The Spatial Structure of Sexual and Cytonuclear Polymorphism in the Gynodioecious *Beta vulgaris* ssp. *maritima*: I/ at a Local Scale

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ABSTRACT

We have analyzed the spatial distribution of the sex phenotypes and of mitochondrial, chloroplast, and nuclear markers within two gynodioecious populations of *Beta vulgaris* ssp. *maritima*. Within both populations, sexual phenotype variation is controlled mainly by the cytoplasmic genotype, although in one study population a joint polymorphism of cytonuclear factors is clearly involved. In spite of contrasts in the ecology (mainly due to different habitats), a clear common feature in both populations is the highly patchy distribution of cytoplasmic haplotypes, contrasting with the wide distribution of nuclear diversity. This high contrast between cytoplasmic *vs.* nuclear spatial structure may have important consequences for the maintenance of gynodioecy. It provides opportunities for differential selection since nuclear restorer alleles are expected to be selected for in the presence of their specific cytoplasmic male sterile (CMS) type, but to be neutral (or selected against if there is a cost of restoration) in the absence of their CMS type. Selective processes in such a cytonuclear landscape may explain the polymorphism we observed at restorer loci for two CMS types.

GYNODIOECY, the coexistence of hermaphrodite and female (male sterile) individuals in natural populations, is relatively frequent in angiosperm species (Delannay 1978). This has motivated numerous studies because the maintenance of this sexual polymorphism is unexpected according to Fisher's sex ratio theory (Fisher 1930), except if male sterile individuals have an increased female fitness that counterbalances the absence of male function. This condition is not fulfilled in most of the cases (reviewed in Gouyon and Couvet 1987). In theory, this inconsistency can be resolved by considering the genomic conflict resulting from a cytonuclear sex determination system (Cosmides and Tooby 1981). Maternally inherited male sterile factors are selected for when associated with increased female fitness, even if this is small (Lewis 1941; Lloyd 1974; Charlesworth and Ganders 1979; Frank 1989). Indeed, in most gynodioecious species, the sexual phenotype is determined by an interaction between cytoplasmic male sterile (CMS) genes and nuclear genes restoring the male function (reviewed in Charlesworth 1981).

Theoretical models, however, have outlined the difficulties of maintaining such a joint cytonuclear polymorphism, since in the absence of a large female advantage, nuclear restorer alleles are expected to sweep to fixation (Charlesworth and Ganders 1979; Delannay *et al.* 1981; Ross and Gregorius 1985; Frank 1989; Gouyon *et al.* 1991; McCauley and Taylor 1997; Couvet *et al.* 1998). Two chief processes were conceived that can explain joint cytonuclear polymorphism. First, additional selective processes, in particular, deleterious effects of the restorer alleles in their alien cytoplasmic type, can explain the maintenance of a sexual polymorphism in a panmictic population (Charlesworth and Ganders 1979; Delannay *et al.* 1981; Frank 1989; Gouyon *et al.* 1991). Second, population structure has long been recognized as a major factor influencing the maintenance and the loss of genetic variation (*e.g.*, Slatkin 1977, 1985, 1987; Wade and McCauley 1988). Indeed, the importance of nonequilibrium metapopulation processes for maintaining gynodioecy includes founder effects (Frank 1989; McCauley and Taylor 1997; Pannell 1997; Couvet *et al.* 1998) and recurrent introduction of cytoplasmic male sterile types through mutations of the mitochondrial genome (Frank 1989).

Cytonuclear sex determination systems have been experimentally well documented (reviewed in Charlesworth 1981). However, relatively few studies have inves-

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tigated empirically the parameters required to maintain a joint cytonuclear polymorphism, although indications of a cost of restoration were obtained in *Thymus vulgaris* (Couvet *et al.* 1986; Manicacci *et al.* 1997; Gigord *et al.* 1998), *Plantago lanceolata* (De Haan *et al.* 1997a), and *P. coronopus* (Koelewijn 1996). Furthermore, the importance of nonequilibrium dynamics was illustrated in *T. vulgaris* (Belhassen *et al.* 1989; Manicacci *et al.* 1996). In particular, few studies have investigated the spatial distribution of genetic diversity in gynodioecious species. A high between-population spatial differentiation of cytoplasmic markers was found in *Beta vulgaris* ssp. *maritima* (Forcioli *et al.* 1998), as well as in two insect-pollinated species, *T. vulgaris* (Tarayre *et al.* 1997) and *Silene vulgaris* (McCauley 1998). In the latter species, comparisons with nuclear markers also suggested considerably higher rates of gene flow for nuclear than for cytoplasmic genes, a situation that would not facilitate the maintenance of a joint cytonuclear polymorphism (Frank 1989; Couvet *et al.* 1990, 1998). Other studies, however, have documented a high between-population differentiation of the nuclear factors involved in the male fertility restoration, as expected under the hypothesis of a cost of restoration (Couvet *et al.* 1986; Manicacci *et al.* 1997; Gigord *et al.* 1998), although a precise quantification and comparison with neutral markers was not possible due to the lack of specific molecular markers. Another very interesting study of the association between sexual phenotypes and cytoplasmic markers within one *T. vulgaris* population provided evidence of the importance of founder effects, leading to a local and temporally cytoplasmic sex determination system, thus favoring the maintenance of gynodioecy (Manicacci *et al.* 1996).

This study is also concerned with the spatial distribution of the genetic diversity at a very local scale within gynodioecious populations. This is, however, the first study that integrates the investigation of sexual phenotypes, mitochondrial, chloroplast, anonymous nuclear markers [both restriction fragment length polymorphism (RFLP) and microsatellites], and nuclear markers loosely linked to selected genes (RFLP). We have examined the spatial distribution of these markers within two gynodioecious populations of *B. vulgaris* ssp. *maritima* with contrasting habitats. First, we determined whether the sexual polymorphism within populations is due to a joint cytonuclear polymorphism, critical information for understanding the maintenance of gynodieocy. Moreover, fine mapping of >100 individuals per population allowed us to quantify and compare the local spatial distribution of phenotypic and molecular markers using spatial autocorrelation, *F*-statistics, and linkage disequilibrium analyses. Through these different analyses, the relative magnitude of pollen *vs.* seed dispersal and the importance of founder effects and selective processes were assessed at this local scale.

MATERIALS AND METHODS

The species: *B. vulgaris* can be divided into the wild form, *B. vulgaris* subsp. *maritima*; the cultivated form, *B. vulgaris* subsp. *vulgaris*; and the weed form that originates from introgression from wild to cultivar populations and infests crop fields (Boudry *et al.* 1993; Desplanque *et al.* 1999). Sea beet, *B. vulgaris* subsp. *maritima*, is a diploid species ($2n = 18$) widely distributed around the Mediterranean Basin and along the coasts of western Europe, but becoming rare in the north of France. In the southern part of its distribution, populations are found all along the coasts as well as inland, although in the north, wild populations are restricted to the sea coasts and are more patchily distributed. This study was carried out in the north of France. In this region, sea beets mainly colonize areas located along estuaries, just at the upper level of the tide and, more rarely, cliffs overhanging the sea.

