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The Dynamics of Gynodioecy in *Plantago lanceolata* L. II. Mode of Action and Frequencies of Restorer Alleles

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ABSTRACT

Male fertility in *Plantago lanceolata* is controlled by the interaction of cytoplasmic and nuclear genes. Different cytoplasmic male sterility (CMS) types can be either male sterile or hermaphrodite, depending on the presence of nuclear restorer alleles. In three CMS types of *P. lanceolata* (*CMSI, CMSIIa,* and *CMSIIb*) the number of loci involved in male fertility restoration was determined. In each CMS type, male fertility was restored by multiple genes with either dominant or recessive action and capable either of restoring male fertility independently or in interaction with each other (epistasis). Restorer allele frequencies for *CMSI, CMSIIa* and *CMSIIb* were determined by crossing hermaphrodites with "standard" male steriles. Segregation of male steriles *vs.* non-male steriles was used to estimate overall restorer allele frequency. The frequency of restorer alleles was different for the CMS types: restorer alleles for *CMSI* were less frequent than for *CMSIIa* and *CMSIIb*. On the basis of the frequencies of male steriles and the CMS types an "expected" restorer allele frequency could be calculated. The correlation between estimated and expected restorer allele frequency was significant.

IN gynodioecious species females (male steriles) and hermaphrodites co-occur. Male fertility in most gynodioecious species is determined by both cytoplasmic and nuclear genes. Male sterility occurs in certain cytoplasmic types, and fertility can be restored by several nuclear restorer genes. In most gynodioecious species two to four cytoplasmic male sterility types (CMS types) have been found; each restored either by several independent restorer loci or by two epistatic loci (VAN DAMME 1983; HANSON and CONDE 1985; BRAUN *et al.* 1992; KOELEWIJN and VAN DAMME 1995).

The distribution of CMS types and/or their restorer alleles among populations has been determined for several species (*Beta maritima,* BOUTIN-SADLER *et al.* 1989; CUGUEN *et al.* 1994; *Plantago coronopus,* KOELEWIJN and VAN DAMME 1995; *P. lanceolata,* VAN DAMME 1986; *Thymus vulgaris,* COUVET *et al.* 1986). However, detailed information about the frequency of different CMS types and their restorer alleles within populations is lacking. Data on the number of CMS types in *P. lanceolata* and their frequencies in eight populations are reported in two parallel studies (DE HAAN *et al.* 1997a,b). In the present study we give data on the frequency of restorer alleles for three CMS types in seven populations.

To assess restorer allele frequencies, the inheritance of male fertility must be determined. A number of characteristics of male fertility makes this difficult. First, the restorer loci are different for each CMS type, and nuclear restorer action can only be studied within each CMS type. Therefore, before the number and action of the nuclear restorer alleles can be determined, the CMS type of the plant must be known (BELHASSEN *et al.* 1991). Second, when epistatic effects occur between restorer loci large numbers of progeny are necessary to detect the loci involved (RAJ and VIRMANI 1988; CONNOR and CHARLESWORTH 1989; FANG and MCVETTY 1989; FRANK 1989; SHIFRISS and PILOVSKY 1993). Third, in different populations of *P. coronopus* different restorer loci were found that were able to restore male fertility for two CMS types (KOELEWIJN and VAN DAMME 1995). Fourth, partially male sterile plants were found, *i.e.,* plants that produce a limited amount of pollen. The inheritance of partial restoration of male fertility is unknown, but the same loci that cause complete restoration seem to be involved (VAN DAMME 1983; KOELEWIJN and VAN DAMME 1995). In most studies, these intermediates are grouped together with the hermaphrodites, and segregation of MS *vs.* not-MS was used to interpret the results, which may underestimate the complexity of the breeding system (VAN DAMME and VAN DELDEN 1982; KOELEWIJN and VAN DAMME 1996). Fifth, expression of male fertility was found to depend on environmental factors. The partially male sterile types appear to be especially sensitive to temperature effects (IZHAR 1978; KIK *et al.* 1993; KOELEWIJN and VAN DAMME 1996).

In *P. lanceolata* there is evidence for four CMS types (DE HAAN *et al.* 1997a). Two phenotypically distinct male sterile types are found: MS1 and MS2 (VAN DAMME and VAN DELDEN 1982). MS1 plants have *CMSI,* while MS2 plants are *CMSIIa* or *CMSIIb.* The fourth *CMSIII* type was found only in hermaphrodites. It could thus

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be a "normal" cytoplasm, or else the genotypes studied may have all been restored for this CMS type. Fertility in *CMSI* is restored by anyone of five loci. Two loci acted in a dominant fashion for restoring male fertility, while restorer alleles at the other three loci showed recessive action (VAN DAMME 1983). In addition, we found evidence for loci with epistatic effects. For the *CMSII* types, VAN DAMME (1983) found three loci with independent dominant restorer effects. In the present study, we determined restorer loci for CMSIIa and CMSIIb separately. Then restorer allele frequencies for CMSI and CMSIIb were estimated in seven populations and compared with data from a previous study (DE HAAN *et al.* 1997b).

MATERIALS AND METHODS

Screening of male fertility: *P. lanceolata* is self-incompatible, so while making crosses no precautions against self-pollinations were needed. Contamination with foreign pollen was prevented by bagging the spikes before female flowering occurred. Pollen was collected and either used immediately for pollination or kept at 4° for a maximum of 2 weeks before pollination. Ripe seed was germinated for each cross in a Petri dish with water agar (0.7%) at day/night temperatures of 22/18° for 9 days. Germination in general was >90%, but in crosses with lower percentages the seeds were cut on one side to stimulate germination. In this way at least 80% germination was obtained for all crosses. Seedlings were precultured in the greenhouse for ~4 weeks in Jiffy pots with potting compost, and then transferred to an experimental garden. The percentage of dead seedlings was <5%.

Plants from crosses were given a cross number and an individual number. The plant codes of the parents refer to the population of origin (first two or three letters) and a number. Sex phenotypes were scored twice in the flowering plants for at least two spikes. Several sex phenotypic classes were distinguished. MS1 showed a brown anther type, whereas MS2 was petaloid (additional petals instead of anthers) (described by VAN DAMME and VAN DELDEN 1982). Moreover, discrimination based on the pollen production was made with plants ranging between MS and H, with several intermediate (partially male sterile) classes. For analysis the different intermediate categories were classified as one sex phenotype (IN). Progeny that did not flower in the first season were taken into the greenhouse and scored after flowering.

