Heredity (1983), **50** (1), 35–45 0018-067X/83/05770035$02.00

SELF-INCOMPATIBILITY IN RYEGRASS

V. GENETIC CONTROL, LINKAGE AND SEED-SET IN DIPLOID *LOLIUM MULTIFLORUM* Lam.

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Received 14.ix.82

SUMMARY

An analysis of four families, whose parents are of very different origin, shows that self-incompatibility in diploid *Lolium multiflorum* is determined, as in *L. perenne* and other self-incompatible grasses that have been investigated, by a pair of multi-allelic genes whose effect in the pollen is gametophytic. Again, as in *L. perenne*, one of these genes, defined as *S*, is linked in its inheritance to the gene, *PGI-2*, which codes for the enzyme phosphoglucoisomerase, the best estimate of the frequency of recombination between these two loci being $\hat{p} = 0{\cdot}2414 \pm 0{\cdot}0795$. However, whereas in all previous experiments *S* and *Z* appear to have assorted independently, in two of these *L. multiflorum* families there is evidence that these genes are linked, the best estimate of their recombination frequency being $\hat{p} = 0{\cdot}2807 \pm 0{\cdot}0595$.

Crosses were made between 27 pairs of plants which included pairs that were expected, on the basis of pollen-stigma reactions, to be incompatible, half-compatible, three-quarters compatible and fully compatible. Fourteen plants were also self-pollinated and ten were allowed to set seed by open pollination. The seed-set obtained from the selfs (0·39 per cent) showed that *L. multiflorum* is no less self-incompatible than *L. perenne*. However, the seed-set obtained from incompatible crosses was significantly higher (3·77 per cent) than that obtained from selfs. On the other hand, there were no systematic differences between the three categories of compatible cross which gave an average seed-set of 20·60 per cent. Though there is some overlap between the distribution of the seed-set of these and incompatible crosses, on average these results confirm the classification of the plants on the basis of their pollen-stigma reactions.

A rather approximate estimate of the number of *S* and *Z* alleles in the *species*, based on the number found in the four parents of the families examined, suggests that at least 40 different alleles are likely to be present at each locus.

1. INTRODUCTION

Italian (*Lolium multiflorum*) and perennial ryegrass (*L. perenne*) are so closely related that fertile interspecific hybrids can be obtained between them, both spontaneously and by hand, as freely as intraspecific progeny. Indeed, some authorities give them no more than sub-specific rank (Clapham, Tutin and Warburg, 1962). Having shown that self-incompatibility in *L. perenne* is determined by a pair of independently inherited, multi-allelic genes, *S* and *Z*, whose effect in the pollen is gametophytic (Cornish, Hayward and Lawrence, 1979*a*, *b*), we might expect *L. multiflorum* to have the same system of self-incompatibility. Hay (1978), however, who investigated a single full-sib family of 26 plants of *L.*

TABLE 1

Parentage and size of families MA – MD

Family	Number of plants	Parents
MA	26	Trident 48/7 × Bb1276/28/2
MB	36	Bb1276/28/2 × Trident 48/7
MC	28	Trident 48/7 × RvP6/6
MD	30	Bb1276/28/2 × Bb1277/189

multiflorum, came to the conclusion that self-incompatibility in this species is controlled by at least three loci. If true, this conclusion would be of very considerable theoretical and practical interest. The investigation described in the present paper was designed to resolve this uncertainty about the genetical control of self-incompatibility in Italian ryegrass.

2. MATERIALS AND METHODS

The parents of the four families of full-sibs which we have examined are of very diverse origin (table 1). Thus "Trident" is a commercial variety produced by the Welsh Plant Breeding Station, Aberystwyth; "RvP Lemtal" is a commercial variety from Belgium; and Bb1276 and Bb1277 (W.P.B.S.) are two varieties which were derived from material collected from the Po valley region in Italy. One plant was chosen as a parent from each of these varieties for the present experiment. Of the three crosses that gave rise to these families, one was made in reciprocal (*MA* and *MB*) and the remaining two families (*MC* and *MD*) have a half-sib relationship with the first. We are greatly indebted to Mr D H Hides of the W.P.B.S. who made these crosses and who kindly supplied the seed from which the plants of these families were raised.