In the north of Europe, sea beets are short-lived perennials (Boudry 1994) and the gene *B*, which controls the vernalization requirement for flowering (Boudry *et al.* 1994), is fixed for the *b* allele, which determines a vernalization requirement (Van Dijk *et al.* 1997). The species is wind pollinated and self-incompatible (Larsen 1977). Fruits are aggregated in an irregular dry body that can contain 1–7 seeds. The aggregated fruits have no specialized dispersal mechanisms and are primarily dispersed by gravity and by tidal water movements for the populations located along the estuaries (Raybould *et al.* 1997). Wild beets are gynodioecious: both female and hermaphrodite individuals coexist frequently within natural populations. The sexual phenotype is determined by cytonuclear interactions (Boutin *et al.* 1987; Cuguen *et al.* 1994).

Study populations and sampling procedures: Two gynodioecious populations, 60 km from each other, were sampled in 1993. Both populations were linear and comprised ~400 individuals. They had contrasted habitats. The population named "Authie" was located along the estuary of the river Authie and was 400 m long. The population named "Wimereux" overhung a small cliff and extended over 150 m. Within each population, different subpopulations were distinguishable in the field by demographic discontinuities (Figure 1). In Wimereux, three subpopulations (W1, W2, W3) were identified; neighboring subpopulations were separated by 15–30 m. In Authie, two subpopulations (A1, A2), 70 m apart, were discriminated.

A total of 242 flowering plants were sampled: 115 in Authie and 127 in Wimereux. Plants were sampled randomly within each subpopulation, regardless of their sex, and all individuals located between the major subpopulations were also sampled (see Figure 1). All sampled plants were mapped, their sexual phenotype (hermaphrodite *vs.* female) was determined, and leaves were collected for molecular studies.

Molecular analyses: *DNA extraction:* Total genomic DNA was extracted using a modified Dellaporta *et al.* (1983) protocol as described in Saumitou-Laprade *et al.* (1991).

Mitochondrial RFLP analyses: Mitochondrial DNA diversity was studied using RFLP markers, as described in Saumitou-Laprade *et al.* (1993). Mitochondrial haplotypes were identified using three diagnostic probes on *Eco*RI DNA digests: ATPase subunit 6 (Dewey *et al.* 1985), pBv4 (Saumitou-Laprade *et al.* 1993), and Nvulg/N2 (Cuguen *et al.* 1994). We followed the nomenclature previously used by Desplanque *et al.* (2000), where 20 mitochondrial haplotypes (named *A* to *U* plus *Nvulg* and *Svulg*) were described.

Chloroplast RFLP analyses: Chloroplast DNA diversity was studied using RFLP techniques, as described in Forcioli *et al.* (1994): the entire chloroplast genome was used as a probe on *Hin*dIII, *Sma*I, and *Eco*RV DNA digests. We followed the nomenclature used by Forcioli *et al.* (1998) where nine chloroplast haplotypes (named *a* to *i*) were described.

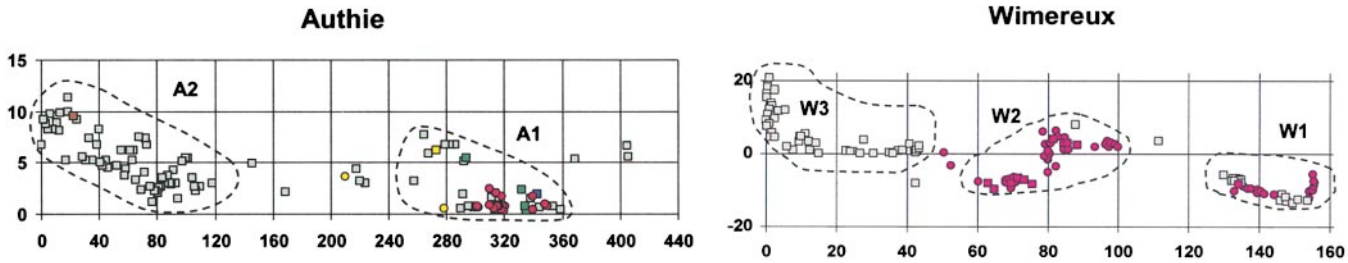

FIGURE 1.—Spatial distribution of the sexual phenotypes and the mtDNA haplotypes within each population. Females are figured by circles and hermaphrodites by squares. The mtDNA haplotypes are indicated by the following colors: *A*, gray; *B*, green; *E*, violet; *G*, red; *Svulg*, yellow; *K*, blue; and *L*, brown. Distances are given in meters. Note that the maps of cpDNA haplotypes (not shown) exactly mirror the maps of mtDNA haplotypes due to the complete linkage disequilibrium between both genomes (see the RESULTS section for details). A table of frequencies is available from the first author upon request.

Nuclear RFLP analyses: Nuclear RFLP analyses were performed as described by DESPLANQUE *et al.* (1999), using five RFLP probes (PILLEN *et al.* 1993): *pKP495* on *Eco*RI and *pKP753*, *pKP967*, *pKP851*, and *pKP826* on *Eco*RV DNA digests. In a previous study of *B. vulgaris* accessions, four of these markers were used and exhibited polymorphism: *pKP495/Eco*RI, *pKP753/Eco*RV, *pKP967/Eco*RV, and *pKP851/Eco*RV (see DESPLANQUE *et al.* 1999). Following the nomenclature of SCHONDELMAIER and JUNG (1997), these nuclear probes are located on chromosomes VII (*pKP495*), IV (*pKP753*), and IX (*pKP826*, *pKP851*, and *pKP967*; PILLEN *et al.* 1993). Probes *pKP826* and *pKP851* are loosely linked with gene *B* (BOUDRY *et al.* 1994): they were estimated to be 5.1 cM and 8.9 cM from the gene *B*, respectively. The probe *pKP967* is >50 cM away from these two probes (PILLEN *et al.* 1993). The probe *pKP753* is closely linked (1.7 cM) to *R1H*, a gene involved in the nuclear restoration of the male fertility of plants carrying the mitochondrial haplotype *H* (LAPORTE *et al.* 1998).

