

ORIGINAL ARTICLE

Genetic determination of male sterility in gynodioecious *Silene nutans*

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Gynodioecy, the coexistence of female and hermaphrodite plants within a species, is often under nuclear–cytoplasmic sex determination, involving cytoplasmic male sterility (CMS) genes and nuclear restorers. A good knowledge of CMS and restorer polymorphism is essential for understanding the evolution and maintenance of gynodioecy, but reciprocal crossing studies remain scarce. Although mitochondrial diversity has been studied in a few gynodioecious species, the relationship between mitotype diversity and CMS status is poorly known. From a French sample of *Silene nutans*, a gynodioecious species whose sex determination remains unknown, we chose the four most divergent mitotypes that we had sampled at the

cytochrome *b* gene and tested by reciprocal crosses whether they carry distinct CMS genes. We show that gynodioecy in *S. nutans* is under nuclear–cytoplasmic control, with at least two different CMSs and up to four restorers with epistatic interactions. Female occurrence and frequency were highly dependent on the mitotype, suggesting that the level of restoration varies greatly among CMSs. Two of the mitotypes, which have broad geographic distributions, represent different CMSs and are very unequally restored. We discuss the dynamics of gynodioecy at the large-scale meta-population level.

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Introduction

Gynodioecy, the coexistence of hermaphrodite and female plants in natural populations, is one of the most common plant-breeding systems after hermaphroditism (Richards, 1997). Male sterility is often under nuclear–cytoplasmic control (Charlesworth and Laporte, 1998; Taylor *et al.*, 2001; van Damme *et al.*, 2004) although purely nuclear determination exists (for example, Ashman, 1999). Cytoplasmic male sterility (CMS) genes in the mitochondrial genome cause male sterility and nuclear restorer genes block their action and restore male fertility (Chase, 2007). As mitochondrial genes are usually maternally inherited, a CMS gene that confers female advantage will invade the population (Lewis, 1941). This female advantage can result from reallocation of the energy not used in pollen production and/or avoidance of inbreeding depression and both have experimental support (Poot, 1997; Shykoff *et al.*, 2003). As CMS invades the population, any nuclear mutation that restores male fertility will be associated with the rare gamete type (that is, pollen) and is selected by the Fisherian selection (Jacobs and Wade, 2003). The molecular mechanisms of male sterility and restoration, as well as the nature of CMS and restorer genes, have been

documented in some crops (Delph *et al.*, 2007), but CMS and restorer genes remain unknown in wild species.

The equilibrium state and dynamics of gynodioecy depend on the genetics of sex determination. Nuclear gynodioecy can only be maintained if females produce at least twice as many seeds as hermaphrodites, whereas, under cyto-nuclear determination, male sterilizing cytoplasms are selected if females have any seed production advantage over hermaphrodites (Lewis, 1941). Conditions that can give rise to stable sex polymorphism within populations have been explored in detail under nuclear–cytoplasmic sex determination and two types of situations can be distinguished. Frank (1989) found transient polymorphisms due to regular invasions of new sterilizing cytoplasms that then go to fixation, and such dynamics can lead to polymorphism at the meta-population level (Couvét *et al.*, 1998). On the other hand, cytoplasmic polymorphisms can be maintained over long periods by frequency-dependent selection (Gouyon *et al.*, 1991; Bailey *et al.*, 2003; Dufay *et al.*, 2007), resulting in the coexistence of several different cytoplasmic types in each population. The conditions for polymorphism differ between models with two sterilizing cytoplasms (Gouyon *et al.*, 1991; Bailey *et al.*, 2003), and models with one sterilizing and one fertile cytoplasm (Jacobs and Wade, 2003; Dufay *et al.*, 2007). Characteristics of restorers, such as dominance (Jacobs and Wade, 2003), cost of restoration (Bailey *et al.*, 2003; Dufay *et al.*, 2007) and number of loci (quantitative vs qualitative restoration; Bailey and Delph, 2007), are also important. Thus, studying the genetic basis of sex determination is essential for understanding the evolution and maintenance of gynodioecy.

