Université Paris Sud XI, UFR scientifique d'Orsay.
École doctorale Sciences du Végétal.

THÈSE

Présentée par

Claire Garraud

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Spécialité : Biologie

Evolution of gynodioecy-gynomonoecy

Experimental approaches in *Silene nutans* & theoretical approach

Soutenue le 11 Mars 2011 devant le jury composé de :

David McCauley, Professor at Vanderbilt University, Nashville, TN	Rapporteur
Sylvain Glémin, Chargé de Recherche CNRS, Université Montpellier II	Rapporteur
Sandrine Maurice, Maître de conférences à l'Université Montpellier II	Examinateur
Joël Cuguen, Professeur à l'Université des Sciences et Technologies de Lille 1	Examinateur
Pierre Capy, Professeur à l'Université de Paris-Sud 11, Orsay	Examinateur
Jacqui Shykoff, Directeur de recherche, CNRS, Orsay	Directrice de thèse

Doubt is an unpleasant mental state but certainty is a ridiculous one.

Voltaire

Warning

I apologize to all non-french readers, as this English version is an incomplete translation of the French version.

Untranslated parts belonged to chapters 2 and 4 :

- In the original French version, chapter 2 presented: (1) The molecular basis of CMS and restorer genes. (2) Theoretical and experimental studies on the conditions of gynodioecy maintenance. (3) The sex determination in gynodioecious species.
  The first part and the methodological aspects of the third part have not been translated. I rewrote the second part in a more concise way, in order to summarize the conditions for gynodioecy maintenance and the different dynamics that are mentioned but not fully explained in Article 1.
- The french version of section 4.5.1 (chapter 4) described evidence of recombination in the mitochondrial genome in gynodioecious plants and the studies carried out since 2005 on paternal leakage and heteroplasmy (Bentley et al., 2010; McCauley et al., 2005, 2007; Pearl et al., 2009; Welch et al., 2006). This part was not translated.

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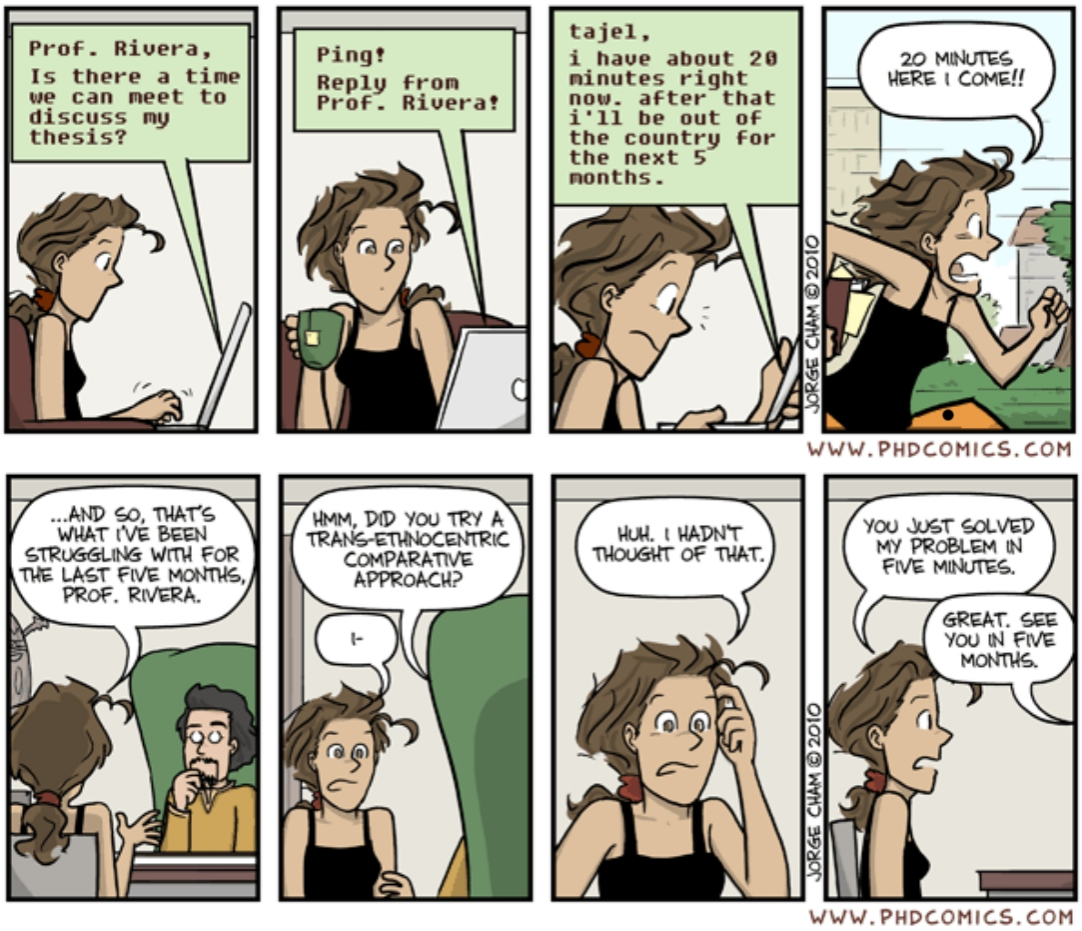

"Piled Higher and Deeper" by Jorge Cham — www.phdcomics.com

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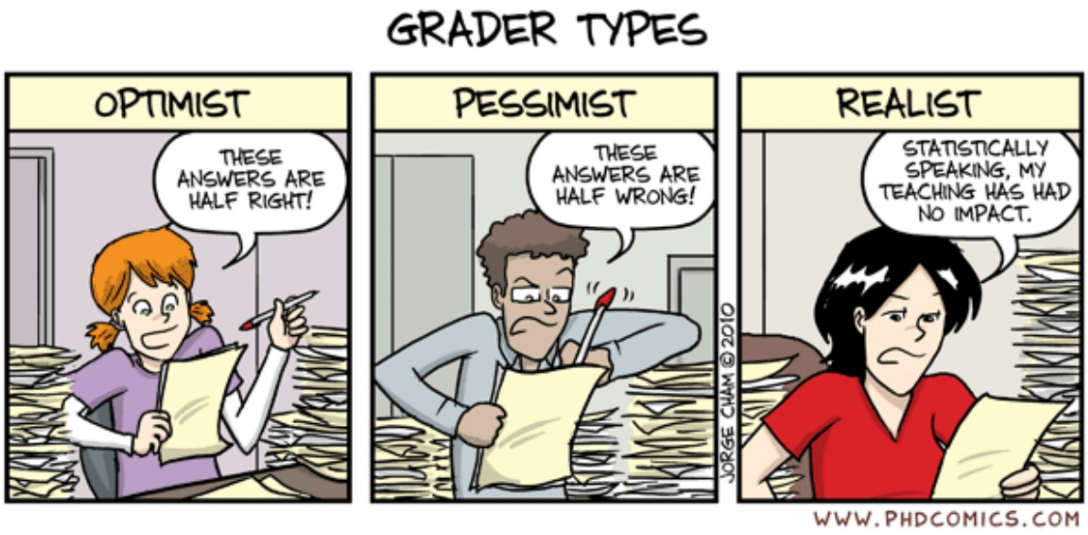

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Contents

Chapter 1

Everything you always wanted to know about sex . . . but were afraid to ask[1]

Because it generates great interest, the evolution of sex and its characteristics benefits from a huge literature, in which it is easy (and delightful) to get lost. One of the difficulties of this literature is linked to the vagueness in definition of some words. Thus, I will define in this introduction some essential words of this manuscript that are related to sex. Some of these words are subject to controversy, but I will keep the definitions simple.

Sex is defined by the exchange of genetic (or epigenetic) information between individuals. In eucaryotes, sex is punctuated by the alternation of meiosis, which reduces the level of ploidy, and fertilization which increases it by bringing together the genomes of two haploid cells called gametes. However, the characteristics of the haploid (gametophyte) and diploid (sporophyte) phases, the meiosis and the fertilization vary greatly. For instance, if fertilization often involves the fusion of two gametes, genetic material can also be exchanged without any fusion (e.g. paramecia conjugation). In the first case, sex creates a new genetic individual; in the second case, the new genotypes are carried by the parents. It is therefore necessary to distinguish sex and reproduction. Reproduction (with or without sex) implies a vertical transmission of information from one generation to the next, while sex without reproduction is a horizontal transmission of information between individuals of the same generation. Sex without reproduction is very well known in procaryotes, but can also be found in eucaryotes (e.g. ciliate conjugation, horizontal gene transfer, gene transfer between nucleus and symbionts or organelles).

The word "sex" has a Latin origin, *secare*, which means "cut, divide" (Gouyon, 2009). This origin puts the emphasis on the separation of sexes. It is important to distinguish the word "sex" from the word "sex (sing.), sexes (plur.)" which means categories of individuals whose characteristics relating to sexuality differ (e.g. males and females). In the absence of sexes, any individual is a potential (sexual) partner.

[1] Allen W. et al, 1972.

On the contrary, if there is more than one sexes, the two partners must differ for their sexes. The existence of sexes is rather counter-intuitive, since it reduces the number of compatible sexual partners within a population and this all the more when the number of sexes is low. The predominance of species with two sexes exacerbates this paradox. The evolution of sex and the evolution of sexes, although closely linked, must therefore be distinguished in order to avoid misunderstandings. The common discussion of the two-fold cost of sex illustrates how much evolution of sex and evolution of sexes have been considered concomitantly. Indeed, it is rarely remembered that this cost only applies to anisogamous species: it is only because the female gamete delivers all the nutrients for the future zygote that reproduction with two parents do not produce more offspring that reproduction with one parent[2].

This thesis will deal with the evolution of sexes (a bit), of sexual systems (a lot), but not of the evolution of sex itself. In this chapter, some key concepts of the evolution of sexes and sexual systems, particularly in plants, will be presented. More detailed (and subject specific) information will be given in the following chapters throughout the presentation of my thesis work.

1.1 The evolution of sexes

The evolution of sexes, which has been less explored than the evolution of sex, has nevertheless been the subject of many models, which differ significantly according to the definition of "sexes" used. These definitions are based on three (non-exclusive) characteristics:

- the size of the gametes
- mitochondrial inheritance
- the mechanisms of recognition between gametes (e.g. pheromone-receptor system in some fungi)

Even if, for a given species, a single criterion may suffice to define groups of compatible gametes, the combination of several criteria is needed to define sexes when all eucaryotes are considered. Hurst and Hamilton (1992) have proposed a classification of species based on mitochondrial inheritance, which defines what they called "sexes" in their publication, and the number of "incompatibility types" (see TABLE 1.1). By this classification, Hurst and Hamilton (1992) show that these two criteria are adequate for defining sexes and can be combined. For instance, in angiosperms, sexes are the male (pollen) and the female (ovule) functions, and "incompatibility types" the system of self-incompatibility.

Let us now see how the features that characterize sexes have evolved.

[2]Here, we neglect post-natal investment, which can also influence this cost.

	Incompatibility types<1	Incompatibility types>1
0 sex		ciliates conjugation, some basidiomycetes
1 sex		basidiomycetes
2 sexes	most organisms (e.g. animals, plants, ascomycetes)	tunicates, self-incompatibles angiosperms
>2 sexes		*Physarum polycephalum* (13 sexes)

Table 1.1: Sexes and "incompatibility types", from Hurst and Hamilton (1992). Sexes are defined by mitochondrial inheritance: 0 sex means that there is no gamete fusion, 1 sex that mitochondrial inheritance is biparental and 2 sexes (or more) that mitochondrial inheritance is uniparental and controlled by 2 (or more) sexes. "Incompatibility types" represent groups of morphologically undifferentiated gametes. Two gametes having two different "incompatibility types" are compatible for sex, two gametes of the same type are not. This table is not exhaustive and does not take account of lots of exceptions within each group (e.g. for fungi, see Billiard et al., 2010).

1.1.1 Evolution of anisogamy

In many eukaryotes, called anisogamous, male and female gametes differ in size, the male gamete being small and generally mobile and the female one being large, full of resources and immobile. In these species, the evolution of sexes could therefore result from the evolution of anisogamy. Evolution of anisogamy is by far the best theoretically studied aspect of the evolution of sexes. In this part, I will present the model of Parker et al. (1972), regarded as the reference model, and the main points of discussion that have emerged in subsequent developments.

1.1.1.1 The PBS model (Parker, Baker & Smith)

If one understands intuitively the advantage of producing small gametes in large numbers at low cost, it is less obvious to explain why the production of large and costly gametes should be able to evolve and be maintained. In other words, why do female gametes accept to be parasitized by male gametes, which transmit their genes without bringing any nutrients for the future zygote? Parker, Baker & Smith suggested a response to this paradox in 1972. Their model, which aims to explain the evolution of sexes via the evolution of anisogamy, is based on two simple ideas:

– The total quantity of resources allocated by an individual to the production of gametes is constant. There are several strategies of distribution of these resources, including two extremes which are the production of a lot of small gametes or a few big ones.

– Zygote fitness depends on the quantity of available resources, i.e. on zygote volume. The relationship between zygote fitness and zygote volume is defined by the following function: $f = V^x$, where f is zygote fitness, V its volume, and x a parameter of the model.

The volume and the number of produced gametes are assumed to be heritable, and the fusion of gametes occurs at random. They also assumed that different strategies of gamete production occurred initially in the population.

Parker et al. (1972) demonstrate that the evolution of gamete volume depends mainly on the relationship between the fitness and the volume of the zygote ($f = V^x$). If zygote fitness does not depend strongly on volume (x small), small gametes are favored. On the contrary, if the fitness increases strongly with increasing volume (x high), large gametes are favored. However, for some intermediate values of x ($x \approx 2$), disruptive selection occurs: producers of large and small gametes are favored at the expense of intermediate gamete producers and a balanced ratio between the two types of producers is observed at equilibrium. In other words, the maintenance of two types of gametes is possible when the advantage of a large zygote compared with a smaller zygote is balanced with the high productivity of small gamete producers. A high initial variability in the population increases the range of x values compatible with disruptive selection.

Although disruptive selection occurs only under restricted conditions ($x \approx 2$), these conditions are selected by natural selection. Consider zygote fitness in relation to volume. In metazoa, the first stages of development are ensured thanks to the egg resources. Therefore, it can be assumed that an increase in zygote volume would have a strong positive effect on zygote fitness when the zygote is small but would have a small effect when the zygote is already quite large, giving a diminishing returns function (see FIGURE 1.1). Thus, when zygotes are small, selection on zygote volume will be very strong (x high), which will favor the large gamete producers and increase *in fine* the volume of zygotes. On the contrary, if the volume of zygotes is almost optimal (x small), the small gamete producers will be favored and the volume of zygotes will decrease. In both cases, the only stable solution as a result of natural selection appears to be one in which gametes of two strongly different sizes coexist in the population.

However, anisogamy does not automatically imply the existence of sexes because the definition of sexes also requires that fusion occurs always between a large and a small gamete (disassortative mating for gamete size). Evolution of disassortative mating is not trivial since it should be more advantageous for both a large or a small gamete to fuse with a large gamete, thereby benefiting from more resources for the future zygote. Parker et al. (1972) suggested that mating preference could have been selected more efficiently by male gametes for two reasons:

– The cost of fusion with a small gamete is likely to be far larger for a small gamete than for a large one, depending on the relationship between fitness and

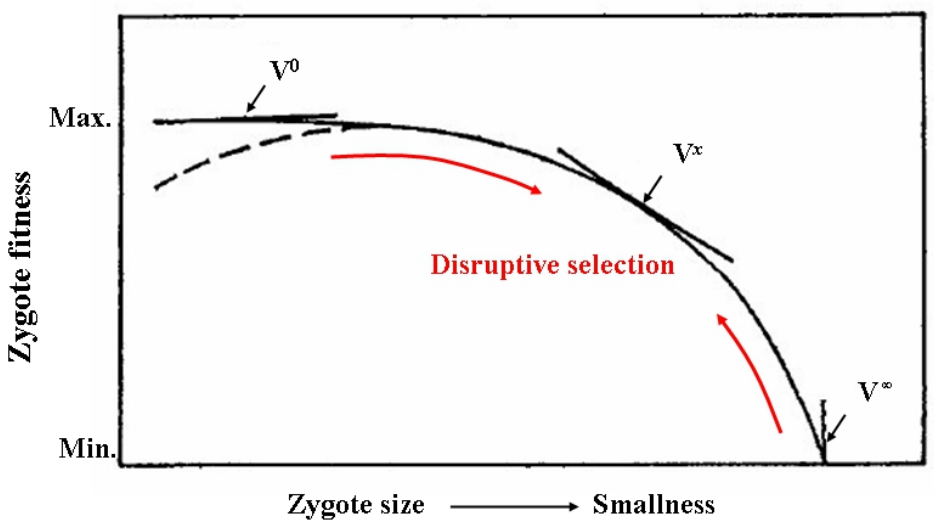

Figure 1.1: Relationship between the fitness and the volume of the zygote, from Parker et al. (1972). The relationship between fitness and volume is defined at each point by $f = V^x$, x being dependent on V. At optimal zygote volume, variation in volume have small effects on zygote fitness and the curve is defined locally by $f = V^0$. On the contrary, when the volume is very small and hence far from the optimum, variation in volume have strong effects on fitness and the curve is defined locally by V^∞.

zygote volume. Therefore, preference for fusion with large gametes could have been selected more efficiently in small male gametes.

– The population of the small gametes could present a higher genetic variability thanks to a higher population size and a higher mutation rate. Thus, if competition for fusion occurs, one should find more mutants able to enforce fusion with a large gamete among small gametes, than mutants able to prevent it among large gametes.

1.1.1.2 Subsequent developments of the PBS model

Many studies on the evolution of anisogamy have their origin in this PBS model. Some subsequent models have only reformulated and clarified the original model, but others have initiated more or less controversial discussions on some aspects of the model. We present here two of these controversial subjects:

– The PBS model requires a particular relationship between the volume and the fitness of the zygote (see FIGURE 1.1). Parker et al. (1972) mentioned that this relationship is plausible for metazoa whose development depends on egg resources but is unrealistic for unicellular species. The predominance of anisogamy among multicellular organisms and of isogamy among unicellular organisms supports their hypothesis. Vertebrates are not good models for

testing this hypothesis, since the development of amniotes does not depend only on zygote resources and egg size varies little in anamniote vertebrates. Therefore, studies focused on algae, and particularly Volvocales (Bell, 1978), and echinoids (i.e. Levitan, 2000). Many studies have provided support to the PBS model but the interpretation of results has sometimes been subject to debate (Randerson and Hurst, 2001) and no consensus is yet established in this regard.

– Parker et al. (1972) proposed that the evolution of motility differences between the two types of gametes could stabilize the two types of gamete producers and disassortative mating. They suggested the role of sperm competition but their demonstration remains purely verbal. More recent models have suggested that anisogamy can be the consequence (and not the origin) of the evolution of motility (for a review see Randerson and Hurst, 2001). Indeed, under sperm limitation, motility differences between male and female gametes would increase gamete encounter rate without decreasing the size of the zygote. In these models, motility is selected under sperm limitation and not under sperm competition as predicted by Parker et al. (1972). More generally, many models on the evolution of anisogamy and motility have been constructed on the idea that the female gamete is a target and that traits increasing gamete encounter rate (larger size, production of pheromones, etc.) should be selected.

1.1.2 Evolution of mitochondrial inheritance

Another criterion used to define sexes is mitochondrial inheritance. Unlike the nuclear genome whose sexual transmission is controlled by meiosis, cytoplasmic genomes are often uniparentally inherited[3] and sexes can therefore be defined by the ability to transmit the cytoplasm. In anisogamous species, cytoplasmic genomes are generally maternally inherited, which is fairly intuitive considering the disparity in volume between the two types of gametes. However, cytoplasmic inheritance is unlikely to be a mere consequence of anisogamy. Indeed, many isogamous species exhibit uniparental inheritance of organelles, the best studied being the green unicellular algae *Chlamydomonas reinhardtii*[4] (Ferris and Goodenough, 1997; Nishimura et al., 2002).

In the nineties, a new category of models arose, based on the idea that the evolution of sexes could have been driven by the evolution of uniparental inheritance of organelles. These models suggested that uniparental inheritance has evolved to prevent invasion by selfish cytoplasmic elements.

Most eucaryotes carry, in addition to the nuclear genome, a mitochondrial genome and possibly a chloroplastic genome. Mitochondria and chloroplasts results from endosymbiotic assimilation of bacteria into the future eucaryotic cell and this origin

[3] Exceptions will be discussed in chapter 4.

[4] Gametes are no longer called male and female in this case, but type a and α.

explains some of their characteristics. Among other things, organelles contain their own DNA and replicate and transmit their genome in a way that differs fundamentally from nuclear genes. Indeed, the transmission of the nuclear genome is highly controlled by the mechanisms of DNA replication and mitosis. In contrast to the "stringent" nuclear genome, organelles have "relaxed" genomes (Birky, 2001). Every cell generally contains several organelles, each one containing several copies of its genome. Organelles replicate independently from each other and from the cellular cycle and are distributed randomly between the two cells at mitosis. Thus, if the cell contains several different mitochondrial genomes, the frequencies of alleles in the lineage are likely to vary. As a consequence, intra-individual selection can occur and is favorable to the emergence of selfish mitochondrial elements. For instance, a mitochondrial mutant with a higher replication rate (e.g. thanks to a DNA deletion) will be selected at the intra-individual level, even if it has a negative impact on the fitness of the individual that carries it. Such selfish elements have been described in yeast (see p.15). This selection leads to an arms race between mitochondrial genomes (inter-cytoplasm conflict).

In eucaryotes, sex almost always involves the fusion of two gametes that allows horizontal (between lineages) transmission of cytoplasmic elements. Such a mixture of cytoplasm is favorable to selfish mitochondrial elements, which may be transmitted more efficiently, but is highly deleterious to the organism, who suffers the cost of these selfish elements. Thus, when selfish elements exist, a nuclear mutation that enforces uniparental inheritance of organelles would be selected. Indeed, uniparental inheritance results *in fine* in homoplasmic lineages in which intra-individual selection is unable to act.

The evolution of uniparental inheritance of organelles is usually modeled by the evolution of a selfish mitochondrial element and a nuclear mutant allowing the destruction of its own cytoplasm or the partner cytoplasm (Hastings, 1992; Hurst, 1994; Hurst et al., 1996; Randerson and Hurst, 1999, 2001; Yamauchi, 2003). Whatever the modeling details, the nuclear mutant spreads thanks to its genetic linkage with the non-selfish cytoplasm. On the contrary, if this linkage is not maintained – for example, when the nuclear mutant allows the destruction of its own cytoplasm, or when inheritance is not strictly uniparental – conditions for the mutant invasion are more restricted. In addition, Yamauchi (2003) has recently shown that recurrent invasions of selfish elements are needed to maintain the uniparental mutant. More generally, selfish cytoplasmic elements (parasites, plasmids, etc.) could be involved in the evolution of sex (and not only sexes), because sex allows horizontal transmission (Hoekstra, 1990). Interestingly, a mitochondrial (non deleterious) plasmid that disables uniparental inheritance in order to increase its own transmission has been discovered in *Physarum polycephalum* (Sakurai et al., 2004).

Some other models assume that mixing different cytoplasms have a cost for the organism *per se* (instead of considering the long-term cost of selfish elements, see Frank, 1996; Hurst and Hamilton, 1992). Molecular incompatibilities may explain

this cost but, to my knowledge, there is only one experimental validation of this cost, whose results were obtained from artificial inter-specific crosses (Ziegler and Davidson, 1981).

Other aspects of mitochondrial inheritance will be discussed in chapter 4.

1.1.3 Evolution of sexes through cellular recognition

The evolution of anisogamy and uniparental inheritance of organelles are often, *in fine*, sustained by the evolution of molecular recognition. However, in order to explain the evolution of sexes in isogamous species or species with biparental inheritance of organelles, some models have studied how the evolution of sexes can be driven by the evolution of molecular recognition.

1.1.3.1 The by-product model

In this model, the emergence of sexes is linked to the molecular structure of the recognition system that allows fusion between gametes (Hoekstra, 1982). The model assumes that, initially, the recognition system is symmetrical, each gamete carrying a pheromone and a pheromone receptor on its cell surface. The evolution of sexes would result from the suppression of the pheromone in some gametes, and the suppression of the receptor in others. The model assumes that this specialization would render the recognition more efficient, for instance by avoiding self-saturation of receptors.

1.1.3.2 The inbreeding depression avoidance model

If selfing rates and inbreeding depression are high enough, sexes may evolve because they prevent fertilization between two gametes bearing the same alleles, and thus prevent selfing. Such models explain the evolution of sexes as well as the evolution of self-incompatibility in plants (Charlesworth and Charlesworth, 1979).

1.1.3.3 The sex advantage enhancer model

Most of the advantages of sex rely on sex occurring between gametes that are genetically different (breakdown of detrimental genetic associations, red queen hypothesis, etc.). Sexes promote the fertilization between gametes that are genetically different and thus could have been selected for by maximizing the advantages of sex mentioned above.

1.1.4 A universal model?

None of the models presented here provides a universal response to the question of the evolution of sexes. Each one brings a solution for species having some characteristics and models that considered the evolution of several traits (anisogamy, motility, organelle inheritance, pheromonal recognition system, etc.) are almost nonexistent.

Yet, sexes are often differentiated by several of these traits and it is difficult to know which trait led to the evolution of sexes and which trait only reinforced pre-existing sexes. Moreover, plants and animals provide little information for answering this question since almost all of them are anisogamous and have a uniparental inheritance of organelles. Other more variable groups, such as algae or fungi, are more interesting, but data are often incomplete (for a review in fungi, see Billiard et al., 2010). Nowadays, it seems that more and more researchers have dropped the idea of a universal explanation to the evolution of sexes, and believe that this issue should rather be studied at the level of taxonomic groups.

1.2 Les "noces végétales" [5]

Flowering plants present a huge diversity of sexual systems. However, on the contrary to groups such as fungi, where almost all combinations of gametes in size, number, mitochondrial inheritance, etc. are observed, flowering plants have a "standard" sexuality, with two anisogamous sexes, male and female, and a maternal inheritance of organelles. So what does this huge diversity mean?

1.2.1 Sexes and sex phenotypes in flowering plants

Sexes are, by definition, categories of individuals that are compatible for sex. But in plants, the definition of the male function (gametes born by pollen) and the female function (gametes born by ovules) is not sufficient for defining compatible partners and other characteristics need to be considered.

Unlike animals, most plant species are hermaphrodites (see TABLE 1.2), i.e. each individual produces both male and female gametes. However, in some species, a self-incompatibility system prevents self-fertilization or fertilization with individuals that carry the same self-incompatibility alleles. This system needs to be taken into account in defining compatible partners. On the other hand, in self-compatible species, the mating system (selfing and/or outcrossing) is subject to diversified strategies that influence the likelihood of sex with compatible partners. Finally, the distribution of male and female functions in time, between individuals, and within individuals is extremely variable. Morphs (sometimes called genders or sex phenotypes) can be defined by the characteristics of this distribution. Here are some examples:

– *Heterostyly.* Heterostyly, which is well known among species in the *Primula* and the *Lythrum* genera, is defined by two or three morphs that differ by the position of the anthers and stigma. Symmetry in the position of the reproductive parts is often observed between morphs (see FIGURE 1.2). This polymorphism allows pollen receipt and deposition that are morph-specific and then promotes

[5] Expression used by Linnæus in the 18th century that translates roughly as "botanical weddings" or "botanical unions". He was the first to propose a plant classification based on the number of (sexual) floral parts.

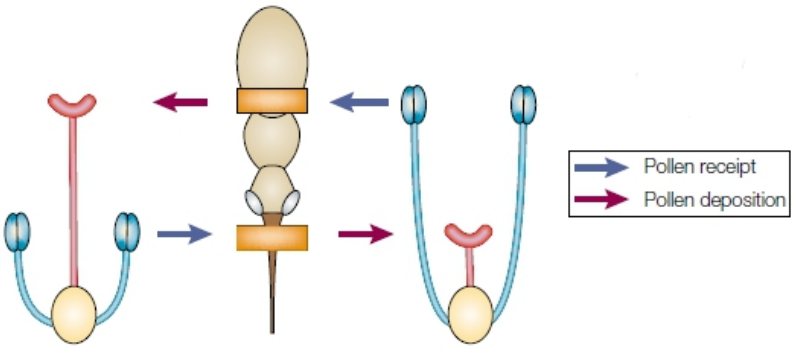

Figure 1.2: Floral design and pollen transfer in a distylous species (heterostyly with two morphs), from Barrett (2002). This diagram illustrates two morphs that differ in the spatial arrangement of female (pistil) and male (stamen) sexual organs. Pollen transfers ensured by pollinators are represented by arrows.

sex between morphs that are different. Its evolution is often explained by selfing avoidance, but other factors such as the optimization of pollen transfer are at least as important (Barrett, 2002).

– *Heterodichogamy.* Dichogamy occurs when the timing of male and female functions differs and is called protandry when pollen is dispersed before stigmas are receptive and protogyny when stigmas are receptive before pollen is dispersed. In heterodichogamous species, two morphs, a protogynous and a protandrous ones, coexist in populations (Kimura et al., 2003) and fertilization between morphs is favored. In some cases, the system is more complex, and the flowers operate alternately as male or female depending on the time of the day (i.e. ginger species). For instance, one morph is male in the morning and female in the afternoon, and another, female in the morning and male in the afternoon. Changes in sex are ensured by a movement of the style (flexistyly) which favors either the deposition or the receipt of the pollen. Here again, crosses between morphs are favored.

– *Separation of sexes.* Although most of the plant species are hermaphrodite, there is a huge variation in the distribution of the male and the female functions within and among individuals. In the following, we will focus on this diversity.

1.2.2 Sexual systems in flowering plants

The main sexual systems of flowering plants are summarized in Table 1.2[6]. Hermaphroditism is, by far, the most common system, but there are also many

[6]There is actually a even higher diversity and almost all combinations of sex phenotypes described in Table 1.2 can be found.

species that are monoecious (including many gymnosperms), dioecious and gynodioecious, each of these systems being distributed among different families (for the distribution of dioecy among families, see Charlesworth, 2002). Sexual systems are often classified in two groups: monomorphic systems, which contain only one sex phenotype, and polymorphic systems, which contain two, or sometimes more, sex phenotypes.

Sexual system	Sex phenotype of individuals	Flower sex		
		pistillate	staminate	perfect
hermaphroditism (80%)	hermaphrodites			X
monoecy (5%)	monoecious	X	X	
gynomonoecy	gynomonoecious	X		X
andromonoecy	andromonoecious		X	X
dioecy (6%)	males		X	
	females	X		
gynodioecy (7%)	hermaphrodites			X
(or gynodioecy-gynomonoecy)	females	X		
	(gynomonoecious)	X		X
androdioecy	hermaphrodites			X
(or androdioecy-andromonoecy)	males		X	
	(andromonoecious)		X	X
trioecy	males		X	
	females	X		
	hermaphrodites			X

Table 1.2: Main sexual systems in flowering plants. The table describes, for each system, the different sex phenotypes and the sex of the flowers produced by each phenotype. In order to avoid confusion, the terminology is different for flowers and individuals. Flowers are perfect (hermaphrodite), pistillate (female) or staminate (male), and individuals are hermaphrodite, female, male, gynomonoecious, etc.

1.2.3 The evolution of separate sexes

The low frequency of polymorphic systems and their scattered distributions among taxa tend to show that hermaphroditism, and more generally monomorphic systems, are ancestral in the evolution of sexual systems in plants. In some families presenting various sexual systems, the distribution of sexual systems in the phylogeny has provided information about the evolution of sexual systems. For instance, in the genus *Silene*, dioecy appeared independently in at least two lineages, probably from a gynodioecious ancestor (see FIGURE 1.3). Reviewing all these works has allowed an estimation of the number of transition between sexual systems, and to propose hypotheses on the conditions that are favorable to these transitions.

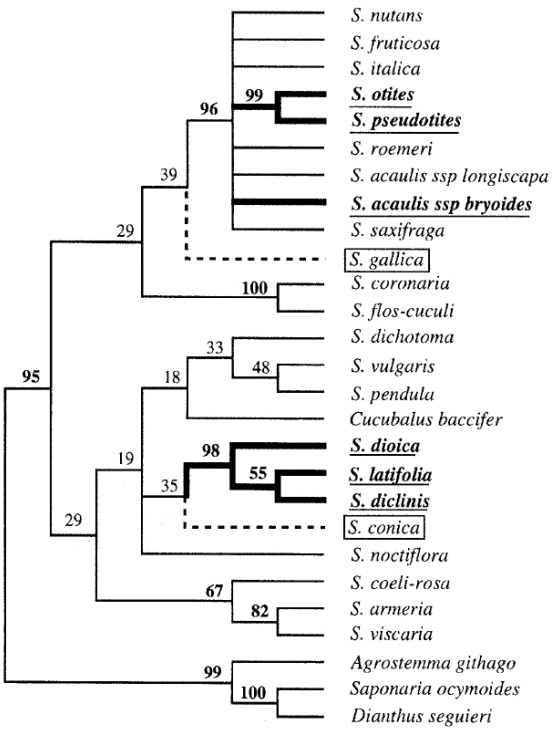

Figure 1.3: Phylogeny of the genus *Silene*, from Desfeux et al. (1996). Dioecious species are underlined and in bold, and hermaphrodite species are framed. All other species may be considered as gynodioecious. The numbers on the nodes are bootstrap estimates. In this genus, it seems that gynodioecy is ancestral and that dioecy evolved twice independently.

Currently, the evolution of dioecy is the most debated. In the main hypothesis, dioecy evolved via gynodioecy (pathway a, FIGURE 1.4). The first step (hermaphroditism→ gynodioecy) involves the appearance and maintenance of a male sterility gene and will be presented in more details in section 1.3. Dioecy could then evolve from gynodioecy through the specialization of hermaphrodites to male function. This specialization can occur because of frequency-dependent selection that favors the rare sex (here, the male function). However, it requires heritability of allocation to male function and a trade-off between allocations to the male and female functions. This hypothesis is supported by the high prevalence of dioecious species in clades that are predominantly gynodioecious (Maurice, 1993).

There is another less studied hypothesis, in which dioecy evolves via monoecy (pathway b, FIGURE 1.4). It has been demonstrated that the transition from hermaphroditism to monoecy had occurred many times, because of the spread of sterile mutants producing unisexual flowers. Then, disruptive selection on quantitative genetic variation in floral sex ratios in monoecious populations has gradually increased gender specialization and led to dioecy.

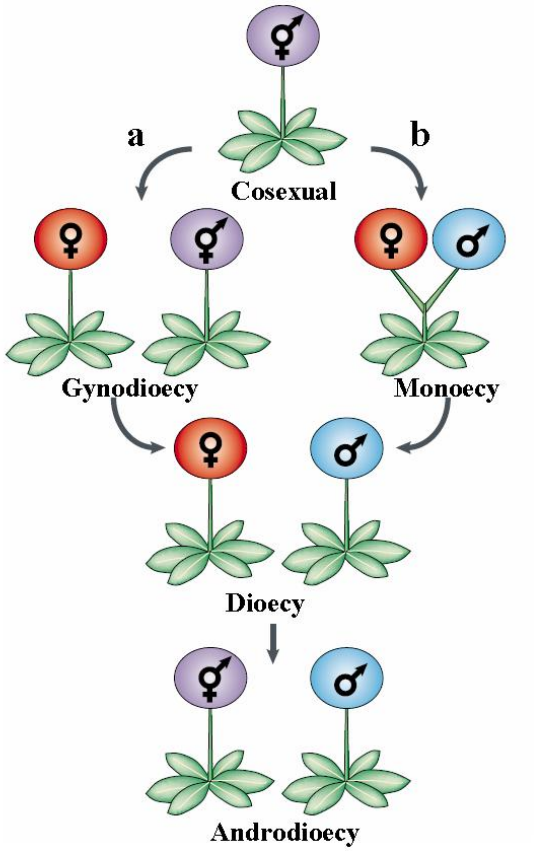

Figure 1.4: Pathways for the evolution of gender dimorphism in flowering plants, from Barrett (2002).

The few studied cases of androdioecy show that it did not evolve by the emergence of female-sterile mutations in a hermaphrodite population, contrary to gynodioecy. It rather seems that it evolved through the (re)emergence of hermaphrodite plants in dioecious populations, because of their selfing advantage during colonization events. Androdioecy would result from the replacement of females by hermaphrodites, and trioecy from the addition of hermaphrodites to the two pre-existing phenotypes (FIGURE 1.4).

1.2.4 Genetic determination of sex in flowering plants

In flowering plants, genetic determination of sex is almost as diverse as sexual systems are. Sex chromosomes, the standard sex determination in animals, are rare in plants but have been found in some dioecious species, in which sex is determined by a XY system (e.g. *Silene latifolia*). Sex chromosomes of plants are very similar to those of animals (structure, inheritance, etc.) and they appear to have evolved through the same steps (genetic linkage between sex loci and gradual suppression of recombination). However, some dioecious species do not have morphologically distin-

guishable sex chromosomes and putative intermediate steps of the sex chromosomes evolution have been observed. Because dioecy has evolved many times independently and quite recently in plants (compared to animals), dioecious plants are a very good model for studying the evolution of sex chromosomes (Charlesworth, 2002). Sex determination of androdioecy seems to be more simple and involves only one nuclear locus, the male allele being generally dominant (Pannell, 2002). In gynodioecious species, sex determination can be nuclear or cytonuclear. This sexual system and its genetic determination will be presented at the end of the introduction and in chapter 2 (p.33).

1.3 Evolution of selfish mitochondrial elements and male sterility

In this part, the evolution of cytoplasmic male sterility will be presented in the context of the evolution of selfish cytoplasmic elements. The evolution of gynodioecy will be the main illustration.

1.3.1 Selfish gene theory and genomic conflicts

If the few thousands of genes carried by an organism seem to interact "in harmony", it is because these genes have often been selected because of their positive effect on the fitness of the organism. However, the interests of genes can differ, and any mutant able to increase its own transmission may be selected, even if it has a deleterious effect on other (not genetically linked) genes and/or on the whole organism. This idea led to the selfish gene theory (Dawkins, 1976). Selfish genes do not need actually to be genes, but can be any small piece of replicating DNA up to an entire chromosome. There is a great diversity of selfish elements and some, such as transposable elements are almost universal. Most of selfish elements follow one of these strategies:

- interference: the selfish element prevents the transmission of the alternative element/allele, for example by destroying the gametes which do not contain the selfish element.
- overreplication: the selfish element escapes the controls of rules regulating the fairness of DNA replication, sometimes by complex mechanisms. ex: transposable elements, some mitochondrial selfish genes.
- gonotaxis: these elements move preferentially toward the germline and away from somatic cells, when presented with the choice.

The spread of a selfish element often induces the selection of genes, within the unfavored part of the genome, that are able to counteract the effect of the selfish gene. Disagreements between parts of the genome have been called intragenomic conflicts, and occur when "the spread of one gene creates the context for the spread

of another gene, expressed in the same individual, and having the opposite effect" (Hurst et al., 1996). The existence of the selfish gene and the suppressor is not needed for genomic conflict to exist, but it renders it explicit and visible. Genomic conflicts are considered as a major evolutionary force and could be involved in the evolution of sex, sexes, sex determination, reproductive isolation, genome structure and recombination (Hurst and Werren, 2001; Hurst et al., 1996).

In this part, I will present only mitochondrial selfish elements, that take advantage of the "relaxed" mitochondrial genome, and conflicts related to these elements. We have already addressed their importance in the evolution of organelle inheritance (see p.6). The following section illustrates mitochondrial selfish elements by three examples of elements with different strategies of transmission.

1.3.2 Examples of selfish mitochondrial elements

1.3.2.1 "Petite" mutation in yeast

In yeast, mitochondrial DNA (mtDNA) transmission is biparental and the fusion of the two gametes (a and α) generates a heteroplasmic cell, i.e. a cell that contains several different mitochondrial genomes. Then, mitochondria segregate rapidly during mitoses and in a few generations, cells become homoplasmic by drift. Mutants that are unable to respire because of a defective mitochondrial DNA have long been known in *Saccharomyces cerevisiae* (Taylor et al., 2002). Although these mutants can still survive and replicate by fermentation, they suffer from a reduced growth rate in an aerobic environment, and were then called "petite".

By crossing "petite" and normal strains and propagating the progeny until they have only one mitochondrial genome, it has been observed than up to 95% of the progeny ends up "petite". This observation shows that, despite its cost on the cell, the mutant is able to transmit very efficiently. In one of these mutants, it has been demonstrated that its replication advantage results from a higher density of replication origins in the mitochondrial genome (MacAlpine et al., 2001). Despite their transmission advantage, "petite" mutants are rarely found in natural populations[7] and several explanations have been suggested:

- high inbreeding rates prevent the spread of the "petite" mutant.
- the mutant is too costly for the organism.
- the rapid segregation of mitochondria just after fusion reduces the period of time during which intra-individual selection is possible.

