No evidence for local adaptation between cytoplasmic male sterility and nuclear restorer genes in the gynodioecious species *Thymus vulgaris* L.

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In *Thymus vulgaris* L., sex determination involves both the nuclear and the cytoplasmic genomes: the cytoplasm is responsible for male sterility (the female phenotype) whereas specific nuclear genes may restore male fertility (the hermaphrodite phenotype). The evolutionary dynamics of cytoplasmic male-sterility genes and nuclear restorer genes represents a coevolutionary conflict. Here we draw a parallel between this conflict and the coevolutionary interaction between hosts and parasites. Local adaptation of parasites to their hosts may be predicted under some situations of relative evolutionary rates of the two partners in this conflict and is often observed in natural populations. We tested for local adaptation between nuclear restorer genes and cytoplasmic male-sterility factors using 12 female plants that were hand-pollinated with pollen from hermaphrodites from more or less distant populations, and measuring progeny sex ratios. No significant effects of the geographical distance between maternal and paternal parents could be detected, although highly significant differences among individual females for the restorability of their cytoplasms were found. We suggest that the same evolutionary processes giving rise to individual differences among cytoplasmic male-sterility types might account for the absence of local adaptation between nuclear and cytoplasmic factors in this system, and also discuss the absence of local adaptation in the light of a recent model of local adaptation in host-parasite metapopulations.

Keywords: cytoplasmic male sterility, geographical distance, gynodioecy, local adaptation, progeny sex ratio, restorer genes.

Introduction

Gynodioecy, the presence of hermaphrodite and male-sterile individuals in the same population, has been the focus of many empirical and theoretical studies both because it is considered a transitory stage from hermaphroditism to dioecy (Bawa, 1980; Schultz, 1994) and because both the nuclear and the mitochondrial genomes are involved in the genetic determination of gynodioecy in many species. Indeed, the existence of cytoplasmic male sterility had been already postulated in 140 species representing 47 genera and 20 families in 1972 (Kheyr-Pour, 1980). More recently, the genetic basis of sex determination has been shown to involve nuclear and cytoplasmic factors in gynodioecious crop species (Saumitou-Laprade *et al*., 1994) as well as in gynodioecious wild species such as *Plantago lanceolata* L. (van Damme, 1983), *Origanum vulgare* L. (Khey-Pour, 1980) and *Thymus vulgaris* L. (Couvet *et al*., 1986).

The evolutionary dynamic of cytoplasmic male-sterility factors with their nuclear restorer genes represents a coevolutionary conflict (Couvet *et al*., 1990; Gouyon *et al*., 1991) as is the relationship between hosts and parasites, although the genetic basis of the interaction may be different. In host-

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parasite interactions virulence factors in parasites select for resistance by their hosts (Frank, 1991), even when virulence and resistance do not follow a simple gene-for-gene relationship, and patterns of local adaptation of parasites to their sympatric hosts are expected (Ebert, 1994) and often observed (Parker, 1985; Lively & Jokela, 1996; but see Burdon & Jarosz, 1992). Recent theoretical developments that consider host-parasite interactions in a metapopulation, where systems of populations are connected by colonization and migration, suggest that locally adapted parasites should only occur under limited conditions. Where parasite populations exchange more genes than do those of their hosts, parasites should be locally adapted. When hosts have higher migration rates than parasites, on the other hand, parasites should be less capable of infecting sympatric than allopatric hosts, i.e. they are locally maladapted (Gandon *et al.*, 1996). When new cytoplasmic factors for male sterility appear by mutation or migration in gynodioecious plant populations this induces selection for specific nuclear genes that restore male fertility (van Damme, 1982). This pattern of selection has been suggested to generate local adaptation of nuclear restorer genes to their particular cytoplasmic factors. Higher rates of restoration (a higher proportion of hermaphrodites) are therefore predicted for the progeny of crosses between two individuals from the same population compared with those between different populations (Couvet *et al.*, 1986). This is consistent with the theoretical expectations outlined by Gandon *et al.* (1996), because nuclear genes, which are carried both in pollen and seeds, migrate more than cytoplasmic genes, which are present only in seeds if the inheritance is maternal as is generally the case (Corriveau & Coleman, 1988; Reboud & Zeyl, 1994). To our knowledge the existence of local adaptation of nuclear restorer genes to cytoplasmic factors has not yet been experimentally tested.

Here we test for this phenomenon using crosses of the gynodioecious species *Thymus vulgaris* L., Labiatae. We compare within-population crosses with those between populations and between regions for their rates of restoration and test for within-population variation in male-sterility cytoplasms in their restorability.

