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New CMS types in *Plantago lanceolata* and their relatedness

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Abstract Mitochondrial variation in *Plantago lanceolata* was used to detect new CMS types. Directional reciprocal crosses were made between plants which differed in mtDNA restriction patterns. Differential segregation of male steriles in reciprocal crosses indicated that the parents differed in CMS type. MtDNA variation revealed nine RFLP patterns, which could be categorised according to the sex phenotype of the plants as MS1 (brown-anther type), MS2 (petaloid flower type) and MS3 (more yellow anthers than MS1). A single mtDNA pattern was found within MS1, six mtDNA patterns were found within the MS2 group, and two mtDNA patterns were found within hermaphrodites which segregated MS3 in the crosses. MS1 and MS2 are known to represent different CMS types, CMSI and CMSII. In reciprocal crosses between plants with different mtDNA patterns within the MS2 group, different ratios of male steriles segregated in the crosses, indicating that the parents differed in CMS type. Within the MS2 group two CMS types were found, designated CMSIIa and b. Finally, the sex phenotype H/MS3 turned out to be a different type. From previous studies it was known that the MS3 phenotype can also occur in CMSI and CMSII types, hence it is unclear whether MS3 is diagnostic for CMSIII. Since the data in this study cannot distinguish between the new type being a fully restored new CMS type or a 'Normal' cytoplasm, it was denoted as CMSIII. In total, four CMS types were found in the material studied. CtDNA variation was screened and three chloroplast haplotypes were identified. Two haplotypes were associated with CMSI plants, and one haplotype with the other CMS types. The ctDNA variation indicated that the CMSI type is widespread within the species, due to migration rather than to recurrent mutation. This may lead to the conclusion that only a limited number of CMS types are successful.

Key words *Plantago lanceolata* · Gynodioecy · CMS · ctDNA · Reciprocal crosses

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Introduction

The involvement of cytoplasmic factors has been shown in many plant species in which male sterility occurs (reviews Hanson and Conde 1985; Braun et al. 1992). In crops like maize (Laughnan and Gabay-Laughnan 1983), *Phaseolus* (Janska and Mackenzie 1993), *Nicotiana* (Håkansson et al. 1988) and *Petunia* (Hanson et al. 1988), it has been shown that this cytoplasmic factor is mitochondrial (Lonsdale 1987). Based on this knowledge, several attempts have been made to link mitochondrial variation with cytoplasmic male sterility (CMS) in several species (maize, Levings and Pring 1977; *Thymus*, Belhassen et al. 1993; *Beta maritima*, Saumitou-Laprade et al. 1993; *Brassica napus*, Handa et al. 1990; Pearl millet, Rajeshwari et al. 1994; *Daucus*, Scheike et al. 1992; *Helianthus annuus*, Crouzillat et al. 1994 and *P. lanceolata*, Rouwendal et al. 1987; Groenendijk et al. 1996).

Within a species several CMS types can occur. In each CMS type, a specific factor causes male sterility. Male fertility can be restored for each CMS type by different nuclear restorer genes. CMS types can therefore be distinguished by the action of restorer loci, as has been most elegantly shown by reciprocal differences (Van Damme 1983; Koelewijn and Van Damme 1995) or by crosses with genotypes that have a known

restorer action (Thompson 1972; Belhassen et al. 1991; Crouzillat et al. 1991).

The first question addressed in the present study is how many CMS types occur in *P. lanceolata*. In maize, one of the most thoroughly studied crops with regard to CMS, four CMS types have been described (Laughnan and Gabay-Laughnan 1983). The number of CMS types influences the dynamics of this sexual system, as shown by theoretical models concerning the maintenance of male sterility (Frank 1989; Gouyon et al. 1991).

In *Plantago lanceolata* two CMS types can be distinguished by the male-sterile phenotype. The male-sterile types MS1 and MS2 differ in the stage at which pollen production is blocked, which is expressed in flower morphology (described by Van Damme and Van Delden 1982). Reciprocal crosses between restored plants showed that the two male-sterile types are restored by different nuclear genes and hence represent different CMS types (named R and P in Van Damme and Van Delden 1982). In *Nicotiana* (Bonnett et al. 1991) and *Daucus* (Pingitore et al. 1989) variation in flower morphology was also coupled with different CMS types. In *P. lanceolata* no evidence for non-sterilizing types, the so-called 'Normal' cytoplasms, has been found (Van Damme and Van Delden 1982; Groenendijk et al. 1996). We made reciprocal crosses in order to uncover new CMS types. A different segregation of male steriles in the reciprocals indicates maternal effects, which were interpreted as different CMS types. Mitochondrial variation was used to make directional reciprocal crosses; the parents of the reciprocal crosses were chosen so that they differed in mtDNA pattern.

The second question addressed in this paper concerns the origin of new CMS types. The widespread occurrence of male steriles in the species could be due to migration or to mutation. In most Angiosperms both mitochondria and chloroplasts are maternally inherited. If a CMS type is widespread because of migration we expect a tight association between mitochondrial and chloroplast variation. If new CMS types frequently arise by mutation in the mitochondrial DNA, only limited association between chloroplast and mitochondrial variation is expected.