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Considerable mtDNA polymorphism has been found in gynodioecious *Silene* species (Städler and Delph, 2002; Houliston and Olson, 2006; Olson *et al.*, 2006; Touzet and Delph, 2009), but the associations between mitotypes and sex phenotypes are not perfect (Olson and McCauley, 2002; Storchova and Olson, 2004; Klaas and Olson, 2006) and crosses are necessary to determine the number of functionally distinct CMS types. In the gynodioecious species *Silene nutans*, nuclear–cytoplasmic sex determination is suspected. Genotyping studies found high polymorphism in the cytochrome *b* (*cob*) and the cytochrome oxidase (*cox1*) mitochondrial genes (Touzet and Delph, 2009) that could be related to CMS types. We set up a crossing experiment to assess this and to answer the following questions:

- (i) Is sex determination nuclear–cytoplasmic in *S. nutans*?
- (ii) How many CMS types and restorers are involved in sex determination?

Materials and methods

Plant material

S. nutans (Caryophyllaceae) is a diploid, long-lived perennial rosette plant, growing in non-acidic open grass communities on hills or forest edges. Its range extends from North-Western Europe to Siberia and the Caucasus. It has been described as gynomonoeious–gynodioecious: female, hermaphroditic and gynomonoeious individuals (with a mixture of perfect (hermaphroditic) and pistillate (female) flowers) are found in natural populations (Dufay *et al.*, 2010). Perfect flowers are protandrous, but self-pollination can occur by geitonogamy. Seeds are dispersed over a short distance from an aperture at the top of the ripe capsule when the stalk is agitated by wind or animals.

Previous studies showed that the mitochondrial genes *cob* and *cox1*, but particularly the former, are highly polymorphic in *S. nutans* (Touzet and Delph, 2009). These two genes are involved in the respiratory chain and probably not directly in male sterility. For our crossing experiment, we chose four divergent sequences at the *cob* locus in an attempt to encompass the maximal variation in CMS types. Three of them were chosen from a previous study (Touzet and Delph, 2009) to be as distant as possible from one another in the haplotype tree. The last one was a divergent sequence from additional sampling from a survey of 13 populations across Belgium and France. All information about the sequences we used is available in Supplementary Table 1. We refer to these four sequences as mitotypes, even if linkage disequilibrium could be incomplete between the *cob* and the CMS loci. One mitotype (AMB) was collected in Ambleteuse (N 50°48' E 1°37'), another (AU) in Auvergne (N 44°43' E 2°21'), and the two others (Q1 and Q9) in Queyras (N 44°46' E 6°44'). The two mitotypes from Queyras were specific to this population, whereas the two others (AMB and AU) were found in several French populations. The Ambleteuse population is located about 700 km from the two other populations. The Auvergne and Queyras populations are 350 km apart. Rosette plants were transplanted from the Ambleteuse population to the greenhouse in March 2006 and used as parents in 2007 (Table 1). In Auvergne

and Queyras, seeds were harvested from open-pollinated plants in 2002 and 2003, respectively, and sown in autumn 2006, giving rise to independent maternal full- or half-sibling families. Table 1 shows the identity of parents used for reciprocal crosses and their verified *cob* genotype. The Queyras population presented intrapopulation mtDNA polymorphism, so we used two different mitotypes (Q1 and Q9) to test for the coexistence of different CMS factors within this population.

Crossing procedure

A difference in sex segregation of progenies from reciprocal crosses means that the two mitotypes tested have distinct CMS types with different specific restorers. CMS types that are poorly restored maximize the probability to find sex segregation differences between reciprocal crosses and are easiest to distinguish. On the other hand, a lack of difference in sex segregation between progenies of reciprocal crosses is ambiguous, because reciprocal crosses between two different CMS types could still lead to similar sex segregation if there is some symmetry in the restorer genotypes of the two parents (van Damme *et al.*, 2004). A complete diallele design was set up, in which each pairwise comparison between our four mitotypes was tested in reciprocal crosses between hermaphrodites (12 crosses). This diallele was replicated three times, using three different parents of each mitotype (Table 1). These replicates increase the power for detecting the different CMSs and limit the risk of misinterpretation owing to the potential maternal effects (van Damme *et al.*, 2004). A total of 36 (12 × 3) crosses were performed.

