Allozyme genetics, self-incompatibility and male sterility in *Plantago lanceolata**

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In *Plantago lanceolata* 14 out of 39 enzyme loci showed genetic variation, as checked by analysis of crosses. Five linkage groups could be formed with these variable loci. They may represent five different chromosomes as $n = 6$ in this species. The *SI* locus, controlling self-incompatibility, appeared to be very tightly linked with *Pgm-1* (recombination = 0.8 per cent) and somewhat more loosely with *6Pgd-1* and *6Pgd-2*. In this linkage group also a recessive male sterility locus could be placed. A different male sterility locus with recessive effect was located in a second linkage group, and a male sterility locus with dominant effect in a third one.

These results, and the finding of differences in germination behaviour when certain chromosomes are substituted, point to the possible further use of allozyme loci in analysing the genetic basis of ecologically important differences in *P. lanceolata*.

INTRODUCTION

Allozyme variation has proved to be a successful tool in the analysis of genetic variation for ecologically important characters within the species *Plantago major* (Van Dijk, 1984). Although *Plantago lanceolata* shows less tendency to ecotypic differentiation, still many interesting examples of genetic variability have been reported: genetic variation for flowering time related to annual time of mowing and for leaf angle and length related to vegetation height has been found by Van der Toorn and Ten Hove (unpublished results). Genetic variation for seed yield is reported by Primack and Antonovics (1981); for photosynthetic response to temperature and irradiance by Teramura and Strain (1979). Sagar and Harper (1964) mention different varieties of *P. lanceolata* which also points to genetic differentiation.

Prior to the use of allozyme loci in analysing quantitative characters, their inheritance should be elucidated. Any linkage relations between the loci must be known. In the present study the inheritance of variable allozyme loci is investigated. Some other genetic differences which were met during these investigations are studied simultaneously:

those connected with the self-incompatibility system, with sex polymorphism and with germination behaviour. The genetics of self-incompatibility in *P. lanceolata* has been investigated by Ross (1973). Gynodioecy in this species has been studied recently by Van Damme (1983a) who succeeded in partially analysing its inheritance.

MATERIALS AND METHODS

Plantago lanceolata plants were collected at seven sites in the east (Br, He, We, Pa, Ud) and the south-west (Wd, Me) of the Netherlands, and were labelled according to origin and serial number. The sample size was about 100 per site. The plants were transferred to an experimental garden. Crosses were made in the greenhouse between plants which were different for many variable enzyme loci. The F_1 seeds were stored in a dry place for a few weeks and then germinated in petri dishes with wet filter paper. Seeds which did not germinate spontaneously were cut open at the side of the radicle. The seedlings were cultured in pots with soil and leaves were used for electrophoresis when the plants were four to six weeks old. After

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transplantation into the experimental garden the F_1 plants were scored for sex phenotype in the next summer. According to Van Damme (1983b) the sex phenotype was described as MS (male sterile), H (hermaphrodite), IN-a (predominantly male sterile), IN-b (intermediate), or IN-c (predominantly hermaphrodite).

Extract preparation, electrophoresis and subsequent staining were performed according to Van Dijk and Van Delden (1981). The best results were obtained with leaves of four to six week old seedlings from the greenhouse and with central leaves from winter-rosettes collected outdoors in winter or spring. Identical electrophoresis patterns were reached in both cases. Older leaves or leaves from plants growing under less optimal conditions needed more mercaptoethanol in the extract mixture to overcome their higher level of phenolic compounds.

Recombination frequencies between loci were calculated by a method which leads to approximately the same results as the maximum likelihood method (see Green, 1981) but which is easier when a computer is available. Advantages are that the 95 per cent intervals can directly be derived and that information about the relative likeliness of the segregation model is obtained if more than one model is possible. The method is based on the determination of the minimal χ^2 value and will be described in Appendix A together with a worked example.

RESULTS

Electrophoretic variation

All plants collected in the field (about 700) were screened for electrophoretic variation. The same 21 enzyme systems as previously used for *Plantago major* (Van Dijk and Van Delden, 1981) were scored for *P. lanceolata*. In general the most probable number of loci derived from each enzyme staining pattern was the same as found in *P. major*, except for the enzymes Glyceraldehyde-3-phosphate dehydrogenase, Amylase and Esterase, where in each case one additional locus is postulated, bringing the most probable number of loci to 39. In two variable loci, Peroxidase-1 and Esterase-3, no clear genetic basis could be determined. Fourteen loci showed genetic variation as proved by crossing results. In table 1 the variable enzyme loci with their alleles are summarised together with other details. Allele frequencies in the sampled populations will not be given here, but form one of the subjects of a next paper (Van Dijk, Wolff and De Vries, in prep.).

Six plants from the populations Br, He and We were selected for making crosses to verify the Mendelian inheritance of the allozyme loci. To detect linkage relations also F_2 's were made by brother-sister crossing within the F_1 ($He_{88} \times We_{44}$). All crosses were made reciprocally. The allozyme genotypes of all plants used for these crosses could

