Heredity (2002) 88, 385–390

www.nature.com/hdy

Self-incompatibility in ryegrass 12. Genotyping and mapping the *S* and *Z* loci of *Lolium perenne* L.

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Perennial ryegrass (*Lolium perenne* L.) is an outcrossing, wind-pollinated species exhibiting a gametophytic two-locus system of self-incompatibility (*S* and *Z*). The two incompatibility loci were genotyped in a cross between a doubled-haploid plant crossed as the female parent with a normal heterozygous plant. The *S* and *Z* loci were found to segregate in the expected 1:1 ratio and also segregated independently. The two loci were mapped to linkage groups one and two respectively, in accordance with the Triticeae consensus map. In addition, there were notable associations between the segregation of particular alleles mapping to the *S* locus region of linkage group 1 and those mapping to the WG889/CDO920 loci region of linkage group 3 which resulted in significant segregation distortions. No such associations were found between the *Z* locus and this region or any other region of the genome. The *L. perenne S* and *Z* loci showed conserved synteny with the equivalent loci in rye (*Secale cereale* L.).
Heredity (2002) **88,** 385–390. DOI: 10.1038/sj/hdy/6800071

Keywords: *Lolium perenne*; gametophytic self-incompatibility; linkage map; genetic markers; synteny

Introduction

Lolium perenne L. (perennial ryegrass) is an outcrossing, wind-pollinated species. Selfing is largely prevented by a gametophytic, two-locus incompatibility system (*SZ*) acting at the stigmatic surface (Cornish *et al*, 1979). The system was first elucidated in rye (*Secale cereale* L.) (Lundqvist, 1954). All eight grass species in which the incompatibility system has been examined in detail (see Baumann *et al*, 2000) exhibit the same two locus system in which the two loci are complementary in action. Both *S* and *Z* alleles must be matched in pollen and style for an incompatible reaction to occur. Both *S* and *Z* loci are highly polyallelic as shown by estimates of allele numbers in a synthetic cultivar (Devey *et al*, 1994) and a native population (Fearon *et al*, 1994).

The *S* and *Z* loci have been mapped in rye (Wricke and Wehling, 1985, Gertz and Wricke, 1989; Fuong *et al*, 1993; Phillip *et al*, 1994; Voylokov *et al*, 1994, 1998). Li *et al* (1994) reported the cloning of a putative *S* gene with a thioredoxin like sequence in the grass species, *Phalaris coerulescens*. This finding was later revised as being a gene linked to *S*, mapping about 2 cM away (Langridge *et al*, 1999). For *L. perenne* the only known linkages are between the *S* locus and the isozyme locus PGI/2 (Cornish *et al*, 1980) and there is also evidence that the *Z* locus is linked to the isozyme locus GOT/3 (Thorogood and Hayward, 1992).

This paper reports the genotyping, based on the functioning of a two locus, gametopytic incompatibility system, and subsequent mapping of the incompatibility loci with a family of 139 plants extensively mapped with RFLP and isozyme markers (Jones *et al*, 2002).

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Received 20 August 2001; accepted 15 January 2002

Materials and methods

Plant material

The *L. perenne* mapping family P150/112 is the core mapping family for the International *Lolium* Genome Initiative (ILGI). It was formed from an anther culture-derived doubled haploid plant (female) crossed with an unrelated heterozygous plant donated from Dr Pete Wilkins' forage breeding programme (IGER) (Jones *et al*, 2002). One hundred and thirty-nine individuals from this family were genotyped for *S* and *Z*. The incompatibility genotype of the female parent was designated $S_{11}Z_{11}$. As the male parent was unrelated, and knowing that the *S* and *Z* loci are highly polyallelic (Devey *et al*, 1994; Fearon *et al*, 1994), it was assumed that it was heterozygous at both loci for two further alleles and was assigned the genotype $S_{23}Z_{23}$.