Each cross was performed on a single flower. Buds were isolated to avoid accidental pollination. A removable mesh cover allowed access to the flower for pollination and seed harvesting. As *S. nutans* is self-compatible, flowers were emasculated by cutting the stamens as soon as they emerged from the corolla. Pollen was collected just before pollination and placed on the receptive sticky stigma with a toothpick. Seeds were harvested at maturity. Within each of the three replicates, all crosses were performed within less than a week. Some crosses failed to produce fruits, and we obtained no seed for the following three crosses: AMB9 × Q1.2, AMB9 × AU4.4 and Q9.5 × Q1.1 (mother × father).

Growing conditions

Seeds were germinated in October 2007, in 9-cm-diameter Petri dishes on 0.8% agar-containing medium. The germination rate was close to 100% for all crosses. Seedlings were transplanted 10 days later into multipots (4.5 cm diameter each) containing a soil mixture of ½ compost and ½ calcareous sand, and placed in the greenhouse with natural light and a daily temperature ranging between 15 and 20 °C. In late February, plants were transplanted to individual pots (7 × 7 cm) filled with compost supplemented with a controlled release fertilizer (Osmocote, 110 g/10l) and placed in a vernalization chamber, at 13 °C for 1 week of acclimation and then at 6 °C (day length 8 h). Plants were planted in the experimental garden in mid-April. Flowering started in mid-May.

Offspring were sexed during the first two flowering seasons, in 2008 and 2009. One-third (339/1113) of the

Table 1 Origin of plants used as parents in the crossing experiment

Population	GPS coordinates		Origin of parents	Parents	Mitotype of parents
	North	East			
Ambleteuse	50°48'	1°37'	Field-collected plants	AMB 5, AMB 9, AMB 13	AMB
Auvergne	44°43'	2°21'	Maternal descent of two plants	AU4.4, AU3.16, AU3.22	AU
Queyras	44°46'	6°44'	Maternal descent of one plant	Q1.2, Q1.1, Q1.7	Q1
			Maternal descent of one plant	Q9.8, Q9.5, Q9.11	Q9

Parents were either directly field collected, or grown from seeds collected from open-pollinated plants. Plants were named by their population of origin, a first number identifying the field-collected plant or the mother of the maternal line, and, when necessary, a progeny number in maternal lines. Thus, AMB5, AMB9 and AMB13 are *a priori* unrelated field-collected plants. Q1.2, Q1.1 and Q1.7 are half- or full-siblings of the same maternal line, as are Q9.8, Q9.5 and Q9.11 from a different maternal line from the same population. Similarly, AU4.4 is from a different maternal line than AU3.16 and AU3.22.

plants flowered in the first year and two-thirds (788/1113) in the second year. About 200 plants did not flower at all. In 2008, newly opened flowers on each plant were sexed every 4 or 5 days during the entire flowering season. The sex phenotype was quantified as the proportion of pistillate flowers. Most gynomonoeious plants were strongly hermaphrodite, bearing more than 80% perfect flowers (data not shown), but some few plants bore few perfect flowers with a majority of pistillate ones. In 2009, the large number of flowering plants made quantitative sex phenotype measurement impossible, so we used a discrete description of sex. Plants were assigned to the female (F), hermaphrodite (H) or gynomonoeious (GM) category by two to five observations during the flowering period. Therefore, in 2009, the number of gynomonoeious plants was underestimated. Indeed, in 2008, about one-third of the plants were found to be gynomonoeious. To take account of the difference in procedure between the 2 years, gynomonoeious plants with fewer than 10% or more than 90% pistillate flowers in 2008 were classified as hermaphrodites or females, respectively. Sex phenotypes, including proportion of pistillate flowers in gynomonoeious individuals, were mostly stable over the years (C Garraud, unpublished data). Although 19 plants out of the 207 that flowered both the years changed their sex phenotype, all changes involved plants that were assigned to the gynomonoeious category during one of the two years and therefore represent a quantitative and not a qualitative change in sex. These plants were excluded from further analysis.