Microsatellite analyses: Amplifications of two microsatellites loci, *Bvm2* and *Bvm3*, were carried out as described by MÖRCHEN *et al.* (1996) and DESPLANQUE *et al.* (1999), respectively. The alleles at the locus *Bvm2*, a trinucleotide repeat, were separated by electrophoresis in 8% polyacrylamide gels and visualized by silver staining. The detection of alleles at the locus *Bvm3*, a dinucleotide repeat, was conducted by using a LI-COR automated sequencer model 4000 L (LI-COR, Lincoln, NB).

Data analyses: *Within-population diversity and linkage disequilibrium:* The frequency of females, the number of mitochondrial and chloroplast haplotypes, and the number of alleles per nuclear locus were estimated. For each cytoplasmic and nuclear marker, the gene diversity was estimated using the program FSTAT v. 1.2 (GOUDET 1995).

The associations between the sexual phenotypes and the cytoplasmic and nuclear markers were investigated. Linkage disequilibrium between the cytoplasmic markers was estimated and normalized according to the haplotype frequencies (LEWONTIN 1964; HEDRICK 1987). Genotypic linkage disequilibria between nuclear loci (WEIR 1996) were studied by using the software GDA (LEWIS and ZAYKIN 2000). To test for deviations from the null hypothesis of unlinked neutral markers and random mating, the estimated values of nuclear disequilibria were compared with the distribution of values obtained from 5000 resamplings with permutations of the single locus genotypes (ZAYKIN *et al.* 1995). Normalized cytonuclear gametic disequilibrium values (ASMUSSEN and BASTEN 1996) were estimated with the program CNDm, developed and kindly provided by C. J. Basten. Since complete linkage disequilibrium was found between the two cytoplasmic genomes, cytonuclear disequilibria were studied considering only mitochondrial genotypes. To test for deviations from the null hypothesis of random associations, exact tests based on a Markov chain method with 100,000 repetitions were performed (BASTEN and ASMUSSEN 1997). Finally, when a sexual polymorphism was observed within a cytoplasmic haplotype, we analyzed the linkage disequilibria between the sexual phenotype and each nuclear marker within this haplotype. For each analysis involving multiple tests, Šidák's correction was applied (SOKAL and ROHLF 1995).

Hierarchical analysis: Hierarchical analyses of the distribution of cytoplasmic and nuclear diversities were conducted using *F*-statistics (WRIGHT 1951) and the program FSTAT v. 1.2 (GOUDET 1995). Analyses were carried out for each population separately by considering the demographic substructure and excluding the individuals located between subpopulations (see Figure 1). The estimators $\hat{f}$, $\hat{\theta}$, and $\hat{F}$ of the parameters F_{is}, F_{st}, and F_{it} were computed according to WEIR and COCKERHAM (1984). All three estimators were obtained for each nuclear locus and for nuclear loci overall. Only $\hat{\theta}$ was estimated for cytoplasmic (haploid) markers. We tested the null hypothesis of Hardy-Weinberg proportions of the nuclear genotypes within subpopulations (H_o: $F_{is} = 0$) and within the whole population (H_o: $F_{it} = 0$). The significance values of the tests were obtained by using the permutation procedures included in FSTAT (5000 resamplings). The test of the null hypothesis of no variance of cytoplasmic and nuclear allelic frequencies between subpopulations was tested using an exact G-test with 5000 resamplings of the data by permuting the individuals (GOUDET *et al.* 1996).

Spatial autocorrelation analyses: The spatial distribution of the sexual phenotypes, cytoplasmic haplotypes, and nuclear alleles was studied by spatial autocorrelation analyses (SOKAL and ODEN 1978a,b; HEYWOOD 1991; EPPERSON and LI 1997), using the programs autocorrF and autocorrI developed and kindly provided by Olivier Hardy and Xavier Vekemans (HARDY and VEKEMANS 1999). Within each population, 15 classes of distance were defined: distance intervals were chosen to give approximately the same number of pairs of individuals within each distance class (532 pairs/class in Wimereux and 437 pairs/class in Authie, on average). The distance bounds used were: 0 m; 5.4 m; 11.3 m; 17.3 m; 25.9 m; 35.7 m; 43.7 m; 53.5 m; 61.1 m; 69.2 m; 75 m; 82.7 m; 95.8 m; 117.8 m; 137.2 m; and 160 m in Wimereux and 0 m; 8.64 m; 18.19 m; 27.33 m; 38.61 m; 52.98 m; 70.73 m; 98.69 m; 166.71 m; 206.11 m; 227.05 m; 245.33 m; 262.71 m; 282.62 m; 308.19; and 410 m in Authie. Within each distance class, three autocorrelation indices were computed for (i) the sexual phenotype, (ii) the mitochondrial haplotypes, and (iii) the nuclear loci. We used different autocorrelation statistics, according to the type of data to be analyzed: (i) Moran's *I* was calculated for the sexual

phenotypes, which were described by a binary variable (*i.e.*, 0 for hermaphrodites, 1 for females; Sokal and Oden 1978a,b), and (ii) the coefficient of relatedness for the mitochondrial and nuclear data (Fr_{mt} and Fr_n, respectively) was estimated using Ritland's (1996) method. This index allowed integration of multiallelic data, or even multiple loci, for the nuclear data (Hardy and Vekemans 1999). The estimates correspond to the inbreeding coefficients for the zero distance class for nuclear data and to kinship coefficients for larger distance classes for both nuclear and cytoplasmic data. The spatial structure of the population was visualized by a graph (autocorrelogram) plotting each autocorrelation index (Moran's *I*, Fr_{mt}, and Fr_n) as a function of the distance between individuals (Sokal and Oden 1978b). The distributions of the statistics under the null hypothesis of no spatial genetic structure were generated from 5000 resamplings of the data by permuting the individuals (for Moran's *I* and kinship coefficients) or the nuclear alleles (for the inbreeding coefficient).

RESULTS

Within-population diversity: Both populations were gynodioecious but with significantly different frequencies of females: 16% in Authie *vs.* 40% in Wimereux (exact test: $P < 0.0001$). In the total sample of 242 individuals, seven mitochondrial and six chloroplast haplotypes were found. Mitochondrial and chloroplast markers were polymorphic within each population (Table 1 and Figure 2). Six mitochondrial and five chloroplast haplotypes were recorded in Authie, but only two mitochondrial and two chloroplast haplotypes in Wimereux. Within each population, mitochondrial and chloroplast markers displayed absolute linkage disequilibrium ($D' = 1$). Overall, seven cytoplasmic haplotypes (*i.e.*, combination of chloroplast and mitochondrial haplotypes) were detected: five in Authie (*B/g*, *G/f*, *K/g*, *L/c*, and *Svulg/j*), one in Wimereux (*E/d*), and one (*A/a*) in both populations. In spite of the different number of haplotypes, both populations had the same level of cytoplasmic diversity, as quantified by Nei's diversity index, since several haplotypes in Authie were quite rare (Table 2 and Figure 2).