Plant pollination and incompatibility genotyping strategy

According to the two locus model of self-incompatibility, the F_1 family derived from this cross segregates into a maximum of four possible incompatibility genotypes ($S_{12}Z_{12}$, $S_{12}Z_{13}$, $S_{13}Z_{12}$, $S_{13}Z_{13}$). Inter-pollination of these genotypes results in the incompatibility reactions shown in Table 1. There are no reciprocal differences in reaction type. There are also no fully compatible reactions as all plants produce 25% S_1Z_1 gametes that never effect successful pollination. At first genotypes needed to be identified that could be used to pollinate each plant of the mapping family. In theory, two pollinator genotypes crossed to the complete set of plants in the mapping

Table 1 Compatibility reactions obtained when inter-crossing the four *SZ* genotypes expected in the F_1 population

	Male parent			
	$S_{12}Z_{12}$	$S_{12}Z_{13}$	$S_{13}Z_{12}$	$S_{13}Z_{13}$
Female parent				
$S_{12}Z_{12}$	–	H	H	T
$S_{12}Z_{13}$	H	–	T	H
$S_{13}Z_{12}$	H	T	–	H
$S_{13}Z_{13}$	T	H	H	–

– = fully incompatible pollination; H = 50% compatible pollination; T = 75% compatible pollination.

family could provide enough information to assign genotypes to every plant in four out of the six possible pairwise combinations of pollinator genotypes. In the two cases where pollinator genotypes have no *S* or *Z* allele pairs in common (ie, $S_{12}Z_{12}$ and $S_{13}Z_{13}$ or $S_{12}Z_{13}$ and $S_{13}Z_{12}$), it would be impossible to distinguish between the two stylar genotypes, which are not the same as the pollinator genotypes (ie, $S_{12}Z_{13}$ and $S_{13}Z_{12}$ or $S_{12}Z_{12}$ and $Z_{13}Z_{13}$) as both would produce a 50%-compatible reaction with both pollinators. If three different genotypes are used as pollinators it is possible to determine which of the four stylar genotypes each of the plants possessed: if the stylar genotype was the same as one of the pollinators an incompatible reaction would occur and the other two reactions would be partially compatible. If all pollinators give a partial reaction with the stylar plant, that plant must belong to the genotype not present in the set of pollinators. It is, however, much more preferable to have at least one representative of each of the four genotypes to use as pollinators. We are then certain to obtain a fully incompatible reaction where the pollinator and stylar parent have the same *SZ* genotype. The incompatible reaction is then confirmed by the 75% compatible reaction of the same stylar plant with the pollinator genotype with no allele pairs in common, and the 50% compatible reaction with the two genotypes that have either the *S* or the *Z* allele pair in common.

In our pollination scheme we initially chose six plants at random which we crossed in diallel fashion. Choosing six plants at random gave us a 96.9% chance of obtaining at least two pollinators with appropriate genotypes that would enable us to classify all stylar plants pollinated with these two plants (ie, two pollinator genotypes with either *S* or *Z* allele pairs in common). Further informative pollinations were determined by the results of this initial diallel.

Pollination methods

Plants were grown in 15-cm diameter pots in Humax John Innes No 3 with wetting agent. Plants were vernalised (short days, low temperature) naturally in an unheated, unlit glasshouse over winter in readiness for pollinations to be made the following spring. Pollination methods were adapted from those of Cornish (1979). Plants to be used as pollinators were chosen, which possessed inflorescences that were about to anthese or had been anthesing for a few days. Plants were still capable of producing pollen 2 or more weeks after the first inflorescences had started anthesis. These plants were placed in a growth room running at 23°C, with a light level at plant height of approximately 300 μmol/m^2/s, on their sides with flowering stems enclosed in a cellophane bag. The plants anthesed around midday. Mature ovaries with stigmas attached were collected from basal florets of spikelets that were close to anthesis. Spikelets were removed from the plants and ovaries were harvested at some distance from flowering plants to avoid contamination from stray pollen. Ovaries were placed on 50-mm diameter petri-dishes plated with agar (2.5% Agar agar, 25% sucrose and 25 ppm Boric acid) which provided a semi-solid medium on which the ovaries could be supported allowing the feathery stigmas to stand proud of the Agar surface in readiness for pollen reception (Lundqvist, 1961). Two ovaries from each of up to 16 stylar plants were placed on a single petri dish. Pollen, which collected in the cellophane bags, was shaken onto the surface of the agar, ensuring that only free-flowing, non-clumped pollen was used. Anthers in the bag were removed prior to pollination to ensure smooth pollen flow onto the plates.