Table 1 The variable enzyme loci

Enzyme	Locus	Number of subunits	Electrophoresis buffer pH	Alleles*
Esterase	<i>Est-4</i>	1	7	A(31), B(33), C(36.5), D(38), E(40), F(42), G(44), 0
	<i>Est-5</i>	1	7	A(48), B(50), C(53), D(55), E(57.5), F(60), 0
Glutamate-oxaloacetate-transaminase	<i>Got-1</i>	2	7	S(19), I(21), F(23)
	<i>Got-2</i>	2	7	S(39), F(41)
Glucose-phosphate-isomerase	<i>Gpi-1</i>	2	6	S_1 (13), S_2 (15), I(17), F(19)
Isocitrate dehydrogenase	<i>Idh</i>	2	7	S(36), N(38), F(40)
Leucine aminopeptidase	<i>Lap-1</i>	1	7	S(26), I(27), F(29)
	<i>Lap-2</i>	1	7	S_1 (34), S_2 (36), N(38), F(40)
Phosphoglucumutase	<i>Pgm-1</i>	1	7	S(27), N(29), F(31)
	<i>Pgm-2</i>	1	7	S_1 (35), S_2 (40), S_3 (42.5), N(45), F_1 (47.5), F_2 (50)
6-Phosphogluconate dehydrogenase	<i>6Pgd-1</i>	2	7	S(25), N(28), F(31)
	<i>6Pgd-2</i>	1	7	S(33), N(35), F(37)
	<i>6Pgd-3</i>	2	7	S(36), N(38), F(45)
Tetrazolium oxidase	<i>To-2</i>	2	6	N(50), F(55)

* S = slow, N = normal, I = intermediate, F = fast, 0 = no activity. The esterase alleles are designated by other characters. The distances from the origin are given in parentheses (bromophenol blue = 100).

Table 2 Allozyme genotypes of the parent plants and of the F_1 plants used for making the F_2 's

Plant	<i>Est-4</i>	<i>Est-5</i>	<i>Got-1</i>	<i>Got-2</i>	<i>Gpi-1</i>	<i>Idh</i>	<i>Lap-1</i>	<i>Lap-2</i>	<i>Pgm-1</i>	<i>Pgm-2</i>	<i>6Pgd-1</i>	<i>6Pgd-2</i>	<i>6Pgd-3</i>	<i>To-2</i>
Br ₁₂	CE	00	II	SS	S ₁ I	NN	IF	FF	NN	F ₂ F ₂	SN	SN	NN	NN
Br ₁₈	CC	B0	II	SS	S ₁ F	NN	IF	FF	NN	NF ₂	SN	SN	NN	NN
Br ₂₃	CC	B0	SI	SF	IF	NN	SF	NN	SN	NN	NN	NN	NN	NN
Br ₇₂	DD	B0	SI	SS	S ₁ F	NN	IF	NN	SN	NN	NN	NN	NN	NN
He ₈₈	E0	A0	SI	SF	S ₁ I	NN	IF	NF	SN	NN	NN	NN	NF	NF
We ₄₄	CE	B0	SS	SS	FF	NF	IF	NF	NN	S ₂ N	NN	NN	NF	NF
HeWe ₇ *	EE	A0	SI	SF	IF	NF	IF	NF	SN	NN	NN	NN	NF	NF
HeWe ₃₁	CE	A0	SI	SF	IF	NF	IF	FF	SN	S ₂ N	NN	NN	NF	NF
WeHe ₉	EE	AB	SI	SS	IF	NN	IF	NF	SN	S ₂ N	NN	NN	NF	NF
WeHe ₈₄	E0	A0	SI	SF	S ₁ F	NF	IF	NF	SN	S ₂ N	NN	NN	NF	NF
HeWe ₁₄	CE	AB	SI	SS	S ₁ F	NN	IF	NF	NN	S ₂ N	NN	NN	NF	NF
HeWe ₁₅	CE	A0	SI	SS	S ₁ F	NF	FF	NF	NN	S ₂ N	NN	NN	NF	NF
WeHe ₂₅	C0	A0	SI	SF	S ₁ F	NF	FF	NF	NN	S ₂ N	NN	NN	NF	NF
WeHe ₈₅	C0	A0	SI	SF	S ₁ F	NF	IF	NF	NN	S ₂ N	NN	NN	NF	NF

* The F_1 plants derived from the cross He₈₈ ♀ × We₄₄ ♂ are designated as HeWe; those from the reciprocal cross as WeHe.

be determined and are described in table 2. The crosses are listed in table 3. The variable allozyme loci were segregating in agreement with the expected ratios with three exceptions: *Pgm-1*, *6Pgd-1* and *6Pgd-2* showed strongly deviating ratios in particular crosses. In these cases the *SI* locus, controlling self-incompatibility, is thought to be responsible for the deviations.

The self-incompatibility locus

Self-incompatibility in *P. lanceolata* is gametophytic: it is governed by one locus, the *SI* locus, which prevents a plant from being fertilised by pollen carrying either of the two *SI* alleles present in the style (Ross, 1973). Self-fertilisation is thus made impossible, but also related plants will often be incompatible or half-compatible. The chance of

unrelated plants not being fully compatible is believed to be low in general, because of the large numbers of *SI* alleles usually present in natural populations.

In the Br population both Br₁₂ and Br₁₈ are carrying the same rare alleles of *6Pgd-1* (S) and *6Pgd-2* (S). Br₂₃ and Br₇₂ share a rare allele of *Pgm-1* (S). These four plants were collected within a distance of 10 m, so plants carrying an identical rare allele may be close relatives. When linkage exists between an enzyme locus and the *SI* locus, the rare enzyme allele has a high chance of being linked with the same *SI* allele in both parents, and will not be able to become homozygous without recombination. Any expected 1:2:1 ratio would change towards 0:1:1, the exact ratio depending on the recombination frequency. Such ratios were indeed found for the crosses *6Pgd-1* SN × SN and

Table 3 The crosses. Percentages of spontaneous germination and the number of segregating sex-phenotypes