Within each population, a strong association was found between the sexual phenotypes and the cytoplasmic haplotypes (Table 2). In Authie, four haplotypes (*A/a*, *B/g*, *K/g*, and *L/c*) were seen only in hermaphrodites, one (*G/f*) only in females, and only the rare haplotype *Svulg/j* was found in both sexual phenotypes. In Wimereux, one haplotype (*A/a*) was strictly associated with hermaphrodites and the second one (*E/d*) was seen in both sexual phenotypes, mainly with females (82% of the individuals carrying the *E/d* haplotype were females).

In each population, at least two alleles per nuclear locus were detected (Table 2 and Figure 2). The microsatellite locus *Bvm3* had the highest number of alleles (9 alleles in Authie and 11 in Wimereux). Nei's diversity index varied between loci from 0.06 (*pKP826*) to 0.74 (*pKP753*) in Authie and from 0.20 (*pKP826*) to 0.82 (*Bvm3*) in Wimereux. Analyses of nuclear disequilibria revealed significant associations at four pairs of nuclear loci in Authie (*pKP753/pKP967*; *pKP753/Bvm2*; *pKP967/Bvm2*; and *pKP826/Bvm3*) and at four other pairs of loci in Wimereux (*pKP753/pKP851*; *pKP753/pKP826*; *pKP967/pKP851*; and *pKP851/pKP826*), but none remained significant after a correction for multiple tests (Sokal and Rohlf 1995). Significant cytonuclear associations were detected only in Wimereux, involving three nuclear loci (*pKP753*, *Bvm3*, and *Bvm2*). Two remained significant after Šidák's correction for multiple tests (*Bvm3*, $P = 0.0007$ and *Bvm2*, $P = 0.004$). Finally, in Wimereux, for the individuals with the cytoplasmic haplotype *E/d* (for which both sexes were found), three nuclear loci (*pKP753*, *pKP826*, and *Bvm3*) were in significant disequilibrium with the sexual phenotype. One association, involving *pKP753*, the marker physically linked to the nuclear restorer locus *R1H* (Laporte *et al.* 1998), remained significant after Šidák's correction ($P = 0.05$). This disequilibrium is mainly due to the most frequent allele at *pKP753*, which was associated with females ($D'^{PK753\text{-}2}{}_{E_F} = +0.62$).

TABLE 1

Cytoplasmic and nuclear diversity within each population, Authie and Wimereux

	Authie ($n = 115$)		Wimereaux ($n = 127$)	
	A	He	A	He
Cytoplasmic haplotypes				
CpDNA	5	0.61	2	0.50
MtDNA	6	0.61	2	0.50
Nuclear loci				
RFLP				
pKP495	4	0.51	4	0.55
pKP753	6	0.74	5	0.66
pKP967	6	0.58	4	0.67
pKP851	5	0.60	5	0.72
pKP826	2	0.06	2	0.20
Microsatellites				
Bvm2	6	0.71	6	0.73
Bvm3	9	0.66	11	0.82

The number of mtDNA, cpDNA haplotypes, and nuclear alleles per locus are presented (A), as well as Nei's diversity index (He), for each marker.

Within-population spatial structure: The frequency of females was extremely different between the local subpopulations: in Wimereux, 52, 73, and 0% of females were found in W1, W2, and W3, respectively; in Authie, female frequencies of 41 and 0% were found in A1 and A2, respectively (see Figure 1). Within each population, cytoplasmic haplotypes were even more patchily structured than sexual phenotypes, and their spatial distribution remarkably followed the spatial discontinuities among subpopulations (suggesting that each subpopulation is the result of different founder events; see Figure

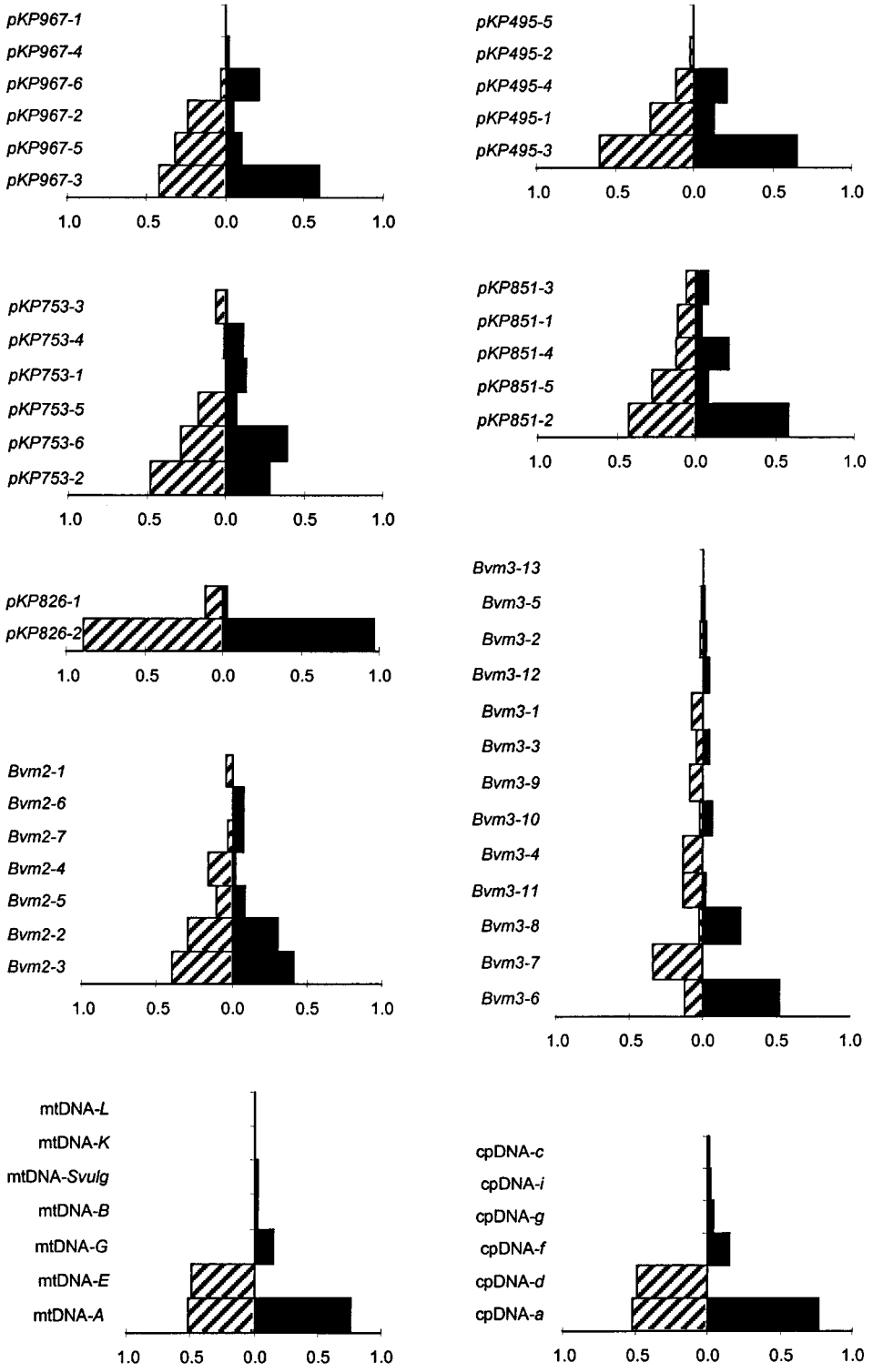

FIGURE 2.—Distribution of the frequencies of the mtDNA and cpDNA haplotypes and nuclear alleles per locus within each population, Authie (solid) and Wimereux (striped).