A minimum of 16 h post-pollination, styles and stigmas were removed from the ovaries with a scalpel on microscope slides. Stigmas were temporarily mounted under a coverslip in a drop of decolourised Aniline Blue (0.1% water-soluble aniline blue in 0.1 M K_3PO_4) (Martin, 1959) in readiness for microscope examination. Pollen-tube growth was observed microscopically at low power (×10) under UV fluorescent light. Incompatible grains produced pollen tubes that were soon arrested at or near the stigma surface and became occluded with callose that fluoresced brightly under UV light. There was also considerable build-up of callose within the grain itself. Compatible grains, on the other hand, were able to germinate successfully, tubes often reaching the base of the style. Sometimes these tubes fluoresced brightly but at other times were relatively difficult to see.

Pollinations were scored by estimating the proportions of compatible and incompatible grains. The fully incompatible pollinations were easiest to score. Mostly it was possible to distinguish 50% and 75% compatible reactions but at other times it was only possible to ascribe a partially compatible reaction where the number of grains was small or large numbers of inviable non-fluorescent, non-germinating grains obscured observations of many of the viable grains. Such problems with scoring were also encountered by Cornish (1979).

Molecular marker generation and linkage analysis

RFLP and isozyme segregation data were collected on the mapping family as described by Jones *et al* (2002). The *S* and *Z* loci were assigned to linkage groups using Joinmap™ v. 2.0. (Stam and Van Ooijen, 1995) using the linkage group numbering system established by Jones *et al* (2002).

Results

Incompatibility genotyping

Pollinations between our six randomly selected plants were classified as in Table 2. Plant 31 was arbitrarily assigned the genotype $S_{12}Z_{12}$. From the table it can be seen that plants 169 and 181 gave the same pollination reactions and the three plants are intra-incompatible.

Table 2 6 × 6 diallel of pollinations of six randomly selected plants

Plant no.	*Male parent*					
	31	59	63	69	169	181
Female parent						
31	–	T	H	H	–	–
59	T	–	H	H	T	T
63	H	H	–	–	H	H
69	H	H	–	–	H	H
169	–	T	H	H	–	–
181	–	T	H	H	–	–

– = Fully incompatible pollination: H = 50% compatible pollination; T = 75% compatible pollination.

Therefore these two plants must have had the same genotype as plant 31. Plant 59 had a unique pollination phenotype and it was 75% compatible with the three $S_{12}Z_{12}$ genotypes. This plant must therefore have had the genotype $S_{13}Z_{13}$. Plants 63 and 69 had the same reactions as each other and are intra-incompatible. They therefore must have had the same genotype as each other. They gave a 50% compatible reaction with both $S_{12}Z_{12}$ and $S_{13}Z_{13}$ genotypes and could therefore be assigned to either of the remaining genotypes. We assigned plants 63 and 69 arbitrarily to genotype $S_{13}Z_{12}$. The fourth genotype segregating in the family, $S_{12}Z_{13}$, was not found in this round of pollinations.

A second round of pollinations was made using the six pollinators genotyped above as pollen parents and a further 48 plants as stylar parents. The reactions obtained were of two types: either only one pollen genotype giving an incompatible reaction and the other two genotypes giving either a 50% or 75% compatible reaction: or all pollen genotypes giving partially compatible reactions, in accordance with expectations given in Table 1. Those plants, which gave partial reactions with all three genotypes so far ascribed, must belong to the remaining genotype, $S_{12}Z_{13}$.