Materials and methods

Materials

The plants used in the reciprocal crosses originate from different populations in the Netherlands (Groenendijk et al. 1996). The plants collected were either hermaphrodites (H) or male-sterile (MS) and intermediate (IN) plants. Since no reciprocal crosses are possible between male-sterile or intermediate plants, hermaphrodites from the first generation crosses were taken as parents in subsequent generations. The plant code employed uses two or three letters referring to the population of origin followed by an individual number. AN67, AN76, VL2-2, AN873 are the same plants as in Groenendijk et al. (1996). Hermaphrodite individuals from the crosses 57, 58, 60, 61, 62, 64 and 81 originate from MS or IN plants named UU3, UU4, AN76, VL2-2, AN67, NA5 and WD875 respectively (Groenendijk et al. 1996). Additional plants were taken from the population Reitma (Rei), which has a relatively high frequency of MS2 compared to other Dutch populations (MS2 + IN2 = 24%), Heteren (Ht) with a high MS1 frequency (MS1 + IN1 = 29%) and Veneweg (Ven) where both types occur (MS1 + IN1 = 7% and MS2 + IN2 = 24%) (unpublished data).

Treatment

P. lanceolata is self-incompatible so that no precautions against self-pollination were needed when making reciprocal crosses. Contamination with foreign pollen was prevented by bagging the spikes before female flowering occurred. Pollen was collected and used immediately for pollination or kept at 4°C for a maximum of 2 weeks before pollination.

Ripe seeds were germinated in a Petri dish with 0.7% water-agar at day/night temperatures of 22/18°C for 9 days. In general, germination was more than 90%, but in crosses with lower percentages the seeds were cut at one side to stimulate germination. In this way at least 80% germination was obtained. Seedlings were pre-cultured in the greenhouse for about 4 weeks in Jiffy pots with potting compost, and then transferred to the experimental garden. A portion of the crosses were grown in the greenhouse for 16/8 h day/night. Reciprocal pairs were always grown under identical environments.

Screening

The sex-phenotype scores were based on at least two spikes per plant, and each plant was scored twice. According to Van Damme and Van Delden (1982) three flower types can be distinguished: a brown-anther type (MS1), petaloid flowers (MS2), and a yellow-anther type (MS3). Several sex-phenotype classes were distinguished within the two extremes MS and H, and these were designated as intermediate types (IN). For analysis purposes the IN category was considered as one sex-phenotype class. Heterogeneity between the reciprocal crosses was tested by chi-square.

Molecular analysis

From at least 1 g of young leaf material total DNA was extracted using the Rogers and Bendich (1988) method. Modifications were that liquid nitrogen was used instead of dry ice, 10 µl of β-mercaptoethanol was used instead of PVP, and centrifuge steps were increased to 10 min. Restriction endonuclease digestion was done with about 5 µg of DNA and 10 units of enzyme; digestion was performed for at least 2 h or overnight at 37°C. After electrophoresis, agarose gels were capillary blotted (Allefs et al. 1990). The Hybond-N nylon membranes were hybridised with AMPPD or CSPD labelled probes (Kreike et al. 1990).

Mitochondrial and chloroplast probes

For restriction fragment length polymorphism (RFLP) analysis the mitochondrial probe pPl311 was used in combination with the restriction enzyme *Hin*dIII. The probe was from a *P. lanceolata* MS2 type, and was cloned in pUC8 (Rouwendal et al. 1987). The insert of the probe was either isolated from the gel and purified with a separation-column (Qiagen), or amplified using two primers based on the plasmid sequence. The digoxigenin labelling was performed according to Boehringer-Mannheim. In order to diminish the background

signal on the autoradiograms, a hybridisation temperature of 62°C was used and the washing buffer was 0.3 × SSC, 0.1%SDS.

Chloroplast variation was detected with the use of heterologous barley chloroplast probes, kindly provided by Dr. T.H.N. Ellis from the John Innes Institute, Norwich, UK (Day and Ellis 1985). Seven plasmids represent the total chloroplast genome; the probes we used were ctP1, 20.7 kb; ctP3, 18.7 kb, and ctP4 13.4 kb. The hybridisation temperature was 65°C, and the washing buffer was 0.3 × SSC, 0.1%SDS.

Size variation for a non-coding region of chloroplast DNA was studied by PCR amplification, using specific primers ct *e* and *f* (Taberlet et al. 1991). The amplified DNA was digested with *Hin*fI.

Definitions as used in this study

Sex phenotype = the result of the CMS type and absence or presence of restorer alleles; characterized by flower morphology (Van Damme 1983): MS1, IN1 (= sex-phenotype 1), MS2, IN2 (= sex-phenotype 2), MS3 and H.

CMS type = CMS types differ from each other if reciprocal differences occur in the segregation of MS/IN/H, or if flower morphology of the MS + IN types is different in the reciprocals.

MtDNA pattern = characterized by the pattern revealed by *Hin*dIII digestion and probed with pPl311 (*P. lanceolata* mtDNA probe, Rouwendal et al. 1987).

Results

Mitochondrial variation

Most of the mitochondrial RFLP variation in *P. lanceolata* was found by a combination of the restriction enzyme *Hin*dIII and the probe pPl311 (Rouwendal et al. 1987). Nine mtDNA patterns, from a total of more than 20 different patterns, were more or less common in the 528 analyzed plants (De Haan 1996). Figure 1 shows the RFLP patterns of the mtDNA patterns from A to H and L. The full sibs H and MS1 both had the same mtDNA pattern A as the mother.

