Theor Appl Genet (1994) 87:641–649

THEORETICAL AND APPLIED GENETICS

Isozyme variation and species relationships in the genus *Lolium* L. (ryegrasses, Graminaceae)

G. Charmet, F. Balfourier

INRA, Station d'Amélioration des Plantes, 63039 Clermont-Ferrand Cedex, France

Received: 10 April 1993 / Accepted: 28 June 1993

Abstract. Thirty-two natural populations belonging to the eight species of the genus *Lolium* (ryegrass) or to *Festuca pratensis* (meadow fescue) were recorded for allelic frequencies at 13 isozyme loci. Cultivated ryegrass (*L. perenne* and *L. multiflorum*), meadow fescue, and the annual *L. rigidum*, are true outbreeders. The other species are true inbreeders, except for *L. canariense*, which shows a moderate level of cross fertilisation (20%). Hierarchical clustering from Nei's unbiased distance leads to four groups. The three self-pollinating, weed species, *L. temulentum*, *L. remotum* and *L. persicum*, belong to the first cluster, which is the most differentiated one. The second cluster comprises *L. multiflorum*, *L. subulatum* and most populations of *L. rigidum*. All *L. perenne* populations belong to the third cluster, as do two of *L. rigidum*. The average genetic distance within the *L. perenne* group is very low. Surprisingly, the fourth cluster groups together *L. canariense* and *Festuca pratensis*. The data suggest that *L. rigidum* is the species with the greatest diversity, and could be a common ancestor of the genus. Knowledge of historical processes of domestication could help to calibrate the molecular clock.

Key words: Taxonomy – Genetic distance – Population genetics – Forage grasses – Isozymes

Introduction

The genus *Lolium* is one of the most important groupings of temperate forage grasses, including the widely cultivated Italian (*L. multiflorum*) and perennial ryegrass (*L. perenne*). Annual *L. rigidum* is also grown under a Mediterranean-type climate, mostly in Australia, for winter grazing. These three species are self-incompatible outbreeders (Cornish et al. 1980; Fearon et al. 1983).

Better knowledge of the genetic relationships between *Lolium* species is required for a more efficient use of genetic resources through intra- and inter-specific hybridization.

Terrell (1968) recognized eight species within the genus *Lolium*: the three outbreeders mentioned above, and five species thought to be self pollinating, namely *L. temulentum*, *L. remotum*, *L. subulatum*, *L. persicum* and *L. canariense*. In his reference paper, Terrell reported many of the synonyms used in the past. Kloot (1983) has subsequently commented on the correspondence between the most popular synonyms, and also indicated the doubtfully-distinct species.

First, it should be noted that the three outbreeding species are all interfertile, providing that flowering dates are compatible. Consequently Naylor (1960) proposed that they should be grouped under a common species name and be regarded as subspecies. These species have a wide distribution area and show a high level of morphological as well as adaptive (= ecotypic) variation.

The outbred *Lolium* species are closely related to members of the genus *Festuca* of the section "*Bovinae*", and they hybridize fairly easily with them. In particular, hybrids between *L. perenne* and *Festuca pratense* have been reported to occur in nature.

In contrast, the inbreeding species generally have a more restricted distribution and/or adaptation. *L. temulentum* and *L. remotum* are known only as weeds of crops, the former in cereals, the latter in flax. As a

Communicated by P. M. A. Tigerstedt

Correspondence to: G. Charmet

consequence of the increasing chemical control of weeds, these two species are progressively disappearing. Since they could be used as sources of self compatibility genes to transfer to outbreeders, their conservation in gene banks must be encouraged (Thorogood and Hayward 1992).

As reported by Terrell (1968), very little data exist about natural populations in the Mediterranean and Southwest Asian regions: *L. persicum* is located in the Middle East countries, *L. subulatum* in the eastern Mediterranean area while *L. canariense* is restricted to North Atlantic Islands (Madeira, Canary and Cape Verde). The relationships between the inbreeding species are not clearly established. They hybridize with difficulty with each other, as well as with the outbreeders, frequently giving sterile hybrids (Jenkin 1954; Terrell 1966).

The aim of the present study was to investigate the relationships between representatives of the eight *Lolium* species and of *F. pratensis* by means of starch-gel electrophoresis of 11 isozymes systems. Particular attention has been paid to the exotic *L. canariense* recently collected in the Canaries. (Charmet et al. 1993).

