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Genetic variation in cultivars of diploid ryegrass, *Lolium perenne* and *L. multiflorum*, at five enzyme systems

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Summary. Samples of approximately 100 plants from each of 22 populations of *Lolium perenne* representing 15 cultivars, and from 13 populations of *Lolium multiflorum* representing six cultivars were scored for isozyme variants in five enzyme systems, PGI, GOT, ACP, PGM and 6-PGD. From the individual banding patterns a genetic interpretation of the variation was formulated and population studies of the resulting six polymorphic enzyme loci were performed. No strong indications of partial selfing was found since at four of the six loci, *Pgi 2, Got 3, Pgm 1* and *Pgd 1*, the genotypic proportions were in correspondence with the Hardy-Weinberg expectations. This indicated, further, that the genetical interpretations of the banding patterns might be correct. Deviations from Hardy-Weinberg proportions for *Acp 1* and *Got 2* indicated presumably selection working on the linkage group including these loci. Gametic phase disequilibrium was observed between *Pgi 2* and *Pgd 1* for populations of one cultivar. These results were discussed in relation to the variation expected within a cultivar.

Key words: Partial selfing – Hardy-Weinberg proportions – Gametic phase disequilibrium – Cultivar identification – Starch gel electrophoresis

Introduction

In plant populations large genetic variation in enzyme loci defined by gel electrophoresis has been observed (Brown 1979). Therefore, electrophoresis is an easy means of obtaining genetic markers for several purposes. An obvious application is to identify cultivars by means of their alleles at monomorphic loci and this is used for cultivated selfing species. For outbreeding species most loci are polymorphic and it might be too laborious to fix certain alleles during the breeding process. In this case, the genotypic composition must be considered together with the population dynamics. For a review see Nielsen 1984.

Prerequisites for using the variation in isozymes for cultivar identification as well as in any population genetical analysis are 1) that the enzyme systems are consistent with respect to banding pattern and easy to score, 2) that the inheritance of each enzyme is well documented, 3) that the mating system is known, and 4) that the genotypic composition is stable i.e. for instance that the genotypic frequencies of each locus under study are in Hardy-Weinberg proportions. In this paper these conditions will be considered in detail for a survey of 5 enzyme systems in diploid cultivars of two incomplete gametophytic self-incompatible ryegrass species (*Lolium*). The utilization of the data for identification of the cultivars appears elsewhere (Nielsen et al. 1984).

Samples and electrophoretic methods

Fifteen cultivars of *Lolium perenne* and six cultivars of *Lolium multiflorum* were investigated (for their designation see Nielsen et al. 1984). Each cultivar was represented by a sample from its standard seed lot (standard population). Further, for three cultivars subpopulations of the standard population representing different generations and environments were included. In total, 22 populations of *L. perenne* and 13 populations of *L. multiflorum* were investigated. Populations were indicated by the abbreviations 'p' (*L. perenne*) or 'm' (*L. multiflorum*) followed

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by a number; the subpopulations were, in addition, indicated by a succeeding letter.

From each population 96 plants, 4–6 weeks old, were used for the electrophoresis. Most individuals were successfully scored for five enzymes: PGI (Phosphoglucoisomerase, E.C. 5.3.1.9), GOT (Glutamate oxaloacetate transaminase, E.C. 2.6.1.1), ACP (Acid phosphotase, E.C. 3.1.3.2), PGM (Phosphoglucomutase, E.C. 2.7.5.1), and 6-PGD (6-Phosphogluconate dehydrogenase, E.C. 1.1.1.43). Due to difficulties with the separation of bands in the earliest part of the investigation, the enzyme ACP was scored in a reduced number of plants in the following cultivars: p1 (84 plants), p2 (75 plants), p4 (69 plants), m1 (76 plants), m2 (74 plants), m3 (85 plants) and m4 (83 plants).

From each plant approximately 8 cm of a leaf was cut into pieces and crushed in buffer solution mixed with a small amount of fine sand. The buffer was Tris-HCl (0.1 M, pH 7.2) containing 0.5% 2-mercaptoethanol. The crude extract was absorbed onto Whatmann 3 MM filter paper wicks 3.5 × 9 mm.

Horizontal starch gel electrophoresis was performed as described by Scandalios (1969) with minor modifications. The gel (300 ml) was made of 13% or 16% hydrolyzed starch (Connaught Lab., Ontario, Canada). The size of the frames was 180 × 180 × 8 mm. Three different buffer systems were used and the gels were run as indicated in Table 1. After running, gels were sliced and stained using the recipes listed in Table 2. Finally, they were incubated in the dark at 37 °C and fixed in 50% glycerol, except for ACP which was fixed in 50% ethanol.

Table 1. Gel and tray buffers

		pH	v/cm
1. Gel:	1 part lithium borate (25 mM LiOH, 180 mM H_3BO_3) and 9 parts Tris-citrate (70 mM Tris, 7 mM citric acid)	8.1	15 for 4–5 h
Tray:	The lithium borate buffer only	8.3	
2. Gel:	14 mM Tris, 4 mM citric acid	7.4	15 for 4–5 h
Tray:	0.3 M H_3BO_3, 0.1 M NaOH	8.7	
3. Gel:	12.5 mM Tris, appr. 4 mM citric acid	7.0	6 for 16 h
Tray:	37.5 mM Tris, appr. 12.5 mM citric acid	7.0	

Table 2. Stain recipes for enzyme systems

Enzyme	Buffer system[b]	Substrate	Additionals
PGI	1	15 mg D fructose-6-phospate	100 ml Tris-HCl (0.1 M, pH 8.0) 1 ml 10% $MgCl_2$ 20 units glucose-6 phosphate-dehydrogenase 5 mg NADP 15 mg MTT 1.2 mg PMS[a]
GOT	1	50 ml Tris-HCl (0.1 M, pH 7.1) 50 mg α-keto-glutaric acid 130 mg L-aspartic acid 250 mg Polyvinylpyrrolidon 7 mg EDTA – Na_2 50 ml 0.4 M Na_2HPO_4 adjusting to pH 7.4 with HCl	3 mg Pyridoxal-5 phosphate[a] 150 mg Fast Blue BB-salt[a]
ACP	2	50 mg Na-α-naphtyl-acid phosphate phosphate	100 ml Na-acetat (0.2 M, pH 5.0) 0.5 ml 10% $MgCl_2$ 25 mg Fast Garnet GBC-salt
PGM	3	25 mg α-d-glucose-1-phosphate	100 ml Tris-HCl (0.1 M, pH 7.1) 0.5 ml 10% $MgCl_2$ 20 units Glucose-6 phosphate-dehydrogenase 5 mg NADP 15 mg MTT 1.2 mg PMS[a]
6-PGD	3	20 mg Na_3-6-phospho-gluconic acid	100 ml Tris-HCl (0.1 M, pH 7.1) 0.5 ml 10% $MgCl_2$ 5 mg NADP 15 mg MTT 1.2 mg PMS[a]