			Reciprocal cross		Combined reciprocals scored for sex-phenotypes									
	Per cent spontaneous germination			Per cent spontaneous germination									Per cent MS	Per cent IN
	<i>n</i>		<i>n</i>		<i>n</i>	MS	IN-a	IN-b	IN-c	H				
Br ₁₂ × Br ₁₈	77	61	57	61	133	26	11	14	21	61	19.5	34.6		
Br ₂₃ × Br ₇₂	90	57	44	57	133	0	0	0	0	133	0	0		
He ₈₈ × We ₄₄	100	39	100	70	197	0	0	3	1	193	0	2.0		
WeHe ₉ × HeWe ₃₁	45	60	45	47	87	7	8	6	6	60	8.0	23.0		
WeHe ₈₄ × WeHe ₉	45	67	45	73	85	6	6	7	4	62	7.1	20.0		
HeWe ₇ × WeHe ₉	45	51	45	51	79	9	4	4	5	57	11.4	16.5		
WeHe ₂₅ × HeWe ₁₄	45	36	40	68	38*	5	0	3	0	30	13.2	7.9		
WeHe ₈₅ × HeWe ₁₅	43	21	45	64	—									
WeHe ₂₅ × HeWe ₁₅	45	38	45	73	—									
WeHe ₈₅ × HeWe ₁₄	44	86	—		—									

* Reciprocal cross not included.

6*Pgd*-2 SN $\times$ SN ($\text{Br}_{12} \times \text{Br}_{18}$) and *Pgm*-1 SN $\times$ SN ($\text{Br}_{23} \times \text{Br}_{72}$).

The linkage of *Pgm*-1 with the *SI* locus was independently confirmed in the F_1 of $\text{He}_{88} \times \text{We}_{44}$. The F_1 plants with genotype *Pgm*-1^{SN} contained two *SI* genotypes (HeWe_7 , HeWe_{31} and WeHe_{84} were not able to fertilise each other, but could be crossed with WeHe_9) and the plants being *Pgm*-1^{NN} behaved in the same way (HeWe_{14} and HeWe_{15} belonged to one *SI* type and WeHe_{25} and WeHe_{85} to the other). When crosses *Pgm*-1 SN $\times$ SN succeeded they were half-compatible as could be concluded by the almost 0:1:1 ratio of SS, SN and NN in the progeny.

From the combined results for *Pgm*-1 (SS:SN:NN = 2:227:234) a recombination percentage of 0.8 (95 per cent confidence limits: 0.3–3.0 per cent) with the *SI* locus could be calculated. The 6*Pgd*-1 locus appeared to be somewhat more loosely linked with the *SI* locus as established by the cross $\text{Br}_{12} \times \text{Br}_{18}$ and several backcrosses of F_1 plants with Br_{12} (combined results: SS:SN:NN = 36:581:525) giving a recombination of 6.4 per cent (4.7–8.8). As no recombination between 6*Pgd*-1 and 6*Pgd*-2 could be detected, 6*Pgd*-2 is at about the same distance to the *SI* locus.

The expected linkage between *Pgm*-1 and 6*Pgd*-1 was qualitatively confirmed in an F_2 starting with the 6*Pgd*-1^{SS} recombinant from the cross $\text{Br}_{12} \times \text{Br}_{18}$ and a *Pgm*-1^{SS} recombinant from the cross $\text{Br}_{23} \times \text{Br}_{72}$. Unfortunately about 20 per cent non-viable albinos segregated in the F_2 , making quantitative conclusions impossible. Also no sequence of the three loci *Pgm*-1, 6*Pgd*-1 and *SI* could be established, as in the F_2 no recombinants between *Pgm*-1 and *SI* were found.

Linkage groups

In addition to the linkage data from the crosses $\text{Br}_{12} \times \text{Br}_{18}$, $\text{Br}_{23} \times \text{Br}_{72}$ and $\text{H}_{88} \times \text{We}_{44}$, the crosses between F_1 plants of the latter cross appeared to be very useful for linkage detection. Twelve loci were found segregating simultaneously in the F_2 : all variable loci except for 6*Pgd*-1 and 6*Pgd*-2. Inbreeding effects like the occurrence of albinos mentioned in the previous section are met frequently in *P. lanceolata*, but appeared to be absent here: germination was almost complete and over 95 per cent of the progeny could be used for electrophoresis. In only one F_2 were the expected Mendelian ratios severely disturbed for the enzyme loci of one linkage group. Those results were discarded.

In table 4 the recombination percentages are summarised. Almost all other possibilities for link-

Table 4 Recombination percentages between enzyme loci, and their 95 per cent confidence intervals. *n* = the combined progeny number

Linkage group	Loci	Recombination percentage	<i>n</i>
I	<i>Gpi</i> -1- <i>Lap</i> -1	13.5 (11.5–15.8)	924
II	<i>Got</i> -1- <i>Idh</i>	5.3 (3.1–9.0)	452
	<i>Idh</i> - <i>Est</i> -5	8.4 (5.1–13.1)	650
	<i>Got</i> -1- <i>Est</i> -5	12.4 (9.7–15.7)	776
III	<i>To</i> -2- <i>Pgm</i> -2	18.6 (16.0–21.7)	742
	<i>Pgm</i> -2- <i>Est</i> -4	30.1 (23.2–38.0)	278
	<i>To</i> -2- <i>Est</i> -4	39.6 (32.0–47.9)	278
IV	6 <i>Pgd</i> -3- <i>Got</i> -2	6.1 (4.1–9.0)	733
	<i>Got</i> -2- <i>Lap</i> -2	32.3 (27.4–37.5)	646
	6 <i>Pgd</i> -3- <i>Lap</i> -2	45.0 (40.1–50.0)	734
V	6 <i>Pgd</i> -1-6 <i>Pgd</i> -2	0 (0–1.0)	133

age relations could be invalidated. The following survey of linkage groups with the sequence of the loci involved can now be made:

Linkage group I: *Gpi*-1...*Lap*-1
 II: *Got*-1...*Idh*...*Est*-5
 III: *To*-2...*Pgm*-2...*Est*-4
 IV: 6*Pgd*-3...*Got*-2...*Lap*-2
 V: 6*Pgd*-1/6*Pgd*-2...*Pgm*-1/*SI*

These linkage groups may represent five different chromosomes as *n* = 6 in this species.