Materials and methods

The origins of the 32 populations of the nine species studied (11 *L. perenne*, three *L. multiflorum*, seven *L. rigidum*, three *L. canariense*, two *L. persicum*, one *L. subulatum*, two *L. temulentum*, one *L. remotum* and two *Festuca pratensis*) are presented in Table 1. On average 75 plants were studied for the outbreeding species, and 30 for the inbreeders which show less within-population diversity.

Eleven enzyme systems were assayed giving 13 polymorphic loci. Staining recipes were adapted from Hayward and McAdam (1977) for PGI, ACP, GOT and SOD, from Pollans and Allard (1985) for PRX, from Greneche et al. (1991) for IDH, SDH and MDH, and from Ostergaard et al. (1985) for PGM and PGD. Diaphorase (synonym lipoamid dehydrogenase) is revealed in 8 mg indophenol, 24 mg NADH and 15 mg MTT in 100 ml. 0.1 *M* Tris HCl, pH 8, buffer (McAdam, personal communication).

Allele nomenclature is that of Hayward and McAdam (1977). Initially alleles were designated in alphabetic order, the faster migrating being *a*. When a new allele was discovered, it became a^+, b^+, c^* as summarized in Table 2.

The BIOSYS 1 programme (Swofford and Selander 1981) was used to compute the following population genetics statistics from genotypic and allelic frequencies: mean number of alleles, average heterozygosity H, within-population fixation index F_{is} for each population, and the standard unbiased genetic distance of Nei (1978) between every pair of populations.

For the self compatible species, the average outcrossing rate was estimated as $t = 1 - F_{is}/1 + F_{is}$ (Brown 1979).

A dendogram was constructed from Nei's distance using the classical UPGMA (unweighted pair group method of aggregation) for clustering.

Assuming the absence of selection (neutral theory of Kimura and Crow 1964) the time of divergence (t) from the last common ancestor is related to Nei's distance (D) by $D = 2\alpha t - \mathrm{Log} I_0$. (Nei 1972) where α is the average mutation rate and I_0 the identity just after the divergence event.

Except with a very strong founder effect, the value of I_0 would be close to one in most cases, thus t could be approximated by $D/2\alpha$.

The main difficulty is to obtain accurate estimates of α. In similar studies of plant speciation, values of α range from 10^{-7} to 5×10^{-6}. Such values need to be related, whenever possible, with archeological or historical data which could suggest realistic dates of colonization and divergence events.

Table 1. Summary data of the 32 *Lolium* accessions

Species	Code	Country of origin
L. perenne	DE 13	Germany
	DE 27	Germany
	AU 03	Austria
	AU 04	Austria
	CSK 1	Czechoslovakia
	CSK 5	Czechoslovakia
	HUN 1	Hungary
	GRE 1	Greece
	GRE 3	Greece
	SP 32	Galicia, Spain
	SP 37	Galicia, Spain
L. multiflorum	DE 28	Germany
	PO 18	Portugal
	IT 32	Italy
L. rigidum	SP 312	Andalusia, Spain
	SP 313	Andalusia, Spain
	TN 17	Tenerife, Spain
	IR 01	Iran
	ISR 2	Israël
	GRE 5	Greece
	FR 57	Corsica, France
L. temulentum	TEM 6	Portugal
	TEM 7	Iran
L. remotum	REM 1	UK?
L. persicum	PER 1	Hungary? (Botanical Garden)
	PER 2	Iran
L. subulatum	SUB 1	Peloponese, Greece
L. canariense	CA 01	Tenerife, Spain
	CA 02	Tenerife, Spain
	CA 14	Gomera, Spain
Festuca pratensis	FEP 1	England, UK
	FEP 2	Romania

Results

Since this paper focuses on between-species differences, only species ranges of allelic frequencies are presented in Table 2. Complete data are available on request.

Phosphoglucose isomerase (PGI). Only the slower migrating locus, *Pgi2*, can be recorded consistently for PGI. In outbreeding species, it is the locus which presents the highest number of alleles: up to seven. Most alleles can be found in the three outbreeders and in