A total of 54 plants had been genotyped: 11 $S_{12}Z_{12}$, 14 $S_{13}Z_{12}$, 13 $S_{12}Z_{13}$ and 16 $S_{13}Z_{13}$ genotypes were ascribed. Ultimately, all 139 plants in the family that were analysed for incompatibility reaction were pollinated with at least two plants of each of the four genotypes. It was possible to unequivocally assign an incompatibility genotype to each plant in the mapping family resulting in the genotyping of all 139 plants.

The number of plants of each *S* and *Z* genotype is given in Table 3. Segregation of the four genotypes was not significantly different from a 1:1:1:1 ratio ($\chi^2 = 0.34$, $P_{[3df]} > 0.95$).

Table 3 Segregation of *S* and *Z* alleles for 139 plants of the mapping family

		S		
		12	**13**	
Z	**12**	34	36	70
	13	35	34	69
		69	70	

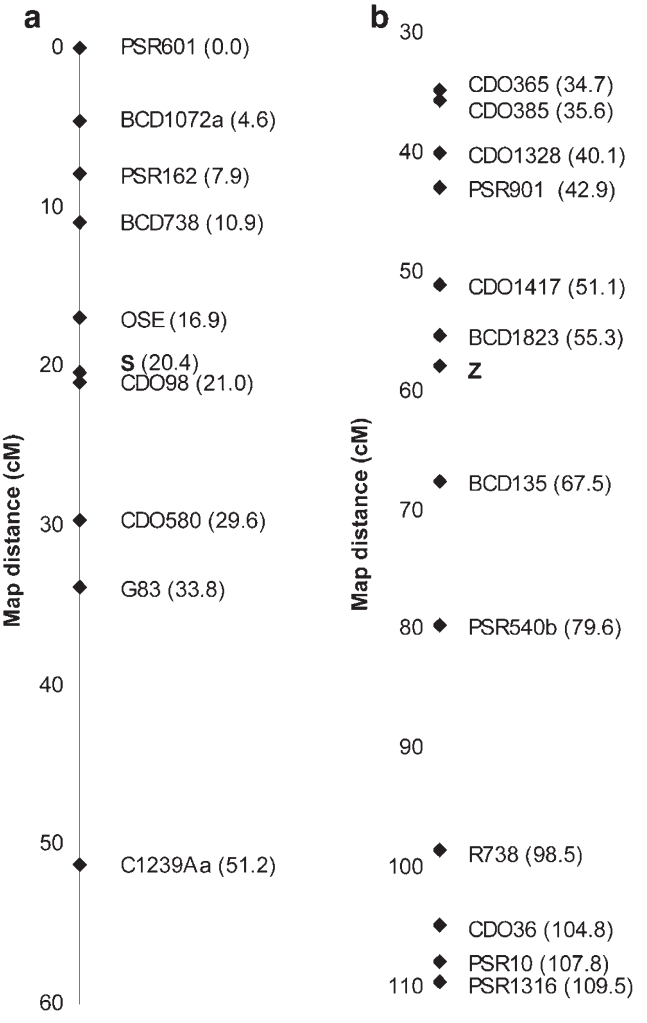

Figure 1 Map positions of the *S* and *Z* loci and closely linked markers. (**a**) Linkage group 1, (**b**) Linkage group 2. Complete marker maps for these linkage groups are given by Jones *et al* (2002).

Mapping the *S* and *Z* loci

The *Z* locus was assigned to linkage group 2 using a LOD threshold of 5 and mapped as indicated in Figure 1. A similar LOD threshold assigned the *S* locus to linkage groups 1 or 3, but failed to split group1 from group 3. The *S* locus could be associated only with linkage group 1 using a LOD threshold >13; it was subsequently mapped as indicated in Figure 1. The 'linkage' between these two groups was associated with significantly distorted segregation ratios for the majority of the markers on linkage group 3 (Jones *et al*, 2002) and LOD scores as high as 13.2 associating markers in the region of the final map position of the *S* locus on linkage group 1 and some of the markers on linkage group 3 (see Table 4). A more detailed analysis of the *S* and *CDO920* allele frequencies (Table 5) revealed that the distorted segregation ratio of *CDO920* alleles ($\chi^2 = 14.7$, $P_{[2df]} < 0.001$) could be accounted for by the excessive number of plants with the

