very weak correlations between CMS frequency and restoration rate: R^2 values were lower than 0.2 in half of the cases, and lower than 0.5 in 90% of the cases (Fig. 3B). It must be noted that when additional dampened cycles generated by the model were analyzed in a similar way, they similarly provided weak correlation between CMS frequency and restoration rates (data not shown).

RATE OF RESTORATION WITHIN FEMALE PROGENIES

The proportion of restored hermaphrodites obtained within female progenies was extremely variable from one population to another. For CMS *E*, it varied from 0.01 to 0.31 and for CMS *G*

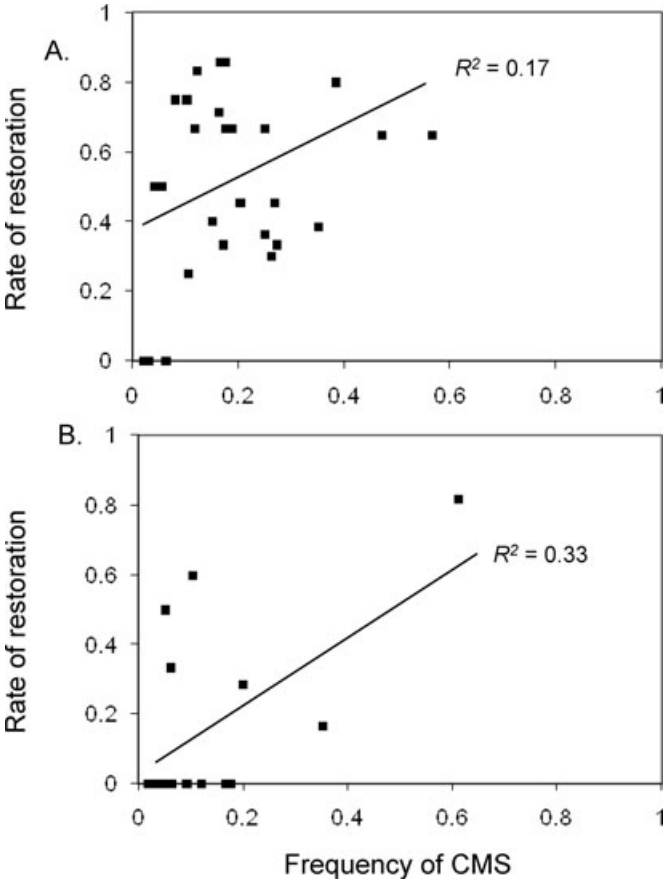

Figure 2. **Frequency of CMS and rate of restoration in populations for CMS *E* (A) and CMS *G* (B); untransformed frequencies were used to build the figures. Each point pictures one population, in which the frequency of CMS was estimated through genotyping, and restoration rate was calculated as the ratio of hermaphrodites carrying the considered CMS cytotype, over the total number of plants carrying this cytotype. The regression line between CMS and restorer frequencies is pictured in both figures, as well as the R^2 value, that was calculated on arcsine-square root transformed frequencies.**