The procedure used to assign the members of each of these families to their incompatibility class was the same as that used by Cornish *et al.* (1979*a*).

We have shown that one of the incompatibility genes, *S*, in perennial ryegrass is linked to another gene, *PGI*-2, which codes for the enzyme phospho-glucoisomerase (Cornish, Hayward and Lawrence, 1980*a*). Using the procedures given by Hayward and McAdam (1977*a*, *b*) and Hayward and Balls (1978), clonal duplicates of each of the plants that had been classified with respect to their incompatibility phenotype were also scored for their *PGI*-2 phenotype in order to ascertain whether these genes are also linked in their inheritance in *L. multiflorum*.

Fifty-four plants from families *MA* and *MC* were crossed reciprocally for seed in order to investigate the relationship between the compatibility of the cross as determined by the observation of pollen-stigma reaction and seed-set. Of a grand total of 27 crosses, 5 were predicted to be incompatible, 6 half-compatible, 5 three-quarters compatible and 11 fully compatible. In addition, 14 plants were also self-pollinated and 10 were allowed to set seed by open pollination in the glasshouse. The procedures used for this part of the investigation were the same as those of Cornish, Hayward and Lawrence (1980*b*).

Full details and results of this work can be found in Scarrott (1981).

3. Results

(i) *The analysis of incompatibility phenotype*

As was the case with perennial ryegrass, it rapidly became apparent that it was necessary to recognise only four kinds of pollination, namely, incompatible, half-compatible, three-quarters compatible and fully compatible, which indicates, of course, that in Italian ryegrass also, self-incompatibility is controlled by two genes only. A summary of the results obtained from each of the four families investigated support this conclusion (table 2).

TABLE 2

Summary of the results obtained from the four families. It should be noted that while the assignation of plants to classes in the present experiment is consistent over reciprocals and families, there is no relationship between the labelling of the incompatibility alleles here and that of the previous experiment with perennial ryegrass

S S Z Z	*MA*	*MB*	*MA+MB*	*S S Z Z*	*MC*	*S S Z Z*	*MD*
1 3 1 3	2	3	**5**	*1 5 1 5*	4	*3 7 3 7*	1
1 3 1 4	3	2	**5**	*1 5 1 6*	0	*3 7 3 8*	2
1 3 2 3	2	0	**2**	*1 5 2 5*	1	*3 7 4 7*	0
1 3 2 4	1	5	**6**	*1 5 2 6*	1	*3 7 4 8*	0
1 4 1 3	1	1	**2**	*1 6 1 5*	1	*3 8 3 7*	0
1 4 1 4	2	2	**4**	*1 6 1 6*	5	*3 8 3 8*	0
1 4 2 3	3	2	**5**	*1 6 2 5*	0	*3 8 4 7*	0
1 4 2 4	0	4	**4**	*1 6 2 6*	2	*3 8 4 8*	6
2 3 1 3	3	2	**5**	*2 5 1 5*	4	*4 7 3 7*	8
2 3 1 4	2	2	**4**	*2 5 1 6*	1	*4 7 3 8*	2
2 3 2 3	1	5	**6**	*2 5 2 5*	3	*4 7 4 7*	1
2 3 2 4	1	1	**2**	*2 5 2 6*	1	*4 7 4 8*	0
2 4 1 3	2	2	**4**	*2 6 1 5*	0	*4 8 3 7*	3
2 4 1 4	0	2	**2**	*2 6 1 6*	0	*4 8 3 8*	3
2 4 2 3	2	0	**2**	*2 6 2 5*	2	*4 8 4 7*	3
2 4 2 4	1	3	**4**	*2 6 2 6*	3	*4 8 4 8*	0
Totals	26	36	**62**		28		29
No. of classes	14	14	**16**		12		9