All the same, "petite" yeasts remain one of the best-known examples of selfish mitochondrial elements and are frequently held up as an example to justify the assumed advantage of uniparental inheritance of organelles used in evolutionary models.

[7] They are more often detected in anaerobic media, where their disadvantage in growth compared to normal strains is reduced.

1.3.2.2 Pathogenic mitochondrial mutants in mouse and human

Even when mitochondrial inheritance is uniparental, selfish mutants can take advantage of the "relaxed" mitochondrial genome. Indeed, biparental inheritance is not the unique cause of heteroplasmy, which can also occur by mutation during development. The frequency of a mitochondrial mutant can therefore increase in the tissues during the lifetime of an individual.

The fate of mitochondrial mutants, and particularly those inducing a pathology, has been studied in the mouse and in humans. Studies on heteroplasmic individuals have shown that the proportion of defective mtDNA increases during development in most of the tissues (but decreases in others) and that the severity of the pathology is correlated with the proportion of defective mtDNA in the organism (Chinnery et al., 2000; Sato et al., 2007). Why does defective mtDNA accumulate is not known with certainty. It was suggested that it has a replication advantage as do "petite" mutants in yeasts. It is also possible that mitochondria bearing the defective mtDNA replicate faster than normal ones. Indeed, replication of mitochondria is up-regulated by the nucleus when the concentration of ATP in the cell is low. Then, replication of mitochondria would be favored in cells containing mainly mutant mitochondria (because these mitochondria are less efficient in the production of ATP), leading to an increased proportion of defective mtDNA (Burt and Trivers, 2006). On the other hand, it seems that the defective mtDNA accumulates in nondividing tissues and is lost from rapidly dividing tissues. This loss would result from mitochondrial segregation during mitoses, which enhances inter-cell selection by increasing the variance between cells (Roze et al., 2005).

The main issue remains, however, the transmission of the defective mtDNA through the germline. Drift seems strongly involved in the segregation of mitochondrial mutants in the germline, thanks to a bottleneck in the population of mitochondria in this lineage, and may enhance the selection against these mutants (Jenuth et al., 1996). Furthermore, Sato et al. (2007) have shown that the proportion of defective mtDNA in ovules declines over the lifetime, probably because of specific elimination.

1.3.2.3 Non-reciprocal nucleus exchange in homobasidiomycetes

An interesting new kind of selfish mitochondrial element has been discovered recently in *Hebeloma crustuliniforme*, a homobasidiomycete. In this taxon, reproduction generally occurs as follows: two monokaryotic (a single haploid nucleus) hyphae, resulting from the germination of haploid spores, exchange their nuclei without karyogamous fusion or exchange of cytoplasm, and give rise to a dikaryon (two haploid nuclei per cell). The inherited nuclei then replicate and migrate within the two parental hyphae, ensuring that all the cells of the dikaryon carry the same nuclear genome. In contrast, half of the dikaryon carry the mitochondria of the first parent, and the other half the mitochondria of the second parent, forming a "cytoplasmic mosaic" (see FIGURE 1.5). This mosaic does not lead to any intracellular conflict since

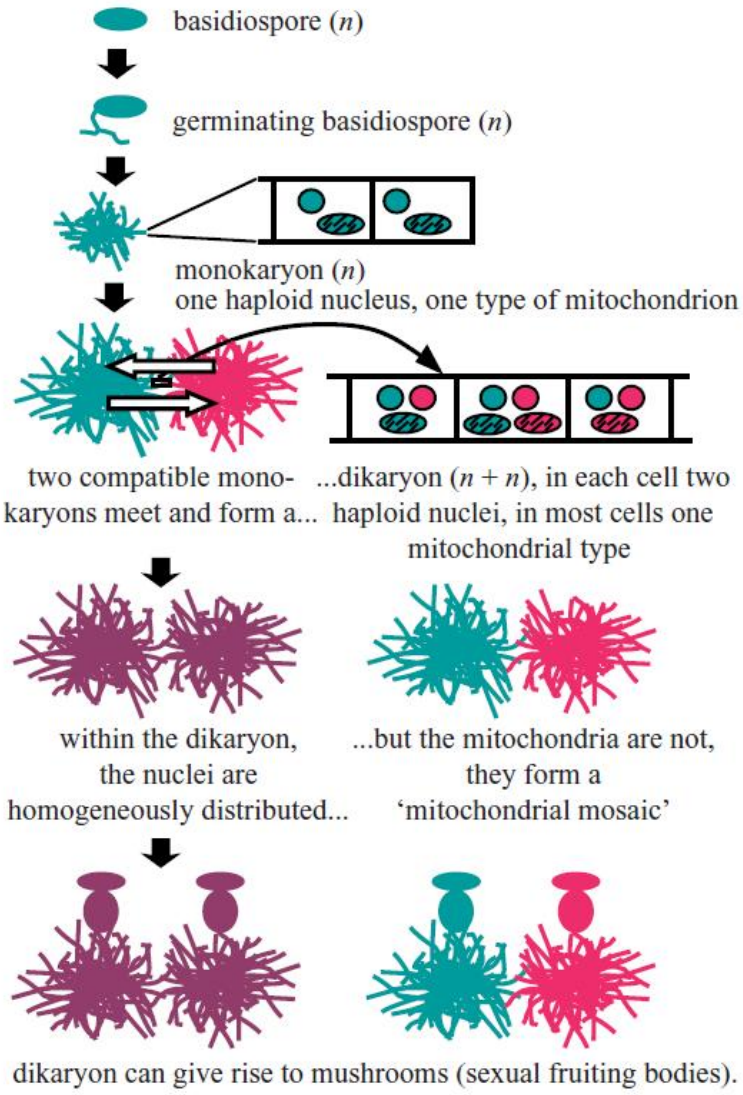

Figure 1.5: General life cycle of a homobasidiomycete, from Aanen et al. (2004).

each cell is homoplasmic but at the dikaryon level, the two types of mitochondria compete over transmission. Therefore, a mitochondrial mutant able to increase its chances of being included in the spores would be selected, even if it reduces the total number of spores produced by the individual and represents a cost for the nuclear genome.

It has been shown that non-reciprocal transfers of nuclei during fertilization commonly occur in some homobasidiomycetes (i.e. Hintz et al., 1988). With non-reciprocal transfer, one monokaryon donates a nucleus and the other accepts it, but not vice versa. This phenomenon is called cytoplasmic male sterility, the "male" function being the nucleus donation. Male sterility increases the transmission rate of the mitochondria carried by the monokaryon that does not donate its nucleus (i.e.

which is male-sterile) but is costly for the nuclear genome because half of the dikaryon contains a single haploid nucleus and is thus unable to reproduce (see FIGURE 1.5).

Aanen et al. (2004) have developed a genetic model in which the exchange of nuclei is determined by:

- the mitochondria, which may be male-sterile (S as selfish) or male-fertile (F as fair).
- the nucleus, which may contain resistant genes (R resistant vs. N non-resistant), that are positively selected when the frequency of male-sterile mitochondria is high.

Non-reciprocal transfers occur when one of the partners have S mitochondria and the other have N nuclear genes (excluding SN-SN fusions that do not lead to any dikaryon). This model explains to a large degree the results obtained from controlled crosses in *Hebeloma crustuliniforme* (Aanen et al., 2004).

Male sterility in homobasidiomycetes thus represents a very good example of conflict, including selfish elements (male sterility) and suppressors (resistant nuclear genes). It is analogous to cytoplasmic male sterility in plants (male sterility $\leftrightarrow$ CMS; resistant nuclear genes $\leftrightarrow$ restorers, see the next section). However, in homobasidiomycetes, selfish mitochondria modify the behavior of nuclei in order to directly increase their representation in propagules or spores while, in plants, selfish mitochondria do not influence mitochondrial inheritance but distort the sex-ratio in order to make the laws of inheritance more favorable to their transmission.

1.3.3 Evolution of cytoplasmic male sterility

Until now, we have shown that uniparental inheritance of organelles limits invasion by selfish mitochondrial elements. Because uniparental inheritance is a way to control the transmission of genes during sex, it can be considered as the analogue to meiosis (see FIGURE 1.6). But solving the inter-cytoplasm conflict by uniparental inheritance generates a new conflict, between the nucleus and the cytoplasm. Indeed, production of male offspring is an evolutionary dead-end for cytoplasmic genes, that are maternally transmitted. Therefore, cytoplasmic genes would profit from a sex-ratio biased toward females (or more generally toward the sex which transmits the cytoplasm) whereas a 1:1 sex-ratio is best for nuclear genes (see FIGURE 1.7). This conflict between the cytoplasm and the nucleus for the sex-ratio is called the cytonuclear conflict.

1.3.3.1 Evolution of the cytoplasmic male sterility (CMS) in plants

Cytoplasmic male sterility (CMS) in plants is the best known example of this conflict. In approximately 7% of angiosperm species, distributed over 39 families, females, or male-steriles, are found in natural populations in coexistence with hermaphrodite individuals. Although some cases of nuclear male sterility are known

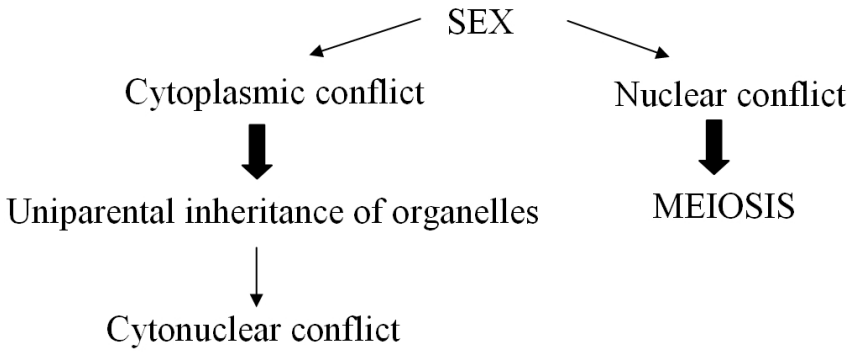

Figure 1.6: Sex and conflicts.

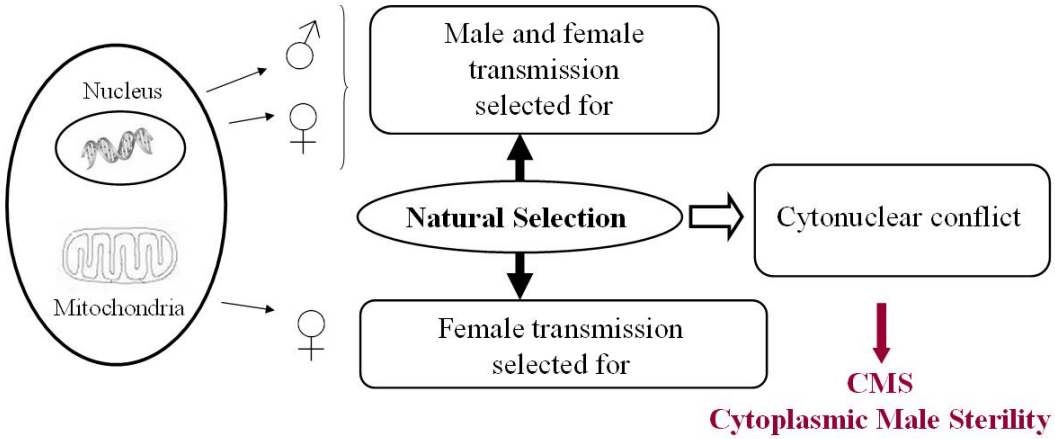

Figure 1.7: The cytonuclear conflict, from Saumitou-Laprade et al. (1994).

(e.g. in *Fragaria*, see Ashman, 1999), male sterility is generally induced by mitochondrial genes. Because they are maternally transmitted, cytoplasmic male sterility genes can spread if females produce more seeds than do hermaphrodites and experimental support to the female advantage has been provided in many gynodioecious species (reviewed in Shykoff et al., 2003). This advantage can result from two phenomena:

- Females produce more ovules because they reallocate the resources not used in the production of pollen to the production of ovules.
- Females do not suffer from inbreeding depression because they cannot self and thus produce better offspring than do hermaphrodites.

The female advantage (s) is usually defined as the quantity of additional ovules produced by females compared to hermaphrodites ($1+s$ vs. 1, see TABLE 1.3). Thus, when s is positive, the mitochondrial male sterility gene is positively selected and if nothing eventually prevents it (like pollen limitation of female reproductive success),

it invades the population, causing its extinction (Lewis, 1941). Under such a model, gynodioecy is transient and lasts during the invasion of the sterility gene.

	hermaphrodites	females
ovule	1	$1+s$
pollen	1	0

Table 1.3: Male et female fitness of hermaphrodites and females.

If mitochondrial genes benefit from the invasion of the male sterility gene, the latter is costly for nuclear genes that are not transmitted through pollen by females. Therefore, any nuclear mutation that restores male fertility will be positively selected. These genes are called restorers and are selected in two ways. First, they benefit from Fisherian selection, which grants advantage to alleles carried by the rare sex. Fisherian selection is frequency-dependent and the restorer allele will be selected only when the frequency of females is high enough, and more precisely when:

$$s(P_h - P_f) < 1$$

with P_h is the frequency of hermaphrodites and P_f the frequency of females (Jacobs and Wade, 2003).
Second, if s <1, hermaphrodites produce more gametes than females do (pollen + ovule: 2 vs. $1+s$, see TABLE 1.3) and the transmission of nuclear genes is better in hermaphrodites than in females. Restorers are therefore selected only by Fisherian selection when $s > 1$ and by Fisherian selection and the advantage in the total quantity of gametes when $s < 1$.

Two simple examples of dynamics are presented in FIGURE 1.8. It may be mentioned that the spread of the restorer allele slows down the invasion of the CMS because the restored hermaphrodites (those who bear the male-sterile allele and the restorer) transmit the CMS without the female advantage. The spread of the CMS stops only when the restorer is fixed. More complex dynamics will be presented in chapter 2.

The occurrence of gynodioecious species probably under-estimates the importance of selfish mitochondrial elements that distort sex-ratio in plants. In hermaphrodite species, male-sterile offspring are frequently observed in progenies of interspecific crosses (or even intraspecific crosses between individuals from different populations). In these hybrids, male sterility is often maternally inherited, suggesting a CMS gene. In most cases, it is assumed that the species carry a cryptic CMS, hidden by fixed restorers, which is revealed by hybridization with populations in which the restorer is not present.

On the other hand, CMS is only an extreme strategy of sex-ratio distortion and genes with a slighter effect (e.g. a reduction in pollen production of y% and an increase in ovule production of x%) must have been selected as well. Such genes

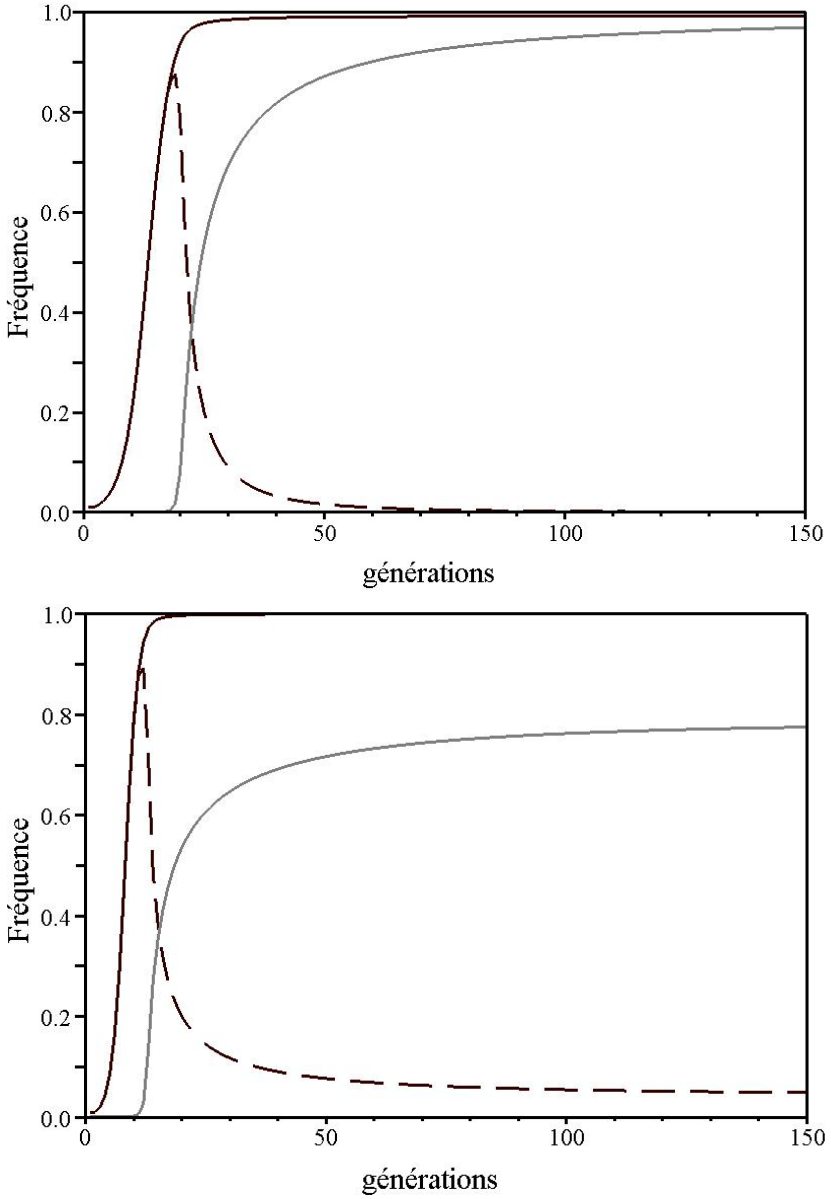

Figure 1.8: Dynamics of gynodioecy in a model with one CMS locus and one biallelic nuclear locus for restoration. The frequencies of the male-sterile allele, the restorer allele and females are plotted in black, gray and dotted lines respectively. Initial frequencies of the male-sterile allele and the restorer allele are 0.01 and 0.01 respectively. **Top**: Dynamic when $s < 1$ (here $s = 0.5$). The male-sterile allele and the restorer allele get fixed. In this example, the male-sterile allele becomes fixed before the restorer allele. For smaller values of s, the restorer allele becomes fixed before the male-sterile allele, which become neutral and stabilizes at an intermediate frequency. In both cases, the population would be gynodioecious transiently before returning to hermaphroditism (cryptic CMS). **Bottom**: Dynamic when $s > 1$ (here $s = 1.1$). In contrast to the previous dynamic, the restorer allele is selected only if $s \times (P_h - P_f) < 1$. Its frequency will thus increase and then stabilize at a frequency such as $s \times (P_h - P_f) = 1$. At equilibrium, the population is gynodioecious with a nuclear determination of sex.

have never been observed but quantitative changes in phenotype are more difficult to detect than qualitative changes, and other selfish mitochondrial elements may exist.

One of the consequences of male sterility is the selection of restorer alleles, which reduce the impact of male sterility genes and sometimes lead to hermaphroditism. However, other nuclear responses to CMS exist, such as masculinization of hermaphrodites which has been shown in some species of *Thymus*, in *Hebe subalpina* and in *Fragaria virginiana*, where half of what appear to be "hermaphrodites" are actually males (Burt and Trivers, 2006). The limit of this process is complete masculinization, that is, dioecy. But dioecy does not solve the cytonuclear conflict and cytoplasmic male killers are expected to be selected. Male killers have been well described in the bacteria *Wolbachia* (see the following section), a symbiot of many nematodes and arthropods. Females biased populations have been observed in dioecious species of the genus *Silene*, but we do not know if the distortion is controlled by the cytoplasm (Taylor, 1994).

1.3.3.2 Is cytoplasmic male sterility a specificity of plants mitochondria?

There is one well studied case of cytoplasmic male sterility in animals which presents many similarities with plants CMS. The sterility is not mitochondrial, but is governed by a endosymbiotic bacteria, *Wolbachia*. This bacterium infects many nematodes and arthropods (e.g. 25% of insects) and, because it lives in the cytoplasm, is maternally transmitted. It has developed various strategies to increase its transmission and some of them involve sex-ratio distortion toward females (Werren et al., 2008):

- *Male killers*. The bacterium kills male embryos and reallocate the energy into the development of female embryos.
- *Diploid feminization*. In some crustaceans (e.g. *Armadillidium*), the bacteria causes genetic males to develop as females. Infected females produce strongly female biased progenies.
- *Haplodiploid feminization*. This strategy is similar to the previous one but the mechanism differs. In some species haplodiploid species, in which males are haploids and females diploids, the bacteria causes the chromosomes to double and thus transform males into females.

Analogies between male sterility in plants and *Wolbachia* will be discussed further in ARTICLE 5 (see p.126).

To my knowledge, there is only one known example of mitochondrial male sterility in animals. Indeed, no mitochondrial male-sterility gene has ever been found in hermaphrodite, androdioecious and gynodioecious species in animals. Similarly, mitochondrial distorters have never been described in dioecious animal species, while

nuclear distorters are common (e.g. in *Drosophila*). The only example of mitochondrial male sterility has been discovered in humans thanks to a study on infertility. One relatively common mitochondrial haplotype (the "T" haplotype) was found to be associated with moderately reduced sperm motility, due to a slightly less efficient oxidative phosphorylation (reviewed in Burt and Trivers, 2006). This slightly reduced efficiency would strongly affect sperm motility, which is extremely sensitive to variation in ATP production.

Why are there so few examples of mitochondrial male sterility in animals? And why, in plants, cytoplasmic male sterility is always mitochondrial and not chloroplastic?

One limit of models on genomic conflicts is linked to the probability of appearance of mutations: it is useless to know that a mutation would be selected if we do not know whether this mutation can appear. This could explain why mitochondrial sterility genes occur more frequently in plants than in animals. Indeed, mitochondrial sterility genes result from rearrangements by recombination of part of the mitochondrial genome and the mitochondrial genome of animals is much more compact than in plants (15-20 kb vs. 200-2500 kb in plants) and does not recombine[8] (Budar et al., 2003). It could also explain why cytoplasmic male sterility is always mitochondrial in plants, since the recombination rate of the chloroplast genome is lower than that of the mitochondrial genome. Moreover, mitochondrial genes may be involved into sensitive functions that are essential for the development of anthers and pollen grains (e.g. functions that require a high ATP production, see Budar et al., 2003).

More generally, the absence of cytoplasmic selfish elements where they are expected shows that the nucleus is often the winner of the cytonuclear conflict. This tendency might be explained by broader opportunities of mutations in the nucleus (larger genome containing more non-coding regions).

1.4 Outlines of the thesis

This manuscript deals with the evolution and maintenance of gynodioecy, when male sterility is cytoplasmic (cases of pure nuclear gynodioecy will not be discussed). Three aspects will be presented in detail. The first part tackles sex determination and its evolutionary consequences (chapter 2). Theoretical models on the evolution of gynodioecy will be presented. In chapter 3, the emphasis will be on the gynomonoecious phenotype. The reproductive characteristics of these individuals will be compared to those of females and hermaphrodites, and their consequences on the evolution of gynodioecy will be discussed. Environmental and genetic control will be the subject of the second part of the chapter. Finally, chapter 4 will deal with mitochondrial inheritance in gynodioecious species, by the light of recent results obtained in *Silene vulgaris*.

[8]The mutation rate is 20 times greater in animal mitochondria than in plant mitochondria but recombination seems to be more likely to create CMS than are point mutations.

These questions will be studied by experimental approaches on gynodioecious *Silene nutans* and by a theoretical approach in chapter 4. Each chapter has its own brief introduction.

Chapter 2

Maintenance and sex determination of gynodioecy

This chapter is an incomplete translation of the original French version (see warning at the beginning of this manuscript).

2.1 Conditions for the maintenance of gynodioecy: theoretical studies and experimental support

Studying the conditions of maintenance of cytonuclear gynodioecy and of the underlying genetic polymorphism represents the greatest part of research on gynodioecy. Indeed, simple models such as those presented in the general introduction do not allow one to explain the maintenance of gynodioecy. Many theoretical studies have thus explored the conditions for the maintenance of cytonuclear polymorphism and some of these conditions have been supported experimentally. These conditions will be summarized in this chapter. In the first part, we will examine the influence of the characteristics of restorers and CMS genes, assuming infinite population and panmixia (sections 2.1.1 and 2.1.2). Genetic structure and pollen limitation will be presented in section 2.1.3. Finally, information on gynodioecy dynamics provided by the genetic diversity of the mitochondrial genome will be discussed.

2.1.1 The characteristics of restorer genes

2.1.1.1 Definitions

In theoretical studies, restoration of a CMS is usually controlled by a biallelic nuclear locus with a restorer and a non-restorer allele. The restorer allele can be dominant, recessive or partially dominant but almost all models assume that it is dominant (experimental data support this choice, see p.27).

We have seen in the general introduction that, without any cost associated with restoration, cytonuclear polymorphism cannot be maintained. Costs have thus been

introduced in models to explain the maintenance of gynodioecy and can be distinguish by:

- The dominance of the cost (different from the dominance of the restorer).
- The cost can be expressed/silent/constitutive: a cost is expressed if it applies when the restorer is associated with a cytotype that it can restore, silent if it applies when the restorer is not associated with a cytotype that it can restore and constitutive if it applies in all individuals that carry the restorer.
- The cost can be paid in production of ovules or pollen.

Tables 2.1 and 2.3 illustrate different costs of restoration.

2.1.1.2 Influence of restoration costs on the maintenance of gynodioecy: theoretical studies

Two main categories of models have looked at the influence of restorers and need to be distinguished because they do not give the same results. The first category assumes a male-fertile cytoplasm (C_f) and a male-sterile cytoplasm (C_s) (Dufay et al., 2007; Jacobs and Wade, 2003; Ross and Gregorius, 1985) whereas the second category assumes two male-sterile cytoplasms (C_1 and C_2), each being restored by a specific restoration locus with a dominant restorer allele (R_1 and R_2) (Bailey et al., 2003; Gouyon et al., 1991; Ross and Gregorius, 1985). TABLES 2.1 and 2.3 illustrate the relationship between genotype and phenotype in the two types of model.

Let us now see the influence of restoration costs in C_s vs. C_f models. In these models, the maintenance of cytonuclear gynodioecy is possible if the restorer and the CMS alleles are selected by frequency-dependent selection. The male-sterile cytoplasm is favored in the absence of the restorer allele because of the female advantage. Cytoplasmic polymorphism is thus maintained when the male-sterile cytoplasm is counter-selected when the frequency of the restorer is high. Similarly, the restorer allele is positively selected when the frequency of the male-sterile cytoplasm is high, and should be counter-selected when the frequency of the male-fertile cytoplasm is high in order to maintain nuclear polymorphism. Briefly, cytonuclear polymorphism is maintained if:

- the hierarchy of female fitness is: C_s associated with r $>$ C_f $>$ C_s associated with R (maintenance of cytoplasmic polymorphism)
- the hierarchy of male or female fitness is: r associated to $C_f$$>$ R associated to C_f (maintenance of nuclear polymorphism)

Two costs must be applied in order to fulfill these conditions. The first one is an expressed seed cost that allows the male-sterile cytoplasm to be counter-selected when the restorer is common (see TABLE 2.1). The second one is a silent or a constitutive cost (it must be paid at least by individuals carrying the male-fertile cytoplasm) and allows the restorer allele to be counter-selected when the male-fertile cytoplasm is common (see TABLE 2.1). The second cost can be paid in ovules or pollen and be recessive or dominant, although cytonuclear polymorphism is maintained in a broader

Cytoplasm	C_s			C_f		
Genotype at the restoration locus	RR	Rr	rr	RR	Rr	rr
Sex phenotype	H	H	F	H	H	H
Female fitness	$1-a_1$	$1-a_1$	$1+s$	1	1	1
Male fitness	1	1	0	$1-a_2$	1	1

Table 2.1: Male and female fitness in a model with a male-fertile cytoplasm (C_f) and a male-sterile cytoplasm (C_s), the latter being restored by a dominant restorer allele (R) with a dominant expressed seed cost (a_1) and recessive silent pollen cost (a_2). These two costs allow the maintenance of cytonuclear polymorphism but other costs are also possible (see details in the text). s: female advantage; H: hermaphrodite; F: female; R: restorer allele; r: non-restorer allele.

range of parameters when the cost is recessive and acts on pollen (Dufay et al., 2007). Indeed, a recessive cost is responsible for over-dominance (heterozygotes advantage) at the restoration locus. A cost acting on ovules decreases the transmission of the male-fertile cytoplasm and enhances the fixation of the male-sterile cytoplasm (i.e. the loss of the cytoplasmic polymorphism).

Restoration costs have a different influence in models with two male-sterile (C_1 and C_2) cytoplasms (Bailey et al., 2003; Gouyon et al., 1991). The main factor is the dominance of the cost. If the cost is recessive, the polymorphism is maintained by over-dominance (see TABLE 2.2). If the cost is dominant (TABLE 2.3), the polymorphism is maintained by frequency-dependent selection and results are similar to those obtained in C_s vs. C_f models (for an example of frequency-dependent dynamics, see FIGURE 2.1).

2.1.1.3 Experimental studies on restorers

Experimental studies have revealed that most of restorers are dominant. Among crops, all restorers were found to be dominant (see TABLE 2.4) and crossing studies in wild species confirmed this trend (see TABLE 2.5 p.34).

The characteristics of restoration costs had been deduced from the mechanisms of restoration which are well known in some crops (see the review of Delph et al., 2007, and TABLE 2.4 from the publication). Costs have been shown to be silent or constitutive, which is in favor of the maintenance of polymorphism in models. Costs seem to apply either exclusively on pollen, either on pollen and ovules simultaneously. To date, no information on the dominance of cost is available.

In wild plants, restoration genes are unknown and the detection of costs requires distinguishing between hermaphrodites that carry the restorer and hermaphrodites that do not. Two direct (Bailey, 2002; de Haan et al., 1997a) and two indirect (Del Castillo and Trujillo, 2009; Dufay et al., 2008) demonstrations of the existence

R/R	R/r	r/r
hermaphrodite	hermaphrodite	female
1-cost	1	0

Table 2.2: Male fitness of individuals carrying a male-sterile cytoplasm. This cytoplasm is restored by the restorer allele R. If the restorer is dominant and restoration cost is recessive (in this example, the cost is expressed and on pollen), the polymorphism at the restoration locus is maintained by over-dominance.

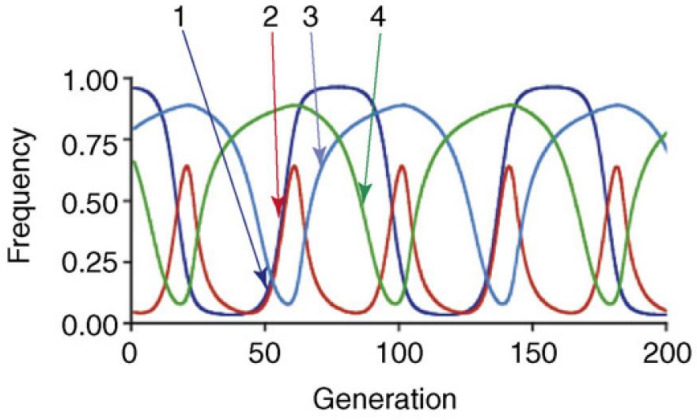

Figure 2.1: Frequency-dependent selection in a model with two male-sterile cytoplasms, C_1 (in dark blue) and C_2 (not represented). The frequencies of R_1, R_2, and females are in light blue, green and red respectively. (1) Initially, C_1 and its restorer R_1 are both rare; (2) C_1 is selected thanks to the female advantage and the frequency of females increases; (3) The R_1 restorer spreads in the population because C_1 is common; (4) The restorer R_2 is counter-selected because C_2 is rare (R_2 have a silent or a constitutive cost in this example). At this point, C_2 and R_2 are both rare in the population, causing the cycle to restart. From Delph et al. (2007).

Cytoplasm	C_1			C_2		
Genotype at the restoration loci	R_1/- r_2/r_2	R_1/- R_2/-	r_1/r_1 -/-	R_2/- r_1/r_1	R_2/- R_1/-	r_2/r_2 -/-
Sex phenotype	H	H	F	H	H	F
Female fitness	1	1	$1+s_1$	1	1	$1+s_2$
Male fitness	1	$1-a_2$	0	1	$1-a_1$	0

Table 2.3: Male and female fitness of the different genotypes in a model with two male-sterile cytoplasms (C_1 and C_2), each being restored by a restoration locus with a dominant restorer allele (R_1 and R_2) which has a dominant silent pollen cost (a_1 for R_1 and a_2 for R_2). "-" means that the identity of the allele does not matter. H: hermaphrodite ; F: female.

Species	CMS gene (cytotype)	Restorer	Restorer dominance	Predicted mode of cost[c]	Predicted effects based on tissue expression[d]
Brassica *Brassica napus*	*orf224* (*pol*)	*Rfp*[a]	Dominant	Silent	Pollen
	orf222 (*nap*)	*Rfn*[a]	Dominant	Constitutive	Pollen
Maize *Zea mays*	*urf13* (CMS-T)	*Rf1*	Dominant	Silent	Seed and pollen
	orf355/orf77 (CMS-S)	*Rf3*	Dominant	Constitutive	Seed and pollen
Bean *Phaseolus vulgaris*	*orf239* (*pvs*)	Fr_2	Dominant	Silent	Pollen
Sunflower *Helianthus annuus*	*orf522* (PET1)	*Rf1*[b]	Dominant	Silent	Pollen
		Rf2[b]	Dominant	Silent	Pollen
Petunia *Petunia* x *hybrida*	*pcf*	*Rf*	Dominant	Silent	Seed and pollen
Radish *Raphanus sativus*	*orf138*	*Rfo*	Dominant	Silent	Pollen
Rice *Oryza sativa*	*orf79* (CMS-BT)	*Rf-1a*[b]	Dominant	Constitutive	Seed and pollen
		Rf-1b[b]	Dominant	Silent	Seed and pollen
Sorghum *Sorghum bicolor*	*orf107* (IS1112C)	*Rf3*[b]	Dominant	Insufficient data	Seed and pollen
		Rf4[b]	Dominant	Insufficient data	Insufficient data

a Restorers are allelic (i.e. occur at the same locus).
b Restorers are epistatic.
c The mode of cost is considered constitutive when the restorer alters expression of necessary mitochondrial genes that are not co-transcribed with the CMS gene and is considered silent when the restorer has no known pleiotropic effects other than producing an unnecessary protein.
d The effects of cost are assumed to be limited to pollen production or pollen viability when the restorer is only expressed in anther tissue and to act on both seeds and pollen when expression of the restorer is not limited to anther tissue.

Table 2.4: Restorer alleles of CMS genes of crops, their predicted mode of cost, and whether the cost would lead to a reduction in either seed and/or pollen quantity or quality, from Delph et al. (2007).

of restoration costs have been provided in wild gynodioecious species. In the first two studies, information on the CMS and restorer alleles carried by individuals were first obtained by crossing experiments while in the two others, correlative approaches were used. All four studies demonstrated the existence of a silent cost, that acts either on the seed production (de Haan et al., 1997a; Del Castillo and Trujillo, 2009) or on pollen quality (Bailey, 2002; Dufay et al., 2008).

2.1.2 CMS and female advantage

In order to explain the maintenance of polymorphism, many models have included restoration costs and/or spatial structure of the population (sections 2.1.1 and 2.1.3), but to my knowledge, only one model included a cost of the CMS. Dufay et al. (2007) assumed that the male-sterile cytoplasm reduces female fitness of all individuals that carry it, whatever their phenotype. This cost reduces the female advantage but, more interestingly, it put restored hermaphrodites (those that carry the CMS) at a disadvantage compared with hermaphrodites that do no carry the CMS. As a consequence, this cost has a similar effect as a expressed ovule restoration cost (see section 2.1.1).

The existence of a CMS cost implies that female fitness depends on the mitochondrial haplotype, which has been demonstrated in *Plantago lanceolata* (van Damme, 1984) and *Silene vulgaris* (McCauley and Olson, 2003). However, the experimental assessment of a CMS cost will require CMS markers in order to discriminate between hermaphrodites that carry the CMS and those that do not, and compare their female fitness. In any case, a CMS cost cannot be distinguish from an expressed ovule restoration cost.

In the general introduction I demonstrated that the female advantage s is the key parameter for the evolution of a CMS gene. The latter can spread when $s > 0$ and the higher the female advantage is, the faster the spread occurs. However, in models that include a male-fertile and a male-sterile cytoplasm, high values of s can prevent the maintenance of the cytonuclear polymorphism. Dufay et al. (2007) have shown by simulations that, even when restoration costs were adequate, it was impossible to maintain cytonuclear gynodioecy when $s > 1$ because the CMS always went to fixation. Jacobs and Wade (2003) had previously demonstrated this result analytically and showed that polymorphism at the CMS locus can be maintain only if $k > s$, s being the female advantage female and k an expressed ovule restoration cost. k being smaller than 1 by definition, the condition cannot be fulfilled if $s > 1$. On the contrary, in models including two male-sterile cytoplasms, $1 + s > 1$ seems to be the only condition on s for the maintenance of cytonuclear polymorphism.

Experimental support for female advantage have been provided in a large number of gynodioecious species (reviewed in Shykoff et al., 2003). Estimates of $1 + s$ in the literature range generally from 1 to 2 (e.g. Agren and Willson, 1991; Asikainen and Mutikainen, 2003; Lafuma and Maurice, 2006; Olson et al., 2006; Poot, 1997), and sometimes reach 3.5 (Morris and Doak, 1998; Ramsey et al., 2006; Thompson

and Tarayre, 2000). However, few studies have taken into account all components of female fitness (fruit set, number of seeds per fruit, germination rate, early survival rate, etc.). Moreover, the mating system (outcrossing vs. selfing) and the genetic structure of the population were not taken in account in studies carried out on artificial populations, although it should have a great impact (Ramsey et al., 2006). Lastly, female advantage may depend on environmental conditions (e.g. soil moisture, see Barr, 2004). In contrast, in some species, no female advantage was detected (e.g. in *Beta vulgaris* spp. *maritima*, see Boutin et al., 1988 or in *Daphne laureola*, see Alonso and Herrera, 2001). In those species, the female advantage may be too low to be detected experimentally though it may still be high enough to select CMS.

I showed that females benefit from a female advantage in *Silene nutans*. The results are presented in ARTICLE 2 (p.62).

2.1.3 Influence of spatial structure on the evolution of gynodioecy

Until now, the models I presented ignored the spatial structure of populations. But true panmixia is rarely verified and genes and phenotypes have been shown to be spatially structured in many gynodioecious populations. This structure can be observed between populations or within populations, but most of studies have focused on between population structure. In this part, the causes and consequences of structure on the evolution of gynodioecy will be discussed.

2.1.3.1 Differentiation between populations: causes and consequences

It has often been observed that the proportion of females varies greatly between populations (Bailey and Delph, 2007a), meaning that the frequencies of one or several genes involved in the sex phenotype vary between populations. This structure can result from several phenomenon.

First, the structure may be due to drift, particularly in small populations or because of extinction/recolonization dynamics (e.g. in thyme, Manicacci et al., 1996). Then, the structure can be maintained over time, particularly for cytoplasmic genes which have reduced gene flow compared with nuclear genes transmitted by pollen (Petit et al., 2005). Second, theoretical studies have shown that oscillations in the frequencies of CMS and restorers genes occur. These oscillations may be damping (this can take hundreds of generations) or persist in limit cycles (e.g. Dufay et al., 2007; Gouyon et al., 1991). Dufay et al. (2009) have shown that in *Beta vulgaris* spp. *maritima*, the variation in sex-ratio between populations could result from such frequency-dependent oscillations. Third, the population sex-ratio depends on the relative fitness of females and hermaphrodites and some factors (moisture, temperature, etc.) influence the value of the female advantage (McCauley and Bailey, 2009, and references therein). Between population variation in the female advantage has been shown in *Plantago maritima* and could explain, at least in part, sex-ratio differences between populations (Nilsson and Agren, 2006). Finally, the relative allocation to male and female functions in hermaphrodites is plastic, the female function

being generally reduced in harsh environments. Therefore, the relative fitness of hermaphrodites compared with females could differ between populations that differ in nutrient or stress levels (reviewed in McCauley and Bailey, 2009).

Between population structure has important implications for the evolution of gynodioecy. Indeed, structure increases the variation in sex-ratio between populations, i.e. gathers females together in some populations and hermaphrodites in others. This can enhance pollen limitation, whose negative effect on females have been demonstrated theoretically (McCauley and Taylor, 1997) and experimentally (McCauley et al., 2000). But structure can also have a negative effect on hermaphrodites. As some populations become biased toward females, hermaphrodites tend to self. Therefore, hermaphrodites suffer from higher inbreeding depression when they are rare. McCauley et al. (2000) have demonstrated this effect in *Silene vulgaris* by observing a negative correlation between the germination rate of seeds produced by the hermaphrodites and population sex-ratio.