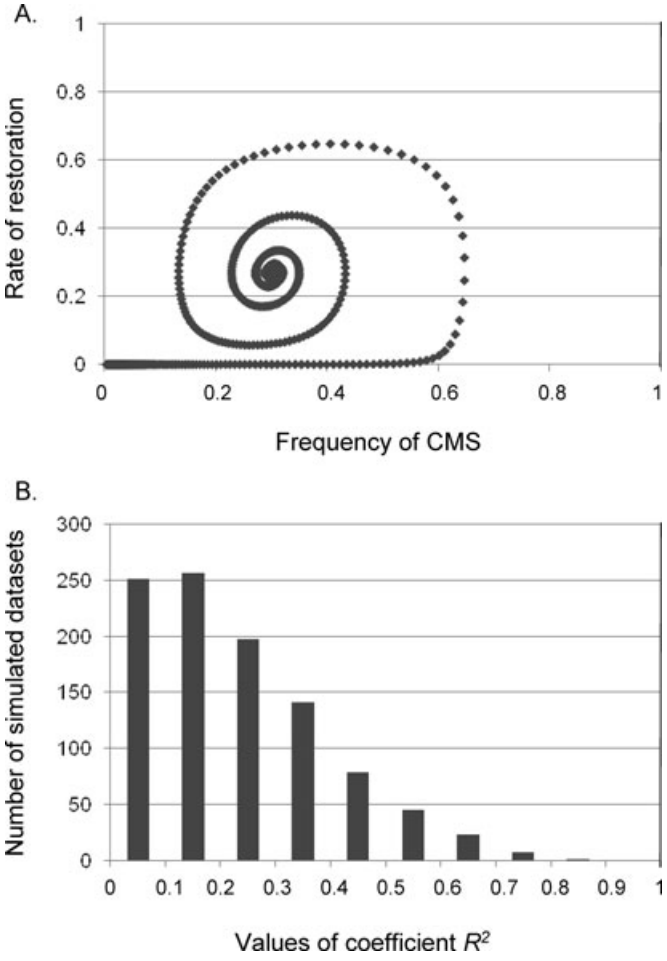

Figure 3. **Correlation between CMS frequency and restoration rate following a theoretical model based on frequency-dependent selection. Figure 3 (A) represents an example of temporal evolution of CMS frequency and restoration rate within a theoretical infinite gynodioecious population. During the first generations, the restoration rate is very low and CMS increases in frequency; restorer alleles are then selected and their frequency increases, which entails a counter selection of CMS. Consequently, the population follows dampened oscillations of both frequencies and finally reaches a point equilibrium, at which CMS frequency is 0.3 and restoration rate equals 0.28. A total of 1000 datasets were generated by randomly sampling 30 points from the 500 points pictured in (A). For each of these datasets, the coefficient of determination R^2 was calculated to measure the correlation between CMS frequency and restoration rate. The distribution of the 1000 values of R^2 is represented in Figure 3 (B).**

from 0 to 0.14 (Table 3). A logistic regression revealed that this proportion was marginally higher for CMS *E* than for *G* ($\chi^2 = 2.79$, df = 1, $P = 0.09$). It did not depend on the frequency of the considered CMS cytotype within the population ($\chi^2 = 0.58$, df = 1, $P = 0.45$) but it did significantly increase with the frequency of restored hermaphrodites carrying the CMS cytotype under consideration ($\chi^2 = 10.73$, df = 1, $P = 0.0011$). In two populations, no in situ restoration had been observed in the plants bearing CMS *G*, but 2% of plants within female offspring were restored hermaphrodites, suggesting that restorers occurred in these populations, but probably at too low frequency to be detected during sampling.

Our results were then compared with values of restoration rates expected under the two scenarios described earlier (see material and methods): scenario (1) under which restorer alleles only occurred in restored hermaphrodites and scenario (2) under which restorer alleles were widespread in the whole hermaphroditic population (Table 3). Considering the populations in which restored hermaphrodites had been observed, in all cases but one, the observed restoration rate within female progenies was higher than the maximum expected value under scenario (1) and lower than

the minimum expected value under scenario (2) whatever the CMS considered (Table 3).