Restorer loci: number and mode of action: Restorer alleles for *CMSI* were studied in two subsequent generations of crosses between standard MS1 and five hermaphrodites each from a different population. The standard MS are described in the next paragraph and the populations in Table 1. Parents of crosses and the number of progeny are shown in the results (Table 2). Hermaphrodites or intermediate (partially male sterile) types from the F_1 progenies were crossed to give F_2 offspring. Parents of the crosses were choosen such that allelism could be determined. At least 125 individuals per F_2 cross were screened to detect epistatic loci. Restorer alleles for *CMSIIa* were studied in four families during several generations, which originally had been made to study new CMS types (DE HAAN *et al.* 1997a). In the first generation in this new CMS type, hermaphrodites originated from either the field or from previous crosses (Tables 2 and 3). Restorer alleles for *CMSIIb* were studied in F_1 and backcross generations. Standard MS2 were crossed with six hermaphrodites from Heteren (HT), five hermaphrodites from Reitma (REI), and one from Koudum (KOU) (Tables 4 and 5). If the F_1 generation segregated mainly intermediates and hermaphrodites, one of those plants was crossed with the male sterile seed parent. If the F_1 generation segregated mainly male steriles, a male sterile was back-crossed with the pollen parent. In this way the mode of action of the restorer alleles could be determined.

Genetic models were fitted for the interpretation of the ratios of MS *vs.* not-MS. The symbols used for the restorer alleles are as follows: *R*, dominant; *r*, recessive; +, the male sterile allele. Epistatic loci are written without spacing *r1r1r2r2*, and independent loci with spacing *r1r1 r2r2*. Two epistatic loci can only restore male fertility, if both loci possess restorer alleles (Figure 1b). Independent loci restore male fertility regardless the allelic composition at other loci (Figure 1a). The models were fitted in the following order of complexity:

1. One locus with dominant restorer action (*R*+)
2. One locus with recessive restorer action (+*r*)
3. One locus with three alleles (*R*, *r*, +)
4. Two or more independent loci, each capable of restoration
5. Two loci with epistatic effects (both loci necessary to restore male fertility: *R1*+*R2*+)
6. Recessive action and epistatic loci (+*r1*+*r2*)
7. A combination of models 1–6.

Furthermore genetic models for the genetic background of intermediate types were fitted against the segregation of three phenotypic classes MS:IN:H. In addition to *R*, *r* and + the symbol, *r* for codominant alleles is used. A codominant allele restores male fertility partially in combination with the + allele and restores male fertility completely if homozygous. The genetic models were fitted in the following order of complexity:

1. Codominance (*R*+)
2. One locus is not enough for complete restoration, *i.e.*, at least one restorer allele at a second locus is needed for complete restoration (IN = *r1r1* ++, H = *r1r1 r2*+).

The expected ratio based on each genetic model was compared with that observed and tested with a chi-square test. Each cross was tested twice. First the frequency of MS *vs.* IN+H plants was tested (correcting for continuity), and second the three classes were fitted separately.

Frequency of restorer alleles: Most of the crosses were performed with male steriles originating from crosses from two lines that produced 100% male sterile progeny, which we call here standard male steriles (J. M. M. VAN DAMME, unpublished results). Family J875 segregated only *CMSI* male steriles (standard MS1) and family J637 segregated only *CMSIIb* male steriles (standard MS2). The restorer allele composition of the standard male steriles was determined in the crosses described above.

To compare restorer allele frequencies among populations, hermaphrodites were collected from seven populations, grown in the greenhouse, brought to flowering, and crossed with standard male steriles. In addition, pollen was collected from several plants separately from Naterseweg (Nat), Leek and Veneweg (Ven), and used to fertilize male steriles in the greenhouse (Table 1). The restorer allele frequency for *CMSI* was estimated from crosses with male steriles *CMSI* (MS1). The restorer allele frequency for *CMSIIb* was estimated from crosses with standard MS2, or second generation male sterile descendants. A limited essay was performed to estimate the frequency of *CMSIIa*. Pollen was collected from five to 11 hermaphrodites per population, mixed and used to fertilize one particular male sterile *CMSIIa* plant. From each cross seeds were grown and the progeny was scored as mentioned above. About 50 progeny per cross were scored. To estimate

TABLE 1

Description of the populations studied

Population	%MS1[a]	%MS2[a]	*n*	%CMSI[b]	%CMSII[b]	%CMSIII[b]	*n*
Reitma (REI)	0.1	15.1	1234	0	64.7	35.2	88
Heteren (HT)	14.8	0.1	514	31.5	62.9	1.1	89
Koudum (KOU)	0.6	0	512	0	18.8	62.5	14
Naterseweg (NAT)	18.1	1.6	1181	34.6	42.3	7.7	49
Leek (LEEK)	18.5	1.3	766	29.3	39.6	0	59
Veneweg (VEN)	13.7	6.5	1561	9.3	66.4	3.6	138
Brummen (BM)	7.2	0	311	57.1	40.1	2.9	35

From DE HAAN *et al.* (1997b).
[a] The remainder percentage of phenotypes (MS1 and MS2) are hermaphrodites.
[b] The remainder of the CMS types (CMSI, CMSII, CSMIII) are unknown types.

