Theor. Appl. Genet. 60, 285-290 (1981)

THEORETICAL AND APPLIED GENETICS

© by Springer-Verlag 1981

Genetic Variability in *Plantago* Species in Relation to Their Ecology

Part 1: Genetic Analysis of the Allozyme Variation in *P. major* Subspecies *

H. van Dijk and W. van Delden
Department of Genetics, Biology Center, University of Groningen, Haren (The Netherlands)

Summary. A survey of enzyme variability in several populations of *Plantago major* in the Netherlands has been made. Nine of 36 loci were found to be polymorphic. The most extensively studies population showed 7 polymorphic loci (19%). The average heterozygosity was 0.005, a low value since *P. major* is predominantly inbreeding; a first estimate of the outcrossing rate is only 10%. All nine variable loci show simple Mendelian inheritance, seven of them could be placed into four different linkage groups. Marked differences in allele frequencies were found between two subspecies: ssp. *major* and ssp. *pleiosperma*. Two enzyme loci possess subspecies-specific alleles, *Pgm*-1 and *Got*-1. The most likely explanation of this phenomenon is the existence of fitness differences, caused either by the enzyme loci themselves or by linked loci.

Key words: Allozyme genetics – Genecology – *Plantago*

Introduction

An answer to the question, why does a given plant species grow in some environments and not in others, can only be given after a multidisciplinary examination of abiotic and biotic environmental factors, the physiology of the examined species and the demographic and genetic parameters of its populations. One of the aims of population genetics in such a project is to reveal the amount, the distribution and the ecological significance of the genetic variation in plant populations. Connections have to be made with biochemistry and plant physiology to explain the mechanism by which genetic variants or ecotypes have different fitnesses in different environments.

The majority of important genetic differences between ecotypes of a given species is probably brought about by genes modulating morphology or development. Unfortunately, the effects of these genes are both genetically and physiologically usually difficult to analyse. This disadvantage does not nold for enzyme loci – one of the reasons for their growing popularity in population genetics. Though genotype-environment correlations have been found for allozyme variants (Clegg & Allard 1972), the functional basis of these relations is still unclear in the majority of cases.

Apart from its functional meaning, if any, variation at enzyme loci proves to be useful for other purposes: to measure genetic distances between populations of the same or of closely related species, to describe the genetic structure of populations, or to calculate such parameters as gene flow between populations, the level of inbreeding, clonesize, etc.

In the present project, where demographic, physiological and genetic characters of grassland plant species have been studied in general, and the genus *Plantago* in particular, we began our examination of genetic variation by analysing the occurrence of allozyme variation. This paper deals with only one of the species, *Plantago major*, but the same methods are already being succesfully applied to the other four indigenous *Plantago* species: *P. lanceolata*, *P. media*, *P. coronopus* and *P. maritima*.

P. major is a very common weed, growing on disturbed sites. It is a self-compatible wind pollinator showing protogyny. A large number of morphologically different varieties have been described (Sagar & Harper 1964) and at least some of them appear to be genetically different (Groot and Boschhuizen 1970). In addition to the most abundant subspecies, *major*, a second subspecies is known: ssp. *pleiosperma* PILGER, formerly *P. intermedia*. These subspecies are rather easy to distinguish morphologically, although transitional forms also exist and are found in different habitats. The ecology of both subspecies has been examined by Mølgaard (1976), who also described a series of morphological differences and started the genetical analysis of these differences by making crosses between the subspecies.

In this first paper, a genetic comparison of both subspecies has been made in order to provide more insight

* Grassland Species Research Group Publication no. 14

0040-5752/81/0060/0285/$1.20

into the genetic basis of ecological differences. The genetic basis of the allozyme polymorphisms is also described.

Materials and Methods

Plants and crosses

Plants were collected at 9 sites throughout the Netherlands (Fig. 1). The sample size was mostly 10, one population (H) being more extensively examined with 47 plants. All plants were kept in a greenhouse at 20-25° C with 16 hr light daily. Self-fertilization was performed by covering spikes with cellophane bags. Cross-fertilization was carried out when the plant used as a female was showing pistils only. After pollination, the upper part of the ear, still without pistils, was removed. Cross-fertilization obtained in this way appeared to be 90-100% when checked by electrophoresis. The seeds were stored at 4° C. After about one month they were capable of a high percentage germination. They were sown in autoclaved (up to 130° C) earth at 25° C with 16 hr light daily.

