Theor Appl Genet (1987) 73:625–629



# Cytoplasmic male sterility and nuclear restorer genes in a natural population of *Beta maritima*: genetical and molecular aspects

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Received June 16, 1986; Accepted November 22, 1986
Communicated by G. Wenzel

**Summary.** One natural population ($F_0$ generation) of *Beta maritima* situated on the French Atlantic coast has been analysed. It was composed of 62% female, 30% hermaphrodite and 8% intermediate plants. The analysis of half-sib progeny ($F_1$ generation) obtained from in situ open pollination demonstrates the cytoplasmic determination of male sterility in *Beta maritima* and the restoration of fertility by nuclear genes. The mitochondrial DNA (mtDNA) and the chloroplast DNA (ctDNA) of sixteen $F_1$ plants, extracted from offspring of the three sexual phenotypes, were analysed using the restriction enzymes *Sal* I and *Bam* HI, respectively. Two cytoplasmic lines with their own peculiar genetic characteristics were distinguished using the restriction enzyme patterns of mtDNA: (i) the S cytoplasmic line was found in segregating progeny of two $F_0$ plants; all three phenotypes were produced (that is, progeny including hermaphrodite, female and intermediate plants); (ii) the N cytoplasmic line was found in the progeny of one $F_0$ hermaphrodite plant; this produced only hermaphrodites. Thus, segregating and non-segregating hermaphrodite $F_0$ plants can be distinguished. The nuclear genes maintaining sterility or restoring fertility are expressed in line S. At the same time the analysis of *Beta vulgaris* material has been carried out at the molecular level : N cytoplasmic lines of *B. vulgaris* and *B. maritima* differed only by 3 fragments of mtDNA; but the S cytoplasmic line of *B. maritima* was very different from Owen's cytoplasmic male sterile line of *B. vulgaris*. No variation in the ctDNA pattern was detected within and between the two taxa.

**Key words:** Wild and cultivated sugar beet (*Beta maritima*, *Beta vulgaris*) – Cytoplasmic male sterility, nuclear restorer genes – Mitochondrial and chloroplast DNAs – Restriction endonuclease fragment analysis

## Introduction

The analysis of male sterility in the genus *Beta* began with the very well known and classical work of Owen (1942, 1945) who focused attention on cultivated varieties of *Beta vulgaris*.

Owen (1942, 1945) demonstrated that male sterility in *B. vulgaris* is based on two components: one cytoplasmic, the other nuclear. Specific nuclear restorer genes restore male fertility in S (male-sterile) cytoplasm. A plant with S cytoplasm is female if at least two nuclear restorers are present in the recessive homozygote state, but hermaphrodite if these genes possess the alternative dominant form. An intermediate sexual phenotype is produced when only one restorer gene occurs in the homozygous recessive state. These nuclear genes are also found in normal cytoplasm, which Owen called the N line. Plants with N cytoplasm are always hermaphrodite irrespective of the nuclear restorer alleles they contain. Among these plants two types are especially useful in selection breeding programs: (i) the maintainer of sterility, which is characterized by the presence of recessive homozygote restorer alleles and which permits the reproduction of female (= male sterile) plants by sexual means; (ii) the restorer of fertility: this type of plant possesses the nuclear restorer genes in the dominant allelic form and in the homozygote state; on crossing with any female plant with S cytoplasm, non-segregating progeny comprised of only hermaphrodite plants are produced. Further work has confirmed the nuclear and cytoplasmic factors of this male sterility but varying results concerning the number of nuclear loci have been reported. Two or three loci of restorer genes have been proposed by some authors (Oldemeyer 1957; Hogaboam 1957; Nagao and Kinochita 1962; Bliss and Gabelman 1965; Bolz 1968) while others have presented evidence for a polygenic determination (Rohrbach 1965; Knapp 1969; Bosemark 1972).