Genetic models for restoration

We generated expected segregation ratios for crosses between two restored hermaphrodite plants for all cases with one or two restorer loci. These are presented in Supplementary Table 2. We tested the segregation ratios from our crosses against these expected ratios. As different mitotypes may represent different CMSs, the number and nature of restorers were studied independently for each mitotype. Heterogeneity was tested with a G-test (1 degree of freedom) with Yates correction for continuity (Sokal and Rohlf, 1995). When several genetic models of restoration gave sex segregation ratios that differed non-significantly from offspring sex segregation ratios, we chose the model with fewer restorer genes. If several models with the same number of genes but different dominance or epistasy characteristics fit the data, we chose the one that minimized the total number

of loci involved over all the crosses. When equivalent models predicted several ratios, none of which differed significantly from the observed ratio, the ratio with the best fit was selected. Restorer genotypes were attributed to the parents, taking into consideration that each parent was involved in different crosses, and the total number of restorer loci was calculated for each mitotype. Gynomonoeious plants were classified as females or hermaphrodites according to their proportion of pistillate flowers. The few gynomonoeious plants for which the proportion of pistillate flowers was close to 0.5 or was not estimated were classified alternatively as females or hermaphrodites and the segregation ratio was tested against different genetic models. For 7 out of 36 crosses, the chosen model depended on how gynomonoeious plants were classified. In those cases, the model that had fewer loci was chosen.

Results

Gynodioecy in *S. nutans* is under nuclear–cytoplasmic control. Reciprocal crosses differed in sex segregation of progenies (Table 2); therefore, cytoplasmic genes are involved in sex determination. Moreover, a nuclear component must also be involved because some hermaphrodite parents generated female offspring.

Sex phenotype segregation

A majority of hermaphrodites was observed in the progenies, with only 17% of females and 7% of gynomonoeious individuals. A few unexpected phenotypes were observed. In 2008, we found three males, one from the cross AU3.16 × Q9.5 and two from the cross Q9.8 × AMB9 (data not shown). Except for the aborted carpels, flower morphology was normal. These plants did not flower again in 2009. We also detected four fully sterile plants, with abnormally small petals and flowers that did not open, but normal vegetative growth. All were from cross AU3.22 × Q9.11, and the two of these that flowered the second year were again sterile. Cyto-nuclear incompatibilities could be responsible for these phenotypes. These plants were excluded from the following analyses.

Cytoplasmic differences and reciprocal crosses

Table 2 presents the numbers of females (F), hermaphrodites (H) and gynomonoeious (GM) individuals segregating in each pair of reciprocal crosses. Differences in sex segregation between reciprocal crosses were tested

Table 2 Sex segregation in progenies of direct and reciprocal crosses

Parent 1	Parent 2	Direct cross			Reciprocal cross			P-value
		F	H	GM	F	H	GM	
		Mitotype AU			Mitotype Q1			
AU4.4	Q1.2	1	27	0	0	10	0	1
AU3.16	Q1.1	0	5	0	0	21	0	1
AU3.22	Q1.7	3	18	5	0	29	4	0.096
		Mitotype AU			Mitotype Q9			
AU4.4	Q9.8	22	3	9	1	26	1	<1e-10
AU3.16	Q9.5	5	15	1	0	15	4	0.041
AU3.22	Q9.11	12	3	3	0	3	0	0.029
		Mitotype AU			Mitotype AMB			
AU4.4	AMB9	40	0	2	—	—	—	—
AU3.16	AMB13	36	1	1	0	32	0	<1e-16
AU3.22	AMB5	27	2	4	0	43	1	<1e-16
		Mitotype Q1			Mitotype Q9			
Q1.1	Q9.5	0	28	1	—	—	—	—
Q1.2	Q9.8	0	30	0	0	30	2	0.492
Q1.7	Q9.11	0	34	2	0	31	1	1
		Mitotype Q1			Mitotype AMB			
Q1.1	AMB13	0	30	0	2	31	3	0.122
Q1.2	AMB9	0	30	1	—	—	—	—
Q1.7	AMB5	0	26	2	0	14	1	1
		Mitotype Q9			Mitotype AMB			
Q9.8	AMB9	0	22	3	4	14	0	0.013
Q9.5	AMB13	0	29	0	0	28	0	1
Q9.11	AMB5	0	19	4	0	20	3	1