2.1.3.2 Fine scale spatial structure

In some gynodioecious populations, structure was found not only between populations but also within populations. In *Silene vulgaris* and *Silene acaulis*, the mitochondrial genome is structured at a fine scale (a few meters) (Klaas and Olson, 2006; Olson et al., 2006; Olson and McCauley, 2002; Storchova and Olson, 2004) but is not always associated with a structure of the sex phenotypes (Klaas and Olson, 2006; Olson et al., 2006). This structure is expected to persist into future generations because seeds often germinate close to their mother, and offspring sex-ratios from females (resp. hermaphrodites) are female-biased (resp hermaphrodite-biased). One of the consequences of fine scale structure is pollen limitation in female patches and the proportion of hermaphrodites in the neighborhood has been shown to be correlated with the fitness of females (De Cauwer et al., 2010; Graff, 1999; Olson et al., 2006) and sometimes the fitness of hermaphrodites (Graff, 1999).

2.1.4 Evolutionary dynamics of gynodioecy and mitochondrial genetic diversity

Dynamics of gynodioecy are often classified into two categories. The first one involves recurrent invasion of new CMS and restorers genes, which result in transient polymorphism. Under this scenario, low genetic variability is expected in the mitochondrial genome of gynodioecious species compared with non-gynodioecious species. On the contrary, a limited number of CMS and restorers can be maintained by balancing selection over long periods of time, which allows the accumulation of mutations and divergence between mitochondrial lineages. In this case, higher mitochondrial diversity is expected in gynodioecious species.

During the last ten years, many studies have compared the mitochondrial genetic diversity of gynodioecious and non-gynodioecious species and results support both

scenario. Städler and Delph (2002) have shown that gynodioecious *Silene acaulis* presented a high level of mitochondrial polymorphism. Similar results were found in *Silene vulgaris* (Houliston and Olson, 2006; Storchova and Olson, 2004) and signatures of frequency-dependent selection have been detected on two mitochondrial genes (Houliston and Olson, 2006). Recently, Touzet and Delph (2009) have compared the mitochondrial diversity at *cob* and *cox1* between three gynodioecious species and seven non-gynodioecious species of *Silene* and have shown that the diversity in gynodioecious species is much higher. On the contrary, Ingvarsson and Taylor (2002) have shown that the diversity in the chloroplast genome of *Silene vulgaris* was not higher than that of a close dioecious species (*Silene latifolia*), and presented signatures of purifying selection. Interestingly, recent publications pointed out that variation in the mutation rate between genes (Barr et al., 2007) and between species (Mower et al., 2007; Sloan et al., 2008) may complicate the relationship between genetic diversity and selection (see also Charlesworth, 2010).

2.2 Genetic determination of gynodioecy

In theoretical studies it appears that the genetic determination of sex (cytoplasmic and/or nuclear, number of CMS and restorer genes, characteristics of restorers, etc.) has a strong influence on the evolution of gynodioecy. Experimental studies on the genetic determination of sex are therefore essential for understanding the evolution of gynodioecy.

Gynodioecious species are often assumed to be under cytonuclear sex determination although evidence is rarely provided. Two to three CMS have generally been detected, up to four for very comprehensive studies performed in *Plantago*. Several CMS have been found to coexist within a population in some studies, supporting the scenario of balancing selection (frequency-dependent selection of the different CMS). Dominant and recessive restorers seem to be involved in restoration, with a slight bias toward dominant restorers, and epistatic relationships between restorers were often found. Quantitative restoration by many additive genes of small effect was shown to be consistent with the results obtained in *Plantago coronopus*, *Thymus vulgaris* and *Silene vulgaris* (Ehlers et al., 2005). The main results are summarized in Table 2.5. It reveals some gaps in theoretical studies, particularly concerning the evolution of epistatic restorers and quantitative restoration (but see Bailey and Delph, 2007b).

During my PhD, I have studied the genetic determination of sex in *Silene nutans*. The results are presented in Article 1 (see p.36).

Species	CMS	Restorers	Ref
Plantago lanceolata	4 CMS (3 coexisting within a population)	1 to 3 restoration loci per CMS; dominant and recessive restorers; epistatic restorers	1,2
Plantago coronopus	4 CMS (3 coexisting within a population)	2 to 5 restoration loci per CMS; dominant (mainly) and recessive restorers; epistatic restorers; also consistent with quantitative restoration	3,4,5,6
Silene vulgaris	2 or 3 CMS (within the same population)	at least 2 dominant epistatic restorers and a independent recessive one; also consistent with quantitative restoration	6,7
Thymus vulgaris	3 CMS	at least 2 recessive restorers and one dominant; epistatic restorers; also consistent with quantitative restoration	6,7,8
Origanum vulgare	2 CMS		9
Beta vulgaris spp *maritima*	3 CMS	at least 2 epistatic restorers	10,11
Lobelia siphilitica	2 CMS	one CMS restored by one dominant restorer; complex restoration for the second CMS	12

Table 2.5: CMS et restorers in wild gynodioecious species. References: 1. de Haan et al. (1997c); 2. de Haan et al. (1997b); 3. van Damme et al. (2004); 4. Koelewijn and van Damme (1995a); 5. Koelewijn and van Damme (1995b); 6. Ehlers et al. (2005); 7. Charlesworth and Laporte (1998); 8. Belhassen et al. (1991); 9. Kheyr-Pour (1981); 10. Cuguen et al. (1994); 11. Touzet et al. (2004); 12. Dudle et al. (2001).

ARTICLE 1

Genetic determination of male sterility in gynodioecious *Silene nutans*

CLAIRE GARRAUD, BENJAMIN BRACHI, MATHILDE DUFAŸ, PASCAL TOUZET AND JACQUI SHYKOFF

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Abstract

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.

Keywords: cytoplasmic male sterility; reciprocal crosses; gynodioecy; gynomonoecy; restorer genes.

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 metapopulation level (Couvet et al., 1998). On the other hand, cytoplasmic polymorphisms can be maintained over long periods by frequency-dependent selection (Bailey et al., 2003; Dufay et al., 2007; Gouyon et al., 1991), resulting in the coexistence of several different cytoplasmic types in each population. The conditions for polymorphism differ between models with two sterilizing cytoplasms (Bailey et al., 2003; Gouyon et al., 1991), and models with one sterilizing and one fertile cytoplasm (Dufay et al., 2007; Jacobs and Wade, 2003). 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, see Bailey and Delph, 2007b) are also important. Thus, studying the genetic basis of sex determination is essential for understanding the evolution and maintenance of gynodioecy.

Considerable mtDNA polymorphism has been found in gynodioecious *Silene* species (Houliston and Olson, 2006; Olson et al., 2006; Städler and Delph, 2002; Touzet and Delph, 2009), but the associations between mitotypes and sex pheno-

types are not perfect (Klaas and Olson, 2006; Olson and McCauley, 2002; Storchova and Olson, 2004) and crosses are necessary to determine the number of functionally distinct CMS types. In the gynodiecious 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 gynomonoecious–gynodioecious: female, hermaphroditic and gynomonoecious 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 2.6). In Auvergne and Queyras, seeds were harvested from open-pollinated plants in 2002 and 2003, respectively, and sown in autumn 2006, giving rise to in-

dependent maternal full- or half-sibling families. TABLE 2.6 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.

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

Table 2.6: Origin of plants used as parents in the crossing experiment.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.

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 2.6). 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 (12x3) 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 selfcompatible, 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 x Q1.2, AMB9 x AU4.4 and Q9.5 x Q1.1 (mother x 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 $\frac{1}{2}$ compost and $\frac{1}{2}$ 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 x 7 cm) filled with compost supplemented with a controlled release fertilizer (Osmocote, 110 g/10 l) and placed in a vernalization chamber, at 13°C for 1 week of acclimation and then at 6°C (day length 8h). 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 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 gynomonoecious 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 gynomonoecious (GM) category by two to five observations during the flowering period. Therefore, in 2009, the number of gynomonoecious plants was underestimated. Indeed, in 2008, about one-third of the plants were found to be gynomonoecious. To take account of the difference in procedure between the 2 years, gynomonoecious 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 gynomonoecious 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 gynomonoecious 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. Gynomonoecious plants were classified as females or hermaphrodites according to their proportion of pistillate flowers. The few gynomonoecious 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 gynomonoecious 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 2.7); 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 gynomonoecious individuals. A few unexpected phenotypes were observed. In 2008, we found three males, one from the cross AU3.16 x Q9.5 and two from the cross Q9.8 x 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 x Q9.11, and the two of these that flowered the second year were again sterile. Cytonuclear incompatibilities could be responsible for these phenotypes. These plants were excluded from the following analyses.

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

Table 2.7: Sex segregation in progenies of direct and reciprocal crosses. Abbreviations: F, female; GM, gynomonoecious; 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.

Cytoplasmic differences and reciprocal crosses

TABLE 2.7 presents the numbers of females (F), hermaphrodites (H) and gynomonoecious (GM) individuals segregating in each pair of reciprocal crosses. Differences in sex segregation between reciprocal crosses were tested by Fisher's exact tests. Our results show that the AU mitotype is clearly functionally distinct from Q9 and AMB (TABLE 2.7). 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 gynomonoecious plants were grouped with hermaphrodites or 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 2.8; $\chi^2 = 452$, 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 gynomonoecious plants with a majority of pistillate flowers and a labile plant that shifted from female in 2008 to gynomonoecious in 2009 (data non shown) in our progenies carrying this mitotype. Gynomonoecious plants were found in all 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)

Table 2.8: Total number of F, H and GM plants carrying each of the four mitotypes. Abbreviations: F, female; GM, gynomonoecious; H, hermaphrodite. Heterogeneity between mitotypes was tested by a chi-square test (6 degrees of freedom) and found to be significant ($P<0.0001$).

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 2.9. Our results suggest at least two independent dominant restorers of the Q9 and AMB mitotypes (TABLE 2.9). 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 gynomonoecious 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 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., 1997c; 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* (Dufay et al., 2009; van Damme et al., 2004). Unfortunately, our crossing design did not permit us to test within-population polymorphism efficiently.

We showed that all cytoplasms but Q1 segregated females and were thus potentially sterilizing. However, this mitotype segregated one gynomonoecious 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 cytoplasms 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., 1997c; Miyake et al., 2009), but are still unknown in the *Silene*

Mother	*Father*	*Offspring*		*Heterogeneity*			*Genetic model for restoration*
		F	*H*	*Fitted ratio*	*G(1)*	P-*value*	
Mitotype AMB							
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
Mitotype AU							
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
Mitotype Q9							
Q9.8	AU4.4	2	26	1:15	0.04	0.842	2 dominant restorers without epistasy

Table 2.9: Offspring sex ratio and fitted genetic models for restoration. 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.

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 (Charlesworth and Laporte, 1998; Koelewijn and van Damme, 1995b) 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., 1997b) 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 (Guitian and Medrano, 2000; Maurice, 1999; Talavera et al., 1996). The frequency of such individuals is difficult to estimate because of sex phenotype plasticity (for example Klaas and Olson, 2006; Koelewijn and van Damme, 1996) and is frequently underestimated because of phenotyping procedures. Two main hypotheses have been proposed for their genetic determination: quantitative or incomplete restoration (Glaettli and Goudet, 2006; Koelewijn and van Damme, 1995b, 1996) and heteroplasmy, which is the coexistence of mitochondria of different genomes in the same individual (Andersson, 1999; Erickson and Kemble, 1993). The most likely mechanism that generates heteroplasmy is paternal transmission of mitochondria, as has been found for *Silene vulgaris* (McCauley et al., 2005; Pearl et al., 2009; Welch et al., 2006). We checked the mitotypes of progenies from our crosses and found no cases suggesting paternal transmission of mitochondria (unpublished data).

As sex determination of gynomonoecious 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 gynomonoecious individuals is lacking, though it is needed for interpreting sex segregation in progenies of controlled crosses (Charlesworth and Laporte, 1998; Koelewijn and van Damme, 1995b). Moreover, the role of gynomonoecious 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 (Houliston and Olson, 2006; Städler and Delph, 2002; Touzet and Delph, 2009), differentiation of CMSs has generally been studied in very nearby populations (Belhassen et al., 1991; Charlesworth and Laporte, 1998; Koelewijn and van Damme, 1995a). 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; Del Castillo and Trujillo, 2009; Dufay et al., 2008), 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* (Bailey and McCauley, 2005; Emery and McCauley, 2002), and the level of restoration does not decrease with geographic distance in between-population crosses (Bailey and McCauley, 2005; Gigord et al.,

1998). 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 2.7). 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 2.7).

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 constrains 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.

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References

See References at the end of the Thesis p.178.

Supplementary Tables

Supplementary Table 1: Polymorphic nucleotide sites on *cob* and *cox1* among the 4 cytoplasms

cob

Haplotype	GeneBank	Ref in Touzet and Delph (2009)	223	735	768	841	927	981	987	997
AMB	EU883274	cob_25	A	T	C	C	A	T	C	T
AU	EU883279	cob_30	C	T	C	T	A	G	C	T
Q1	EU883278	cob_29	C	T	C	T	G	T	A	T
Q9	EU883277	cob_28	A	G	A	T	A	T	A	G

cox1

Haplotype	GeneBank	Ref in Touzet and Delph (2009)	199	751	763	775	940	954	957	1000
AMB	HM775207	-	A	A	T	G	T	G	G	G
AU	EU883306	cox1_19	A	A	G	T	C	G	C	C
Q1	EU883299	cox1_12	C	A	G	G	C	C	C	G
Q9	EU883301	cox1_14	A	G	T	G	C	G	C	G

Supplementary Table 2: Models of restoration and expected sex segregation

Supplementary Table 2: Models of restoration and expected sex segregation. Expected sex segregation (female:hermaphrodite) in progenies and associated genotypes of parents are presented under different models of restoration, involving one or two restorers, with different dominance and interactions. The two parents are hermaphrodites: therefore, some sex-ratios (in bold) can be obtained only on the assumption that the two hermaphrodite parents have different CMS. "-" means that the identity of the allele does not matter.

number of loci	relation between loci	dominance	Expected sex segregation (female:hermaphrodite)						
one locus	-	dominant	0:1 R/R x -/-	1:3 R/+ x R/+	**1:1** **R/+ x +/+**				
	-	recessive	0:1 r/r x r/r	**1:1** **r/r x r/+**	**1:0** **r/r x +/+**				
two loci	independent	both dominant	0:1 R1/R1 -/- x -/- -/- or -/- R2/R2 x -/- -/-	1:15 R1/+ R2/+ x R1/+ R2/+	1:7 R1/+ +/+ x R1/+ R2/+ or +/+ R2/+ x R1/+ R2/+	1:3 R1/+ +/+ x R1/+ +/+ or +/+ R2/+ x +/+ R2/+ or R1/+ +/+ x +/+ R2/+ or **R1/+ R2/+ x +/+ +/+**	**1:1** **R1/+ +/+ x +/+ +/+ or +/+ R2/+ x +/+ +/+**		
		one dominant and one recessive	0:1 R1/R1 -/- x -/- -/- or -/- r2/r2 x -/- r2/r2	1:7 R1/+ r2/+ x R1/+ r2/r2	3:13 R1/+ r2/+ x R1/+ r2/+	1:3 R1/+ +/+ x R1/+ -/- or R1/+ r2/+ x +/+ r2/r2 or **R1/+ r2/r2 x +/+ r2/+**	**3:5** **R1/+ r2/+ x +/+ r2/+**	1:1 R1/+ +/+ x +/+ -/- or **R1/+** -/- **x +/+ +/+ or +/+ r2/r2 x +/+ r2/+**	**1:0** **+/+ r2/r2 x +/+ +/+**
		both recessive	0:1 r1/r1 -/- x r1/r1 -/- or -/- r2/r2 x -/- r2/r2	1:3 **r1/r1 r2/r2 x r1/+ r2/+** or r1/r1 r2/+ x r1/+ r2/r2	**3:5** **r1/+ r2/+ x r1/r1 r2/+ or r1/+ r2/+ x r1/+ r2/r2**	1:1 -/- r2/+ x +/+ r2/r2 or **+/+ r2/+ x -/- r2/r2** or r1/+ -/- x r1/r1 +/+ or **r1/+ +/+ x r1/r1 -/-**	**3:1** **r1/r1 r2/+ x +/+ r2/+ or r1/+ r2/r2 x r1/+ +/+**	1:0 r1/r1 +/+ x +/+ -/- or **r1/r1** -/- **x +/+ +/+** or +/+ r2/r2 x -/- +/+ or -/- **r2/r2 x +/+ +/+**	
	with epistasy	both dominant	0:1 R1/R1 R2/R2 x -/- -/- or R1/R1 R2/+ x -/- R2/R2 or R1/+ R2/R2 x R1/R1 -/-	1:3 R1/R1 R2/+ x -/- R2/+ or R1/+ R2/R2 x R1/+ -/-	7:9 R1/+ R2/+ x R1/+ R2/+	**1:1** **R1/- R2/+ x R1/R1 +/+ or -/- +/+ x R1/R1 R2/+ or R1/+ R2/R2 x +/+ -/- or R1/+ R2/- x +/+ R2/R2**	**5:3** **R1/+ R2/+ x +/+ R2/+ or R1/+ R2/+ x R1/+ +/+**	**3:1** **R1/+ R2/+ x +/+ +/+**	
		one dominant and one recessive	0:1 R1/R1 r2/r2 x -/- r2/r2	1:3 R1/+ r2/r2 x R1/+ r2/r2	**1:1** **R1/R1 r2/r2 x -/- r2/+ or +/+ r2/r2 x R1/+ r2/r2 or R1/R1 r2/+ x R1/- r2/r2**	**5:3** **R1/+ r2/r2 x R1/+ r2/+**	**3:1** **R1/+ r2/r2 x +/+ r2/+**	**1:0** **R1/- r2/r2 x -/- +/+**	
		both recessive	0:1 r1/r1 r2/r2 x r1/r1 r2/r2	**1:1** **r1/r1 r2/r2 x r1/+ r2/r2 or r1/r1 r2/r2 x r1/r1 r2/+**	**3:1** **r1/r1 r2/r2 x r1/+ r2/+**	**1:0** **r1/r1 r2/r2 x +/+ -/- or r1/r1 r2/r2 x -/- +/+**			

Summary of chapter 2

* In most gynodioecious species, sex determination is cytonuclear: cytoplasmic male sterility (CMS) genes turn hermaphrodites into male-steriles (females) while nuclear restorers counter-act the CMS action and restore fertility.

* Females benefit from an advantage in the production of seeds, which allows the spread of the CMS gene. Restorers are selected by Fisherian selection because of their association with the rare gamete.

* Theoretical studies have shown that stable gynodioecy can be obtained thanks to adequate restoration costs or spatial structure of populations.

* Evolutionary dynamics depend on the number and characteristics of genes involved in sex determination. Signatures of selection in the mitochondrial genome provide information about the dynamics.

* The molecular basis of sterility and restoration are unknown in wild species but have been well explored in crops. CMS are chimeric genes, co-transcribed with essential genes, but their mechanisms remain uncertain. Restoration involves a few dominant restorers, that act on the expression of the CMS gene.

* In wild species, several CMS and restorers genes are often involved in sex determination. In *Silene nutans*, we detected 2 to 3 different CMS and up to 4 restorers per CMS. Two of these CMS have a broad geographical distribution in Europe and are very unequally restored.

Chapter 3

The gynomonoecious phenotype

3.1 Close encounters of the third kind

In chapter 2, we defined gynodioecy as the coexistence of females and hermaphrodites in populations. Yet in many gynodioecious species a third sex phenotype exists, composed of pistillate (females) and perfect (hermaphrodites) flowers, called the gynomonoecious phenotype (see FIGURE 3.1). There are pure gynomonoecious species, such as *Silene noctiflora* (Davis and Delph, 2005) or many species among Asteraceae (Bertin and Kerwin, 1998), in which all individuals are gynomonoecious. However, this chapter focuses on the description of the gynomonoecious phenotype in gynodioecious species.

The gynomonoecious phenotype is often described as marginal and few publications have reported it, except in Caryophyllaceae. However, it is likely that the frequency of gynomonoecious individuals has been largely under-estimated (Koelewijn and van Damme, 1996) and gynomonoecy could be general in gynodioecious species. Standard phenotyping procedures are indeed based on the observation of a few flowers while all the flowers must be phenotyped to determine definitively if the plant is female, hermaphrodite or gynomonoecious. Such comprehensive phenotyping is time-consuming and has been carried out in only a few species. Thus, the proportion of gynomonoecious individuals within populations and the proportion of gynodioecious species with some gynomonoecious individuals need to be estimated more precisely.

Literature on the frequency of the gynomonoecious phenotype is summarized in TABLE 3.1. This summary shows that the frequency of gynomonoecious individuals is actually high, often higher than that of females. In many studies, comprehensive phenotyping has allowed the estimation of the proportion of perfect and pistillate flowers on gynomonoecious plants and to determine the distribution of femaleness (proportion of pistillate flowers) among these individuals (TABLE 3.1). Studies showed unanimously that femaleness is very variable across individuals ($[0; 100\%[$) but is biased toward perfect flowers (TABLE 3.1). The reason for this bias and its possible evolutionary implications are unknown.

Species	Proportion of gynomonoecious plants	Femaleness of gynomonoecious plants	Refs
Silene littorea	50% (vs. 6% of females)		1
Silene italica	13 to 39% depending on populations		2
Silene nutans	34-47% (vs. 18-28% of females) depending on years	great range of femaleness but many "almost hermaphrodite"	3,4
Silene vulgaris	11%	mainly perfect flowers	5
Silene acaulis	up to 35% (vs. 23% of females)	no more than 15% of pistillate flowers	6,7
Silene stockenii	40% (vs. 7% of females)		8
Plantago coronopus	2 to 37% depending on populations (20% on average)	great range of femaleness but bias toward perfect flowers	9,10
Plantago lanceolata	0 to 25% depending on populations (6 to 18% on average depending on studies)		11,12
Glechoma hederacea	30% on average (0 to 100% depending on populations)	great range of femaleness but 50% of "almost hermaphrodite'	13
Dianthus sylvestris		60 to 70% of gynomonoecious carry less than 40% of pistillate flowers	14
Geranium maculatum	about 20% (more frequent than females)	many "almost hermaphrodite"	15

Table 3.1: Gynodioecious species in which the occurrence of gynomonoecious individuals have been reported. The proportion of gynomonoecious plants has been measured in natural populations or in progenies from controlled crosses. In some cases, the femaleness (proportion of pistillate flowers) has also been estimated. For studies prior to 1980, see Table 6 of Koelewijn and van Damme (1996). 1. Guitian and Medrano (2000); 2. Maurice (1999); 3. Dufay et al. (2010); 4. Desfeux (1996); 5. Charlesworth and Laporte (1998); 6. Shykoff (1992); 7. Delph and Mutikainen (2003); 8. Talavera et al. (1996); 9. Koelewijn and van Damme (1995b); 10. Koelewijn and van Damme (1996); 11. van Damme and van Delden (1982); 12. Poot (1997); 13. Widén and Widén (1999); 14. Collin and Shykoff (2003); 15. Agren and Willson (1991).

Figure 3.1: Two gynomonoecious individuals in *Silene nutans*. In this species flowers are protandrous and have two rows of five stamens; the first juts beyond the corolla opening as the flower opens, the second matures and the filaments lengthen to also extend beyond the corolla opening one or two days later. If the first reproductive organ that is visible beyond the corolla opening is the style, the flower is pistillate (aborted anthers can be observed within the corolla). On the two pictures, we can see a pistillate flower (below) and one or several perfect flowers at different levels of maturity (male or female phase).

These studies show that the gynomonoecious phenotype is not a unique phenotype but must rather be defined in a quantitative way. Moreover, the quantitative variation in these individuals does not depend only on the proportion of pistillate flowers because intermediate flower phenotypes can also be observed. In *Plantago lanceolata* and in *Plantago coronopus*, two gynodioecious species in which gynomonoecy has been described, the femaleness of a gynomonoecious plant depends on the number of perfect flowers, on the number of normal vs. aborted stamen per flower, and on the level of abortion of anthers (see FIGURE 3.2).

In other species (particularly in *Silene*), gynomonoecious individuals were reported to carry perfect and pistillate flowers, with almost no variation in the number of normal anthers and in the level of abortion of anthers (e.g. Maurice, 1999). In *Silene nutans* however, I have observed many flowers with 1 to 9 normal anthers (instead of 10, see FIGURE 3.3) and to a lesser extent, partially aborted anthers.

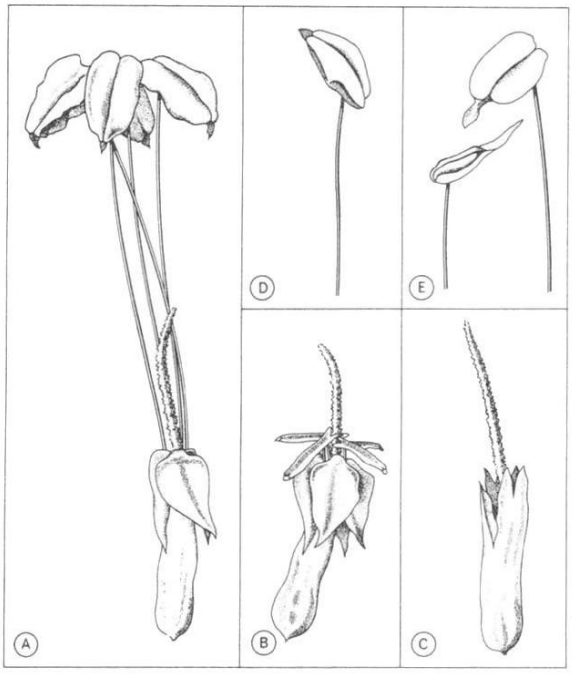

Figure 3.2: Flower and anther types in *Plantago lanceolata*, from van Damme and van Delden (1982). (A) Flower of an hermaphrodite plant, with 4 normal anthers; (B) flower from a male-sterile plant (type 1); (C) flower from a male-sterile plant (type 2); (D) abnormal anther (type 1); (E) abnormal anther (type 2). In spite of slight morphological differences, partially aborted anthers produce significantly less viable pollen than normal ones (van Damme and van Delden, 1982).

For instance, flowers having 1 to 5 normal stamens accounted for 5% of flowers of gynomonoecious individuals. These observations are marginal in comparison with what has been described in *Plantago*. However, intermediate flowers may have been under-estimated in other species, because their detection requires careful observation of each flower.

Figure 3.3: Flower of *Silene nutans* having a single normal stamen (in light green). We cannot see if the flower will carry only one normal stamen or if a second row of stamen will emerge from the corolla.

3.2 Floral and reproductive traits of gynomonoecious plants

More and more studies show that gynomonoecious individuals represent a significant part of gynodioecious populations and are thus likely to influence the evolution of gynodioecy. To understand their evolutionary impact, a good knowledge of their reproductive characteristics and genetic determination is essential. This section will be devoted to the reproductive traits and the next section to the genetic and environmental control of the phenotype.

Literature on the floral and reproductive traits of the gynomonoecious phenotype has been summarized and reorganized by traits in TABLE 3.2. General conclusions are difficult to draw because results are scarce, scattered across species, and sometimes inconsistent. In *Silene italica*, *Plantago lanceolata* and *Geranium maculatum*, it has been shown that gynomonoecious individuals produce more seeds than hermaphrodites but fewer than females (TABLE 3.2). Agren and Willson (1991) quantified the seed production and showed that gynomonoecious individuals produce 1.4 more ovules than hermaphrodites (the female advantage was 1.6). This advantage may result from different fitness components (number of flowers, fruit set, number of seeds per fruit) whose effects vary according to populations (Agren and Willson, 1991; Poot, 1997). Contrary to what is sometimes found in females, the seed advantage of gynomonoecious plants may not be explained by a reduced selfing rate, but only one study has measured selfing rate of gynomonoecious plants (see TABLE 3.2). The quality of pollen grains produced by gynomonoecious plants is different from that of hermaphrodites and is rather lower (reduced viability). The quantity of pollen grains per flower seems to be identical for the two phenotypes, but because gynomonoecious plants carry fewer perfect flowers, their pollen production at the individual level would be reduced.

Beyond the characterization of the gynomonoecious phenotype, some studies have compared the different types of flowers produced by gynomonoecious plants, between them and/or with those produced by females and hermaphrodites. The results suggest a continuum ranging from pistillate flowers of females to perfect flowers of hermaphrodites, through pistillate flowers and perfect flowers of gynomonoecious individuals, but very few studies managed to show significant differences for all steps of this continuum.

During my PhD, I studied the floral and reproductive traits of the gynomonoecious phenotype in *Silene nutans*. The results are presented in ARTICLE 2 (p.62).

Species	Nb of flowers	Flower size	First flowering day	Nb of seeds, fruit set	Germination rate, early survival	Pollen	Selfing rate	Ref
Silene littorea	$GM > H = F$			fruit set: $H = GM = F$ and $H_{GM} = F_{GM}$				1
Silene italica	$H \geq GM \geq F$; GM: negative correlation nb fl/femaleness	$F_F = F_{GM} < H_{GM} = H_H$		fruit set: $F \geq GM > H$ and $H_H = H_{GM} \leq F_{GM} = F_F$ mass/fruit: $F \geq GM \geq H$		qt/fl: $H = GM$ size: $H > GM$ or $GM > H$ depending on families		2,3
Silene nutans	GM: no correlation femaleness/nb fl	$F_F \leq F_{GM} \leq H_{GM} \leq H_H$ GM: negative correlation flower size/femaleness		fruit set: $F_F = F_{GM}$ and $H_{GM} = H_H$	germination: crossp H>selfp H= crossp GM=selfp GM	size: $H > GM$ qt/fl: $H = GM$ qt/ind: $H \geq GM$		4,5
Silene acaulis[a]				mass/seed : $H = F_{GM} \geq F_F$ (NS trend)	germination : $H = F_{GM} = F_F$ early survival: $H = F_{GM} < F_F$			6
Dianthus sylvestris		$F_F = F_{GM} < H_{GM} = H_H$		nb of seed/fruit: NS mass/seed : $F_F \geq F_{GM} \geq H_{GM} > H_H$	germination and early survival: NS		$F < GM = H$ (ind) $F_F < H_{GM} < F_{GM} = H_H$ (fl)	7,8
Plantago lanceolata	$F \geq GM \geq H$ (nb of spikes)		$F = GM \leq H$	qt of seed/ind: $F \geq GM > H$		qt of viable pollen correlated with anther abortion		9,10
Geranium maculatum	$GM > H = F$	$F < GM < H$	$F = GM < H$	fruit set: $F \geq GM \geq H$ nb seed/fruit: $F > GM = H$ nb seed/ind: $F > GM > H$ seed size: $H = GM = F$				11
Glechoma hederacea		$F < GM < H$						12

Table 3.2: Floral and reproductive traits of gynomonoecious plants (GM). Femaleness is defined as the proportion of pistillate flowers and the size of pollen by the proportion of large (viable) pollen grains. H: hermaphrodites; F: females; F_F pistillate flowers of females; H_H: perfect flowers of hermaphrodites; H_{GM}: perfect flowers of GM; F_{GM}: pistillate flowers of GM; selfp: self-pollinated; crossp: cross-pollinated; qt: quantity; ind: individual ; NS: non significant; fl: flowers; nb: number. a: perfect flowers of H and GM were not distinguished in this study. References: 1.Guitian and Medrano (2000); 2.Maurice (1999); 3.Lafuma and Maurice (2006); 4.Desfeux (1996); 5.Dufay et al. (2010); 6.Delph and Mutikainen (2003); 7.Collin and Shykoff (2003); 8.Collin et al. (2002); 9.Poot (1997); 10.van Damme and van Delden (1982); 11.Agren and Willson (1991); 12.Widén and Widén (1999).

Article 2

Characterisation of sex phenotypes in the gynomono-gynodioecious *Silene nutans*

Claire Garraud, Mathilde Dufaÿ and Jacqui Shykoff

(in prep.)

Abstract

Gynodioecy, the coexistence of female and hermaphrodite plants in populations, is often associated with the occurrence of mixed individuals carrying both perfect (hermaphrodite) and pistillate (female) flowers, a phenotype called gynomonoecy. Although quite common, these individuals are poorly characterized for both reproductive traits and genetic determination. We used the progenies of controlled crosses between parents from three French populations to characterize some floral and reproductive traits of female, hermaphrodite and gynomonoecious plants of *Silene nutans*. Our phenotyping procedure revealed that gynomonoecious individuals were more frequent than females. Their proportion of pistillate flowers varied greatly although plants with a high proportion of perfect (hermaphrodite) flowers were predominant. Gynomonoecious plants often displayed reproductive traits that were intermediate between those of females and hermaphrodites but more interestingly, some of these traits depended on the proportion of pistillate flowers of the plant. Pistillate and perfect flowers on gynomonoecious plants differed significantly for none of the traits measured. Gynomonoecious presented reduced compensation (i.e. female advantage) compared with females and lower male fitness than hermaphrodites, a consequence of their reduced number of perfect flowers. Implications for the evolution of gynodioecy are discussed.

Introduction

In gynodioecy, a breeding system in which hermaphrodite and female (male-sterile) plants coexist within the same species, sex phenotype is often determined by both cytoplasmic male sterility (CMS) genes, carried by the mitochondrial genome, and nuclear restorer genes that restore the male function (Belhassen et al., 1993; Charlesworth and Laporte, 1998; Cuguen et al., 1994; Garraud et al., 2011; Taylor et al., 2001; van Damme et al., 2004). The maintenance of females and of the underlying genetic polymorphism has puzzled evolutionary biologists since the 19^{e} century (Darwin, 1877). Addressing this question requires a careful comparison of reproductive traits between sex phenotypes. Females generally produce more but smaller flowers compared to hermaphrodites (reviewed in Shykoff et al., 2003), benefit from a higher outcrossing rate (Collin and Shykoff, 2003) and higher seed production (Lafuma and Maurice, 2006; Olson et al., 2006; Shykoff et al., 2003), and produce heavier seeds that germinate better (Collin et al., 2002; Olson et al., 2006; Shykoff et al., 2003). These traits contribute to the advantage of females in seed fitness (called female advantage or compensation), a condition required for the invasion of maternally inherited CMS genes (Lewis, 1941).

Both experimental and theoretical studies have focused on the dimorphism between females and hermaphrodites, although many gynodioecious species also contain individuals carrying a mixture of pistillate (female) and perfect (hermaphrodite) flowers, called gynomonoecious. Because their detection require intensive phenotyping procedures, their frequency must be usually underestimated (Koelewijn and van Damme, 1996), though, gynomonoecious plants can be at least as common as females, with frequencies varying from 10% to 50% among populations (Guitian and Medrano, 2000; Koelewijn and van Damme, 1996; Maurice, 1999; Shykoff, 1992; Talavera et al., 1996; Widén and Widén, 1999).

Gynomonoecious plants usually bear a majority of perfect flowers, but proportion of pistillate flowers from almost zero to almost one have been found (Agren and Willson, 1991; Collin and Shykoff, 2003; Dufay et al., 2010; Koelewijn and van Damme, 1996; Maurice, 1999; Shykoff, 1992; Widén and Widén, 1999). Flowers with intermediate phenotype (e.g. reduced number of anthers) have also been recorded in *Plantago coronopus* (Koelewijn and van Damme, 1996). In addition to detection difficulties, studying gynomonoecious plants is hampered by their high phenotypic plasticity, compared with females and hermaphrodites (Delph and Mutikainen, 2003; Klaas and Olson, 2006; Maurice, 1999; Shykoff, 1988). Most plasticity involves only small changes in the proportion of pistillate flowers (Agren and Willson, 1991), and because of the high proportion of gynomonoecious plants with only few pistillate flowers, this can occasionally lead to a change in sex category, mainly between hermaphrodites and gynomonoecious plants (Agren and Willson, 1991; Delph and Mutikainen, 2003; Koelewijn and van Damme, 1996; Maurice, 1999). Despite the strong influence of environmental factors on phenotype, gynomonoecy is thought to be at least partially genetically determined, involving quantitative restoration

(Ehlers et al., 2005; Glaettli and Goudet, 2006; Koelewijn and van Damme, 1995b, 1996) or heteroplasmy, which is the coexistence of different mitochondrial genomes within the same individual (Andersson, 1999; Erickson and Kemble, 1993).

Floral and reproductive traits of gynomonoecious individuals tend to be intermediate between pure females and hermaphrodites (Agren and Willson, 1991; Dufay et al., 2010; Lafuma and Maurice, 2006; Widén and Widén, 1999 but see Guitian and Medrano, 2000). In detail, gynomonoecious plants have been shown to enjoy only reduced compensation in *Geranium maculatum* and *Plantago lanceolata*, with seed production per plant higher than hermaphrodites but lower than females (Agren and Willson, 1991; Poot, 1997). In *Silene nutans* and *Silene italica*, per flower pollen production appears independent of the sex phenotype (Dufay et al., 2010; Lafuma and Maurice, 2006) but the proportion of viable pollen can be higher in hermaphrodite than in gynomonoecious plants (Dufay et al., 2010), although not for all sibships (Lafuma and Maurice, 2006). As expected under sex-allocation theory (Charnov, 1982), a trade-off between investment in male and female functions was sometimes found in hermaphrodite and gynomonoecious individuals indicating reallocation of saved resources (Agren and Willson, 1991; Kikuzawa, 1989; Koelewijn, 2003 but see Glaettli and Goudet, 2006). Furthermore, sex ratio within families sometimes covaries with reproductive traits of hermaphrodites (Gigord et al., 1999; Glaettli and Goudet, 2006; Koelewijn, 2003) suggesting that genes responsible for male fertility may also influence sex allocation, through pleiotropic effects or genetic linkage to those genes directly controlling sex allocation.

Because gynomonoecious individuals can represent a large proportion of the population, their reproductive potential will influence the dynamics of cytoplasmic male sterility factors and nuclear restorers in populations. Therefore, studies of maintenance of sex polymorphism cannot be complete without including the gynomonoecious contribution. Here we investigate the reproductive characters of all three sex phenotypes of *Silene nutans*, a species described as gynomonoecious-gynodioecious (Desfeux, 1996; Dufay et al., 2010; Jurgens et al., 1996) and whose sex is under cytonuclear determination (Garraud et al., in press). To date, there is no evidence of female advantage for this species. As in most gynodioecious species, pistillate flowers are smaller than perfect flowers, here for both female and gynomonoecious plants (Dufay et al., 2010), which could lead to reduced pollinator attractiveness but also allow for reallocation of saved resources to seed production at the plant or flower level. For this reason, we characterized pollen and seed production of gynomonoecious plants at both the plant and flower levels and we looked for trade-offs between male and female functions. We used a large sample of individuals from controlled crosses in order to answer the following questions:

i How frequent are gynomonoecious plants? How are pistillate flowers distributed in terms of frequency and timing in these plants?

ii How do gynomonoecious plants compare with females and hermaphrodites in terms of pollen and seed production?

iii Do reproductive traits covary with the proportion of pistillate flowers in gynomonoecious plants? Do pistillate and perfect flowers of gynomonoecious plants differ in seed production?

iv Does the mean sex ratio in a family, a measure of the degree of restoration of that family, influence pollen or seed production of gynomonoecious and hermaphrodite plants?

v Is there a trade-off between investment in male and female functions within plants and within families?

Material and Methods

Plant material

Silene nutans (Caryophyllaceae) is a diploid, self-compatible 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. Perfect (hermaphrodite) flowers possess ten anthers and are protandrous. 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. We used seeds issued from controlled crosses using parents from three French populations (Ambleteuse N 50°48' E 1°37, Auvergne N 44°43' E 2°21', Queyras N 44°46' E 6°44'), distant of 350 km to 700 km from each other. Parents were collected as seeds in Auvergne and Queyras and as rosettes in Ambleteuse, between 2002 and 2006. From four hermaphrodite plants (one from Auvergne, one from Ambleteuse and two from Queyras), we set up a diallele crossing design, in which each pairwise comparison was tested in reciprocal crosses (twelve crosses). This diallele was replicated three times, using different parents from the same populations (for details about the crossing design see Garraud et al., in press). A total of 36 (12×3) crosses were performed. Each cross was performed on a single flower and 30 to 40 seeds were generally obtained. Three crosses failed to produce fruits. Seeds were germinated in October 2007 on 0.8% water-agar and the germination rate was close to 100% for all crosses. Seedlings were transplanted 10 days later, placed in the greenhouse at 20°C until late February, and then in a vernalization chamber at 6°C (day length 8h). Plants were planted in the experimental garden in mid-April and started to flower at the end of May.