TABLE 2

Crosses with *CMSI* as seed parent

Cross	Seed parent	Pollen parent	Observed number MS:IN:H	Expected ratio MS:IN+H MS:IN:H	χ^{2a}	d.f.
1	stand-MS1-1 +***r1*** ++ +*r3R4R4*	HT109 +***r1*** ++ ++*R4*–	34:3:6	3:1 3:0:1	0.19	1
F_1 generation						
2	stand-MS1-2 +– ++ +***r3***R4R4	HT134 +– ++ ***r3r3***R4+	26:17:9	1:1 2:1:1	0.00 2.46	1 2
3	stand-MS1-3 +*r1* +***r2*** +*r3R4R4*	REI141 ++ +***r2*** ++++	57:7:7	3:1 3:1:0	0.79	1
4	stand-MS1-4 +*r1* ++ +***r3***R4+	NAT9 ++ ++ ***r3r3***R4R4	9:5:3	1:1 2:1:1	0.00 0.53	1 2
5	1-1 MS1 +***r1*** ++ +***r3***R4R4	LEEK3 +***r1*** +– +***r3***R4+	28:16:16	9:7 18:3:11	1.87 21.15***	1 2
6	stand-MS1-6 +***r1*** +*r2* +***r3***R4R4	BM103 +***r1*** ++ ***r3r3***R4+	26:13:12	3:5 6:3:7	3.40 8.47*	1 2
F_2 generation						
7	4-1 H +***r1*** ++ ***r3r3***R4R4	5-1 H ***r1r1*** ++ +***r3***R4+	24:15:91	1:3 2:1:5	2.63 3.49	1 2
8	3-1 IN1 +***r1*** *r2r2* ++*R4*+	5-1 H ***r1r1*** ++ +*r3R4*+	103:15:67	1:1 1:0:1	2.16	1
9	3-2 IN1 +– ***r2r2*** +***r3***R4+	6-1 H +*r1* +***r2*** ***r3r3***R4R4	71:53:155	1:3 1:1:2	0.01 5.77	1 2
10	3-2 IN1 +– *r2r2* +***r3***R4+	2-1 H +– ++ ***r3r3***R4R4	84:13:70	1:1 2:1:1	0.00 38.92***	1 2
11	3-2 IN1 +– *r2r2* +***r3R4***+	2-2 IN1 +– ++ ***r3r3R4***+	100:21:29	5:3 5:2:1	0.94 13.28**	1 2
12	6-1 H +***r1*** +– ***r3r3***R4R4	5-1 H ***r1r1*** ++ +***r3***R4+	42:36:132	1:3 2:1:5	2.54 5.73	1 2
13	4-2 H +***r1*** ++ ***r3r3***R4R4	6-2 H ***r1r1*** +– +***r3***R4+	43:19:67	1:3 2:1:5	4.34 6.40*	1 2

In the F_1 generation standard-MS1 (*CMSI*) is the seed parent, the CMS type of the pollen parents is unknown. In the F_2 both parents are *CMSI*. For each cross, the proposed genotype (at the loci involved in restoration of male fertility) is given. The symbols are explained in Table 7. Loci that determine the segregation in the progeny are printed in bold. The differences between observed and expected ratios were tested with a chi-square test; the upper value is for MS *vs.* IN + H, and the lower for MS:IN:H.
[a] Significant chi-square values are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

TABLE 3

Crosses with *CMSIIa* as seed parent, explained with a two-locus model

Cross	Seed parent	Pollen parent	Observed number MS:IN:H	Expected ratio MS:IN+H MS:IN:H	χ^{2a}	d.f.
	Family 1a					
14	Vel H	J1	0:2:23	0:1	—	
	R1+ R2+	*R1+ R2R2*		0:1:7	0.14	1
15/16	14-1 H	A1	4:5:12	1:3	0.14	1
	++ *R2+*	++ *R2+*		1:2:1	11.86**	2
17	15-1 H	16-1	0:2:9	0:1	—	
	++ *R2R2*	++ *R2+*		0:1:1	3.27	1
18	17-1 IN2	A2	6:20:9	1:3	0.77	1
	++ *R2+*	++ *R2+*		1:2:1	1.23	2
	Family 1b					
19/20	14-1 H	An1	2:3:8	1:3	0.23	1
	++ *R2+*	++ *R2+*		1:2:1	9.31**	2
21	19-1 IN2	20-1	4:12:19	1:3	2.75	1
	++ *R2+*	++ *R2+*		1:2:1	16.31***	2
	Family 2					
22/23	Vel H	31-1	0:0:12	0:1	—	
	R1+ R2+	*R1R1 R2+*		0:0:1		
24	22-1 H	23-1	0:1:24	0:1	—	
	R1R1 ++	*R1+ R2+*		0:0:1		
25	22-2 H	23-1	4:11:45	1:15	0.00	1
	R1+ R2+	*R1+ R2+*		1:2:13	1.94	2
26	22-2 H	23-2	4:13:19	1:15	0.74	1
	R1+ R2+	*R1+ R2+*		1:2:13	21.01***	2

Pollen parents have another CMS type than *CMSIIa*. Two cross numbers per line indicates reciprocal (*i.e.*, the same nuclear loci are involved). The reciprocal cross has a different CMS type than *CMSIIa*. See Table 7 for symbols and interpretation.

[a] Significant chi-square values are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

the restorer frequency for *CMSI* and *CMSIIb*, respectively, the average of the IN+H percentage of all the crosses between the standard male sterile and the hermaphrodites from a particular population was calculated. This estimate reflects the number of loci (and not only of individuals) that carry restorer alleles for a particular CMS type. The following example may explain this. A hermaphrodite carries restorer alleles at two loci for *CMSI, r1r1 r2r2*. The cross with standard MS1 (*+r1 +r2*) segregates 75% IN+H, whereas a cross with hermaphrodite *r1r1* ++ segregates 50% IN+H. A hermaphrodite carries restorer alleles for its own CMS type, but may or may not carry restorer alleles for other CMS types, and crosses with a hermaphrodite ++ ++ will only segregate MS. Therefore the restorer percentages indicate the restorer allele frequency as an average over loci. The difference in restorer percentages was tested with the procedure Genmod in SAS, by fitting a log-linear model with a binomial error distribution to the data.

Restorer allele frequency was estimated assuming either dominant or recessive restorer action. For the dominant case, the estimate was $1 - \sqrt{q}$, where q is the mean MS frequency in the crosses; for the recessive case the estimate was $\sqrt{p}$, where p is the mean IN+H frequency.

In addition to the crosses with standard MS, intra- and interpopulation crosses were made between male steriles and hermaphrodites from seven populations (Heteren, Reitma, Junner Koeland, Anloo, Westduinen, Vlietlanden, Merrevliet; VAN DAMME and VAN DELDEN 1982). On average 35 progeny was scored per cross (minimum six and maximum 61 plants). The crosses were divided into four categories: first by intra- or interpopulation, second by crosses with MS1 (*CMSI*) as a seed parent or MS2 (taking *CMSII* types together). Differences in the proportion of MS were tested by fitting a log-linear model to the data with three factors; intra-/interpopulations, CMS type, and seed parent within CMS type.