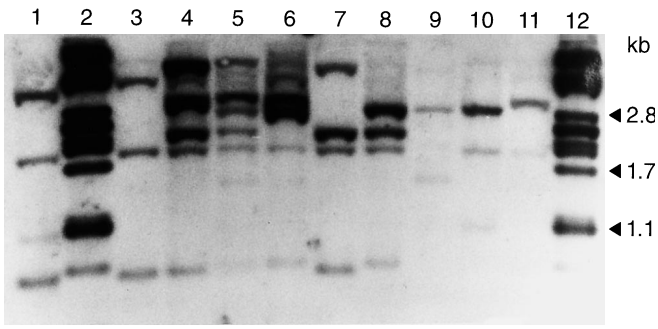

Fig. 1 Southern-blot hybridization of *P. lanceolata* total DNA digested with *Hin*dIII and probed with mtDNA clone pPl311. *Lane 1* mtDNA pattern A from a hermaphrodite; *lane 3* mtDNA pattern A from a MS1 plant; *lane 4* mtDNA pattern B; *lane 5* pattern H; *lane 6* pattern D; *lane 7* pattern E; *lane 8* pattern F; *lane 9* pattern C; *lane 10* pattern L; *lane 11* mtDNA pattern G. *Lanes 2 and 12* are lambda DNAs digested with *Pst*I

Table 1 The distribution of nine mtDNA patterns A to L (*Hin*dIII with *P. lanceolata* mt probe pPl311) over populations in the Netherlands and other countries. For each mtDNA pattern the number of populations in which it occurred in the Netherlands and/or worldwide is given. The total number of populations screened, and the total number of analyzed plants, are shown at the bottom of the Table

MT	# Pop-Netherlands	# Pop-other countries than Netherlands
A	6	5
B	5	0
C	8	0
D	8	0
E	7	2
F	5	0
G	5	4
H	4	0
L	1	0
Total # populations	12	7
# Analyzed plants	528	13

Eight mtDNA patterns (A–H) occurred within 4–8 of the 12 populations, while mtDNA pattern L occurred only in one population at a high frequency (Table 1). These nine mtDNA patterns cover 87% of all patterns from more than 500 plants. Most of the plants were sampled in eight populations within the Netherlands, but a small sample (13 plants) came from seven locations in Germany, Poland, France, Greece, and the USA. Those plants did not reveal new mtDNA patterns.

The mtDNA patterns can be classified according to the male-sterile phenotypes they are associated with. Non-restored genotypes with mtDNA pattern A show the MS1 phenotype. The mtDNA patterns B, C, D, G, H and L were linked with the MS2 phenotype. The mtDNA patterns E and F are predominantly found in hermaphrodites, but some plants which had reduced fertility resembled the MS3 phenotype (Van Damme and Van Delden 1982).

Reciprocal crosses

In order to identify new CMS types in *P. lanceolata*, reciprocal crosses were made between plants which differed in mtDNA pattern. In Table 2 the results from crosses are shown between hermaphrodites with mtDNA pattern A (associated with MS1) and hermaphrodites with the mtDNA patterns B, C, D and L (associated with MS2). In all pairs in this table, reciprocal differences are present in morphology, MS1 and IN1 versus MS2 and IN2. In the two pairs of reciprocal crosses between plants with mtDNA patterns A and B, a significantly different segregation of male steriles, intermediates and hermaphrodites was found. Of the seven pairs of reciprocals between plants

Table 2 Segregation of male steriles (MS), intermediates (IN) and hermaphrodites (H) in reciprocal crosses between plants with mtDNA patterns (Mt) A (sex-phenotype 1) and B, C, D, L (sex-phenotype 2). For each pair of reciprocal crosses both parents are given as the seed parent, but each acted as the pollen parent in the reciprocal cross. Heterogeneity between the reciprocals is tested with a chi-square test. The chi-square value, degrees of freedom (*df*) and its significance level (*P*) are given

No.	Parent 1	Mt	MS1	IN1	H	Parent 2	Mt	MS2	IN2	H	Chi	*df*	*P*[a]
1	81-29	A	3	1	11	58-7	B	0	12	29	10.6	2	**
2	549-2.2	A	3	4	31	569-1.1	B	13	13	8	24.4	2	***
3	81-29	A	0	2	29	60-5.2	C	2	7	33	3.5	2	n.s.
4	81-29	A	11	6	2	283-1	C	3	5	26	22.8	2	***
5	81-29	A	2	3	11	283-4	C	0	0	40	13.7	2	***
6	284-2	A	1	0	38	60-5.2	C	0	1	73	2.4	2	n.s.
7	284-4	A	2	4	21	60-5.2	C	3	9	23	1.2	2	n.s.
8	284-1	A	19	11	20	283-3	C	11	10	12	0.7	2	n.s.
9	284-2	A	0	3	38	283-4	C	0	5	56	0	1	n.s.
10	HT128	A	6	3	8	REI146	D	0	2	16	8.9	2	**
11	357-22	A	4	5	10	358-5	D	2	7	38	6.7	2	*
12	358-6	A	0	6	30	357-1	D	0	12	51	0.1	1	n.s.
13	534-1.1	A	3	4	34	VENB29	L	0	6	17	4.4	2	n.s.