Sex phenotype

All flowering plants were sexed during the two first flowering seasons, in 2008 and 2009. In 2008, all flowers of each plant were sexed, by screening newly opening flowers every four or five days during the entire flowering season, and were classified as perfect (hermaphrodite), pistillate (female) or intermediate (1 to 5 normal anthers). Flowers on gynomonoecious plants were marked according to their sex with a small piece of coloured thread tied around the pedicel. Then, we defined the sex of each

plant by a quantitative sex measurement (hereafter called femaleness) calculated as follows:

$$\text{Femaleness} = \frac{\text{number of pistillate flowers} + 0.5 \times \text{number of intermediate flowers}}{\text{total number of flowers}}$$

In 2009, the large number of flowering plants made quantitative sex phenotype measurement impossible and we used a discrete description of sex phenotype. Plants were sexed by two to five observations during flowering and assigned to one of the following categories: females (F), hermaphrodites (H), gynomonoecious with fewer than 10% pistillate flowers, more than 90% pistillate flowers or from 10% to 90% pistillate flowers. Therefore the number of gynomonoecious plants was underestimated in 2009 because a rare floral sex phenotype could have been missed.

Floral and reproductive traits measurement

In 2008, the number of flowering spikes, the total number of flowers, the first flowering day and the proportion of flowers that set fruits (fruit set) were measured on all flowering plants. Fruit set of gynomonoecious plants was calculated not only for the entire plant, but also separately for pistillate, intermediate and perfect flowers. In 2009, because plants produced up to 300 flowers, we only recorded the number of flowering spikes (as a proxy of the number of flowers) and the first flowering day. The number of seeds per fruit, seed weight and germination rate were recorded in 2009 for a subset of plants that were transplanted into the greenhouse after the 2008 flowering season. These plants were manually pollinated with an excess of pollen from unrelated donors. Because *Silene nutans* is self-compatible, flowers were emasculated to avoid self pollination. Because we had cloned these plants by splitting up the rosette for other purposes, crosses were performed indiscriminately on one or another clone. Fruit set was close to 100%. In total, fruits were obtained for 62 flowers from 5 females, 8 flowers from 5 hermaphrodites and 42 pistillate flowers, 23 perfect flowers and 17 intermediate flowers from 10 gynomonoecious plants. Seeds were harvested at maturity, counted and weighed. Except for those from intermediate flowers that were for other purposes, seeds were germinated on 0.8% water-agar and the germination rate was recorded.

Pollen production for almost all plants producing pollen (96 gynomonoecious and 178 hermaphrodite plants) was analyzed in 2008. The protocol was identical to the one described in an earlier study on *Silene nutans* (Dufay et al., 2010) but we used a larger sample size from a number of different families and included the number of normal anthers in our analyses. For each plant, one bud was collected during the two first weeks of flowering. All anthers from each bud were collected and stored in ethanol at 95%. When the bud did not contain 10 normal anthers, aborted anthers were also collected and the number of normal anther was recorded. The ethanol was then evaporated and samples were placed at 56°C for 24 hours to force anther dehiscence. One mL of distilled water was added to each pollen sample and sonicated to separate pollen grains and remove them from the anthers.

Samples were diluted in isotonic CASY®ton, and the number of pollen grains was estimated using a particle counter CASY®model TT (Innovatis, Bielefeld). The number of detected particles was determined for 400 size classes ranging from 0.125 to 100μm using the software CASY®excel 2.1. Appropriate corrections for dilution were applied in order to obtain the total quantity of pollen grains in each class for each sample. Our measurement showed that pollen grains clearly fell into two size classes: from 23 to 35μm (hereafter called small pollen grains) and from 35 to 60μm (large pollen grains). A previous study on *Silene nutans* showed that the proportion of large pollen grains is positively correlated with the proportion of viable pollen grains, estimated with Alexander stain (R=0.89; see Dufay et al., 2010). We thus used the number of large pollen grains as a proxy for the number of viable pollen grains.

Because the sequence of opening of perfect vs. pistillate flowers may be important for plant fitness, e.g. by affecting the probability of self-pollination by geitonogamy or pollen limitation, we decided to record the sequence of newly opening flowers in gynomonoecious plants in order to determine if pistillate flowers open generally before or after perfect flowers. For each gynomonoecious plant phenotyped in 2008, days of observation were numbered from 1 (first observation) to n (last observation). The average opening day of perfect flowers was calculated as follows:

$$R_h = \frac{\sum_{i=1}^{n} \frac{i}{n} \times \text{number of newly opened perfect flowers on day i}}{\text{total number of perfect flowers}}$$

where i is the day of observation.
The distribution of this variable under random opening of flowers of the different sexes (null hypothesis) was obtained for each plant by calculating R_h on 100 000 random permutations of the sequence of opening flowers (respecting the number of newly opened flowers of each observation day and the total number of flowers of each sex). The null hypothesis was rejected when the two-tailed P-value (probability of obtaining R_h values at least as extreme as the R_h calculated from the data) was inferior to 0.05. In those cases, perfect flowers were described as early-perfect, when R_h<mean(simulated R_h), or late-perfect, when R_h>mean(simulated R_h). Although identical measurements can be done for pistillate and intermediate flowers, analyses were performed on perfect flowers because almost all gynomonoecious plants had at least one perfect flower. Repeatability of R_h values was tested on a subset of 88 cuttings (clones) from 25 plants, for which the sequence of flowering was recorded again in 2009.

Statistical analysis

All statistical analyses were conducted using the JMP statistical program (JMP® 8.0.2, SAS Institute Inc.). Since the phenotyping procedure differed between the two years, analyses were conducted independently for 2008 and 2009 data. We defined sex phenotype as a qualitative variable with four categories: females (F), hermaphrodites

(H), gynomonoecious with less than 10% of pistillate flowers ($GM_{<10\%}$), and gynomonoecious with more than 10% of pistillate flowers ($GM_{>10\%}$). The rationale was to use a similar definition of sex phenotype for both years and to take into account the distribution of the femaleness in gynomonoecious plants (see results). We used general linear models (ANOVA) to test the effect of sex phenotype on the number of flowers and flowering spikes, the fruit set, the first flowering day and the quantity of pollen grains. Family was included in these models as a block effect but since the distribution of sex phenotypes among families was very unequal (see results), we were generally not able to test the interaction between sex phenotype and family. The number of seeds per fruit, seed weight and germination rate were compared for the different plant sexes and for flower sex nested within plant sex. Because of the reduced sample size, the effect of family was not considered in these latter analyses. Transformations were performed for some variables to ensure homoscedasticity (see TABLE 3.5 for details). Correlations were calculated using the Pearson correlation coefficient (r), except when data were far from normal. In these cases, we used the Spearman correlation coefficient (ρ), calculated on ranks (Zar, 1984). Intra-class correlations coefficients (R_I) were calculated to test for the repeatability of pollen measurements (Zar, 1984).

Results

Sex phenotype

Of the 1113 plants, 339 flowered in 2008 and 788 in 2009. We phenotyped 29 females, 189 hermaphrodites and 113 gynomonoecious in 2008 and 131 females, 567 hermaphrodites and 70 gynomonoecious individuals, including 31 with fewer than 10% of pistillate flowers, in 2009. Of all plants that flowered at least once, more hermaphrodites than females flowered in 2008 (36.3% vs. 23.4%; $\chi^2 = 9.14$, $p = 0.003$), thereby gaining a head start on reproduction. The proportion of females varied greatly over the 36 families, ranging from 0% to 70%, and almost all females belonged to six families (Garraud et al., in press). Sex phenotypes were mostly stable over the 207 plants that flowered both years: considering those gynomonoecious plants from 2008 with fewer than 10% (resp. more than 90%) of pistillate flowers as hermaphrodites (resp. females) in order to limit detection artefacts, 8 plants shifted from hermaphrodite to gynomonoecious, 9 from gynomonoecious to hermaphrodite and 2 from gynomonoecious to female. These changes of category mainly represented small modification in the plant femaleness. We also noticed a few unexpected phenotypes: in 2008, three males that exhibited aborted carpels but normal flower morphology, and four fully sterile plants, with abnormal flowers that did not open but normal vegetative growth, were detected. Apart from two of the sterile plants that flowered, also sterile, in 2009, these plants did not flower again in 2009. These plants were excluded from the following analyses.

Gynomonoecious plants

Altogether, gynomonoecious plants bore 15% pistillate flowers and 5% intermediate flowers, leading to an average femaleness of 0.23. Half of the gynomonoecious plants had a femaleness lower than 0.1, though the entire range of femaleness was found (FIGURE 3.4). No correlation was found between the proportions of gynomonoecious and female plants in the different families in 2008 ($\rho = 0.08$, $n = 33$, $p = 0.64$) or in 2009 ($\rho = 0.30$, $n = 33$, $p = 0.09$) but the femaleness of gynomonoecious plants tended to increase with the proportion of females in 2008 ($\rho = 0.36$, $n = 28$, $p = 0.057$). Almost half of the analysed sequences of flower opening deviated from a random distribution of pistillate and perfect flowers across the sequence, far more than the 5% expected by chance. Perfect flowers appeared more often late than early in the flowering sequences allowing us to reject the hypothesis that females are always late (60 random, 17 early-perfect and 35 late-perfect individuals). The timing of anthesis of perfect flowers, R_h, was repeatable among clones within genotypes ($R_I = 0.38$, $F_{24,63} = 3.10$, $p = 0.0002$).

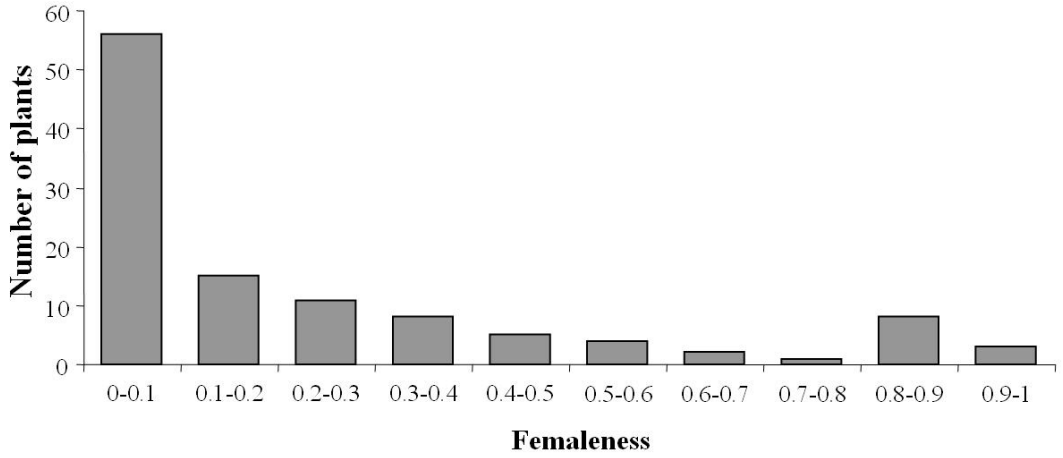

Figure 3.4: Distribution of the sex ratio of gynomonoecious plants in 2008. The femaleness was defined as (number of pistillate flowers + 0.5 x number of intermediate flowers)/total number of flowers; $n = 113$. We defined $GM_{<10\%}$ (resp. $GM_{>10\%}$) as gynomonoecious plants having a sex ratio lower (resp. higher) than 0.1.

We showed that among gynomonoecious plants having a femaleness higher than 0.1 ($GM_{>10\%}$), none of the traits measured in 2008 (first flowering day, number of flowers, fruit set and pollen production per flower) covaried with plant femaleness. However $GM_{>10\%}$ differed from gynomonoecious plants with a femaleness lower than 0.1 ($GM_{<10\%}$) for some floral and reproductive features. Thus, we chose to consider mainly hermaphrodite gynomonoecious plants ($GM_{<10\%}$) and others gynomonoecious plants ($GM_{>10\%}$) as two different phenotypes in our analyses of floral and reproductive traits. We did not define a symmetrical category with plants having a femaleness higher than 0.9 because these plants were too rare to provide enough

power in statistical analyses.

Trait measurement

All traits tested varied among the families (TABLE 3.5), revealing high genetic variation among families. Unfortunately, almost all female plants were found in only 6 families, confounding the effect of sex phenotype and the genetic effect of family. Since these effects are inseparable in this study, below we describe mean effects of sex phenotype without considering variation due to family. Sex phenotypes differed for first flowering day, fruit set, number of flowers and flowering spikes, number of seeds per fruit and germination rate (TABLE 3.5). Flowering started earlier in 2009 than in 2008 (131 vs. 180 julian days, $F_{1,1088} = 2650$, $p < 0.0001$), and both years, hermaphrodite and $GM_{<10\%}$ flowered earlier than $GM_{>10\%}$ and female plants (FIGURE 3.4). In 2008, females produce marginally more flowers than hermaphrodites while gynomonoecious plants carried either many ($GM_{<10\%}$ category) or only few flowers ($GM_{>10\%}$ category) (FIGURE 3.4). In 2009, females produce more spikes than hermaphrodites, gynomonoecious being intermediate (FIGURE 3.4). Furthermore the proportion of females within a family and the number of flowers (or spikes) of females from this family were positively correlated (FIGURE 3.3). Plants, and particularly females, produced more spikes in 2009 than in 2008 (year effect: $F_{1,1060} = 471$, $p < 0.0001$; interaction year*sex phenotype: $F_{3,1060} = 4.58$, $p = 0.003$). Among plants that flowered in 2009, plants that had not flowered in 2008 produced globally more spikes than those that had already flowered in 2008, but this effect was significant only for females and $GM_{>10\%}$ (significant interaction sex phenotype*2008 flowering, see TABLE 3.5). For plants that flowered the two years, flowering time varied among individuals ($F_{198,192} = 1.76$, $p < 0.0001$) when year was included in the analysis but number of spikes did not ($F_{198,197} = 1.19$, $p = 0.11$).

Females showed higher fruit set and seed number per fruit than hermaphrodites. For fruit set, $GM_{>10\%}$ were intermediate and $GM_{<10\%}$ not significantly different from hermaphrodites while for seeds per fruit all gynomonoecious plants were intermediate. Pistillate and perfect flowers on gynomonoecious plants did not differ in fruit set (paired t-test, $t_{87} = 0.92$, $p = 0.36$) or seed number per fruit (paired t-test, $t_5 = 1.30$, $p = 0.24$), although pistillate (resp. perfect) flowers from gynomonoecious plants tended to resemble pistillate (resp. perfect) flowers from female (resp. hermaphrodite) plants (FIGURE 3.4). No effect of sex phenotype or flower sex was detected on seed weight and germination rate (TABLE 3.5).

We tested the repeatability of our pollen measurements on a few individuals for which repeated measurements were done. For a given sample, measurements were found to be repeatable for both quantities of all and large pollen grains (total pollen grains: $R_I = 0.75$, $F_{20,25} = 6.91$, $p < 0.0001$; large pollen grains: $R_I = 0.75$, $F_{20,25} = 7.69$, $p < 0.0001$). Similarly, pollen quantities per normal anther were repeatable between buds from the same plant (total pollen grains: $R_I = 0.52$, $F_{29,32} = 3.26$, $p = 0.0007$; large pollen grains: $R_I = 0.55$, $F_{29,32} = 3.52$, $p = 0.0004$). Quantities

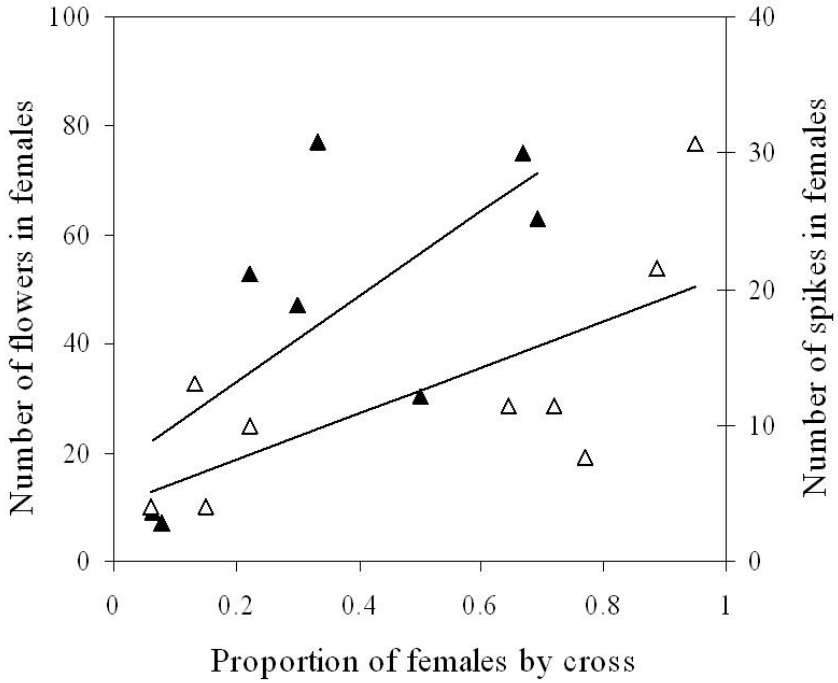

Table 3.3: Correlation between the mean number of flowers (2008) or spikes (2009) of females and the proportion of females in each cross. 2008 and 2009 data are represented by full and empty triangles respectively. The Pearson correlation coefficient was marginally significant in 2008 ($r = 0.6944$; $n = 8$, $p = 0.0560$) and significant in 2009 ($r = 0.7020$; $n = 9$, $p = 0.0350$).

of both all and large pollen grains varied among families (TABLE 3.5). We found no correlation between the total quantity of pollen grains per bud and the proportion of large pollen grains ($r = 0.022$, $n = 274$, $p = 0.72$). The number of normal anthers in buds varied with the sex of the plant (Wilcoxon's rank test: $p < 0.0001$; means: H: 9.9, $GM_{<10\%}$: 9.4, $GM_{>10\%}$: 8.3). The number of normal anthers had no effect on the total quantity of pollen per bud ($F_{1,270} = 0.20$, $n = 272$, $p = 0.66$), but buds with more normal anthers had more large pollen grains ($F_{1,270} = 4.96$, $n = 272$, $p = 0.027$). This suggests that aborted anthers contained as many pollen grains as did normal ones but that they were immature or aborted. The sex phenotypes did not differ in total quantity of pollen grains per bud but $GM_{>10\%}$ tended to produced fewer large pollen grains than $GM_{<10\%}$ and hermaphrodites, although this effect was not significant (TABLE 3.5). This tendency was not found for the number of large pollen grains per normal anther (TABLE 3.5).

No correlations were found between pollen and seed production, viewed here as proxies for male and female fitness. In hermaphrodites, the fruit set and the quantity of large pollen grains were not significantly correlated either at the individual or at the family level ($r = 0.064$, $n = 178$, $p = 0.40$; $r = 0.24$, $n = 27$, $p = 0.42$ respectively).

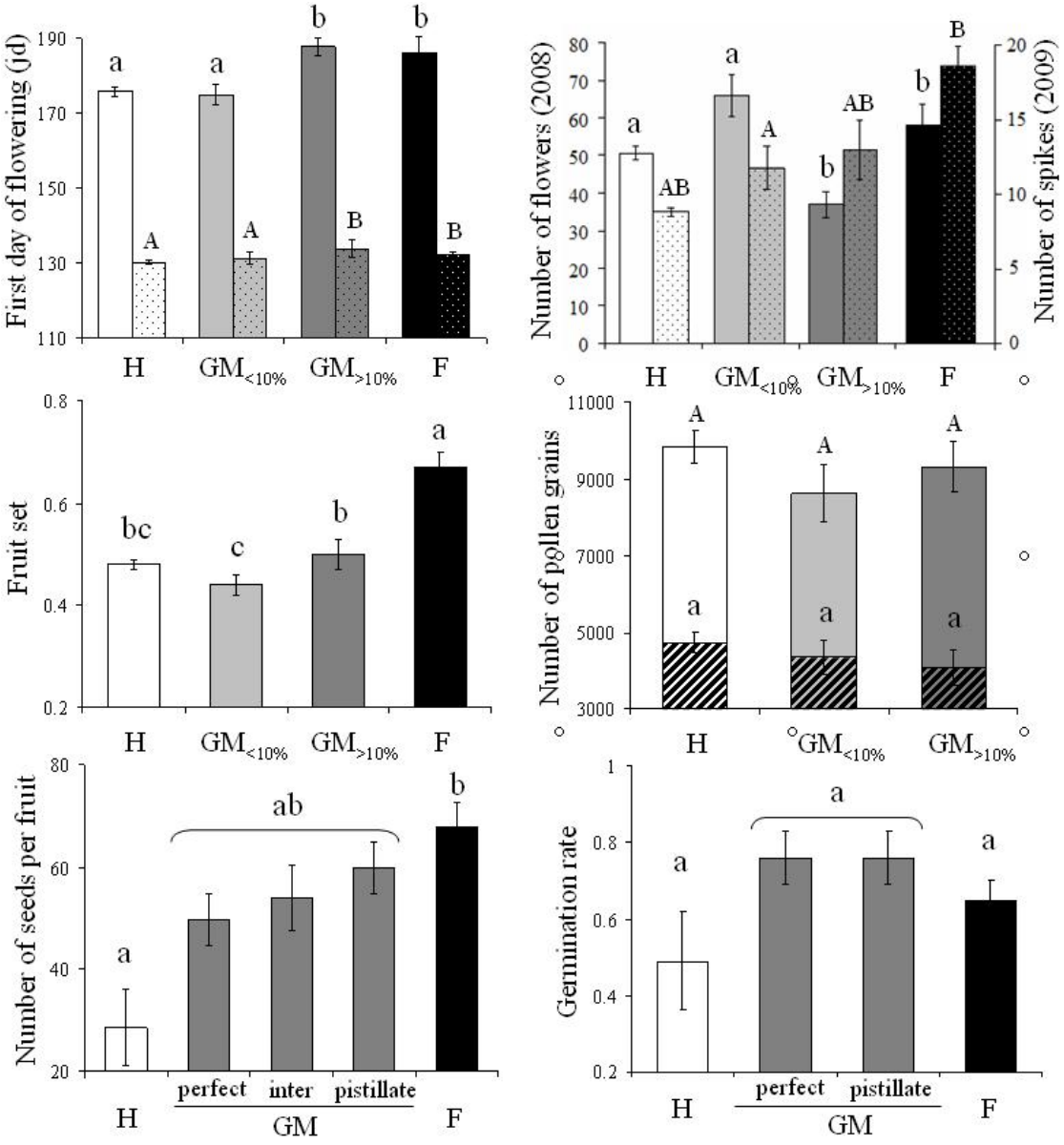

Table 3.4: Floral and reproductive traits by sex phenotype (mean ± SE). The results of the full statistical analyses are given in TABLE 3.5 and in the text. When data were collected during the two flowering seasons, 2008 and 2009 data are represented in the same graph by full and dotted area respectively. The total number of pollen grains and the number of large pollen grains are in full and hatched area respectively. Flowers of different sexes (perfect, intermediate and pistillate) from gynomonoecious plants were graphed separately for the number of seeds per fruit and the germination rate. Results of post-hoc Tukey tests are mentioned by letters and refer to ANOVAs presented in TABLE 3.5. H: hermaphrodites; F: females; $GM_{<10\%}$ (resp. $GM_{>10\%}$): gynomonoecious plants (GM) with a sex ratio lower (resp. higher) than 0.1; Inter: intermediate; jd: julian day.

Variable	Source of variation	dF	MS	F	p
First flowering day	cross	32	0.037	5.15	**<0.0001**
in 2008[a]	sex phenotype	3	0.093	13.00	**<0.0001**
	error	293	0.007		
First flowering day	cross	32	0.089	23.01	**<0.0001**
in 2009[a]	sex phenotype	3	0.023	5.89	**0.0006**
	error	725	0.004		
Number of flowers	cross	32	0.886	2.78	**<0.0001**
(2008)[a]	sex phenotype	3	6.255	19.51	**<0.0001**
	error	295	0.321		
Number of flowering	cross	32	1.711	7.04	**<0.0001**
spikes (2009)[b]	sex phenotype	3	1.121	4.62	**0.0033**
	2008 flowering	1	2.002	8.24	**0.0042**
	2008 f. x sex phenotype	3	1.298	5.34	**0.0012**
	error	697	0.248		
Fruit set	cross	32	0.104	4.50	**<0.0001**
	sex phenotype	3	0.186	8.07	**<0.0001**
	error	295	0.023		
Number seeds per fruit	sex phenotype	2	4152	4.19	**0.0264**
	flower sex (sex phenotype)	2	786	0.79	0.4627
	error	26	12873		
Seed weight	sex phenotype	2	0.011	0.25	0.7833
	flower sex (sex phenotype)	2	0.003	0.06	0.9411
	error	26	0.559		
Germination rate[c]	sex phenotype	2	0.189	0.65	0.5340
	flower sex (sex phenotype)	1	0.001	0.00	0.9472
	error	15	2.169		
Total pollen quantity	cross	31	3148	6.11	**<0.0001**
per bud[d]	sex phenotype	2	508	0.99	0.3743
	error	240	515		
Quantity of pollen	cross	31	1933	4.89	**<0.0001**
grains >35μm per bud[d]	sex phenotype	2	994	2.51	0.0832
	error	240	396		
Quantity of pollen	cross	31	211	5.06	**<0.0001**
grains >35μm per	sex phenotype	2	14.984	0.36	0.6983
normal stamen[d]	error	239	41.656		

a : ln transformation; b: cube root transformation;

c: arcsine(square root) transformation; d: square root transformation.

Table 3.5: Analysis of variance of floral and reproductive traits. For all analyses but the number of seeds per fruit, the seed weight and the germination rate, sex phenotype was defined as a qualitative variable with four categories: females, hermaphrodites, $GM_{<10\%}$ and $GM_{>10\%}$. For the three remaining analyses, the sex of the plant (sex phenotype) was defined as female, hermaphrodite or gynomonoecious, and the sex of the flower was nested within the sex phenotype. Significant p-values are in bold. Results of post-hoc Tukey tests are available in FIGURE 3.4.

Similarly, in gynomonoecious plants, we found no correlation at the flower level between the number of normal anthers and the number of seeds produced ($r = -0.14$, $n = 82$, $p = 0.21$) nor at the individual level between the femaleness and the fruit set of the plant ($r = 0.14$, $n = 113$, $p = 0.15$). We also tested for a correlation between the mean sex ratio by family (sum of quantitative sex measurement of all plants divided by the number of plants) and the mean male or female fitness by family. For hermaphrodite as for gynomonoecious plants, no correlations were found between the fruit set and the family sex ratio ($r = 0.036$, $n = 27$, $p = 0.86$ for hermaphrodites and $r = 0.24$, $n = 28$, $p = 0.23$ for gynomonoecious plants) nor between the quantity of large pollen grains per normal anthers and the family sex ratio ($r = 0.28$, $n = 26$, $p = 0.17$ for hermaphrodites and $r = 0.21$, $n = 26$, $p = 0.31$ for gynomonoecious plants).

Discussion

We observed a clear female advantage in the number of flowers and flowering spikes, the fruit set and the number of seeds per fruit. Combining these three traits, females produced 3.7 (2008) to 6.9 (2009) more seeds than hermaphrodites. These estimations are consistent with female advantage estimates for other species of *Silene* (Lafuma and Maurice, 2006; Morris and Doak, 1998; Shykoff, 1988) and more than suffice to explain the maintenance of females in sex-polymorphic populations. However, we may have over-estimated compensation for two reasons: First, pollen limitation may occur in the field especially since females produce smaller flowers (Dufay et al., 2010) that are expected to be less attractive for pollinators (Delph, 1996). Our plants grew at high plant density and a sex ratio largely biased toward hermaphrodites, so females did not lack for pollen. Moreover, we observed that, as in others gynodioecious species (Agren and Willson, 1991; Collin et al., 2002 but see Poot, 1997), females and hermaphrodites differed in their phenological traits, with females initiating flowering after hermaphrodites. Since the larger females flowered for longer than the hermaphrodites (data not shown) late-produced female flowers may bloom when no more pollen is available, so sexual differentiation in phenology may affect reproductive success. Second, we noticed that hermaphrodites flowered better than females from the first year, which could have a strong impact on their lifetime fitness though females may live longer than hermaphrodites (van Damme and van Delden, 1984 but see Morris and Doak, 1998). A comprehensive estimation of compensation in *Silene nutans* would require additional information on survival and flowering during the entire lifetime.

In accordance with the study of Dufay et al. (2010), gynomonoecious plants, and particularly those with a high proportion of perfect flowers, were frequent. We showed that intensive phenotyping can treble the detection of gynomonoecious plants compared with a standard phenotyping procedure with only a few observations during flowering. Gynomonoecious plants were very heterogeneous, not only for plant femaleness but also for floral and reproductive traits. This has rarely been stud-

ied although it has been suggested for fruit set (Philipp, 1980), number of flowers (Maurice, 1999) and flower size (Dufay et al., 2010). We found that $GM_{<10\%}$ were generally more similar to hermaphrodites and $GM_{>10\%}$ to females but surprising results were found for the number of flowers in 2008, with $GM_{<10\%}$ producing the most flowers and $GM_{>10\%}$ the fewest. Thus, only large plants successfully expressed the minority pistillate phenotype, possibly because, if the probability of each flower being pistillate is low, such flowers will only be seen on plants with many flowers. We only observed this phenomenon in the first year of flowering, during which plants may not yet have fully developed their proper flowering phenotype, particularly in long lived perennials such as *Silene nutans*. Indeed, the first flowering was highly delayed and reduced compared with the two following years (data for 2010 not shown). The literature reveals all patterns for the relative number of flowers of female, hermaphrodite and gynomonoecious plants: gynomonoecious plants have either more flowers than both females and hermaphrodites (Agren and Willson, 1991; Guitian and Medrano, 2000) or are intermediate (Lafuma and Maurice, 2006; Poot, 1997) and within the gynomonoecious class, inconsistent effects of the femaleness on the number of flowers have been found (Desfeux, 1996; Maurice, 1999).

Previous studies on gynodioecious-gynomonoecious species have found that flower characteristics depend both on the plant and the flower sex, and suggested a continuum from perfect flowers of hermaphrodites to pistillate flowers of females through perfect flowers and pistillate flowers of gynomonoecious for traits such as fruit set and flower size (Agren and Willson, 1991; Collin and Shykoff, 2003; Desfeux, 1996; Dufay et al., 2010; Lafuma and Maurice, 2006; Maurice, 1999; Widén and Widén, 1999). We found such a tendency, albeit non-significant, for seed number per fruit. Unfortunately we have not the statistical power to conclude with confidence about the relative performance of pistillate and perfect flowers of gynomonoecious plants. They did not differ for fruit set, nor did fruit set covary with plant femaleness. In particular, pistillate flowers that were produced early in flowering on mainly hermaphrodite gynomonoecious plants set fruit normally, contrary to what has been suggested in other species (Koelewijn and van Damme, 1996; Philipp, 1980).

We showed that the number of normal anthers in buds was a reliable estimation of the quantity of large (i.e. viable) pollen grains. Buds from gynomonoecious plants contained (nonsignificantly) fewer large pollen grains than buds from hermaphrodite plants, similar to Dufay et al. (2010), but this difference was totally explained by the reduced number of normal anthers in gynomonoecious buds. The production of viable pollen by gynomonoecious plants was thus proportional to the number of normal anthers produced by the entire plant. We calculated the total production of viable pollen grains per plant for the flowering season of 2008 as the number of normal anthers per plant times the quantity of viable pollen grains per normal anther. $GM_{<10\%}$ produced a factor 1.23 more viable pollen than hermaphrodites thanks to their higher total number of flowers, and $GM_{>10\%}$ produced 0.47 as many viable pollen as hermaphrodites due to their small overall number of flowers of which

several were female flowers. Obviously, pollen counting is only a proxy of male fitness and additional information on the contribution to the pollen cloud (Philipp et al., 2009), pollen competition success for fertilization (Morand-Prieur et al., 2003), and the fine scale spatial structure of sex phenotypes (Olson et al., 2006) would be necessary to provide a reliable estimate of the relative male fitness of gynomonoecious and hermaphrodite plants.

Some gynomonoecious plants ($GM_{>10\%}$) thus presented a reduction in potential male fitness compared with hermaphrodites and all of them benefited from a reduced compensation compared with females, with a seed production only 1.3 to 3 times higher than hermaphrodites. This result is consistent with the reallocation of the resources saved from pollen production and reduced petal size (Dufay et al., 2010) into the production of seeds, as is supposed to occur in females. This hypothesis rests on the existence of a trade-off between male and female functions, demonstrated in hermaphrodites of some gynodioecious species (Agren and Willson, 1991; Koelewijn, 2003 but see Glaettli and Goudet, 2006) but we found no evidence for such a trade-off in *Silene nutans*. Alternatively, the compensation of gynomonoecious plants observed in fruit and seed production could result from the avoidance of self-pollination (geitonogamy) and associated inbreeding depression. Collin and Shykoff (2003) showed that the selfing rate was similar in hermaphrodite and gynomonoecious plants of *Dianthus sylvestris*. In fact, perfect flowers on gynomonoecious plants showed lower selfing rates than those from hermaphrodites but pistillate flowers on gynomonoecious plants had the highest selfing rates. The authors suggested that this pattern could be due to preferences of pollinators that visit perfect (larger) flowers before pistillate (smaller) flowers on gynomonoecious plants. We showed that, in *Silene nutans*, pistillate flowers tend to appear early in flowering and that this trait is at least partially genetically determined. Precocity of pistillate flowers has already been reported in gynomonoecious plants, although the trait was not quantified (Dufay et al., 2010; Maurice, 1999; Philipp, 1980; Widén and Widén, 1999). This trait may be involved in the avoidance of geitonogamy, particularly in *Silene nutans*, that has moderate to severe inbreeding depression (Dufay et al., 2010; Thiele et al., 2010). However the impact of selfing could be limited by maternal discrimination against self-pollen (Hauser and Siegismund, 2000). Further investigations will be needed to estimate the consequences of selfing rate on the compensation of female and gynomonoecious plants.

The influence of compensation and potential restoration costs on the evolution of gynodioecy has been the subject of many theoretical studies (e.g. Bailey et al., 2003; Dufay et al., 2007) but to our knowledge, gynomonoecious plants have never been included in such models. Bailey and Delph (2007b) investigated the evolution of gynodioecy under quantitative restoration but, since sex determination was a threshold trait, the gynomonoecious phenotype was not considered. Our study provides essential input on the relative fitness of female, hermaphrodite and gynomonoecious plants, but information on the genetic determination of gynomonoecious individuals

is still lacking in order to predict their influence on the dynamics of genes involved in sex determination. Koelewijn and van Damme (1995b) suggested that gynomonoecious individuals could be heterozygous at restorer loci, inducing incomplete restoration, and could maintain polymorphism at restorer loci through overdominance if these plants were good females and good hermaphrodites. However, we and other studies of gynodioecious species found no evidence for overdominance of gynomonoecy (Agren and Willson, 1991; Lafuma and Maurice, 2006; Poot, 1997). On the other hand, we showed that the mean femaleness of gynomonoecious plants within a family was correlated with the proportion of females in the family, as has already been found in *Plantago coronopus* (Koelewijn and van Damme, 1995b) and *Silene vulgaris* (Glaettli and Goudet, 2006). This suggested a quantitative restoration of male fertility implying many restorer loci, a hypothesis that is also upheld by data on sex segregation in cross progenies of several species (Ehlers et al., 2005). Under that case, gynomonoecy could be a simple by-product of the quantitative determination of sex. On the other hand, Desfeux (1996) proposed that gynomonoecy could represent a bet-hedging strategy, because gynomonoecious individuals might benefit from compensation while ensuring fertilisation by selfing in case of pollen limitation. However there is no evidence for differential pollen limitation of females in gynodioecious species (Lopez-Villavicencio et al., 2003; Shykoff et al., 2003 but see Alonso, 2005) though highly female patches can suffer reduced seed production (McCauley and Brock, 1998; Widén and Widén, 1990).

We remain with a number of unanswered questions:

1. Do gynomonoecious individuals represent a strategy or a developmental error? Distinguishing between strategy and developmental error is not an easy task: One might predict more developmental errors under more stressful conditions, for example, and here we found that only our most vigorous plants fell in the category of highly hermaphrodite gynomonoecious individuals, which we would not predict were this a response to stress. On the other hand, the ability to flexibly express different phenotypes as a response to environmental variation may itself be a strategy, so it may not be useful to attempt to differentiate between strategy and developmental error.
2. Is gynomonoecy an expression of heteroplasmy or of partial restoration? Our data on the repeatability of sex ratio and reproductive characters within genotypes are, in fact, consistent with both genetic models for the determination of gynomonoecy. These data indicate a genetic basis, which could be due to a particular cytoplasm or particular combination of nuclear genes, or their interaction, for all these traits.
3. Is it useful to group all gynomonoecious individuals as a single category? We find a clear bimodality in the expression of gynomonoecy, with strongly hermaphrodite individuals behaving differently than the others, suggesting that gynomonoecy is not a single phenomenon. On the other hand, the gynomonoecious category is different from the female or hermaphrodite one, and it would

be adequate to consider the overall contribution of all gynomonoecious individuals to future generations in order to predict the dynamics of cytoplasmic factors and nuclear restorer genes. For such a prediction, however, we need a clear understanding of the genetic basis of this phenotype. Additional controlled crossing studies and testing for heteroplasmy will be necessary before we can answer this question.

References

See References at the end of the Thesis p.178.

3.3 Determination of the gynomonoecious phenotype

3.3.1 Plasticity of the gynomonoecious phenotype

In the majority of gynodioecious species having gynomonoecious individuals, it has been demonstrated that gynomonoecious plants show a high level of plasticity compared with females and hermaphrodites (Agren and Willson, 1991; Andersson, 1999; de Haan, 1996; Desfeux, 1996; Klaas and Olson, 2006; Koelewijn and van Damme, 1996; Maurice, 1999) and some general features of their plasticity have emerged:

- Gynomonoecious can change sex over years, becoming female or hermaphrodite, but these changes reflect in general small quantitative changes in the proportion of pistillate flowers (Agren and Willson, 1991; Andersson, 1999; Desfeux, 1996; Widén and Widén, 1999). Plastic quantitative modifications of femaleness has never been quantified, except in *Glechoma hederacea* (Lamiaceae) where repeated measures over years of the quantitative sex phenotype (estimated by the number of normal stamens) were correlated (Widén and Widén, 1999). Interestingly, sex change between hermaphrodite and female sometimes occurs in *Thymus vulgaris*, but only for females having the largest flowers (which is a hermaphrodite feature) and for hermaphrodites having the smallest flowers (female feature) (Atlan, 1991). Again, plasticity involved limited phenotypic changes.
- Sex changes occur mainly between the hermaphrodite and the gynomonoecious phenotypes and less frequently between female and gynomonoecious (Agren and Willson, 1991; Koelewijn and van Damme, 1996; Maurice, 1999). Indeed, because a high proportion of gynomonoecious plants carry only a few pistillate flowers (see TABLE 3.1), small quantitative changes in sex are more likely to produce a sex change with the hermaphrodite phenotype than with the female phenotype.
- In general, plants produce more pistillate flowers in a controlled environment (greenhouse, experimental garden), which result in a greater proportion of gynomonoecious plants and fewer hermaphrodites (de Haan, 1996; Koelewijn and van Damme, 1996; Maurice, 1999). Temperature and soil have been suggested as two explanatory factors.

In order to understand the causes of plasticity, a few studies have tested the effect of environmental factors such as light, soil nutrient or temperature. To my knowledge, only two studies on gynodioecious plants looked at the influence of environmental factors on the plasticity of the gynomonoecious phenotype. For this reason, I include here some results on pure gynomonoecious species. In *Plantago coronopus*, Koelewijn and van Damme (1996) have shown that increasing temperature had no influence on females and hermaphrodites but increase the femaleness of gynomonoecious plants, by reducing the number of normal anthers per flower or

increasing the proportion of pistillate flowers. Temperature has not been tested in other gynodioecious species but a similar result has been obtained in *Silene noctiflora*, a pure gynomonoecious species (Folke and Delph, 1997).

Nutrient stress was also found to increase femaleness in some pure gynomonoecious species (Olesen et al., 1998; Wise et al., 2008). In addition, this observed plastic response is likely to be adaptive in *Solidago altissima* since individuals with the smallest femaleness were the most fit in rich environments whereas individuals with the highest femaleness were the most fit in poor environments (Wise et al., 2008). In contrast, in *Plantago lanceolata*, nutrient availability and light were found to have no effect (de Haan, 1996). More generally, environmental cues has been suggested to act on sex expression via hormonal changes (Folke and Delph, 1997).

3.3.2 Genetic determination of the gynomonoecious phenotype

Although gynomonoecious plants are described as very plastic, the phenotypic variance within gynomonoecious individuals has been shown to be smaller than the phenotypic variance between gynomonoecious individuals (Widén and Widén, 1999). It suggests that a genetic component is involved in the determination of the gynomonoecious phenotype (and the femaleness) but currently there is no direct evidence of this genetic control and *a fortiori* of its nature (number of genes, cytoplasmic or nuclear genes, etc.). Two hypotheses of genetic determination have nevertheless been formulated and there are more or less direct arguments for both of them.