In order to improve the biological understanding of male sterility in *Beta vulgaris*, we began a study of natural populations of the ancestral form, *Beta maritima*. First, populations of *B. maritima* along the Atlantic coast of France were surveyed for the occurrence of female plants. Females were found to comprise 5% of the population in Mont-Saint-Michel Bay (Varinard unpubl.) and were found in even greater frequency in the Canche Estuary at the southern end of the English Channel. The presence of females in these numbers has to be explained and cannot be

considered as an adaptation promoting outcrossing since *B. maritima* is self-incompatible. However, the population-genetic model proposed by Gouyon (Gouyon et al. 1983; Couvet et al. 1985; Gouyon and Couvet 1985) explains the occurrence of female plants by interaction between nuclear and cytoplasmic genes. It also explains the presence of several cytoplasmic sterility and nuclear restorer genes within a single population (Khey-Pour 1976, in *Origanum vulgare;* Van Damme 1984, in *Plantago lanceolata;* Dommée *et al.* 1983; Dommée and Jacquard 1985, in *Thymus vulgaris*).

In addition to these genetic studies, molecular analysis of mitochondrial DNA (mtDNA) was undertaken in the genus *Beta* because some work in other taxa had shown an association between mtDNA and male sterility. Powling (1981) first reported the existence of short circular DNA molecules in *Beta vulgaris* mitochondria. Then, with Owen's material and using restriction enzymes, he demonstrated that mtDNA from sterile cytoplasm differed from normal mtDNA (Powling 1982). The same author tested the 'US1' variety and found an exact concordance between the digestion patterns of mtDNA and the sexual phenotypes (Powling and Ellis 1983). Moreover, he observed no variation between the chloroplast DNA (ctDNA) of the S and of the N lines. Kinoshita's team (Mikami et al. 1985) extended the molecular analysis to wild beets from Oldemeyer's collection (USDA) without knowing the precise origins and genetic composition of the wild populations from which they were taken. The same authors distinguished four mtDNA groups within the S cytoplasm, one of them being identical to the mtDNA of Owen's strain of *Beta vulgaris.* Finally, they also found a variability of the chloroplast genome among S plants.

This work represents a joint genetic and molecular analysis of one *Beta maritima* population and of the plants obtained from it. *Beta vulgaris* material is included for comparison. The genetic part of the study was carried out at Lille University (France), and the molecular genetic part at Düsseldorf University (FRG). The cytoplasmic and nuclear factors determining the observed male sterility in *Beta maritima* are characterized. The mitochondrial genome of sterile plants is demonstrated to be different from that of *Beta vulgaris.*

## Materials and methods

### *The population and its offspring*

The *B. maritima* population forming the $F_0$ generation is situated in the Canche estuary near the town of Etaples, South of Boulogne-sur-Mer (France). It was composed of about 60 plants that covered an area approximately 40 m long and 10 m wide. The plants belonged to one of the three sexual phenotypes defined at anthesis by the characters of anthers and pollen: 1. hermaphrodite plants (H), with yellow stamens containing viable and functional pollen; 2. intermediate plants (I), with more or less dehiscent anthers containing little pollen, a part of which was empty; plants of this class were heterogeneous because of large variations in viable pollen; 3. female plants (Fe), with white stamens lacking pollen.

Pollen was analysed in June 1984 in situ using a binocular microscope. Ten plants were examined again in June 1985 and thus the stability of sexual phenotypes was established over two years.

Fruits were harvested between 10–20 August 1984. Their seeds were sown in February 1985, and seedlings were individually planted in small pots in the experimental garden of the University of Lille, and these formed the $F_1$ generation. Plants obtained from 1 $F_0$ plant represent a half-sib family whose size varied from 4 to 80 $F_1$ plants. In the different families not all plants reached the flowering state, and several families with fewer than 10 plants were not taken into account. Thus, 926 $F_1$ plants, shared between 41 families, were available for the genetic analysis.

### *Plant material used for biochemical analysis*

*Beta maritima.* 16 $F_1$ plants from the Canche estuary, 1 hermaphrodite plant from the botanical garden of Düsseldorf University. Its natural origin is not known.

*Beta vulgaris.* Clone no. 0049Y1, cytoplasmic male fertile, clone no. 0052A1, cytoplasmic male sterile; both clones were kindly provided by Kleinwanzlebener Saatzucht AG, Einbeck (FRG).