[a] Added just before use
[b] According to Table 1

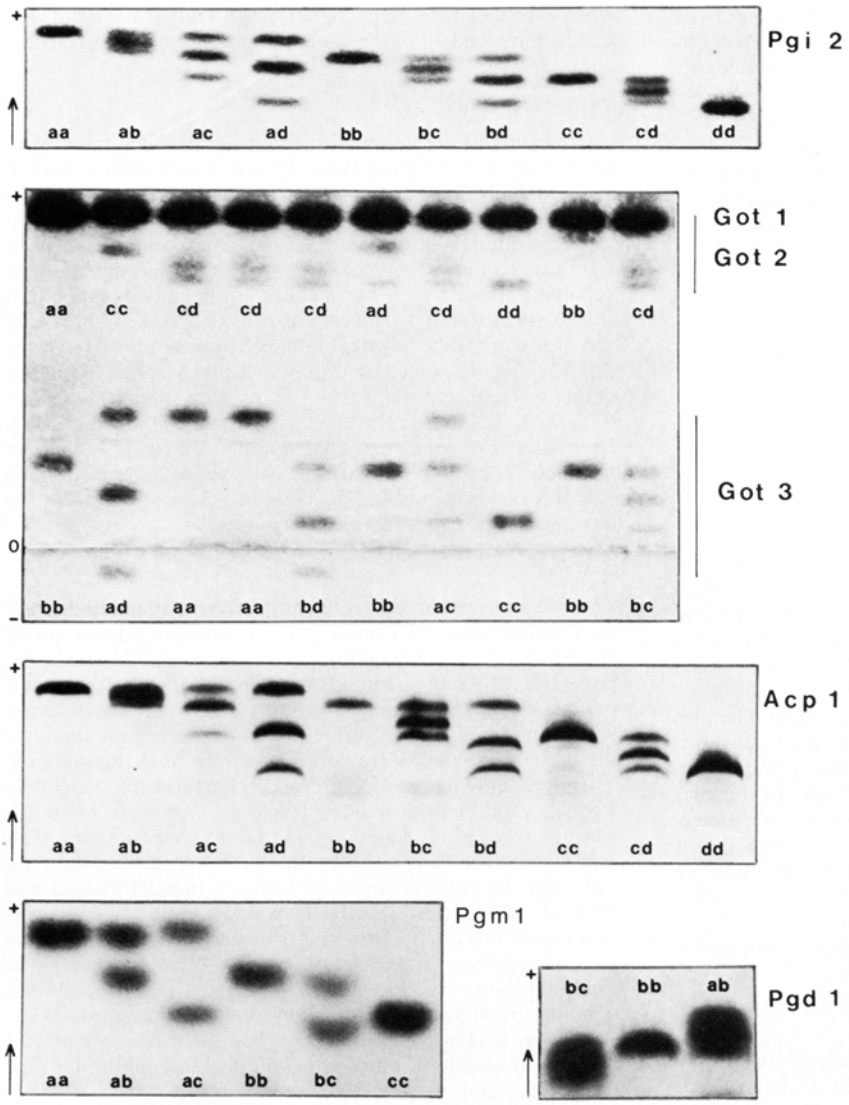

Fig. 1. Selected zymograms from each of the five enzyme systems studied

Genetic interpretation of banding patterns

A genetic interpretation of the banding patterns for three of the five enzymes studied, i.e. PGI, GOT, and ACP has been given previously by Hayward and McAdam (1977a, b). The interpretation was deduced from the banding pattern in observed zymograms. It implied non-Mendelian segregation for the isozymes of GOT in the few crosses for which segregation ratios were examined (Hayward and McAdam 1977a). The zymograms given in Fig. 1 show the variation found within the banding groups considered in this study. Each group is assumed to represent one locus. In ACP several other groups have been observed but the genetics could not be deduced because too many bands were observed to allow simple interpretation. The same is true for an additional group observed in PGI and PGM, respectively. Finally, the enzyme ACO (Aconitate hydratase, E.C. 4.2.1.3) interfered with an additional banding group of 6-PGD so this group has been omitted as well.

The variation found within a locus consists of either one-banded and two-banded patterns (*Pgm 1*) or one-banded and three-banded patterns (*Pgi 2, Got 2, Got 3, Acp 1,* and *Pgd 1*). The corresponding isozymes are, therefore, assumed to be monomers or dimers, respectively. However, since *Got 1* and *Got 2* overlap (Fig. 1), two-banded patterns appear in *Got 2* as well.

Each single-banded pattern is assumed to represent a homozygote for a certain allele. According to the position of the band, the corresponding allele is denoted 'a', 'b', 'c', etc. In addition to the alleles indicated in Fig. 1, a rare allele faster than 'a' was observed in *Got 3* in two cultivars. Further, four individuals from different cultivars did not show any band for *Acp 1* and we concluded that they were homozygous for a non-active allele, a silent allele. Finally, the alleles of *Got 2* with banding positions 'c' and 'd' were previously designated 'a' and 'b'. This was concluded from the cultivar p12 which has been scored previously (S23 in Hayward and McAdam 1977b).

Estimation procedure

Allele frequencies

In analyses of allelic and genotypic distributions it is often necessary to pool the rarest alleles to obtain powerful tests. In this analysis two strategies were followed: (1) to consider only

Table 3. Rule for pooling rare alleles for HW-tests. The chosen number of classes is the one for which both conditions on the expected numbers assuming HW-proportions are satisfied

No. of allelic classes with active alleles	Maximal no. of genotypic classes with expected nos. smaller than 1	Maximal no. of genotypic classes with expected nos. smaller than 5
4	2	5
3	1	3
2	1[a]	1

[a] The number expected has, however, to be larger than 0.5

two allelic classes, one consisting of the most frequent allele and one consisting of the remaining alleles, or (2) to consider as many allele classes as reasonable according to ad hoc rules as for instance that given in Table 3.

Allele frequencies were calculated as the relative number of alleles observed from each allele class at the loci *Pgi 2, Got 3, Pgm 1,* and *Pgd 1.* In *Acp 1* the presence of a silent allele was deduced for the cultivars p2, p5, p11 and m2. A silent allele produces no electrophoretically detectable protein, so heterozygotes between an active and a silent allele cannot be distinguished from the homozygote for the active allele. The presence of a silent allele implies that the allele frequencies can be calculated only when assuming Hardy-Weinberg proportions. Further, iteration is necessary to obtain estimates of the allele frequencies and in this study the "gene-counting" method (Rust 1972) was used. For details see Appendix. In a specific case, a silent allele was assumed to be present in *Got 2* (see 'Results') but an additional problem arose here since the "silent homozygote" could not be distinguished from the homozygote 'aa'. However, the allele frequencies could still be estimated by "gene counting" (see 'Appendix').

One locus

In the one-locus analysis deviations from Hardy-Weinberg proportions (HW-proportions) was expressed by F defined from the following general description of genotypic frequencies at a locus with active alleles $A_1, ---, A_n$ and corresponding gene frequencies $p_1, ---, p_n$:

A_iA_i: $p_i^2 + p_i(1-p_i)F$ and
A_iA_j: $2p_ip_j(1-F)$ for $i \neq j$

with the restriction

$$F > -p_i/(1-p_i) \quad \text{for all i.} \tag{1}$$

F can be estimated in various ways each with its own characteristics (Curie-Cohen 1982). The estimates, $\hat{F}$, used were found by comparing the observed number of homozygotes of each allele (allele class) with the HW-proportions, i.e. f2 defined by Curie-Cohen (1982). The condition (1) might not always be satisfied for $\hat{F}$ especially if one of the allele frequencies is small. If (1) was not satisfied for $\hat{F}$ calculated from a certain number of alleles, the estimate was omitted and the two rarest alleles were pooled to obtain another estimate of F. If only non-negative values of F are considered, F can be interpreted as the average inbreeding coefficient of a population; negative values of F, on the other hand, indicate any homozygous deficiency compared to HW-proportions.

