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Complex determination of male sterility in *Thymus vulgaris* L.: genetic and molecular analysis

E. Belhassen [1], B. Dommée, A. Atlan, P.H. Gouyon [2], D. Pomente, M.W. Assouad and D. Couvet

Laboratory of the Evolution of Genetic Systems, CEPE, CNRS Route de Mende, BP 5051, F-34033 Montpellier Cedex, France
[1] Station of Genetics and Plant breeding, INRA Center of Montpellier, Domaine de Melgueil, F-34130 Mauguio, France
[2] Laboratory of the Systematics and Evolution of Plants, Bldg. 362, University of Paris-Sud, F-91405 Orsay Cedex, France

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Summary. Nucleocytoplasmic determination of male sterility in *Thymus vulgaris* L. has been assumed in all papers attempting to explain the remarkably high frequencies of male steriles found in natural populations of this species. This paper provides strong evidence that both nuclear and cytoplasmic genes are involved in the determination of male sterility of this species, giving a complex inheritance. Interpopulation and intrapopulation crosses have shown that the ratio of females versus hermaphrodites among offsprings varied widely from 1:0 to 1:1. Furthermore, interpopulation crosses consistently yielded a higher frequency of females than intrapopulation crosses. Nucleocytoplasmic inheritance was demonstrated by an absence of male fertiles in backcrosses and asymmetrical segregation in reciprocal crosses. Molecular analysis of the mitochondrial DNA of some of the parents used in crosses suggested the involvement of different cytoplasms in the inheritance of male steriliy.

Key words: Male sterility – Nucleocytoplasmic interaction – Complex inheritance – *Thymus vulgaris*

Introduction

Gynodioecy is the occurrence of female and hermaphrodite individuals together in natural populations of a species. The females can be considered as hermaphrodites that have lost their male function, and are called "male steriles", while hermaphrodites are "male fertiles." The maintenance of male sterility has been a topic of interest for a long time. Correns (1908) first showed that the determination of male sterility in several plant species was maternally inherited, and Lewis (1941) analyzed the conditions for maintenance of male sterility under purely nuclear and purely cytoplasmic inheritance.

Later studies on wild plant species considered the determination of male sterility to be purely nuclear. For instance, it was claimed that only nuclear genes were involved in the determination of male sterility in *Origanum vulgare* (Lewis and Crowe 1956) and in *Plantago lanceolata* (Ross 1969). In fact, results from wild species were based on only a few selected crosses, and increasing the number of parents of crosses leads frequently to very complicated results, which are not always suitable for publication. Spectacular examples have been provided more recently for both of the above wild species, when extending the number of parents in the analysis of determination of male sterility. Kheyr-Pour (1980) for *Origanum vulgare* and Van Damme (1983) for *Plantago lanceolata* showed that cytoplasmic genes were also involved in determining male sterility in those species. Furthermore, both works noted that the number of loci involved in the determination was highly dependent on the number and origin of the parents used in the crosses.

Nucleocytoplasmic determination of male sterility has been inferred by crossing experiments in many cultivated species, such as sugar beet (Owen 1942), maize (Duvick 1965), wheat (Maan and Lucken 1972), barley (Ahokas 1979), chives (Tatlioglu 1987), and sorghum (Bailey-Serres et al. 1986). Crop species have been extensively studied because of the economic importance of male sterility. Indeed, for self-compatible species, the production of hybrid seed is made much easier when using male-sterile plants. Studies on wild species (Ganders 1978; Kheyr-Pour 1980, 1981; Duc 1982; Thiellement 1982; Van Damme 1983; Stevens and Richards 1985) are less frequent, but are of major importance for the understanding of the evolution of gynodioecy.

Thymus vulgaris (thyme) is a species where male sterile plants exist spontaneously among male fertiles in natural populations. The male sterility is expressed by the absence of stamens on flowers (Assouad 1972). Using this perennial plant, more than 160 genetic crosses have been carried out among parents coming from eight natural populations (Assouad 1972; Dommée 1973). Results were very complex, depending on which cross was examined and where the parents were originally located. Although a hypothesis involving nuclear and cytoplasmic inheritance was proposed (e.g., Delannay et al. 1981; Couvet et al. 1986; Belhassen et al. 1989, 1990), it was impossible to produce any overall explanation or a formal proof of cytoplasmic inheritance. Here, we wish to show that male sterility inheritance in *T. vulgaris* is complex and that it is controlled by a large number of both nuclear and cytoplasmic loci. This species is remarkable for the very high frequencies of females found in natural populations (up to 95%; Dommée and Jacquard 1985; Belhassen et al. 1989). All the models that have been proposed to explain such high female frequencies (e.g., Delannay et al. 1981; Charlesworth 1981; Couvet et al. 1986; Frank 1988) deal with nucleocytoplasmic determination of male sterility. However, no results have been published to date showing that both nuclear and cytoplasmic genes are involved in thyme.