Estimated *vs.* expected frequency of restorer alleles: In the experiment mentioned above, an estimate of restorer allele frequencies in each population was obtained. This estimate can be compared with the expected in DE HAAN *et al.* (1997b), *i.e.*, the difference between the assessed percentage of a CMS type and its male sterile percentage assessed in the population. The difference between estimated and expected restoration percentages was tested with a paired *t*-test, and the Pearson correlation coefficient between the two was calculated.

RESULTS

Restorer loci for *CMSI*: number and mode of action: In Table 2 crosses for restorer loci for *CMSI* are shown. In cross 5 a descendant of standard MS1 was used and is shown in the first line of Table 2. Hermaph-

TABLE 4

Crosses with *CMSIIa* as the seed parent, explained with a one-locus model with three alleles

Cross	Seed parent	Pollen parent	Observed number MS:IN:H	Expected ratio MS:IN+H MS:IN:H	χ^{2a}	d.f.
	Family 1a					
14	Vel H	J1	0:2:23	0:1	—	
	RR	*R+*		0:1:3	3.0	1
15/16	14-1 H	A1	4:5:12	1:3	0.14	1
	R+	*R+*		1:1:2	0.52	2
17	15-1 H	16-1	0:2:9	0:1	—	
	RR	*R+*		0:1:3	0.03	1
18	17-1 IN2	A2	6:20:9	1:3	0.77	1
	R+	*R+*		1:2:1	1.23	2
	Family 1b					
19/20	14-1 H	AN1	2:3:8	1:3	0.23	1
	R+	*R+*		1:1:2	0.85	2
21	19-1 IN2	20-1	4:12:19	1:3	2.75	1
	R+	*R+*		1:1:2	3.91	2
	Family 2					
22/23	Vel H	31-1	0:0:12	0:1	—	
	RR	*R+*		0:1:3	2.78	1
24	22-1 H	23-1	0:1:24	0:1	—	
	R+	*RR*		0:1:3	4.81*	1
25	22-2 H	23-1	4:11:45	0:1	—	
	R+	*RR*		0:1:3	0.02	1
26	22-2 H	23-2	4:13:19	1:3	3.00	1
	R+	*R+*		1:1:2	4.61	2

Two cross numbers per line indicates reciprocal (*i.e.*, the same nuclear loci are involved). The reciprocal cross has a different CMS type than *CMSIIa*. See Table 7 for symbols and interpretation.

[a] Significant chi-square values are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

rodites or intermediate types from the F_1 progeny of crosses 2–6 were crossed among each other to produce the F_2. Crosses 3, 8 and 13 show that three recessive loci are necessary to explain the data. The segregation ratio of cross 11 was significantly different from 1:1 ($\chi^2 = 16.67***$) and could not be explained with one locus. It did not show deviation from 5:3, which indicates epistatic effects (Figure 1a). In total, four restorer loci for *CMSI* were proposed, three with recessive action, which can restore male fertility independently of each other, and one locus with a codominant restorer allele, which is epistatic and can only restore male fertility together with the third locus. Hence, at least two recessive restorer alleles are necessary for restoration of male fertility in *CMSI*.

The inheritance of the intermediate types cannot be fully explained. Locus 2 may only restore partially, as proposed in the interpretation of cross 3. Crosses 2, 4, 7 and 12 were best explained by assuming that at locus three and four *r3r3R4+* would be an intermediate type. Furthermore, it was assumed that partial restoration at both loci (*r2r2* and *r3+*) together leads to complete restoration (additive effects), which explains the ratio in cross 9. The genetic model explains all the MS *vs.* IN+H ratios and most of the MS *vs.* IN *vs.* H ratios. Five crosses showed significant differences between the observed and expected ratios, when IN is taken as a separate phenotype. In crosses 5, 6 and 13 more IN types segregated than expected, whereas crosses 10 and 11 segregated fewer intermediates than expected.

Restorer loci *CMSII*: number and mode of action: Table 3 shows four families in which the restorer loci for *CMSIIa* can be determined. In most crosses IN and H segregated, with a very limited number of MS. The segregation patterns can be explained by either two loci with dominant restorer alleles (Table 3) or one locus with three alleles (Table 4). The second model (with one dominant *R* allele, codominant R, and a ms-allele +) gave the better fit. Two loci with a dominant (locus 1) or codominant (locus 2) restorer allele were necessary to explain the expected ratios in crosses 25 and 26 under the two-locus model (Table 3). The presence of intermediate phenotypes in four crosses can be explained if one assumes that the codominant restorer allele at locus 2 only partially restores male fertility. Compared to the two-locus model, an excess of IN types was observed in the cross 26, whereas more IN would have been expected in crosses 15/16, 19/20 and 21.

TABLE 5

Crosses between seed parents with *CMSIIb* and their backcrosses on the seed parent

Cross	Seed parent	Pollen parent	Observed number MS:IN:H	Expected ratio MS:IN+H MS:IN:H	χ^2	d.f.
F_1 generation						
27	stand MS2-1	REI138	2:32:7	0:1	—	
	++++ +*r3*	*R1R1R2R2 +r3*		0:3:1	0.98	1
28	stand MS2-2	REI149	2:27:18	0:1	—	
	++++ +*r3*	*R1R1R2R2 r3r3*		0:1:1	2.13	1
29	stand MS2-2	HT103	1:13:27	0:1	—	
	++++ +*r3*	*R1R1R2R2 r3r3*		0:1:1	3.51	1
30	stand MS2-2	HT104	1:14:18	0:1	—	
	++++ +*r3*	*R1R1R2R2 r3r3*		0:1:1	0.12	1
31	stand MS2-2	HT106	1:4:18	0:1	—	
	++++ +*r3*	*R1R1R2R2 r3r3*		0:1:1	6.26*	1
32	stand MS2-2	KOU109	0:5:19	0:1	—	
	++++ +*r3*	*R1R1R2R2* ++		0:1:0	—	
Back cross generation						
33	stand MS2-2	27-1 H	16:21:5	3:5	0.01	1
	++++ +*r3*	*R1+R2+ r3r3*		3:2:3	17.84***	2
34	stand MS2-2	28-1 H	11:18:8	3:5	0.65	1
	++++ +*r3*	*R1+R2+ r3r3*		3:2:3	11.36**	2
35	stand MS2-1	29-1 IN	13:10:8	9:7	2.03	1
	++++ +*r3*	*R1+R2+ +r3*		9:3:4	4.15	2
36	stand MS2-1	30-1 H	12:16:7	3:5	0.05	1
	++++ +*r3*	*R1+R2+ r3r3*		3:2:3	8.96*	2
37	stand MS2-1	31-1 H	15:5:13	3:5	0.58	1
	++++ +*r3*	*R1+R2+ r3r3*		3:2:3	1.87	2
38	stand MS2-2	32-1 IN	40:6:3	3:1	0.82	1
	++++ +*r3*	*R1+R2+* ++		3:1:0		

Pollen parents in the first generation can have a different CMS type than *CMSIIb*. See Table 7 for symbols and interpretation.