Estimates of F are difficult to compare over loci and over populations since the sampling variance changes considerably when F or the allele frequencies change. Only if $F = 0$ the variance is independent of the allele frequencies and in that case it approximates

$$V(\hat{F}) = 1/(n-1)N \tag{2}$$

where N equals the sample size and n equals the number of allele classes (Curie-Cohen 1982). In case of two allele classes, $\hat{F}$ is identical with the maximum likelihood estimate (Curie-Cohen 1982).

Tests for HW-proportions of the genotypes at each locus were carried out by comparing the observed and the expected genotypic frequencies by means of likelihood ratio tests (Bishop et al. 1980). In the case of two allele classes, these tests are approximately equivalent to the obvious test for $F = 0$ using (2). Alleles were pooled according to Table 3. As an example 4 alleles were observed for *Got 3* in p1 with allele frequencies 0.76, 0.20, 0.04 and 0.01, respectively (Table 4). Since 5 genotypic classes have an expected number less than 1, the two rarest alleles are pooled. Then the expected number is less than 1 in only one class and less than 5 in two classes. Therefore, the test is performed with three allele classes.

Two loci

The two-locus genotypes were analysed by sequentially testing for random union of gametes (RUG) and for gametic phase equilibrium (linkage equilibrium) by means of likelihood ratio tests (Hill 1974); the gametic phase disequilibrium, D, was estimated during the testing procedure. Only two allele classes were considered but, nevertheless, the expected numbers for the nine genotypes were low, especially in *L. multiflorum.* Therefore, only pairs of loci for which the most frequent allele frequency on both loci were less then 0.96 were included. Further, when more than two expected numbers were less than 1 or when more than five were less than 5 then the likelihood ratio test for random union of gametes (the RUG-test) was omitted, cf. Table 3. If the RUG-test was omitted or if it was significant, the disequilibrium parameter, Δ, defined by Burrows (Cockerham and Weir 1977; Weir 1979) as a combined disequilibrium was used. This parameter measures both disequilibrium within the gametes and between gametes and coincides with D when populations practice random union of gametes. A standard test for no combined disequilibrium is in obvious notation

$$N\Delta^2/p_1q_1p_2q_2$$

which is χ^2-distributed with 1 degree of freedom.

Combining tests

During the analysis it was useful at several times to apply an overall test for evaluating a hypothesis when tested in many populations. This test statistic was calculated by summing the corresponding χ^2-distributed test statistics. This gives a χ^2-distributed test statistic with degrees of freedom equal to the sum of degrees of freedom for each.

Results

The degree of genetic variation of the six polymorphic enzyme loci *Pgi 2, Got 2, Got 3, Acp 1, Pgm 1* and *Pgd 1* was high in the 22 populations of *Lolium perenne* (Table 4) and in the 13 populations of *Lolium multiflorum* (Table 5). The only exceptions are that *L. perenne* was monomorphic at *Pgd 1* and *L. multiflorum* was nearly monomorphic at *Got 3* and *Pgd 1.*

Table 4. Number of plants with various banding patterns[a] at the six loci in the 22 populations of *Lolium perenne*. The banding patterns are listed in decreasing frequencies

Enzyme locus	Population																					
	1	2	2a	2b	2c	2d	3	4	5	6	7	7a	7b	7c	8	9	10	11	12	13	14	15
Pgi 2																						
bb	16	42	41	44	41	45	10	40	55	17	33	26	23	30	14	36	9	8	51	21	21	13
ab	35	38	42	39	43	45	35	33	21	29	31	11	31	21	40	20	30	29	22	34	34	43
aa	13	13	10	8	9	4	16	12	1	12	3	3	3	5	14	9	17	31	3	15	28	30
bc′	15	3	1	2	1	1	9	6	12	17	23	36	30	27	15	20	14	11	17	8	4	4
ac′	15	0	0	3	2	1	19	5	6	16	3	15	8	9	7	8	21	16	3	15	7	5
c′c′	0	0	2	0	0	0	6	0	1	5	2	5	1	4	3	2	5	1	0	3	1	0
Got 2																						
dd	38	62	56	55	57	53	34	68	74	64	72	72	71	72	39	55	28	43	45	30	46	48
cd	31	24	29	35	29	30	35	11	18	28	15	18	20	17	31	19	46	32	42	42	37	33
cc	19	7	9	6	10	11	18	6	2	4	3	1	3	2	20	15	21	20	7	24	12	14
bd	0	0	2	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0	0
bc	1	0	0	0	0	0	0	0	0	0	2	0	0	0	1	1	0	0	0	0	0	0
bb	0	1	0	0	0	0	2	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
ad	2	2	0	0	0	0	4	7	0	0	1	3	0	2	0	0	0	0	0	0	0	0
ac	0	0	0	0	0	0	1	1	0	0	0	0	2	1	0	1	0	0	0	0	0	0
ab	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aa	4	0	0	0	0	1	0	2	2	0	0	2	0	1	2	2	0	1	0	0	0	0
Got 3																						
bb	53	25	28	16	21	29	62	67	69	70	48	55	53	36	77	57	35	61	46	32	56	48
bc	30	40	36	39	41	40	16	19	21	21	11	16	15	23	12	34	5	13	10	30	13	31
cc	3	13	15	18	15	11	2	1	2	2	4	0	0	6	0	3	0	2	3	5	5	6
bd	6	9	8	11	10	7	13	0	3	2	15	14	13	12	0	1	17	11	8	11	11	5
cd	1	5	3	8	6	7	0	0	0	0	0	0	0	3	0	0	1	3	0	4	3	2
dd	0	1	0	0	0	0	2	0	0	0	0	0	2	1	0	0	2	0	0	0	0	1
ab	0	0	4	4	1	1	0	3	1	1	11	7	10	13	4	0	26	6	24	9	6	2
ac	0	3	2	0	2	0	0	2	0	0	2	1	1	0	0	0	0	0	1	5	1	0
ad	1	0	0	0	0	0	0	0	0	0	0	3	1	2	0	0	8	0	2	0	1	0
aa	0	0	0	0	0	0	0	2	0	0	0	0	1	0	0	0	2	0	1	0	0	0
Acp 1																						
aa	17	3	6	13	9	6	12	14	27	48	29	24	26	28	78	41	57	50	25	41	49	67
ab	24	16	17	13	22	14	24	13	16	27	20	22	32	28	9	29	28	20	25	22	24	15
bb	16	23	14	24	21	19	44	24	8	16	13	15	12	13	0	18	10	13	35	14	18	4
ac	4	6	23	9	9	13	6	3	22	2	15	17	13	13	5	5	1	2	1	7	3	3
bc	12	14	20	17	15	17	2	8	1	2	4	12	3	11	0	1	0	2	6	3	0	0
cc	2	7	4	8	4	12	2	2	9	0	10	3	3	3	1	1	0	1	1	1	0	0
ad	4	4	3	3	10	6	0	4	7	0	1	2	4	0	0	0	0	6	0	5	1	6
bd	2	0	6	5	3	6	0	1	2	0	1	0	1	0	0	0	0	1	1	3	1	0
cd	3	1	2	4	2	2	0	0	2	0	1	0	2	0	0	0	0	0	0	0	0	0
dd	0	1	1	0	1	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
00	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	1	0	0	0	0
Pgm 1																						
aa	42	27	32	28	31	19	58	44	48	34	59	57	66	58	61	59	74	44	54	55	48	60
ab	38	48	39	43	48	59	31	42	27	48	27	29	23	26	24	26	22	45	34	36	42	30
bb	4	21	24	24	15	17	6	7	6	12	4	3	2	4	2	5	0	6	7	4	6	4
ac	12	0	0	0	1	0	1	3	9	2	5	7	3	7	6	4	0	1	1	1	0	1
bc	0	0	0	1	1	1	0	0	5	0	0	0	2	1	1	0	0	0	0	0	0	0
cc	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Pgd 1																						
bb	96	96	95	96	96	95	95	96	96	96	95	96	96	95	94	95	96	96	96	96	96	95