The sexual phenotypes and the provenance of the 16 plants originating from the Canche population were as follows:
6 hermaphrodite plants were taken among the 57 $F_1$ plants of the B21 family composed only of hermaphrodite plants; the B21 mother plant belonged to the "non-segregating hermaphrodite" type; material from the six plants was pooled for the DNA preparation; 3 hermaphrodite and 2 intermediate plants were taken from 20 $F_1$ plants of the B62 family; this family is composed of 5 hermaphrodite, 13 intermediate and 2 female plants; the B62 mother plant belonged to the "segregating hermaphrodite" type; 5 intermediate plants were taken from 63 $F_1$ plants originating from the female B9; this family is composed of 4 hermaphrodite, 31 intermediate and 28 female plants.

### *DNA analysis*

*a) Isolation of mitochondrial DNA.* Mitochondrial DNA was isolated from 400 g of leaves and shoots from *Beta maritima* or from about 800 g of taproot tissue from *Beta vulgaris,* according to the method of Boutry and Briquet (1982) with the following modification: a sucrose cushion (0.6 M sucrose, 0.01 M TES, 0.02 M EDTA, pH 7.2), instead of a discontinuous sucrose gradient, was used as the last step in the purification of mitochondria. Centrifugation was performed at 9,000 rpm for 20 min in a Sorvall SS-34 rotor. One gram of mitochondria was lysed in 4 ml lysis buffer (5% SDS, 2% sarkosylate, 150 mM NaCl, 20 mM EDTA, 10 mM Tris-HCl, pH 8.0) for 10 min at room temperature. After phenol extractions the DNA was precipitated by ethanol. The CsCl density gradient centrifugation was replaced by centrifugation through a discontinuous CsCl gradient, following the method of Kolodner and Tewari (1975).

*b) Isolation of chloroplast DNA.* All steps of the preparation were performed at 4 °C. Leaves (20 g) were blended in 10 volumes of homogenization medium (Boutry and Briquet 1982). After filtration through one layer of gauze and two layers of miracloth the chloroplasts were pelleted in a Sorvall GSA rotor at 5,000 rpm for 5 min. They were washed twice in 200 ml of the same buffer. The chloroplast fraction was resuspended in 10 ml of homogenization medium, layered on a discontinuous sucrose gradient (15 ml 20% sucrose, 15 ml 40% sucrose in homogenization buffer) and centrifuged in a Sorvall HB-4 rotor at 5,000 rpm for 15 min. The chloroplast fraction was diluted with the same volume of TE buffer (50 mM Tris-HCl, 20 mM EDTA, pH 7.2). After centrifugation the chloroplast pellet was lysed for 1 h at 37 °C with three volumes of the following medium: 2% sodium sarkosylate, 1% SDS, 0.1 M NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 8.4, supplemented with 0.1 mg/ml

proteinase K. The chloroplast DNA was purified in a discontinuous CsCl gradient according to Kolodner and Tewari (1975).

*c) Restriction and gel electrophoresis of DNA.* Restriction endonuclease digestions were carried out under conditions suggested by the suppliers. The DNA was electrophoresed on agarose slab gels buffered with 40 mM Tris-HCl, 20 mM Na-acetate, 2 mM EDTA, 18 mM NaCl, pH 8.0.

## Results

*Description of the in situ population and of its offspring*

The Canche population contained a high proportion of females. Of 66 plants studied, 62% were females, 8% intermediates and 30% hermaphrodites. From these 66 plants, 41 half-sib progeny were analysed. Female and intermediate plants (i.e. 27/41) always generated segregating progeny (composed of two or three phenotypes). Offspring of hermaphrodites are separated into two groups: (i) segregating hermaphrodites (5 plants) which generate two or three sexual phenotypes as do the female and intermediate plants mentioned above; (ii) non-segregating hermaphrodites (9 plants) which produce only hermaphrodite plants. The problem is to understand the genetic basis of the two types of progeny. They cannot be explained by maternal inheritance because female plants should produce only females. We have therefore to distinguish between pure nuclear inheritance and interaction between nuclear and cytoplasmic factors. The molecular analysis might help us to choose between the alternatives.