The detailed outcome of the examination of the crosses, on which the results in table 4 are based, are not given in this paper but will be available on request.

Male sterility

Substantial numbers of male steriles (MS) and intermediate types (IN) were met in the population samples with percentages ranging from 1.5 to 18.8 for MS and from 3.1 to 30.0 for MS + IN. In some of the crosses male steriles and intermediates segregated too; in all cases plasmon type *R* was involved (MS1 and IN1) as described by Van Damme and Van Delden (1982). Among the parents used, Br_{18} , Br_{23} , Br_{72} and We_{44} proved to be stable hermaphrodites, whereas Br_{12} and He_{88} , although producing enough pollen to function as a male parent, could be considered as IN-c. The numbers of sex phenotypes appearing in each of the progenies are given in table 3.

The inheritance of male sterility has been studied extensively by Van Damme (1983*b*), using plants of the Wd population. To be MS1, at least two recessive and three dominant genes should all be in the appropriate allelic state. He designated

these male sterility loci in the *R* plasmon type as mr_1 , mr_2 (recessives), Mr_3 , Mr_4 and Mr_5 (dominants). The same designation will be used here, without suggesting that the same subscript means the same locus in both studies. Intermediates IN1 were concluded by Van Damme to occur in an MS genotype by substitution of one arbitrary mr^{mm} by mr^{m+} , and perhaps also by substitution of one arbitrary Mr^{M+} by Mr^{++} .

In the F_1 resulting from $Br_{12} \times Br_{18}$ a strong association existed between the *6Pgd-1* genotype and the sex phenotype. The combined reciprocal crosses (between which no differences could be found) segregated as shown in table 5.

Table 5 The numbers of sex phenotypes for each *6Pgd-1* genotype class in the F_1 of $Br_{12} \times Br_{18}$

		H	IN	MS	Totals
<i>6Pgd-1</i>	NN	17	20	24	61
	SN	43	26	2	71
	SS	1	0	0	1
		61	46	26	133

Because *6Pgd-1* is linked with the *SI*-locus ($r = 6.4$ per cent), an involved male sterility locus will be linked with the *SI*-locus too. The simplest model to explain the male sterile frequencies assumes two loci: mr_1 with sterility alleles linked with the *6Pgd-1*^N alleles in both parents, and a second locus mr_x or Mr_x , not linked with *6Pgd-1*, which reduces the MS fraction by a factor 2. Because Br_{12} is assumed to be genetically IN, Br_{12} is mr_x^{mm} or Mr_x^{M+} , whereas Br_{18} is mr_x^{m+} or Mr_x^{++} . The parental chromosomes for linkage group V are:

	mr_1	<i>6Pgd-1</i>	<i>SI</i>
	+	S	a
Br_{12} :	<hr/>		
	m	N	b
and	<hr/>		
	mr_1	<i>6Pgd-1</i>	<i>SI</i>
	+	S	a
Br_{18} :	<hr/>		
	m	N	c

with the remark that the sequence of *SI* and *6Pgd-1* is still uncertain. In the progeny the mr_1^{mm} fraction is given by $(1-r)/2$, r being the recombination frequency between mr_1 and *SI*. The other locus mr_x (or Mr_x) reduces the number of male steriles to $(1-r)/4$. The 19.5 per cent MS observed corresponds to an r -value of 22 per cent. In this

model the fraction of IN genotypes is $\frac{1}{2} - \frac{1}{4}r$ which gives an expectancy of 59 IN's. Because only 46 IN phenotypes are observed, about 22 per cent of the IN genotypes would be phenotypically H, assuming perfect segregation ratios. Using this model a recombination percentage of 13.4 (5.9–23.7) could be calculated between mr_1 and *6Pgd-1* (see Appendix B).

A still more complicated situation was met in the crosses between F_1 plants of the cross $He_{88} \times We_{44}$. A significant association between sex phenotype and enzyme genotype was established here with *6Pgd-3*, *To-2* and *Pgm-2*, so two different linkage groups (III and IV, see table 4) are involved. For these linkage groups identical chromosomes were concerned in the four F_1 crosses scored for sex phenotype (see table 3), which allows the combination of their progenies for this purpose. The linkage relations between sex phenotype and the enzyme genotype were indeed found to be similar in all these progenies, although individually not always statistically significant due to the low numbers in each separate cross. The combined numbers of MS, IN and H plants in each of the *6Pgd-3/To-2* genotype classes are shown in table 6. The *6Pgd-3*^{NF} plants could be divided into two classes by making use of the linked *Got-2* locus, one class containing the *6Pgd-3*^F allele derived from He_{88} and the other class with the *6Pgd-3*^F allele from We_{44} . In *To-2* both F-alleles were from He_{88} origin, both N alleles from We_{44} origin.