Materials and methods

Plant materials

Thymus vulgaris (thyme) is a small perennial entomophilous shrub characteristic of dry open Mediter-

ranean vegetation. It is a pioneer species found in abandoned fields (Dommée *et al.*, 1978) and burnt habitats (Belhassen *et al.*, 1989) but remains in later stages of succession. In thyme the frequency of females varies between populations from 5 to 95% and decreases with population age (Assouad *et al.*, 1978; Dommée *et al.*, 1983; Belhassen, 1987). Plants are mainly pollinated by bees, which carry pollen over short distances (Dommée *et al.*, 1983), but butterflies have been reported also to participate in pollination over longer distances (Tarayre & Thompson, 1997). Flowering occurs every year and lasts for two to four weeks. Each plant produces hundreds of small flowers with an ovary enclosing four ovules. The hermaphrodite flowers, which are larger than female flowers, also bear four stamens. These flowers are protandrous, the pistil becoming receptive a few days after the stamens' dehiscence. Sex in thyme is determined by the interaction of cytoplasmic male-sterility genes and nuclear restorer genes (Belhassen *et al.*, 1991). The restoration of male fertility of a given cytoplasmic male sterility requires the presence of its specific restorer gene(s).

Geographical origin of parental plants

Seeds were collected from three natural populations of the region of Languedoc (Vialaret: 3°40′E, 43°49′N; Pompignan Haut: 3°52′E, 43°54′N; La Jasse: 3°46′E, 43°47′N) and two populations of Provence (Rustrel: 5°29′E, 43°55′N; Le Barroux: 5°05′E, 44°07′N) in the south of France. The three populations from the Languedoc are 10–50 km apart. The two Provence populations are situated more than 300 km from the Languedoc populations. The frequencies of hermaphrodites in these populations at the time the seeds were collected were 0.90, 0.50 and 0.55 for Pompignan Haut, La Jasse and Vialaret and 0.77 and 0.58 for Rustrel and Le Barroux, respectively, whereas the average frequency of hermaphrodites within the species is about 0.4. The frequency of hermaphrodites was measured by recording the sex of all individuals in each population. All populations contained between 100 and 200 plants. We chose populations with higher than average frequencies of hermaphrodites because hermaphrodite frequency is known to increase with increasing population age (Manicacci *et al.*, 1996), and coevolutionary processes that could lead to local adaptation should be evident in old populations where male-sterility cytoplasms have coevolved with their nuclear restorer genes.

Seeds were sown in the greenhouse and seedlings were transplanted into an experimental field. When

these plants flowered, 12 females and 50 hermaphrodites were chosen for the experiment as follows. Four females originating from each of the three Languedoc populations (12 altogether), each from a different mother, were chosen at random and transplanted to an insect-free greenhouse. Fifty hermaphrodites (10 from each of the five populations) were chosen at random to be used as pollen donors the following spring.

Crossing protocol

Three types of crosses were performed on each of the 12 females (for exact protocol see Fig. 1): (i) within-population crosses with pollen from the same population as the female; (ii) between-population crosses with pollen from a population different from the female but of the same region (Languedoc); (iii) between-region crosses with pollen from a population located in a different region (Provence).

Each female individual was divided into three equivalent parts using screens. The flowers of each part received pollen from the 10 hermaphrodites from each chosen paternal population, to simulate the pollen cloud of this population. To avoid pollen competition, each female flower was pollinated with pollen from a single hermaphroditic flower. Approximately 200 flowers were pollinated per female per treatment.

Seed families

Seeds produced from each of the 36 (12 females × three treatments) combinations were collected two months later, germinated and cultivated in pots in the greenhouse. Individuals originating from each combination shared a common mother and 10 fathers coming from a single population; they will be referred to as a family. We obtained 20–50 offspring per family. One year later, at flowering, the frequency of hermaphrodites and females was scored in each family.

Statistical analysis

All statistical analyses were conducted using the GLIM statistical package (Baker, 1987). We performed two independent analyses. The first tested the effects of cross type (within population, between populations within region, and between regions), maternal population, maternal individual and their interactions on the numbers of hermaphrodites and females within each progeny using a logistic regression. The second analysis investigated the effect of paternal population, testing for specific interactions between maternal individual, maternal population and paternal population, without considering the distance between populations. In both analyses maternal individual was nested within maternal population. Cross type effect was declared as a fixed factor. It was possible to calculate the interactions between maternal plant and cross type or paternal population to determine whether different mothers reacted differently to the three types of cross and to the five paternal populations, respectively.