3.3.2.1 The heteroplasmy hypothesis

Under this hypothesis, gynomonoecious plants carry two different mitochondrial genomes, one male-sterile and one male-fertile, and the sex of flowers is determined by the mitochondrial genome carried by the surrounding tissues. Therefore, branches containing mainly male-sterile mitochondria carry pistillate flowers and branches containing mainly male-fertile mitochondria carry perfect flowers. The mixture of different cytoplasmic genomes within an individual is called heteroplasmy and will be presented in full details in chapter 4.

Two studies support this hypothesis. The first was carried out in *Silene vulgaris* and consisted of crosses between on one hand, pistillate and perfect flowers of gynomonoecious plants and on the other hand, a non-related pollen donor (Andersson, 1999). The two types of crosses (pistillate flowers x pollen and perfect flowers x same pollen) produced female, hermaphrodite and gynomonoecious offspring but perfect flowers produced more gynomonoecious plants and fewer females than pistillate flowers did. Because nuclear genes are *a priori* equally transmitted by perfect and pistillate flowers, Andersson (1999) suggested that the two types of flowers did not transmit the same cytoplasmic genome and that the pistillate flowers produced more female offspring because they carried mainly male-sterile mitochondria. Furthermore, pistillate flowers carried by chimeric branches (containing both perfect and pistillate flowers) were shown to produce fewer female offspring than pistillate

flowers carried by non-chimeric branches, which suggest that branches containing only pistillate flowers contain a higher proportion of male-sterile mitochondria than chimeric branches. Andersson (1999) mentioned however that other mechanisms, such as maternal effects or epigenetics, cannot be excluded.

The second study is less famous. It has been carried out in *Brassica napus* by crossing two laboratory strains, one used as a father and having a male-sterile cytoplasm (restored by a identified restorer) and the other used as a mother, carrying a male-fertile cytoplasm and without the restorer of the father's cytoplasm (Erickson and Kemble, 1993). The aim was to study the cytoplasmic transmission from parents to offspring thanks to male-sterility, used as a phenotypic marker, and to genetic markers (restriction fragment length polymorphism). The genetic markers allowed the detection of three offspring carrying a mixture of male-sterile and male-fertile cytoplasms (on 290 offspring). One of them was completely male-fertile, another entirely male-sterile and the last one carried both pistillate and perfect flowers. The authors explained the two first phenotypes by a unbalanced stoichiometry of the two cytoplasms resulting in the expression of the more frequent one. In the mixed individual, crosses have shown that its pistillate (resp. perfect) flowers produced female (resp. hermaphrodite) offspring, suggesting a link between the flower phenotype and the mitochondrial genome carried by surrounding tissues.

These studies brought arguments for the heteroplasmy assumption but were unfortunately performed on very small sample sizes and their repeatability needs to be tested in future studies. On the other hand, heteroplasmy is generally considered as a rare event (but see chapter 4) which is inconsistent with the high proportion of gynomonoecious plants observed in populations.

3.3.2.2 The quantitative restoration hypothesis

The second hypothesis of genetic determination of gynomonoecious individuals is quantitative restoration. Until now, we only mentioned restorers that act in a qualitative way (restored vs. non restored). Indeed, almost all theoretical studies assumed that a single restoration locus is required to restore a CMS and, even if in crossing studies several restorers were often needed to explain the progenies segregation-ratios, genetic models rarely involved more than three loci. However, there is much indirect evidence for quantitative restoration, i.e. restoration by many genes with small and additive effects. Under this assumption, gynomonoecious individuals would carry only some restorer alleles that make the production of pollen possible but are non sufficient to express a normal hermaphrodite phenotype. Quantitative restoration does not necessarily imply the existence of gynomonoecious individuals and is supported by evidence that is sometimes not related to the gynomonoecious phenotype.

Quantitative restoration and segregation-ratio in controlled crosses

Koelewijn and van Damme (1995b) were among the first to express the quantitative restoration hypothesis that they called incomplete restoration in the original publication. In a study on the number of restorers involved in the restoration of

a few CMS, they showed that it was possible to build a genetic model of restoration explaining the proportion of females and hermaphrodites in progenies (as it is usually done), but also the proportion of gynomonoecious plants. Assuming that the gynomonoecious individuals were heterozygotes at the restoration locus, thus partially restored, their model explained the segregation-ratios of progenies in about 50% of the crosses.

More recently, Ehlers et al. (2005) published a quantitative restoration model in which the level of restoration is a quantitative variable but results in a discrete phenotype, female or hermaphrodite, determined by a threshold. By re-analyzing the results of published crossing studies, they showed that this model was able to explain the proportion of females and hermaphrodites in progenies (Belhassen et al., 1991; Charlesworth and Laporte, 1998; Koelewijn and van Damme, 1995b). In order to take into account gynomonoecious offspring, Ehlers et al. (2005) generalized their model by including two thresholds defining three phenotypes (females, gynomonoecious and hermaphrodite) and have shown that this model successfully fitted data obtained in *Plantago coronopus* (Koelewijn and van Damme, 1995b).

Quantitative restoration and inbreeding

Again by crossing experiments, Glaettli and Goudet (2006) provided other evidence in a study on inbreeding in *Silene vulgaris*. Generally, the proportion of females is higher in inbred progenies (including selfing) than in outbred progenies (Bailey and McCauley, 2005; Emery and McCauley, 2002; Glaettli and Goudet, 2006), which is generally explained by an increased homozygosity in inbred progenies and the dominance of most restorers. Glaettli and Goudet (2006) showed that the proportion of females, of gynomonoecious individuals, and the proportion of pistillate flowers on these gynomonoecious individuals were higher in inbred than in outbred progenies. These results are consistent with quantitative restoration in which restoration loci are mostly dominant. Indeed, the proportion of individuals homozygous for these restoration loci increases with inbreeding which reduces the level of restoration, since non-restored homozygotes segregate, and explains the higher proportion of females, gynomonoecious and pistillate flowers on gynomonoecious plants. Glaettli and Goudet (2006) mentioned however that their results could also be explained by inbreeding depression (Bailey and McCauley, 2005). On the contrary, their results are inconsistent with the heteroplasmy hypothesis if we assume that heteroplasmy results from the transmission of the mitochondrial genome via the pollen (see chapter 4). Indeed, it is expected to find less heteroplasmy (and thus fewer gynomonoecious plants) in inbred progenies since the two parents must carry different cytoplasms for heteroplasmy to occur.

Restorers and reproductive allocation toward male and female functions

Many studies suggested that restorer genes may be involved in reproductive allocation toward male and female functions in gynomonoecious and hermaphrodite plants. For instance, Philipp (1980) showed in *Stellaria longipes* that gynomonoecious offspring from a hermaphrodite mother carried mainly perfect flowers whereas

those from a female mother produce about the same number of perfect and pistillate flowers. This result suggests that genes involved in restoration also influence the relative male/female allocation in gynomonoecious individuals (or are genetically linked to genes having that effect). In addition, it has been shown in *Plantago coronopus* that the proportion of females was strongly correlated to the femaleness of gynomonoecious plants within families (Koelewijn and van Damme, 1995b). This correlation has however not been found in *Silene italica* (Maurice, 1999).

The link between restorer genes and genes having a quantitative action on reproductive allocation was also observed in hermaphrodite plants of gynodioecious species. Indeed, a continuum of relative male/female allocation is sometimes found in hermaphrodites (Koelewijn, 2003) and reproductive allocation appeared to depend on the level of restoration of the family, suggesting a link between restoration and reproductive allocation. For instance, it has been shown in *Thymus vulgaris* that hermaphrodites from well restored families (high proportion of hermaphrodites within the family) had higher male and female fitness than those from poorly restored families (Gigord et al., 1999). On the contrary, in *Plantago coronopus*, hermaphrodites from well restored families invested more in the male function and less in female function compared with those from poorly restored families (Koelewijn, 2003). Lastly, in *Silene vulgaris*, a positive correlation between the level of restoration of the family and the male fertility of hermaphrodites has been observed but no correlation has been found between the level of restoration and female fertility (Glaettli and Goudet, 2006). All these studies suggest an epistatic effect of restorers on quantitative reproductive allocation.

Molecular evidence

Very few restorers with a quantitative effect have been found in crops where the genes and mechanisms of restoration are well known. To my knowledge, only one case was reported in rice (*Oryza sativa* L.), with three restorers inducing partial and additive restoration (Tada, 2007). Scarce evidence for quantitative restoration genes could however result from a detection artifact, genes with slight effect being more difficult to detect than genes with large effect.

In conclusion, many studies support quantitative restoration but direct evidence for the link between gynomonoecy and quantitative restoration are rare. Furthermore, the quantitative restoration hypothesis, which easily explains the continuum of sex phenotype observed in natural populations[1], does not explain why gynomonoecious plants carry fully male-sterile and male-fertile flowers and not intermediate flowers (except from *Plantago*).

ARTICLE 3 (p.88) presents my PhD works on environmental and genetic determination of the gynomonoecious phenotype in *Silene nutans*.

[1]The continuum in sex phenotype was often used as an argument for quantitative restoration (quantitative traits are indeed generally controlled by many genes with slight effects), but the heteroplasmy hypothesis would also lead to a quantitative phenotype if the proportion of male-sterile mitochondria determines the femaleness of the plant.

Article 3

Environmental and genetic factors controlling the sex determination of the gynomonoecious phenotype in the gynomono-gynodioecious *Silene nutans*

(ongoing work)

Introduction

In gynodioecious species, the gynomonoecious phenotype has been reported to be very plastic. Gynomonoecious plants often change into hermaphrodites (and sometimes into females) from one year to another, but these changes are generally due to small modifications in the proportion of pistillate flowers. The environmental factors controlling this phenotype remain largely unknown although some effect of temperature and nutrient availability have been detected in the gynodioecious *Plantago coronopus* and some purely gynomonoecious species. Moreover, the relative impact of genetic and environmental factors has been poorly studied and even if genetic control has been suggested, its nature remains controversial.

In this study, we set out to determine some of the genetic and environmental factors that control the gynomonoecious phenotype in *Silene nutans*. First, we compared the quantitative phenotype (proportion of pistillate flowers) of clones over two years and under two different environmental conditions in order to determine whether there is phenotypic plasticity and to detect of possible genetic control of the phenotype. We tested two environmental conditions that differed in soil nutrient availability since this factor has already been suggested to modify sex ratio in some purely gynomonoecious species but has not been confirmed in gynodioecious species (de Haan, 1996). Then, we set up two crossing experiments to determine the main features of sex determination and inheritance of the gynomonoecious phenotype. More particularly, these experiments were designed to disentangle the two main hypothesis of sex determination, *i.e.* the heteroplasmy and the quantitative restoration hypotheses.

Material and Methods

Plant material

The plants we used in the plasticity experiment and as parents in the genetic determination experiments were offspring from 36 inter-populations crosses performed in 2007 for other purposes (see ARTICLE 1 p.36). These offspring were first grown as seedlings in the greenhouse at 20°C, then vernalized for two months and finally transplanted as young rosettes into an experimental garden in spring. They were sexed in the summer of 2008 by screening newly opening flowers every four or five days during the entire flowering season. Flowers were classified as perfect (hermaphrodite), pistillate (female) or intermediate (1 to 5 normal anthers) and the sex of each plant was defined by a quantitative sex measurement (hereafter called femaleness) calculated as follows:

$$\text{Femaleness} = \frac{\text{number of pistillate flowers} + 0.5 \times \text{number of intermediate flowers}}{\text{total number of flowers}}$$

Gynomonoecious individuals have been shown to represent up to 33% of offspring and about 50% of them were mainly hermaphrodite although all proportion of pistillate flowers were found (see Article 2).

Plasticity experiment

In this experiment, we tested whether the gynomonoecious phenotype is stable under different soil nutrient conditions. Among the 113 gynomonoecious plants phenotyped in 2008, we selected individuals with a femaleness higher than 0.1 in 2008 from as many families as possible. The rationale was to exclude almost hermaphrodite gynomonoecious, for which even very small changes in the sex phenotype could lead to sex change to hermaphrodite individuals, and to represent as broad a range of genotypes as possible in the tested plants.

Forty-one individuals were transplanted from the experimental garden to the greenhouse in November 2008 but only 30 survived the transplantation. After three weeks of recovery, plants were cloned by taking rosette cuttings that included a piece of the central tap root. These cuttings were placed in pots filled with compost. In February, cuttings were randomly assigned to the low or high soil nutrient treatment. The low nutrient treatment was defined by a soil mixture of 1 part compost and 2 parts calcareous sand and the high nutrition treatment by a soil mixture of 3 parts compost and 2 parts calcareous sand, supplemented with a controlled release fertilizer (Osmocote, 200 g/65 l). After transplantation to their new soil substrate, plants were randomized and vernalized for six weeks in order to enhance further flowering. Plants were placed at 20°C for flowering in early April and phenotyped using the same protocol as previously described.

Genetic determination experiments

In order to determine the genetic determination of gynomonoecious individuals, we carried out two crossing experiments. In the first one, we crossed female individuals with pollen from gynomonoecious and hermaphrodite plants to test whether restoration abilities of gynomonoecious plants are reduced compared with those of hermaphrodites. Different flowers on three female plants were pollinated with pollen from full-sibling plants, some flowers using pollen from hermaphrodite pollen donors, others using pollen from their gynomonoecious full-siblings. In one case we used two gynomonoecious plants of different femaleness (Table 3.6). If the female and the pollen donors carry the same cytoplasm, we expect that progenies from female x hermaphrodite crosses will be better restored than progenies from female x gynomonoecious crosses under the quantitative restoration hypothesis, but not under the heteroplasmy hypothesis. If cytoplasms differ between the female and the pollen donors, we expect on average no difference in progenies, whatever the hypothesis of sex determination of gynomonoecy. Thus, we crossed each female genotype with a group of donors carrying the same cytoplasm as the female and a group of donors carrying a different cytoplasm (see Table 3.6).

In the second experiment, we crossed pistillate and perfect flowers of gynomonoecious plants with pollen from an unrelated hermaphrodite donor, in a crossing design similar to the one of Andersson (1999). As far as possible, several pollen

mother \father	27#15 f08: 0.55 f09: 0.5	27#29	29#33 f08: 0.1 f09: 0.22	29#29	9#14 clone 1 f08: 0.58 f09: 0.51	9#16 f08: 0.12 f09: 0.32	9#5
2#3	183	320	80	113			
3#11 clone 1	115	143					
3#11 clone 2					29	86	92

Table 3.6: First sex determination experiment. Each female was crossed with two groups of full-sibling plants, each group containing one hermaphrodite plant and one or two gynomonoecious plant(s). One group bore the same cytoplasm as the mother, the other, a different one. Families 2, 3, and 27 carry the same cytoplasm, which is different from the cytoplasm of families 29 and 9. The number of seeds obtained for each cross is indicated. Parents are numbered by their family of origin and a plant number within family (e.g. 27#15 is the 15th offspring of the 27th family). 27#15 and 27#29, 29#33 and 29#29, and 9#14, 9#16 and 9#5 are thus groups of full-sibling plants. 27#29, 29#29 and 9#5 are hermaphrodite plants. Femaleness (f) of gynomonoecious plants used as parents is indicated for both 2008 and 2009 flowering seasons. 3#11 was cloned by taking cuttings and two clones were used in the crosses.

donors were used for each gynomonoecious plant, and pollen donors were used to pollinate several gynomonoecious plants (see TABLE 3.7). This experiment tested whether the sex phenotype of gynomonoecious plants was determined by a factor that varied within plants. Indeed, if progenies of pistillate and perfect flowers of gynomonoecious individuals segregate different proportions of the three sex phenotypes, sex determination should be at least partly determined at the flower or branch level. This would be consistent with the heteroplasmy hypothesis or with other complex systems such as maternal effects or epigenetic inheritance but would be inconsistent with the quantitative restoration hypothesis. If, on the contrary, no difference can be detected between the progenies of perfect and pistillate flowers, no conclusion can be drawn. Indeed, under the heteroplasmy hypothesis, a pollen donor could be equally able to restore the two cytoplasms carried by the heteroplasmic gynomonoecious plant.

For both sex determination experiments, parents were transplanted from the experimental garden to the greenhouse during the 2008-2009 winter, and some of them were cloned by taking cuttings to ensure sufficient ovules and pollen supply for the crosses. Plants were transplanted into a soil mixture of 3 parts compost and 2 parts calcareous sand supplemented with a controlled release fertilizer (Osmocote, 200 g/65 l), put in a vernalization chamber for six weeks, and placed at 20°C for flowering in early April.

Crosses were performed from late May to early July. In order to synchronize the flowering of parents, we cut the early flowering spikes of some hermaphrodite plants to enhance further flowering. Each cross was performed on several flowers and these

GM (ovule donor) \H (pollen donor)		17#19	23#36	33#5	1#21	34#32
27#34 clone 1	pistillate fl.	225				
f08: 0.58, f09: 0.43	perfect fl.	73				
27#34 clone 2	pistillate fl.		64			
f08: 0.58, f09: 0.41	perfect fl.		171			
27#34 clone 3	pistillate fl.			213		
f08: 0.58, f09: 0.70	perfect fl.			119		
9#14 clone 2	pistillate fl.				97	104
f08: 0.58, f09: 0.56	perfect fl.				109	43
27#25	pistillate fl.				111	
f08: 0.33, f09: 0.62	perfect fl.				102	
3#31	pistillate fl.			77		
f08: 0.44, f09: 0.42	perfect fl.			10		
3#25 clone 1	pistillate fl.			56		
f08: 0.14, f09: 0.45						
3#25 clone 2	perfect fl.			36		
f08: 0.14, f09: 0.49						

Table 3.7: Second sex determination experiment. Pistillate and perfect flowers (fl.) were crossed with the same pollen. Number of seeds obtained for each cross are indicated. Parents are numbered by their family of origin (first number) and a plant number within family (second number). Some gynomonoecious (GM) parents were cloned by taking cuttings and different clone members were sometimes used for the crosses. Femaleness (f) of gynomonoecious plants used as parents is indicated for both 2008 and 2009 flowering seasons.

replicates were performed within ten days. Buds were isolated to avoid accidental pollination. As *Silene 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. Seeds were harvested at maturity.

Seed were germinated in September 2009, in 9cm diameter Petri dishes on 1% water agar. The germination rate was about 85% for most of the crosses but germination was slow and the Petri plates became contaminated with fungi for the following crosses: 9#14 x 1#21, 9#14 x 34#32, 2#3 x 27#15, 2#3 x 27#29, 3#11 x 9#14, 3#11 x 9#16, 3#11 x 9#5. After germination, seedlings were transplanted into multipots (4.5cm diameter each) filled with compost, and placed in the greenhouse at 20°C with natural light for six weeks. Then, plants were vernalized at 5°C (day length 8h) for three months, transplanted in individual pots (8cm diameter) filled with compost supplemented with a controlled release fertilizer (Osmocote, 500 g/60 l) and placed randomly in the greenhouse (daily temperature: 15-20°C, natural light).

Results

Plasticity experiment

Among the 163 clones, 90 flowered in 2009 and the rate of flowering was significantly higher in the high soil nutrient treatment than in the low soil nutrient treatment (61.9% vs. 42.3%, $\chi^2 = 5.41$, $df = 1$, $p = 0.02$). Moreover, in the low treatment, the number of flowers per plant was much reduced than in the high nutrition treatment (6 vs. 50 flowers, $F_{1,40} = 61$, $p < 0.0001$) and the flowering was delayed (179 vs. 149 julian days, $F_{1,40} = 24.5$, $p < 0.0001$).

We showed that the femaleness of gynomonoecious plants did not differ between 2008 and 2009 (paired t-test, $t_{25} = 0.99$, $p = 0.33$). Nor was the difference between 2008 and 2009 femaleness significant when the two treatments were analyzed separately. The level of plasticity between years, calculated as the difference between 2008 and 2009 femaleness, varied among genotypes ($F_{18,61} = 3.2$, $p = 0.0004$). However, this effect was attributable to only one plant, that was mainly female in 2008 (femaleness 2008: 0.88) and mainly hermaphrodite in all clones in 2009 (femaleness 2009: 0→0.25). Overall, the femaleness was repeatable over years within genotypes (intra-class correlation coefficient: 0.73, $F_{25,26} = 3.53$, $p = 0.0011$).

The soil nutrient treatment had no influence on the femaleness of plants (paired t-test, $t_{16} = -0.62$, $p = 0.54$, cf. FIGURE 3.5) although plants bore more pistillate flowers in the high soil nutrient treatment ($t_7 = -2.55$, $p = 0.0436$) when we excluded plants with fewer than 7 flowers, for which the estimation of femaleness is rather uncertain. Considering all genotypes for which more than one clone flowered in 2009, degree of femaleness was repeatable among clones within genotype (intra-class correlation coefficient: 0.56, $F_{18,64} = 6.41$, $p < 0.0001$).

Genetic determination experiments

Almost no plants flowered during their first summer (2010 summer) so we were not able to collect enough data to analyze the results on segregation of sex phenotypes from experimental crosses. *Silene nutans* is a long-lived perennial and as such, few individuals flower in their first season. We had managed to obtain quite a high flowering rate in the first season in a previous study (see Article 1), by placing rosettes outside at the end of winter when they experienced late frosts and snowfall. However, even under these conditions only about $\frac{1}{3}$ of plants flowered in the first season. In this study, with plants in the greenhouse, appropriate environmental cues that allowed the triggering of flowering appear to have been lacking. Data will hopefully be collected next summer (2011).

Conclusion

We found that, although the gynomonoecious phenotype in *Silene nutans* exhibited some phenotypic plasticity over years and among clones, the level of plasticity was limited for all but one genotype. Degree of femaleness was repeatable over years

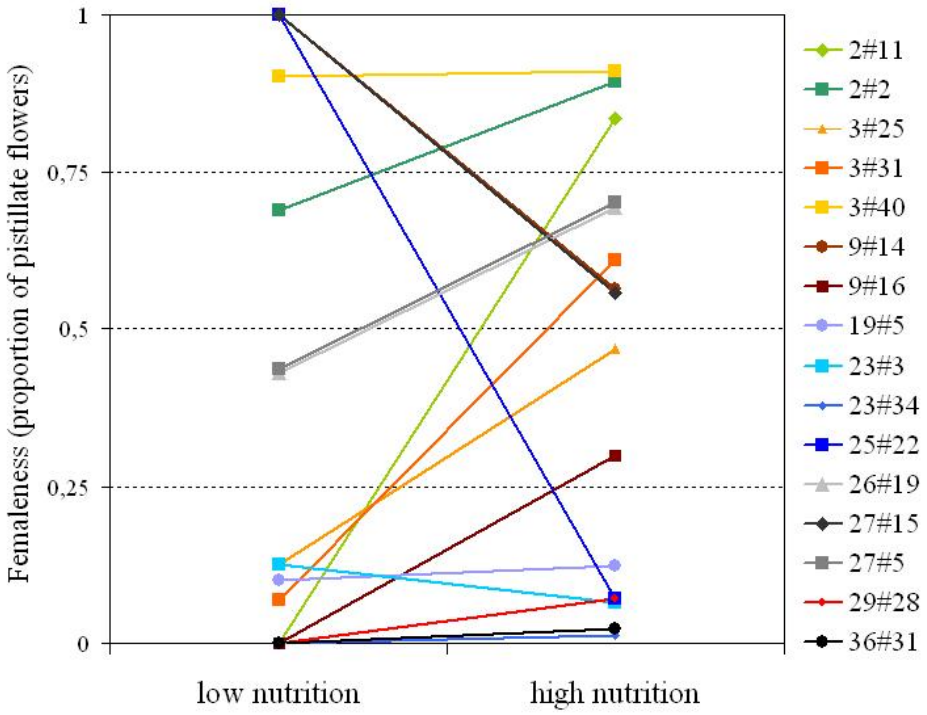

Figure 3.5: Mean femaleness of clones in the low and high soil nutrient treatments. Data are given for the 16 genotypes for which some clones flowered in both treatments.

and among clones of the same genotype, which suggests that the proportion of pistillate flowers of gynomonoecious plants is under some genetic determination. To our knowledge, such an effect of the genotype on the proportion of pistillate flowers in gynomonoecious plants have been demonstrated in only one another gynodioecious species (Widén and Widén, 1999). More information on the genetic determination of gynomonoecious plants will be available next summer.

Sex-allocation theory predicts that environmental stresses, such a low nutrient availability, will increase the sex allocation towards the male function because male gametes are cheaper than female gametes (Charnov, 1982). This expectation has been supported in many monoecious and andromonoecious species but little attention have been paid to gynodioecious or gynomonoecious species (see Wise et al., 2008, and references included). Nevertheless, expectations in species having pistillate and perfect flowers are not straightforward since the smaller pistillate flower type is likely to be cheaper. Thus, one can expect that stresses will either increase the production of the cheaper flower type by increasing the proportion of pistillate flowers, or increase the production of the cheaper gamete (pollen production) by increasing the proportion of perfect flowers (and decreasing the total number of flowers to ensure adequate resources). Shift toward the production of pistillate flowers under nutrient

stress have been demonstrated in some purely gynomonoecious plants (Olesen et al., 1998; Wise et al., 2008) and have been shown to be adaptive in *Solidago*.

In *Silene nutans*, we found no effect of nutrient availability on the proportion of pistillate flowers of gynomonoecious plants, and plants responded to the low nutrition treatment by a reduction in flower number. This resulted in our femaleness estimation on these plants being imprecise because of the small number of flowers on which it was based. Thus the imprecision in the femaleness estimate could have hampered our detection of a soil nutrient effect. On the other hand, de Haan (1996) also found no effect of nutrient availability on sex phenotype in the gynodioecious *Plantago lanceolata*. Again, this suggests a strong genetic control of femaleness in gynomonoecious plants. Because phenotypic plasticity appears quite low in gynomonoecious individuals of *Silene nutans*, it seems unlikely that plasticity, by allowing fine adjustment of the floral sex ratio to local conditions, could provide a selective advantage to gynomonoecious plants compared with females and hermaphrodites. However, further experiments will be needed to better estimate the influence of other factors and their potential interactions on phenotypic plasticity in the gynomonoecious phenotype.

References

See References at the end of the Thesis p.178.

Summary of chapter 3

* In many gynodioecious species, intermediate individuals carrying both perfect and pistillate flowers are found to coexist with females and hermaphrodites in natural populations. These individuals are called gynomonoecious.

* The proportion of pistillate flowers in gynomonoecious individuals (=femaleness) can vary from 0 to 1 (excluded), but is often biased toward the production of perfect flowers.

* Floral and reproductive traits of gynomonoecious plants are poorly described. It seems however that gynomonoecious plants produce more seeds than hermaphrodites but fewer than females and produce pollen of low quality compared with hermaphrodites. In *Silene nutans*, we have shown that gynomonoecious plants enjoyed an advantage in seed production compared with hermaphrodites, but that this advantage is more reduced than that of females. No difference in the production of pollen (quantity and quality) has been detected at the anther level between hermaphrodites and gynomonoecious. In contrast, at the individual level, gynomonoecious plants tend to produce less pollen.

* In gynodioecious species, gynomonoecious individuals are often described as very plastic in their proportion of pistillate flowers. Our observations in *Silene nutans* tend to temper this affirmation since the plastic variance within individuals was smaller than the variance among individuals. These data suggest a genetic control of the gynomonoecious phenotype.

* Two hypotheses of genetic determination of gynomonoecious individuals have been proposed: the heteroplasmy hypothesis — a male-fertile and a male-sterile cytoplasms coexist within gynomonoecious individuals — and the quantitative restoration hypothesis — gynomonoecious individuals carry only some restorers and are partially restored.

* In order to study the impact of gynomonoecious individuals on the evolution of gynodioecy, a better knowledge of the genetic determination and male and female fitness of gynomonoecious is essential. It is currently unknown if the gynomonoecious phenotype is adaptive or is only the consequence of the genetic determination of gynodioecy.

Chapter 4

Evolution of mitochondrial inheritance and gynodioecy

Over the past few years, a series of studies on mitochondrial inheritance in *Silene vulgaris* have revealed some surprises. These results call into question previous thought on mitochondrial (or more generally organelle) inheritance. Although these results are very specific to date, they open many doors for further studies. If these observations should be generalized, some major principles of the evolution of the mitochondrial genome and of gynodioecy would need a revision. In this chapter, we will present the current knowledge on organelle inheritance, particularly in plants. The specificity of gynodioecious species will be the subject of the last part.

Mitochondria in everyday life.

4.1 Why? Causes and consequences of the evolution of uniparental inheritance of organelles

Historically, it was the non-mendelian nature of inheritance of the cytoplasmic genomes that revealed their presence within organelles, first in 1909, with the observations of Baur and Correns of the transmission of green and white chloroplasts in variegated plants (*Pelargonium* and *Mirabilis*) and then in 1933, the transmission of mitochondrial male sterility genes in maize by Rhoades. Since then, exceptions to uniparental inheritance of cytoplasmic genomes have been found more and more often but uniparental inheritance remains the rule. However, there is no consensus as to the causes of this widespread trait whose consequences on the evolution of cytoplasmic genomes are fundamental.

4.1.1 Evolution of uniparental inheritance of organelles: Theoretical studies

We have shown in chapter 1 that the evolution of sexes could be a consequence of the evolution of uniparental inheritance of organelles (see p.6). According to those models, uniparental inheritance would be enforced by the nucleus to avoid the cost induced by the arms race between cytoplasms. These models give a satisfactory answer to the question of the evolution of uniparental inheritance but they are highly dependent on the "allowed" mutations in the model. For instance, if once uniparental inheritance is controlled by a nuclear gene, the model allows the appearance of a new mitochondrial mutation able to break uniparental inheritance to increase its own transmission, the mutant will spread and mitochondrial inheritance will become biparental again. Thus, the conflict between the nucleus and the cytoplasm is similar to a host-parasite Red Queen, except that one of the protagonists, here the nucleus, almost always prevails. The question of uniparental inheritance can thus be rephrased as follows: why does the nucleus generally win the cytonuclear conflict? To my knowledge, this question is still unsolved[1] but the larger size of the nuclear genome and higher opportunities of mutation have been mentioned as two explanatory factors.

Models involving selfish mitochondrial elements are the most widely accepted for explaining the evolution of uniparental inheritance. There are however some others, such as the two following examples:

– *The specialization of organellar transmission by one sex* (Godelle and Reboud, 1995). This model requires the pre-existence of two sexes. The authors assume that the transmission rate of cytoplasmic genes can vary according to the replication efficiency in the male and female germlines. Under certain conditions (shape of the trade-off between the replication rate in the male and the female

[1] More often, research aimed at determining the factors (e.g. level of consanguinity) that affect the outcome of the conflict.

germlines), the model predicts a specialization of the transmission of cytoplasmic genes by one of the germlines. This specialization requires an asymmetry in the conditions of replication between the two germlines. Anisogamy is thus particularly prone to a specialization of the transmission via the female germline, which is more adapted to an efficient replication of organelles. This model could also apply to isogamous species but requires asymmetry between the gametes in organelle replication or transmission efficiency.

– *The STOR model - Salvage, TurnOver, Repair* (Sears and VanWinkle-Swift, 1994). This model assumes that the degradation of the DNA of organelles is a source of nutrients for the metabolism and of nucleotides for recombination, DNA repair and RNA synthesis. This model, initially designed from data in *Chlamydomonas*, applies only to isogamous species in which the destruction of the DNA brings a substantial amount of resources.

4.1.2 Consequences of uniparental inheritance of organelles

We will not present here the consequences of uniparental inheritance of organelles for genomic conflicts (see general introduction p.14) but rather the consequences for the evolution of the mitochondrial genome. Basically, uniparental inheritance makes the mitochondrial genome asexual. Uniparental inheritance with vegetative segregation of organelles during mitoses leads to homoplasmic lineages, i.e. lineages that carry only one mitochondrial genome. Recombination thus become inefficient since it takes place between identical genomes. Cytoplasmic genomes should therefore be unable to eliminate their deleterious mutations and become less and less functional (Müller's ratchet). In addition, linkage disequilibrium between mitochondrial genes is almost complete (the entire mitochondrial genome is a single linkage group), which reduces the effect of selection. Lastly, uniparental inheritance and haploidy of the mitochondrial genome reduces the effective population size, which favors the accumulation of deleterious mutations (Neiman and Taylor, 2009).

Yet, uniparental inheritance is widespread in eucaryotes which suggests that it is not such a heavy handicap (Birky, 1995). Lots of hypotheses have been proposed to explain this paradox:

– Mitochondrial genes are not subjected to changing selective pressures because they are linked to basic functions of the cell. Therefore they can bear a lower adaptation rate (Birky, 1995).

– The functions of these genes being essential, mitochondrial mutations are often lethal or highly deleterious and must be eliminated very rapidly (Birky, 1995).

– Even without sex, cytoplasmic genomes can repair their DNA thanks to the many copies within each cell (or even within each organelle).

– In the mitochondria of plants, high fidelity polymerases ensure a mutation rate ten times lower than the one of nuclear genes and may compensate the lack of recombination. High fidelity polymerases do not exist in animals.

- Müller's ratchet can be avoided by low recombination rates (Neiman and Taylor, 2009) and more and more studies have shown that the mitochondria of plants, fungi and to a lesser extent animals experience occasional recombination because of occasional paternal leakage of mitochondria. According to Barr et al. (2005), recombination rates reported in these studies would effectively eliminate deleterious mutations while having little effect on the susceptibility to selfish elements.
- Cytoplasmic genomes are selected at the intra- and intercellular levels, which can eliminate deleterious mutations. This selection is of course not effective if individuals are homoplasmic but it could play a role in early stages of the appearance of a mutation.
- According to some authors, the bottleneck in the mitochondrial population in the germline, which is well described in mammals, would increase the intercellular variance and enhance selection against deleterious mutations (Roze et al., 2005). However, according to others, the bottleneck would also increase drift, which makes selection less efficient (Neiman and Taylor, 2009). The relative importance of these two effects, which depends on the size of the bottleneck and the selection coefficients of mutations, is still debated.

Overall, mitochondrial genomes accumulate more deleterious mutations than nuclear genomes in plants, animals and fungi (Lynch and Blanchard, 1998). Gene transfers from organelles to the nucleus could have been favored for this reason.

4.2 How? Mechanisms and genetic control of organelle inheritance

4.2.1 Mechanisms of organelle inheritance

If anisogamy is obviously favorable to asymmetrical inheritance of organelles, it does not ensure uniparental inheritance (see p.104). Various exclusion mechanisms have been described (Birky, 2001; Korpelainen, 2004; Xu, 2005) and can take place from gametogenesis to the early stages of development. By this criterion, three categories of mechanisms are sometimes distinguished:

- *Those that act during gametogenesis.* In multicellular organisms, gametogenesis implies many mitotic and/or meiotic divisions. Asymmetrical distribution of organelles during these divisions can lead to the exclusion of organelles from the gamete. Active mechanisms such as the degradation of organelle DNA have also been reported.
- *Those that act at fertilization.* These often imply an active destruction of mitochondria.
- *Those that act in the early stages of development.* Physical exclusion by stochastic or controlled segregation of organelles during cell divisions or active degradation of organelles may be involved.

These different mechanisms are more or less common depending on the taxa. In the unicellular species that have been studied for this trait (e.g. *Cryptococcus neoformans*, *Physarum polycephalum*and *Chlamydomonas reinhardtii*), active destruction of mitochondrial DNA (mtDNA) from one parent occurs just after the fusion. Nucleases are involved in DNA digestion (see genetic determination below) but the causes of the specific degradation of the mtDNA from only one parent are still unknown. In animals, mitochondria from the sperm that reached the ovule cytoplasm degenerate progressively during the first stages of development. mtDNA degradation is activated by ubiquitination of the paternal mtDNA during spermatogenesis. In angiosperms, the combination of several mechanisms ensures uniparental inheritance. Physical exclusion of organelles as well as digestion by enzymes occurs during the formation of the sperm in the pollen grain (Mogensen, 1996). Contrary to what happens in unicellular species, mtDNA digestion always occurs before fertilization. In some species, it has been shown that mitochondria in the pollen tube no longer contain mtDNA and Corriveau and Coleman (1991) and Nagata et al. (1999) showed that organelle inheritance was correlated with the detection of mtDNA in the male gamete. However, the presence of mtDNA does not imply efficient transmission of the genome (Zhang et al., 2003) and inheritance should be checked by genetic markers. In gymnosperms, the localization of organelles within the zygote often determines organelle inheritance (Mogensen, 1996) and a single mechanism seems to be involved for each species. In multicellular algae, the destruction of mtDNA and cpDNA (chloroplastic DNA) takes place during spermatogenesis and not at fusion as in other unicellular algae (for an example in *Bryopsis maxima*, see Kuroiwa and Hori, 1986).

4.2.2 Genetic determination of organelle inheritance

Contrary to the mechanisms, the genetic determination of organelle inheritance is almost totally unknown. Except from a few unicellular species, studies are scarce and sometimes give contradictory results, maybe reflecting a complex determination.

In the unicellular alga *Chlamydomonas reinhardtii*, the mating type (mt+ or mt−) determines the parent who transmits organelles and the degradation of the DNA from one parent occurs within a few hours after cell fusion. It has been shown that a Ca^{2+}-dependant nuclease produced by the mt+ mating type targets specifically the cpDNA of the mt− parent but the position of the gene coding for this nuclease is unknown (Nishimura et al., 2002). The DNA from the parent transmitting its organelles may be protected by methylation. In the slime mold *Physarum polycephalum*, DNA digestion is similar to that described in *Chlamydomonas*; but *Physarum* having not two, but thirteen sexes, the ability of a parent to transmit its mitochondria depends on its partner's sex (Moriyama and Kawano, 2003). In this species, the digestion involves at least two nucleases (one Ca^{2+}-dependent, another Mn^{2+}-dependent), which are imported into the mitochondria for DNA digestion (Moriyama et al., 2005). In the basidiomycete *Cryptococcus neoformans*, the mating type also determines organelle inheritance and Yan et al. (2007) have determined two

genes, SXI1α, expressed in the mating type α and SXI2a, expressed in the mating type a, that coordinate the control of mitochondrial inheritance. In the yeast *Saccharomyces cerevisiae*, whose inheritance is biparental, the segregation of mitochondria during budding is linked to the actin cytoskeleton (Frederick et al., 2008). More generally, cytoskeleton genes could be involved in organelle inheritance in species whose organelle inheritance is determined by the segregation of organelles during pre- or post-fertilization mitoses.

To my knowledge, the nature and mechanisms of the genetic determination of organelle inheritance is unknown in animals, probably because it raises no interest since organelle inheritance is almost always maternal. In plants in contrast, exceptions are less rare (especially for the chloroplasts) and a few genetic studies have been performed. The variegated plants are the historical model for studying chloroplast inheritance. These plants, which present a half green half white foliage, are heteroplasmic and carry green and white chloroplasts. Since these two types of chloroplast can be used as phenotypic markers, paternal leakage of chloroplast has been easily noticed. In *Pelargonium*, it has been shown that chloroplast inheritance depends on the plastid genome (Tilney-Bassett and Birky, 1981), on the maternal nuclear genome (Tilney-Bassett and Birky, 1981) and on the paternal nuclear genome acting in a gametophytic way (Derepas and Dulieu, 1992; Dulieu et al., 1990). The influence of the plastid and nuclear genomes has also been found in *Oenothera* (Chiu and Sears, 1993) but in this species, chloroplast inheritance is determined by the level of compatibility between the chloroplast genomes and the nuclear genome. The transmission rate of paternal plastids is also negatively correlated to the style length (i.e. to the time necessary for pollen tube elongation). Information about mitochondrial DNA is rarer because of the lack of obvious phenotypic markers. However, recent studies in *Silene vulgaris* have shown that the paternal leakage rate of mitochondria was very cross-dependent (McCauley et al., 2005; Welch et al., 2006), with a major role of the pollen donor (Bentley et al., 2010). On the contrary, in *Brassica napus*, maternal and paternal nuclear genomes seem to be involved, suggesting a control by both pollen and ovules (Erickson and Kemble, 1993).

4.3 Examples of unusual uniparental inheritance

Uniparental inheritance exhibits rare but amazing variations. For instance, in *Chlamydomonas reinhardtii*, organelle inheritance is uniparental but the mitochondrial genome is transmitted by the mt− parent and the chloroplast genome by the mt+ parent. Unfortunately, it is not known whether the two mechanisms of DNA digestion have evolved independently, nor whether this inheritance is adaptive. Such a crossed inheritance is also found in most of gymnosperms, which transmit their chloroplasts paternally and their mitochondria maternally (with some exceptions such as the Sequoia which have paternal inheritance of both organelles). This example illustrates that anisogamous taxa do not always transmit mitochondria via the macrogamete.