The simplest model to explain the results is the assumption of a dominant gene Mr_3 linked with *6Pgd-3* and a recessive gene mr_2 linked with *To-2*. The parental genotypes are (considering that He_{88} is IN-c):

	Mr_3	<i>6Pgd-3</i>	<i>Got-2</i>	mr_2	<i>To-2</i>	<i>Pgm-2</i>
	M	F	S	m	F	N
He_{88} :	<hr/>			<hr/>		
	+	N	F	+	N	N
	+	F	S	+	F	N
We_{44} :	<hr/>			<hr/>		
	+	N	S	+	N	S ₂

and the two genotypes of the F_1 plants used as parents for the F_2 are:

	+	F	S	m	F	n
	<hr/>			<hr/>		
	+	N	F	+	N	S ₂ (N)
and	<hr/>			<hr/>		
	M	F	S	m	F	N
	<hr/>			<hr/>		
	+	N	S	+	N	S ₂

Table 6 The numbers of MS-IN-H plants for each 6*Pgd-3*/*To-2* genotype in the combined F_2 's from $He_{88} \times We_{44}$

		<i>To-2</i> ^{NN}			<i>To-2</i> ^{NF}			<i>To-2</i> ^{FF}			Total numbers		
		MS	IN	H	MS	IN	H	MS	IN	H	MS	IN	H
6 <i>Pgd-3</i> ^{NN}		0	0	14	1	4	49	1	4	25	2	8	88
6 <i>Pgd-3</i> ^{NF}	<i>Got-2</i> ^{SS}	0	0	14	0	2	42	1	3	18	1	5	74
6 <i>Pgd-3</i> ^{NF}	<i>Got-2</i> ^{SF}	0	2	10	7	9	14	8	10	1	15	21	25
6 <i>Pgd-3</i> ^{FF}		1	3	6	3	11	13	4	5	3	8	19	22
Total numbers		1	5	44	11	26	118	14	22	47	26	53	209

with the restriction that the sequences in both linkage groups are not absolutely certain, although the lack of any linkage effects between sex phenotype and *Lap-2* or *Est-4* makes the alternative sequences unlikely.

The recombination percentage between *Mr₃* and 6*Pgd-3* can be calculated as 11.9 (5.9–19.8), taking into account the disturbing influence on the division of 6*Pgd-3* genotypes by the use of the *Got-2* locus, which showed about 6 per cent recombination with 6*Pgd-3*. In Appendix B the detailed calculation of the recombination percentage is given. It is not possible to detect any recombination between *Mr₃* and *Got-2* in these crosses because no combined heterozygotes were involved. From table 6 it can be concluded that no IN's appear when the *Mr₃* locus is in the ++ state. The few IN's still observed in the two upper rows can fully be explained by the recombination between *Mr₃* and 6*Pgd-3*.

In the other linkage group (III) the recombination percentages of *mr₂* with both *To-2* and *Pgm-2* could be calculated as 32.2 per cent (24.4–41.6) between *mr₂* and *To-2* (see Appendix B) and 33.4 per cent (24.4–44.8) between *mr₂* and *Pgm-2*. The observed number of IN plants was almost in agreement with the expected number of *mr₂*^{m+}, *Mr₃*^{M+}. The expression of this genotype appeared to be 85 per cent IN and 15 per cent H.

The above made assumptions about the inheritance of male sterility in the F_2 of $He_{88} \times We_{44}$ are consistent with the absence of MS plants in the F_1 (see table 3). This F_1 contained, however, 63 plants with an enzyme genotype predicting an IN genotype. To explain the only 4 plants showing the IN phenotype an expression of 6 per cent IN and 94 per cent H in the IN genotype has to be assumed.

Germination

A variable fraction of the seeds produced in each cross germinated spontaneously under the conditions used (table 3). Germination is a character which is very susceptible to environmental conditions, in particular humidity. Alternation of wet and dry periods enlarge the germination fraction, and the germination increases also with longer periods of storage after ripening (Blom, 1978). Because of the strong influence of germination conditions, no conclusions will be drawn from the differences in germination percentages, ranging from 21 to 86. When however within one cross, of which the seeds have always been germinated in the same petri dish, significant differences in germination behaviour between the enzymes genotypes could be established, genetic variation is made probable.

Table 7 Significance levels of the relations between germination behaviour and enzyme genotype (– $P > 0.05$, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$)

Linkage group	Crosses			
	Br ₁₂ × Br ₁₈	Br ₂₃ × Br ₇₂	He ₈₈ × We ₄₄	F ₁ × F ₁ He ₈₈ × We ₄₄
I (<i>Gpi-1</i> , <i>Lap-1</i>)	—	—	**	—
II (<i>Got-1</i> , <i>Idh</i> , <i>Est-5</i>)	—	***	***	***
III (<i>To-2</i> , <i>Pgm-2</i> , <i>Est-4</i>)	—	—	—	—
IV (6 <i>Pgd-3</i> , <i>Got-2</i> , <i>Lap-2</i>)	—	—	—	*
V (6 <i>Pgd-1</i> , 6 <i>Pgd-2</i> , <i>Pgm-1</i>)	*	—	*	*

Such differences between enzyme genotypes were found to some extent in all crosses. A survey of the differences (table 7) shows a strong effect of linkage group II in crosses with plants from different origin, and also linkage group V shows an effect in different crosses, although much weaker. The distinctly significant effect of linkage group I in the cross $He_{88} \times We_{44}$ was not repeated in the $F_1 \times F_1$ crosses, but this can be explained by regarding the F_1 plants chosen: in each of those crosses the same linkage group I chromosome from He_{88} origin was present in both parents, whereas the different influence on germination behaviour in the F_1 was due to a difference between both He_{88} chromosomes. By similar considerations linkage group II and linkage group V chromosomes could be divided into those with relatively strong and those with less strong effects on spontaneous germination. Not shown here is that within linkage group I, *Gpi-1* acted more strongly on germination than *Lap-1*; within linkage group II the effects of *Idh* and *Est-5* were stronger than the effect of *Got-1*.