A proportion of the pollinations in each of these families, however, was initially inconsistent with the overall classification of the plants into their classes. In all cases, these were confined to the misclassification of half-compatible pollinations as three-quarters compatible and *vice versa*. The accuracy of the initial classification of the plants in each family was confirmed when additional pollinations were made between plants of the same class, which in all cases turned out to be incompatible as expected. The proportion of misclassified pollinations in the present material (17·25 per cent of partially compatible or 6·61 per cent of all pollinations) was very similar to that encountered in perennial ryegrass (16·85 per cent and 7·53 per cent respectively), which were resolved in the same way (Cornish *et al.*, 1979*a*).

One plant in family *MD* could not be accommodated in a two-locus model for it was fully compatible with five plants and three-quarters compatible with a sixth. The six tester plants in question were known to

be of different genotype. The leucine aminopeptidase phenotype of all other members of this family turned out to be *bb* at the *LAP*-2 locus, whereas that of the anomalous plant was *ab*. It is clear, therefore, that the latter cannot be a legitimate member of the family and that it probably originated as the result of contamination of the cross by foreign pollen.

No differences between reciprocal pollinations were observed in any of the families, which indicates that the cross giving rise to each was of the type $S_{1.2}Z_{1.2} \times S_{3.4}Z_{3.4}$. However, because the members of families *MC* and *MD* have a half-sib relationship with those of families *MA* and *MB*, it is possible in these circumstances to go further than this and to deduce the genotypes of all four parents. Thus if three appropriately chosen testers from family *MA* (*e.g.*, $S_{1.3}Z_{1.3}$, $S_{1.4}Z_{1.4}$ and $S_{1.4}Z_{2.3}$) are used to pollinate one plant from each class of both families *MC* and *MD*, those plants of the latter which carry the same *S* or *Z* alleles as the former can be recognised and hence the genotypes of the parents determined. Defining the genotype of Trident 48/7 as $S_{1.2}Z_{1.2}$, those of Bb1276/28/2, RvP6/6 and Bb1277/189 turned out to be, on the basis of these pollinations, $S_{3.4}Z_{3.4}$, $S_{5.6}Z_{5.6}$ and $S_{7.8}Z_{7.8}$ respectively. However, because progeny of the cross between RvP6/6 and Bb1277/189 were not examined, it is possible (though unlikely) that these parents possess one or more alleles in common at the *S* and/or *Z* loci. In all, therefore, these four parents carry a minimum of six and a maximum of eight different alleles at each locus.

(ii) *The joint segregation of S and Z*

The results of an analysis of the joint segregation of *S* and *Z* in each family are shown in table 3, the procedure used being that of Lawrence,

TABLE 3

Tests for linkage between S and Z on the data from each of the four families taken separately and on the pooled data of the pair of reciprocal crosses, MA and MB. Each χ^2 *has 1 degree of freedom*; **P* = 0·05 – 0·01; ***P* = 0·01 – 0·001 *and* ****P* < 0·001. *Other details are as follows*: 1 = χ^2 *for Trident* 48/7; 2 = χ^2 *for Bb*1276/28/2; *and* 3 = *heterogeneity* χ^2*s calculated on* $p = \hat{p}$, *rather than* 0·5

Item	*MA*	*MB*	*MA* + *MB*	*MC*	*MD*
Female	0	1·000	0·065[1]	3·571	7·759**
Male	0·615	0·111	0·065[2]	9·143**	2·793
Joint	0·308	0·222	0	12·071***	9·931**
Heterogeneity	0·308	0·889	0·129	0·820[3]	0·749[3]