*Analysis of mitochondrial and chloroplast DNA (mtDNA and ctDNA)*

*a) Restriction patterns of mtDNA.* Mitochondrial DNA was isolated from the various lines of *Beta maritima* and *Beta vulgaris* described in Materials and methods. The *Sal* I digests of mtDNA from *Beta maritima* plants of the Canche population are compared in Fig. 1. Two types of restriction patterns can be distinguished. One type is represented by family B62 (lanes 3 and 4 of Fig. 1). This family comprises plants with different sexual phenotypes (H and I). An identical *Sal* I restriction pattern is obtained with mtDNA of the progeny from the female plant B9 (lane 1 of Fig. 1). The second type of mitochondrial DNA is found in the B21 non-segregating hermaphrodite family (lane 2 of Fig. 1; lane 4 of Fig. 2) and in the hermaphrodite *Beta maritima* from the botanical garden of the University of Düsseldorf (lane 3 of Fig. 2).

As described already in the literature, hermaphrodite and female plants of *Beta vulgaris* possess different *Sal* I restriction patterns (lanes 5 and 6 of Fig. 2). These patterns differ from those of *Beta maritima*, as shown in Fig. 2. While the differences between hermaphrodite and female plants of *B. vulgaris* involve many fragments, the homologous restriction patterns of *B. maritima* show fewer variations. The hermaphrodite plants of *Beta vulgaris* (lane 5 of Fig. 2) and the hermaphrodite non-segregating plants of *Beta maritima* (lane 4 of Fig. 2) differ only by three DNA fragments.

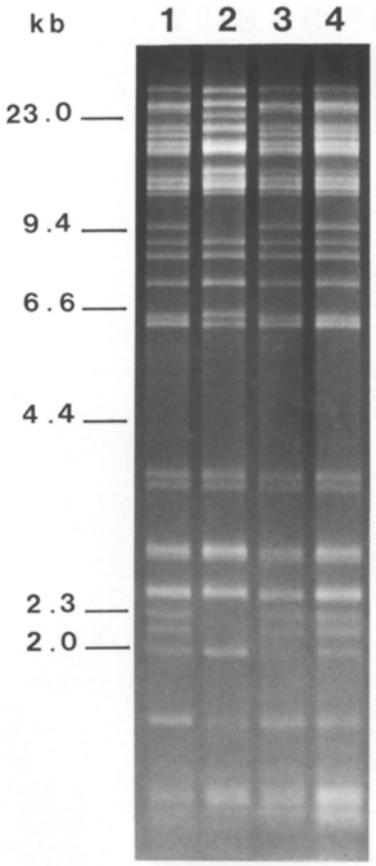


**Fig. 1.** *Sal* I digests of mitochondrial DNA from *Beta maritima* plants (origin: Canche population). Mitochondrial DNA from progeny of: *1* female plant (B9): intermediate offspring (mtDNA from 5 plants); *2* hermaphrodite non-segregating plant (B21): mtDNA from 6 hermaphrodite plants; *3* hermaphrodite segregating plant (B62): hermaphrodite offspring (mtDNA from 3 plants); *4* hermaphrodite segregating plant (B62): intermediate offspring (mtDNA from 2 plants)

From these data it can be concluded that the wild population of *B. maritima* contains two mitochondrial genomes: the genome of female, intermediate and hermaphrodite segregating plants and the genome of non-segregating hermaphrodite plants. These two mitochondrial genomes can be distinguished from those of *Beta vulgaris*. Thus, the male sterile cytoplasms of *Beta vulgaris* and *Beta maritima* reveal characteristic differences.