[a] The notation follows Fig. 1 except for *Pgi 2:* c′ = c + d

Table 5. Number of plants with various banding patterns[a] at six loci in the 13 populations of *Lolium multiflorum*. The banding patterns are listed in decreasing frequencies

Enzyme locus	Population												
	1	2	3	4	5	6	6a	6b	6c	6d	6e	6f	6g
Pgi 2													
dd	40	32	19	31	28	35	30	41	38	45	34	34	29
bd	40	42	37	40	51	48	40	43	38	34	49	44	46
bb	14	17	8	10	17	13	20	11	15	14	13	13	17
c'd	2	3	26	10	0	0	1	0	4	0	0	1	3
bc'	0	2	5	5	0	0	4	1	1	3	0	3	0
c'c'	0	0	1	0	0	0	0	0	0	0	0	0	0
Got 2													
dd	14	21	15	18	20	23	25	25	21	15	23	21	26
cd	17	21	23	27	15	24	18	23	29	20	20	25	22
cc	21	28	27	10	7	25	21	26	20	24	17	16	16
bd	8	7	8	6	4	7	2	0	2	5	5	6	3
bc	13	6	11	13	2	1	3	0	3	0	2	1	0
bb	10	3	2	7	10	3	5	4	3	5	5	3	6
ad	1	3	3	8	11	5	7	7	8	9	8	0	10
ac	2	2	1	1	13	2	9	5	7	11	8	16	9
ab	1	0	0	0	0	0	0	0	0	0	0	2	0
aa	8	5	6	6	14	6	5	6	3	7	8	5	3
Got 3													
bb	91	88	87	85	93	95	93	96	90	94	93	94	95
ab	2	2	7	0	0	1	2	0	3	1	1	0	0
aa	0	0	0	0	0	0	0	0	0	0	0	0	0
bc	0	5	2	11	3	0	0	0	2	0	2	0	0
ac	0	0	0	0	0	0	0	0	0	0	0	0	0
cc	1	0	0	0	0	0	0	0	0	0	0	0	0
Acp 1													
bb	41	27	21	17	57	56	45	49	56	46	48	51	50
ab	13	16	15	16	21	24	37	34	18	30	35	29	24
aa	4	13	1	2	11	9	8	7	5	8	3	7	5
bc'	9	12	20	21	1	3	3	6	10	9	8	4	10
ac'	1	4	14	9	2	2	1	0	6	3	2	2	5
c'c'	3	2	14	18	4	1	0	0	1	0	0	2	1
00	0	1	0	0	0	0	0	0	0	0	0	0	0
Pgm 1													
aa	80	80	43	51	76	79	90	85	83	84	75	75	78
ab	12	13	44	40	17	14	4	10	13	10	18	16	15
bb	0	0	9	5	3	1	0	1	0	0	1	1	0
ac	3	2	0	0	0	1	0	0	0	2	1	2	0
bc	1	0	0	0	0	0	0	0	0	0	0	1	0
cc	0	0	0	0	0	0	0	0	0	0	0	0	0
Pgd 1													
bb	96	88	96	96	92	91	86	81	89	81	83	86	86
a'b	0	6	0	0	4	4	8	15	7	15	11	9	6
a'a'	0	2	0	0	0	0	0	0	0	0	1	0	1

[a] The notation follows Fig. 1 except for *Pgi 2:* c' = a + c, *Acp 1:* c' = c + d, *Pgd 1:* a' = a + c

At a few loci, one allele was very rare within a species and this allele has been pooled with the next most rare allele (Tables 4 and 5). A null genotype was observed in *Acp 1* in four populations (p2, p5, p11, m2) and at first it was assumed that the corresponding silent allele was not present in the remaining populations.

The analysis of the genetic variation consists of 1) an evaluation of the degree of selfing by means of the parameter F, 2) a description of deviations from Hardy-Weinberg proportions (HW-proportions) at one locus and 3) a description of deviations from genetic equilibrium at two loci.