A even more astonishing case has been found in some bivalves (e.g. *Mytilus edulis* or *Anodonta grandis*) in which females carry mtDNA from their mother while males carry mtDNA from their mother in somatic tissues and mtDNA from their father in the germline (reviewed by Korpelainen, 2004; Xu, 2005). The mitochondrial genome is thus transmitted from mother to daughters and from father to sons and the two lineages of mitochondrial genomes evolve more or less independently, leading to up to 30% of sequence divergence (recombination events occasionally occur). The mechanism of this phenomenon, called doubly uniparental inheritance (DUI), is not yet fully clarified but it seems that asymmetrical segregation of mitochondria during the first divisions of the zygote are involved (Cao et al., 2004; Cogswell et al., 2006). The possible adaptive value remains unknown even if the existence of two lineages of mitochondria could have favored the specialization of each genome to one sex, for example through the selection of mutations that are beneficial for one sex but deleterious for the other (Burt and Trivers, 2006).

These observations raise the question of maternal inheritance: since anisogamy does not seem to constrain the transmission of organelles, why is inheritance always, or at least usually maternal and not paternal? A first hypothesis is linked to the metabolism of two gametes: the gamete having the highest metabolic requirements would be more adapted to the transmission of the cytoplasmic organelles because the presence of organelles in the gamete would be essential. Because the resources are carried by the female gamete, the latter could be more dependent on mitochondrial activity than is the male gamete (this assumption may not be true in animals in which the male gamete motility requires a lot of energy). This hypothesis may explain why gymnosperms more often transmit their organelles paternally. Indeed, pollen tube growth lasts for one to two years in this group, which might require a high metabolic activity in the male gametophyte. Another hypothesis is based on the mutation rates in the two germlines. In animals, paternally inherited genomes have a higher mutation rate that maternally inherited genomes, possibly because of the many cell divisions occurring in the male germline that lead to accumulation of errors in the DNA sequence. This trend has been confirmed in gymnosperms, in which paternally transmitted cytoplasmic genomes have a higher mutation rate than maternally transmitted cytoplasmic genomes[2] (Whittle and Johnston, 2002). Under this hypothesis, maternal transmission of cytoplasmic genomes would limit the accumulation of deleterious mutations, already high in these genomes.

4.4 Heteroplasmy

Although uniparental inheritance of organelle is thought to be general in eucaryotes, biparental inheritance is frequent in some taxa and is even the rule in some species. On the other hand, even in species whose organelle inheritance was thought

[2]This difference is rather surprising in plants since the germline and the somatic lineages separate very late during the development.

to be uniparental, more and more exceptions are detected. The first consequence of regular or occasional biparental inheritance of organelles is the coexistence within an individual of two genomes that can be different. This phenomenon, called heteroplasmy, is rather poorly known, partly because the best studied species have overwhelmingly uniparental inheritance of organelles and are thus homoplasmic. For this reason and in order to have a more comprehensive view of this phenomenon, I have included in this section cases of heteroplasmy that do not result from biparental inheritance. Let us first see what are these other origins.

4.4.1 Origins of heteroplasmy

When heteroplasmy is detected in a species, one of these four origins is generally invoked:

- Regular or occasional biparental transmission of cytoplasmic genomes. In this case, heteroplasmy depends on mating rules (i.e. selfing, population structure), on the proportion of DNA transmitted by each of the parents, etc.
- Point mutations that can appear during replication or DNA repair. These mutations occur in coding or non-coding sequences but will be more quickly eliminated – and therefore less probably detected – if they occur in coding sequences.
- Modification of the genome structure by recombination, leading to duplications or deletions (for details, see below).
- Maintenance of a pre-existing heteroplasmy. Even if this is not an origin, the maintenance of heteroplasmy over generations is often invoked to explain heteroplasmy when the origin is ancient. This aspect will be treated in detail in section 4.4.3.

In animals, heteroplasmy is most often due to point mutations. Indeed, the mutation rate in the mitochondrial genome of animals is 15 to 20 times higher than that of the nuclear genome, partly because of the many ROS (reactive oxygen species) present in the mitochondria. In contrast, in plants, the mutation rates in the mitochondrial and chloroplastic genomes are respectively 6 and 2 times lower than that of the nucleus thanks to high fidelity polymerases[3]. Thus point mutation explains very few cases of heteroplasmy in plants (but see the example of *Olea europeae*, Garcia-Diaz et al., 2003). On the other hand, the mitochondrial genome of plants has a labile and fragmented structure and contains many introns and inter-gene non-coding sequences that increase the genome size (200 to 2500kb versus 15-20kb in animals). Frequent intragenomic recombination events occur at large repeats (Backert et al., 1997) and create subgenomic molecules. Thus the mitochondrial genome is often divided into several DNA molecules, sometimes redundant, and this fragmentation can

[3]Because of this reduced mutation rate, coding sequences of the mitochondrial genome in plants present a low polymorphism compared those of animals.

lead to a different organelle DNA content between cells (Kmiec et al., 2006). For this reason, one can read that heteroplasmy is common in plants but this heteroplasmy has nothing to do with paternal leakage of mitochondria.

In fungi, biparental inheritance is almost as frequent as uniparental inheritance but generally results in only transient heteroplasmy because cells (i.e. individuals for unicellular species) rapidly become homoplasmic thanks to the segregation of mitochondria during mitoses. On the contrary, the detection of paternal inheritance of organelle DNA in plants or animals remains exceptional although many cases have been found recently (for reviews, see Barr et al., 2005; Kmiec et al., 2006; Korpelainen, 2004; White et al., 2008; Xu, 2005). Most frequent exceptions concern chloroplast inheritance in plants (e.g. *Medicago sativa*, *Pelargonium zonale*). In animals, cases have been detected in species as various as human, mouse, salmon, lizards and some insects and arachnids including drosophila, cicadas, silkmoths, mites and scorpions. However, most of these observations were made in interspecific crosses (see Figure 1 in Barr et al., 2005), a trend than could be interpreted in two ways:

- To detect paternal leakage, the mitochondrial genomes of the two parents must be different. Since the intraspecific diversity in the mitochondrial genome of animals is very low, it is easier to detect paternal leakage in interspecific crosses.
- The mechanisms of uniparental inheritance could be specific and thus not be functional in interspecific crosses. To date, information is too scarce to allow comparison among species of mechanisms controlling mitochondrial inheritance. However, regulations between the nucleus and organelles are known and although they are almost ubiquitous in eucaryotes, mechanisms are poorly conserved, which might explain a dysfunction in interspecific crosses (Woodson and Chory, 2008).

4.4.2 The different levels of heteroplasmy

Heteroplasmy can be observed in each biological structure containing several copies of the cytoplasmic genome i.e. organelles, cells and individuals. Even if very few studies have been experimentally able to determine at what level heteroplasmy occurred, these levels should be distinguished (at least theoretically) because their evolutionary consequences differ:

- *Heteroplasmy within organelles.* Each organelle contains several copies of the genome, which may differ. These different DNAs are potentially subject to selection (e.g. via differential DNA replication rates). In plants, different DNA molecules can recombine (in animals, recombination is still debated, see Barr et al., 2005).
- *Heteroplasmy within cells.* The genome can vary among organelles when a cell contains several organelles, which is usually true (e.g. plants have in average 200 mitochondria per cell). These differences are essentially due to an asymmetrical distribution of DNA during organelle divisions or events of fu-

sion/scission of organelles that are known to occur in plants (Arimura et al., 2004; Sheahan et al., 2005). This level is subject to drift and selection.

– *Heteroplasmy within individuals.* In multicellular species, cells can carry different cytoplasmic genomes because of the stochastic distribution of organelles during mitoses. This level is also subject to drift and selection but, contrary to intracellular selection (inter-molecules or inter-organelles), there will always be selection for functional DNA.

It should be mentioned that only the first level – and possibly the second level if organelles can fuse – allows recombination between different genomes. Heteroplasmy may exist simultaneously at several levels, as has been shown in a variegated mutant of cotton that had cells with normal pastids, cells with white plastids and cells with a mixture of plastids (Lax et al., 1987).

4.4.3 The maintenance and transmission of heteroplasmy

In humans, mitochondrial mutations are responsible for many diseases (blindness, myopathy, Parkinson and Alzheimer diseases, etc.) and these pathogenic mutations are almost always carried in a heteroplasmic way (otherwise they are lethal). Except from one case of paternal inheritance in humans (Schwartz and Vissing, 2002), these cases of heteroplasmy are explained by the emergence of mutations in the soma or the germline and/or by the maintenance of heteroplasmy over generations. These pathologies have stimulated research on variation in heteroplasmy among tissues, over individual lifetimes and between generations in humans and some other mammal models. In this section, I will present those data, as well as occasional observations from other animals and plants.

4.4.3.1 Variation in heteroplasmy over the lifetime and among tissues

Despite the many studies having considered the variation in heteroplasmy over animal lifetimes, there is still no consensus. In humans, defective mtDNA seems to accumulate with age (White et al., 2008), while in other animals, the proportion of defective mtDNA increases in some tissues and decreases in others. This segregation of the defective mtDNA could be under the control of nuclear genes (Battersby et al., 2003, and references included). Since most of the studied heteroplasmic mutants were *a priori* neutral, the role of selection has been neglected until recently (Chinnery et al., 2000). However, in heteroplasmic mice carrying a deleterious mutation, the defective mtDNA was shown to accumulate in most of the tissues except the female germline, where intracellular selection progressively eliminated the defective mtDNA (Sato et al., 2007). Selection between mtDNA has also been demonstrated in the genus *Drosophila* using heteroplasmic individuals generated by paternal leakage in interspecific crosses. Selection favors the mtDNA with the best compatibility with the nuclear genetic background (see the review of Korpelainen, 2004). On the contrary, in the mite *Tetranychus urticae*, Van Leeuwen et al. (2008) have shown that

there was no intra-individual selection between the different mtDNA in heteroplasmic individuals though the mutation, coding for insecticide resistance, was positively selected at the individual level (advantage of resistant individuals).

In plants, heteroplasmy would be likely to vary among tissues but this variation has been demonstrated only once, in *Senecio vulgaris*, in heteroplasmic individuals from natural populations carrying a mutation coding for resistance to triazine (Frey et al., 2005). The role of drift and selection in this variation is unknown. However, experiments carried out in protoplast-derived cells explored the cellular mechanisms in more details. For instance, Albert et al. (2003) have demonstrated in *Nicotiana* that selection differs for the different mtDNA and that the nuclear genome plays a role in the regulation of their replication.

4.4.3.2 Transmission of heteroplasmy over generations

Studies on the transmission of heteroplasmy over generations are more univocal. In cow, mouse and human, it has been shown that heteroplasmic mothers for *a priori* neutral mutations produced offspring with varied levels of mtDNA heteroplasmy, from the fixation to the loss of the mutant (reviewed in Chinnery et al., 2000; Kmiec et al., 2006). This variation could result from drift due to the bottleneck in the population of mitochondria in the female germline. Such a bottleneck has been demonstrated in mammals even if its timing is still debated (Cao et al., 2007; Cree et al., 2008). It could result from a reduction of the quantity of mtDNA per cell but also from the compartmentalization of mtDNA molecules into nucleoids which could reduce the number of segregation units, or from a preferential replication of some mitochondria (for instance, mitochondria close to the nucleus replicate faster, see Davis and Clayton, 1996). The average heteroplasmy level in progenies being often close to the heteroplasmy level of the mother, selection might be less important than drift (Chinnery et al., 2000; Jenuth et al., 1996). Similar results have been found in the mite *Tetranychus urticae* in which heteroplasmic individuals transmit their resistant and susceptible haplotypes to progeny in highly variable ratios but on average equivalent to the ratio in the mother. Van Leeuwen et al. (2008) estimated a sampling bottleneck of approximately 180 copies.

Heteroplasmy is expected to be less efficiently transmitted in plants than in animals because soma and germline separate later in plant development, possibly allowing a complete segregation of organelles before the differentiation of the germinal meristems. This expectation has been confirmed in *Medicago truncatula* and cultivars of *Pelargonium*, where the segregation of chloroplasts was complete within a generation (Matsushima et al., 2008; Tilney-Bassett and Birky, 1981). Tilney-Bassett and Birky (1981) suggested a major role of drift in the first divisions of the zygote and in particular during the separation of the basal cell (future suspensor). On the contrary, in *Senecio vulgaris*, Frey (1999) has observed that heteroplasmy was maintained from one generation to the next even when the plants were not treated with triazine (this maintenance is surprising because the resistant mutation

is costly and should be quickly eliminated). Intermediate transmission has also been observed in a variegated mutant of cotton: Lax et al. (1987) have shown that heteroplasmy was transmitted from mother to offspring but was unlikely to persist since inter-generational variation in the proportion of two types of chloroplast was large. In addition, the role of sexual reproduction in the segregation of heteroplasmy has been shown in two studies, one on mitochondrial heteroplasmy in olive (*Olea europaea*) and the other on chloroplast heteroplasmy in kiwifruit (*Actinia deliciosa*). These two studies found that asexual offspring from cuttings presented higher levels of heteroplasmy than sexual offspring from seeds from the same parent tree (Chat et al., 2002; Garcia-Diaz et al., 2003). Again, a bottleneck in the female germline was suggested to explain these results.

Finally, there is a special case of heteroplasmy in plants that deserves attention. The mitochondrial genome of plants is often described as multipartite with a master DNA molecule and many smaller circular molecules, called subgenomic molecules that result from reversible recombination events. In addition, there are also short circles of DNA, called sublimons, which derived from irreversible recombination events of the master molecule (Kmiec et al., 2006). In general, sublimons are present in very low copy number (approximately 1 copy for 100 cells) but can reach a high number of copies by abrupt changes in the proportions of genomic molecules. These changes appear to be controlled by nuclear genes which may prevent or enhance the replication of some of the molecules (Shedge et al., 2007; Zaegel et al., 2006). In *Brassica napus* and *Phaseolus vulgaris*, it has been shown that sublimons contain male sterility genes (CMS) and that abrupt changes in their frequencies were responsible for sexual phenotype reversions (Bellaoui et al., 1998; Janska et al., 1998).

4.5 Gynodioecious species and mitochondrial inheritance

Gynodioecious species have some particular features concerning mitochondrial inheritance (McCauley and Olson, 2008). First, male sterility is a phenotypic marker and the gynomonoecious phenotype has opened doors for studies on mitochondrial heteroplasmy in gynodioecious species (Andersson, 1999; Erickson and Kemble, 1993), just as variegated plants have drawn the attention to unusual chloroplast inheritance. Gynomonoecy could thus be a consequence of heteroplasmy. On the other hand, mitochondrial inheritance is involved in the transmission of the sex phenotype in gynodioecious species and can influence the stability of gynodioecy. This aspect will be treated in the last part of this chapter (see p.123). Third, occasional biparental inheritance, by enhancing efficient recombination, could favor the creation of new CMS genes [4] and can explain the high genetic variability found in the mitochondrial genomes of several gynodioecious species (Touzet and Delph, 2009).

[4] CMS genes are chimeric genes that result from rearrangements in the mitochondrial genome by recombination.

Finally, all experimental studies on the genetic determination of gynodioecy assumed that mitochondrial inheritance is maternal. If it were not the case, these data must be reinterpreted.

4.5.1 Experimental support for paternal leakage of mitochondria in gynodioecious species

NB: In the French version, I described here evidence of recombination in the mitochondrial genome in gynodioecious plants and the studies carried out since 2005 on paternal leakage and heteroplasmy (Bentley et al., 2010; McCauley et al., 2005, 2007; Pearl et al., 2009; Welch et al., 2006).

Since almost all studies on heteroplasmy and paternal leakage have been performed in *Silene vulgaris*, we wondered whether the high paternal leakage rates that have been observed reflects a very specific phenomenon or can be more general in gynodioecious species. Therefore, we tested the mitochondrial inheritance in *Silene nutans* and preliminary results are presented in the following article.

ARTICLE 4

Mitochondrial inheritance in *Silene nutans*

(ongoing work)

Introduction

In angiosperms, the mitochondrial genome is generally thought to be maternally inherited but the occurrence of non-maternal inheritance and heteroplasmy and their evolutionary consequences have been reconsidered recently (see Barr et al., 2005; Kmiec et al., 2006; Korpelainen, 2004; White et al., 2008; Xu, 2005 and section p.101 for further details).

Inheritance of the mitochondrial genome is of particular importance in gynodioecious species for several reasons (McCauley and Olson, 2008). First, mitochondrial genes are implicated in sex determination and their transmission should influence the evolution of gynodioecy. More precisely, paternal transmission of mitochondria is expected to reduce the advantage of the male-sterile cytoplasm (see Wade and McCauley, 2005 and section p.123). Second, heteroplasmy and recombination could enhance the emergence of new CMS genes, that result from recombination, and could explain why gynodioecious species present a higher mitochondrial diversity than closely related non-gynodioecious species (Touzet and Delph, 2009). Third, lots of crossing studies have assumed maternal inheritance and paternal leakage can provide a new framework for interpreting empirical results. Lastly, heteroplasmy could be implicated in gynomonoecy, the co-occurrence of pistillate and perfect flowers on the same plant (Andersson, 1999). Therefore a good knowledge of the transmission of the mitochondrial genome is needed to understand the evolutionary dynamics of gynodioecy (McCauley and Olson, 2008).

Recently, evidence for paternal leakage and heteroplasmy has been found in the genus *Silene*. Particularly in *Silene vulgaris* paternal transmission of mitochondria has been clearly demonstrated (Bentley et al., 2010; McCauley et al., 2005; Pearl et al., 2009; Welch et al., 2006) and because this is a gynodioecious species, this poses important questions about the role of mitochondrial transmission, recombination and heteroplasmy in the evolution and maintenance of this plant reproductive system. Furthermore, evidence of mitochondrial recombination, which can only occur if there is heteroplasmy, has been found in *Silene acaulis* and *Silene nutans* (Städler and Delph, 2002; Touzet and Delph, 2009), making these species logical models for testing for the occurrence of non-maternal mitochondrial transmission. Here we characterise the mitochondrial genotypes of offspring from a series of crosses between parents of *Silene nutans* that differ in mitochondrial markers to investigate the occurrence and frequency of paternal mitochondrial transmission.

Material and methods

In order to determine the transmission of the mitochondrial genome in *Silene nutans*, we genotyped offspring from crosses between parents of different *cox1* (cytochrome oxidase) haplotypes in a crossing procedure described in Article 1 (p.36). If the inheritance of the mitochondrial genome is maternal and if the mother carries a unique mitochondrial genome, mother and offspring should carry the same haplotype, excluding mutation. Moreover, we were able to distinguish paternal inheritance

from mutation because parents differed by at least 3 sites (all information about the sequences we used is available in the Supplementary Table 1 of ARTICLE 1, p.51).

Paternal leakage is expected to be a rare event and was shown to be highly cross-dependent or population-dependant when detected (Bentley et al., 2010; McCauley et al., 2005; Welch et al., 2006). For this reason, we used a large sample (n=1113) of offspring from 36 different crosses. Assuming that paternal leakage occurs at a rate of 4%, which is consistent with most of studies on paternal leakage in *Silene vulgaris* (Bentley et al., 2010; McCauley et al., 2005; Pearl et al., 2009; Welch et al., 2006), our sample size allowed us to detect with a 95% probability paternal leakage rates higher than 0.3% (Milligan, 1992). However, if paternal leakage occurs only in some crosses, we should considered the probability of detection independently for each cross and paternal leakage rate would need to be higher than 8.5% to be detected with a 95% probability (cross sample size is approximately n=35). The crossing design and the number of offspring obtained for each cross are summarized in TABLE 4.1. Detailed description of the crossing protocol and growing conditions are available in ARTICLE 1. Offspring were named by a first number identifying the cross (see TABLE 4.1) and a progeny number within crosses.

We collected one leaf per plant on young rosettes of approximately 7 cm diameter and stored them in Silica gel (desiccant). Because mitochondrial heteroplasmy has been suggested to be linked with gynomonoecy (Andersson, 1999), we also collected leaves from different spikes of 16 gynomonoecious plants during the first flowering season (44 samples). For each sample (n=1113+44=1157), total genomic DNA was extracted from a leaf piece using Chelex© (Biorad). Samples were frozen at -80°C for at least two hours and then immediately mixed with 100μl of a boiling 5% Chelex© (Biorad) suspension. Samples were vortexed and incubated at 96°C for 10 min, twice. Then samples were put on ice and spun down for 10 min at 6000rpm. 50μl of supernatant were taken and stored at -20°C. The *cox1* gene was amplified by polymerase chain reactions (PCR) on the genomic DNA using the primers described in TABLE 4.2. PCR was performed in a total volume of 15μl containing 1XPCR buffer with 2mM MgCl2, 0.2mM of each dNTP, 0.2μM of reverse and forward primers, 0.2mg/mL BSA, 0.375U Taq polymerase (Biolabs) and 6ng of template DNA. PCR conditions were 5 min at 95°C; 30 cycles of 40s at 95°C, 45s at 58°C and 60s at 72°C; followed by 10min at 72°C.

Samples were genotyped by the CE-SSCP technique – Single Strand Conformation Polymophism on Applied Biosystems Capillary Electrophoresis Systems – on a ABI3100 DNA Sequencer. Denaturation and CE-SSCP fractionation were as described in the ABI3100Avant Reference Guide (Applied Biosystems). The labelled fragments were visualized on an Applied Biosystems DNA analyzer (see Figure 4.1). The GeneScan^{TM}-500 LIZ size standard used in all samples served as an internal ladder to align data from different capillaries and eliminate capillary-to-capillary variability. Parents were included as samples in all runs in order to eliminate run-to-run variability. Samples that were not assigned to the mother haplotype (outliers or assignation to another haplotype) or were not successfully visualized were genotyped

Mother/Father	AU4.4	Q1.2	Q9.8	AMB 9
AU4.4		1 (n=38)	2 (n=38)	3 (n=43)
Q1.2	4 (n=11)		5 (n=36)	6 (n=35)
Q9.8	7 (n=31)	8 (n=39)		9 (n=38)
AMB 9	10 (n=0)	11 (n=0)	12 (n=39)	

Mother/Father	AU3.16	Q1.1	Q9.5	AMB 13
AU3.16		13 (n=5)	14 (n=33)	15(n=38)
Q1.1	16 (n=22)		17 (n=31)	18 (n=35)
Q9.5	19 (n=29)	20(n=0)		21 (n=38)
AMB 13	22 (n=38)	23 (n=39)	24 (n=37)	

Mother/Father	AU3.22	Q1.7	Q9.11	AMB 5
AU3.22		25 (n=36)	26 (n=38)	27 (n=38)
Q1.7	28 (n=37)		29 (n=38)	30 (n=38)
Q9.11	31 (n=5)	32 (n=35)		33 (n=38)
AMB 5	34 (n=45)	35 (n=37)	36 (n=35)	

Table 4.1: Crossing design. Each of the three replicates include twelve reciprocal crosses between four parents carrying four different haplotypes: AU, Q1, Q9 and AMB (self-replications were not performed). Parents are named by their population of origin (Auvergne AU, Queyras Q, and Ambleteuse AMB), 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. Crosses were numbered and the number of offspring we genotyped for each cross is indicated. Three crosses failed to produce fruits.

	primer sequence 5' to 3'	fluorescent dyes
cox1-sscp-F (763)	GTGGTTGCACATTTCCATTATGTA	HEX
cox1-sscp-R (763)	TGCATCGGGAAGAAGGTAAG	6-FAM

Table 4.2: Primers for *cox1* amplification by PCR.

again.

Samples that were not assigned to the maternal haplotype by SSCP-genotyping were sequenced at the *cox1* locus. *Cox1* was amplified with two pairs of primers, cox1F1/cox1R663 and cox1F473/cox1R1077 (described in Touzet and Delph, 2009), generating overlapping fragments. PCR conditions were similar to those used for SSCP-genotyping except that the total volume was 25μL and TM was 53°C. PCR products were purified using a NucleoFast 96 PCR Kit (MACHEREY-NAGEL) and directly sequenced on both strands, using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI3130Avant DNA Sequencer (Applied Biosystems).

Results

Of the 1157 samples, 111 were not assigned to the mother's haplotype (outlier or misassignment) by the first SSCP-genotyping run, and among them, 21 were misassigned again by the second run. For both runs, the samples that were not assigned to the mother's haplotype were mostly outliers, not corresponding to any of the parental haplotypes, but a few plants were assigned to an existing parental haplotype that was not the mother's. The first individual, 31#1, had a Q9-mother and a AU-father and was assigned to the Q1 haplotype by the two SSCP-genotyping runs. Because of this unexpected result, a new DNA-extraction was performed on the leaf sample, and another leaf was collected on the plant at the end of the first flowering season. These two additional samples were SSCP-genotyped and assigned to the Q9 maternal haplotype. The second individual, 27#24, was assigned to the Q1 haplotype instead of the AU haplotype, expected under maternal inheritance. This plant was phenotyped as gynomonoecious during the first flowering year, and three additional leaves from three spikes were taken and genotyped. One of them (27#24(1)) was assigned to the AU haplotype, another (27#24(3)) to the Q1 haplotype and the last one (27#24(2)) was a null. On the second SSCP-genotyping run, Q1 and AU haplotypes were not clearly distinguishable and we were able to analyze none of the four samples of 27#24.

We decided to sequence at the *cox1* locus all 21 individuals that had not been assigned to their mother's haplotype on the second SSCP-genotyping run, as well as the four 27#24 samples and the second leaf sample from 31#1. Among the 20 samples that were successfully sequenced, four samples carried a haplotype that differed from the mother's (see TABLE 4.3). Paternal transmission of the mitochondrial genome was shown on one offspring from cross number 21 (21#19) that carried the paternal haplotype AMB. Consistent with what we found by SSCP-genotyping, the first DNA extraction of 31#1 carried the Q1 haplotype and the other leaf carried the Q9 haplotype. Finally, the 27#24 individual gave surprising results. 27#24(1) was shown to carry the maternal haplotype AU but 27#24 and 27#24(3) carried new haplotypes that could have resulted from recombination between the AU and the Q1 haplotypes or mutation. 24#24(2) was not successfully sequenced.

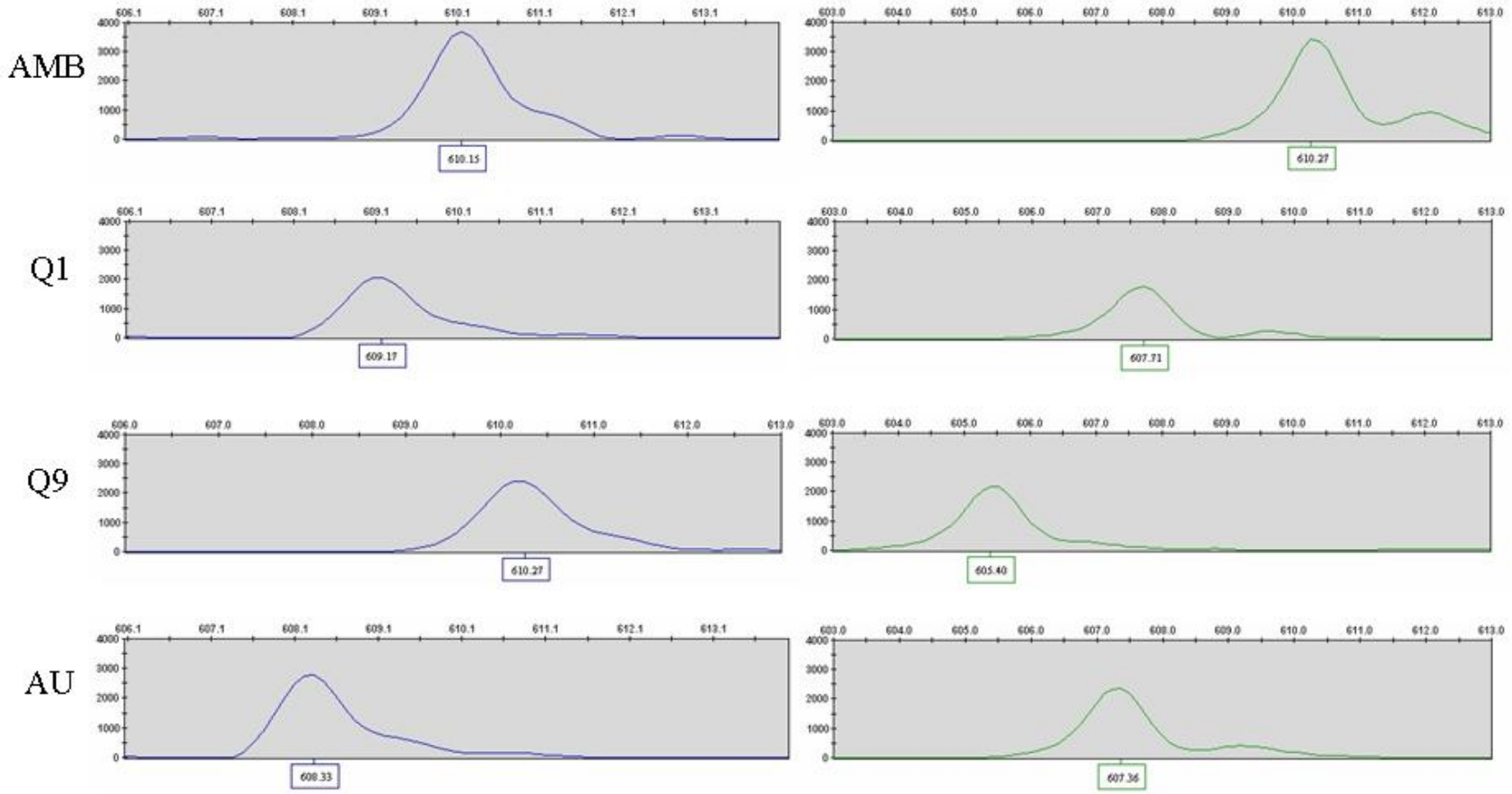

Figure 4.1: Examples of chromatograms obtained from SSCP. Both fluorescent dyes (blue and green) are needed to distinguish the four parental haplotypes.

Haplotype	199	751	763	775	940	954	957	1000	Samples
Q1	C	A	G	G	C	C	C	G	5#23, 6#1, 6#5, **31#1a**
AMB	A	.	T	.	T	G	G	.	34#28, 34#29, 34#45, 36#2, **21#19**
Q9	A	G	T	.	.	G	.	.	31#1b, 7#13
AU	A	.	.	T	.	G	.	C	1#3, 2#3, 2#8, 3#30, 15#17, 15#22, 27#24(1)
27#24	.	.	.	T	.	.	.	.	
27#24(3)	.	.	.	.	.	G	.	.	

Table 4.3: *Cox1* sequences of samples that were misassigned by SSCP genotyping. 31#1a represent the first DNA extraction performed on one leaf and 31#1b a DNA-extraction performed on a second leaf. 27#24 represent the leaf sampled at the rosette stage and 27#24(1), 27#24(2) and 27#24(3) the leaves sampled on different flowering spikes. We failed to sequence 6 samples (5#6, 5#10, 21#15, 27#12, 27#24(2), 36#22).

Discussion & Future work

We detected only very few cases of non-maternal inheritance. First, 21#19 was shown to carry the paternal haplotype AMB (instead of the maternal Q9 haplotype). Second, 31#1 was shown to carry different sequences on different leaves and different SSCP genotypes for different DNA-extractions from the same leaf, and could therefore be heteroplasmic. Third, a complex case of heteroplasmy was detected on a gynomonoecious plant, 27#24, which carried a mix of the maternal haplotype and two new, original haplotypes. These new haplotypes are likely to result from recombination between the maternal haplotype AU and the Q1 haplotype although mutation cannot be ruled out.

We showed in a previous study (Article 1) that the AU haplotype and the three other haplotypes represent two distinct CMS. We can thus expect than, in some crosses, non-maternal inheritance would lead to a different sex phenotype than what is expected under maternal inheritance and we looked for such phenotype abnormalities in the three cases of non-maternal inheritance. Unfortunately, for 21#19 and 31#1, the detected haplotype and the maternal haplotype did not carry distinct CMS and we were not able to detect sex phenotype modification due to non-maternal inheritance. However, the individual 27#24 carried a mixture of the AU haplotype and recombinants between the AU and the Q1 haplotypes, that were shown to be two distinct CMS with very different level of restoration (AU was poorly restored and Q1 well restored, see Garraud et al., 2011, in press). Therefore, we could expect that, in the 27#24 individual, heteroplasmy would result in gynomonoecy, and that spikes containing mitochondria with different sequences would vary in sex phenotype. Spikes were roughly similar in their proportion of pistillate flowers. However, 27#24

was mainly hermaphrodite (femaleness: 0.09) whereas females were largely predominant within the 27th family, which can reflect the presence of the well restored CMS corresponding to the Q1 haplotype. We observed among the 32 offspring belonging to the 27th family three other mainly hermaphrodite gynomonoecious plants and one hermaphrodite and we will examined in the future if they could not reflect other cases of non-maternal inheritance. Indeed, two of them were outliers in at least one SSCP-genotyping run and one of these two was 27#12 that we planned but failed to sequence.

This work is still in progress and the cases of non-maternal inheritance that we detected need to be check by further samples on plants (if these are still alive) or at least by new DNA extractions. In particular, the result obtained for 31#1 should be reexamined carefully since it could be explained by contamination of the first DNA-extraction. On the other hand, these preliminary analyzes could have missed some cases on non-maternal inheritance. Indeed, the second run of SSCP-genotyping, performed on individuals that were not clearly assigned to the maternal haplotype by the first run, suffered from some technical problems, probably linked to PCR-product age. Analyzes were still possible but the AU and Q1 haplotypes were not clearly distinguishable, which could have lead us to miss some cases on non-maternal inheritance. Therefore, SSCP-genotyping will be repeated on these individuals and sequencing will be performed on potential new outliers as well as on the 6 individuals that we failed to sequence the first time.

Our second perspective is to evaluate if our SSCP-genotyping method can detect heteroplasmy. Indeed, in this study, only the most frequent fragment was recorded by the DNA analyzer (higher fluorescent peak). However, several fluorescent peaks can sometimes be visualized and could represent different haplotypes coexisting within individuals. In order to known how to interpret multiple peaks, we mixed DNA from different parents (12 different mixes), and performed the PCR and SSCP-genotyping as usual. For some mix, we were able to visualized double peaks that clearly reflect heteroplasmy. For others, only one haplotype was detected which suggest that either the stoichiometry was under the detection threshold or one haplotype was preferentially amplified during the PCR. We plan to determine the detection threshold by measuring the concentrations of the parents DNA-solutions (NanoDrop^{TM}) and mixing parents DNA in controlled stoichiometry (e.g. 1:1, 1:4 and 1:10). Mix of DNA would be performed before and after PCR to disentangle the detection threshold of SSCP-genotyping and possible PCR bias.

References

See References at the end of the Thesis p.178.

4.5.2 Theoretical studies on mitochondrial inheritance in gynodioecious species

During the presentation of the cytonuclear conflict (see p.18), we showed that cytoplasmic male sterility can select for a diverse array of nuclear responses, including restorers of fertility and masculinization of hermaphrodites. This thesis ends up with a quite intuitive possible response of the nucleus to cytoplasmic male sterility, a response to the very thing from which it springs: uniparental inheritance of mitochondria. In a population containing cytoplasmic male sterility genes, the transmission of mitochondria via the pollen benefits male fertility because mitochondria in pollen are less likely to carry CMS genes than mitochondria in ovules. The nuclear genes may therefore be selected to promote biparental or even paternal inheritance of mitochondria as a way of reducing the transmission of the selfish CMS genes (Burt and Trivers, 2006). In term of genetic conflict, this would reduce the cytonuclear conflict (inheritance laws of nuclear and mitochondrial genomes are closer) but would increase the risk of invasion by other selfish mitochondrial elements (see FIGURE 4.2). Since support for the existence of this expected nuclear response has been found only very recently, theoretical studies on paternal leakage and gynodioecy are almost non-existent to date. Wade and McCauley (2005) explored the influence of paternal leakage on the maintenance of gynodioecy under several sex determination systems and showed that paternal leakage can contribute to the maintenance of the cytonuclear polymorphism. Because the evolution of paternal leakage itself was not considered in their model, I developed another model to explore whether cytoplasmic male sterility can enhance the evolution of paternal leakage of mitochondria (see ARTICLE 5).

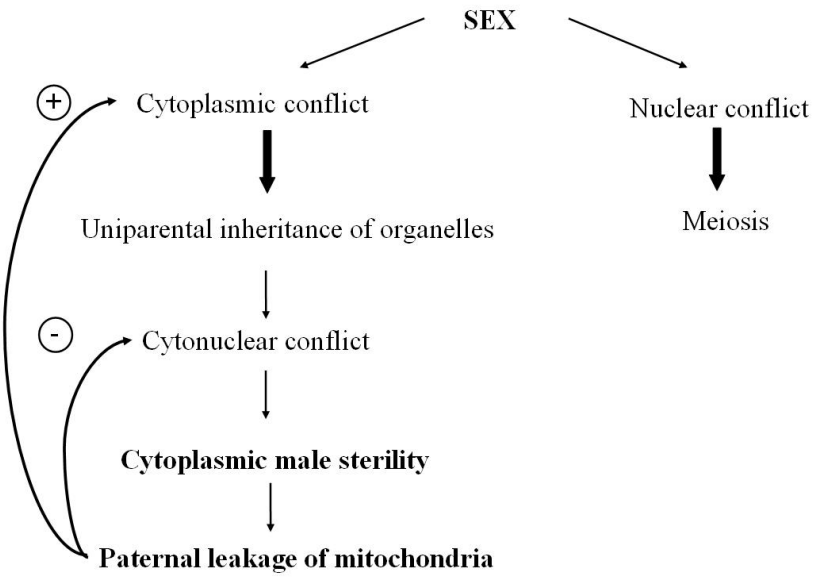

Figure 4.2: Intra-genomic conflicts and evolution of paternal leakage of mitochondria in gynodioecious species.

Article 5

Does cytoplasmic male sterility (CMS) enhance the evolution of occasional transmission of mitochondria through pollen? A theoretical approach.

Claire Garraud and Jacqui A. Shykoff

(submitted to American Naturalist)

Abstract

Gynodioecy, the coexistence of female and hermaphrodite plants within a species, is generally under cytonuclear sex determination, involving cytoplasmic male sterility (CMS) and nuclear restorers. Many models assuming strict maternal inheritance of mitochondrial genes explain the maintenance of gynodioecy and of the underlying polymorphism. However, occasional transmission of mitochondria through pollen, paternal leakage, has been observed recently in some gynodioecious species. A previous theoretical study showed that a fixed rate of paternal leakage can sustain polymorphism at the CMS locus but the evolution of paternal leakage itself was not considered. We tested whether CMS can select for paternal leakage of mitochondria. We modeled the evolution of a mutant that allows paternal leakage under pure cytoplasmic sex determination. We showed that (i) a population containing a CMS gene is susceptible to invasion by a nuclear gene allowing paternal transmission of mitochondria; (ii) paternal leakage maintains male-fertile mitochondria by allowing their transmission via pollen. The results were robust for four different underlying systems of inheritance we tested. We discuss our results in terms of genetic conflict. We compare the way by which paternal leakage and restorer genes are selected and propose analogies with other sterilizing cytoplasmic elements, such as *Wolbachia* bacteria.

Introduction

Genetic conflicts, an important evolutionary force, are implicated in a large number of fundamental evolutionary processes including the evolution of sex, sex determination, reproductive isolation, species extinction, genome structure, recombination, and uniparental inheritance of organelles (Hurst and Werren, 2001; Hurst, 1992; Hurst et al., 1996). Genetic conflict is caused by divergent interests between genes, and occurs when "the spread of one gene creates the context for the spread of another gene, expressed in the same individual, and having the opposite effect" (Hurst et al., 1996). Genes that are not genetically linked are more susceptible to come into conflict and genes with different inheritance systems are particularly favourable to the emergence of conflicts. In eukaryotes, nuclear genes are inherited in a Mendelian way, following the segregation rules of mitosis and meiosis. These two stringent mechanisms, by constraining the transmission of the genome, reconcile the interests of all nuclear genes and limit conflicts among those genes. On the contrary, there is no control for the segregation of organelles during mitosis and meiosis and the multiple copies of cytoplasmic DNA of each cell replicate independently of the cellular cycle. Cytoplasmic genes are thus submitted to drift and selection, both within and among individuals. If selection acts in opposite ways on the different levels of selection, which can be viewed as sets of genes sharing the same interest, selfish genes that follow their own interest at the expense of others genes can evolve. For instance, any cytoplasmic mutant with an increased transmission rate (increased replication, segregation advantage, cytoplasm killer at syngamy, etc.) would be selected at the organelle level, even if it were costly for the host cell (for a example of mitochondrial selfish element see MacAlpine et al., 2001).