[a] Significance levels: * < 0.05; ** < 0.01; *** < 0.001

Table 3 Segregation of male steriles (MS), intermediates (IN) and hermaphrodites (H) in reciprocal crosses among plants with sex-phenotype 2 (see Table 2)

No.	Parent 1	Mt	MS2	IN2	H	Parent 2	Mt	MS2	IN2	H	Chi	*df*	*P*
14	AN67	B	12	6	12	AN76X	C	16	13	11	1.8	2	n.s.
15	57-2	B	3	15	31	60-5.2	C	0	2	17	4.7	2	n.s.
16	58-7	B	4	2	5	60-5.2	C	9	21	28	3.1	2	n.s.
17	62-3.1	B	5	13	8	60-5.2	C	4	4	5	1.4	2	n.s.
18	211-18	B	14	33	33	210-4	C	9	9	49	16.9	2	***
19	206-24	B	17	19	7	207-10	D	0	2	9	18.6	2	***
20	58-2.2	B	2	1	14	VL2-2	D	1	3	29	1.6	2	n.s.
21	58-2.2	B	0	8	0	61-2.2	D	1	13	12	6.5	2	*
22	62-3.1	B	22	20	15	61-2.2	D	4	5	12	6.6	2	*
23	62-3.1	B	6	12	2	56-17	G	3	17	4	2.2	2	n.s.
24	AN76X	C	13	12	5	61-2.2	D	2	3	8	8.8	2	**
25	278-6.3	C	11	7	4	279-1.3	D	4	12	19	12.0	2	**
26	64-2.1	C	0	2	9	VL2-2	D	0	0	12	2.4	1	n.s.
27	200-2	C	9	13	14	199-7	D	4	13	19	2.7	2	n.s.
28	200-7	C	0	4	24	199-5	D	0	1	24	1.6	1	n.s.
29	200-7	C	0	7	18	199-7	D	4	11	45	2.5	2	n.s.
30	AN76X	C	6	4	22	VL2-2	D	6	22	41	5.4	2	n.s.
31	202-1	C	11	17	21	201-1	D	8	33	28	3.3	2	n.s.
32	202-1	C	3	24	45	201-2	D	0	7	57	13.3	2	***
33	HT104	C	0	4	37	HT121	H	1	12	19	9.8	2	**

with mtDNA patterns A and C, two are significantly different (pairs 4, 5). For pairs 3 and 6–9 the segregation is the same for both sides, implying that the two parents have a similar constitution of restorer alleles for each other's CMS type. Between plants with mtDNA patterns A and D three pairs of reciprocal crosses were made, two of which were significantly different. The reciprocal crosses between plants with mtDNA patterns A and L did not segregate different frequencies of MS or IN. However, the MS and IN types from both sides had a different flower morphology; if mt A was used as the seed parent the male steriles were MS1 and the intermediates were IN1; if a plant with mtDNA pattern L was used as the seed parent the intermediates were IN2. The phenotypic differences were indicative of different CMS types. The results in Table 2 clearly show that plants with mtDNA pattern A and plants with mtDNA patterns B, C, D and L represent different CMS types, showing variation by either different segregation ratios or by the morphological differences between the male steriles in the reciprocal crosses, which corresponds with the observations of Van Damme and Van Delden (1982) and Van Damme (1983).

Several mtDNA patterns were found within the MS2 category, namely B, C, D, G, H and L. In order to discover to what extent these mtDNA patterns represent different CMS types, reciprocal crosses were made. Plants with mtDNA patterns B, C and D were included in most of the realised combinations, while plants with mtDNA patterns G and H were used only in a limited number of crosses (Table 3). In five pairs of crosses

Table 4 Segregation of male steriles (MS), intermediates (IN) and hermaphrodites (H) in reciprocal crosses between plants with mtDNA patterns (Mt) A (sex-phenotype 1) and E, F (sex-phenotype 3) (see also Table 2)

No.	Parent 1	Mt	MS1	IN1	H	MS3 + Inb	Parent 2	Mt	MS3	INb	H	Chi	*df*	*P*
37	HT128	A	23	10	18	0	REI138	E	0	0	45	44.4	2	***
38	534-1.1	A	34	10	4	0	527-1.4	E	0	0	45	78.3	2	***
39	361-5	A	7	12	19	9	359-6	F	0	1	27	16.6	2	***
40	361-9	A	1	5	18	0	359-5	F	0	3	7	0.7	2	n.s.
41	362-12	A	7	13	29	0	359-10	F	0	0	24	13.5	2	***
42	364-14	E	0	5	45	0	359-7	F	1	1	22	2.8	2	n.s.
43	364-2	E	0	6	30	0	359-9	F	0	4	22	0.0	1	n.s.
44	364-4	E	3	6	23	0	359-5	F	0	12	35	4.8	2	n.s.
45	527-1.1	E	0	0	36	0	266-3	F	0	0	31	–		n.s.

between plants with mtDNA patterns B and C, only one shows a significant difference (pair 18). All crosses segregate MS2 and IN2 types from both sides. In pairs 14–17 it is evident that mtDNA patterns B and C either reflect the same CMS type or else are different CMS types with a combination of alleles at restorer loci which result in equal segregation ratios.