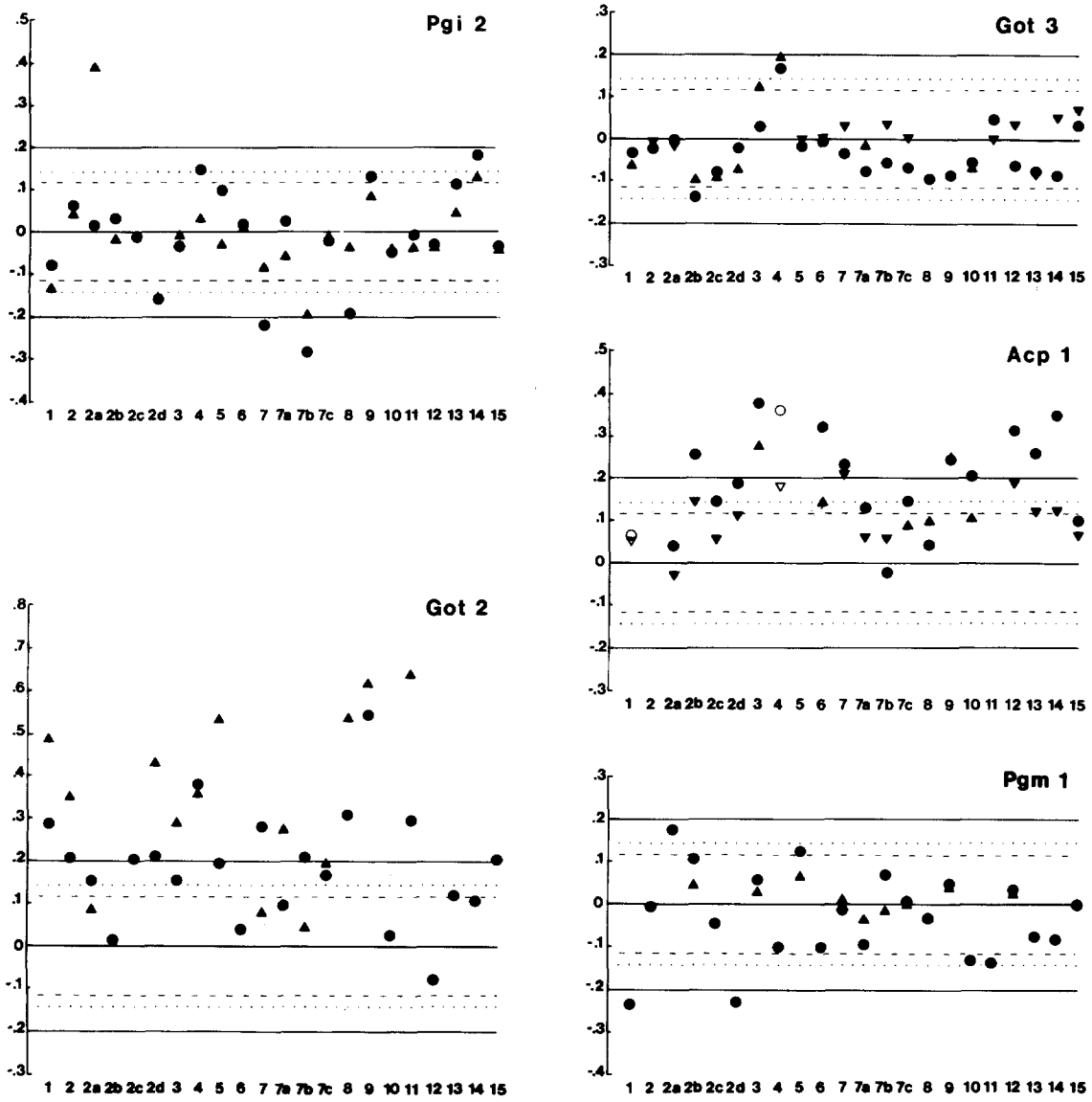

Fig. 2. Estimates of the parameter F for 22 populations of *L. perenne* based on 2 alleles (●), 3 alleles (▲) or 4 alleles (▼) and about 96 plants. Open symbols indicate estimates on basis of about 70–85 plants. Missing values are due to missing estimates of F. Lines indicate 95% confidence limits for F = 0 on basis of 93–96 plants in case of 2 alleles (–), 3 alleles (. .) and 4 alleles (- -)

Average inbreeding coefficient

The degree of selfing in each cultivar is evaluated by the parameter F. If the values $\hat{F}$ for all loci are larger than zero, partial selfing (or a mixture of subpopulations) is indicated; on the contrary, for a single locus selection might give rise to positive $\hat{F}$-values. The values obtained for all loci and all populations of *L. perenne* and *L. multiflorum*, are shown in Figs. 2 and 3, respectively. For each population two values (which may coincide) are given for each locus; one calculated for the maximal number of alleles and one for two allele classes. In none of the populations the values of $\hat{F}$ indicated strong partial selfing so random mating is assumed in the following. On the other hand, many negative $\hat{F}$-values were observed especially for *Got 3* and *Pgd 1;* true negative values of F indicate the presence of selection but with rare alleles $\hat{F}$ tends to be negative due to sampling.

The loci can be partitioned into two groups. For *Got 2* and *Acp 1* $\hat{F}$-values were significantly larger than zero except for very few populations. On the contrary, for *Pgi 2, Got 3, Pgm 1* and *Pgd 1* $\hat{F}$-values were spread around zero with few significant deviations but, in general, with more negative than positive values. These two classes of loci will now be considered in more detail using all the information from the genotypic distributions.

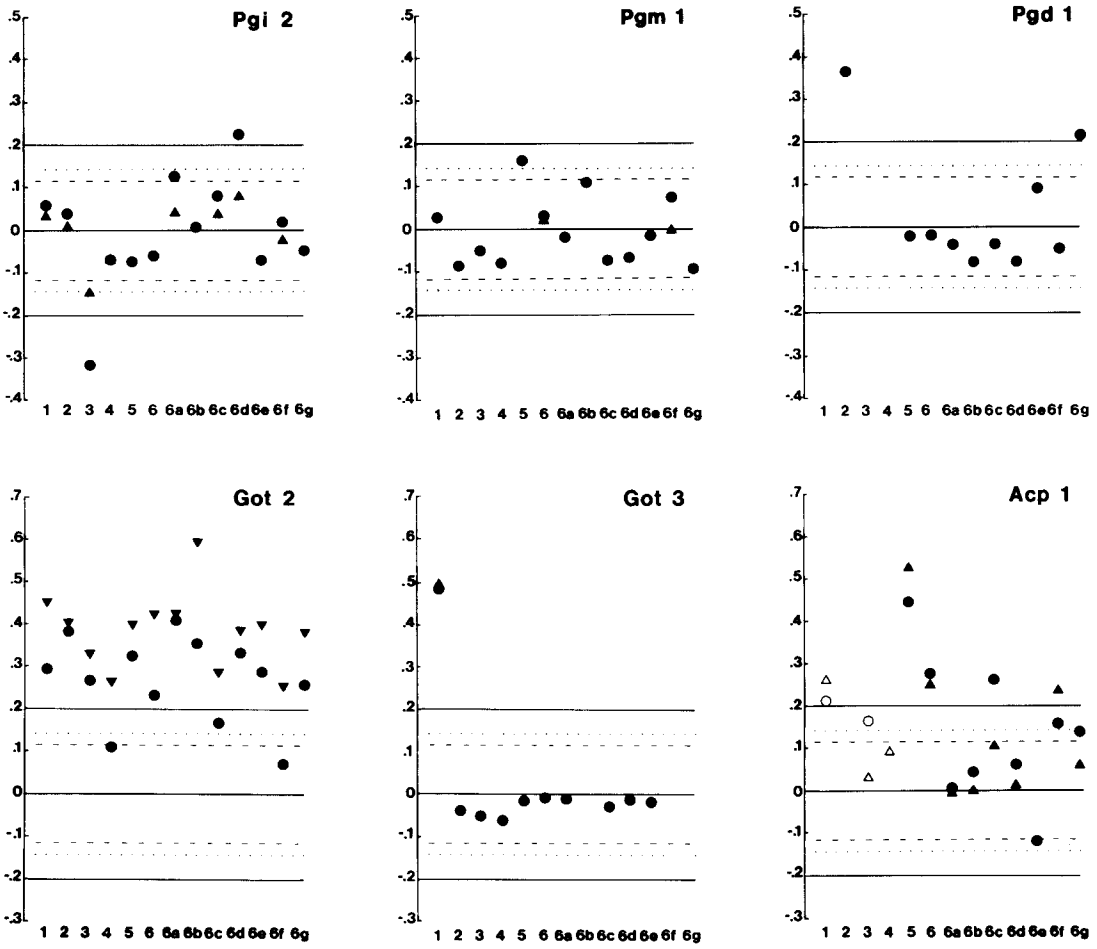

Fig. 3. Estimates of the parameter F for 13 populations of *L. multiflorum* based on 2 alleles (●), 3 alleles (▲) or 4 alleles (▼) and about 96 plants. Open symbols indicate estimates on basis of about 70–85 plants. Missing values are due to missing estimates of F. Lines indicate 95% confidence limits for F = 0 on basis of 93–96 plants in case of 2 alleles (–), 3 alleles (. .) and 4 alleles (- -)

Genotypic distribution at one locus

The parameter F is just one of the parameters describing deviations from the HW-proportions to be expected in a large random mating population not influenced by selection. In case of two alleles, F is sufficient for describing the deviations but otherwise additional parameters are necessary. It appeared, however, that significant deviations from HW-proportions in case of more than two alleles were found only when $\hat{F}$ was correspondingly numerically large, except for one population (p7) at the locus *Got 3*. This indicates that deviations from HW-proportions were due to deviations between the observed and the expected number of homozygotes.