Inheritance of cytoplasmic genes is constrained, however, by uniparental inheritance of organelles, maternal inheritance being the most common case. Uniparental inheritance of cytoplasmic genes is very widespread and applies to most anisogamous species including animals, plants, fungi (Barr et al., 2005; Korpelainen, 2004; Xu, 2005) and algae (Adams et al., 1990; Kuroiwa et al., 1991; Miyamura and Nagumo, 2007). However uniparental inheritance cannot be considered merely as a by-product of anisogamy. Indeed, numerous examples of uniparental inheritance in isogamous species are known, for example in fungi (Barroso and Labarere, 1995; Nishimura et al., 2002; Yan and Xu, 2003), algae (Hamaji et al., 2008; Kuroiwa et al., 1985), and protists (Moriyama and Kawano, 2003), and active mechanisms by which the mtDNA of the non-transmitting sex is eliminated have been demonstrated (Moriyama and Kawano, 2003; Nishimura et al., 2002). Furthermore some anisogamous species show paternal inheritance of organelles (*e.g.* gymnosperms, see Reboud and Zeyl, 1994). Models have been developed to explain why uniparental inheritance has evolved and why it is so widespread. The most widely accepted suggests that uniparental inheritance was driven by genomic conflicts. Under this hypothesis, nuclear genes enforcing uniparental inheritance evolved in order to prevent the spread of selfish cytoplasmic elements (Hastings, 1992; Hurst and Hamilton, 1992; Randerson and Hurst, 1999).

Other models have been proposed, such as the specialization of organelle transmission in the germ-line of one of the two pre-existing sexes or mating types (Godelle and Reboud, 1995), or the STOR model (Salvage-TurnOver-Repair) in which the degradation of a part of the DNA is advantageous by providing nutriments for the development of the zygote (Sears and VanWinkle-Swift, 1994). However, these models are unlikely to apply for both isogamous and anisogamous species.

While uniparental inheritance of cytoplasmic genes solves the conflict between cytoplasmic genes, it creates a new and different conflict between cytoplasmic and nuclear genes. Production of male offspring is an evolutionary dead-end for mitochondrial genes that are maternally inherited. Mitochondrial genes therefore would profit from a sex-ratio biased toward females whereas a 1:1 sex-ratio profits nuclear genes. The best known illustration of the nucleo-cytoplasmic conflict is cytoplasmic male sterility (CMS) in plants (Cosmides and Tooby, 1981). Cytoplasmic male sterility (CMS) genes are present in the mitochondrial genome and cause male sterility (*i.e.* generate females by suppressing male function of hermaphrodites). Because of its maternal inheritance, a CMS gene that allows females to produce more seeds than hermaphrodites will invade the population (Lewis, 1941). As CMS invades the population, any nuclear mutation that restores male fertility will be associated with the rare gamete type (*i.e.* pollen) and selected by Fisherian selection (Jacobs and Wade, 2003). This conflict results in gynodioecy, the coexistence of hermaphrodite and female plants in natural populations, which is the most common mating system after hermaphroditism in angiosperms.

Recently, strict maternal inheritance of cytoplasmic organelles has been questioned in a few gynodioecious species. Signatures of recombination in the mitochondrial genome have been observed in several species of *Silene* (Houliston and Olson, 2006; McCauley et al., 2005; McCauley and Ellis, 2008; Städler and Delph, 2002; Touzet and Delph, 2009), indicating that heteroplasmy (*i.e.* the coexistence of different cytoplasmic genomes within an individual) must have occured at the evolutionary scale. Phylogenetic patterns of mitochondrial and chloroplastic genes were found to be incongruent in *Silene vulgaris*, suggesting exceptions in the strict maternal inheritance of these two genomes (Houliston and Olson, 2006). Gynomonoecious individuals, a phenotype described in many gynodioecious species, that present both pistillate and perfect flowers within a plant, provide additional evidence for heteroplasmy. Using controlled crosses, Andersson (1999) showed that pistillate and perfect flowers of gynomonoecious plants crossed with pollen from the same non-related individual produced full-siblings of the three sexes (females, hermaphrodites and gynomonoecious) but in different proportions. Since nuclear genes are transmitted to ovules in the same way by a pistillate or a perfect flower, this suggests that the two flower sexes of gynomonoecious plants bore different, more or less male-fertile and male-sterile cytoplasms. A link between heteroplasmy and gynomonoecy has also been suggested by heteroplasmic hydrids obtained by crossing experiments on laboratory lines of rapeseed (Erickson and Kemble, 1993). Some heteroplasmic hybrids were shown to exhibit both perfect and pistillate flowers and those pistillate (re-

spectively perfect) flowers produced male-sterile offspring (respectively male-fertile), suggesting that only local mitochondria determine the local phenotype. However, the association between heteroplasmy and gynomonoecy was not perfect and the sample size limited. More recently, genetic marker studies revealed transmission of mitochondrial genes via the pollen, a process called paternal leakage. In *Silene vulgaris*, 2.5 to 14% of offspring presented a haplotype that differed from the mother's (Bentley et al., 2010; McCauley et al., 2005; Welch et al., 2006) and 15% to 23% were shown to be heteroplasmic using q-PCR (Pearl et al., 2009; Welch et al., 2006) or a method that amplified low-copy number haplotypes (McCauley et al., 2005). Pearl et al. (2009) demonstrated that heteroplasmy in S. vulgaris was mainly due to paternal leakage, other cases being explained by the transmission of heteroplasmy from a heteroplasmic mother. When leakage did occur, the paternal contribution ranged from 0.5% in some offspring (minimal threshold for detection) to 100% in others, with an average of 12 to 28% (Bentley et al., 2010; Pearl et al., 2009). The distribution was bimodal with many occurrences of very low leakage or complete paternal inheritance.

Since paternal leakage and heteroplasmy in the mitochondrial genome has been described only very recently, their treatment in theoretical studies is almost nonexistent to date. However, Wade and McCauley (2005) studied the influence of paternal leakage on a gynodioecious system, assuming a low percentage of paternal transmission and no heteroplasmy. They showed that paternal leakage can prevent the invasion of CMS and sustain a stable mitochondrial polymorphism when restorers are not present. With some mechanisms of nuclear restoration, paternal leakage also enhances the stability of joint cytonuclear polymorphism by reducing oscillations to extreme frequencies. Although these results indicate the influence of paternal leakage on CMS and restorer genes, they do not explore the evolution of paternal leakage itself, since the rate of leakage was a constant parameter of each simulation and had no genetic basis. It has already been proposed that paternal leakage could be favoured in populations with CMS as a way of reducing the transmission of the selfish male-sterile cytoplasm (Burt and Trivers, 2006; McCauley and Bailey, 2009; McCauley and Olson, 2008) but no theoretical framework has been proposed so far.

In this paper, we explore whether cytoplasmic male sterility can enhance the evolution of paternal leakage of mitochondria. Because our model assumes that mitochondrial inheritance is under genetic control and can generate heteroplasmy when paternal leakage occurs, it requires a wealth of information on the mechanisms and the genetics of organelle inheritance that are briefly summarized here. In plants, uniparental transmission of organelles is ensured by one or several mechanisms including physical exclusion by controlled segregation, active degradation or differential replication, and can occur from gametogenesis to the first stages of post-zygotic development (Mogensen 1996; Zhang et al. 2003; Xu 2005). Most assumptions concerning heteroplasmy in this model were inspired by data on fungi, in which biparental inheritance is common and more data are available (Birky 2001; Barr et al. 2005). In

yeast and some basidiomycetes with biparental inheritance of mitochondria, fusion of gametes results in heteroplasmic individuals but mitochondria segregate rapidly, giving rise to homoplasmic individuals in yeast (Birky 2001), and heteroplasmic mycelia composed of homoplasmic cells in multicellular fungi (Barr et al. 2005; Xu 2005). In plants, simple mathematical models showed that, when heteroplasmy occurs, vegetative sorting of organelles should be complete within one plant generation (Birky 2001). But experimental studies are rare and results are controversial: Some showed a complete sorting in one generation that occurred during development (Erickson and Kemble 1993; Matsushima et al. 2008) or from a bottleneck in the germ line (Chat et al. 2002) but heteroplasmy could also be maintained, by drift or selection, and transmitted to offspring (Lax et al. 1987; Frey 1999). In *Silene vulgaris*, heteroplasmic mothers produced on average 17% of heteroplasmic offspring, showing that vegetative sorting occurred but was not always complete in one generation (Pearl et al. 2009; Bentley et al. 2010). When transmission occurred, heteroplasmy level tended to decrease from one generation to the next.

The genetic determination of mitochondrial inheritance remains poorly understood, and the underlying genes have been identified for only a few species, such as *Chlamydomonas reinhardtii* (Nishimura et al., 2002) or *Crytococcus neoformans* (Moriyama and Kawano, 2003). In Angiosperms, few data are available and concern mainly chloroplasts, whose inheritance is less strict. The historical case of variegated plants (Baur, 1909) that are heteroplasmic with green and white plastids, remains one of the best studied cases of heteroplasmy in plants. For instance, in *Pelargonium*, chloroplast inheritance depends on the plastid genome (Tilney-Bassett and Birky, 1981), the maternal nuclear genome (Tilney-Bassett and Birky, 1981), or the paternal nuclear genome, acting in a gametophytic way (inheritance depends only on the pollen genome and not on the diploid parent genome, see Derepas and Dulieu, 1992; Dulieu et al., 1990). Information is rarer on mitochondrial inheritance, probably because of the lack of phenotypic markers compared to chloroplasts. However, the degree of paternal leakage is highly cross-dependent in *Silene vulgaris* (McCauley et al., 2005; Welch et al., 2006), and the population from which the pollen donor was collected had an effect on the leakage rate whereas the origin of the ovule donor had no effect (Bentley et al., 2010). In *Brassica napus*, both paternal and maternal genomes were involved in the control of mitochondrial inheritance, suggesting a mechanism operating in both pollen and ovule (Erickson and Kemble, 1993). The compatibility between the plastid and the nuclear genomes of the progeny also modified the transmission of paternal plastid (Chiu and Sears, 1993). More generally, nuclear genes that control the replication of sublimons and other small fragments of mtDNA might influence the transmission and expression of mtDNA (Battersby et al., 2003; Kmiec et al., 2006; Mackenzie and McIntosh, 1999; Woodson and Chory, 2008; Zaegel et al., 2006). Overall, data on the genetics of organelle inheritance remain scarce and are sometimes contradictory, and for this reason we tested several systems of genetic control for the inheritance of mitochondria in our model.

The model

We considered a diploid out-crossed species with non-overlapping generations. The population is infinite, panmictic and without pollen limitation. We modeled the evolution of a gene that controls the inheritance of mitochondria in a gynodioecious population with pure cytoplasmic sex determination. Plants that carry the male-sterile allele (C1) at the CMS locus of their mitochondrial genome are females, and those that carry the male-fertile allele (C0) are hermaphrodites. Females benefit from a female advantage (s) that increases their ovule production relative to hermaphrodites from 1 to $1+s$. We also defined a biallelic B locus that controls mitochondrial inheritance, and that will be either mitochondrial or nuclear depending on the scenario tested. The maternal inheritance allele (b) codes for strict maternal inheritance whereas the paternal leakage allele (B) allows transmission of the mitochondrial genome through pollen. When paternal leakage, *i.e.*, biparental inheritance, occurs, the proportion of mitochondria from paternal origin in the zygote is set to a parameter (α), hereafter called paternal leakage rate. Since females cannot produce pollen, paternally transmitted mitochondria are always male-fertile at the CMS locus. We thus expect that the B allele will slow down and maybe prevent the invasion of the male-sterile cytoplasm C1 (Wade and McCauley, 2005).

Assumptions on heteroplasmy

Because of paternal leakage of mitochondria, zygotes can be heteroplasmic. We assumed complete vegetative sorting during plant development such that heteroplasmic zygotes develop into heteroplasmic plants with homoplasmic cells. Since gametes are formed late in plant development, all gametes must then be strictly homoplasmic. We assumed no selection between mitochondrial genomes within individuals at the CMS locus. Thus, a zygote with a proportion α of mitochondria from paternal origin will give rise to a plant with a proportion α of homoplasmic cells carrying mitochondria of paternal origin. Heteroplasmic individuals that carry both male-sterile and male-fertile mitochondria are considered to be gynomonoecious, with pistillate flowers produced on branches bearing the male-sterile mitochondria, and perfect flowers on branches bearing the male-fertile mitochondria. We also assumed that pistillate (respectively perfect) flowers on gynomonoecious plants have the same fecundity and fertility phenotype as do pistillate (respectively perfect) flowers on female (respectively hermaphrodite) individuals (TABLE 4.6), as has been observed in *Silene nutans* and *Silene italica* (Desfeux, 1996; Lafuma and Maurice, 2006).

Adding a cost of heteroplasmy should render more stringent the conditions under which biparental mitochondrial inheritance can evolve. The existence of such a cost is supported by three main arguments: First, if biparental mitochondrial inheritance occurs regularly, individuals are susceptible to invasions by selfish elements that can be transmitted between maternal lines (see introduction). Then, on the shorter term, cytoplasmic mixing could be detrimental to host fitness *per se* because of negative interactions between cytoplasmic elements (Ziegler and Davidson, 1981).

In our model, this cost would be limited to early development, when cells are still heteroplasmic. Finally, recombination between mitochondrial genomes might generate deleterious associations of alleles (Birky, 1995). A cost of heteroplasmy was added as a third parameter (Φ), independent from α, such that male and female fitness of all heteroplasmic individuals was reduced by a factor $1-\Phi$ (with $\Phi > 0$, see TABLE 4.6). For instance, the fitness of a gynomonoecious (heteroplasmic) individual will depend on α, the proportion of mitochondria of paternal origin (male-fertile mitochondria), on the female advantage s, and on the cost of heteroplasmy, Φ:

Female fitness$= (1-\Phi)((1-\alpha)(1+s)+\alpha)$

Male fitness$= \alpha(1-\Phi)$

Note that heteroplasmic individuals with two mitochondrial types that bear the same CMS allele also suffer this cost (TABLE 4.6) since their mitochondria can differ at other loci, and be vectors of selfish elements. A summary of all parameters of the model is available in TABLE 4.4.

Parameters	Description
C1	Male-sterile allele at the CMS mitochondrial locus
C0	Male-fertile allele at the CMS mitochondrial locus
B	Paternal leakage allele at the locus that determines mitochondrial inheritance
b	Maternal transmission allele at the locus that determines mitochondrial inheritance
f(C1), f(C0), f(B), f(b)	Frequencies of C1, C0, B and b, respectively, in the population
s	Female advantage in ovule production (the relative ovule production of females compared with hermaphrodites is 1+s)
Φ	Cost of heteroplasmy
α	Paternal leakage rate: proportion of mitochondria from paternal origin in zygotes when paternal leakage occurs
d	Dominance of the B allele in sporophytic models
r	Recombination rate (in mitochondrial models only)

Table 4.4: Parameters of the model of gynodioecy with paternal leakage.

Genetic determination of mitochondrial inheritance

The main novelty of this model compared to the analytical developments of Wade and McCauley (2005) is the introduction of genetic control for mitochondrial inheritance. Because little is known about the genetics of mitochondrial inheritance, we developed six models with different genetic determination systems. Two models with mitochondrial determination were considered. The first one assumes that the mito-

chondrial gene acts in pollen by enforcing mitochondrial transmission (mitochondrial pollen model, see TABLE 4.5). The mitochondrial genotype of pollen at the B locus determines whether there is paternal leakage or not. In the second one, the gene acts in ovules by increasing susceptibility to invasion by pollen mitochondria, so the mitochondrial genotype of the ovule determines whether paternal leakage can occur (mitochondrial ovule model, see TABLE 4.5). Because recombination has been described between mitochondria of heteroplasmic cells (Belliard et al., 1979; Mackenzie and McIntosh, 1999; Raineri et al., 1992), the rate of recombination between the B and the CMS loci (r) was included as a parameter in mitochondrial models. In addition, four models with nuclear control of mitochondrial inheritance were developed, considering that (i) as in mitochondrial models, the gene can act in pollen by enforcing mitochondrial transmission or in ovules by allowing invasion by paternal mitochondria; (ii) genetic determination can be gametophytic (the genotype of the gamete determines mitochondrial inheritance) or sporophytic (the genotype of the parent determines mitochondrial inheritance). In this last case, we added a dominance parameter (d). All combinations were studied in four nuclear models (TABLE 4.5). All of these models present the advantage of being consistent with several mechanisms of mitochondrial inheritance, even if ovule models require the presence of functional mitochondria in the pollen.

Paternal leakage locus	Paternal gene expression		Model
Mitochondrial	Pollen	-	Mitochondrial pollen model
	Ovule	-	Mitochondrial povule model
Nuclear	Pollen	Gametophytic	Gametophytic pollen model
		Sporophytic	Sporophytic pollen model
	Ovule	Gametophytic	Gametophytic ovule model
		Sporophytic	Sporophytic ovule model

Table 4.5: Summary of the different models of paternal leakage.

Simulations

For each generation, the allelic frequencies in the gametes were calculated by adding the contributions of each adult genotype, using its frequency and its female and male fitness (TABLE 4.6). Allelic frequencies in zygotes of the next generation were calculated based on the frequencies of all ovule and pollen genotypes, taking into account paternal leakage and assuming random mating. Initial conditions for simulations were set to 1% of male-sterile cytoplasm C1 and 0.1% of the paternal leakage allele B, with no linkage disequilibrium. These conditions simulate the arrival of a mutant that allows paternal leakage in a gynodioecious population susceptible to female invasion. Other possible initial conditions were examined and provided the same results at equilibrium and so are not presented here. Simulations were

run for about 4000 combinations of parameters, with $s \subset [-0.1, 2.5]$, $\Phi \subset [0, 1]$, and $\alpha \subset]0, 1[$. Indeed, estimates of female advantage in the literature range from 0 to 1 (*e.g.* Agren and Willson, 1991; Asikainen and Mutikainen, 2003; Lafuma and Maurice, 2006; Olson et al., 2006; Poot, 1997), exceptionally reaching 2.5 (Thompson and Tarayre, 2000). Strict maternal and paternal inheritance (α=0 and α=1 respectively) were not considered because the existence of a cost of heteroplasmy is inconsistent with uniparental inheritance. When $\Phi = 0$, α could also be considered as the frequency of offspring that inherit paternal mitochondria in a model with strict uniparental inheritance and no heteroplasmy. In mitochondrial models, simulations were run for r=0.1, r=0.01 and r=0.001. Simulations were run for at least 10000 generations. We considered that frequencies were stable when they varied less than 0.01% over the 2000 last generations. All simulations led to stable equilibria. At equilibrium, we considered that an allele was fixed (respectively lost) when its frequency was higher than 99% (respectively lower than 1%). In particular, the population was considered gynodioecious when female frequency fell between 1% and 99%.

	Hermaphrodites				Females		Gynomoecious	
	C0 bb	C0 Bb	C0C0 Bb	C0C0 BB	C1 bb	C1 Bb	C0C1 Bb	C0C1 BB
Ovules								
C1b	-	-	-	-	1+s	(1+s)/2	(1-Φ)(1-α)(1+s)/2	-
C1B	-	-	-	-	-	(1+s)/2	(1-Φ)(1-α)(1+s)/2	(1-Φ)(1-α)(1+s)
C0b	1	0.5	(1-Φ)/2	-	-	-	α(1-Φ)/2	-
C0B	-	0.5	(1-Φ)/2	(1-Φ)	-	-	α(1-Φ)/2	α(1-Φ)
Pollen								
C0b	1	0.5	(1-Φ)/2	-	-	-	α(1-Φ)/2	-
C0B	-	0.5	(1-Φ)/2	(1-Φ)	-	-	α(1-Φ)/2	α(1-Φ)

Table 4.6: Genotypic contributions to pollen and ovule gamete frequencies in the gametophytic pollen model.

Results

Mitochondrial determination of mitochondrial inheritance

Calculations with the mitochondrial pollen model showed that the paternal leakage allele (B) was able to spread in the population and led to maintenance of polymorphism at the CMS locus, except when $\Phi = 1$ where B was lost and the male-sterile mitochondria invaded the population. At equilibrium, B was always fixed in the male-fertile background, and, if mitochondria were allowed to recombine ($r \neq 0$), invaded the entire population. These results were expected since B is neutral in females and advantageous in hermaphrodites because of its additional transmission through pollen. When recombination occurred ($r \neq 0$), the B allele was able to invade the male-sterile background and went to fixation. The value of the recombination rate

(when $r \neq 0$) did not influence the equilibrium state but determined the time needed for the B allele to become fixed in the male-sterile background. Similarly, the cost of heteroplasmy (Φ) did not influence the equilibrium state (except when $\Phi = 1$) but only modified the selection coefficient of B and thus, the dynamics of invasion. Equilibrium at the CMS locus depended on the female advantage (s) and the paternal leakage rate (α): When $s > 0$ and $1 + s > \frac{1}{1-\alpha}$, polymorphism at the CMS locus was maintained and the population consisted of hermaphrodite and gynomonoecious individuals (there were no females because B was fixed in the male-fertile background and all hermaphrodites transmitted their mitochondria through pollen). Otherwise, the male-sterile allele C1 was lost and the population evolved to hermaphroditism. The mitochondrial ovule model gave contrasting results: All simulations showed the invasion of the male-sterile mitochondrion (C1) and the loss of B, leading to purely female populations (and then, to extinction). Indeed, in this model, the B allele allowed its own mitochondria to be invaded by mitochondria from pollen and thus had reduced transmission through ovules. Values of parameters did not influence equilibrium states and only modified the dynamics of fixation or loss: As expected, increased values of s accelerated the fixation of C1 whereas increased values of α and Φ accelerated the loss of B.

Nuclear determination of mitochondrial inheritance

Since the results of the four nuclear models (TABLE 4.5) differed only quantitatively and not qualitatively, we will present those of the gametophytic pollen model in detail and only mention deviations from this model for the three others. Simulations generally proceeded as follows: (i) If there was a female advantage ($s > 0$), the male-sterile mitochondrion (C1) began to spread; (ii) when the frequency of C1 became high enough, the paternal leakage allele (B) started to spread because of its association with pollen that bears the rare male gametes (see explanations below); (iii) the spread of the paternal leakage allele B prevented further spread of the male-sterile mitochondrion C1 because some females produced gynomonoecious heteroplasmic individuals with a proportion $(1-\alpha)$ of male-sterile mitochondria instead of producing only female individuals (see Supplementary Figure 1). This led to a reduction in frequency of the male-sterile mitochondrion C1, this reduction being greater for higher values of α ; (iv) because the frequency of the male-sterile mitochondrion decreased, the advantage of the paternal leakage allele B decreased and further spread of B is prevented or slowed down. For some parameter values, we observed oscillations and steps (i) to (iv) occurred several times before equilibrium. The range and duration of oscillations were shown to increase with Φ and α, and when s decreased. However, oscillations were always damping and no limit cycles were found.

Depending on the parameters values, the dynamics described above led to different equilibrium states (FIGURE 4.3). In most simulations, the CMS locus was polymorphic at equilibrium and the mitochondrial inheritance locus was either poly-

morphic or fixed for the paternal leakage allele (zones (2) and (3) in FIGURE 4.3a). This corresponds to a gynodioecious population including a number of gynomonoecious individuals that depends on the frequency of B (when B is fixed, only gynomonoecious and hermaphrodite individuals are found in the population). As expected, populations were hermaphroditic when $s < 0$, and neither C1 nor B were able to spread (zone (1) in FIGURE 4.3a). These simulations ($s < 0$) will not be considered further. A very high cost of heteroplasmy prevented the spread of B and led to the fixation of C1 (such pure female populations are of course destined to extinction, zone (4) in FIGURE 4.3a). On the contrary, when $\Phi = 0$, B was maintained at intermediate frequencies but C1 was lost, leading to hermaphroditism (zone (5) in FIGURE 4.3a).

As already demonstrated by Wade and McCauley (2005), paternal leakage allowed the maintenance of male-fertile mitochondria by allowing their transmission through pollen (FIGURE 4.4). Here, we also showed that the spread of B relies on the presence of female flowers and occurs only when the male-sterile mitochondrion (C1) is common enough. Then, B can go to fixation or remain polymorphic, depending on parameter values (FIGURE 4.3). As restorers of fertility, the paternal leakage allele spreads because of its genetic association with the rare gamete (*i.e.* pollen). Indeed, the transmission of male-fertile mitochondria through pollen is enforced by hermaphrodites carrying B, leading to linkage disequilibrium between the paternal leakage allele B and the male-fertile mitochondrion C0 (Supplementary Figure 1). This linkage increases with the proportion (α) of paternal mitochondria transmitted. B thus takes advantage of Fisherian selection, and, when $s < 1$, of the higher total gamete production of hermaphrodites compared with females. Because Fisherian selection is frequency-dependent, the more females there are, the more B is advantageous (FIGURE 4.4).

Both dynamics and equilibria were influenced by female advantage (s), cost of heteroplasmy (Φ) and paternal leakage rate (α). As expected, increasing Φ delayed the spread of B (data not shown) and reduced its frequency at equilibrium, leading to a higher frequency of C1 (FIGURE 4.5a and 4.5c). Because of the linkage disequilibrium between B and C0, Φ also favors the male-sterile mitochondrion directly (FIGURE 4.4). The effect of α was more complex: Higher α led to an earlier spread of B but decreased frequencies of B and C1 at equilibrium. Although increasing α increased the linkage disequilibrium between C0 and B (data not shown), which should facilitate the spread of B, a higher α also prevented the spread of C1, that in turn limited the spread of B (FIGURE 4.4). This indirect effect of α via the decreasing of the frequency of C1 seemed to be stronger than the direct effect. The total amount of leakage in the population at equilibrium, defined by ($\alpha \times$ the frequency of B in the C0 background), was shown to increase with α (data not shown), explaining the decrease in the C1 frequency. Finally, when s increased, C1 and B showed earlier spread and both frequencies at equilibrium generally increased (FIGURE 4.5). However, in some rare cases with low Φ and high α and s, the frequency of B slightly decreased when s increased (see FIGURE 4.5d). Indeed, we expected contrasting ef-

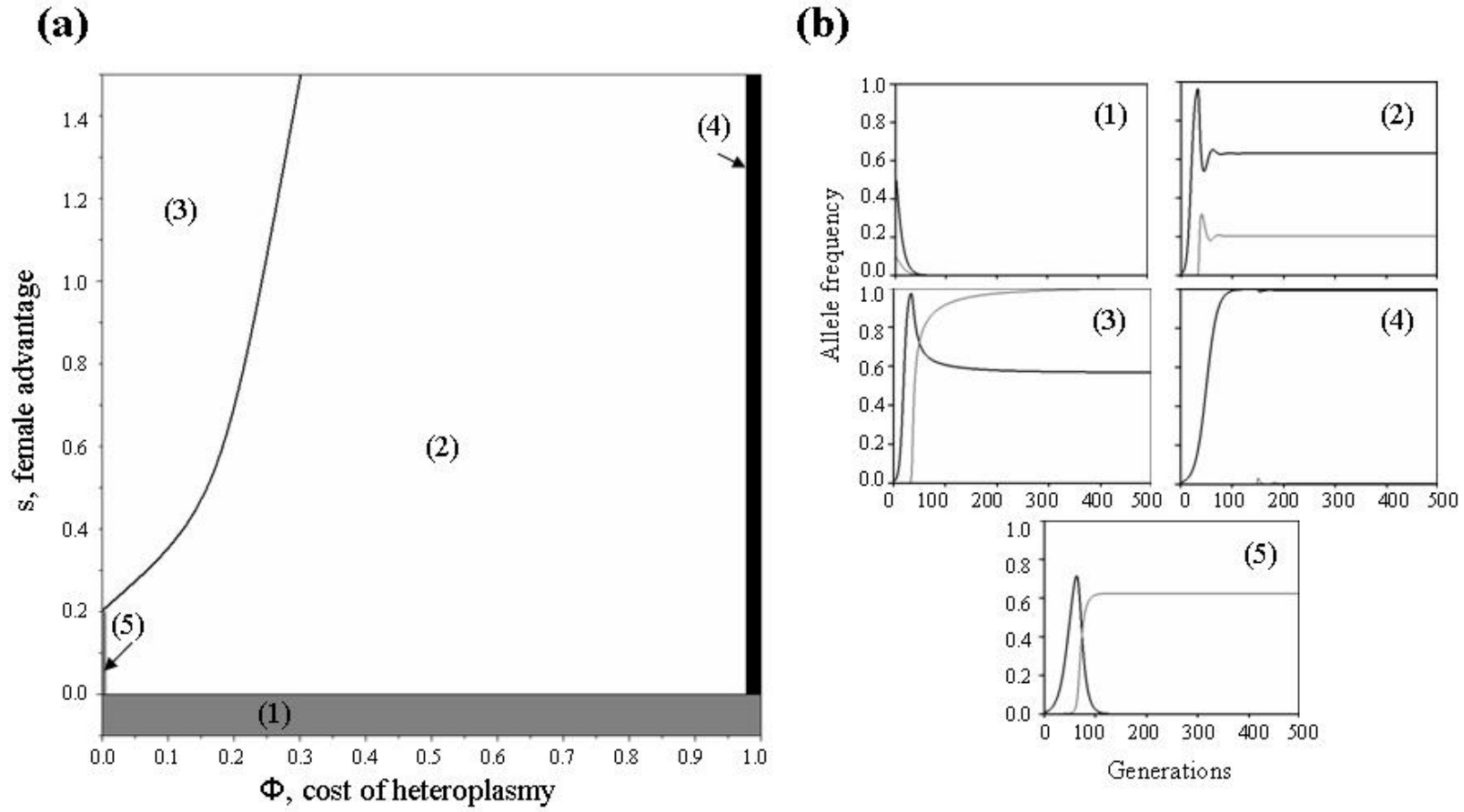

Figure 4.3: **(a)** Equilibrium states of the gametophytic pollen model, for $\alpha = 0.1$. Five main scenarios can be defined, based on the frequencies of the male-sterile mitochondrion (C1) and of the paternal leakage allele (B) at the equilibrium: (1) C1 and B alleles are lost; (2) both loci are polymorphic; (3) the B allele is fixed and the CMS locus is polymorphic; (4) B is lost and C1 is fixed; (5) C1 is lost but the B locus is polymorphic. Increasing α reduces zone (3) and extends zone (5). Female populations are in black, hermaphrodite populations in grey and gynodioecious and/or gynomonoecious populations in white. **(b)** For each scenario, we give an example of the dynamics of the frequencies of the male-sterile mitochondrion C1 (in black) and of the paternal leakage allele B (in grey). Simulations were run with the following parameter values : (1) $s = -0.1$; $\Phi = 0.2$; $\alpha = 0.2$; for illustration purposes initial frequencies were exceptionally set to 0.5 for C1 and to 0.1 for B, in order to show the decrease in the frequencies of B and C1 alleles (2) $s = 0.3$; $\Phi = 0.4$; $\alpha = 0.4$ (3) $s = 0.3$; $\Phi = 0.1$; $\alpha = 0.1$ (4) $s = 0.1$; $\Phi = 0.95$; $\alpha = 0.1$ (5) $s = 0.1$; $\Phi = 0$; $\alpha = 0.3$.

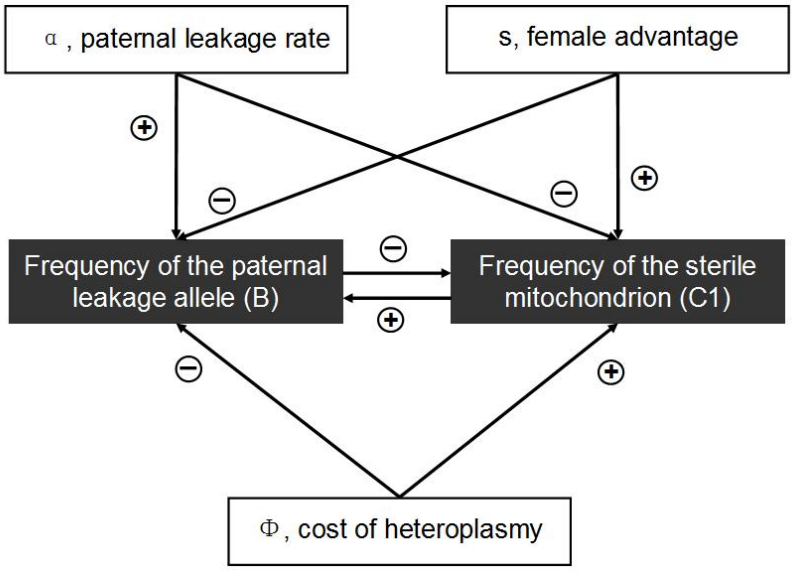

Figure 4.4: Direct effects of the three parameters s, α, and Φ on the frequencies of the paternal leakage allele B and the male-sterile mitochondrion C1 and frequency-dependent relationships between the two. Positive effects are denoted by a (+) and negative effects by a (-).

fects of s on the frequency of B: First, s should help the spread of paternal leakage because it increases the frequency of C1. But increasing s has also a negative impact on B because of the linkage disequilibrium between C0 and B. Lastly, when $s > 1$, the total number of gametes produced by females is higher than by hermaphrodites, so B does not benefit from a higher gamete production thanks to its linkage with C0 and benefit only from Fisherian selection. FIGURE 4.4 summarizes the complex dynamics of B as a function of s, Φ and α.

The gametophytic ovule model gave very similar results with only quantitative differences. Dynamics followed the same steps as the gametophytic pollen model: Invasion of male-sterile mitochondria because of female advantage, spread of the paternal leakage allele thanks to its association with the male-fertile mitochondrion C0 (Fisherian selection and higher total gamete production when $s < 1$), decrease in the frequency of the male-sterile mitochondrion C1, and weaker selection of the paternal leakage allele B when C0 is common. However, B spread more efficiently and frequencies of B at equilibrium were always higher in the ovule model than in the pollen model (mean difference over all parameter combinations=0.24). Frequencies of C1 at equilibrium were similar, although on average slightly higher in the pollen model (mean difference=0.009). These differences between pollen and ovule models were largely due to the fact that the B alleles that prevented the spread of the male-sterile mitochondrion, by either transmitting through pollen or accepting by ovules male-fertile mitochondria, were those associated with C0 in the pollen model but with C1 in the ovule model. Thus, because linkage disequilibrium arises between

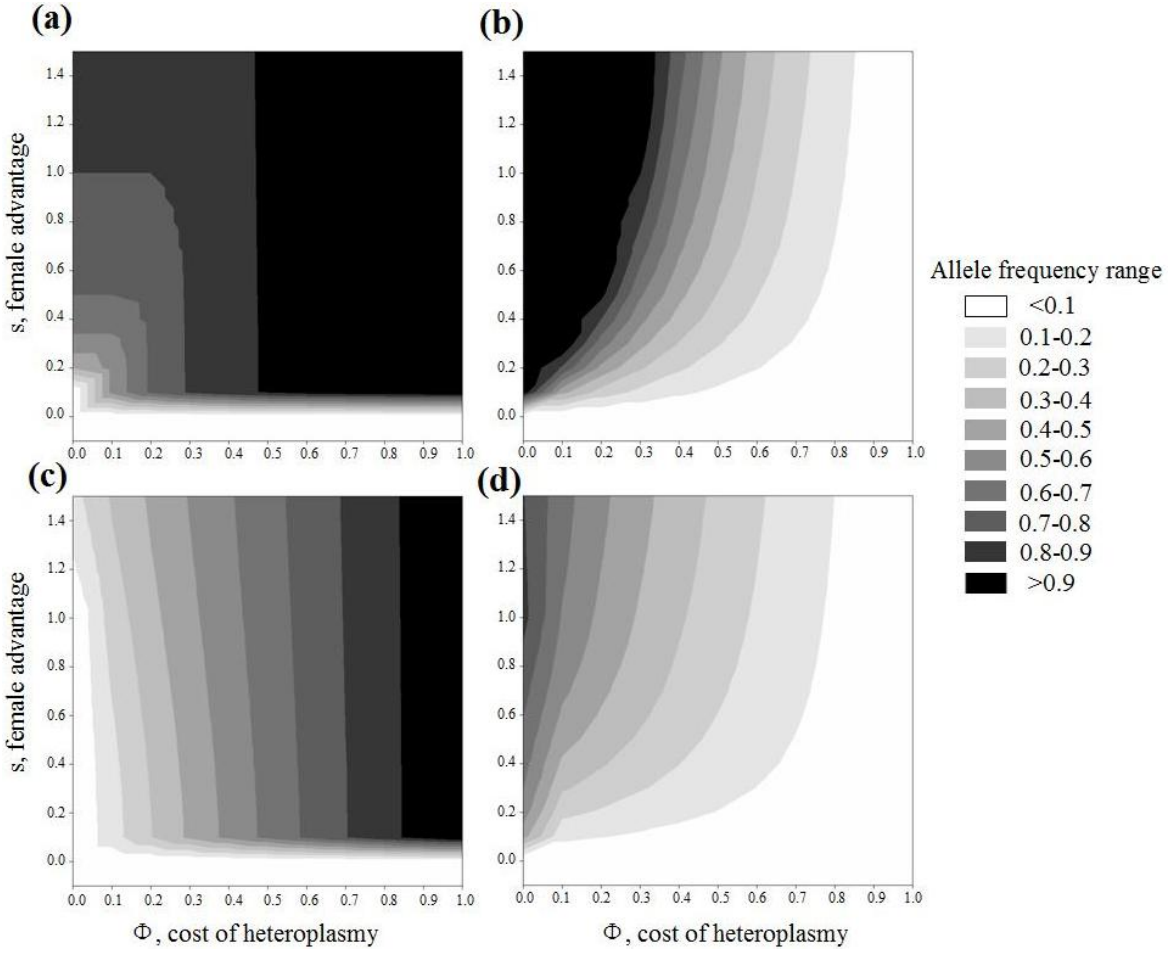

Figure 4.5: Frequencies of the male-sterile mitochondrion C1 (a and c), and of the paternal leakage allele B (b and d) at equilibrium, for $\alpha = 0.1$ (a and b) and $\alpha = 0.6$ (c and d) in the case of the gametophytic pollen model. Equilibrium frequencies are represented as a function of s, the female advantage and Φ, the cost of heteroplasmy.

B and C0 in both models, at the same population frequency of B, those B alleles that actively contribute to paternal leakage (associated with C0 in the pollen model but C1 in the ovule model) are necessarily more common in the pollen model. As a consequence, B limited its own spread by decreasing the frequency of C1 (thereby decreasing its own advantage) more strongly in the pollen model, thus explaining why B reached higher frequency in the ovule model. However, at equilibrium, the frequency of B in the C0 background in the pollen model was still slightly lower than the frequency of B in the C1 background in the ovule model (mean difference=0.07), which requires further exploration. Oscillations were larger in the ovule model. The effects of Φ, α and s were similar except from some cases where the frequency of C1 decreased with increasing s, probably because of the more efficient spread of B. The sporophytic (dominant and recessive) and gametophytic pollen models all led to

the same frequency of C1 at equilibrium, and the frequencies of pollen transmitting mitochondria were the same in the three models. We did not find an equivalent relationship between the three ovule models, but, again, no qualitative changes in the dynamics nor in the influence of parameters existed.

Discussion

Despite some superficial similarities, nuclear and mitochondrial models provided very different results. In mitochondrial models, paternal leakage behaved as a selfish element and was selected only when it increased its own transmission, *i.e.*, when the gene enforced transmission by pollen (mitochondrial pollen model). The allele allowing self-invasion in ovules (mitochondrial ovule model) was always lost because it allowed its own invasion by paternal mitochondria. A model with mitochondrial determination of mitochondrial inheritance proved inadequate for explaining the evolution of paternal leakage in a population being invaded by a male-sterile mitochondrion because: (i) The model did not account for the presence of females at equilibrium; (ii) our models account for either fixation of the paternal leakage allele (in the pollen model) or its loss (in the ovule model), which is inconsistent with the intermediate rates of paternal leakage that have been observed in crossing studies (see introduction); (iii) The invasion of the paternal leakage allele in the mitochondrial pollen model does not require the presence of male-sterile mitochondria, and should then evolve in all species having uniparental mitochondrial inheritance; (iv) Uniparental inheritance is usually considered to have evolved as a nuclear control of the arms race between mitochondria (Hastings, 1992; Hurst and Hamilton, 1992; Randerson and Hurst, 1999). Under this hypothesis, mitochondrial mutants that break the rules of uniparental inheritance should not be maintained in the long term, because we expect that the nucleus will counteract and enforce uniparental inheritance again.