In Table 3 four pairs of reciprocal crosses are shown between genotypes with mtDNA patterns B and D; three of them are significantly different, whereas pair 20 segregates equal numbers of male steriles and intermediates. MtDNA patterns B and D probably represent different CMS types. Pair 23 shows that no difference could be detected between plants with mtDNA patterns B and G. Between genotypes with mtDNA patterns C and D, nine pairs of reciprocal crosses were made. Pairs 24, 26 and 30 are crosses between genotypes taken from the field (see Materials and methods). An76X and VL61-2.2 (pair 24) show a significantly different segregation of MS2 and IN2 among reciprocals, as also does pair 25, which is a pair of crosses among their F_1 progeny. Pair 26 and the crosses among their F_1 progeny (pairs 27–29) do not show significant differences. All crosses predominantly segregate hermaphrodites and intermediates, and when they do segregate male steriles (pair 27) they occur in equal numbers. Pairs 31 and 32 are reciprocal crosses between the F_1 progeny of pair 30 and in the case of pair 32 reciprocal differences are found. Out of the nine pairs, three are found to be significantly different. A pair of crosses between mtDNA patterns C and H also shows a significant difference. It is concluded that the different mtDNA patterns (within the sex-phenotype 2 group) represent at least two different CMS types, namely mt B and C, differing from D and possibly from each other. For G, H and L the results are not adequate to categorize them.

Two more mtDNA patterns were distinguished, E and F. In the Dutch populations these two patterns have been found only in hermaphrodites. Segregating intermediates and male steriles in the crosses resemble mostly type-3 male sterility as described by Van Damme (1983). The expression of MS3 is independent of CMS type and can segregate from both sides. In Table 4 reciprocal crosses between mt E and F versus mt A, as well as among genotypes with mt E and F, are shown. Four out of five crosses between mt A versus E or F differ significantly. If a parent with mtDNA pattern A is the seed parent, high numbers of MS1 and IN1 segregate, while crosses with mt E or F as seed parent predominantly produce hermaphrodites. In crosses between plants with mtDNA patterns E and F no differences are found; both infrequently segregate male steriles, and when present they occur in equal frequencies.

In Table 5 it is shown that about half of the crosses between genotypes with mtDNA patterns B, C, D and genotypes with mt E and F are significantly different. Pairs 46 and 47 both show that plants with mtDNA patterns B and F are different. For plants with mtDNA pattern C versus E only a difference at the 5% significance level was found. Most of the segregating progeny are hermaphrodite and the parents are probably restored for each other's CMS types. The reciprocal crosses between plants with mtDNA patterns C and F are all significantly different (parents pairs 50, 51; backcrosses 52, 53; among F_1 progeny 54–56). Crosses between plants with mtDNA patterns D and E predominantly segregate hermaphrodites (pair 58 only hermaphrodites; crosses among F_1 progeny 59–62 some intermediate sex phenotypes, no significant differences). Pairs 57 and 63 show significant differences between plants with mtDNA patterns D and E. Reciprocal crosses between genotypes with mtDNA patterns D and F showed that no differences could be detected, because no male steriles segregated in the crosses (neither between genotypes 64, 65 from the field, nor among crosses in the F_1 progeny 66, 67). Taken together the results from Tables 4 and 5 show that the plants with mtDNA patterns E and F are not different from each other, but that they differ from those with mtDNA pattern A (sex-phenotype 1) as well as those from with mtDNA patterns B, C and D (sex-phenotype 2).

Table 5 Segregation of male steriles (MS), intermediates (IN) and hermaphrodites (H) in reciprocal crosses between plants with mtDNA patterns (Mt) B, C, D, L (sex-phenotype 2) and E, F (sex-phenotype 3) (see Table 2)

No.	Parent 1	Mt	MS2	IN2	H	MS3 + IN3	Parent 2	Mt	MS1	IN1	H	Chi	*df*	*P*
46	260-2	B	1	3	0	1	259-1	F	0	3	13	10.6	2	**
47	260-5	B	11	21	6	1	259-1	F	0	3	13	21.7	2	***
48	HT132	C	0	10	65	0	REI133	E	0	2	74	5.9	1	*
49	REI131	C	0	0	46	1	REI133	E	0	2	62	1.5	1	n.s.
50	60-5.2	C	8	17	3	0	AN873-2	F	0	0	17	34.2	2	***
51	64-1.2	C	7	4	19	0	AN873-3	F	0	0	13	6.4	2	*
52	265-7	C	1	14	42	0	AN873-3	F	0	0	55	16.7	2	***
53	265-8	C	0	5	12	0	AN873-3	F	0	1	51	12.2	1	***
54	265-8	C	7	20	2	7	266-1	F	0	16	20	21.7	2	***
55	265-9	C	29	36	2	0	266-1	F	6	9	30	53.6	2	***
56	265-9	C	9	8	0	8	266-4	F	1	4	25	18.8	2	***
57	REI137	D	1	9	37	0	REI133	E	0	0	42	10.1	2	**
58	REI132	D	0	0	37	0	REI138	E	0	0	49	–		n.s.
59	363-27	D	0	0	20	0	364-10	E	0	6	60	2.0	1	n.s.
60	363-27	D	0	9	44	0	364-9	E	0	1	11	0.6	1	n.s.
61	363-35	D	0	3	70	0	364-2	E	0	2	48	0	1	n.s.
62	363-36	D	0	0	34	0	364-4	E	0	0	48	–		n.s.
63	556-1.3	D	6	18	9	2	529-3.1	E	0	2	17	20.2	2	***
64	REI141	D	0	7	63	1	REI127	F	0	9	68	0.0	1	n.s.
65	REI132	D	0	3	46	0	REI127	F	0	0	54	3.4	1	n.s.
66	360-9	D	0	0	26	0	359-6	F	0	3	48	1.6	1	n.s.
67	360-9	D	0	1	28	0	359-9	F	0	2	25	0.4	1	n.s.