In conclusion, the genotypic distributions were in general found to be in HW-proportions at the loci *Pgi 2, Got 3, Pgm 1* and *Pgd 1* but not at the loci *Acp 1* and *Got 2* (Table 6). The few significant deviations from HW-proportions (numerically large $\hat{F}$-values) in the first group of loci might be due to seed contamination, sampling or selection. A likely example of the first type is in *Got 3* for m1 (Table 5).

The large positive $\hat{F}$-values (or large HW-test values, Table 7) observed for *Acp 1* (Figs. 2 and 3) might be caused by the presence of a silent allele. In few cases, homozygotes for a silent allele were observed; if the silent allele was present in all populations but not taken into account in the calculation of the expected proportions, positive values of $\hat{F}$ are expected even if the populations show random mating (Brown 1979). The expected allele frequencies in case of HW-proportions are calculated as described in the Appendix and the frequencies of the silent allele are shown in Table 7. Some of these values are of a considerable magnitude and cannot be neglected. However, in *L. perenne* they cannot account for the deviations from HW-proportions. This can be concluded from tests calculated ac-

Table 6. Chi-squared test[a] of overall evaluation of fit to HW-proportions, shown for each locus within species

Locus	*L. perenne*		*L. multiflorum*	
	$\Sigma \chi^2$	df	$\Sigma \chi^2$	df
Pgi 2	86.39	62	36.46	29
Got 2	168.18***	32	528.13***	78
Got 3	78.84	61	–	–
Acp 1	210.06***	86	95.07***	45
Pgm 1	34.26	32	8.88	13
Pgd 1	–	–	2.54	2

[a] The significance levels are
* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$ and *** $P < 0.001$

cording to the pooling rules of Table 3. Even if the number of populations with significant deviations is halved, from 9 to 5 in *L. perenne* and from 3 to 0 in *L. multiflorum,* the deviations are still so large than the combined test in *L. perenne* shows a large deviation from HW-proportions when the silent allele is included (Table 7).

In *Got 2* the fastest moving band partly overlaps the bands of *Got 1* so the large excess of homozygotes (positive $\hat{F}$-values (Figs. 2 and 3) or large HW-test values (Table 8)) might be due to misclassifications resulting in, for instance, too few observations of 'ab'. This is investigated by considering only the genotypes 'dd', 'dc', and 'cc' (Table 8). Again deviations from HW-proportions due to excess of homozygotes are found. The excess of homozygotes might, in stead, be due to the presence of a silent allele. This cannot be tested reasonably in *L. perenne* due to the small number of observations in the classes including 'a' and 'b' and a silent allele cannot account for the excess of homozygotes in *L. multiflorum* (Table 8). Due to the uncertainties regarding the interpretation of the banding patterns in *Acp 1* and *Got 2,* these loci are omitted from further analysis.

Genotypic distribution at two loci

In a large random mating population the genotypes on two loci will show equilibrium proportions or Robbins' proportions (Robbins 1918) in the absence of selection on the polymorphism, and after a sufficient number of generations for the linkage disequilibrium to decay. The number of generations necessary increases with increasing linkage. A population in Robbins' proportions automatically shows marginal HW-proportions whereas the opposite is not true. From the available data it was possible to test both for random union of gametes (RUG) and for gametic phase equilibrium (Tables 9 and 10). Assuming random mating the former test is a test for the absence of selection. It is likely that populations showing deviations from "random union of gamete"-proportions at one locus (i.e. deviations from HW-proportions) will also show deviations at two loci when this locus is included in the pair. This was not always the case partly due to a statistically less powerful test with two loci. In a few cases, however, large deviations from RUG could not be ascribed to this one-locus effect.

The observed gametic phase disequilibria (Tables 9 and 10) can be summarised as follows. Gametic phase disequilibrium between *Pgi 2* and *Got 3* was observed in 2 of 25 populations (p4 and m2), between *Pgi 2* and

Table 7. Characteristics of the *Acp 1* locus. For further explanations see text

Population	HW-test without a silent allele (df)[c]	Frequency of the silent allele	HW-test with a silent allele (df)[c]
p1	9.29 (6)	0.03	6.51 (3)
p2[a]		0.09	12.02 (6)
p2a	8.19 (6)	0.00	8.19 (6)
p2b	14.23 (6)*	0.08	8.08 (6)
p2c	8.71 (6)	0.03	7.94 (6)
p2d	8.05 (6)	0.05	5.18 (6)
p3	17.08 (3)***	0.10	9.36 (3)*
p4	11.85 (3)**	0.11	5.88 (3)
p5[a]		0.11	9.74 (6)
p6	9.84 (3)*	0.09	4.71 (1)*
p7	18.66 (3)***	0.12	7.81 (3)*
p7a	4.55 (3)	0.04	3.23 (3)
p7b	7.51 (6)	0.03	5.32 (3)
p7c	2.13 (3)	0.04	0.96 (3)
p8	0.15 (1)	0.03	2.27 (1)
p9	10.94 (3)*	0.10	1.13 (1)
p10	3.81 (1)	0.07	–
p11[a]		0.13	2.83 (3)
p12	19.20 (3)***	0.12	3.95 (1)*
p13	9.12 (3)*	0.08	5.25 (3)
P14	15.05 (3)**	0.11	6.99 (1)**
p15	7.11 (3)	0.05	–
Σ[b]	185.47 (71)***		92.65 (53)***
m1	7.94 (3)*	0.09	0.02 (1)
m2[a]		0.13	3.13 (3)
m3	9.92 (6)	0.02	5.48 (3)
m4	9.04 (6)	0.04	4.84 (3)
m5	33.05 (3)***	0.14	0.22 (1)
m6	8.70 (3)*	0.09	0.77 (1)
m6a	0.14 (3)	0.00	–
m6b	3.95 (3)	0.00	–
m6c	6.42 (3)	0.05	2.93 (1)
m6d	1.68 (3)	0.01	–
m6e	1.99 (3)	0.00	0.24 (1)
m6f	7.26 (3)	0.05	0.34 (1)
m6g	1.85 (3)	0.03	–
Σ[b]	91.94 (42)***		14.84 (12)

[a] In these populations the silent homozygote is observed
[b] Populations showing a silent allele are not included in the summation
[c] The significance levels are
* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$ and *** $P < 0.001$