Cornish and Hayward (1979) and Cornish *et al.* (1979*b*). None of the linkage χ^2's are affected by disturbed single factor ratios, because only one of a total of twenty was significant ($S_3 : S_4$ in family *MD*; $\chi^2_{(1)} = 4{\cdot}172$, $P = 0{\cdot}05 - 0{\cdot}02$). Since the genotypes of the parents are known, only one set of linkage χ^2's need be calculated for each family and the appropriate rejection levels for the null hypothesis of no linkage are, in these circumstances, the customary 5 per cent and 1 per cent respectively. However, the phase of linkage, if any, is not known in these parents before it has been detected. For reasons given in Cornish *et al.* (loc. cit.), though the

male and female linkage χ^2's are independent of the phase of linkage, the designation of the remaining pair of items in the analysis as the joint and heterogeneity χ^2's is arbitrary. Once linkage has been detected in a family, however, it is possible by an inspection of the data to deduce the phase of linkage in each parent and hence to decide which of these items provides a joint test of linkage and which is the heterogeneity item.

While there is no evidence of linkage between *S* and *Z* in the progeny of the cross between Trident 48/7 and Bb1276/28/2 (families *MA* and *MB*), there is clear evidence of such linkage in both families *MC* and *MD*. An inspection of the data from the latter pair of families suggested that the phase of linkage in their parents was:

Trident 48/7	S_1Z_1/S_2Z_2
Bn1276/28/2	S_3Z_4/S_4Z_3
RvP6/6	S_5Z_5/S_6Z_6
Bb1277/189	S_7Z_7/S_8Z_8

Since neither of the heterogeneity χ^2's of families *MC* or *MD* are significant, linkage appears to be homogeneous on the female and male side of each cross and the joint estimates of linkage are $\hat{p}_{MC} = 0{\cdot}2679 \pm 0{\cdot}0592$ and $\hat{p}_{MD} = 0{\cdot}2931 \pm 0{\cdot}0598$. Finally, since these estimates are similar, it is worth combining them to obtain an overall joint estimate of linkage in these families which is $\hat{p} = 0{\cdot}2807 \pm 0{\cdot}0595$ (heterogeneity $\chi^2_{(1)} = 0{\cdot}180$, $P = 0{\cdot}70 - 0{\cdot}50$, so that linkage is also homogeneous over families).

That we have been able to detect linkage between *S* and *Z* in families *MC* and *MD*, but not in the others (*MA* + *MB*), is puzzling, both because two of the parents of the former are also the parents of the latter (Trident 48/7 and Bb1276/28/2) and because, this family, when the data is pooled over reciprocal crosses, is twice the size of the others. Taken as a whole, however, these data suggest that while *S* and *Z* are carried on the same chromosome, their locations are sufficiently far apart for them to display linkage in some but not all crosses, the intensity of this linkage being susceptible to modification by the environment.

(iii) *The joint segregation of S and PGI-2*

Only one of the families, *MD*, segregated at the *PGI-2* locus, the cross being $dd \times bd$. We can, therefore, test for linkage between *S* and *PGI-2* in the male parent of this cross, Bb1277/189, whose incompatibility genotype we have just shown to be S_7Z_7/S_8Z_8. Since *S* and *Z* are interchangeable until linkage has been detected between one or other and *PGI-2*, it is necessary to test for linkage between the latter and each of the incompatibility genes in turn. The analysis is shown in table 4. There is very little doubt that *PGI-2* is linked to the incompatibility gene labelled *S* in this family. The phase of linkage in the male parent of this family is dS_7/bS_8 and the estimate of linkage, $\hat{p} = 0{\cdot}2414 \pm 0{\cdot}0795$.

(iv) *Seed-set*

The seed-set of the 14 self-pollinated plants ranged from 0 per cent (three plants) to 1·65 per cent, with an average of 0·39 per cent. Italian ryegrass is thus a highly, though not completely, self-incompatible species.