The total model was somewhat underdispersed, as demonstrated by the error mean deviance of less

Hermaphrodites		Paternal populations		
Females	♀ / ♂ ♀	Region LANGUEDOC		Region PROVENCE
		Within-population 10 ♂	Between-population 10 ♂	Between-region 10 ♂
Pompignan	Mother 1	Pompignan	La Jasse	Rustrel
	Mother 2		Vialaret	Rustrel
	Mother 3		La Jasse	Le Barroux
	Mother 4		Vialaret	Le Barroux
La Jasse	Mother 5	La Jasse	Pompignan	Rustrel
	Mother 6		Vialaret	Rustrel
	Mother 7		Pompignan	Le Barroux
	Mother 8		Vialaret	Le Barroux
Vialaret	Mother 9	Vialaret	Pompignan	Rustrel
	Mother 10		La Jasse	Rustrel
	Mother 11		Pompignan	Le Barroux
	Mother 12		La Jasse	Le Barroux

Fig. 1 Design of the experiment specifying the paternal populations used for each cross type.

than one. Because testing against this error term would artificially inflate the F-value, for those effects that would be tested against the error mean deviance we compared the deviance directly against the χ^2 expectation with appropriate degrees of freedom. These effects were Mother (Maternal population) and the interactions between this factor and Type of cross in the first analysis and Paternal population in the second analysis.

In addition, to examine the relationship between sex ratios produced by each mother in the different cross types we calculated the correlation between sex ratio produced by the within-population cross and sex ratio produced by the other crosses. This tests whether the restoration by one cross type is a good predictor of restoration by other cross types.

Results

The proportion of hermaphrodites (the sex ratio) observed in the 36 families was highly variable (Table 1), ranging from 0 to 1. Although, on

Table 1 The number of hermaphrodites produced by each mother in each cross type of *Thymus vulgaris*

	Within population	Between population	Between region
	6 (59)	9 (64)	2 (40)
Mother 1	*0.10*	*0.14*	*0.05*
	12 (19)	10 (22)	17 (27)
Mother 2	*0.63*	*0.45*	*0.63*
	4 (21)	14 (59)	2 (30)
Mother 3	*0.19*	*0.24*	*0.07*
	10 (27)	0 (30)	4 (48)
Mother 4	*0.37*	*0*	*0.08*
	11 (47)	0 (21)	1 (41)
Mother 5	*0.23*	*0*	*0.02*
	9 (26)	3 (31)	9 (40)
Mother 6	*0.35*	*0.10*	*0.23*
	0 (34)	1 (44)	10 (37)
Mother 7	*0*	*0.02*	*0.27*
	0 (33)	3 (45)	1 (27)
Mother 8	*0*	*0.07*	*0.04*
	14 (34)	0 (45)	1 (33)
Mother 9	*0.41*	*0*	*0.03*
	30 (30)	25 (25)	50 (50)
Mother 10	*1*	*1*	*1*
	17 (29)	11 (36)	5 (67)
Mother 11	*0.59*	*0.31*	*0.07*
	14 (30)	24 (45)	23 (39)
Mother 12	*0.47*	*0.53*	*0.59*

Numbers in parentheses represent the total number of progeny per mother and cross. Sex ratio values (proportion of hermaphrodites) are given in italics.

average, the proportion of hermaphrodites was larger in within-population crosses than in between-population or between-region crosses (0.36 ± 0.08, 0.26 ± 0.09 and 0.24 ± 0.09, respectively; mean $\pm$ SE; see Table 1), the logistic regressions showed that variation in numbers of hermaphrodites and females within families was explained by differences between mothers within maternal populations and the interaction between mothers within maternal populations and both the type of cross in the first analysis and the particular paternal population in the second (Table 2). However, no main effects of cross type (the distance between maternal and paternal populations), paternal nor maternal population of origin were found.

Progeny sex ratios generated in the within-population crosses were very good predictors of progeny sex ratios from both other cross types (Fig. 2). This demonstrates that some maternal parents were well restored in all crosses whereas some were poorly restored overall. Between-population crosses restored better than between-region crosses for some maternal plants although for others the opposite was true, as demonstrated by the significant interactions in Table 2. In addition, most data points lay below the diagonal for equal restoration for all three cross types, showing that most within-population crosses restored somewhat better than between-population or between-region crosses (Fig. 2).