Chloroplast variation

Chloroplast variation was found by using the RFLP technique and by PCR-amplification with specific primers. Figures 2 and 3 demonstrate the polymorphic patterns obtained. The restriction enzyme *Ava*I shows two patterns in combination with probes ctP1 and ctP3 (Fig. 2a, b). *Xho*I also gives two patterns in combination with ctP1 (Fig. 2c). The three combinations (*Ava*I-ctP1, -ctP3 and *Xho*I-ctP1) reveal two haplotypes, designated as I and II. Further haplotypes II and III could be distinguished by a RFLP (*Eco*RI-ctP4; Fig. 3a) and a PCR-RFLP (ct*e* + *f* **Hin*fI, according to Taberlet et al. 1991; Fig. 3b). The RFLP pattern (*Eco*RI-ctP4) showed a fragment of 0.82 kb in haplotype II. In haplotype III this fragment was absent, but here a new

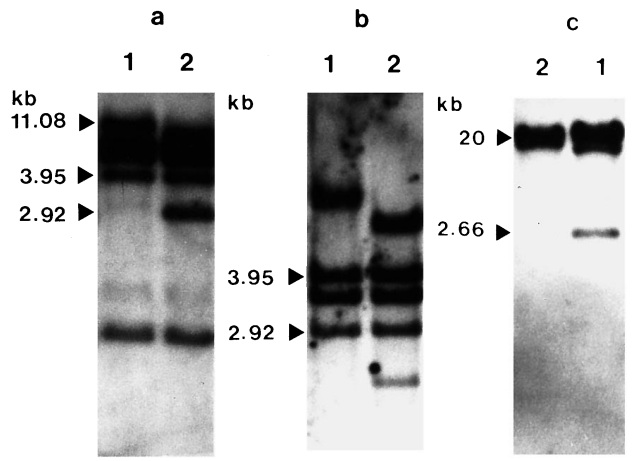

Fig. 2a–c CtDNA variation which distinguished ctDNA haplotype I (*no. 1*) from II and III (*no. 2*). Southern-blot hybridization of *P. lanceolata* total DNA of two plants of the Veneweg population, which had been screened for mtDNA variation: mtDNA patterns C (*no. 1*) and A (*no. 2*). **a** digested with *Ava*I and probed with ctP1; **b** digested with *Ava*I and probed with ctP3; **c** digested with *Xho*I and probed with ctP1

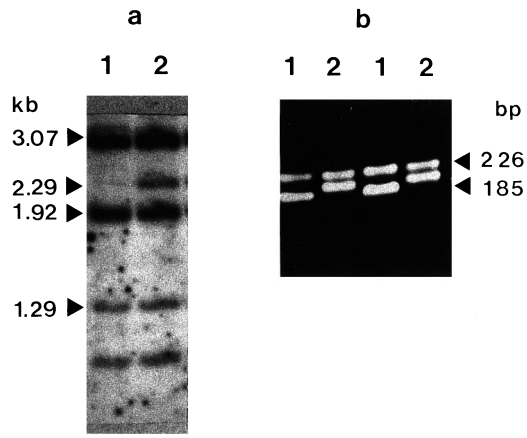

Fig. 3a, b CtDNA variation which distinguished ctDNA haplotype II (*no. 1*) from III (*no. 2*). **a** Southern-blot hybridization of two plants with mtDNA pattern A of the Heteren population. Total DNA digested with *Eco*RI, probed with ctP4. **b** *Hin*fI digestion pattern of PCR amplification products using ct primers *e* and *f* and total DNA from plants of the populations Durham and Montpellier which showed the mtDNA patterns G (*no. 1*) and A (*no. 2*)

Table 6 The association between chloroplast (CtDNA) haplotypes I, II and III found in plants from different populations, with different mtDNA patterns (Mt A and L versus mtDNA patterns B, C, D, E, F, G, J) is shown. For each population, the country and the sample size (n) is given

Population	Country	*n*	Mt	CtDNA haplotype
Heteren	Netherlands	9	B C G	I
Veneweg	Netherlands	6	C G J	I
Junner Koeland	Netherlands	1	A	I
Junner Koeland	Netherlands	9	C D E G	I
Reitma	Netherlands	11	D E F	I
Nürnberg	Germany	1	G	I
Montpellier	France	1	G	I
Katowice	Poland	1	E	I
Kos	Greece	2	E	I
Ithaca	U.S.A	1	G	I
Durham	U.S.A	1	G	I
Heteren	Netherlands	2	A	II
Veneweg	Netherlands	4	A	II
Veneweg	Netherlands	3	L	II
Junner Koeland	Netherlands	3	A	II
Nürnberg	Germany	1	A	II
Zywiec	Poland	1	A	II
Durham	U.S.A	1	A	III
Montpellier	France	1	A	III
Heteren	Netherlands	2	A	III

fragment of 2.3 kb occurred. A strict linkage was found with a PCR-RFLP marker, an amplification product with the primers *e* and *f*. The observed fragment of about 400 bp does not show variation among the genotypes, but after restriction with *Hin*fI two variants were evident.

The three ct haplotypes were found not only in plants from different populations (Table 6) but also together in a single population (Heteren). A strong association between mtDNA patterns and ct haplotypes was evident (Table 6). A new mtDNA variant, J, was also used in the analysis (data not shown). MtDNA pattern A was associated with ctDNA haplotypes II and III, with one exception: in a single plant from Junner Koeland mtDNA pattern A was associated with ctDNA haplotype I. The other mtDNA patterns (except mt L) were associated with ctDNA haplotype I.