There are two ways of inferring nucleocytoplasmic inheritance. First, crossing experiments can provide direct evidence for interactions of nuclear and cytoplasmic genes. Second, molecular analysis of cytoplasmic genes can readily furnish correlations of cytoplasmic DNA patterns with phenotypic expression of sex.

When using crossing experiments, a first classical genetic method to prove cytoplasmic inheritance is to carry out reciprocal crosses between hermaphrodites (where the hermaphrodites are used as male and female, successively). A difference of segregation between the two reciprocal crosses indicates maternal inheritance (Grun 1976). A second method is to show that the progeny of a hermaphrodite and a female is entirely composed of females as are the successive backcrosses. The reason for this is that progeny of backcrosses for n generations will bear $1-(1/2)^n$ of the fathers' genome, i.e., the progeny's successive generations will bear a nuclear genome more and more identical to the father. Thus, if successive backcrosses between a hermaphrodite and a female line give only female offspring, cytoplasmic inheritance of male sterility must be involved.

Molecular analyses of several species reveal that cytoplasmic genes involved in male sterility are located in mitochondria (see Newton 1988, for a review). Mitochondria of females are different from those of hermaphrodites in their DNA structure (Bailey-Serres et al. 1986; Dewey et al. 1987; Boutin et al. 1987; Rouwendal et al. 1987), their transcript RNA, and their protein synthesis (Ford and Leaver 1980; Dixon and Leaver 1982; Boutry et al. 1984; Dewey et al. 1987).

In this paper, we describe experiments showing that the determination of male sterility in *T. vulgaris* is controlled by both nuclear and cytoplasmic genes. For this, crossing experiments were conducted, including reciprocal and backcrosses. Furthermore, a molecular analysis of the mitochondrial DNA of some of the parents used in crosses was performed, in order to (1) correlate male sterility with mitochondrial patterns, and (2) allow unambiguous characterization of the female lines in crosses and in populations.

Materials and methods

Thyme is a perennial *Labiateae* in which hermaphrodites and females can easily be distinguished when in flower. Crosses were performed to determine male sterility inheritance, and the mitochondrial DNA pattern of some of the parents used was also analyzed.

Crosses

Four types of crosses (interpopulation crosses, intrapopulation crosses, backcrosses, and reciprocal crosses) were carried out in order ot determine the inheritance. Furthermore, some selected crosses from the 160 crosses performed by Assouad (1972) and Dommée (1973) were repeated in order to show the typical nuclear segregation obtained in thyme. All crosses were carried out in a greenhouse, each plant being protected from contamination with an "insect-proof cage," which prevents unwanted pollinations (thyme is pollinated essentially by bees). After harvest, seeds were germinated in sterile petri dishes containing moistened cotton wool and filter paper. Seedlings were grown in the greenhouse and transplanted to an experimental garden when sufficiently established.

Interpopulation crosses

Fourteen hermaphrodites from three populations (J2, P1, P2) from Languedoc in southern France were crossed with 14 isofemales [all 14 females were daughters of the same female (J1-44) and thus had the same cytoplasmic genes].

Intrapopulation crosses

Ten hermaphrodites from population J1 from Languedoc were crossed with ten isofemales (J1-44 cytoplasm).

Back crosses

Hermaphrodites and females were taken from the J1 population. Six hermaphrodites were crossed with nine isofemales descended from the same grandmother (J1-44 female). In the first generation of offspring, one of the hermaphrodites (H168) gave almost all female progeny (see Table 3). Three females – F201, F202, and F203 – from one of these crosses were backcrossed with this hermaphrodite. A second generation of backcrosses was performed with two females (F301, F302, offspring from F201 and F202, respectively and the father (H168).

Reciprocal crosses

Eight hermaphroditic plants from several populations were each divided into two halves, one used as a pollen donor (male) and

the other as a pollen receptor (female). In order to avoid contaminations by self-pollination, branches were enclosed in bags before flowering. Because *T. vulgaris* has protandrous flowers (hermaphroditic flowers are functionally male before being functionally female), hand-pollinations of hermaphrodite flowers were carried out when the four stamens opened (at this time, the stigmas began to be receptive). No parasitic pollinations were detected on control flowers that were not hand-pollinated.