[a] Significant chi-square values are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

In contrast, only cross 24 gave too few IN types compared to the expected ratio for the one-locus model (Table 4).

Tables 5 and 6 show crosses with *CMSIIb* as a seed parent. Table 5 shows crosses that segregated mainly IN and H in the F_1, which were backcrossed with the male sterile seed parent. Table 6 shows crosses that segregated MS in the F_1, which were backcrossed with the pollen parent. Cross 32 and its backcross 38 showed that two epistatic loci were involved (Table 5). In addition, one locus with a recessive mode of action was necessary to explain cross 40 and its backcross 46 (Table 6). In cross 50 the segregation of MS *vs.* IN+H cannot be explained with this genetic model. More epistatic loci have to be assumed, or else there could be linkage between the restorer loci, or the MS2 seed parent may have been genotypically hermaphroditic. To explain the occurrence of partial male steriles (IN), the restorer alleles at the two epistatic loci were assumed to be codominant, with *R1+R2+* giving an IN phenotype. In this way the segregation of MS:IN:H could be explained in 16 out of 24 crosses. Crosses 33, 34, 36 and 41 showed a surplus of observed IN types; cross 31 segregated fewer IN types than expected on the basis of the three-locus model. Crosses 32, 38 and 40 segregated H when only IN were expected. Table 7 summarizes the genetic models for all three CMS types.

Frequency of restorer alleles: We used standard male steriles to estimate restorer allele frequencies of seven populations. Based on the results of the number and mode of action of the restorer alleles, the standard male steriles can be assessed for their ability to detect restorer alleles. The standard MS1 is heterozygous for the four restorer loci and will be able to detect variation for recessive alleles. From the proposed genetic model for *CMSIIa* the restorer allele composition for the standard MS2a (14-1) can be deduced. The standard MS was used to screen restorer allele variation among populations and must be homozygous ++ at the restorer locus for *CMSIIa*. Crosses with this standard MS2a can be expected to detect dominant restorer alleles for this locus, as well as codominant alleles. The standard MS2b

a

	r1r1	r1+	+r1	++
r2r2	H	H	H	H
r2+	H	MS	MS	MS
+r2	H	MS	MS	MS
++	H	MS	MS	MS

b

	r3r3	r3+	+r3	++
r4r4	H	MS	MS	MS
r4+	MS	MS	MS	MS
+r4	MS	MS	MS	MS
++	MS	MS	MS	MS

FIGURE 1.—Illustration of independent (a) and epistatic (b) restorer gene interaction for a model with two genes having recessive restorer alleles (after KOELEWIJN and VAN DAMME, 1995).

(family J637) was used to estimate the restorer allele frequency for *CMSIIb* in the field, and variation for all three proposed loci can be detected. Although the genetic basis of restoration of male fertility is not simple, in many crosses only one locus was variable. Therefore we assume that one locus is sufficient to restore male fertility. Segregation ratios of IN+H types in crosses between standard male steriles and hermaphrodites from seven populations yields estimates of the frequency of restoration of male fertility (Figure 2). In all populations the restoration frequency for *CMSI* was lower than for *CMSIIb*. The differences in restoration level between CMS types, populations, and the interaction between CMS type and population were all significant (Table 8). The lowest restoration frequencies for *CMSI* and *CMSIIb* were found in Naterseweg (NAT) and Reitma (REI), respectively, whereas in Brummen (BM) a high level of restoration for *CMSI* was found.

Additional crosses were made between male steriles and hermaphrodites from seven other populations. In these cases the male steriles originated from field populations and were possibly different from the standard male steriles used in the previous crosses. These crosses confirmed the asymmetrical segregation of male steriles of the CMS types (Figure 3, $\chi^2 = 89.2^{***}$, d.f. = 1).

Based on the segregation of IN+H in crosses between standard male steriles and hermaphrodites from populations, an estimate of the frequency of restoration of male fertility was made and the allele frequencies calculated (Table 9). The restorer allele frequency is necessarily higher if a recessive mode of action was assumed. The restorer allele frequency of *CMSIIb* was higher than for *CMSI*. However if the restorer alleles for *CMSI* are assumed to be recessive and for *CMSIIb* dominant, then the allele frequencies were about the same (0.51 and 0.49). The restorer allele frequency for *CMSIIa* appears to be high, based on the limited data.

Estimated *vs.* expected frequency of restorer alleles: In DE HAAN *et al.* (1997b) male sterile and CMS type frequencies were assessed in eight populations. Without restorer alleles the frequency of a MS type would be equal to the frequency of its CMS type. Differences between the occurrence of MS in a population and the observed CMS type frequencies must therefore be due to the presence of restorer alleles. Seven of the same eight populations were screened in the present study and gave estimates of the restorer frequencies.

Figure 4 shows the correlation between the "estimated" restoration level and the "expected" restoration level for seven populations for both the *CMSI* and *CMSII* types. The estimated restoration level in the present study was based on segregation of IN+H in crosses between standard male steriles and hermaphrodites from the field, and estimates the frequency of restorer alleles in the pollen pool. The expected restoration level in the previous study was based on the difference between the frequency of the CMS type and its male sterile type (MS1 for *CMSI*, MS2 for the *CMSII* types). The surplus of plants with that CMS type must be intermediate or hermaphrodite in the population. Therefore the expected restoration level includes the male sterile plants in the population and gives an estimate of the restoration level in both ovules and pollen. With a paired *t*-test, a significant difference between estimated and expected was found. The estimated frequency was higher than for both CMS types in all populations. However, the correlation between the estimated and expected values was significant.