Discussion

Cytoplasmic male sterility is caused by mutations in the mitochondrial DNA (Lonsdale 1987; Saumitou-Laprade et al. 1994). Mutations in mitochondrial DNA are thought to originate by two possible mechanisms. First, frequent recombination events between large repeats raise a subset of molecules within the cell (Lonsdale et al. 1988; André et al. 1992); selection of a limited number of molecules in the germ line could then lead to mutations (Atlan and Couvet 1993). Secondly, rare recombinational events between small repeats can give rise to new pseudogenes and chimeric genes; some of these new genes are possibly involved in blocking pollen production (Conklin and Hanson 1994). The mechanisms described can lead to large mitochondrial variation and mutation, but only a limited number of mt mutations will spread. Such mutations have first to overcome selection within the cell, and in order to be transmitted the mutant mitochondrial DNA has to occur in the germ line. Finally, the mutant should have a selective advantage at the plant level to be able to spread more easily in the species than by drift alone (Cosmides and Tooby 1981).

In order to examine the number of CMS types in natural populations, variation in mtDNA was screened in *P. lanceolata* with a single combination of a restriction enzyme and mt probe pPl311. More variation would be expected if the mtDNA was to be digested with several restriction enzymes and additional probes were utilized. However, the probe-enzyme combination we used has been shown to demonstrate maximum variation compared to other probe-enzyme combinations in *P. lanceolata* (Groenendijk et al. 1996). A morphological difference had already been found between two CMS types in *P. lanceolata* (Van Damme and Van Delden 1982). Associated with MS1 and IN1, only one mtDNA pattern is found, here designated as A (Groenendijk et al. 1996). Within the MS2 and IN2 group the latter authors found more mtDNA patterns (P1–P7) which correspond to patterns B, C, D, G and H. Types P4 and P7 were not used in this study. Furthermore AN6, here mt F, was found only in hermaphrodites, and could not be categorized in either sex-phenotype 1 or 2 (Groenendijk et al. 1996).

With the molecular techniques applied in the present study, much more variation is found than at the morphological level (sex phenotypes), which makes it possible to investigate whether there are more than the two known CMS types in *P. lanceolata*. Specific genotypes, which differ in mtDNA pattern, were used in a series of reciprocal crosses. Reciprocal crosses can reveal possible CMS type differences by differential segregation of male steriles in the progenies. The inheritance of mitochondria in *P. lanceolata* has been shown to be maternal, and although some paternal leakage cannot be excluded it is assumed that its frequency is so low that it will not interfere with segregation ratios (Corriveau and Coleman 1988). The analysis of ten individuals from two sets of reciprocal crosses did not violate this assumption (data not shown). Furthermore the inheritance of the two male sterile types MS1 (CMSI) and MS2 (CMSII) was strictly maternal, as shown in Table 2. The reciprocal crosses between genotypes with mtDNA pattern A and the other patterns clearly show that former represents a distinct CMS type, which we call CMSI and corresponds with R in Van Damme and Van Delden (1983). CMSI is characterized (Table 7) by its morphology in the male-sterile

Table 7 Four cytoplasmic male sterility types (CMSI, IIa, IIb and III) in *P. lanceolata* are shown with their markers, the mtDNA patterns (Mt), and flower morphology when they are male sterile. MS1 is a brown-anther type, MS2 shows petaloid flowers, MS3 is a yellow-anther type. Brackets (MS3) mean that the sex phenotype is not strictly associated with CMSIII, but also occurs in CMSI and CMSII types. CMSIIb/c and CMSIIa/b/c are different CMS types than CMSI and CMSIII, showing sex phenotype 2, but the exact classification is not known

CMS type	Mt pattern	Sex phenotype
CMSI	A	MS1
CMSIIa	D	MS2
b	C	MS2
b/c	B	MS2
a/b/c	G, H, L	MS2
CMSIII	E, F	(MS3)

and intermediate sex phenotypes MS1 and IN1, the mtDNA pattern A, and has specific nuclear loci that can restore male fertility as shown by the reciprocal differences.

Within the sex phenotype-2 group several mtDNA patterns were found. Reciprocal crosses among genotypes with these mtDNA patterns showed differences. The mtDNA patterns D and C represent different CMS types, and we designate the two CMS types as CMSIIa and CMSIIb (Table 7). Differences between reciprocal crosses were shown not only by the first generation of crosses, but also by crosses between F_1-progenies; therefore maternal effects other than CMS are unlikely to have caused the differences (Koelewijn and Van Damme 1995). One pair of crosses (pairs 18) between mtDNA patterns B and C showed a reciprocal difference in the segregation of male steriles, whereas the other reciprocal crosses between B and C always segregate male steriles in equal frequencies to both sides. In such cases mirrored restoration could be the explanation, i.e. both parents have the same allelic combination for each other's restorer loci, e.g. heterozygous for a dominant restorer allele. Another explanation would be that the two mtDNA patterns are both markers for the same CMS type. In that case pair 18 presents a problem. Table 3 shows that this reciprocal difference is mainly due to a difference in the intermediate class, and it is known that the expression from intermediate types is under the influence of the environment (data not shown; also Tracey et al. 1991; Ruffio-Chable et al. 1993; Koelewijn and Van Damme 1996). The evidence for a difference in CMS type is thus inconclusive in this case. The mtDNA patterns G, H and L all belong to the sex phenotype-2 group, but the number of reciprocal crosses between genotypes with these mtDNA patterns are not sufficient to classify them.