Abbreviations: F, female; GM, gynomonocious; H, hermaphrodite. Numbers of F, H and GM individuals are given in a single row for each cross and its reciprocal. In direct crosses, parent 1 was used as mother and parent 2 as father and conversely in reciprocal crosses. Crosses were performed between three representatives of each mitotype but three crosses failed, leaving only two replicates for three mitotype combinations. Heterogeneities between direct and reciprocal crosses were tested with Fisher tests. Significant probability *P*-values are indicated in boldface.

by Fisher's exact tests. Our results show that the AU mitotype is clearly functionally distinct from Q9 and AMB (Table 2). Although Q1 cannot be distinguished from any other mitotype in our crosses, because almost no females segregate from crosses involving this mitotype as either mother or father, Q1 cannot be the same CMS as both AU and Q9. The Q1 parents (Q1.1, Q1.2 and Q1.7) must therefore carry all restorers needed to restore male sterility induced by the three other mitotypes. The AMB and Q9 mitotypes probably carry different CMS factors, because one of the three replicates showed sex segregation differences between reciprocal crosses. Indeed, it is not inconsistent to find significant heterogeneity between reciprocal progenies for some but not all replicates because two distinct CMSs can give similar sex segregation in reciprocal crosses. However, as this difference was no longer significant when gynomonocious plants were grouped with hermaphrodites or

Table 3 Total number of F, H and GM plants carrying each of the four mitotypes

Mitotype	Number (proportions within rows) of		
	F	H	GM
AMB	6 (0.03)	182 (0.93)	8 (0.04)
AU	146 (0.60)	74 (0.30)	25 (0.10)
Q1	0 (0.00)	238 (0.96)	10 (0.04)
Q9	1 (0.01)	175 (0.91)	15 (0.08)

Abbreviations: F, female; GM, gynomonocious; H, hermaphrodite. Heterogeneity between mitotypes was tested by a chi-square test (6 degrees of freedom) and found to be significant ($P < 0.0001$).

females according to their proportion of pistillate flowers, this result must be interpreted carefully.

Female frequencies in our families depended on the cytoplasmic background (Table 3; $\chi^2 = 452$, degree of freedom = 6, $P < 0.0001$). Very few females appeared in cytoplasms other than AU. No females were found on the Q1 background, which may represent a fertile or a very well restored cytoplasm. The sterilizing ability of the AMB mitotype was confirmed by field observation: seven females carrying this mitotype were found in different populations in France and Belgium (data not shown). Q9 must be sterilizing, even if very well restored, because we found one female, two gynomonocious plants with a majority of pistillate flowers and a labile plant that shifted from female in 2008 to gynomonocious in 2009 (data non shown) in our progenies carrying this mitotype. Gynomonocious plants were found in all mitotypes.

Restoration of CMS types

Different levels of restoration can result from different numbers of restorer loci with different dominance and epistasy relationships. The genetic models, involving the fewest loci that give theoretical segregation ratios consistent with our observations, are listed for each cross in Table 4. Our results suggest at least two independent dominant restorers of the Q9 and AMB mitotypes (Table 4). As we obtained no females in the Q1 mitotype, any model of restoration can fit the data for this mitotype. Restoration of the AU mitotype is much more complex. Explaining a 7:1 ratio requires a genetic model with three epistatic loci that can be either recessive or dominant. Taking all progenies carrying the AU mitotype into account, four restorers are necessary to explain our results: one dominant independent locus, and three epistatic loci with at least one dominant and one recessive locus among them. Our estimates of the number of restorer loci are minimum because (i) we chose the fitted model with the fewest loci and not the model with the best fit; (ii) our offspring sample size did not allow us to test very complex models; and (iii) information on the segregation of gynomonocious plants cannot be used, as the genetic basis of this sex phenotype remains unknown.