Extract Preparation

From each plant, about 400 mg fresh leaf material was frozen at −30° C together with a mortar and a pestle. Just prior to electrophoresis, the frozen leaf material was crushed and ground with 4 mg Polyclar AT (BDH). The icy powder was supplemented with 200 μl 2% mercaptoethanol in gel buffer and was again ground to an (almost melting) powder. After centrifuging for 2 min at 2000 g, the supernatant was applied directly to the gel.

Fig. 1. Location of the sampled *Plantago major* populations in the Netherlands

Electrophoresis

Electrophoretic runs were carried out at 4° C and 15 V/cm in horizontal 6% polyacrylamide slabs, containing electrophoresis buffer diluted 10 ×. The following electrophoresis buffers, indicated by their pH, were used: pH 6: Tris-maleate: 0.05 M maleic anhydride. Tris is added to pH 6.0; pH 7: Tris-citrate: 0.05 M citric acid. Tris is added to pH 7.0; pH 9: sodium borate: 0.1 M boric acid. NaOH is added to pH 9.0.

Staining Methods

New methods were developed for the following enzymes: shikimate dehydrogenase: 25 mg shikimic acid in 30 ml 0.1 M Tris-HCl buffer, pH 7.1 with 5 mg $NADP^+$ and 6 mg MTT. After $\frac{1}{2}$ hr, 1 mg PMS was added. The gel was incubated at 37° C. Glycollate oxidase: 60 mg glycollic acid in 30 ml 0.1 M sodium phosphate buffer, pH 7.0 with 0.6 mg FMN, 3 mg peroxidase and 6 mg o-dianisidine. The gel was incubated at 37° C. Amylase: when preparing the gel, 0.5% soluble starch was added to the monomer solution. After electrophoresis, the gel was incubated in water for 30 min at 37° C and 1 ml iodine-potassium iodide solution was added. After 10 min, the excess of iodine was removed, giving white bands on a deep blue background. Malic enzyme and tetrazolium oxidase were assayed according to Ayala et al. (1972), all other enzymes according to Shaw & Prasad (1970); however, in all dehydrogenase stains, MTT was used instead of NBT.

Results

Electrophoretic Variation

The plants described in Materials and Methods were all screened for variation in 21 enzyme systems, presusumably representing 36 loci. These enzyme systems were selected from a total of 50; the other 29 have been rejected because they gave no or non-reproducible activity. The remaining enzyme systems were: alcohol dehydrogenase (ADH), shikimate dehydrogenase (SHDH), malate dehydrogenase (MDH, 2 loci), malic enzyme (ME, 2 loci), isocitrate dehydrogenase (IDH), 6-phosphogluconate dehydrogenase (6PGD, 3 loci), glucose-6-phosphate dehydrogenase (G6PD), glycollate oxidase (GLYCOX), glyceraldehyde-3-phosphate dehydrogenase (GA-3-PD), xanthine dehydrogenase (XDH), glutamate dehydrogenase (GDH, 2 loci), tetrazolium oxidase (TO, 2 loci), peroxidase (PER, 2 loci), glutamate-oxaloacetate-transaminase (GOT, 2 loci), phosphoglucomutase (PGM, 2 loci), esterase (EST, 4 loci), acid phosphatase (ACPH, 2 loci), fructose-1,6-diphosphatase (FDPH), amylase (AMY), leucine aminopeptidase (LAP, 2 loci) and glucosephosphate isomerase (GPI, 2 loci).

Nine of these 36 loci showed genetic variation. Number, mobility and designation of the alleles are presented in Table 1, together with other details.

Table 1. The variable enzyme loci

Locus	Number of subunits	pH of electrophoresis buffer used	Alleles[a]
Acph-2	2	9	N (24), F (26)
Est-4	1	6	N (23), F (26)
Got-1	2	7	S (21), I (26), F (31)
Got-2	2	7	S (33), N (37), F (41)
Gpi-1	2	6	N (23), F (26)
Me-1	⩾ 4	7	S (16), N (18)
6Pgd-2		7	N (31), O[b]
Pgm-1	1	6	S_1(27), S_2(33), N (36)
Shdh	1	7	S_1(33), S_2(34), N (36), F (38)

[a] S = slow, N = normal, I = intermediate, F = fast, O = no activity. The distances are given in parentheses (bromophenol blue = 100)
[b] The NO heterozygotes are distinguishable by giving fainter bands than the NN homozygotes