Table 2. Species range of allele frequencies

Locus		Species								
		L. per.	*L. multi.*	*L. rigid.*	*L. tem.*	*L. rem.*	*L. pers.*	*L. subul.*	*L. canar.*	*F. prat.*
Pgi-2	a^+	0.00–0.01	0.00–0.01	0.00–0.03	0	0	0	0	0	0
	a	0.14–0.58	0.00–0.05	0.00–0.033	1.00	1.00	0	0	0–0.66	0.06–0.12
	b	0.42–0.71	0.06–0.25	0.07–0.68	0	0	0	0	0.53–1	0.48–0.78
	*b**	0	0	0	0	0	0–1.00	0	0	0
	c	0.00–0.07	0.00–0.27	0.00–0.12	0	0	0–1.00	0	0–0.24	0.09–0.28
	*c**	0.00–0.20	0.00–0.01		0	0	0	0	0	0.02–0.06
	d	0.00–0.10	0.46–0.87	0.22–0.90	0	0	0	1.00	0	0.00–0.07
	e	0.00–0.11	0.00–0.05	0.00–0.10	0	0	0	0	0	0.00–0.03
Acp 1	*a*	0.00–0.02	0.00–0.04	0.00–0.09	0	0	0	0	0.98–1	0.00–0.02
	b	0.00–0.16	0.17–0.24	0.04–0.35	0	0	0	1.00	0–0.02	0.77–0.94
	c	0.20–0.71	0.48–0.69	0.35–0.74	0	0	0–100	0	0	0.06–0.22
	d	0.19–0.77	0.07–0.25	0.09–0.60	1.00	1.00	0–1.00	0	0	0
Acp 2	*a*	0.10–0.77	0.00–0.18	0.02–0.34	0	0	0	0	0.98–1	0
	b	0.14–0.62	0.56–0.67	0.50–0.70	1.00	0.90	1.00	1.00	0–0.02	0
	c	0.01–0.22	0.02–0.38	0.05–0.26	0	0	0	0	0	0.00–0.92
	d	0.00–0.20	0.06–0.12	0.00–0.22	0	0.10	0	0		0.08–0.50
	e		0	0.00–0.02	0	0	0	0	0	0.00–0.50
Got 1	*a*	0.00–0.04	0	0	1.00	1.00	0	0	0	0
	b	0.94–1.00	0.98–1.00	0.91–1.00	0	0	1.00	1.00	1.00	0.97–1.00
	c	0.00–0.06	0.00–0.02	0.00–0.09	0	0	0	0	0	0.00–0.03
Got 2	a^+	0	0.16–0.40	0.06–0.91	0	0	0	0	0	0.00–0.05
	a	0.10–0.55	0.30–0.40	0.09–0.85	1.00	1.00	1.00	1.00	1.00	0.77–0.88
	b	0.44–0.90	0.30–0.52	0.00–0.33	0	0	0	0	0	0.00–0.23
	c	0.00–0.02	0	0.00–0.29	0	0	0	0	0	0.00–0.07
Got 3	a^+	0.00–0.001	0	0		0	0	0	0	0
	a	0.00–0.18	0.01–0.02	0.00–0.15	0	0	0	0	0	0
	b	0.26–0.97	0.75–0.93	0.60–1.00	0	0	0	1.00	1.00	0.98–1.00
	c	0.03–0.36	0.05–0.22	0.00–0.39	1.00	1.00	1.00	0	0	0.00–0.02
	d	0.00–0.13	0.00–0.02	0.00–0.01	0	0	0	0	0	0
Prx	a^+	0.00	0.00–0.01	0.00–0.35	0	0	0	0.62	0	0
	a	0.00–0.08	0.09–0.32	0.10–0.75	0	0	0	0	0	0
	b	0.92–1	0.67–0.91	0.16–0.88	0	0	1.00	0.38	1.00	1.00
	c		0	0.00–0.16	1.00	1.00	0	0	0	0
Sod	a^+	0	0.00–0.05	0	0	0	0	0	0.00–0.37	0
	a	0.00–0.09	0.65–0.83	0.05–0.68	1.00	1.00	1.00	1.00	0.63–0.98	0.92–0.95
	b	0.91–1	0.17–0.32	0.32–0.95	0	0	0	0	0.00–0.09	0.05–0.08
	c	0.00–0.01	0.00–0.01	0.00–0.02	0	0	0	0	0	0
Idh	*a*	0.00–0.06	0.18–0.41	0.00–0.36	0	0	0	0	0.00–0.91	1.00
	b	0.29–0.69	0.47–0.80	0.34–0.88	1.00	1.00	1.00	1.00	0.09–100	0
	c	0.25–0.77	0.02–0.12	0.01–0.47	0	0	0	0	0	0
	d	0	0	0.00–0.06	0	0	0	0	0	0
Dia 1	*a*	0	0.00–0.02	0.00–0.31	0	0	0	0	0.20–0.56	0
	b	0.03–0.30	0.56–0.84	0.34–0.89	0	1.00	0	0.88	0.44–0.80	0.88–1.0
	c	0.70–0.97	0.14–0.43	0.00–0.66	1.00	0	1.00	0.12		0.00–0.12
Sdh	*a*	0.00–0.24	0.08–0.17	0.04–0.52	0	0	1.00	0	0–0.35	0.00–0.11
	b	0.76–1	0.47–0.78	0.46–0.89	1.00	1.00	0	1.00	0.65–1.00	0.77–0.97
	c	0.00–0.12	0.05–0.35	0.00–0.10	0	0	0	0	0	0.00–0.07
	d	0	0	0.00–0.01	0	0	0	0	0	0.00–0.05
Pgm	*a*	0.42–0.70	0.62–0.81	0.63–0.83	1.00	1.00	1.00	1.00	0.90–1.00	0.43–1.0
	b	0.30–0.58	0.18–0.37	0.03–0.36	0	0	0	0	0–0.10	0.00–0.57
	c	0	0.00–0.01	0.00–0.05	0	0	0	0		0
Mdh	a^{++}	0	0	0.00–0.05	0	0	0	0	0	0
	a^+	0	0	0.00–0.32	0	0	0	0	0	0
	a	0.91–1	0.45–0.69	0.47–1.0	1.00	1.00	1.00	1.00	1.00	1.00
	b	0.00–0.09	0.03–0.31	0.00–0.13	0	0	0	0	0	0
	c	0	0.00–0.52	0.00–0.20	0	0	0	0	0	0