1). In Wimereux, all individuals sampled in subpopulation W3 had the mitochondrial haplotype (*A*); 80% of the individuals sampled in subpopulation W2 displayed another mitochondrial haplotype, namely, *E*, while the third subpopulation, W1, was a mosaic of four monomorphic patches (*A* or *E*). In Authie, subpopulation A2 was nearly monomorphic for the mitochondrial haplotype *A* but the second one, A1, was polymorphic.

The *F*-statistic analyses revealed significant spatial substructuring within each population (Table 3). Overall, nuclear markers revealed a slight but significant deficiency of heterozygotes ($\hat{F} = 0.06$ in Authie, $\hat{F} = 0.05$ in Wimereux). This was mainly due to a low differentiation between subpopulations ($\hat{\theta} = 0.019$ in Authie and 0.028 in Wimereux, respectively, $P < 0.001$) with no significant heterozygote deficiencies within the subpopula-

TABLE 2

Associations between the cytoplasmic haplotype (MtDNA/CpDNA) and the sexual phenotype within each population, Wimereux and Authie

	Wimereux (n = 127)		Authie (n = 115)		Total (n = 242)	
MtDNA/CpDNA	H	F	H	F	H	F
A/a	65	0	88	0	153	0
B/g	0	0	4	0	4	0
E/d	11	51	0	0	11	51
G/f	0	0	0	18	0	18
K/g	0	0	1	0	1	0
L/c	0	0	1	0	1	0
Svul/j	0	0	1	2	1	2
Total	76	51	95	20	171	71

H, hermaphrodite; F, female.

tions ($\hat{f}$ = 0.038 and 0.021 in Authie and 0.028 in Wimereux, respectively). Examination of the individual nuclear locus *F*-statistics are in agreement in Wimereux: no locus showed significant heterozygote deficiencies within the subpopulations and all but one revealed a significant differentiation between the subpopulations (with $\hat{\theta}$ varying from 0.001 to 0.058). In Authie, the overall pattern is slightly less clear, with two loci showing significant heterozygote deficiencies (including one locus with very little polymorphism, *pKP826*) and three loci showing no significant differentiation between the subpopulations (with $\hat{\theta}$ varying from −0.007 to 0.066).

Within each population, cytoplasmic markers were considerably more differentiated between the subpopulations than nuclear loci, with 25-fold larger $\hat{\theta}$. Moreover, the Wimereux population had a clearly greater degree of spatial structure than Authie ($\hat{\theta}$ = 0.75 *vs.* 0.42).

In Wimereux, we also investigated the cytonuclear associations at the subpopulation level for the two loci that displayed significant cytonuclear disequilibria over the whole population (Table 4). At *Bvm2*, one nuclear allele (*Bvm2-5*) was preferentially associated with the mitochondrial haplotype *A* over the whole population. Within each subpopulation, the same tendency was observed. By contrast, the disequilibrium at *Bvm3* was an among-subpopulation effect as the individuals with the mitochondrial haplotype *A* were associated with different nuclear alleles at *Bvm3*, depending on their location.

The significant spatial structure can also be visualized through the autocorrelograms (Figure 3). Within each population, all three correlograms of the sexual phenotypes, the cytoplasmic haplotypes, and the nuclear alleles revealed a decrease from significantly positive autocorrelations (individuals separated by <17 m in Wimereux and <100 m in Authie) to significantly negative values at greater distance intervals (>44 m in Wimereux and >227 m in Authie). Similar patterns were obtained for sexual phenotypes and cytoplasmic haplotypes, as expected due to their close association. Conversely, there was a considerable difference between the cytoplasmic and nuclear autocorrelograms: the estimated kinship coefficients were 10-fold lower for nuclear markers compared to cytoplasmic haplotypes. Cytoplasmic autocorrelograms also revealed a striking difference between the populations, with correlation coefficients in Wimereux more than twice as large as in Authie (*e.g.*, in the first distance class, Fr_{mt} = 0.82 *vs.* 0.30).

DISCUSSION

The sexual polymorphism and the sex determination system within populations: We studied two sea beet gynodioecious populations, namely, Wimereux and Authie, with 40 and 16% of females, respectively. The first major result is the highly clumped distribution of the sexual phenotypes. Within each population, we observed a succession of patches containing exclusively (or predominantly) a single sexual phenotype, either hermaphrodite or female (see Figure 1). This grouping contrasts with the pattern described in the well-studied gynodioecious species *T. vulgaris* (Thompson *et al.* 1998): only patches of females were observed in recently founded populations, hermaphrodites being rare and isolated (Manicacci *et al.* 1996). This was explained by the increased relative fecundity of female individuals associated with a (local) cytoplasmic sex determination system. The similar sizes of the patches of females and hermaphrodites observed here may reflect the fact that in *B. vulgaris* females have only a slightly increased female fitness compared with hermaphrodites (Boutin *et al.* 1988).