Discussion

SEX RATIO VARIATION AMONG WILD BEET POPULATIONS AND CMS CYTOTYPE POLYMORPHISM

Beta vulgaris spp. *maritima* is a gynodioecious species, 15% of plants in this study being females. A large variation of sex ratios was reported among gynodioecious populations in wild beet: female frequencies varied from 2% to 42%. Except for some species, in which very high female frequencies can be found in some natural populations, such as *Thymus vulgaris*, *Lobelia spicata*, *Plantago maritima, Silene vulgaris*, or *Lobelia siphilitica* (Thompson et al. 1998; Byers et al. 2005; Nilsson and Ågren 2006; Olson et al. 2006; Caruso and Case 2007), this range was comparable to what was found in many other gynodioecious species, such as *Saxifraga granulata*, *Chionochloa bromoides*, *Cirsium chikushiense*, *Plantago coronopus*, *Plantago lanceolata*, *Geranium sylvaticum*, *Nemophila menziesii*, *Raphanus sativus*, *Daphne laureola*, or *Kallstroemia grandiflora* (Stevens and Richards 1985; Connor 1990; Kawakubo 1994; Koelewijn et al. 1996; De Haan et al. 1997; Asikainen and Mutikainen 2003; Barr 2004; Murayama et al. 2004; Alonso 2005; Cuevas et al. 2006).

We showed that the frequency of females was positively correlated with the frequency of plants carrying a CMS, as expected when sex is partially determined by cytoplasmic genes. Additionally, the frequency of females increased with the number of CMS cytotypes occurring within the population. This effect of the number of CMS cytotypes on the sex ratio was suggested by theoretical works of Gouyon et al. (1991), Manicacci (1993), and Dufay et al. (2007), all based on FDS. The fact that cytoplasmic diversity could directly influence female frequency shows the critical importance of knowing the genetic determination of sex and cytotypes diversity to better understand how sex ratio varies among populations.

In the gynodioecious populations of *Beta vulgaris* spp. *maritima*, we found high frequencies of male fertile cytotypes. This result confirms what was already known about this species (Cuguen et al. 1994; Forcioli et al. 1998; Laporte et al. 2001) and constitutes a key difference with other well-studied gynodioecious species, such as *Silene vulgaris*, *Silene acaulis*, *Plantago coronopus*, or *Thymus vulgaris* which appear to only contain CMS cytotypes (Manicacci et al. 1997; Olson and McCauley 2002; Van Damme et al. 2004; Klaas and Olson 2006). Moreover, in this study, three wild beet populations appeared to be nongynodioecious and to contain only male fertile cytotypes. Consequently, these populations differed from what is sometimes found in some other gynodioecious species, in which purely hermaphroditic populations are composed by CMS cytotypes for which restorer alleles have been fixed.

THREE CMSS WITH DIFFERENT FEATURES

In the studied region, male sterility in wild beet was due to three different CMS cytotypes, *E*, *G*, and *Svulg* that presented very different features. First, the three CMSs were found at very different frequencies, *E* being the most frequent CMS, present in all but one gynodioecious population, and *Svulg* being rare and being found in only five populations. It must be noted, that *Svulg* (also called CMS *Owen*) is carried by all cultivated sugar beet varieties and its occurrence in wild populations is generally considered as the result of gene flow from cultivated fields to natural sea beet populations (Viard et al. 2004; Fénart et al. 2008). At this stage, it is not possible to establish the source of CMS *Svulg* found in the analyzed populations: they may be either suggestive of ancient seed escape from former cultivated beet fields or genuine wild cytotypes.

The low frequency of CMS *G* compared to *E* may be due to a lower magnitude of female advantage, as predicted by theoretical models (Bailey et al. 2003; Dufay et al. 2007), but comparative data on female advantage in wild beet are currently lacking to confirm this hypothesis. Alternatively, the difference in frequency between *G* and *E* may be due to a birth-age difference. Unfortunately, the low resolution of the haplotype network built between the different cytotypes found in wild beet accessions does not enable us to confirm or reject the hypothesis of a more recent origin for CMS *G* compared to CMS *E* (Fénart et al. 2006).