DISCUSSION

Restorer loci: number and mode of action: In each population, restorer allele variation for different loci was found, but populations also shared restorer alleles. In *P. coronopus* evidence was found for variation among populations (KOELEWIJN and VAN DAMME 1995). We found recessive alleles or interacting loci with epistatic effects (loci 3 and 4), whereas VAN DAMME (1983) found two loci with dominant restorer action for this CMS type. The apparent differences between the study of VAN DAMME (1983) and this study could reflect variation in restorer loci for *CMSI* within and/or among populations. It is possible that with a larger sample from

TABLE 6

Crosses between seed parents with *CMSIIb* and their backcrosses on the pollen parent

Cross	Seed parent	Pollen parent	Observed number MS:IN:H	Expected ratio MS:IN+H MS:IN:H	χ^{2a}	d.f.
F_1 generation						
39	stand MS2-1	REI124	12:21:5	3:5	0.34	1
	++++ +*r3*	*R1R1R2*+ +*r3*		3:3:2	5.68	2
40	stand MS2-1	REI127	21:7:7	3:1	3.44	1
	++++ +*r3*	−−−− +*r3*		3:0:1		
41	stand MS2-1	REI141	56:38:13	9:7	0.52	1
	++++ +*r3*	*R1*+*R2*+ +*r3*		9:3:4	23.4***	2
42	stand MS2-1	HT128	14:8:16	3:5	0.01	1
	++++ +*r3*	*R1*+*R2*+ *r3r3*		3:2:3	0.46	2
43	stand MS2-1	HT129	21:34:60	1:3	2.55	1
	++++ +*r3*	*R1R1R2*+ *r3r3*		1:1:2	3.16	2
44	stand MS2-1	HT130	13:12:10	3:5	0.02	1
	++++ +*r3*	*R1*+*R2*+ *r3r3*		3:2:3	1.95	2
Back cross generation						
45	39-1 MS2	REI124	13:8:4	1:1	0.00	1
	R1+++ ++	*R1R1R2*+ +*r3*		2:1:1	1.32	2
46	40-1 MS2	REI127	25:2:0	1:0	—	
	−−−− ++	−−−− +*r3*		1:0:0		
47	41-1 MS2	REI141	13:11:9	15:17	0.47	1
	R1+++ +*r3*	*R1*+*R2*+ +*r3*		15:6:11	4.62	2
48	42-1 MS2	HT128	1:4:9	5:11	2.75	1
	R1+++ +*r3*	*R1*+*R2*+ *r3r3*		5:2:9	5.66	2
49	43-1 MS2	HT129	10:3:29	1:3	0.00	1
	R1+++ +*r3*	*R1R1R2*+ *r3r3*		2:1:5	1.28	2
50	44-1 MS2	HT130	0:1:18	5:11	7.24**	1
	R1+++ +*r3*	*R1*+*R2*+ *r3r3*		5:2:9	11.74**	2

Pollen parents in the first generation can have a different CMS type than *CMSIIb*. See Table 7 for symbols and interpretation.

[a] Significant values are indicated by $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

more populations, dominant restorer alleles would also have been found. A second possibility could be that the proposed genetic models are too simple. In both studies the most parsimonious model was proposed that could explain the results. Reexamination of the results VAN DAMME (1983) showed those segregation ratios could also be explained with recessive loci. Moreover, some of his crosses can be better explained by two epistatic loci, as proposed in the present study (A. A. DE HAAN, unpublished results).

Recently variation in CMS types within *CMSII* was found (DE HAAN *et al.* 1997a). It therefore became important to be able to distinguish between the restorer loci for *CMSIIa* and *CMSIIb*. The *CMSII* tester line used appeared to be *CMSIIb*. Because this line was used in most crosses, we could present a genetic model for the *CMSIIb* type, but only preliminary models for *CMSIIa*. Compared with the genetic model of VAN DAMME (1983) for *CMSII* with three loci each with dominant restorer action, we now found, in addition, loci with recessive action and loci with epistatic effects on each other. We can conclude that many loci seemed to be involved in restoration of male fertility for *CMSI* and for the two *CMSII* types together. A number of restorer loci have also been found in other gynodioecious species (CHARLESWORTH and CONNOR 1989; BELHASSEN *et al.* 1991). Both recessive and dominant restorer alleles were found at loci that could restore male fertility independently, and loci with epistatic effects have also been found in earlier studies with other gynodioecious species (LAUGHNAN and GABY-LAUGHNAN 1983; KOELEWIJN and VAN DAMME 1995).

VAN DAMME (1983) found that the sex expression of the intermediate types can partly be traced to genetic factors. We also have tried to explain the inheritance of the intermediate types. For most crosses the genetic models were satisfactory, but for all CMS types deviations in expected and observed ratios were found. Environmental effects on the expression of male fertility in the progeny of the *CMSI* crosses 7–13 (Table 2) occur (DE HAAN 1996). It is thus possible that different circumstances in the experimental garden between years

TABLE 7

Proposed genetic models for the restoration of male fertility in *CMSI*, *CMSIIa* and *CMSIIb*

CMSI
Three recessive loci *+r1 +r2 +r3;* one codominant locus *R4+*; epistatic loci *+r3R4+*;
Partial restoration: locus 2 or locus 3+4 but interaction between the loci
MS: +− +− +−−−
IN: +− *r2 r2* +−−− or +− +− *r3r3R4+*
H: *r1r1* −− −−−− or −− *r2r2 r3r3R4+* or −− −− *r3r3R4R4* or −− *r2r2 r3+R4R4*

CMSIIa
Model 1. Two loci: *R1* dominant restorer allele; *R2* codominant
MS: ++ ++
IN: ++ *R2+*
H: *R1*− −− or −− *R2R2*
Model 2. Three alleles on one locus: *R*, *R*, +
MS: ++
IN: *R+*
H: R*R* or *R*−

CMSIIb
Two epistatic loci with codominant restorer alleles
One locus with a recessive restorer allele *+r3*
MS: *+R1*++ +− or +++*R2* +−
IN: *R1+R2+* +−
H: *R1R1R2*− +− or *R1*−*R2R2* +− −−−− *r3r3*

R, dominant restorer allele; *R*, codominant; *r*, recessive restorer allele; +, male sterile allele. If two loci have epistatic effects on each other, the loci are written together without spacing. For each phenotype the possible genotypes are given. The possible phenotypes are MS, male sterile; IN, intermediate; and H, hermaphrodite. The possible genotypes show the allele composition at all proposed loci. If at a locus either one of the alleles does not affect the phenotype, it is indicated as (−).