In beets, the sex determination system can be investigated by analyzing the association between the sexual phenotypes and the mitochondrial haplotypes revealed by molecular markers (Boutin *et al.* 1987; Cuguen *et al.* 1994). A very strong association was found within each population. Several mtDNA haplotypes are found exclusively in hermaphrodite phenotypes (*A*, *B*, *K*, and *L*) and the other mtDNA haplotypes are mainly or exclusively associated with male sterility (*E*, *G*, and *Svulg*). These associations agree with those found in previous studies of natural populations and cultivated material (Boutin *et al.* 1987; Saumitou-Laprade *et al.* 1991; Cuguen *et al.* 1994). This suggests that the main genetic component of the sexual variation is cytoplasmic. This also agrees with observations in other gynodioecious species, including *P. lanceolata* (De Haan *et al.* 1997b), *P. coronopus* (Koelewijn and Van Damme 1995), *S. vulgaris* (Charlesworth and Laporte 1998), and in recently founded populations of *T. vulgaris* (Manicacci *et al.* 1997). This finding is essential because it suggests that the maintenance of gynodioecy depends mainly on the maintenance of a cytoplasmic polymorphism that is theoretically possible under fewer constraints than the

TABLE 3

***F*-statistics within each population**

	Authie (n = 104)			Wimereux (n = 123)		
	$\hat{F}$	$\hat{\theta}$	$\hat{f}$	$\hat{F}$	$\hat{\theta}$	$\hat{f}$
			Cytoplasmic haplotypes			
MtDNA	—	0.420 ***	—	—	0.750 ***	—
CpDNA	—	0.423 ***	—	—	0.750 ***	—
			Nuclear loci			
RFLP						
pKP495	0.031 ns	−0.007 ns	0.037 ns	0.078 ns	0.027 **	0.052 ns
pKP753	0.022 ns	0.025 ***	−0.004 ns	0.057 ns	0.013 ***	0.044 ns
pKP967	0.119 *	−0.005 ns	0.123 *	−0.026 ns	0.001 ns	−0.026 ns
pKP851	0.031 ns	0.001 ns	0.030 ns	0.040 ns	0.022 **	0.018 ns
pKP826	0.280 **	0.041 *	0.249 **[a]	0.052 ns	0.046 **	0.006 ns
Microsatellites						
Bvm2	0.000 ns	0.016 **	−0.017 ns	0.021 ns	0.032 ***	−0.011 ns
Bvm3	0.120 *	0.066 ***	0.058 ns	0.107 **	0.058 ***	0.052 ns
			Overall loci			
	0.057 *	0.019 ***	0.038 ns	0.048 *	0.028 ***	0.021 ns

Three and two subpopulations were identified in Authie and Wimereux, respectively; see Figure 1. ns, $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

[a] Within the single polymorphic subpopulation, A2.

maintenance of a joint cytonuclear polymorphism. Indeed, pure cytoplasmic gynodioecy can be maintained provided there is pollen limitation of female fertility, since the cytoplasm controlling hermaphroditism is protected by frequency dependence (Lewis 1941). By contrast, the maintenance of a joint cytonuclear polymorphism requires additional selective processes (*e.g.*, pleiotropic deleterious effects of the restorer alleles in their alien cytoplasmic type; see Charlesworth and Ganders 1979; Delannay *et al.* 1981; Frank 1989; Gouyon *et al.* 1991) or nonequilibrium metapopulation processes (Frank 1989; McCauley and Taylor 1997; Pannell 1997; Couvet *et al.* 1998).

However, within both study populations, a sexual polymorphism was also observed for a given mitochondrial haplotype. In Wimereux, 20% of the individuals with haplotype *E* were hermaphrodites. In Authie, out of three plants with the haplotype *Svulg*, one was a hermaphrodite. This suggests that the maintenance of gynodioecy depends on both cytoplasmic and nuclear factors. These observations were confirmed by the presence of hermaphrodites in the progenies of females pollinated *in situ* (Laporte 1998). Because the number of nuclear loci involved in the restoration of male fertility and the mode of action of the restorer alleles are poorly understood in *B. vulgaris* ssp. *maritima*, we cannot estimate the frequency of restorer alleles within the populations. However, these observations clearly pro-

TABLE 4

Significant cytonuclear associations in Wimereux

		Nuclear loci					
		Bvm2			*Bvm3*		
Population	MtDNA	No. of plants with *Bvm2-5*/n	P	$D'^{\text{Bvm2-5}}_{A}$	No. of plants with *Bvm3-4*/n	P	$D'^{\text{Bvm3-4}}_{A}$
W	*A*	20/130	0.007	+0.53	7/120	0.002	−0.55
	E	6/124			24/120		
W1	*A*	9/30	0.029	+0.54	7/30	0.29	−0.24
	E	3/36			14/38		
W2	*A*	1/4	0.165	+0.28	0/4	1.00	−1.00
	E	3/88			10/82		
W3	*A*	10/96		—	0/86	—	—

Associations are given for the total population (W) and within subpopulations (W1, W2, W3). *P*, results of Fisher's exact test.