Genetic Analysis of the Electrophoretic Variation

To demonstrate the genetic basis of the observed electrophoretic variants, a number of crosses was made between plants that were supposed to be homozygotes for a different allele. For all nine varying loci F_1 staining patterns contained both parental bands, sometimes with additional heteromultimeric bands (see Table 1). Two pairs of F_2 progenies were analysed. They were obtained by selfing one F_1 plant of each reciprocal of two pairs of crosses ($G_1 \times Z_2$ and $Z_2 \times G_1$; $H_{19} \times H_{44}$ and $H_{44} \times H_{19}$, see Fig. 1). In the P-generation G_1 and Z_2 differed for seven loci. Plant G_1 (subspecies *major*) was *Shdh*^{NN}, *Me*-1^{NN}, *6Pgd*-2^{OO}, *Pgm*-1^{S_2S_2}, *Got*-1^{SS}, *Got*-2^{NN} and *Est*-4^{NN}. Plant Z_2 (ssp. *pleiosperma*) was *Shdh*^{S_2S_2}, *Me*-1^{SS}, *6Pgd*-2^{NN}, *Pgm*-1^{NN}, *Got*-1^{II}, *Got*-2^{FF} and *Est*-4^{FF}. The second pair of crosses was made between H_{19} (ssp. *major*): *Shdh*^{S_2S_2}, *Pgm*-1^{S_2S_2}, *Got*-1^{FF}, *Got*-2^{NN}, *Est*-4^{NN} and H_{44} (ssp. *pleiosperma*): *Shdh*^{NN}, *Pgm*-1^{NN}, *Got*-1^{II}, *Got*-2^{FF}, *Est*-4^{FF}. Table 2 shows the genotype numbers of both

Table 2. Genotype numbers in both pairs of F_2's, with χ^2 values for the deviation from the expected 1:2:1 ratio and for heterogeneity of reciprocal crosses

Locus	Homozyg. like ssp. *major* parent	Hetero-zygotes	Homozyg. like ssp. *pleiosp.* parent	χ^2(2)	Homozyg. like ssp. *major* parent	Hetero-zygotes	Homozyg. like ssp. *pleiosp.* parent	χ^2(2)	χ^2(2) combined	χ^2(2) for heterogeneity
	F_2 from G_1 ♀ × Z_2 ♂				F_2 from Z_2 ♀ × G_1 ♂					
Est-4	24	40	31	3.40	28	35	28	4.85	7.49*	0.71
Got-1	26	48	21	0.54	24	51	16	2.74	2.59	0.76
Got-2	23	40	32	4.07	25	37	29	3.53	7.27*	0.26
Me-1	30	45	20	2.37	25	48	18	1.35	3.11	0.57
6Pgd-2	27	45	23	0.60	24	40	27	1.53	1.39	0.70
Pgm-1	25	51	19	1.27	35	42	14	10.23**	7.84*	3.21
Shdh	27	51	17	2.62	22	55	14	5.37	7.12*	0.87
	F_2 from H_{19} ♀ × H_{44} ♂				F_2 from H_{44} ♀ × H_{19} ♂					
Est-4	25	55	34	1.56	33	60	14	8.33*	1.27	9.44**
Got-1	25	61	27	0.79	25	58	23	1.02	1.65	0.17
Got-2	30	51	23	0.98	34	58	15	7.50*	6.64*	2.34
Pgm-1	33	49	29	1.81	28	49	21	1.00	1.97	0.88
Shdh	22	60	24	1.92	32	49	18	3.97	2.23	3.58

* $p < 0.05$
** $p < 0.01$

Table 3. Genotype numbers for the linked loci in the combined F_2's from $G_1 \times Z_2$ and $Z_2 \times G_1$

Me-1	*Shdh*	n	*6Pgd*-2	*Got*-2	n	*Got*-2	*Est*-4	n	*6Pgd*-2	*Est*-4	n
NN	NN	47	OO	NN	43	NN	NN	40	OO	NN	35
NN	S_2N	8	OO	NF	7	NN	NF	7	OO	FF	13
NN	S_2S_2	–	OO	FF	1	NN	FF	1	OO	FF	3
SN	NN	2	NO	NN	5	NF	NN	11	NO	NN	17
SN	S_2N	91	NO	NF	64	NF	NF	56	NO	NF	47
SN	S_2S_2	–	NO	FF	16	NF	FF	10	NO	FF	21
SS	NN	–	NN	NN	–	FF	NN	1	NN	NN	–
SS	S_2N	7	NN	NF	6	FF	NF	12	NN	NF	15
SS	S_2S_2	31	NN	FF	44	FF	FF	48	NN	FF	35