DISCUSSION

The original aim of analysing crosses of *P. lanceolata*: verifying the Mendelian inheritance of the enzyme loci and grouping them on chromosomes, has been achieved satisfactorily. The five concluded linkage groups may well correspond to five different chromosomes although the possibility of two linkage groups on the same chromosome cannot be totally excluded. Like this *6Pgd-3* and *Lap-2* would not have been placed in the same linkage group without the interjacent *Got-2* locus which is linked with both other loci. Also the linkage between *Est-4* and *To-2* would have been questionable without the presence of the intermediate *Pgm-2* locus. The estimates of the recombination frequencies presented are not only of limited accuracy due to sampling effects, but may also be influenced by genetic variation in recombination. Because recombination frequencies have been reported to be different in oogenesis and spermatogenesis in several plant species (see Bell, 1982), the values determined may be considered to represent mean recombination frequencies for both sexes.

A lot of additional information has been obtained during these investigations. The important *SI* locus, controlling self-incompatibility, could be placed in one of the linkage groups. The tight linkage with *Pgm-1* provides the possibility of "labelling" *SI* alleles by means of *Pgm-1* alleles,

avoiding in this way elaborate incompatibility tests. The *6Pgd-1* and *-2* loci, although less closely linked, may also be useful for such purposes.

Three male sterility loci have been placed in three different linkage groups. To explain the segregating numbers of male steriles and intermediates, assumptions about the inheritance of male sterility made by Van Damme (1983b) appeared to be very useful. In his model a male sterile phenotype will arise when the multi-locus genotype is at least mr_1^{mm} , mr_2^{mm} , Mr_3^{M+} , Mr_4^{M+} , Mr_5^{M+} . When one *m*-allele is missing the phenotype will be IN. The possibility that instead of one *m*-allele, one *M*-allele could be missed still resulting in an IN phenotype is not in agreement with the behaviour of the locus Mr_3 in this study.

Considering only the male sterility loci noticed in the particular crosses, the most probable parental genotypes are: $Br_{12} = mr_1^{m+}, mr_x^{mm}$; $Br_{18} = mr_1^{m+}, mr_x^{m+}$; $He_{88} = mr_2^{m+}, Mr_3^{M+}$; $We_{44} = mr_2^{++}, Mr_3^{++}$. The numbers of MS plants in the various progenies are in full agreement with the model, and so are the parental phenotypes. The IN frequencies however are always lower than predicted. It was calculated that the genotypes mr_1^{mm} , mr_x^{m+} and mr_1^{m+} , mr_x^{mm} in the cross $Br_{12} \times Br_{18}$ gave rise to only 78 per cent IN while 22 per cent was H. In the crosses $He_{88} \times We_{44}$ the genotype mr_2^{m+} , Mr_3^{M+} resulted in 6 per cent IN and 94 per cent H in the F_1 and in 85 per cent IN and 15 per cent H in the F_2 . Especially this difference between F_1 and F_2 with the same variable male sterility loci involved, and scored for sex phenotype under exactly the same environmental conditions, suggests that additional loci exist which modify the fraction of IN expressed in the IN genotype.

As these experiments were not primarily set up to analyse male sterility, some questions still remain. So it is unclear whether mr_x is indeed a recessive gene and whether it is identical to mr_2 . The cross $Br_{12} \times Br_{18}$ could have answered this question when many more offspring were studied: *Pgm-2* is segregating too and a double heterozygote mr_x^{m+} , *Pgm-2*^{NF₂} is involved. With the numbers used, however, the results were compatible with both $mr_x = mr_2$ and $mr_x \neq mr_2$ although somewhat closer to the first possibility. Furthermore two male sterility loci are not met in the crosses at all: Mr_4 and Mr_5 . A series of experiments adjusted to the problem would undoubtedly bring about a more complete knowledge of the genetic basis of male sterility in *P. lanceolata*. The merit of the use of allozyme marker loci exists in making male sterility loci with similar effects distinguishable which makes it possible to study them separately.

Because the aim is a further use of allozyme variation in studying the genetics of ecological differences, the effects of certain linkage groups on germination behaviour as found here are interesting. In nature germination in *P. lanceolata* is observed to occur largely during spring in most vegetation types whereas germination in autumn is predominant in some very dense vegetations (Van der Toorn, unpublished). Whether this difference is genetically determined is not known thus far. If so, the genetic factors determining this difference are not necessarily the same as the loci responsible for the observed germination differences in the petri dishes. Nevertheless, the conclusion that the existing ecological differences within the species *P. lanceolata* can be analysed in a successful way by making use of allozyme variation seems to be correct. When compared with *P. major* (Van Dijk, 1984) such an analysis will be more difficult because of the larger intrapopulational genetic variability of *P. lanceolata*, but the greater number of variable enzyme loci allows a good ultimate result.

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APPENDIX A

The calculation of recombination frequencies

The observed numbers of each possible two-locus genotype (see table 8(a)) or enzyme genotype/sex phenotype combinations are compared with the expected numbers belonging to the segregation model chosen and being a function of the recombination frequency r (table 8(c)). With the aid of a computer the value of r which gives the minimal χ^2 value is easily found, together with the 95 per cent confidence interval which includes all r 's leading to a χ^2 less than $\chi^2_{\min} + 3.84$. An improvement of this method is attained when the observed genotype numbers are previously corrected in order to obtain row and column totals which are in accordance with the expected segregation ratios (table 8(b)). This correction is possible without disturbing any deviations caused by the eventual linkage: alternately the genotype numbers are changed to obtain correct row totals and to obtain correct column totals. This iterative process is stopped when no further change is observed. Disturbing influences other than those originating from linkage are removed partially in this way.