TABLE 8

Differences in restorer allele frequencies

	d.f.	χ^2	*P*
CMS type[a]	1	1469.1	0.000
POP[b]	6	521.4	0.000
CMS type * POP	6	141.4	0.000

Degrees of freedom (d.f.), χ^2-value and the significance level (*P*) from fitting a log-linear model to the data are given.
[a] *CMSI* and *CMSIIb*.
[b] Seven populations.

may have caused deviant segregation of intermediates in the *CMSI* series.

Frequency of restorer alleles: We found that levels of restoration for the CMS types differ. In all populations the frequency of restorers for *CMSI* was lower than for the other CMS types. This could have been an artifact due to the standard male steriles used. The composition of the restorer loci in the male steriles has a great effect on the detection capacity for restorer allele frequencies in the populations. However, crosses between randomly sampled male steriles and hermaphrodites both within and among populations showed the same differences among CMS types. Furthermore in DE HAAN *et al.* (1997b), a limited assessment of the restoration level for the CMS types was carried out and the level of restoration for *CMSI* types was also found to be significantly lower than for the other CMS types. In several other gynodioecious species, there are indications that differences in the occurrence of restorer alleles for different CMS types exist. In *P. coronopus* the two CMS types segregated male steriles in different rates (KOELEWIJN and VAN DAMME 1995). CUGUEN *et al.* (1994) found that in *B. maritima* some mitochondrial

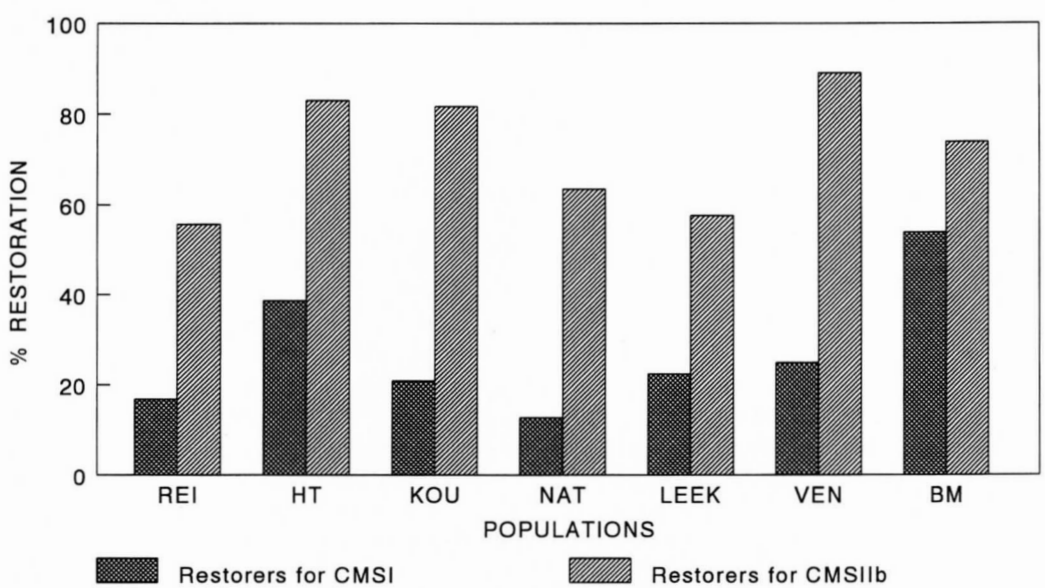

FIGURE 2.—Restoration level for two CMS types in seven populations. The first bar represents the restoration level for *CMSI*, the second for *CMSIIb*. The restoration level was estimated by crosses between hermaphrodites from the populations with standard male steriles and represented as the mean percentage of intermediates and hermaphrodites in the crosses per population.

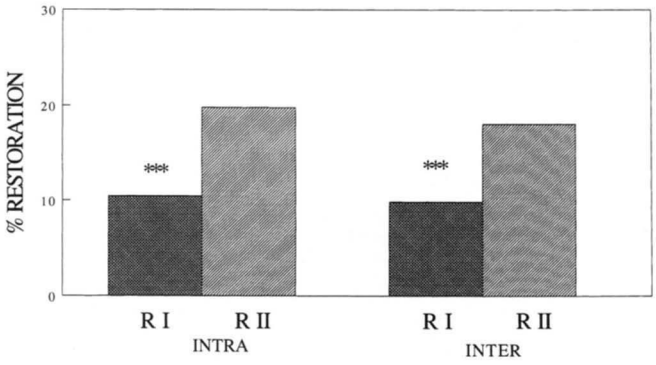

FIGURE 3.—Restoration level for two CMS types estimated from crosses between male steriles and hermaphrodites within (intra) or between (inter) populations. The restoration level is represented as the percentage of intermediates and hermaphrodites in the crosses for the two CMS types separately (RI and RII) and for intra- and interpopulation crosses.

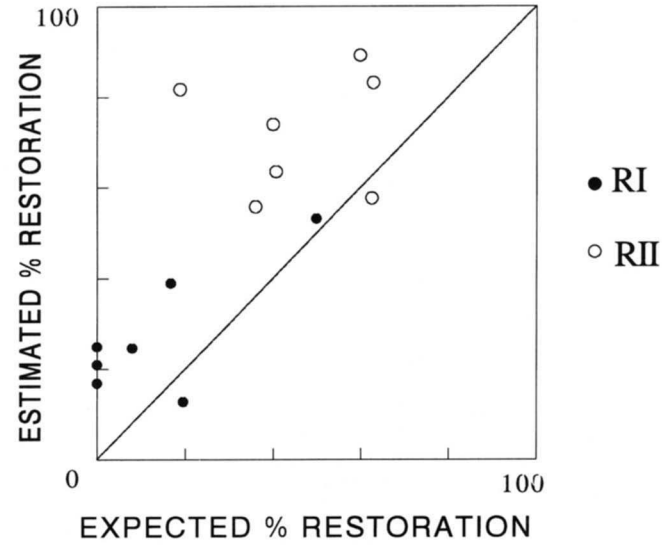

FIGURE 4.—The estimated and expected frequency of restorer alleles for seven populations is shown. "Estimated" was based on segregation of intermediates and hermaphrodites in crosses between hermaphrodites and standard male steriles (see Figure 1). "Expected" was based on the difference between the percentage of a CMS type and its male sterile percentage (based on DE HAAN 1997b). The line represents estimated = expected. A paired *t*-test showed a significant deviation (difference in mean = 20.31; *t*-value = 4.43; d.f. = 13, $P = 0.0007$) of the data from this line. The correlation between estimated and expected is 0.78** (14 cases).