Table 4. Allele frequencies for the polymorphic loci in the H-population (47 plants)

Locus	Allele	Frequency	Locus	Allele	Frequency
Acph-2	N	0.89	*Me*-1	S	0.39
	F	0.11		N	0.61
Est-4	N	0.80	*6Pgd*-2	N	1.00
	F	0.20		O	–
Got-1	S	0.39	*Pgm*-1	S_1	–
	I	0.36		S_2	0.09
	F	0.25		N	0.91
Got-2	S	0.01	*Shdh*	S_1	0.05
	N	0.86		S_2	0.11
	F	0.13		N	0.82
				F	0.02
Gpi-1	N	1.00			
	F	–			

pairs of F_2's. 2 × 3 contingency tests for each of the loci indicated in only one case a significant difference between reciprocal F_2's. The observed genotype ratios were generally in agreement with the expected 1:2:1 ratio; although, in some cases, there was a significant deviation (χ^2 test, $P < 0.05$). In the G × Z crosses, *Pgm*-1 and *Shdh* showed a lack of homozygotes from Z_2 origin, whereas *Got*-2 and *Est*-4 had a lack of heterozygotes. In the H × H crosses, *Got*-2 and *Est*-4 showed a lack of homozygotes from H_{44} origin.

Linkage Groups

The seven enzyme loci in the F_2's derived from $G_1 \times Z_2$ and $Z_2 \times G_1$ were positioned into four linkage groups. *Pgm*-1 and *Got*-1 are not linked to each other nor to one of the other enzyme loci. As indicated in Table 3, a rather strong linkage exists between *Me*-1 and *Shdh* (4.7% recombination). The remaining three loci are also linked: *Got*-2 and *6Pgd*-2 with 10.5% recombination, *Got*-2 and *Est*-4 with 12.6% recombination and *6Pgd*-2 and *Est*-4 with 21.4% recombination. The sequence on the chromosome is: *6Pgd*-2 ⟷ *Got*-2 ⟷ *Est*-4. Recombination between *Got*-2 and *Est*-4 could also be determined in the second pair of F_2's. Here, too, a value of 12.6% was calculated.

Allele and Genotype Frequencies

In only a single population (H) was the sample large enough (n = 47) to calculate allele frequencies (see Table 4). It was striking that the 'N' (normal) alleles were also the most frequent in all other population samples, except for *Got*-1. But, in contrast with the H-population, the individual plants of these samples were collected over a too great area to be sure that no different subpopulations may have been sampled.

Genotype frequencies were obviously not in agreement with the Hardy-Weinberg law. Homozygotes were found almost exclusively, even at loci with intermediate allele frequencies. In the H-population, the value of F, Wright's fixation index, averaged over the polymorphic loci, was 0.84 ± 0.12. In the ssp. *pleisperma* part of the H-population, the F value was 0.93 ± 0.06; in the ssp. *major* part, 0.77 ± 0.19.

Differences Between the Subspecies of *P. major*

Two easily recognizable subspecies of *P. major* have been described: ssp. *major* and ssp. *pleiosperma* PILGER. The ssp. *major* is found on roadsides and is resistant to heavy trampling; *P. major* ssp. *pleiosperma* is found at unstable sites with respect to the water level (Blom 1979). The best criterion for distinction is the seed number per capsule: ⩽ 13 for ssp. *major* and > 13 for ssp. *pleiosperma* (Mølgaard 1976).

The majority of the populations sampled contained members of both subspecies. Only the S-population was entirely composed of ssp. *major* and only the A-popula-

tion was entirely composed of ssp. *pleiosperma*. It is only possible, with our data, to detect large differences in allele frequencies that may exist between the two subspecies. Indeed we did find such differences within the H-population (15 plants being ssp. *major* and 32 plants being ssp. *pleiosperma*), but the results of all populations taken together (81 ssp. *major* plants and 44 ssp. *pleiosperma* plants) gave a still more convincing picture of these differences, especially with respect to the loci *Got*-1 and *Pgm*-1 (see Table 5). Significant differences in allele frequencies between the subspecies (χ^2 2 × N contingency test, $P<0.05$) were found for these loci and also for *Me*-1.