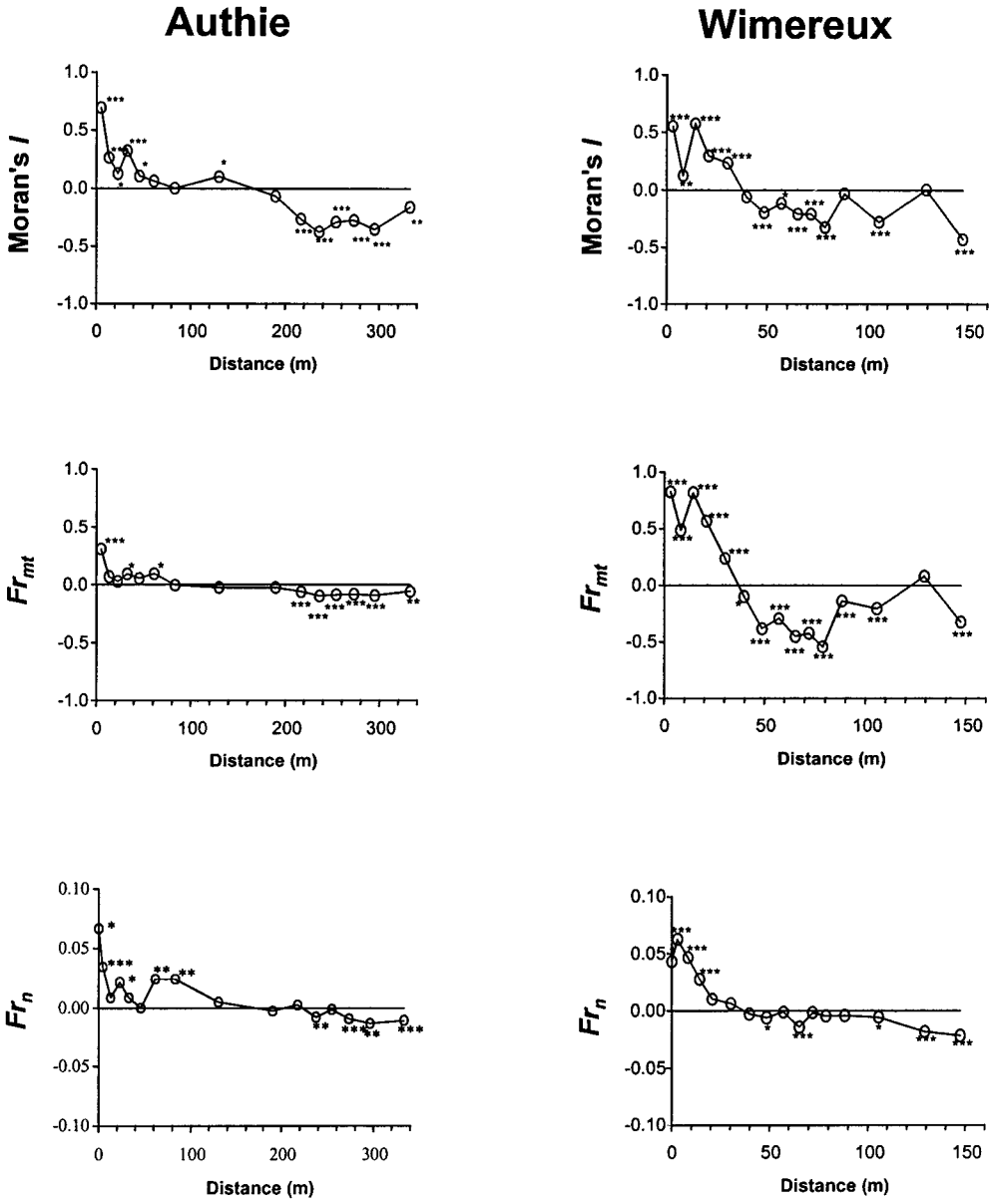

FIGURE 3.—Autocorrelograms of the sexual phenotypes (Moran's *I*), mtDNA haplotypes (Fr_{mt}), and nuclear alleles from seven nuclear loci (Fr_n) within each population. Significant deviations from values expected under random distribution are indicated: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

vide evidence that the nuclear restorer loci are polymorphic within the populations. Since complex systems involving several loci are suggested by data from best-studied gynodioecious species (reviewed in CHARLESWORTH and LAPORTE 1998), we here refer to "restorer alleles" to designate the possibly multiple nuclear restorer alleles specific to a given cytoplasmic male sterile type.

The spatial structure of cytonuclear diversity and the founder effect hypothesis: In both populations we found high cytoplasmic diversity, but the populations differed by their number of haplotypes: six and two cytoplasmic haplotypes were detected in Authie and Wimereux, respectively. None of the three mitochondrial haplotypes in which male sterility occurred (*E, G,* and *Svulg*) is unique to these populations (SAUMITOU-LAPRADE *et al.* 1991; CUGUEN *et al.* 1994; FORCIOLI *et al.* 1998; DESPLANQUE *et al.* 2000). Moreover, mitochondrial and chloroplast haplotypes are in linkage disequilibrium, as they were at a larger spatial scale (DESPLANQUE *et al.* 2000). These observations (*i.e.*, of a large geographical distribution of this cytoplasm together with strong linkage disequilibrium between organellar genomes) suggest that cytoplasmic haplotypes did not arise recently and imply a slow mutational dynamics of the mitochondrial genome in *B. vulgaris* ssp. *maritima.* Therefore, we do not expect gynodioecy in *B. vulgaris* ssp. *maritima* to be due to the recurrent occurrence of male sterile mutations. This is similar to the situation reported in the gynodioecious *P. lanceolata* (DE HAAN *et al.* 1997c), but contrasts with the pattern observed in *T. vulgaris* (BELHASSEN *et al.* 1993; TARAYRE *et al.* 1997).

We also found a high nuclear diversity using both RFLP and microsatellite markers. There is, however, a

major contrast between the spatial structure of cytoplasmic and nuclear markers within each population (see Figure 3). Cytoplasmic diversity is very low within subpopulations and high between closely adjacent subpopulations (see Figure 1). Conversely, the nuclear diversity is largely distributed within the subpopulations, and the nuclear differentiation between the subpopulations is considerably lower than the cytoplasmic differentiation. This is quantified through the estimates (i) of the kinship coefficients in the first class of distance of the autocorrelograms and (ii) of the F_{st} between subpopulations that are both 10 times lower for nuclear than for cytoplasmic genes. These observations are in agreement with the few other studies that have compared the spatial structure of cytoplasmic and nuclear markers at a local scale within angiosperm plant populations, including the gynodioecious species *T. vulgaris* (Tarayre *et al.* 1997) and *Phacelia dubia* (Levy and Neal 1999) and the dioecious species *S. alba* (McCauley *et al.* 1996). Such a contrast is not unexpected because the genes differ in their effective population size and their mode of dispersal. Founder effects are expected to generate this pattern (McCauley 1994, 1995). Moreover, the dynamics of the approach to equilibrium will be slower for cytoplasmic genes (Petit *et al.* 1993), especially in a wind-pollinated self-incompatible species like *B. vulgaris* ssp. *maritima*. An increased contrast is expected in the case of gynodioecy with cytoplasmic sex determination since this reproductive system results in a greater deviation between cytoplasmic and nuclear effective population sizes (Laporte *et al.* 2000).

Therefore, our observations suggest that (i) very local subpopulations may have originated from different founder events; (ii) seed dispersal is very restricted, almost never occurring between subpopulations, so that subpopulations expand until they nearly merge without losing their initial cytoplasmic differentiation; and, finally, (iii) founder events affect the nuclear polymorphism and its spatial structure only slightly, and/or sufficient pollen dispersal quickly erases any nuclear differentiation due to founder effects. Little is known about the population dynamics of natural populations of *B. vulgaris* ssp. *maritima*. However, the sea shore, its typical habitat, is potentially highly disturbed due to both human activities and natural perturbations (Boutin-Stadler *et al.* 1989). Indeed, we observed that half of the population Authie was destroyed in 1994, the year following this study. The following year we observed that the population Wimereux had also experienced a considerable size reduction: less than one-quarter of the individuals remained. This high disturbance regime associated with the long life time of the individuals (up to 7 years in the north of France, H. Van Dijk, personal communication) is expected to result in nonequilibrium patterns, as suggested by these results.

The processes sustaining the sexual polymorphism: This major contrast between cytoplasmic and nuclear diversity patterns may have important consequences for the maintenance of gynodioecy. The high cytoplasmic differentiation provides a landscape for differential selection, since restorer alleles are selected for in the presence of their specific cytotype but are either neutral or even selected against (under the hypothesis of a cost of restoration) in its absence. High nuclear gene flow between locations subject to different selection pressures could account for the polymorphism at nuclear restorer loci. This may occur at a very local scale, since very local subpopulations are highly cytoplasmically differentiated. This may also occur between populations since wild beet populations show different patterns of cytoplasmic diversity (Cuguen *et al.* 1994; Forcioli *et al.* 1998; Desplanque *et al.* 2000).