Discussion

Cytoplasmic types

We found at least two distinct CMS types that were involved in sex determination in *S. nutans*. Two to three

Table 4 Offspring sex ratio and fitted genetic models for restoration

Mother	Father	Offspring		Heterogeneity			Genetic model for restoration
		F	H	Fitted ratio	G(1)	P-value	
<i>Mitotype AMB</i>							
AMB13	Q1.1	2	34	1:15	0.029	0.865569	2 dominant restorers without epistasy
AMB9	Q9.8	4	14	1:3	0	1	1 dominant restorer
<i>Mitotype AU</i>							
AU3.16	Q9.5	5	16	1:3	0.016	0.900	1 dominant restorer
AU3.16	AMB13	36	2	7:1	1.440	0.230	3 epistatic restorers (either recessive or dominant)
AU3.22	Q1.7	4	22	1:3	0.889	0.346	1 dominant restorer
AU3.22	AMB5	29	4	3:1	2.586	0.108	2 loci, either 2 recessive restorers with or without epistasy, either 1 recessive and 1 dominant or 2 dominant with epistasy
AU4.4	Q1.2	1	27	1:15	0.04	0.842	2 dominant restorers without epistasy
AU4.4	Q9.8	30	4	3:1	2.873	0.090	2 loci, either 2 recessive restorers with or without epistasy, either 1 recessive and 1 dominant or 2 dominant with epistasy
AU4.4	AMB9	42	0	1:0	0	1	1 recessive restorer
<i>Mitotype Q9</i>							
Q9.8	AU4.4	2	26	1:15	0.04	0.842	2 dominant restorers without epistasy

Abbreviations: F, female; H, hermaphrodite.

Fitted ratio is the expected ratio of females:hermaphrodites according to the genetic model. We chose the genetic model with the fewer genes over the several models having an expected ratio non-significantly different from the offspring ratio. Heterogeneity was tested with a G-test with Yates correction for continuity (1 degree of freedom). Gynomonoecious individuals were classified as females or hermaphrodites according to their majority sex ratio. Sex ratio of 0:1 and 1:1 can fit any genetic model. For this reason, crosses that present a sex ratio of 0:1 or 1:1 were not informative and are not represented here.

CMSs have often been found to coexist at the species level (Belhassen *et al.*, 1991; Charlesworth and Laporte, 1998; Delph *et al.*, 2007; Dufay *et al.*, 2009) and only extensive studies managed to collect genetic evidence for four distinct CMSs (de Haan *et al.*, 1997b; van Damme *et al.*, 2004). Indeed, demonstrating the differences between CMSs suffers from methodological limitations (see Materials and methods) that can explain why so few CMSs were found. Coexistence of different CMSs within populations has been shown, with two CMSs in *Silene vulgaris* (Charlesworth and Laporte, 1998) and three in *Plantago coronopus* and *Beta vulgaris* ssp. *maritima* (van Damme *et al.*, 2004; Dufay *et al.*, 2009). Unfortunately, our crossing design did not permit us to test within-population polymorphism efficiently.

We showed that all cytoplasm but Q1 segregated females and were thus potentially sterilizing. However, this mitotype segregated one gynodioecious individual with 85% pistillate flowers, demonstrating that suppression of male function occurs at the flower level. Nonetheless, we cannot rule out that other non-CMS mutations or environmental effects may have generated such a plant and the very small number of female plants we found for the Q9 mitotype. To date, fertile cytoplasm have been demonstrated mainly in gynodioecious crops (*Brassica napus*, *Zea mays* and *Beta vulgaris*; reviewed in Delph *et al.*, 2007) and in some wild species (Cuguen *et al.*, 1994; de Haan *et al.*, 1997b; Miyake *et al.*, 2009), but are still unknown in the *Silene* genus. Self-crosses would help us to determine whether Q1 is a fertile or a very well restored cytoplasm.