Mitochondrial DNA analysis

The mitochondrial DNA (mtDNA) of the test female (F125, which carries the J1-44 cytoplasm) and of three hermaphrodites (H168, H142, H174) used in the backcross experiment was characterized (Table 5).

The mtDNA was analyzed according to the minipreparation of total DNA of Dellaporta et al. (1983), with the following modification. Four grams of leaf tissue was used instead of 0.75 g. The extraction buffer was highly enriched in mercaptoethanol (up to 50 m*M*) because of quick oxydation of the ground tissue. Precipitated DNA was incubated at −70 °C for 30 min. Proteins were extracted three times with a mixture of phenol (1 vol.), chloroform (1 vol.), and metacresol (1/5 vol). Restriction endonuclease digestion of the total DNA was carried out under conditions suggested by the suppliers, however, with an addition of spermidine (final concentration = 4 m*M*) to the digestion medium. The DNA was electrophoresed on 0.8% agarose slab gels buffered with TEA.

Digested total DNA was transferred on Zeta-Probes (Bio-Rad) membranes using a vacuum blot. mtDNA was hybridized with heterologous probes under stringent conditions. A ribosomal mtDNA probe from wheat (18S–5S) (Falconet et al. 1984), the fragment *Xho*I-*Sal*I issued from the fragment of the subunit II of cytochrome oxidase from wheat (*COX II;* see Bonen et al. 1984), and the subunit a of the ATPase from *Oenothera* (α-ATPase; see Schuster and Brennicke 1986) were used. The detection was carried out with the nonradioactive system of Chemiprobe-Orgenics (see Lebacq et al. 1988).

Results

Selected crosses

The 15 crosses shown in Table 1 support a purely nuclear determination of male sterility. A segregation of 1:1 could be interpreted most easily as the results of a single nuclear locus with a recessive restorer gene. However, in addition to those 15 crosses, more than 200 crosses gave much more complicated results (Assouad 1972; Dommée 1973).

Interpopulation and intrapopulation crosses

The ratio of females versus hermaphrodites varied widely in both interpopulation and intrapopulation crosses (Tables 2 and 3). The most frequent segregations observed were 1:0 (females : hermaphrodites), followed by 5:3, 3:1; and 1:1. These results are in agreement with those of Assouad (1972) and Dommée (1973).

Interpopulation crosses consistently yielded a higher frequency of females than intrapopulation crosses. Indeed, while in the interpopulation crosses 6 out of 14 hermaphrodites (Table 2) were nonrestorer, all the

Table 1. Selected crosses where 1:1 segregations were observed

Cross F × H	Offspring F:H	χ^2 ($df=1$)
C54 × L18	77:77	0.00
M2 × P4	12:14	0.07
C43 × C19	57:58	0.00
33 × 971	38:31	0.35
V2 × C503	44:33	0.93
C40 × C502	14:18	0.24
F3 × F37	15:21	0.49
C583 × C503	33:25	0.55
C43 × O2	15:10	0.49
C5 × C2	19:14	0.37
412 × 414	12:15	0.16
212 × 214	30:18	1.44
1419 × 1421	21:25	0.17
C40 × C56	43:31	0.97
C440 × C49	10:10	0.00

Chi-square was computed between observed segregation ratio and 1:1

Table 2. Interpopulation crosses

Cross F × H	Father origin	Offspring F:H	Expected ratio	χ^2
F1 × H2	P1	33: 0	1:0	–
F5 × H7	P1	52: 1	63:1	0.00
F6 × H8	P1	48:34	5:3	0.55
F10 × H12	P1	41:45	1:1	0.19
F8 × H13	P1	60: 0	1:0	–
F11 × H14	P1	64: 0	1:0	–
F12 × H17	P1	57: 0	1:0	–
F14 × H21	P1	68:18	3:1	0.76
F15 × H20	P1	61: 3	15:1	0.40
F16 × H23	P1	33: 0	1:0	–
F3 × H4	P2	36: 7	7:1	0.56
F9 × H11	J2	49:30	5:3	0.01
F13 × H16	J2	25:15	5:3	0.00
F17 × H24	J2	87: 0	1:0	–

Fourteen daughters of J1–44 female (issued from population J1) with 14 hermaphrodites issued from three populations (P1, P2, J2). F and H indicate female and hermaphrodite, respectively