If the χ^2 value, with the remaining degrees of freedom, is not significant at the 5 per cent level for $r = 0.5$, linkage is assumed to be absent unless there is other evidence for its existence e.g., when data for a third locus, linked with the ones under study, are available. The minimal χ^2 value gives information about the relative probability of the segregation model used, which is of particular importance in cases where different male sterility

Table 8 A worked example of the calculation of recombination frequencies

a. Observed numbers					b. Corrected numbers				
		6Pgd-3							
		NN	NF	FF	Totals	NN	NF	FF	Totals
Got-2	SS	10	212	129	351	6.8	190.9	168.9	366.6
	SF	213	160	9	382	176.5	175.7	14.4	366.6
		223	372	138	733	183.3	366.6	183.3	733.2
c. Expected numbers									
	NN	NF	FF	Totals					
SS	$\frac{1}{4}rN$	$\frac{1}{4}N$	$\frac{1}{4}(1-r)N$	$\frac{1}{2}N$					
SF	$\frac{1}{4}(1-r)N$	$\frac{1}{4}N$	$\frac{1}{4}rN$	$\frac{1}{2}N$					
	$\frac{1}{4}N$	$\frac{1}{2}N$	$\frac{1}{4}N$	N					

inheritance models are possible. When the same linkage relationship is studied in crosses producing different segregation ratios, the results are combined by reducing the more complex ratios to the most simple ratio e.g., 1:1:1:1 to 1:2:1 or 1:2:1 to 1:1. As this is only a matter of making different alleles indistinguishable by giving them the same name, the recombination frequencies calculated will not be affected. The confidence intervals will be broadened, however, because some information is lost.

The correctness of the described method of calculating recombination frequencies and confidence intervals has been checked by computer simulation. The worked example in table 8 is based on the real data of the crosses made. The minimal $\chi^2_{(1)}$, applied on the figures of table 8(b), is 3.45 at $r=6.1$ per cent. The $\chi^2=7.29$ at $r=4.1$ per cent and at $r=9.0$ per cent. The maximum likelihood method (see Green, 1981) would give here 5.8 per cent (3.4–8.2), also applied on the corrected numbers.

only, because the expected numbers of *6Pgd-1^{SS}/MS* or *IN* are too low. Table 9 gives a survey of the procedure to calculate the expected numbers in each class. The 6.4 per cent recombination between *6Pgd-1* and *SI*, and the assumed 78 per cent expression of the *IN* genotype have been taken into account.

The minimal $\chi^2_{(3)}=1.77$ at $r=13.4$ per cent. The χ^2 value is 5.61 at $r=5.9$ per cent and at $r=23.7$ per cent.

Mr₃ and *6Pgd-3*.

The calculation of the expected numbers is given in table 10, taking into account the 6.1 per cent recombination between *6Pgd-3* and *Got-2* and the assumed 85 per cent expression of the *IN* genotype. The observed numbers, as shown in table 6, have to be corrected for unequal row and column totals. Because *6Pgd-3* and *To-2* are not linked to each other, the expected frequency of each two-locus enzyme genotype is equal to the product of the

Table 9 Recombination frequency between *mr₁* and *6Pgd-1*: survey of calculation steps

<i>a. Expected gamete frequencies (double crossing overs neglected)</i>			
<i>mr₁^m/6Pgd-1^N</i>	Pollen M1 = 0.936(1 - <i>r</i>)	Ovuli F1 = 0.5 - 0.468 <i>r</i>	
<i>mr₁⁺/6Pgd-1^N</i>	M2 = 0.936 <i>r</i>	F2 = 0.468 <i>r</i>	
<i>mr₁^m/6Pgd-1^S</i>	0	F3 = 0.468 <i>r</i>	
<i>mr₁⁺/6Pgd-1^S</i>	M3 = 0.064	F4 = 0.5 - 0.468 <i>r</i>	
<i>b. Expected mr₁/6Pgd-1 genotype frequencies</i>		<i>c. Expected phenotype frequencies</i>	
mm/NN: G1 = M1F1		MS/NN: P1 = $\frac{1}{2}$ G1	
m+/NN: G2 = M1F2 + M2F1		IN/NN: P2 = 0.78 ($\frac{1}{2}$ G1 + $\frac{1}{2}$ G2)	
+/NN: G3 = M2F2		H/NN: P3 = 0.22 ($\frac{1}{2}$ G1 + $\frac{1}{2}$ G2) + $\frac{1}{2}$ G2 + G3	
mm/SN: G4 = M1F3		MS/SN: P4 = $\frac{1}{2}$ G4	
m+/SN: G5 = M1F4 + M2F3 + M3F1		IN/SN: P5 = 0.78 ($\frac{1}{2}$ G4 + $\frac{1}{2}$ G5)	
+/SN: G6 = M2F4 + M3F2		H/SN: P6 = 0.22 ($\frac{1}{2}$ G4 + $\frac{1}{2}$ G5) + $\frac{1}{2}$ G5 + G6	
<i>d. Phenotype numbers</i>			
MS/NN	Expected P1 × 61 / (P1 + P2 + P3)	Expected at $r=13.4$ per cent 23.1	Observed 24
IN/NN	P2 × 61 / (P1 + P2 + P3)	23.4	20
H/NN	P3 × 61 / (P1 + P2 + P3)	14.5	17
MS/SN	P4 × 71 / (P4 + P5 + P6)	3.6	2
IN/SN	P5 × 71 / (P4 + P5 + P6)	24.4	26
H/SN	P6 × 71 / (P4 + P5 + P6)	43.0	43

APPENDIX B

The calculation procedures of the recombination frequencies between allozyme loci and male sterility loci.

mr₁ and *6Pgd-1*

The data are given in table 5. The χ^2 is calculated from the *6Pgd-1* NN and SN genotype numbers

expected single-locus genotype frequencies.