TABLE 4

(a) *The data from family MD arranged so as to allow tests of linkage between the PGI-2 alleles, b and d, and each of the two pairs of incompatibility alleles in turn.* (b) *Linkage χ^2's for each of these two comparisons*

(a)

$b:d$ with				
$S_7:S_8$	bS_7 4	dS_7 10	bS_8 12	dS_8 3
$Z_7:Z_8$	bZ_7 8	dZ_7 8	bZ_8 8	dZ_8 5

(b)

$b:d$ with	χ^2	P
$S_7:S_8$	7·759	0·01 – 0·001**
$Z_7:Z_8$	0·310	0·70 – 0·50

The seed-set of the five incompatible crosses, each of which was made in reciprocal, was significantly higher than that of the selfs with a mean of 3·77 per cent and a range of 0·57–17·37 per cent. This latter figure, though high, is probably genuine. However, even if this is omitted from the data, the difference between selfs and incompatible crosses is still highly significant, the mean seed-set of the remaining crosses being 2·26 per cent.

The analysis of variance of the seed-set of the twenty-two compatible crosses showed that these data were homogeneous over both families and categories of cross (*i.e.*, half, three-quarters and fully compatible). The mean seed-set of these crosses was 20·60 per cent and the range, 0·83–65·13 per cent. Though there is some overlap between the distribution of seed set of these crosses and that of the incompatible crosses (fig. 1), on average, the seed set of the former is, of course, significantly higher than that of the latter.

Lastly, the seed-set of the open pollinations ranged from 42·11 per cent to 80·00 per cent, with an average of 57·50 per cent. Again, though there is a considerable overlap between the distribution of seed-set of these open-pollinated crosses and that of the compatible crosses, the difference between their means is highly significant.

4. DISCUSSION

These results leave little doubt that self-incompatibility in *L. multiflorum* is, as in *L. perenne*, determined by a pair of multi-allelic loci; that control of the pollen phenotype is gametophytic; and that one of the incompatibility genes, defined as *S* is linked to a gene that codes for the enzyme phosphoglucoisomerase. Furthermore, while the intensity of this linkage in *L. multiflorum* appears, at $\hat{p} = 0{\cdot}2414 \pm 0{\cdot}0795$, to be less than in *L. perenne*, for which $\hat{p} = 0{\cdot}1583 \pm 0{\cdot}0252$, these estimates are, in fact, homogeneous ($\chi^2_{(1)} = 0{\cdot}993$, $P = 0{\cdot}50 - 0{\cdot}30$).

There are four further points worth making about these results. First, *S* and *Z* appear to be linked in their inheritance in *L. multiflorum* and hence to be carried on the same chromosome. However, some caution should be exercised about this conclusion. Thus no comparable evidence was obtained from an analysis of *L. perenne* crosses in which *S* and *Z* appeared to segregate independently (Cornish *et al.*, 1979*b*). On the other