*b) Restriction pattern of ctDNA.* The variation of mitochondrial DNA contrasts with the uniform *Bam* HI

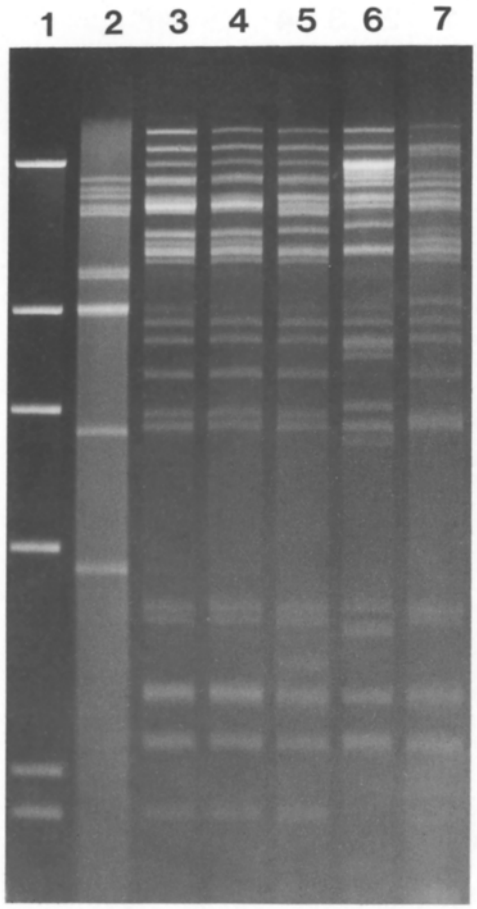


**Fig. 2.** *Sal* I digests of mitochondrial DNA from various *Beta maritima* (*B.m.*) and *Beta vulgaris* (*B.v.*) plants. *1* Lambda DNA digested with Hind III as marker; *2 Sal* I digest of chloroplast DNA from *Beta maritima* (Düsseldorf) as a control; *3 B.m.* from Düsseldorf botanical garden (origin and progeny unknown); mtDNA of one hermaphrodite plant; *4 B.m.* from Canche population:hermaphrodite offspring (mtDNA from 6 plants) of the hermaphrodite non-segregating plant B21 (idem Fig. 1: lane 2); *5 B.v.* from KWS cytoplasmic male fertile (=hermaphrodite clone 0049Y1; *6 B.v.* from KWS cytoplasmic male sterile (=female) clone 0052A1; *7 B.m.* from Canche population:intermediate offspring (mtDNA from 2 plants) of the hermaphrodite segregating plant B62 (idem Fig. 1: lane 4)

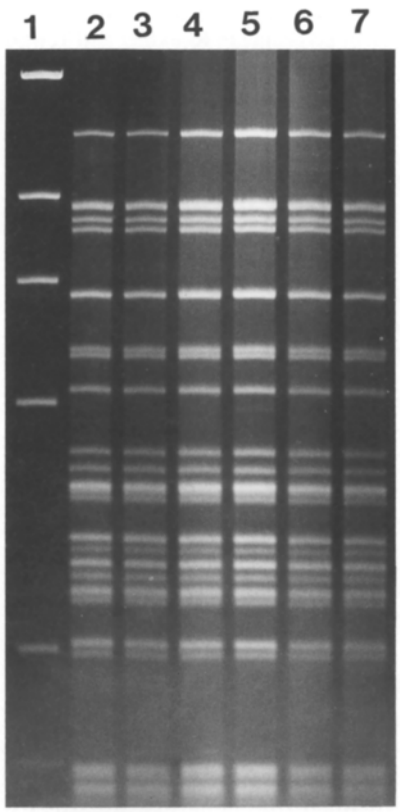


**Fig. 3.** *Bam* HI digests of chloroplast DNA from various *Beta maritima* (*B.m.*) and *Beta vulgaris* (*B.v.*) plants. *1* Lambda DNA digested with Hind III as marker; *2 B.m.* from Canche population:intermediate offspring of the female plant B9; *3 B.m.* from Canche population:intermediate offspring of the hermaphrodite segregating plant B62; *4 B.m.* from Canche population:hermaphrodite offspring of the hermaphrodite segregating plant B62; *5 B.m.* from Canche population:hermaphrodite offspring of the hermaphrodite non-segregating plant B21; *6 B.v.* from KWS cytoplasmic male fertile (=hermaphrodite) clone 0049Y1; *7 B.v.* from KWS cytoplasmic male sterile (=female) clone 0052A1

restriction pattern of ctDNA (Fig. 3). Not a single deviation could be observed when the various chloroplast DNAs of *B. maritima* and *B. vulgaris* were compared.