Table 4 LOD scores for the two-point comparison of each marker on linkage group 1 (L1) with each marker on linkage group 3 (L3). LOD scores ≥10 are indicated in bold. The relative genetic distances (cM) of the markers on L1 and L3 and the number of genotypes scored (*n*) for each marker are indicated

			L3													
			WG110	*CD0345*	*GOT/3*	*BCD828*	*WG889*	*CD0920*	*CDO328a*	*CDO244*	*PSR370*	*PSR394*	*R1613*	*CDO328b*	*CDO460*	*C390*
		cM	0.0	7.6	13.9	20.8	20.8	22.9	25.4	27.0	28.4	30.9	32.2	37.7	45.6	64.9
L1	cM	*n*	86	138	151	77	141	156	82	86	128	85	109	93	79	105
PSR601	0.0	118	2.1	1.0	2.4	2.4	4.8	4.8	5.6	3.0	2.9	4.6	3.7	3.2	0.9	0.4
BCD1072a	4.6	73	0.7	0.0	1.4	2.6	3.4	3.2	3.4	3.5	4.2	4.2	1.6	3.4	0.8	0.0
PSR162	7.9	87	1.6	0.2	2.0	2.5	4.1	4.4	5.3	3.7	3.9	5.5	3.4	2.6	0.6	0.4
BCD738	10.9	97	2.3	1.3	2.5	3.0	5.7	5.1	6.9	4.6	4.9	3.9	2.8	3.7	1.6	0.6
OSE	16.9	93	2.2	7.3	8.1	6.4	9.6	**10.2**	4.0	1.8	5.4	1.6	5.8	1.4	1.3	0.7
S	20.4	139	2.5	5.2	7.0	6.9	**13.2**	**12.3**	6.9	4.2	8.1	4.5	5.0	4.5	0.6	1.0
CDO98	21.0	139	1.5	4.2	6.1	6.2	**10.0**	**12.2**	5.2	2.8	5.9	4.5	4.8	3.5	0.6	0.9
CDO580	29.6	144	1.6	4.2	6.2	5.3	8.9	**10.2**	5.6	2.4	7.3	3.5	5.9	4.4	0.1	0.3
G83	33.8	146	2.3	6.6	7.4	5.6	8.7	**10.0**	3.5	1.2	5.0	2.8	6.2	2.4	0.1	0.7
C1239Aa	51.2	103	0.3	1.1	1.7	0.8	1.3	1.9	1.0	0.8	2.4	1.5	1.8	0.7	0.3	0.1

Table 5 Numbers of individuals recovered for the four genotype classes associated with the segregation of the *S* alleles (*2* and *3*) on linkage group 1 and the *CDO920* alleles (*a* and *b*) on linkage group 3

		S	
		2	3
CDO 920	*a*	58	23
	b	3	36

S_2/*CDO920a* alleles and the low number of plants with the S_2/*CDO920b* alleles which resulted in a significant deviation from the expected 1:1 ratio ($\chi^2 = 49.6$, $P_{[2df]} < 0.001$). The segregation ratio for plants with the S_3/*CDO920a* and S_3/*CDO920b* allele combinations did not depart significantly from a 1:1 ($\chi^2 = 2.9$, $P_{[2df]} > 0.05$).

The *Z* locus had no such association with linkage group 3 or any other linkage group.