No reciprocal differences between plants with mtDNA patterns E and F were found, but both differed from CMSI, CMSIIa and b. Therefore the two mtDNA patterns E and F represent another type whose morphological characterization is not clear. The MS3 types found in the segregating crosses are probably not typical for this type, since they were also found in combination with CMSI and CMSII types. Van Damme (1983) showed the existence of nuclear male sterility genes for MS3. Since no *cytoplasmic* male-sterile types with mtDNA patterns E and F were found, one could conclude that this type is a normal (N) cytoplasm without sterility inducing genes. This would be a new type in *P. lanceolata* since no N-cytoplasm is known. However, it is also possible that the genotypes used in this study were all fully restored for this CMS type. Since we cannot distinguish between these two alternatives, we designate this type CMSIII.

In addition to the two CMS types found by Van Damme and Van Delden (1982) two new CMS types were found in the present study. The four CMS types are named I, IIa, IIb and III (Table 7). The segregation ratios of all four types cannot be explained by only one locus for each type. The restoration of male fertility in *P. lanceolata* has been shown to be rather complex, which accounts for the different segregation of sex phenotypes in crosses with genotypes having the same mtDNA patterns (Van Damme 1983; De Haan 1996). The question arises whether the four CMS types found here represent the upper or lower limits of the variation for CMS types in *P. lanceolata*. Frank (1989) stated that, due to the inevitable limited sample sizes in genetic studies, only a small part of the potential variation will be sampled. On the other hand it has been found that mtDNA variation also exists within CMS types (Kemble et al. 1983; McNay et al. 1983; Pring et al. 1987; Pingitore et al. 1989; Komarnitsky et al. 1990; Saumitou-Laprade et al. 1993).

In theoretical models concerning the maintenance of male sterility it is evident that the number of CMS types influences the conditions under which male sterility can be maintained. Large variation in CMS types and stochasticity requires less stringent conditions for the maintenance of male sterility (lower advantage of the male-sterile fitness and lower costs of restorer alleles; Frank 1989) than a model assuming two CMS types (Gouyon et al. 1991). The lower limit of the number of CMS types in *P. lanceolata* is the four found in our study. The upper limit is more difficult to estimate. There is a lot of mitochondrial variation, which could be taken as the ultimate upper limit, but it is unlikely that each mtDNA pattern represents a distinct CMS type.

Chloroplasts and mitochondria are both maternally transmitted in *P. lanceolata* (Corriveau and Coleman 1988). Therefore, a tight association between ct haplotypes and mtDNA patterns is expected. But if new CMS types frequently arise by mutation, the association between mtDNA variants and ct haplotypes will be broken down. The association between ctDNA haplotypes and mtDNA variants was clearly non-random in specimens of *P. lanceolata* collected from European

and American populations. Three ctDNA haplotypes, I, II and III, were found. In general, chloroplast DNA shows a low rate of mutations, which are mostly base substitutions (Palmer 1990). The difference between ctDNA haplotype I versus II and III was revealed by a combination of two restriction enzymes and the probes ctP1 and ctP3. These combinations showed three RFLPs which were completely linked. The restriction enzymes we used are known to give polymorphism in *P. lanceolata* (Wolff and Schaal 1992; Hooglander et al. 1993). CtDNA haplotypes II and III were associated with the mtDNA pattern-A group, while ctDNA haplotype I was nearly always associated with the other mtDNA variants. Two exceptions were found to the association between the mtDNA pattern A and two ctDNA haplotypes. First, one out of 19 mt A genotypes was associated with ctDNA haplotype I; second, mtDNA pattern L was associated with ctDNA haplotype II. One explanation for the genotype from the population Junner Koeland, with mt A and the 'wrong' ctDNA haplotype, might be paternal leakage from either the mitochondrion or the chloroplast (Reboud and Zeyl 1994). It is also possible that mtDNA pattern A arose before the ctDNA haplotype variation. In that case ctDNA haplotype I would be the ancestral ctDNA haplotype; ctDNA haplotype II arose subsequently in a genotype with mt A and then later ctDNA haplotype III was produced. In that way all three observed ctDNA haplotypes could be found associated with mt A. An event of paternal inheritance seems to be more likely because the other 13 plants of the Junner Koeland population show the expected associations. The existence of mtDNA variant L (sex-phenotype 2) in the population Veneweg seems to be unique for that population. The association with the same ctDNA haplotype as mtDNA pattern A suggests that mtDNA pattern L is descended from a mt A = CMSI genotype (sex-phenotype 1).

In *P. lanceolata*, CMSI appeared to be the most frequent male-sterile type (MS1) in the field, while MS2 (CMSII) is relatively rare in populations (Van Damme and Van Delden 1982). MtDNA pattern A, which is a marker for CMSI, was associated with ctDNA haplotypes II and III. This strong association suggests that the CMSI type became widespread by migration, and not by recurrent mutation. Evidence for migration was also found in *Beta maritima* (Forcioli 1995). The original distribution of *P. lanceolata* was thought to be in Europe (Meusel et al. 1978) and it then became widespread over the five continents as a result of human activity. Possibly CMSI also became widespread coincident with the spread of the species over the continents. The results to-date do not exclude the possibility that there could be a large potential of CMS types in *P. lanceolata*. However, in the field only one of them is the dominant male sterile, which has also been suggested to be the case in *P. coronopus* (Koelewijn and Van Damme 1995).

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