Table 3. Intrapopulation crosses

Cross F × H	Offspring F:H	Expected ratio	χ^2
F1 × H1	39: 1	15:1	0.42
F2 × H3	25:11	3:1	0.59
F4 × H5	30:29	1:1	0.02
F5 × H6	28:17	5:3	0.00
F7 × H9	57:22	3:1	0.34
F8 × H10	75: 7	15:1	0.73
F12 × H15	36:15	3:1	0.53
F14 × H18	33:48	1:1	2.77
F17 × H19	55:15	3:1	0.48
F16 × H22	39:32	1:1	0.69

Ten daughters of J1–44 female with ten hermaphrodites issued from population J1. F and H indicate female and hermaphrodite, respectively

Table 4. Number of crosses with no restoration and with restoration

Crosses	No restoration	Restoration
Intrapopulation	37	65
Interpopulation	71	68

No restoration occured with crosses giving only female offspring, and restoration occured with crosses giving both hermaphrodite and female offsprings. Comparison between inter- and intrapopulation crosses shows that no restoration occurred more frequently for interpopulation crosses ($G=5.22$; $df=1$; $P<0.025$)

Table 5. First generation of crosses in population J1

Cross F × H	Offspring F:H
F107 × H168	44: 0
F115 × H168	20: 2
F125 × H168	31: 1
F117 × H168	9: 0
Total	104: 3
F107 × H171	1: 1
F114 × H171	44: 7
F117 × H171	53: 4
F124 × H171	28: 3
Total	126:15
F108 × H142	13: 0
F116 × H142	40: 4
F118 × H142	25:22
F125 × H142	27:11
Total	105:37
F107 × H143	7: 2
F116 × H143	41: 0
F124 × H143	22: 9
F117 × H143	9: 2
Total	79:13
F108 × H169	18: 3
F115 × H169	5: 2
F118 × H169	17: 7
F125 × H169	16: 4
Total	56:16
F108 × H174	18:11
F115 × H174	30:11
F118 × H174	18:11
F125 × H174	20:14
Total	86:47

Nine daughters of J1–44 female were crossed with six hermaphrodites issued from population J1

Table 6. First and second generations of backcrosses

Cross F × H	Offspring F:H
First backcross generation	
F201 × H168	62:0
F202 × H168	48:0
F203 × H168	72:1
Second backcross generation	
F301 × H168	5:0
F302 × H168	6:0

Table 7. Reciprocal crosses between hermaphrodites

Cross F × H	Offspring F:H	Offspring of reciprocal cross F:H	Heterogeneity χ^2 ($df=1$)
98 × 122	6:17	10: 0	18.28***
2 × T4	43: 0	75: 5	3.96*
3 × 1	50:24	11: 0	7.48**
6 × T4	95: 4	66:10	4.70*
6 × 1	19:12	54: 4	12.88***
3 × 2	7: 2	35: 0	4.71*
4 × 1	4: 4	48: 2	8.85**
1 × T4	57: 0	21: 2	3.87*

Significance levels of heterogeneity test:
* $P<0.05$
** $P<0.01$
*** $P<0.001$

hermaphrodites of the intrapopulation crosses restored male fertility (Table 3). Hermaphrodites of different populations were nonrestorer significantly more frequently than hermaphrodites of the same population as the test female [G-test, Sokal and Rohlf (1981), p735; $G=7.19$, $df=1$, $P<0.01$]. Only one hermaphrodite of the J1 population was found to be a nonrestorer (H168) in the backcross experiment (Table 5). In other crosses not detailed here, the same influence due to the origin of the father remains (Table 4; $G=5.22$, $df=1$, $P<0.025$).

Backcrosses

All the segregations of the first generation of crosses were female biased (Table 5). The H168 hermaphrodite gave a very female-biased segregation (97% of females). This hermaphrodite was used for backcrosses. The first and second generations of backcrosses gave a segregation of 1:0, except for one cross, where one hermaphrodite was found among 72 females (Table 6).

Reciprocal crosses

Reciprocal crosses gave significant asymmetrical segregations (Table 7). In nine other crosses not shown here, segregations were not significantly different.