Discussion

The results from the pollinations in this mapping family confirm the two-locus control of self-incompatibility. Disturbance of expected *S* and *Z* allele ratios and linked markers has been suggested in *Lolium* (Hayward *et al*, 1998) and in rye (Philipp *et al*, 1994) based on the hypothesis that some *S* or *Z* alleles, or *SZ* allele combinations, are less effective than others at preventing self-pollination. However, in the present study, both male and female gametes that form progeny from the original fully compatible cross are equally likely to contain either of the two segregating *S* and *Z* alleles. There is no evidence in this family that any *S* or *Z* incompatibility allele is more or less likely to effect pollination. The most likely cause of segregation distortion of *S*, *Z*, or linked markers would be in crosses resulting in partially compatible pollination where some *SZ* allele combinations produce an incompatibility reaction and are excluded from effecting pollination; or in progeny of selfed plants which are heterozygous for a self-fertility mutation at one of the incompatibility loci or other loci as demonstrated by Thorogood (1991) in *Lolium* and Voylokov *et al*, (1994) in rye.

The position of *S* on linkage group 1 is endorsed by the observation of Cornish *et al* (1980) who found that *S* and *PGI/2* are linked with a recombination frequency of 15.8%. Although *PGI/2* did not segregate in the ILGI mapping family, a F_2 *L. perenne* mapping family positions *PGI/2* on linkage group 1 (Armstead, Turner and Humphreys, personal communication). In addition, the map positions of *S* and four common RFLP markers on linkage group 1 in *L. perenne* and rye indicate a high degree of conserved synteny for this region of the genome between the two species (Voylokov *et al*, 1998; Korzun *et al*, 2001) (Figure 2). The positions of markers flanking the *S* locus are not entirely co-linear but this may be as a result of sampling effects, rather than genuine chromosomal re-arrangements. The RFLP marker,

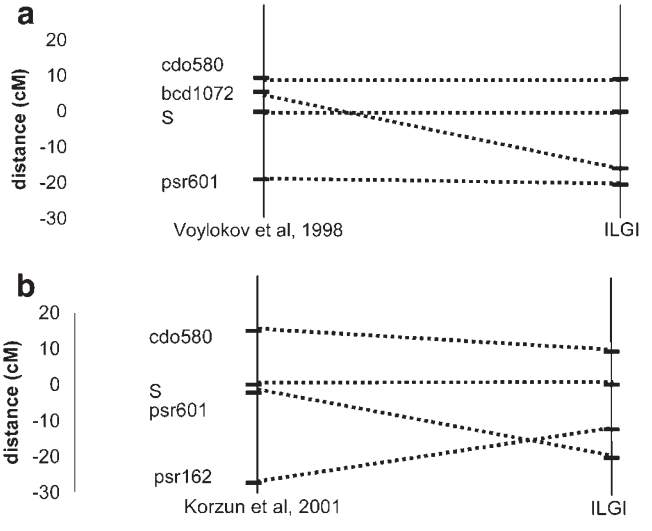

Figure 2 Syntenic relationships between rye and ryegrass. (**a**) Voylokov *et al*, (1998) *vs* ILGI population (**b**) Korzun *et al*, (2001) *vs* ILGI population.

CDO98, mapped within 0.6 cM of the *S* locus and is probably closer than the *Bm2* gene isolated by Langridge *et al* (1999) which was estimated to lie up to 2-cM away from the *S* locus.

Markers from the linkage groups of the mapping family used in this study have enabled alignment of the *Lolium* map with those of other members of the *Poaceae* family including rye and members of the *Triticeae* tribe (Jones *et al*, 2002). The *Z* locus was found to be on the linkage group 2 coinciding with the location of *Z* in rye (Fuong *et al*, 1993). This again indicates conserved synteny between the species though none of the markers in the ILGI map were used in the construction of the rye map.