Table 8. Characteristics of the *Got 2* locus. For further explanations see text

Population	HW-test with respect to a, b, c and d (df)[a]	HW-test with respect to c and d (df = 1)[a]	HW-test with a silent allele (df)[a]
p1	26.36 (3)***	5.98*	
p2	3.78 (1)	3.57	
p2a	2.07 (1)	2.79	
p2b	0.02 (1)	0.02	
p2c	3.81 (1)	3.81	
p2d	4.06 (1)*	3.68	
p3	10.52 (3)*	2.43	
p4	16.85 (3)***	12.36***	
p5	3.11 (1)	0.45	
p6	0.17 (1)	0.17	
p7	5.91 (1)*	2.60	
p7a	0.76 (1)	0.01	
p7b	3.57 (1)	0.96	
p7c	2.20 (1)	0.57	
p8	23.17 (3)***	7.00**	
p9	46.39 (3)***	18.10***	
p10	0.07 (1)	0.07	
p11	8.42 (1)**	7.65**	
p12	0.59 (1)	0.45	
p13	1.42 (1)	1.42	
p14	1.06 (1)	1.06	
p15	3.83 (1)	3.83	
Σ	168.18 (32)***	78.98 (22)***	
m1	48.95 (6)***	5.88*	1.82 (5)
m2	36.81 (6)***	11.12***	2.48 (5)
m3	34.82 (6)***	4.66*	7.83 (5)
m4	35.45 (6)***	0.00	19.26 (5)**
m5	53.56 (6)***	1.82	19.72 (5)**
m6	42.95 (6)***	8.13**	5.60 (2)
m6a	40.33 (6)***	12.52***	8.37 (5)
m6b	56.46 (6)***	10.86***	13.13 (5)*
m6c	17.76 (6)**	2.06	4.96 (5)
m6d	41.75 (6)***	5.57*	15.69 (5)**
m6e	37.23 (6)***	6.50*	5.93 (5)
m6f	41.28 (6)***	2.01	28.07 (5)***
m6g	40.78 (6)***	5.62*	18.28 (5)**
Σ	528.13 (78)***	76.75 (13)***	151.10 (62)***

[a] The significance levels are
* $0.01 < P < 0.05$; ** $0.001 < P < 0.01$ and *** $P < 0.001$

Pgm 1 in 1 of 34 populations (m6g), between *Pgi 2* and *Pgd 1* in 3 of 7 populations (m6b, m6f, m6g), between *Got 3* and *Pgm 1* in 2 of 25 populations (p9 and p10) and in the remaining two pairs of loci, in none of one and six populations, respectively. If a group of populations were under the same influence with respect to gametic phase disequilibrium, e.g., if two loci were linked or if the populations were newly founded, an overall test might show whether the deviations were true or merely due to random fluctuations. A general tendency of gametic phase disequilibrium between *Pgi 2* and *Pgd 1* showed up in subpopulations of m6.

Discussion

The use of electrophoretic variation in cultivar identification as well as in any population genetic analysis requires that the banding pattern is consistent and that the relationship between bands and alleles is well defined. The six polymorphic loci under study *Pgi 2, Got 2, Got 3, Acp 1, Pgm 1* and *Pgd 1* have been chosen to satisfy the former prerequisite but unfortunately the latter prerequisite was only partially satisfied in *Acp 1, Got 2* and *Got 3*. In *Acp 1* a homozygote for a silent allele was observed in a few populations; the a-band in *Got 2* could not be distinguished from the band in *Got 1;* and finally, apparently non-Mendelian segregation has been observed in the few crosses made on segregation at *Got-2* and *Got-3* (Hayward and McAdam 1977b). One of the purposes of the present analysis was to investigate whether the genetic interpretation was reasonable, judged from the expected genotypic proportions.

The populations assayed were samples of standard seed lots of 15 cultivars of *Lolium perenne* and 6 cultivars of *Lolium multiflorum*. These cultivars are based on a selection of a dozen of plants which are intercrossed and continuously selected for some generations. Before cultivars are introduced, they must be shown to be phenotypically stable from generation to generation for morphological characters; this analysis should, therefore, investigate the stability on the genotypic level. Thirteen additional populations were investigated. They represented seed lots taken from different generations of some of the cultivars and grown in different environments to investigate the possible influence of sampling, contamination and selection.

The population genetic consequences of constructing a new cultivar are expected to be comparable with those in a natural population caused by founder effects, i.e. diversity between populations and even loss of different alleles. Nevertheless, the same alleles were found in nearly all cultivars within a species. A possible explanation for this could be that some gene flow occurs among populations.

Self-incompatibility in *L. perenne* and *L. multiflorum* is presumably determined by two multi-allelic loci with gametophytic control of the pollen phenotype (for a discussion see Lawrence et al. 1983). Loci in gametic phase disequilibrium with one of the self-incompatibility loci might tend to show increased heterozygosity since no homozygotes are present at the self-incompatibility locus. One of the loci investigated, *Pgi 2,* has this potential since it is linked to one of the self-incompatibility loci (S) (Cornish et al. 1980a; Fearon et al. 1983). The data do not indicate any effect of this linkage.

Table 9. Deviations from "random union of gamete"-proportions (RUG) measured as likelihood ratio statistics (df=5) and deviations from gametic phase equilibrium (GPE) measured as likelihood ratio statistics or Burrows' tests (df=1) for pairs of the loci *Pgi 2*, *Got 3* and *Pgm 1* in *Lolium perenne*. For the test procedure see text

	Pgi 2/Got 3		*Pgi 2/Pgm 1*		*Got 3/Pgm 1*	
	RUG	GPE	RUG	GPE	RUG	GPE
1	10.39	0.53	11.56*,+	0.42	7.54+	0.16
2	0.99	1.08	4.81	2.79	5.23	1.24
2a	3.46	0.01	3.15	0.45	5.98	0.75
2b	3.36	0.14	2.61	0.70	7.10	0.12
2c	4.46	3.12	2.84	3.30	1.55	1.79
2d	3.36	0.20	11.44*,+	0.01	12.88*,+	1.48
3	8.83	1.49	11.49*	2.11	5.72	0.67
4	5.95	8.95**	7.43	0.37	4.87	1.31
5	1.42	0.00	10.55	0.12	4.32	0.64
6	–[a]	0.04	5.03	0.84	–	0.22
7	8.71+	0.61	8.34+	0.81	5.07	1.68
7a	0.84	0.02	2.03	0.99	1.68	0.78
7b	20.08**,+	0.69	14.49*,+	0.09	3.25	1.28
7c	9.94	0.05	2.27	1.72	2.21	0.17
8	–	0.01	5.69	1.05	4.53	2.60
9	5.53	0.60	4.04	1.20	–	4.67*
10	2.82	1.41	–	1.21	–	5.25*
11	2.51	0.11	8.70	0.08	3.64	0.48
12	3.51	2.01	2.27	1.06	1.62	0.05
13	2.50	3.79	2.96	2.04	2.57	0.93
14	9.79	0.61	5.76	2.93	6.98	0.17
15	5.92	0.27	1.20	0.00	7.92	0.00
Test value	114.37	25.74	128.66	24.29	94.66	26.44
(df)	(110)	(22)	(105)	(22)	(95)	(22)