For instance, *Svulg* had never been reported in French natural sea coast populations (Cuguen *et al.* 1994; Forcioli *et al.* 1998; Desplanque *et al.* 2000). Specific restorer alleles for *Svulg* should then be neutral or selected against in coastal populations. However, this haplotype, exploited in plant breeding programs (Saumitou-Laprade *et al.* 1991), is nearly fixed in weed beet populations infesting crop fields in the north of France (Boudry *et al.* 1993). In these populations, restorer alleles are expected to be highly selected for. Consequently, the presence of the *Svulg* haplotype and its nuclear restorer alleles within Authie could result from gene flow from weed beet populations. Indeed, infested sugar beet fields are found only a few kilometers away from the coastal population Authie. The importance of gene flow from wild to cultivated beets has been studied and suggested to influence the nuclear diversity of weed beets (Boudry *et al.* 1993; Desplanque *et al.* 1999). In contrast, the impact of gene flow from weed to nearby sea beet populations has received little attention. It may, however, influence the nuclear diversity in sea beet populations, as recently suggested by Bartsch *et al.* (1999).

The haplotype *G*, by contrast, appears completely male sterile, at least from *in situ* observations. This haplotype is rare in France and Spain (Cuguen *et al.* 1994; Desplanque *et al.* 2000). Therefore, there is little opportunity for selection of its restorer alleles. A study at a larger spatial scale is required to determine whether the nuclear restorer alleles specific to this haplotype are generally rare in the landscape. This is expected under the hypothesis of a cost of restoration. Alternatively, founder effects causing a reduction of the nuclear diversity could lead to the local absence of certain nuclear restorer factors in a recently founded population (*e.g.*, Manicacci *et al.* 1996), even if these alleles are relatively frequent in the landscape. Although large nuclear gene flow within populations is suggested by this study, a comparative study at a larger spatial scale is required to investigate the importance of founder effects on nuclear differentiation between populations.

Finally, in Wimereux, the mitochondrial haplotype *E* was clearly associated with polymorphic restorer loci but

the low level of restoration is striking given the relatively high frequency of this haplotype within the population. The genetic diversity also revealed an unexpected pattern: in spite of high nuclear gene flow, as evidenced by individual nuclear markers, we found three significant linkage disequilibria between markers. This is unexpected in a highly outcrossed population at equilibrium, thus suggesting either selective processes or a nonequilibrium metapopulation dynamics (Asmussen *et al.* 1987; Asmussen and Schnabel 1991; Babcock and Asmussen 1996, 1998). The analyses of linkage disequilibria suggests that both processes may have shaped this diversity pattern.

The linkage disequilibrium involving the microsatellite *Bvm3* is due to the cytonuclear structure among the farthest subpopulations: individuals carrying the mitochondrial haplotype *A* are associated with different nuclear alleles at *Bvm3*, depending on their location. Such a pattern can be expected after founding if individuals from different subpopulations originated from founders with different nuclear backgrounds, despite their cytoplasmic similarity. Although the persistence of this disequilibrium is unexpected with high nuclear gene flow, pollen flow between the farthest subpopulations may be restricted because they are separated by a clump of females.

By contrast, at the microsatellite *Bvm2*, one nuclear allele is preferentially associated with the mitochondrial haplotype *A* independently of the plants' location. Such a pattern is expected under cytonuclear selection (Asmussen *et al.* 1987; Asmussen and Schnabel 1991; Babcock and Asmussen 1996, 1998).

At the RFLP *pKP753*, one allele, frequent in females, is rare in hermaphrodites with the haplotype *E*. Because *pKP753* is physically linked to nuclear restorer loci of the *H* and *Svulg* cytoplasmic haplotypes (Laporte *et al.* 1998), this *in situ* linkage disequilibrium between *pKP753* and the sexual phenotype is impressive. It raises the question of its physical linkage to a nuclear restorer locus of the *E* cytoplasmic haplotype, either due to (i) a pleiotropic (generalist) locus involved in the restoration of male fertility of different cytoplasmic male sterility types or (ii) a cluster of restorer loci. More detailed mapping analyses are required to test this assumption.

This disequilibrium could reflect a recent migration of restorer alleles in the population. Nonequilibrium dynamics between migration and selection pressures would also explain the low level of nuclear restoration. Such a disequilibrium due to metapopulation dynamics is expected to be very transient, unless the marker is physically linked with nuclear restorer loci. Moreover, the allele *pKP753-2* that is frequent in females (*i.e.*, associated with nonrestorer alleles) is also frequent in hermaphrodites carrying the *A* mitochondrial haplotype, such that we observe a significant cytonuclear disequilibrium within hermaphrodite individuals ($D' = 0.58$, $P = 0.011$). Under the hypothesis that *pKP753* is physically linked to a restorer locus of the *E* male sterility type, this cytonuclear disequilibrium could indicate a cost of restoration as well as a recent migration event. Indeed, differential selection between haplotypes will generate permanent cytonuclear disequilibria between the mitochondrial haplotype and the nuclear restorer loci, as well as transient cytonuclear disequilibria with other nuclear loci physically linked to the restorer loci. Although a cost of restoration has not been studied in *B. vulgaris* ssp. *maritima*, it has been suggested in several gynodioecious species including *T. vulgaris* (Couvet *et al.* 1986; Manicacci *et al.* 1997; Gigord *et al.* 1998, 1999), *P. lanceolata* (De Haan *et al.* 1997a), and *P. coronopus* (Koelewijn 1996). Our results therefore suggest that both metapopulation dynamics and selective processes may be involved in the maintenance of gynodioecy in *B. vulgaris* ssp. *maritima* at this very local scale.

Habitat differences, gynodioecy, and founder effects: The two study populations show different patterns regarding gynodioecy: they differ in their frequency of females, the mtDNA haplotypes that are associated with male sterility, and the level of nuclear restoration associated with these cytoplasmic haplotypes. As previously discussed, the cytoplasmic component is the main determinant of the sex determination system in both populations. Therefore, these differences appear mainly due to cytoplasmic differentiation between the populations. Other differences between the populations concern (i) the plant density (higher in the cliff population than in the estuary population), (ii) the number of cytoplasmic haplotypes (higher in the estuary population than in the cliff population), and (iii) the cytoplasmic differentiation between subpopulations (extremely high in the cliff population, lower in the estuary population).

A likely explanation for these differences is an effect of the habitat occupied by the two populations. Indeed, the Authie population is located along the estuary. The Wimereux population, however, overhangs a small cliff. Since seeds of sea beet are dispersed by the tide (Raybould *et al.* 1997), the estuary population is expected to experience seed dispersal over long distance within the population and high seed immigration rate. In the cliff population, seed migration events are certainly rarer and the seeds are mainly dispersed by gravity over shorter distances. These observations thus further agree on the importance of founder effects on genetic diversity in wild beet populations, which here appear to be stronger in the cliff population than in the estuary population.

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