## Discussion

Only one cytoplasm conferring male sterility is so far available for sugar beet breeding and cultivation (for review see Barocka 1985). Therefore, it would be interesting to obtain new male sterile material which could be used as an alternative to Owen's S line. Studying the natural variability of the ancestral form of *Beta maritima* along the Atlantic coast of France, we were able to identify male sterile plants in the wild population. The genetic factors of this male sterility in *Beta maritima* can be clearly deduced from the present results. The nuclear component is demonstrated by the fact that females segregate and give hermaphrodites among their progeny. The number of nuclear restorer genes involved requires a further analysis. The existence of the cytoplasmic component was shown by mtDNA differences among maternal offspring. On one hand, the female, the segregating hermaphrodite and their progeny plants (whatever their sexual phenotype) all present the same mtDNA, while on the other hand, the non-segregating hermaphrodite plants present a clearly different mtDNA pattern. Thus, the joint genetic and molecular analysis of these three plants and their progeny allow the detection of two cytoplasmic types within the

population. These results can be extended to all plants of the population. The more abundant one, the S cytoplasm, in which the male sterility genes and their restorers are expressed, gives the population its gynodioecious character. The second one, the N cytoplasm, gives all the plants and their progeny the hermaphrodite phenotype. Thus, the population contains two types of hermaphrodites which cannot be separated by their sexual phenotypes but can be distinguished either by the sexual phenotype of their progeny or by their mtDNA pattern.

The cytoplasmic determination of male sterility combined with nuclear restorer genes appears more frequently than a pure nuclear one like that observed in particular *B. maritima* material by Kinoshita and Takahashi (1972). However, their study was based on only one family and so it is possible that the cytoplasmic component escaped the attention of the experimenters. Finally, genetic data obtained by authors on different species (Kheyr-Pour 1976; Van Damme 1984), molecular data obtained by Mikami et al. (1985) on *Beta*, and theoretical models (Gouyon and Couvet 1985) suggest that more than two cytoplasmic types differing by mtDNA and/or ctDNA could exist.

Mitochondrial and chloroplast DNAs from *Beta maritima* and cultivated *Beta vulgaris* plants were compared in our study. No variability of chloroplast DNA could be detected between the two taxa. Fertile plants of *Beta maritima* and *Beta vulgaris* gave closely similar restriction patterns of mitochondrial DNA. *Beta maritima* hermaphrodites from two different provenances present the same pattern and differed from *Beta vulgaris* mtDNA only by three bands. The interesting results of the present study of the Canche population demonstrate a novel cytoplasmic male sterility system which differs from that described by Owen.

*Acknowledgements.* We would like to thank Dr. K. H. Barocka, Kleinwanzlebener Saatzucht AG (Einbeck, FRG) and Strube-Dieckmann Saatzucht (Schöningen/Braunschweig, FRG) for advice and plant material. This work was supported by the GFP/BMFT grant Fr Zr 17/83-RP 2/83 to G. Michaelis, and a ATP "Biologie des Populations" no. 09 84 93 from CNRS to Ph. Vernet and R. Jean. The first author receives a research allocation from the "Ministere de l'Industrie et de la Recherche". We are also indebted to Alexis Ducousso, Daniel Petit and Myriam Valero for their help in the field and to the latter, for her useful comments. The manuscript has been improved by Dr. Doyle McKey (University of Miami, USA) and Professor David A. Jones (University of Hull, UK).