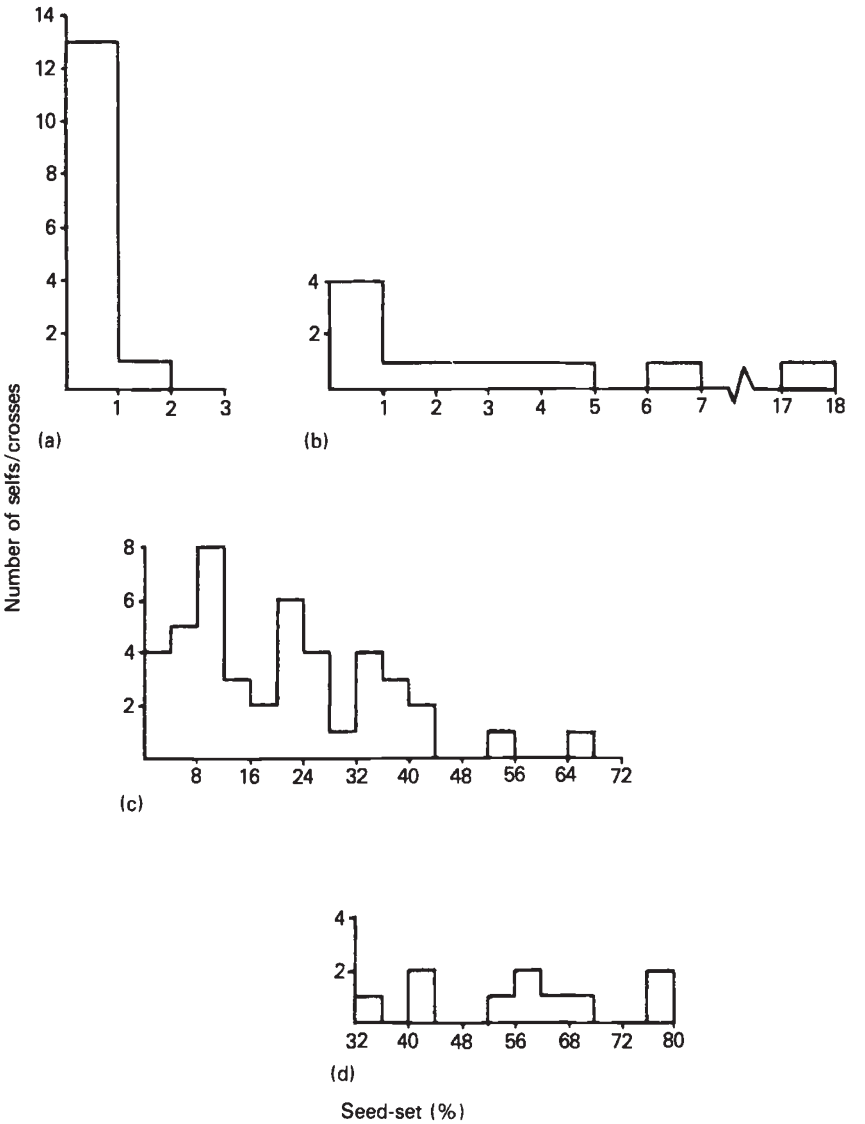

FIG. 1. The distribution of seed-set in (a) selfs; (b) incompatible crosses; (c) compatible crosses; and (d) open pollinations.

hand,, in one of the *L. perenne* crosses (families *H* and *I*) there was an indication that *S* and *Z* might be carried on the same chromosome, because both were linked to the *PGI*-2 gene, the former tightly ($\hat{p} = 0{\cdot}1114 \pm 0{\cdot}0289$) and the latter loosely ($\hat{p} = 0{\cdot}3645 \pm 0{\cdot}0552$). This suggests, of course, that the position of the *PGI*-2 locus is intermediate to that of the two incompatibility loci. But if this is the case, then we might expect to detect linkage between *PGI*-2 and *Z* in the *L. multiflorum* family *MD* in which linkage was detected between *S* and both *Z* and *PGI*-2 ($\hat{p} = 0{\cdot}2931 \pm 0{\cdot}0598$ and $\hat{p} = 0{\cdot}2414 \pm 0{\cdot}0795$ respectively); in fact, no evidence of

linkage between *Z* and *PGI-2* was found in this family. Taken as a whole, therefore, the data are not consistent in respect of the information they provide about the joint segregation of *S* and *Z*.

Part of our difficulty here arises from the fact that the size of the families examined are smaller than is desirable for the purpose of the detection and estimation of linkage. Although family size could be increased, an alternative approach would be to examine a number of primary trisomics, whose diploid and tetraploid parents are of known incompatibility genotype. If both *S* and *Z* are located on chromosome 6, double triallelics are expected to occur (Scarrott, 1981, after Lewis, Humphreys and Caton, 1980). An experiment with this objective in mind is in hand.