Restoration of CMS types

The different mitotypes that we found present great variation in restoration level, as has already been shown in *P. coronopus* (Koelewijn and van Damme, 1995b), and show different genetic systems of restoration. Multiple-

restorer systems seem to be common in gynodioecious species, with up to five different restorers acting on the same CMS (Koelewijn and van Damme, 1995b), and quantitative restoration fits well to data on sex segregation in cross progenies of several species (Ehlers *et al.*, 2005). Dominant restorers seem to be more frequent than recessive ones (reviewed in Delph *et al.*, 2007) and benefit from an increased selective advantage because of a stronger association with the minority gamete (Jacobs and Wade, 2003). Epistatic interactions between restorers are often invoked in crossing studies (Koelewijn and van Damme, 1995b; Charlesworth and Laporte, 1998) and have been found in molecular studies of maize (Chase, 2007). But evolution of such restorers remains difficult to understand as they would produce hermaphrodites, and would therefore benefit from a selective advantage, only when all epistatic factors are present. Their role in the dynamics of gynodioecy has never been studied theoretically. Variation in the number of restorers between CMS types (de Haan *et al.*, 1997a) and even between the populations carrying the same CMS (Charlesworth and Laporte, 1998) has already been reported.

Gynomonoecious individuals

Gynomonoecious individuals are a common feature in gynodioecious species (Talavera *et al.*, 1996; Maurice, 1999; Guitian and Medrano, 2000). The frequency of such individuals is difficult to estimate because of sex phenotype plasticity (for example, Koelewijn and van Damme, 1996; Klaas and Olson, 2006) and is frequently underestimated because of phenotyping procedures. Two main hypotheses have been proposed for their genetic determination: quantitative or incomplete restoration (Koelewijn and van Damme, 1995b, 1996; Glaetli and Goudet, 2006) and heteroplasmy, which is the coexistence of mitochondria of different genomes in the same individual (Erickson and Kemble, 1993;

Andersson, 1999). The most likely mechanism that generates heteroplasmy is paternal transmission of mitochondria, as has been found for *Silene vulgaris* (McCauley *et al.*, 2005; Welch *et al.*, 2006; Pearl *et al.*, 2009). We checked the mitotypes of progenies from our crosses and found no cases suggesting paternal transmission of mitochondria (unpublished data).

As sex determination of gynomonocious plants remains uncertain, we tried to minimize their influence on the choice of the genetic model for restoration. We classified them by their majority flower type, whereas most previous studies have excluded them, classified them as hermaphrodites (Charlesworth and Laporte, 1998; van Damme *et al.*, 2004) or built genetic models with the assumption that they are heterozygous at restorer loci (Koelewijn and van Damme, 1995b). Information on the sex determination of gynomonocious individuals is lacking, though it is needed for interpreting sex segregation in progenies of controlled crosses (Koelewijn and van Damme, 1995b; Charlesworth and Laporte, 1998). Moreover, the role of gynomonocious individuals in the maintenance of gynodioecy is still unknown, and theoretical developments for addressing this question will require understanding the genetic basis of such individuals. Unfortunately, this study is unable to provide new insights into their genetic determination or their role in breeding-system evolution.