The three CMS cytotypes also exhibited differences in terms of restoration rate, CMS *G* being significantly less restored than the two others. Because CMS *Svulg* was very rare in our populations, the focus was directed on CMSs *E* and *G*. Variations in restoration rate between CMSs *E* and *G* may be linked to their differences in frequency: because *E* is more widespread and more frequent than *G*, one can assume that selection for restorers of CMS *E* is stronger compared to restorers of CMS *G*. A second, nonexclusive, hypothesis, would be that CMSs *E* and *G* differ in their physiological ease of restoration due to the nature of the corresponding sterilizing genes. Indeed, in the case of CMS *G*, variants of subunits of mitochondrial respiratory complexes have been found, that could affect their activity and be possibly the cause of CMS through energy limitation during pollen production (Ducos et al. 2001). Therefore, one might expect restoration to be more constrained (involving a gene coding for a complementary subunit of the same respiratory complex) than in the case of a mitochondrial chimeric gene, often described in crop CMS, for which a large nuclear family, coding for PPR proteins, seems to be a natural reservoir (Touzet and Budar 2004; Geddy and Brown 2007).

WHY DOES SEX RATIO VARY AMONG POPULATIONS?

Sex ratio in a population is the combined output of CMS frequency and restoration rate for each CMS type. In this study, we found that both CMS frequencies and restoration rate could strongly vary from one population to another. As a consequence, sex ratio also varied strongly, from 0% to 42% of females. In gynodioecious species, sex ratio variation among populations can be due to stochastic processes, environmental variation, or deterministic oscillations due to frequency-dependent selection. In this study, natural populations were all located in the same geographic area, and plants were found in the same habitat (coastal populations, just at the upper level of the tide). The large variation of sex ratio was thus unlikely to be only explained by an environmental gradient. An effect of stochasticity, however, could be suggested by the fact that for CMSs *E* and *G*, respectively, no and moderate correlations were found between restoration rate and CMS-related cytotype frequencies. This is at least how Murayama et al. (2004) interpreted a similar result, found on *Raphanus sativus*, in which CMS frequency was correlated to female frequency but not to restorer frequency. Because one intuitively expects restorers to be selected when CMS is frequent in a population, no correlation between CMS frequency and restoration rate could indeed be interpreted as an effect of stochastic processes that would hide the effect of selection.

However, no other clue for an effect of stochasticity on sex ratio was found in our study. We found no effect of population size on sex ratio, nor on sex ratio variance. The same result was found for CMS frequency and rate of restoration. This contrasts with what was found on two other gynodioecious species, *Plantago maritima* (Nilsson and Ågren 2006) and *Lobelia siphilitica* (Caruso and Case 2007). In *P. maritima*, females were often absent from small populations, whereas in *L. siphilitica* females were more common in small populations. These two contrasted results can be both interpreted as the possible consequence of drift, which may alter female frequencies by causing the loss of either CMS or restorer alleles. In *Beta vulgaris* spp. *maritima*, however, no such result was found. Besides, analyses of genetic markers suggested that these natural populations were at genetic equilibrium and had not suffered from a recent reduction in population size. Hence, our results suggest that studied populations of *Beta vulgaris* spp. *maritima* are quite stable, well-established populations, and stochastic processes occurring within these populations are not strong enough to be detected. As a consequence, it appears that stochasticity may not play a major role in the variation of sex ratio in wild sea beet in the studied region.