[a] The expected numbers are too small
+ Significant deviation at one locus (two allele classes)
*, ** Test significant at 5% and 1% level, respectively

Table 10. Deviations from "random union of gamete"-proportions (RUG) measured as likelihood ratio statistics (df=5) and deviations from gametic phase equilibrium (GPE) measured as likelihood ratio statistics or Burrows' tests (df=1) for pairs of the loci *Pgi 2*, *Got 3*, *Pgm 1* and *Pgd 1* in *Lolium multiflorum*. For the test procedure see text

	Pgi 2/Got 3		*Pgi 2/Pgm 1*		*Pgi 2/Pgd 1*		*Got 3/Pgm 1*		*Got 3/Pgd 1*		*Pgm 1/Pgd 1*	
	RUG	GPE	RUG	GPE	RUG	GPE	RUG	GPE	RUG	GPE	RUG	GPE
1			–	0.94								
2	–[a]	4.74*	–	0.56	–+	0.09	–	0.01	–+	0.10	+	0.49
3	–+	0.05	19.11**,+	0.00			–	0.04				
4	–	0.14	1.61	0.01			–	1.86				
5			–	0.01								
6			–	1.14								
6a					–	0.53						
6b			–	0.52	–	4.87*					–	0.47
6c			–	0.00								
6d			–+	0.05	–+	0.02					–	0.47
6e			–	1.11	–	0.00					–	0.01
6f			–	0.50	–	6.78**					–	0.00
6g			–	7.09**	–+	6.73**						
Test value		4.92	20.72*	11.93		19.02***		1.91		0.10		
(df)		(3)	(10)	(12)		(7)		(3)		(1)		

[a] The expected numbers too small
+ Significant deviation at one locus (two allele classes)
*, ** Test significant at 5% and 1% level, respectively

The self-incompatibility is, however, not complete (Cornish et al. 1980b; Fearon et al. 1983) so selfing could be an important population parameter. The parameter used for describing the degree of selfing is the average inbreeding coefficient or Wright's fixation index, F (Brown 1979). This parameter can be estimated in different ways by means of data from one generation (Curie-Cohen 1982). In all cases the sampling error is very much influenced by the allele frequencies except when $F=0$; therefore, average values of $\hat{F}$ over loci should be applied carefully. For this reason no overall estimates of selfing have been calculated in this study. Since the interpretation of negative F-values is dubious, averaging large negative and large positive values (Kidd et al. 1980) should be avoided.

Partial selfing does not seem to be important in the considered cultivars although a considerable rate of selfing has been reported in a natural population of *L. multiflorum* (Allard et al. 1977). This difference might be due to a low statistical power of the test applied using information from one generation only (Ward and Sing 1970). A much more efficient method is to sample two successive generations (Allard et al. 1977; Shaw and Allard 1982).

The six loci under study were partitioned into two groups with respect to genetic equilibrium (HW-proportions) at one locus. At *Got 2* and *Acp 1* large deviations from HW-proportions indicating excess of homozygotes were observed. Hayward et al. (1978) have observed the same phenomenon in three cultivars, one of which equals p12. A likely explanation in case of *Acp 1* is the presence of silent alleles but this is not enough to account for the deviations found. For *Got 2* technical problems might give rise to misclassifications but again this does not give a full explanation for the deviations. These two loci were, therefore, omitted in the two-locus analysis. Preliminary results have indicated that *Got 2* and *Acp 1* are linked (Hayward et al. 1979) so their genotypic distributions might be altered by a selection force working on that part of the genome.

At the remaining four loci (*Pgi 2, Got 3, Pgm 1,* and *Pgd 1*), genetic equilibrium at one locus was obtained. The abnormalities in segregation at *Got 3* (Hayward and McAdam 1977a) have not been supported except for a somewhat skewed distribution of the estimates of F towards negative values. Only two of the four loci have been located i.e. *Pgi 2* and *Got 3* and they are found on different chromosomes (Lewis et al. 1980). Genetic equilibrium at two loci (Robbins' proportions) were found except in the subpopulations of the cultivar m6. Here a small degree of gametic phase disequilibrium was found between *Pgi-2* and *Pgd-1*. Due to founder effects, gametic phase disequilibrium was expected more often but with the small number of observations the disequilibrium has to be numerically large, 0.03 to 0.05, for a 50% certainty of rejecting the hypothesis of $D=0$ at a 5% level (Brown 1975).

In the present analysis insufficient statistical power of the genetic tests have been an overwhelming problem. Pooling of rare alleles is unsatisfactory but necessary. Even then, zero-observations and small expectations cause problems when using the asymptotic test results, either for the likelihood ratio test or for Pearson's χ^2-test (Larntz 1978). However, it seems as if the likelihood ratio test for HW-proportions is reliable even for small sample sizes (Elston and Forthofer 1977).

In conclusion, the stability of cultivars observed in quantitative morphological characters was also found in most of the studied isozymes as judged from their fit in one generation to "random union of gamete"-proportions in one and two loci and to gametic phase equilibrium. Indications of instability were, however, found in subpopulations exposed to new environmental conditions and this has elsewhere been supported by tests for homogeneity (Nielsen et al. 1984).

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Appendix

Maximum likelihood estimation of allele frequencies when a silent allele is present (Rust 1972).

In Table A.1 the expectation in each observational class is described in case of two or three active alleles. The procedure can easily be extended to more alleles. The iteration equations for simultaneous estimation of allele frequencies can be calculated from the first derivative of the log-likelihood function or from conditional expectations. The right-hand side of the equations gives the new value of the parameters in each iteration cycle. In the case of three alleles the equations are:

$$p'_a = \tfrac{1}{2}(O_3 + O_5)/N + (p_a + p_0)/(p_a + 2\,p_0) * O_2/N$$

$$p'_b = \tfrac{1}{2}(O_3 + O_6)/N + (p_b + p_0)/(p_b + 2p_0) * O_4/N$$

Table A 1. Expectation of proportion of individuals belonging to each observational class in case of a silent allele and two or three functional alleles

Banding pattern	Expectation	Observation	
00	p_0^2	O_1	
aa	$p_a^2 + 2p_0p_a$	O_2	2 alleles
ab	$2p_ap_b$	O_3	
bb	$p_b^2 + 2p_0p_b$	O_4	3 alleles
ac	$2p_ap_c$	O_5	
bc	$2p_bp_c$	O_6	
cc	$p_c^2 + 2p_0p_c$	O_7	
	1	N	

$$p'_c = \tfrac{1}{2}(O_5 + O_6)/N + (p_c + p_0)/(p_c + 2p_0) * O_7/N$$

$$p'_0 = 1 - p'_a - p'_b - p'_c$$

and the equations in the case of two alleles are given by cancelling the equation for p'_c.

The fit of observations to expectations (Table A.1) is evaluated by a likelihood ratio test which is χ^2-distributed with $[n(n+1)/2 - n]$ degrees of freedom (n equals the number of non-silent alleles).

For the locus *Got-2* the observational classes O_1 and O_2 are pooled so the iteration equation for p'_a becomes

$$p_a' = \tfrac{1}{2}(O_3 + O_5)/N + p_a/(p_a + p_0) * (O_1 + O_2)/N$$

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