Discussion

In this study, using females of *T. vulgaris* crossed with pollen from sympatric and more or less distant allopatric populations, we found no evidence for local adaptation but strong differences between cytoplasms in their restoration ability. The existence of different cytoplasmic male-sterility types within a single population has been reported in several gynodioecious species: *Plantago lanceolata* (van Damme, 1982), *Plantago coronopus* (Koelewijn & van Damme, 1995) and *Beta vulgaris* ssp. *maritima* (Boutin *et al.*, 1987; Saumitou-Laprade *et al.*, 1993). In thyme, the existence of at least four cytoplasmic male-sterility types within populations has been demonstrated (Manicacci *et al.*, 1997), but this probably underestimates population variation. Even more polymorphism is suggested by the distribution of sexual phenotypes within natural populations, and by the high level of molecular polymorphism observed for cytotypes (Belhassen *et al.*, 1993; Manicacci *et al.*, 1996). Although it is difficult to establish a clear relationship between molecular and functional diversity, several studies (Belhassen *et al.*,

Table 2 Mixed-model hierarchical logistic regression of the number of hermaphrodites and females in the 36 progenies of *Thymus vulgaris*

Source of variation	d.f.	MD	F-value	P
Maternal population	2	76.24	2.13	NS
Mother (Maternal population)†	9	35.72	—	***
(a)				
Type of cross	2	12.65	2.35	NS
Type of cross × Maternal population	4	3.30	0.61	NS
Type of cross × Mother (Maternal population)†	18	5.38	—	***
(b)				
Paternal population	4	9.28	3.16	NS
Paternal population × Maternal population	8	7.86	2.68	NS
Paternal population × Mother (Maternal population)†	12	2.93	—	***
Error	1299	0.72		

All factors sequentially fitted to the model are specified in the left column. The first analysis (a) tested for Type of cross effect and interactions with other factors. The second analysis (b) tested for the Paternal population effect and interactions with other factors. The Maternal population effect was tested using the Mother (Maternal population) as denominator, Type of cross and the interaction between the Type of cross and the Maternal population effects were tested using the interaction between the Mother (Maternal population) and the Type of cross effects as denominator.
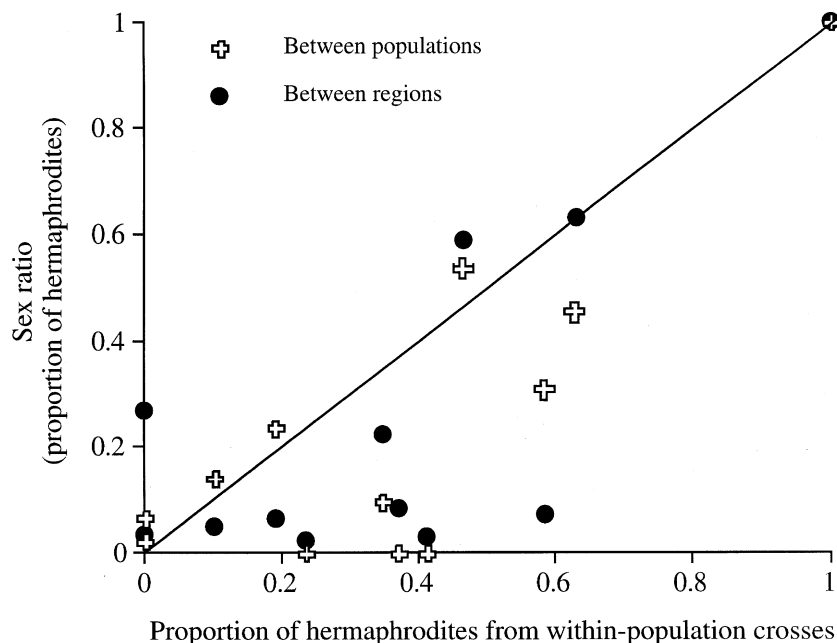†Because of underdispersion of the model (error mean deviance <1), these effects were tested by comparing the deviance against the χ^2. MD, mean deviance.
NS, $P>0.05$; ***, $P<0.0001$.

1993; Cuguen *et al.*, 1994; Ronfort *et al.*, 1995) have shown that they are linked.