This led us to investigate whether an absence of correlation between CMS frequency and restoration rate (i.e., the only one result that could suggest an effect of stochasticity on sex ratio) could also be explained by the alternative dynamics, that is, frequency-dependent selection. When 30 points were randomly chosen among 500 simulated generations following deterministic oscillations of CMS and restorer frequencies (see Fig. 3), it provided weak correlation between CMS and restorer frequencies. In half the cases, values of coefficient of determination R^2 were lower than 0.2, corresponding to what was found for CMS *E* ($R^2 = 0.17$). Besides, correlation between CMS frequency and restoration rate were sometimes slightly better (R^2 values comprised between 0.2 and 0.5 for about 40% of simulated datasets); in these cases, at least one point with high values of both CMS frequency and restoration rate was included in the dataset, increasing the correlation. Such points correspond to a transitory step of oscillating dynamics, during which the restorer allele has been strongly selected for and has increased in frequency, and CMS has not been counter-selected yet. This actually fits what was observed in the case of CMS *G*, for which the significant correlation and the moderate value of R^2 (0.33) was entirely explained by the population Saint Clement Bay (CL), in which 82% of individuals were carrying the CMS G, with a restoration rate equal to 0.61 (Fig. 2B). Hence, both the results obtained for CMS *E* and *G* (i.e., either no correlation or a weak correlation that relies on a single population) are situations expected under deterministic oscillations of frequencies. Finally, it should be stressed that the first phase predicted by selection models shows a very fast increase in frequency of unrestored CMS (carried by females); selection of restorer alleles actually takes place quite late, during a second phase that leads to a decrease of female frequency (Gouyon et al. 1991; Bailey et al. 2003; Dufay et al. 2007). Consequently, even without considering the deterministic oscillations that could follow these two first phases, the patterns of selection of CMS and restorer alleles actually do not predict CMS and restorer frequencies to be strongly and positively correlated with each other. This suggests that stochasticity is not the only process that must be invoked to explain that CMS and restorer frequencies do not covary; indeed, we would further suggest that a strong correlation between these two frequencies should be rarely observed, even if natural selection were the main process that acted on these frequencies in populations.

As emphasized by Olson and McCauley (2002), a way to gain further insight into the evolution of sex ratios in such a network of populations would be to determine the joint population genetic structure of both nuclear restorer genes and CMS genes and to compare them to neutral genetic variation. The population structure of CMS and nuclear restorer genes is probably affected by both stochastic and selective factors, but the relative magnitude of these factors may be very difficult to assess, especially through a dynamic equilibrium when populations differ in sex ratio because they are not located in the same position along cycling evolutionary trajectories. Moreover, to what degree the possible difference in genetic structuring at neutral nuclear and cytoplasmic genes compared to the interacting set of nuclear

restorers and CMS genes may arise as a consequence of selection is not supported by clear theoretical expectations. For instance, high F_{ST} estimates might result either from limited seed flow among populations or from localized selection favoring different CMS haplotypes, making it difficult to disentangle the stochastic and selective effects (McCauley 1998). One potential way to determine whether population genetic structure can differentially affect cytoplasmic genes involved in sex determination would be to compare levels of genetic diversity and associated structure among gynodioecious and nongynodioecious populations in a similar demographic situation.

RESTORATION WITHIN FEMALE PROGENIES

The last variable on which this study concentrated, was the frequency of restored hermaphrodites observed in female's progenies, which was measured in a subsample of populations. This provided some information about how restorer frequency, and subsequently sex ratio, could vary across generations. The values that we obtained for *RFo* (restoration in female offspring) did match with what we know about the restoration of male fertility in the study area. First, this restoration rate was positively and significantly related to the frequency of restored hermaphrodites occurring within the population and carrying the considered CMS cytotype: assuming random mating, the more restored hermaphrodites within the population, the more the females are susceptible to receive a pollen grain carrying a restorer allele. As a consequence, *RFo* values were marginally higher for CMS *E* than for CMS *G*, being presumably consistent with the fact that restorers of CMS *G* are less frequent than those of CMS *E* within populations. Second, *RFo* was not correlated with the CMS-related cytotype frequency. This fits the idea, discussed earlier, that the CMS frequency is not a reliable predictor of restorer frequency within a population: if a CMS type is frequent, but mainly carried by females, this will clearly not give rise to a high *RFo* frequency.