The nature of the observed differences is analysed in Fig. 2, which shows the partition of the most distinguishing alleles over the seed number classes. *Pgm*-1 shows an absolute difference; the S_2-allele is totally absent in ssp. *pleiosperma*, while the ssp. *major* is polymorphic with about 20% S_2. *Got*-1 contains two subspecies-specific alleles; the F-allele is predominant in ssp. *major*, but absent in ssp. *pleiosperma*, except for some plants with relatively low seed numbers. The I-allele is the most frequent allele in ssp. *pleiosperma*, but is absent in ssp. *major*, except for some plants with higher seed numbers. *Me*-1^S has a higher frequency in ssp. *pleiosperma*, just like *Got*-1^S, but, ssp. *major* plants with lower seed numbers can also bear these alleles.

Discussion

Electrophoretic variation is present in *P. major* in normal amounts for a plant species (see for a review Nevo 1978). Nine of 36 loci showed variation. The best examined population (H) is variable for seven of 36 loci (= 19%), and the mean number of alleles per locus is 1.31. The heterozygosity is low (about 0.005), but this has to be seen in the view of the mating system. *P. major* individuals are readily self-fertilizing when standing isolated, in spite of a distinct protogyny. The shortage of heterozygotes, as expressed in the afore-mentioned high F values, may be largely caused by self-fertilization, although other reasons can not be totally excluded. Among these other reasons (Brown 1979), selection and Wahlund effect are the most important. Selection, however, seems unlikely, because no large differences between loci could be found. The Wahlund effect may have a limited influence because no clear pattern in

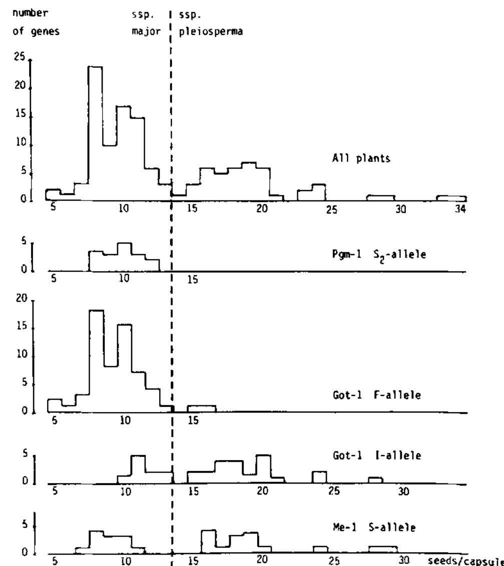

Fig. 2. The distribution of some alleles over the seed number classes

Table 5. Allele frequencies for the polymorphic loci in the subspecies *major* (81 plants) and *pleiosperma* (44 plants)

Locus	Allele	Frequency in: ssp. *major*	ssp. *pleio.*	Locus	Allele	Frequency in: ssp. *major*	ssp. *pleio.*
Acph-2	N	0.93	0.91	*Me*-1	S	0.16	0.35
	F	0.07	0.09		N	0.84	0.65
Est-4	N	0.83	0.80	*6Pgd*-2	N	0.98	1.00
	F	0.17	0.20		O	0.02	–
Got-1	S	0.14	0.44	*Pgm*-1	S_1	0.01	–
	I	0.13	0.51		S_2	0.20	–
	F	0.73	0.05		N	0.79	1.00
Got-2	S	0.05	–	*Shdh*	S_1	0.08	0.07
	N	0.83	0.81		S_2	0.07	0.11
	F	0.12	0.19		N	0.79	0.80
					F	0.06	0.02
Gpi-1	N	0.99	0.98				
	F	0.01	0.02				

the spatial partition of the genotypes was observed. If selfing is the only cause of heterozygote shortage, t (= the amount of cross-fertilization), can be calculated as $(1-F)/(1+F)$. For the whole H-population t would be 0.09 ± 0.07; in the ssp. *pleiosperma* part of this population $t = 0.04 \pm 0.03$; in the ssp. *major* part $t = 0.14 \pm 0.12$.

The genetic analysis of the electrophoretic variants suggests simple Mendelian inheritance, although some significant deviations of the 1:2:1 ratio in the F_2's are found. These deviations may be caused by unequal fitness of the genotypes in the haploid or early diploid stage, as germination was almost complete, and no plants died before being analysed.

Four linkage groups can be found for seven of the polymorphic enzyme loci. Chromosome length is not known, so it is not certain that these linkage groups correspond to different chromosomes (n = 6 in this species). Further mapping of the chromosomes is possible with the use of other loci. This may be helpful for the location of loci contributing to morphological and developmental characters.