In our thyme populations some mothers carried a cytoplasmic male-sterility type that was readily restored whereas others carried a poorly restored cytoplasmic male-sterility type (Table 1). Indeed, some mothers (mothers 10 and 2, for example) produced a high rate of hermaphrodites regardless of the paternal population they were mated to, but some others (mothers 8 and 3, for example) always

Fig. 2 Sex ratios from between-population and between-region crosses of *Thymus vulgaris* plotted against the sex ratio from the within-population cross for each mother. The diagonal line represents the line of equal restoration for the three cross types. Pearson product-moment correlations were significant both for between-population and between-region crosses ($N = 12$, $r = 0.81$, $P = 0.0014$ and $N = 12$, $r = 0.74$, $P = 0.0056$, respectively).

produced a low sex ratio (Table 1). This was observed in all maternal populations and no significant differences were obtained. In addition to this difference in general restoration ability, mothers also differed in the nature of their response to the different cross types. Some mothers were equally well or poorly restored by all cross types whereas others varied greatly (Table 1). For the latter, restoration was generally better by sympatric pollen (Fig. 2), but because few females showed this pattern, there was no significant overall effect of local adaptation. Comparable results have been obtained for a larger selection of populations from the Languedoc Region (Manicacci *et al.*, 1997).

This experiment therefore did not support the hypothesis of local adaptation between nuclear and cytoplasmic sex determination factors in *T. vulgaris* (Couvet *et al.*, 1986). We found no evidence that nuclear restorer genes were better at restoring their sympatric cytoplasms than they were at restoring allopatric cytoplasms. Nor was there an effect of distance between the populations of origin for maternal or paternal parents. Unfortunately our statistical power was limited for this analysis, with only four maternal plants for each of three populations. Indeed, we would have been able to find local adaptation only if at least 10 of the 12 females had been better restored by sympatric than allopatric pollen, using a conservative sign test. Possibly therefore our inability to find local adaptation might have resulted from low detection ability. Alternatively, we might have sampled at an inappropriate geographical scale, and local effects may have been found if we had chosen a finer one.

If local adaptation is truly lacking in this system, this could result from several causes. First, as stated above, the fact that different females responded differently to the three cross types suggests that there is no general pattern of local adaptation. These differences between mothers could result from the fact that some cytoplasmic types, on account of the biochemical basis of male sterility, are more easily restored than others, as has been shown for *Plantago lanceolata* (van Damme, 1983). Such male-sterility cytoplasms may be restored by many different unspecific restorer genes and therefore by a diversity of cross types or paternal populations. Another possibility is that if there is little cost of restorer genes, the restorer genes for cytoplasmic types that have existed for a long time can be expected to spread even to populations where the specific cytoplasm is absent. This process generates a loss of local adaptation with increasing age of a cytoplasmic type. Sampling a range of ages of cyto-

plasmic male-sterility types would therefore generate the large differences among mothers for their response to cross type and thus their degree of local adaptation.

A different possibility to explain the lack of local adaptation between nuclear restorer and cytoplasmic male-sterility factors is that the same cytoplasmic types exist over a broad geographical range, as has been shown for *Beta maritima* (Boutin *et al.*, 1987; Saumitou-Laprade *et al.*, 1993). In this case appropriate restorer genes would also be selected throughout, leading to an absence of geographical pattern when pollinations are performed with pollen mixtures. This situation might result from repeated mutation to the same male-sterility factor, or from a high rate of migration of cytoplasms, through high seed flow. In fact, in coevolutionary conflicts local adaptation is only predicted when migration rates or, similarly, mutation rates have particular restricted values per generation for both partners (Gandon *et al.*, 1996). Even if migration rates are appropriate for the evolution of local adaptation, observations from a particular moment during the coevolutionary cycle can show lag phases where the more rapidly evolving player trails the slower one in a transitory manner (Nee, 1989). However, the parallel between host-parasite and cytoplasmic male-sterility restorer genes warrants further examination, because cytoplasmic male sterility and restorer genes modify the fitness of their bearers in a different way than do virulence and resistance genes.

Conclusion

Here we present a number of hypotheses that might explain the lack of local adaptation between male-sterility cytoplasms and their nuclear restorer genes. These hypotheses are not mutually exclusive, and a combination of these effects could generate the patterns observed. High variation for neutral markers associated with male-sterility cytoplasms has been found in thyme (Manicacci *et al.*, 1996), suggesting that widespread cytoplasms are uncommon. Therefore, it is unlikely that local adaptation is absent because the same cytoplasm is found over a large geographical area. Clearly the strong maternal effect together with the highly significant interactions of maternal effect with both distance between maternal and paternal populations and paternal population itself indicate that cytoplasms differ in how easily they are restored by different nuclear restorer genes. At the same time, low cost of restoration could allow restorer alleles to accumulate. Estimates of the real costs of restoration have

proved difficult to obtain, so low or even absent costs can reasonably be supposed (de Haan *et al.*, 1997). Therefore, we consider that a combination of the last two factors would be the most likely explanation for a lack of local adaptation.

Acknowledgements

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