The second point worth making about these results concerns the seed-set data, a summary of which is shown in table 5, which also shows summaries

TABLE 5

Percentage seed-set in L. multiflorum and L. perenne. Subscripts show the number of plants on which each entry in the table is based

Category of cross	*L. multiflorum* (this paper)		*L. perenne* (Cornish *et al.*, 1980*b*)		*L. perenne* (Scarrott, 1981)	
Selfs	$0{\cdot}39_{(14)}$		$0{\cdot}26_{(56)}$		$0{\cdot}72_{(10)}$	
Incompatible crosses	$3.77_{(10)}$		$0{\cdot}36_{(56)}$		$0{\cdot}32_{(10)}$	
Half-compatible crosses	$24{\cdot}00_{(12)}$		$6{\cdot}68_{(14)}$		$18{\cdot}89_{(30)}$	
Three-quarters compatible crosses	$19{\cdot}00_{(10)}$	$20{\cdot}60_{(44)}$	$10{\cdot}33_{(18)}$	$9{\cdot}56_{(52)}$	$17{\cdot}13_{(32)}$	$18{\cdot}64_{(96)}$
Fully compatible crosses	$19{\cdot}47_{(22)}$		$10{\cdot}88_{(20)}$		$19{\cdot}84_{(34)}$	
Open pollinations	$57{\cdot}50_{(10)}$		—		$29{\cdot}38_{(15)}$	

of two experiments involving seed-set in *L. perenne* (Cornish *et al.*, 1980*b*; Scarrott, 1981). The results of the present experiment clearly show that the expected relationship between pollen-stigma reaction and seed-set holds in *L. multiflorum*, for compatible crosses yield, on average, much more seed than incompatible ones. These results confirm the early studies of Jenkin (1931) that *L. multiflorum* is as self-incompatible as *L. perenne*. In other respects, however, the results obtained from Italian ryegrass differ from those obtained from the experiments with perennial ryegrass. First *L. multiflorum* appears to be somewhat less cross-incompatible than *L. perenne*, though whether the difference between incompatible crosses and selfs in the former species is due to the circumstances of this particular experiment (an environmental effect) or is due to a less than perfect matching of *S* and *Z* combinations in crosses because of differences in the background genotype of pairs of plants (a genetical effect) is not clear at present. Second, there are no systematic differences between categories of compatible cross in *L. multiflorum*, whereas for the first experiment with *L. perenne*, these differences were very nearly significant and the percentage seed-set of the crosses was in the expected order (Cornish *et al.*, loc. cit.).

However, we note that the average seed-set of the compatible *L. multiflorum* crosses in the present experiment is more than twice that of compatible crosses of the first *L. perenne* experiment. In discussing the results from the latter experiment, we argued that differences between categories of compatible crosses could only appear when the amount of viable cross pollen that was delivered to, or able to function on, receptive stigmas was very small and that, accordingly, if the amount of such pollen could be increased, such differences would vanish. The results obtained from the *L. multiflorum* experiment support this argument, as indeed do those of the second *L. perenne* experiment, in which the average seed set of compatible crosses was also twice as high as that of the first experiment and in which differences between categories of cross were again no greater than that expected by chance alone. Lastly, the results obtained from open-pollinated heads confirm that conditions in the crossing bags are still far from ideal (see Foster, 1968 and Cornish *et al.*, 1980*b*).

The third point worth making about these results concerns the information they provide on the number of alleles at the *S* and *Z* loci. It will be recalled that each of the four parents of the families analysed were heterozygous for both *S* and *Z*. Hence between them they could contain as many as eight different alleles at each locus. However, because we have not examined progeny of the cross between RvP6/6 and Bb1277/189, it is possible that only six different alleles were present at each locus.