The minimal $\chi^2_{(3)}=2.60$ at $r=11.9$ per cent. The χ^2 value is 6.44 at $r=5.9$ per cent and at $r=19.8$ per cent.

mr₂ and *To-2*.

Table 11 shows the calculation procedure of the expected numbers, assuming 85 per cent

Table 10 Recombination frequency between Mr_3 and $6Pg d-3$: survey of calculation steps*a. Expected gamete frequencies*

	First parent (F from He)	Second parent (F from We)
$Mr_3^M/6Pg d-3^F$	$\frac{1}{2}(1-r)$	—
$Mr_3^M/6Pg d-3^N$	$\frac{1}{2}r$	—
$Mr_3^+/6Pg d-3^F$	$\frac{1}{2}r$	$\frac{1}{2}$
$Mr_3^+/6Pg d-3^N$	$\frac{1}{2}(1-r)$	$\frac{1}{2}$

b. Expected $Mr_3/6Pg d-3$ genotype frequencies

M+/FF or NF (F from He):	$G1 = \frac{1}{2}(1-r)$
M+/NN or NF (F from We):	$G2 = \frac{1}{2}r$
+/+/FF or NF (F from He):	$G3 = \frac{1}{2}r$
+/+/NN or NF (F from We):	$G4 = \frac{1}{2}(1-r)$

c. Expected phenotype frequencies

MS/FF or NF (F from He):	$P1 = \frac{1}{4}G1$
IN/FF or NF (F from He):	$P2 = 0.85 \times \frac{1}{2}G1$
H/FF or NF (F from He):	$P3 = 0.15 \times \frac{1}{2}G1 + \frac{1}{4}G1 + G3$
MS/NN or NF (F from We):	$P4 = \frac{1}{4}G2$
IN/NN or NF (F from We):	$P5 = 0.85 \times \frac{1}{2}G2$
H/NN or NF (F from We):	$P6 = 0.15 \times \frac{1}{2}G2 + \frac{1}{4}G2 + G4$

d. Phenotype numbers

	Expected	Exp. at $r = 11.9$ per cent	Observed (corrected)
MS/FF or NF (<i>Got-2</i> SF)	$(0.9695P1 + 0.0305P4)288$	30.9	27.8
IN/FF or NF (<i>Got-2</i> SF)	$(0.9695P2 + 0.0305P5)288$	52.5	50.8
H/FF or NF (<i>Got-2</i> SF)	$(0.9695P3 + 0.0305P6)288$	60.6	65.4
MS/NN or NF (<i>Got-2</i> SS)	$(0.0305P1 + 0.9695P4)288$	5.1	2.1
IN/NN or NF (<i>Got-2</i> SS)	$(0.0305P2 + 0.9695P5)288$	8.7	9.2
H/NN or NF (<i>Got-2</i> SS)	$(0.0305P3 + 0.9695P6)288$	130.2	132.7

Table 11 Recombination frequency between mr_2 and *To-2*: survey of calculation steps*a. Expected gamete frequencies in both parents*

$mr_2^m/To-2^F$:	$H1 = \frac{1}{2}(1-r)$
$mr_2^m/To-2^N$:	$H2 = \frac{1}{2}r$
$mr_2^+/To-2^F$:	$H3 = \frac{1}{2}r$
$mr_2^+/To-2^N$:	$H4 = \frac{1}{2}(1-r)$

b. Expected $mr_2/To-2$ genotype frequencies

mm/FF:	$G1 = H1^2$
mm/NF:	$G2 = 2H1H2$
mm/NN:	$G3 = H2^2$
m+/FF:	$G4 = 2H1H3$
m+/NF:	$G5 = 2H1H4 + 2H2H3$
m+/NN:	$G6 = 2H2H4$
+/+/FF:	$G7 = H3^2$
+/+/NF:	$G8 = 2H3H4$
+/+/NN:	$G9 = H4^2$

c. Expected phenotype frequencies

MS/FF:	$P1 = \frac{1}{2}G1$
IN/FF:	$P2 = 0.85 \times \frac{1}{2}G4$
H/FF:	$P3 = \frac{1}{2}G1 + 1.15 \times \frac{1}{2}G4 + G7$
MS/NF:	$P4 = \frac{1}{2}G2$
IN/NF:	$P5 = 0.85 \times \frac{1}{2}G5$
H/NF:	$P6 = \frac{1}{2}G2 + 1.15 \times \frac{1}{2}G5 + G8$
MS/NN:	$P7 = \frac{1}{2}G3$
IN/NN:	$P8 = 0.85 \times \frac{1}{2}G6$
H/NN:	$P9 = \frac{1}{2}G3 + 1.15 \times \frac{1}{2}G6 + G9$

d. Phenotype numbers

	Expected	Expected at $r = 32.2$ per cent	Observed (corrected)
MS/FF	288P1	16.5	15.0
IN/FF	288P2	13.4	21.8
H/FF	288P3	42.1	35.2
MS/NF	288P4	15.7	13.1
IN/NF	288P5	34.5	29.8
H/NF	288P6	93.8	101.1
MS/NN	288P7	3.7	1.8
IN/NN	288P8	13.4	8.4
H/NN	288P9	54.9	61.8

expression of the IN genotype. The observed numbers have been corrected as described above.

The minimal $\chi^2_{(5)} = 12.02$ at $r = 32.2$ per cent. The χ^2 value is 15.86 at $r = 24.4$ per cent and at $r = 41.6$ per cent. The recombination frequency between mr_2 and $Pgm-2$ has been calculated in an analogous way (details not given).

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