The association of the *S* locus with some of the markers on linkage group 3 (Table 4) indicates that there is likely to be a gene(s) on linkage group 3 that interacts with the *S* locus (or a gene tightly linked to it). The result of this interaction is that pollen or plants formed after pollination which possess the S_2 allele and allele(s) from one of the linkage phases on group 3 are virtually inviable, whereas pollen or plants possessing the S_2 allele and allele(s) from the other linkage phase on group 3 are super-viable (ie, more progeny with these genotypes are recovered than expected). The inviable/super-viable balance results in the restoration of the expected 1:1 ratio for the segregation of the *S* alleles. The action of this locus had no effect on the classification of incompatibility genotypes according to the classic two locus, gametophytic incompatibility model and therefore must act downstream from the initial pollen-stigma surface interaction. Whether it acts gametophytically or effects viability of post fertilisation products is unknown. The isozyme locus, *GOT/3*, is positioned on linkage group 3 (see Table 4) and a previous study (Thorogood and Hayward, 1992) found an association between a self-fertility locus segregating in *L. perenne*/*L. multiflorum* × *L. temulentum* first generation back-crosses to *L. perenne*/*L. multiflorum* and the *GOT/3* locus with a recombination frequency of 18%. Furthermore, progeny of selfed plants heterozygous for self-fertility (50% self-compatible) were found to reveal significantly skewed ratios at the *GOT/3* locus (Thorogood, 1991). Controlled pollinations confirmed that the self-fertility locus was either *S* or *Z* (or a locus closely linked to either of these loci (Thorogood and Hayward, 1992). As it was already known that *S* was linked to the isozyme locus *PGI/2* (Cornish *et al*, 1979), and as *PGI/2* and *GOT/3* were also known to segregate independently (Hayward and McAdam, unpublished) it was concluded that the self-fertility trait from *L. temulentum* was likely to be a mutation of the *Z* locus linked to *GOT/3*. In the current paper, *GOT/3* maps to linkage group 3 and therefore is not linked to either *S* or *Z*. The significant co-segregation between self-fertility and *GOT/3*, and the skewed ratios of *GOT/3* allele segregations observed on selfing plants that were heterozygous for self-fertility (Thorogood, 1991) was probably due to interaction between the *S* locus and the locus identified on linkage group 3 in the present study. However, the action of this locus on *S* or *Z* is in contrast to other loci, unlinked to *S* and *Z*, identified in *L. perenne* (Thorogood and Hayward, 1991), *Phalaris coerulescens* (Hayman and Richter, 1992) and rye (Voylokov *et al*, 1993) which in all cases override the *S* and *Z* incompatibility response to produce self-fertile plants. In rye, a self-fertility locus has been mapped to chromosome 5R (Fuong *et al*, 1993).

Commercial turf and forage ryegrass cultivars are produced, exploiting the self-incompatibility system, as synthetics made from polycrossing a number of selected mother plants. The production of synthetics is an effective way of exploiting heterosis yet, inevitably, selection for important traits can lead to homozygosity of regions of the genome and homogeneity of these regions across the breeding population. Clearly, if selection for traits that are linked to *S* and *Z* occurs, seed production ability of these populations could be seriously compromised.

Genome mapping in *L. perenne* is well enough advanced (Jones *et al*, 2002; Armstead, Turner and Humphreys, personal communication) to be used as a tool for identifying and mapping traits of interest to forage and turfgrass breeders.

Quantitative trait loci (QTL) have been identified for forage quality traits such as water-soluble carbohydrate (Turner *et al*, 2000), crown rust (*Puccinia coronata* Corda) resistance (Thorogood *et al*, 2001) and delayed leaf senescence (Thorogood *et al*, 1999). Molecular markers associated with QTL can be used to aid selection for such complex, difficult to measure traits. Identifying the map positions of *S* and *Z* now means that marker-assisted selection strategies can be devised to select for favourable combinations of incompatibility alleles and linked QTL. Ultimately, the aim will be to produce allele specific probes for both *S* and *Z* loci in order to monitor and maintain heterogeneity at these loci in any future marker-assisted selection programmes.

Acknowledgements

Danny Thorogood is funded by Germinal Holdings Ltd, and William Kaiser received a Nuffield Foundation Undergraduate Student Bursary (NUFF-URB01) enabling the incompatibility genotyping to be done. The authors acknowledge the contribution of the International Lolium Genome Initiative (ILGI) partners at IGER, Aberystwyth, UK, AV-PBC, Melbourne, Australia, YPDES/HNAES, Sapporo, Japan, and INRA, Clermont Ferrand, France, who produced the molecular marker data for this mapping family.

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