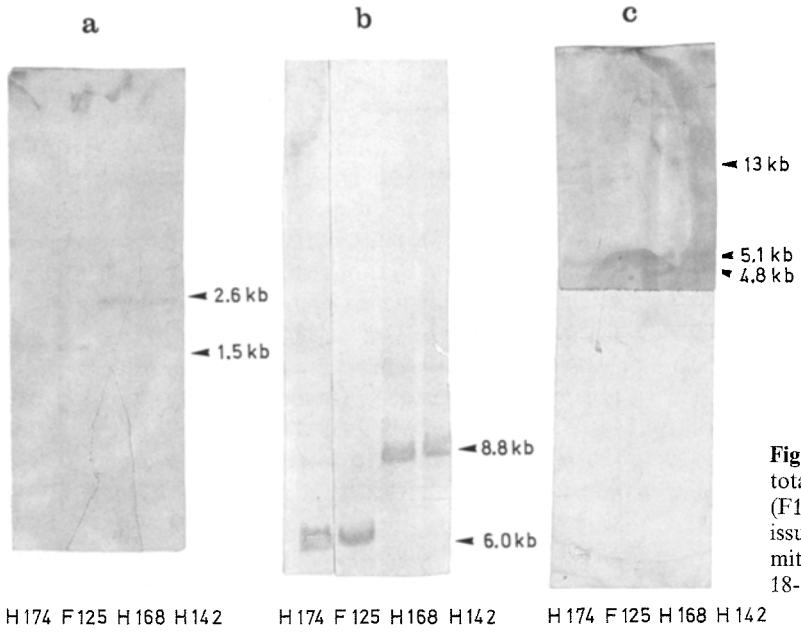

Fig. 1a–c. Southern blot hybridizations of HindIII total DNA digested with HindIII, of the test female (F125) and three hermaphrodites (H174, H168, H142) issued from population J1. The three heterologous mitochondrial probes (**a**: *CoxII*, **b**: a-ATPase, **c**: 18-5S) were immunoenzymatically labelled

Molecular analysis

MtDNA partial patterns were always different between the test female (F125) and the nonrestorer hermaphrodite (H168) for all probes (Fig. 1). Clearly, these individuals had different mitochondrial genomes. The restorer hermaphrodite H174 (Table 5) apparently had similar mtDNA to F125, since none of the partial patterns distinguished them (Fig. 1). Hermaphrodites H168 and H142 had virtually identical patterns (Fig. 1).

Discussion

The data reported here show that the determination of male sterility in *Thymus vulgaris* is controlled by both nuclear and cytoplasmic genes. The most frequent segregations observed were 1:0 (females:hermaphrodites), followed by 5:3, 3:1, and 1:1. Such results as 1:1 segregation ratios attested to the existence of nuclear genes controlling male sterility expression. If only these crosses were considered from the results, the genetic determination of male sterility in thyme would have been described as a simple nuclear system (see Introduction).

The reciprocal crosses demonstrate that cytoplasmic genes act on the determination of male sterility in *T. vulgaris*. If only nuclear genes are responsible for male sterility, no differences between reciprocal crosses would be expected. The significant heterogeneity found between crosses (Table 7) indicates that non-Mendelian inheritance occurs and that the hermaphrodites do not have the same cytoplasmic genes.

Backcrosses further support the hypothesis of a cytoplasmic inheritance of sex. Indeed, the backcrosses gave only female offspring, which indicates that the hermaphrodites and females tested had different cytoplasmic genes. One of the backcrosses produced one hermaphrodite among 72 females (Table 6). This segregation is not different from 1:63 ($\chi^2 = 0.00$) or 1:32 ($\chi^2 = 0.34$), which would require at least five or six loci in the determination of male sterility. It is highly probable that this hermaphrodite is either a contamination or a cytoplasmic revertant (as, e.g., in maize – Singh and Laughnan 1972; Pring and Lonsdale 1985). Unfortunately, this individual died during flowering and further investigations are not possible.

The analysis of mtDNA patterns confirmed the results found with crosses. For a cross in which a hermaphrodite does not restore a female, the cytoplasmic genes of these individuals are expected to be different. Mitochondria, which could contain genes involved in the expression of male sterility are, in most angiosperm species, maternally inherited (for a review, see Birky 1978). In thyme, we have shown that mitochondria are maternally inherited (Belhassen 1989; Belhassen et al. submitted). We showed that these genetic differences are correlated with different mtDNA patterns. For example, the nonrestorer hermaphrodite H168 and the female F125 have different mtDNA patterns. Furthermore, the mtDNA analysis allows some predictions concerning the nuclear restorer genes. Assuming that identical mtDNA structure is correlated with similar genetic behavior of cytoplasmic genes, we expect that if two hermaphrodites have identical mtDNA patterns, and if one is restorer while the other is nonrestorer, differences in nuclear restorer genes are probably involved. For instance, the nonrestorer hermaphrodite H168 and the restorer hermaphrodite H142 had the same mtDNA pattern. Therefore, H142 may have additional restorer genes of a

cytoplasm other than its own, those of the F125 cytoplasm. Further analysis should be carried out to determine if mitochondrial DNA is the only source of cytoplasmic genes involved in the male sterility of *T. vulgaris*.