The results of nuclear models were shown to be very robust to changes in the genetic determination of paternal leakage (gametophytic/sporophytic action on pollen/ovule). All models demonstrated that a population containing a CMS gene is susceptible to being invaded by a nuclear gene allowing paternal transmission of mitochondria through pollen over a wide range of parameters. The evolution from strict uniparental inheritance to paternal leakage (*i.e.* partial biparental inheritance) when CMS is present can be understood in the light of genetic conflicts. Paternal leakage renders the laws of inheritance of the mitochondrial genome closer to the ones of the nuclear genome. As a consequence, it partially reconciles the interests of the two sets of genes and limits the invasion of CMS but, in return, increases the risk of invasion by other mitochondrial selfish elements (a cost represented by the cost of heteroplasmy in our models). Paternal leakage could thus imply a trade-off between two conflicts, by reducing the cost of the conflict between the cytoplasm and the nucleus but reactivating the conflict between cytoplasms. Importantly, the final outcome of the conflict strictly depends on the stochastic occurrence of mutations, as

in all genetic conflicts (Hurst et al., 1996). Despite our model having demonstrated that paternal leakage can be selected because of CMS, this will occur only if a paternal leakage mutant can appear within the time window of CMS spread. Indeed, in a stochastic model in finite population, the male-sterile cytoplasm sometimes became fixed before paternal leakage began to spread (particularly for certain values of α and Φ such that a very high proportion of females is necessary for the invasion of the paternal leakage allele).

Our model did not attempt to obtain precise predictions of rates of paternal leakage or female frequencies in natural populations and our purpose was only to demonstrate that CMS induces a selective pressure for the evolution of paternal leakage of mitochondria. Considering the increasing number of studies on paternal leakage of mitochondria, and particularly in gynodioecious species (Bentley et al., 2010; McCauley et al., 2005; Pearl et al., 2009; Welch et al., 2006), better knowledge of paternal leakage (genetic determination, frequency in natural populations) and heteroplasmy (sex phenotype, fitness, vegetative sorting and inheritance) are likely to be available soon and will allow testing the underlying assumptions of this model as well as the construction of more predictive models. Indeed, our models make strong assumptions related to heteroplasmy. Heteroplasmic individuals were assumed to complete vegetative sorting early in development, such that at flowering they were gynomonoecious, the sex of each flower being determined by its resident mitochondrion. Although it is possible that vegetative sorting might sometimes be incomplete in one generation, experimental data are too rare to justify including alternative modes of heteroplasmy inheritance in our models. Nonetheless other ideas for how heteroplasmic mitochondria segregate do exist. For example, a quantitative determination based on the stoichiometry of male-sterile versus male-fertile mitochondria, in the flower or in the entire individual, cannot be ruled out. Quantitative models of restoration fit data on sex segregation in progenies of controlled crosses in several species (Ehlers et al., 2005) but these data may also be consistent with quantitative male sterilization.

Nuclear restorer and paternal leakage alleles are quite similar in the mechanism of their spread. Both allow females to produce at least some hermaphrodite offspring, leading to a genetic linkage between restorer/paternal leakage and pollen production. Thus, both benefit from Fisherian selection and a higher total gamete production than females if $s < 1$. However, paternal leakage always limits the spread of the male-sterile mitochondria by replacing them by male-fertile mitochondria, whereas restorers only disable male-sterile mitochondria (male-sterility segregates when the association between the restorer and the CMS is broken). However male-sterility mitochondria may still decrease in frequency if they impose a cost or if there is a cost associated with restoration (Bailey et al., 2003; Dufay et al., 2007). Nuclear restoration of cytoplasmic male sterility has been clearly demonstrated in numerous gynodioecious species and paternal leakage to date in one. Indeed, the initial appearance of new restorer mutants is probably more common than that of paternal leakage mutants. For instance, there may be more nuclear genes controlling mito-

chondrial gene expression (that are the putative precursors of restorers, see Delph et al., 2007) than genes controlling mitochondrial inheritance. On the other hand, paternal leakage may occur more often than it is detected, particularly when the paternal contribution of mitochondria is very low. Lastly, differences in evolutionary dynamics of restoration versus paternal leakage and potential interactions between the two phenomena may favor restoration, but this last point deserves future theoretical development. Wade and McCauley (2005) have shown that a fixed rate of paternal leakage cannot prevent the invasion of the CMS and its restorer. A positive effect of paternal leakage in maintaining polymorphism applied only when two CMSs and two restorers were modeled under the matching allele assumption (specificity between CMS and restorers). Future developments should also consider the influence of spatial structure of CMS and restorer genes on the evolution of paternal leakage (for experimental demonstration of structure see Olson et al., 2006; Olson and McCauley, 2002; Storchova and Olson, 2004). Complex results can be expected because: (i) Paternal leakage will be less efficient because of inbreeding, since it will occur more often between individuals having the same mitochondrial genome; (ii) because the spread of the paternal leakage allele is based on the production of gynomonoecious plants by female plants (*i.e.* on crosses between females and hermaphrodites), the patchiness of females will make the spread of paternal leakage more difficult because hermaphrodites will more probably pollinate other hermaphrodites; (iii) inbreeding might increase female advantage if hermaphrodites suffer inbreeding depression; (iv) Pollen limitation will occur in female patches and will prevent the invasion by the male-sterile mitochondria.

Our model of paternal leakage invasion presents some analogies with host-pathogen models. Mitochondria are very similar to parasites or symbionts that live in the cytoplasm of their host, and are even sometimes considered as such. While biparental transmission of mitochondria is supposed to be responsible for an arms race between mitochondria, horizontal, compared with vertical transmission of parasites is expected to increase virulence (Stewart et al., 2005). With vertically-transmitted parasites (or symbionts) as with maternally transmitted mitochondria, female-biased sex-ratio distortion is often observed in hosts and we discuss whether a phenomenon analogous to paternal leakage of mitochondria in plants can be found in those symbionts. Because a non perfect vertical transmission of the parasite would have an equivalent effect and may be selected in an equivalent manner to the leakage of some male-fertile mitochondria in the descent of a female plant, we examined information about the vertical-transmission rate of such symbionts. One of the best-known examples of sex-ratio distortion by symbionts is the case of the alphaproteobacteria *Wolbachia*, which is the most widespread symbiont of arthropods and nematodes. *Wolbachia* is maternally transmitted and its transmission is increased by various mechanisms including feminization, which turns genetic males into functional females, called neo-females (Werren et al., 2008). *Wolbachia* is analogous to the male-sterile mitochondria in plants and the paternal leakage of mitochondria would be equivalent to a non-perfect vertical transmission of the symbiont. Rigaud and

Juchault (1992) showed that, in *Armadilidium vulgare*, most neo-females produce highly female-biased progenies but a few produce male-biased progenies. The high rate of males was attributed to a low vertical transmission rate of the bacteria, this rate being influenced by the host genome. Further investigations will tell us whether paternal leakage in plants and non-perfect vertical transmission of *Wolbachia* are two congruent responses to similar selfish sterilizing elements.

More generally, in addition to its direct influence on CMS dynamics, paternal leakage could have interesting evolutionary consequences. Because paternal leakage increases the occurrence of heteroplasmy, it also raises the potential of intragenomic recombination. Fusion and fission of mitochondria in plants are commonly admitted (Arimura et al., 2004; Sheahan et al., 2005) and intragenomic recombination within the mitochondrial genome is facilitated by the presence of large ($>$ 4kb) direct repeats. Evidence for both intra- and inter-genic recombination at the evolutionary scale have been found in the genus Silene (Houliston and Olson, 2006; McCauley and Ellis, 2008; Städler and Delph, 2002; Touzet and Delph, 2009) and intergenomic mtDNA recombination has been shown in protoplast-derived plants (Belliard et al., 1979; Raineri et al., 1992). Just as does sexual reproduction, heteroplasmy and recombination are expected to accelerate the evolution of the genome. Particularly, recombination could increase the rate at which new genotypes, and particularly potential CMS, are generated. Indeed, CMS is often caused by chimeric genes that are thought to arise from recombination within the mitochondrial genome (for a review, see Delph et al., 2007). However, efficient recombination requires intra-individual variation at the recombining loci, *i.e.* paternal leakage between individuals with different cytoplasms. Recombination within the mitochondrial genome of *Silene vulgaris* was shown to generate new multilocus genotypes (McCauley and Ellis, 2008), but its effect was limited by the strong spatial population structure. More generally, the very high diversity of the mitochondrial genome in gynodioecious species (*e.g.* Touzet and Delph, 2009), often attributed to balancing selection at CMS loci (Städler and Delph, 2002), might also be due to paternal leakage and recombination.

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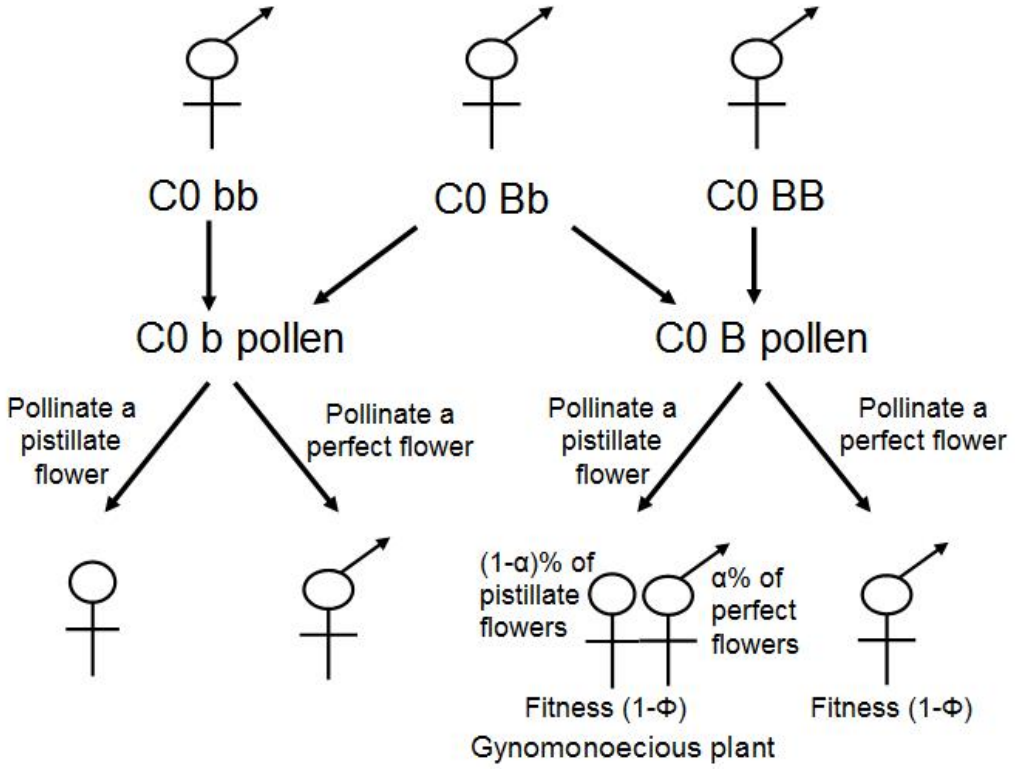
References

See References at the end of the Thesis p.178.

Supplementary Table 1: Transmission of the paternal leakage allele (B) in the gametophytic pollen model



Supplementary Figure 1: Transmission of the paternal leakage allele (B) in the gametophytic pollen model. Since the B allele acts in pollen, it has an effect when carried by hermaphrodites and only pollen grains that carry the paternal leakage allele (C0B) are able to transmit their mitochondria. When these pollen grains pollinate a perfect (hermaphrodite) flower, they give rise to hermaphrodite heteroplasmic individuals carrying mitochondria that are similar at the CMS locus but can differ at other loci. When these pollen grains pollinate a pistillate (female) flower, offspring are gynomonoecious individuals, which are heteroplasmic with α% of male-fertile mitochondria associated with the production of perfect flowers and $(1-\alpha)$% of male-sterile mitochondria associated with the production of pistillate flowers. Therefore, the B allele rapidly develops linkage disequilibrium with the C0 background and is selected by Fisherian selection (rare gamete advantage). On the contrary, when females are rare, Fisherian selection is weak and the B allele is counter-selected because of the cost of heteroplasmy (Φ).

Summary of chapter 4

* In almost all plants and animals and in many eucaryotes, cytoplasmic (mitochondrial and chloroplastic) genomes are uniparentally inherited, most often maternally.

* Uniparental inheritance is controlled by various mechanisms (active destruction by enzymes, organelles segregation during cell divisions, etc.), occurring from gametogenesis to the first stages of development. The genetic determination is poorly known.

* Uniparental inheritance of cytoplasmic genomes could have been selected by nuclear genes as a way of preventing the invasion by mitochondrial selfish elements.

* In a few *Silene* species and particularly in *Silene vulgaris*, the mitochondrial genome has been shown to be occasionally transmitted through pollen (=paternal leakage), generating heteroplasmy which is the coexistence of more than one cytoplasmic genome within an individual.

* Heteroplasmy is quite rare and is consequently poorly known. In plants, heteroplasmy could be transmitted from mother to offspring but may not be able to persist in lineages for a long time.

* To date, paternal leakage and heteroplasmy have been observed in too few species to conclude on a link between sexual system and paternal leakage. However, evolution of cytoplasmic male sterility and paternal leakage are theoretically linked in two ways : (1) Cytoplasmic male sterility could favor the evolution of paternal leakage of mitochondria; (2) Paternal leakage sustains the cytonuclear polymorphism in gynodioecious species.

Chapter 5

Conclusion

> Did you ever answer any really interesting question ?
>
> A student (pers.comm.)

There were a few major questions (and the hope of providing an answer) that prompted me to start this PhD: what is the influence of sex determination on the dynamics of evolution of gynodioecy? What is the contribution of gynomonoecious individuals to the evolution of gynodioecy? Is there a link between heteroplasmy and gynomonoecy? Are the evolution of paternal leakage and that of gynodioecy linked? etc. After three years, I must admit that I have not found a clear answer to all of these questions but rather many small answers to more precise questions that are sometimes a little bit dissociated from the initial question. This conclusion will therefore give me the opportunity to make the link between these incomplete responses and the major issues which motivated my research, to bring together the different aspects of this thesis and to mention a few perspectives.

5.0.3 Mitochondrial inheritance and gynodioecy

5.0.3.1 Are the evolution of paternal leakage and cytoplasmic male sterility linked?

It has been demonstrated theoretically that the evolution of paternal leakage and the evolution of gynodioecy were linked in two ways. Indeed, it has been shown recently than paternal leakage could stabilize the cytonuclear polymorphism underlying gynodioecy (Wade and McCauley, 2005) and I showed that the occurrence of a cytoplasmic male sterility gene favored the evolution of paternal leakage (ARTICLE 5). But is there experimental support to these theoretical expectations?

Regarding the stabilizing effect of paternal leakage, it seems that it can play a role in *Silene vulgaris*, since paternal leakage rates from 5 to 10% produced significant effects in the model and the observed rates of non-maternal transmission ranged from 2.5 to 14% according to the studies (Bentley et al., 2010; McCauley et al., 2005; Welch et al., 2006). In contrast, in other gynodioecious species in which occasional paternal leakage is suspected (e.g. *Silene acaulis*), signatures of recombination in the mitochondrial genome are the only evidence for paternal leakage and can be explained by paternal leakage rates much lower than those needed to stabilize gynodioecy. It is therefore difficult to know if paternal leakage could enhance the maintenance of gynodioecy in other species. In *Silene nutans* we found only one case of paternal inheritance (1/1113 individuals) and a rate of this order of magnitude must be insufficient to contribute significantly to the maintenance of polymorphism (Wade and McCauley, 2005).

On the other hand, how can we determine experimentally if cytoplasmic male sterility enhances the evolution of paternal leakage? And how can we know if the observation of paternal leakage in some gynodioecious species is a coincidence, results from a bias in studied species or reveals an interesting evolutionary phenomenon? Testing this hypothesis requires precisely estimating paternal leakage rates in gynodioecious and non-gynodioecious species and determining if sexual system and paternal leakage are correlated. Large sample sizes are needed to detect rare events such as paternal leakage and the quantification of paternal leakage rates in a number of species high enough to obtain a reliable correlation would be a tremendous amount of work. In addition, this approach requires knowing the evolutionary histories of sexual systems within the studied taxa. For instance, in the genus *Silene*, gynodioecy is ancestral and one could find paternal leakage in all species, including those that are derived non-gynodioecious, without invalidating the hypothesis. Similarly, in species that evolved recently as gynodioecious, the absence of paternal leakage would not be informative. Finally, this approach has the disadvantage of all correlative approaches, i.e. that no causal link can be drawn. In particular, if cytoplasmic male sterility may theoretically enhance the evolution of paternal leakage, it is also possible that paternal leakage (or rather the heteroplasmy and recombination associated) has favored the emergence of CMS (see next paragraph).

5.0.3.2 Paternal leakage, gynodioecy and evolution of the mitochondrial genome

An important consequence of paternal leakage is that it can lead to heteroplasmy and possibly recombination between different mitochondrial genomes. This recombination, whose consequences are important for the evolution of the mitochondrial genome in general, takes a particular dimension in gynodioecious species (see chapter 4). Indeed, CMS genes result from recombination events within the mitochondrial genome and even if this does not require that recombination occurs between two different molecules, recombination between different genomes can favor the creation of new variants and increase the probability of CMS appearance. In addition, recombination events that occur between CMS loci could recreate male-fertile cytoplasms or produce new multilocus complexes of male sterility. Such complexes could explained the frequent epistatic relationships between restorers whose evolution is non trivial and has never been explained. Indeed, the restoration of a multilocus complex would need the restoration of all the CMS genes of the complex and the restorers of these CMS genes would seem to be epistatic.

Contrary to the effect of paternal leakage on the maintenance of gynodioecy, the impact of the recombination on the evolution of the mitochondrial genome can be important even if paternal leakage rates are very low. Events of intra- and inter-locus recombination have been shown to occur at the evolutionary scale in several gynodioecious species of the genus *Silene* (see chapter 4). Thus, even if recombination rates are insufficient to ensure a linkage disequilibrium between the mitochondrial loci (McCauley and Ellis, 2008), they could influence the evolution of cytoplasmic male sterility.

One can be surprised that despite the high rates of heteroplasmy observed in *Silene vulgaris*, recombination is not more frequent. This paradox is explained by the many conditions that are needed for the appearance and detection of a recombinant. Indeed, heteroplasmy would have needed to exist within the organelles and not only within the individual (this requires the fusion of organelles from paternal and maternal origins if we assume that heteroplasmy results from paternal leakage) and recombination should occur between two different molecules within the organelles – which has a low probability when the stoichiometry of the different molecules is unbalanced, which could often be the case. Lastly, recombination will only be detected if two sites located on the two sides of the site where recombination occurred are genotyped. Therefore, recombination rates probably greatly under-estimates the frequency of paternal leakage and heteroplasmy.

In *Silene nutans*, we detected, within a gynomonoecious individual, two non-parental haplotypes that could have resulted from the recombination between the AU (Auvergne) maternal haplotype and the Q1 (Queyras1) haplotype, the latter being *a priori* not carried by the parents (Article 4). This recombination event probably did not occur in the offspring and it is more likely that these two recombinants were

already present in low stoichiometry in a cryptic heteroplasmic mother. According to this hypothesis, the recombination between the Q1 and the AU haplotypes would have occurred in the Auvergne population, where the mother was sampled. Unfortunately, the Auvergne population seems to be monomorphic (50 genotyped individuals) and the two haplotypes only coexist in the Queyras population. Thus for the moment, the origin of these two recombinants remains mysterious.

5.0.3.3 Paternal leakage and analysis of controlled crosses

All the experimental studies that investigated the genetic determination of gynodioecy to date have assumed maternal inheritance of organelles. But ignoring a non-maternal transmission in some offspring can lead to misinterpretation regarding the number of CMS and/or the number of restorers. The non-maternal transmission rates observed in *Silene vulgaris* are high enough to lead to such misinterpretations, especially if we consider that paternal leakage is highly cross-dependent and that some crosses showed 50% of non-maternal inheritance (McCauley et al., 2005).

Our study on the genetic determination of sex in *Silene nutans* is not an exception and assumes maternal cytoplasmic inheritance in order to interpret the segregation-ratio in progenies from controlled crosses (see ARTICLE 1). However, we tested the mitochondrial inheritance of all offspring and showed that the non-maternal transmission rate was very low (3/1113 individuals, see ARTICLE 4). Because the three exceptions belonged to three different crosses, their influence on the segregation-ratio of the progeny is lower than sampling fluctuations (n=30 by cross). There are however two particular cases for which small variation in segregation-ratio are likely to affect the results significantly: adding a hermaphrodite offspring to a pure female progeny or adding a female offspring to a pure hermaphrodite progeny. Indeed, the sex ratio 0:30 and 30:0 (female:hermaphrodite) can be explained by a small number of restorer genes while the sex ratio 29:1 and 1:29 require complex models of restoration (particularly for 29:1). I thus checked the genotypes of all individuals having the minority sex within their crosses. All carried a maternal haplotype with the exception of one individual from the 27th cross (27#24, see ARTICLE 4) and two suspicious individuals from the same cross. Depending on additional genotyping results on these individuals, the number of restorers needed to explain the segregation-ratio of this cross may need to be modified. This modification could only lead to an increase, and not a reduction, in the number of restorers needed to explain the restoration of the AU haplotype.

5.0.4 The unanswered questions on gynomonoecy

5.0.4.1 The genetic determination of gynomonoecy

Our data showed that the proportion of perfect and pistillate flowers in gynomonoecious individuals is at least partially genetically determined (ARTICLES 2 and 3). Although our data do not allow us to choose between the two assumptions of

genetic determination (heteroplasmy or quantitative restoration), we will discuss the consistency of some of our results with the two assumptions.

It has been suggested that the gynomonoecious phenotype results from the segregation of male-sterile and male-fertile cytoplasms within individuals and recent evidence of paternal leakage and heteroplasmy in *Silene vulgaris* provided indirect support to this hypothesis. Indeed, in this species, the proportion of gynomonoecious individuals and the proportion of heteroplasmic individuals are of the same order of magnitude (Charlesworth and Laporte, 1998; Pearl et al., 2009; Welch et al., 2006), even if no direct link has been established between gynomonoecy and heteroplasmy. In *Silene nutans* however, the non-maternal transmission rate appeared to be very low (0.3%) while the proportion of gynomonoecious individuals reached 30% when comprehensive phenotyping was carried out. Thus, even if the heteroplasmic individuals carrying a maternal majority haplotype were not detected by our genotyping method, the heteroplasmy assumption does not fit our data. In addition, on 16 gynomonoecious plants we genotyped several floral stems that sometimes varied in their proportion of pistillate flowers and we only detected one case of heteroplasmy but without detecting any relationship between the local genotype and the local phenotype. Although this experiment was carried out with a small sample size, it shows that it is unlikely that all gynomonoecious individuals are heteroplasmic.

Hence, our data are difficult to reconcile with the heteroplasmy hypothesis although no evidence can clearly refute it. On the contrary, our data are consistent with the quantitative restoration hypothesis. The many genes involved in restoration (Article 1) suggest a complex, perhaps quantitative restoration but no link with gynomonoecy has been found. All these arguments remain indirect. The ongoing experiments on the genetic determination of the gynomonoecious phenotype will hopefully provide direct evidence in favor of one of the hypotheses (the two are not necessarily exclusive).

5.0.4.2 Is gynomonoecy a adaptive strategy?

A main question about gynomonoecy is to know whether producing both perfect and pistillate flowers within a plant is an adaptive strategy or only results from the quantitative determination of sex or from a defective canalization that causes developmental errors. Distinguishing among those three phenomena is not obvious but an adaptive strategy should imply some selective advantage while the two others could lead to phenotypes with low fitness.

Two evolutionary advantages of the gynomonoecious phenotype are often mentioned in the literature. The first one is linked to the advantage of phenotypic plasticity. Indeed, gynomonoecious individuals are generally described as very plastic, which could be adaptive for several reasons[1]:

[1]However, the plasticity of the gynomonoecious phenotype could also be explained by the fact that it is more difficult to canalize a heterogeneous phenotype than a homogenous one.

– Plasticity could allow gynomonoecious plants to produce more perfect flowers when pollen availability is low – in order to ensure fertilization of their ovules – and to produce more pistillate flowers when pollen availability is high – in order to take advantage of outcrossing.

– Plasticity could promote the production of the type of flowers or gametes that cost fewer resources when environmental conditions are harsh (see discussion of ARTICLE 3).

In gynodioecious species having gynomonoecious individuals, few studies focused on plasticity and to date there is no evidence that phenotypic plasticity for plant or flower sex is adaptive. Moreover, the plasticity of the gynomonoecious phenotype has rarely been quantified and the two studies (including our own) that investigated this showed that intra-individual variation in the sex phenotype is lower that inter-individual variation (Widén and Widén, 1999, ARTICLE 3). Therefore overall plasticity of the gynomonoecious phenotype may have been generally over-estimated, perhaps because phenotyping only a sample of the flowers borne by an individual increases the variance of the estimated phenotype. Quantifying phenotypic plasticity in other gynodioecious species is thus needed before studying the possible adaptiveness of plasticity.

However, some interesting results have been obtained in pure gynomonoecious species and motivate further investigations in gynodioecious species. For instance in the gynomonoecious *Silene noctiflora*, Folke and Delph (1997) have shown that the proportion of pistillate flowers increased with temperature. The authors suggested that this plasticity could be adaptive since pollinator activity increases with temperature and would ensure the fertilization of pistillate flowers. But to my knowledge, plasticity has been shown to be adaptive in only one study: In *Solidago altissima*, a low availability in nutrients in the soil enhances the production of pistillate flowers and plants with the smallest femaleness are the most fit in rich environments while plants with the highest femaleness are the most fit in poor environments (Wise et al., 2008). Nevertheless, the evolutionary impact of plasticity was likely to be low because the amplitude of the plasticity was small.

Gynomonoecy might also be adaptive *per se*, independently of plasticity. This advantage belongs to bet-hedging strategies that take advantage of the reduction of variance around the mean fitness. Gynomonoecious plants that produce both perfect and pistillate flowers could theoretically benefit from a bet-hedging strategy between minimizing inbreeding depression by producing many pistillate flowers and minimizing pollen limitation by producing more perfect ones. Gynomonoecious *Silene noctiflora* could benefit from this strategy (Davis and Delph, 2005). On the contrary, Collin and Shykoff (2003) have shown in gynodioecious *Dianthus sylvestris* that pistillate flowers do not benefit from a higher outcrossing rate than perfect flowers in gynomonoecious plants. Moreover, pollen limitation has rarely been found in gynodioecious species (see chapter 3).

If there is no adaptive advantage to gynomonoecy, one may also imagine that the gynomonoecious phenotype results from developmental errors. By definition, developmental errors are rare events (if not, it is rather called polymorphism) and can hardly explain gynomonoecious plants carrying pistillate and perfect flowers in similar proportions. It can however explain the gynomonoecious plants that produce only a few pistillate flowers, and particularly when these flowers are produced at the beginning of flowering. The initialization of the flowering physiology (hormones, etc.) could indeed be characterized by early dysfunction. On the other hand, it is expected that stressful conditions would enhance developmental errors and therefore increase the proportion of gynomonoecious plants. None of our data suggest that the gynomonoecious phenotype can result from a biotic or an abiotic stress (see the end of the discussion in ARTICLE 2) but this hypothesis requires proper testing.

5.0.4.3 Influence of gynomonoecy on the evolution of gynodioecy

In *Silene nutans*, as in most species in which the gynomonoecious phenotype has been studied carefully, the proportion of gynomonoecious individuals is high and this phenotype is therefore likely to play a role in the dynamics of evolution of gynodioecy. However, no theoretical study has taken into account the existence of these individuals. The reasons for this are simple:

- First, the reproductive traits of gynomonoecious plants are poorly known (Chapter 2) and in this point of view, our study in *Silene nutans* contributes to a better understanding of the gynomonoecious phenotype (ARTICLE 2). On the other hand, it also tends to make it more complex since we have shown that gynomonoecious individuals were divided into two classes with different reproductive characteristics. This distinction, although often mentioned, has never been included in the analysis of floral and reproductive traits in other studies and the generalization of these two categories to gynomonoecy in general remains to be demonstrated.
- The second and main reason is the lack of information about the genetic and environmental control of the gynomonoecious phenotype. We have demonstrated that the femaleness of gynomonoecious plants was at least partially genetically determined in *Silene nutans* (ARTICLE 3). However, neither the literature nor our data allow elimination of one of two hypotheses of genetic control.

To study the impact of gynomonoecious plants on the evolution of gynodioecy, it is therefore necessary to choose one of the two genetic determination hypotheses and to define the male and female fitnesses of gynomonoecious individuals.

Let us first assume a determination by quantitative restoration. Even if the impact of the gynomonoecious phenotype has never been modeled, there is a model of gynodioecy that assumes that restoration is quantitative (Bailey and Delph, 2007b). In this model, the level of restoration is a quantitative variable with 6 values (0 to 5). Above a given threshold of restoration (set to 3 in the model), the plant is

hermaphrodite, below, it is female. Two CMS, each restored independently and according to this principle, were modeled and different restoration costs tested. Bailey and Delph (2007b) showed that the results were similar to those obtain in equivalent models in which restoration is controlled by a single locus (Bailey et al., 2003; Gouyon et al., 1991). What would the presence of gynomonoecious individuals change in the model?

Let assume that the phenotype is no longer determined by one threshold, but by two thresholds that define females, hermaphrodites and gynomonoecious plants (see FIGURE 5.1A). The impact of gynomonoecious plants will depend on their male and female fitnesses at the individual level (the characteristics of perfect or pistillate flowers have no importance since all flowers transmit the same genetic information). If the male and female fitnesses of gynomonoecious plants are intermediate to those of females and hermaphrodites, which is plausible (see chapter 3 and ARTICLE 2), the addition of gynomonoecious plants in the original model should have a similar effect on the evolution of CMS and restorer genes as that of a shift of the restoration threshold in a model without gynomonoecious plants (see FIGURE 5.1A). Unfortunately, variation in the threshold values has not been tested in the model of Bailey and Delph (2007b) and these effects are difficult to predict. Indeed, a shift of the restoration threshold to the right produces immediately more females but should also change the selection on restorers and could move the curve to the right. On the other hand, the difference $T_1 - T_2$ should also influence the proportion of gynomonoecious plants.

Speculation under the heteroplasmy assumption is more difficult because no model has ever considered that the sterilizing effect of a CMS can be quantitative. Moreover, it requires knowing the reproductive traits of the pistillate and perfect flowers and not only the overall fitness of the individual. Let us first imagine that gynomonoecious plants are rare and negligible and that the proportions of male-fertile and male-sterile mitochondria [2] within an individual determines if it is female or hermaphrodite (see FIGURE 5.1B). If heteroplasmy is rare, the proportion of male-fertile mitochondria of an individual is almost always equal to 0 or 1 and the model is similar to standard models of gynodioecy. If heteroplasmy is not rare, females can carry some male-fertile mitochondria and hermaphrodites some male-sterile mitochondria, which can imply several consequences:

- Male-fertile mitochondria carried by females benefit from female advantage in seed production. This will decrease the relative advantage of male-sterile mitochondria compared with male-fertile ones.
- On the contrary, male-sterile mitochondria carried by hermaphrodites no longer benefit from female advantage, which strengthens the effect mentioned above.
- Assuming that heteroplasmy is generated by paternal leakage, hermaphrodites transmit their (mainly male-fertile) mitochondria through pollen, which gives

[2] Male-sterile and male-fertile genomes are defined relative to the restorers carried by the individual.

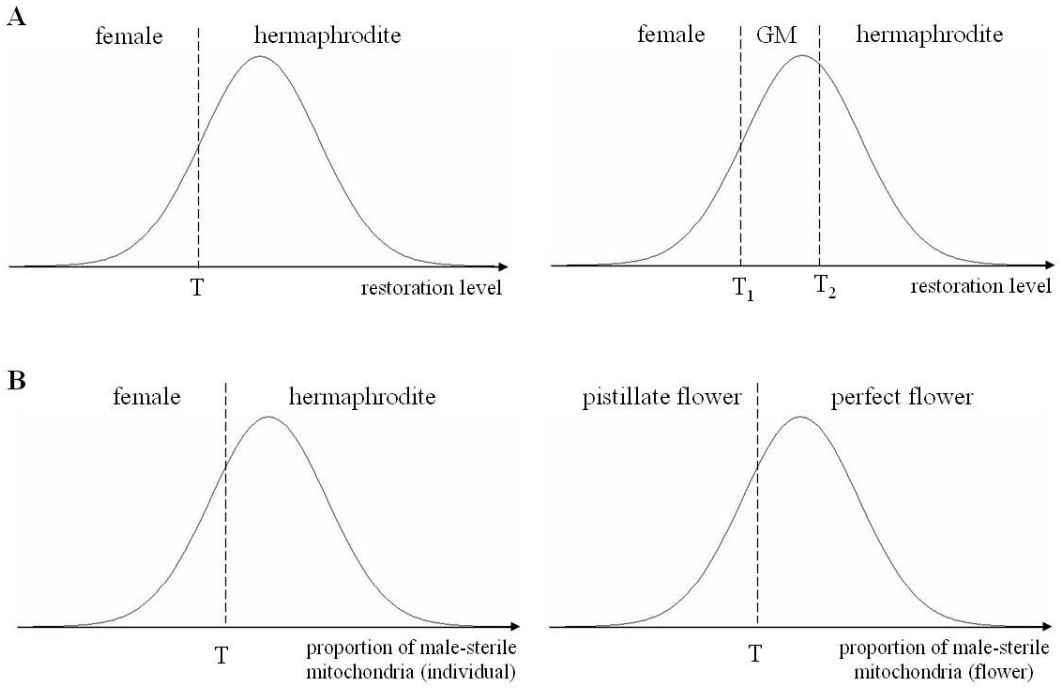


Figure 5.1: Sex determination under the quantitative restoration hypothesis (A) or the heteroplasmy hypothesis (B). **(A)** Restoration is a quantitative variable. On the left, gynomonoecious individuals are not considered. Below a given threshold of restoration, named T, individuals are females, above, they are hermaphrodites. On the right, gynomonoecious individuals are added thanks to two thresholds that define the three sex phenotypes. If gynomonoecious plants have reproductive traits similar to those of females (resp. hermaphrodites), this model is identical to the previous one (one the left) with $T \approx T_2$ (resp. $T \approx T_1$). If gynomonoecious plants have intermediate characteristics between those of females and hermaphrodites, intermediate results between these two extremes ($T = T_1$ and $T = T_2$) are expected. **(B)** Under the heteroplasmy hypothesis, sex is also a quantitative variable but is determined by the proportions of male-sterile and male-fertile mitochondria. On the left, gynomonoecious individuals are not considered and the proportion of male-fertile mitochondria in the individual determines if it is female or hermaphrodite. On the right, this determination occurs at the flower level, which allows the addition of gynomonoecious plants to the model. GM: gynomonoecious plants. The distributions have been assumed to be normal for illustration purposes.

an advantage to male-fertile mitochondria (see the effect of paternal leakage on the maintenance of the CMS, described in Wade and McCauley, 2005).

It is thus expected that considering the CMS as a quantitative variable leads to a reduction of the male-sterile mitochondria advantage. Including gynomonoecious plants in such a model does not fundamentally change its principle but requires that sex determination occurs at the flower level (see FIGURE 5.1B). However, the level of sex determination (flower or individual) has no influence if the pistillate flowers of gynomonoecious plants and females (resp. the perfect flowers of gynomonoecious plants and hermaphrodites) have the same reproductive characteristics. Otherwise, gynomonoecious individuals must be modeled as such and a better knowledge of their origin (maintenance of heteroplasmy over generations, paternal leakage, etc.) is essential for constructing the model.

5.0.5 Conclusions & Perspectives

Since two of the studies undertaken during this thesis are still ongoing, the perspectives for the near future have already been mentioned in the discussions of ARTICLES 3 and 4, and the expected data on the genetic determination of gynomonoecy (ARTICLE 3) should constitute a good starting point for the construction of models including gynomonoecious individuals.

Regarding further perspectives, I have already widely discussed in this conclusion perspectives on gynomonoecy and on paternal leakage and heteroplasmy. Other perspectives, that are perhaps more conventional but not less important are related to aspects that I have left aside during my thesis, such as the impact of pollen limitation, mating system and all other spatial effects. Indeed, all experiments were carried out in artificial populations composed of progenies from controlled crosses. If this approach has the advantage of taking into account the effects of family and better controlling the environmental conditions, it does not mimic the spatial heterogeneity of natural populations. In particular, estimations of fitness (ARTICLE 2) could be significantly different in natural populations, for instance because of pollen limitation and/or inbreeding depression. Similarly the non-maternal transmission rate was estimated on crosses designed to maximize the probability of detection of paternal leakage and this rate could be much lower in structured natural populations.

Finally, the identification of different CMS genes and the associated restorers (ARTICLE 1) constitutes an interesting basis for the study of the dynamics of gynodioecy at the large spatial scale. A study is in progress on the distribution of mitochondrial haplotypes in Europe and includes the haplotypes studied in ARTICLE 1. Associated with experimental studies on the proportion of females and the relationship between phenotype and mitochondrial haplotype in natural populations, our work could permit a better understanding of the dynamics of CMS and restorers at large spatial scale.

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Résumé

Chez les plantes à fleurs, la gynodioécie – système dans lequel coexistent des individus femelles et des individus hermaphrodites – est le système de reproduction le plus commun après l'hermaphrodisme. La question de l'évolution et surtout du maintien de la gynodioécie et du polymorphisme génétique sous-jacent a intrigué les chercheurs depuis le XIX^{e} siècle. Aujourd'hui, les grands principes de son évolution sont posés mais beaucoup de zones d'ombres persistent. Durant ma thèse, j'ai exploré trois aspects de la gynodioécie en utilisant une approche expérimentale chez l'espèce *Silene nutans* et une approche théorique. Je me suis en premier lieu intéressée au déterminisme génétique de la gynodioécie grâce à la réalisation de croisements contrôlés qui m'ont permis de montrer que le déterminisme génétique du sexe était cytonucléaire, c'est à dire contrôlé par plusieurs gènes de stérilité mâle cytoplasmique (CMS) et plusieurs restaurateurs nucléaires de fertilité. En parallèle, j'ai porté une attention particulière aux plantes gynomonoïques – celles où coexistent sur le même pied des fleurs pistillés (femelles) et des fleurs parfaites (hermaphrodites) – fréquentes chez *Silene nutans* comme chez d'autres espèces gynodioïques. J'ai montré que les caractéristiques reproductrices et florales de ce troisième phénotype sexuel étaient souvent intermédiaires entre celles des femelles et des hermaphrodites mais pouvaient dépendre de la proportion de fleurs pistillées sur la plante. Par ailleurs et contrairement à ce qui avait été suggéré, la plasticité du phénotype gynomonoïque s'est révélée être relativement réduite, suggérant un déterminisme génétique dont la caractérisation est encore en cours. La troisième partie de ma thèse a été motivée par les preuves récentes d'hétéroplasmie – coexistence de différents génomes mitochondriaux au sein d'un individu – et de la transmission occasionnelle du génome mitochondrial par le pollen chez *Silene vulgaris*. J'ai montré théoriquement que la présence d'un gène de stérilité mâle cytoplasmique favorisait l'évolution de la fuite paternelle de mitochondries. J'ai également vérifié expérimentalement l'hérédité mitochondriale chez *Silene nutans* par le génotypage des descendances de croisements contrôlés.

Mots clés: gynodioécie | gynomonoécie | Silene nutans | hérédité mitochondriale | stérilité mâle cytoplasmique | restaurateurs nucléaires | plasticité phénotypique | déterminisme génétique du sexe.

Abstract

In flowering plants, gynodioecy – a system in which females and hermaphrodites coexist within populations – is the most common sexual system after hermaphroditism. The evolution and maintenance of gynodioecy and its underlying polymorphism have puzzled evolutionary biologists since the 19^{th} century. The main principles of its evolution are well known but some points remain vague. During my PhD, I explored three aspects of gynodioecy using an experimental approach in the species *Silene nutans* and a theoretical approach. First, I studied the genetic determination of gynodioecy using controlled crosses that showed that the genetic determination of sex was cytonuclear, i.e. controlled by several cytoplasmic male sterility (CMS) genes and several nuclear restorers of fertility. Second, I focused on gynomonoecious plants – those that carry both pistillate (female) flowers and perfect (hermaphrodite) flowers – that are frequently found in *Silene nutans* as in other gynodioecious species. I showed that the floral and reproductive traits of this third sex phenotype were often intermediate between those of females and hermaphrodites but varied with varying proportions of pistillate flowers on the plant. Contrary to what was previously thought, the plasticity of the gynomonoecious phenotype was found to be limited, suggesting a genetic determination whose characterization is still in progress. The third part of my PhD was motivated by recent evidences of heteroplasmy – the coexistence of different mitochondrial genomes within an individual – and occasional transmission of the mitochondrial genome through pollen in *Silene vulgaris*. I showed theoretically that the occurrence of a cytoplasmic male sterility gene can favor the evolution of paternal leakage of mitochondria. I also investigated mitochondrial inheritance in *Silene nutans* by genotyping progenies from controlled crosses.

Keywords: gynodioecy | gynomonoecy | Silene nutans | mitochondrial inheritance | cytoplasmic male sterility | nuclear restorer | phenotypic plasticity | genetic determination of sex.