A distinct difference in allozyme frequencies exists between the two subspecies of *P. major*. Two loci have alleles that are specific for only one of the subspecies: *Pgm*-1^{S_2} and *Got*-1^{F} are nearly only found in the ssp. *major*, while *Got*-1^{I} is nearly only present in ssp. *pleiosperma*. A third enzyme locus, *Me*-1, has different frequencies in the two subspecies.

An explanation for the absence in one subspecies of alleles which are frequent in the other can be given in two ways. The first possibility is a total reproductive isolation of the subspecies, either by geographical isolation, by incompatibility or by a very low hybrid fitness. Genetic drift and/or selection can then cause genetic differences. However the subspecies are growing together at several places, fertilization takes place both between and within subspecies and the hybrids are healthy looking, normally seed producing plants, at least when grown in a greenhouse or garden (in fact, a plant possessing all characteristics of a F_1 hybrid was present in the H-population sample). The only indication of a lowered fitness in the descendants of hybrids was found in our F_2's. In addition to the already mentioned shortage of some genotypes in the F_2's, we found several male steriles in the F_2 derived from the cross G_1 ♀ × Z_2 ♂, possibly caused by certain homozygous Z_2 genes in G_1 cytoplasm.

A second and more probable explanation is that either the enzyme loci, themselves, or linked loci are concerned with the fitness of individuals in the specific environmental conditions of the subspecies. Hybrids will occasionally be formed, but they (or their descendants) will be less fit in either of both environments. Because they can, to some extent, segregate homozygous descendants with normal fitness, chromosomes or parts of chromosomes that do not greatly influence fitness can be exchanged between the subspecies. The striking similarity in allele frequencies of the loci *Shdh*, *Got*-2, *Acph*-2 and *Est*-4 supports this possibility (Table 5).

The question whether the enzyme loci *Pgm*-1 and *Got*-1, themselves, are subject to selection or that linked loci are responsible cannot be answered yet. The existence of correlations between ecologically important quantitative characters and these enzyme loci has to be investigated. Furthermore, different allozymes should be examined for differences in biochemical properties.

Acknowledgement

We are grateful to Mr. P. Dubbeldam for preparing the gels and to Drs. G. van Nigtevecht, R. Bijlsma and J. van Damme for their comments on this manuscript.

These investigations were supported by the Foundation for Fundamental Biological Research (BION), which is subsidized by the Netherlands Organization for the Advancement of Pure Research (ZWO).

Literature

Ayala, F.J.; Powell, J.R.; Tracey, M.L.; Mourão, C.A.; Pérez-Salas, S. (1972): Enzyme variability in the *Drosophila willistoni* group. IV. Genetic variation in natural populations of *Drosophila willistoni*. Genetics **70**, 113-139

Blom, C.W.P.M. (1979): Effects of trampling and soil compaction on the occurrence of some *Plantago* species in coastal sand dunes. VI. The vegetation of two dune grasslands in relation to physical soil factors. Thesis, University of Nijmegen

Brown, A.H.D. (1979): Enzyme polymorphism in plant populations. Theor. Popul. Biol. **15**, 1-42

Clegg, M.T.; Allard, R.W. (1972): Patterns of genetic differentiation in the slender wild oat species *Avena barbata*. Proc. Nat. Acad. Sci. (USA) **69**, 1820-1824

Groot, J.; Boschhuizen, R. (1970): A preliminary investigation into the genecology of *Plantago major* L. J. Exp. Bot. **21**, 835-841

Mølgaard, P. (1976): *Plantago major* ssp. *major* and ssp. *pleiosperma*. Morphology, biology and ecology in Denmark. Bot. Tidskrift 71, 31-56

Nevo, E. (1978): Genetic variation in natural populations: patterns and theory. Theor. Popul. Biol. **13**, 121-177

Sagar, G.R.; Harper, J.L. (1964): Biological Flora of the British Isles. *Plantago major* L., *P. media* L. and *P. lanceolata* L. J. Ecol. **52**, 189-221

Shaw, C.R.; Prasad, R. (1970): Starch gel electrophoresis of enzymes – A compilation of recipes. Biochem. Genet. **4**, 297-320

Received April 1, 1981
Communicated by R.W. Allard

Dr. H. van Dijk
Dr. W. van Delden
Department of Genetics
Biology Center
University of Groningen
9751 NN Haren (Gn), (the Netherlands)