Our results were then compared to expected values under two extreme theoretical scenarios. Scenario (1) involved a population with restorer alleles carried only by restored hermaphrodites, meaning that there were no silent restorers in the populations (restorer alleles in individuals that do not bear the corresponding CMS). This is expected if the cost associated is of high magnitude in normal hermaphrodites (or with another CMS) and thus restorer alleles are highly counter selected. Such cost of silent restorers, paid by the plants that do not carry the corresponding CMS, has been theoretically showed to be a necessary condition for FDS to maintain gynodioecy (Gouyon et al. 1991; Bailey et al. 2003; Dufay et al. 2007). However, we found that *RFo* was usually higher than the expected values under scenario (1). In contrast, scenario (2) hypothesized that restorers were widespread in the population and could be provided by other hermaphrodites, in particular normal hermaphrodites (no cost hypothesis). Besides the fact that *RFo* values were always lower than expected values under scenario (2), we found arguments against this scenario: first, restorer alleles occurring at the same frequency in CMS and male fertile cytotypes would mean that restorer alleles ensure no silent cost, which is not consistent with theoretical studies (Gouyon et al. 1991; Bailey et al. 2003; Dufay et al. 2007); second, a recent empirical study carried out on CMS *E*, in two natural populations of *Beta vulgaris* spp. *maritima*, strongly suggested a silent cost of restoration on male function (Dufay et al. 2008).

The fact that values of *RFo* were intermediate between expected values under the two scenarios can be explained by two nonexclusive causes. First, restorer alleles also occur in normal hermaphrodites (contrarily to scenario (1)) as the result of within population gene flow, as expected in outcrossed wind-pollinated species, but at lower frequency than under scenario (2). This would be expected if restorer alleles are associated with a moderate fitness cost. Second, populations are not perfectly panmictic and pollen flow would be higher among neighboring plants carrying the same CMS cytotype owing to short-range pollen dispersal. This may be a common phenomenon in gynodioecious species, in which CMS cytotypes are often spatially aggregated, due to spatially restricted seed dispersal (McCauley 1997, 1998; Olson and McCauley 2002; McCauley et al. 2003; Olson et al. 2005, 2006; Klaas and Olson 2006; Fievet et al. 2007). In *Beta vulgaris* spp. *maritima*, such spatial aggregation of CMSs has been shown in natural populations (Laporte et al. 2001) and pollen flow indeed appears to preferentially occur within patches of genetically related plants sharing the same CMS cytotype (unpubl. data). This could explain why the values of *RFo* were always higher than expected values under scenario (1): even when restored hermaphrodites are relatively rare, females located in their vicinity would preferentially receive pollen grains from them.

RFo, the frequency of restored hermaphrodites in female's progenies, is an estimate of restorer frequency in the pollen cloud that is deposited on females and thus directly affects the temporal evolution of sex ratio in populations. Thus, our results emphasize the critical importance of precisely assessing patterns of pollen flow within populations to gain further insights into the evolutionary dynamics of gynodioecy and sex ratio evolution. In our studied populations, *RFo* values were sometimes of relatively high magnitude, suggesting that sex ratio may rapidly change in some gynodioecious populations of wild beet, due to the selection of restorer alleles, as previously suggested by Boutin-Stadler et al. (1989).

Conclusion

This study, by bringing together data on CMS identity, CMS frequency, restoration rate, and female frequency, allowed a better understanding of the various processes affecting sex ratio in

gynodioecious populations. We showed that three different CMS cytotypes occurred in populations and differed for their frequency and for their restoration rate. Consequently, the various CMS cytotypes did not equally contribute to the expression of gynodioecy in wild beet. We found no obvious impact of drift on sex ratios, CMS frequencies, and restoration rates in populations. On the other hand, we showed that the values of sex ratios in wild beet, as well as the weak correlation between CMS frequency and restorer frequency were consistent with what would be expected under frequency-dependent selection. However, future studies should focus on other geographical regions, with more variable demographic and ecological conditions, to assess the possible impact on sex ratio evolution of other evolutionary forces, such as genetic drift and environmental-mediated variation of the strength of natural selection. Finally, what we found about restoration of male fertility in females' progenies could suggest that effective gene flow may preferentially occur among neighboring plants carrying the same cytotype. Because such processes will have crucial importance for the evolutionary dynamics of gynodioecy, this work underlines the necessity of studying both seed and pollen flow within populations to understand the spatial and temporal variation of sex ratio among populations.

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