MtDNA analysis reveals that significant variation in mtDNA patterns seems to exist within populations. For the four individuals analyzed that issued from population J1, we discerned two mitochondrial DNA types. This important intrapopulation polymorphism has been confirmed in a complete study of several natural populations of *T. vulgaris* (Belhassen et al. submitted). Such a polymorphism will certainly act on the dynamics of male sterility (Belhassen 1989).

The results here point out the existence of a differentiation among populations of nuclear genes causing male sterility (Tables 2–4). Hermaphrodites coming from the same population of a test female more frequently have the specific restorer genes than do hermaphrodites from different populations. Genetic differentiation of populations coupled with colonization and extinction has been suggested as an important factor for the maintenance of high proportions of females in the wild (Couvet et al. 1985; Dommée and Jacquard 1985; Gouyon and Couvet 1985; Couvet et al. 1986; Belhassen et al. 1989, 1990).

In this paper, we provide arguments using both genetic and molecular analysis that male sterility in *T. vulgaris* is due to nucleocytoplasmic interactions. This was assumed in all the models describing the evolution of gynodioecy in *T. vulgaris* (Gouyon and Couvet 1985; Couvet et al. 1986; Belhassen et al. 1989).

Furthermore, evidence is presented that the inheritance in *T. vulgaris* is complex and that it is a general feature of male sterility inheritance. In thyme, certain models dealing with at least five loci have been proposed to explain some of the observed segregations (Couvet 1984). However, these models always fail to explain all the observed results and thus are only of passing interest. The interactions of nuclear genes are very complex and prevent the establishment of a general model in *T. vulgaris* for the present.

In fact, it is often not possible to propose a general genetic model that correctly interprets all the results (Kheyr-Pour 1980; Van Damme 1983; Stevens and Richards 1985; J. Antonovics, personal communication). The complexity of the genetics of male sterility systems seems to be very common. One of the most carefully studied cases in *Plantago lanceolata* (Van Damme 1983), where the possibility of distinguishing the two different cytoplasms by the morphology of the anthers has permitted the proposal of a model with at least five nuclear loci. Here, the parents of the crosses were sampled in an area of only 20 m^2, and yet here too the conclusion was that "not all results can be explained this way, which emphasizes the complexity of nuclear inheritance" (Van Damme 1983).

As mentioned in the Introduction, crosses performed with small sets of individuals can lead to oversimplification of the system, as demonstrated in *Origanum vulgare* (Kheyr-Pour 1980) and *Plantago lanceolata* (Van Damme 1983). Thus, the simple nuclear determination proposed by Horovitz and Dulberger (1983) on *Silene vulgaris* might be demonstrably insufficient if other crosses are performed. This is actually acknowledged by the authors (A. Horovitz, personal communication), who advanced this possibility in the paper itself: "Yet we ask whether there is also room for the suggestion that male-sterility-conferring factors may be present secondarily in cytoplasmic DNA."

Another kind of oversimplification is the description given by plant breeders, who defined a normal cytoplasm in relation to a sterile one. The so-called normal cytoplasm is, in fact, likely to be a "sterile cytoplasm," for which all restorer genes are present. This has been demonstrated in Colza (Thompson 1972), in which an exotic strain ("Bronowsky") possesses a nuclear genome that produces male sterility when associated with the previously "normal" cytoplasm.

Investigations can be made easier by the introduction of the molecular tools in such studies, which produce a large amount of new informations, not accessible with classical methods. Here, the analysis of mitochondrial DNA coupled with results from crosses allows us to define the cytoplasmic and nuclear genotype of several hermaphrodites. It is clear that the systematic use of the RFLP tools in a crossing program could improve genetic investigations in such complex systems.

Gynodioecy results from long-conflicting coevolutionary processes (Cosmides and Tooby 1981; Gouyon and Couvet 1985). Our results, together with the results in the literature, show that the process results in high complexity of the genetic determination of the male sterility.

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