variants (maybe indicative of different CMS types) were found mostly in hermaphrodites, whereas others were found mainly in male steriles. MANICACCI (1993) found different levels of restoration for different mitochondrial variants among populations of *T. vulgaris*. In crop species "normal" cytoplasms occasionally segregate male steriles, indicating that a normal cytoplasm could be a highly restored cytoplasm, whereas CMS types would have low levels of restoration (KENNELL *et al.* 1987; CLARK *et al.* 1988; HÅKANSSON *et al.* 1988; DUDAREVA *et al.* 1990).

Several explanations are possible for the differential distribution pattern of the restorer alleles over the CMS types. First, a technical point should be mentioned. The four-locus model for *CMSI* proposed only recessive restorer alleles, or epistatic loci. Therefore at least two recessive alleles are necessary to restore male fertility in *CMSI*. The three-locus model for *CMSIIb* consists of both dominant and recessive restorer alleles. As a consequence of the difference in mode of action, the estimation of the restorer frequency for *CMSI* will be lower than for *CMSIIb* even if allele frequencies were equal. This, however, does not explain the differences in male sterile frequencies, while male fertility in *CMSIIb* could only be restored either by two recessive alleles or by two dominant alleles at two epistatic loci. An explanation for the differential frequency of restorer alleles over the CMS types could be that the restorer alleles for one CMS type have a higher "cost" (negative pleiotropic effects) than others (GOUYON *et al.* 1991). If the

TABLE 9

Allele frequencies of restorer loci

	R CMSI[a]			*R CMSIIb*			*R CMSIIa*			
Population	If dominant[b]	If recessive[b]	*n*	If dominant	If recessive	*n*	If dominant	If recessive	No.[c]	n[d]
Reitma	0.09	0.41	25	0.33	0.75	29	0.72	0.96	11	4
Heteren	0.22	0.62	26	0.59	0.91	25	0.91	1.0	6	3
Koudum	0.11	0.46	7	0.57	0.90	11				
Naterseweg	0.07	0.36	24	0.40	0.80	25	1.0	1.0	5	1
Leek	0.12	0.50	18	0.35	0.76	17	0.78	0.97	5	2
Veneweg	0.13	0.50	45	0.67	0.94	52				
Brumen	0.32	0.73	16	0.49	0.86	17	1.0	1.0	5	2
Mean	0.14	0.51		0.49	0.86		0.80	0.98		

[a] *R* is the restorer allele frequency in seven populations for three CMS types (*CMSI, CMSIIb, CMSIIa*) and is based on the segregation of male steriles in crosses of hermaphrodites from the populations with standard male steriles.
[b] The restorer alleles were assumed to be either dominant or recessive.
[c] *n* is the number of crosses used to estimate *R*.
[d] For *CMSIIa* the number of crosses (*n*) was different from the number of used hermaphrodites (No. 1) (mixed pollen).

cost of restoration is higher for restorer alleles for *CMSI* than for restorer alleles for *CMSII* types, the frequency of the *CMSI* restorer alleles is expected to be lower. Alternatively, CMS types might have invaded populations at different times during the course of evolution: a CMS type arises, male steriles spread within a population until restorer alleles spread, and male steriles will be found each time as a new CMS type arises. The results presented here are not conclusive for any of the scenarios.

Estimated *vs.* expected frequency of restorer alleles: In the present study the restoration level was estimated in the pollen pool, whereas the expected restoration percentage was based on all plants, including the male steriles. The comparison of both methods could lead to four possible outcomes: a good fit, deviations per population, or a general over- or underestimation in this study compared to the results of DE HAAN *et al.* (1997b). A good fit is expected if male steriles in the population have the same restorer allele composition as the standard male steriles, meaning heterozygous and homozygous at the same loci. We found that the frequency of restorer alleles in the pollen pool was higher than in the total gene pool. This means that the male steriles in the studied populations possessed fewer restorer alleles than the standard male steriles we used to assess the restoration level. If the standard male sterile was heterozygous at loci for which many male steriles in the studied populations were homozygous wild type, then crosses with the standard male sterile will segregate IN+H, whereas crosses with the male steriles in the field will generate MS. An additional factor is that we used only H to assess the restoration level and did not include IN. Possibly IN possesses less restorer alleles than H, which may also lead to an overestimation in this study. However based on the pollen production of IN in the greenhouse (A.A. DE HAAN, personal observations), the contribution of IN to the pollen pool is expected to be small.

Despite the overestimation of the restoration level in this study, a significant positive correlation with the expected restoration percentage, based on a parallel study, was found (DE HAAN *et al.* 1997b). This implies that the differences between the populations were due to differences in frequencies of the CMS types in restorer allele frequencies, and thus in male sterile frequencies in those populations. For example, in Brummen a low frequency of male steriles with *CMSI* was found, whereas the frequency of *CMSI* was high (DE HAAN *et al.* 1997b), suggesting a relatively high frequency of restorer alleles for *CMSI* in Brummen.

In DE HAAN *et al.* (1997b) the occurrence of MS1 could be partly predicted by the occurrence of *CMSI*. A high restoration level for *CMSIIb* was found, compared to that for *CMSI*, that can explain the observed low frequency of the MS2 phenotype together with the observed high frequency of *CMSII* types. In the present study, only restorer alleles for *CMSIIb* were determined, which seemed to be a good indicator for restorer alleles for *CMSII* types together. Altogether, the significant positive correlation between the estimated and expected restoration level indicates that the frequency of male steriles in the populations studied can be fairly well predicted from the underlying gene frequencies of the CMS types and their restorer alleles.

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