Geographical pattern in CMS diversity and restoration

Our study found well-differentiated CMSs at a large geographic scale and differences in restoration capacities of the different populations. Although studies on mtDNA polymorphism have sometimes been carried out at the continental scale (Städler and Delph, 2002; Houlston and Olson, 2006; Touzet and Delph, 2009), differentiation of CMSs has generally been studied in very nearby populations (Belhassen *et al.*, 1991; Koelewijn and van Damme, 1995a; Charlesworth and Laporte, 1998). Here, we demonstrated that the AU mitotype is clearly distinct from the Q9 and AMB mitotypes. Preliminary results on the mitotypes' geographical distributions suggest that the AU and AMB mitotypes are common in Europe and exhibit wide geographic distributions that are largely overlapping (S Le Cadre, unpublished data). On the other hand, the Q1 and Q9 mitotypes are specific for the Queyras population, which showed extreme mtDNA polymorphism (Touzet and Delph, 2009). Interestingly, the AU and AMB mitotypes were found to coexist in one population in Belgium and in the Queyras population, meaning that two different CMSs may coexist in these populations. However, as mitotypes were defined only by the *cob* sequence, it should be checked by further reciprocal crosses or sequencing.

As our tested mitotypes were chosen from distant populations and were highly differentiated at our marker loci, we expected to find more females in our progenies. Indeed, crosses between distant individuals are expected to yield higher proportions of female offspring than crosses between moderately related individuals, because of spatial correlations between CMS types and their associated restorers (Bailey and McCauley, 2005). Such a structure can be achieved when restorers suffer a silent cost of restoration, that is, a cost expressed only when the

restorers are not active (silent) in sex determination. Indeed, these restorers are expected to co-occur with the CMS they restore, because they are counter-selected when the CMS is absent. Such costs have found experimental support in some gynodioecious species (for example Bailey, 2002; Dufay *et al.*, 2008; Del Castillo and Trujillo, 2009), but spatial match between CMS and associated restorers is still debated. Better restoration is found in within- compared with between-population crosses in *Thymus vulgaris* (Belhassen *et al.*, 1991; Gigord *et al.*, 1998) but not *Silene vulgaris* (Emery and McCauley, 2002; Bailey and McCauley, 2005), and the level of restoration does not decrease with geographic distance in between-population crosses (Gigord *et al.*, 1998; Bailey and McCauley, 2005). Moreover, restorers were found to be maintained outside of the geographical distribution of their associated CMS in *P. coronopus* (van Damme *et al.*, 2004) and no correlation was found between CMS and restorer frequencies in *Beta vulgaris* ssp. *maritima* (Dufay *et al.*, 2009).

We found some evidence for co-occurrence between CMSs and their associated restorers at a large geographical scale, which could suggest the existence of a silent cost of restoration. Restorers of the AU mitotype are present in Queyras but not in Ambleteuse, at least in the sample of nuclear genotypes used for the current study, since individuals from Queyras (especially Q1 but also Q9) were able to restore the AU mitotype, whereas parents from Ambleteuse were not (Table 2). This is consistent with the geographical distributions of the AU mitotype, which was found in the Queyras but not in the Ambleteuse population (S Le Cadre, unpublished results). On the other hand, restorers of the AMB mitotype must present a wide geographic distribution: both Auvergne and Queyras individuals were able to restore it (Table 2).

Therefore, restorers associated with the two more frequent and widely distributed mitotypes that we found in Europe (AMB and AU) have not been equally successful in their expansion. This variation could be explained by the differences in the expansion dynamics of the two mitotypes, or by different costs and molecular constraints that can affect restorer evolution and maintenance, but genetic drift could also have a role. Although we found the Q9 mitotype in only one population, it was well restored in all crosses, suggesting that its restorers are widely distributed, which is unexpected for a rare CMS. However, we cannot be completely sure that the Q9 and AMB mitotypes represent different CMS, so Q9 could be well restored as AMB is widespread. If, on the other hand, Q9 were a different CMS than AMB, its high degree of restoration would be more difficult to explain (but see the case of CMS Sv in beet; Dufay *et al.*, 2009). The identity of the Q9 mitotype should be confirmed by further crosses. To understand the dynamics of restorer distributions and frequencies, the proportion of females in the populations and the association between mtDNA mitotype and sex phenotype need to be determined in the field. The better knowledge of large-scale geographical distributions of CMSs and restorers will offer new perspectives for the study of gynodioecy.

Conflict of interest

The authors declare no conflict of interest.

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