Suppose there are a total of k equally frequent *S* (or *Z*) alleles in the populations from which these parents were obtained. Then the probability of drawing a sample of n alleles, each of which is different, from a total of k is

$$P(n \text{ different alleles}) = \frac{(k-1)!}{(k-n)!\, k^{n-1}}.$$

If the two incompatibility loci are in linkage equilibrium, the joint probability of n different *S* and n different *Z* alleles is the square of the above expression. Values of this joint probability for $n = 6$ and $n = 8$ for various values of k are shown in table 6 from which it can be seen that we are unlikely to have obtained the observed result unless k is at least 40. Using a slightly different procedure, Cornish (1979) came to a similar conclusion with *L. perenne*.

TABLE 6

Probabilities of obtaining six and eight different S and Z alleles for the indicated number of alleles in the species

Total number of alleles	*P* (6 different *S* and *Z* alleles)	*P* (8 different *S* and *Z* alleles)
k		
10	0·0229	0·0000
20	0·1901	0·0394
30	0·3439	0·1294
40	0·4552	0·2239
50	0·5362	0·3071
60	0·5969	0·3773
70	0·6439	0·4360

Now these estimates are much higher than expected on theoretical grounds or on the basis of such empirical evidence as is available on this matter. First, it is intuitively obvious that the number of alleles required for a given level of cross-compatibility in a population of a species with a two-locus system is less than that required for a species with a one-locus system. Thus, whereas the number of different pollen phenotypes in the latter is equal to the number of alleles, the number of pollen phenotypes in the former is the product of the number of alleles present in the population at each locus. Second, Lundqvist (1962, 1964, 1969) found only eleven *S* and twelve *Z* alleles in a population of *Festuca pratensis* derived from the variety "Svalof Late"; and Larsen's (1978*b*) evidence from *Beta vulgaris*, which has a four-locus gametophytic system of self-incompatibility, indicates that the number of alleles at these loci could be quite small. On the other hand, it must be emphasised that these estimates from ryegrass are *species* estimates that are based on material of diverse genetic origin whereas that for *Festuca pratensis* is from a bred cultivar of restricted parentage; the number of alleles in a population is almost certainly less than this. Furthermore, the procedure we have used is clearly less satisfactory than a direct assay of the number of *S* and *Z* alleles in a population (Cornish, loc. cit.). Again, an experiment with this objective in mind is currently underway.

The fourth and final point worth making about these results is to consider why Hay's (1978) results and, hence conclusions, differ from ours. Hay investigated a single full-sib family of 26 plants of *L. multiflorum* and found that each plant was of a different incompatibility genotype; 18 per cent of cross-pollinations were reciprocally incompatible and 24 per cent were compatible one way, but incompatible the other way. She concluded that *L. multiflorum* has a complex self-incompatibility system that is controlled by at least three loci. Since Hay's procedure, results and conclusions are similar to those of Spoor (1976), who examined a single full-sib family of 28 plants of *L. perenne*, the criticisms we have made about the latter (Cornish *et al.*, 1979*a*) apply also to the former.

It is doubtful whether systems of the complexity proposed by Spoor (loc. cit.) and Hay (loc. cit.) can be resolved with only one family or in only one generation, for they require analytical procedures which allow the recognition of hierarchies of relationships of the kind used by Østerbye (1975, 1977) with *Ranunculus acris* and by Larsen (1977, 1978*a*) with *Beta vulgaris* (see also Lundqvist, Østerbye and Larsen, 1981). Until such procedures have been used with *L. perenne* and *L. multiflorum* and have shown beyond all reasonable doubt that these species possess a complex, multi-locus system, we prefer the more simple hypothesis that self-incompatibility in these species is determined by just two, multi-allelic loci and that claims to the contrary are based on data in which misclassification of pollinations cannot be ruled out.

Acknowledgements.—We wish to express our thanks to Mr N. J. McAdam of the Welsh Plant Breeding Station, Aberystwyth; to Mrs Danielle Edgerton and the garden staff of the Department of Genetics, University of Birmingham; and to Dr J. S. Gale for advice on statistical matters. One of us (C. H. Fearon née Scarrott) also wishes to acknowledge receipt of a Science Research Council CASE award which enabled the work described in this paper to be carried out.

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