## References

Barocka KH (1985) Zucker- und Futterrüben. In: Hoffman W, Mudra A, Plarre W (eds) Lehrbuch der Züchtung landwirtschaftlicher Kulturpflanzen, vol 2, Spezieller Teil. Parey, Berlin Hamburg, pp. 245–287

Bliss FA, Gabelman WH (1965) Inheritance of male sterility in beets, *Beta vulgaris* L. Crop Sci 5:403–406

Bolz G (1968) Monohybride Vererbung der plasmatisch-genisch bedingten Pollensterilität bei *Beta vulgaris* L. Z Pflanzenzücht 60:219–234

Bosemark NO (1972) Studies of cytoplasm male sterility in sugar beet. J Inst Sugar Beet Res 6:232–251

Boutry M, Briquet M (1982) Mitochondrial modifications associated with cytoplasmic male sterility in faba beans. Eur J Biochem 127:129–135

Couvet D, Gouyon PH, Kjellberg F, Valdeyron G (1985) La différenciation nucléocytoplasmique entre populations: une cause de l'existence de male-stériles dans la population de Thym. C R Acad Sci Paris 300:665–668

Dommée B, Guillerm JL, Valdeyron G (1983) Régime de reproduction et hétérozygotie des populations de Thym, *Thymus vulgaris* L., dans une succession postculturale. C R Acad Sci Paris 296:111–114

Dommée B, Jacquard P (1985) Gynodioecy in thyme, *Thymus vulgaris* L.: evidence from successional populations. In: Jacquard P, Heim G, Antonovics J (eds) Genetic differentiation and dispersal in plants. Springer, Berlin Heidelberg New York Tokyo, pp 141–164

Gouyon PH, Couvet D (1985) Selfish cytoplasm and adaptation: variations in the reproductive system of thyme. In: Haeck J, Woldenhorp JW (eds) Structure and functioning of plant populations. North-Holland, Amsterdam Oxford New York, pp 299–319

Gouyon PH, Lumaret R, Valdeyron G, Vernet Ph (1983) Reproductive strategies and disturbance by man. In: Mooney H, Godron M (eds) Disturbance and ecosystems. Ecological studies, vol 44. Springer, Berlin Heidelberg New York Tokyo, pp 213–224

Hogaboam GJ (1957) Factors influencing phenotype expression of cytoplasmic male sterility in the sugar beet. J Am Soc Sugar Beet Technol 9:457–465

Kheyr-Pour A (1976) Nucleocytoplasmic polymorphism for male sterility in *Origanum vulgare* L. J Hered 71:253–260

Kinoshita T, Takahashi M (1972) Genic male sterility found in *Beta maritima*. Jpn J Breed 22:11–19

Knapp E (1969) Zur Genetik der plasmatisch kontrollierten Pollensterilität der Zuckerrübe. IIRB 4:145–159

Kolodner R, Tewari KK (1975) The molecular size and conformation of the chloroplast DNA from higher plants. Biochim Biophys Acta 402:372–390

Mikami T, Kishima Y, Sugiura M, Kinoshita T (1985) Organelle genome diversity in sugar beet with normal and different sources of male sterile cytoplasms. Theor Appl Genet 71:166–171

Nagao S, Kinoshita T (1962) Causal genes and character expression of male sterility in beets. J Fac Agric Hokkaido Univ 52:51–69

Oldemeyer RK (1957) Sugar beet male sterility. J Am Soc Sugar Beet Technol 9:381–386

Owen FV (1942) Male sterility in sugar beets produced by complementary effects of cytoplasmic and mendelian inheritance. Am J Bot (abstr) 29:692

Owen FV (1945) Cytoplasmically inherited male sterility in sugar beets. J Agric Res 71:423–440

Powling A (1981) Species of small DNA molecules found in mitochondria from sugar beet with normal and male sterile cytoplasms. Mol Gen Genet 183:82–84

Powling A (1982) Restriction endonuclease analysis of mitochondrial DNA from sugar beet with normal and male sterile cytoplasms. Heredity 49:117–120

Powling A, Ellis THN (1983) Studies on the organelle genomes of sugar beet with male fertile and male sterile cytoplasms. Theor Appl Genet 65:323–328

Rohrbach U (1965) Beiträge zum Problem der Pollensterilität bei *Beta vulgaris* L. 2. Untersuchungen über die Wirkung der Herkunft auf den Phänotyp des Merkmals „Pollensterilität". Z Pflanzenzücht 54:23–45

Van Damme JMM (1984) Gynodioecy in *Plantago lanceolata* L. 3. Sexual reproduction and maintenance of male steriles. Heredity 52:77–93