F. pratensis, except for *Pgi_c**, which is absent from *L. rigidum*. Clear differences do exist for the frequency of *Pgi_d*, which is much more common in *L. rigidum* and *L. multiflorum* than in *L. perenne* and can thus be used as a species marker. Four of the self-pollinating species are monomorphic and homozygous, although not the same allele is fixed: *Pgi_a* in *L. temulentum* and *L. remotum*, *Pgi_b** or *c* in *L. persicum*, and *Pgi_d* in *L.*

subulatum. *L. canariense* is the only inbreeder which shows some within-population variation at *Pgi2*, its alleles being those which are most common in *L. perenne*.

Acid phosphatase (ACP). There are two loci for this enzyme, *Acp1* with four alleles and *Acp2* with five alleles. Again most inbreeders are fixed for both loci while the outbreeders display a range of allelic variation. There are less differences among species for allele frequencies within the genus *Lolium*, while *F. pratensis* can be distinguished by its lack of *Acp2_a* and *Acp2_b* which are the most frequent in *Lolium*.

Glutamate oxaloacetate transaminase (GOT). Three loci are found for GOT, of which *Got1* is quite monomorphic in the outbreeders. It should be noted however that a very rare allele, *Got1_a*, found only in *L. perenne*, has been fixed in the weedy *L. temulentum* and *L. remotum*. *Got2* has four alleles, the most common being *a* and *b*. The five inbreeders are fixed for *Got2_a*, and *Got2_a*$^+$ is absent for *L. perenne*, rare in *F. pratensis*, and quite common in *L. multiflorum* and *L. rigidum*. The inbreeders are fixed and the outbreeders show few differences in allele frequencies for *Got3*.

Peroxidase (PRX). The locus for peroxidase shows its greatest polymorphism in *L. rigidum* with four alleles. Some of these are absent from *L. perenne* but can be found, and are sometimes fixed, in the self-pollinating species: *Prx_a*$^+$ in *L. subulatum*, *Prx_c* in *L. temulentum* and *L. remotum*.

Superoxide dismutase (SOD). In the case of SOD the relative frequencies of the alleles *Sod_a* and *Sod_b* allows one to discriminate *L. perenne* from all other species. The highest degree of polymorphism is found again in the *L. multiflorum-L. rigidum* group. Note that the rare allele *Sod_a*$^+$ can be quite frequent in *L. canariense*.

Isocitric dehydrogenase (IDH). For this enzyme three to four (in L. rigidum) alleles occur in the outbreeders. *Idh_a* is much more frequent in *L. rigidum*, *L. multiflorum*, *L. canariense* and *F. pratensis* than in *L. perenne*. The other inbreeders are fixed for *Idh_b*.

Diaphorase (DIA). There are at least three loci for diaphorase of which *Dia1* is the most easy to read. The other two loci have not been analyzed although *Dia2* has been recorded in some *L. perenne* populations. The ratio *Dia1_a + b/Dia1_c* allows one to distinguish between *L. perenne* and the other outbreeders including *F. pratensis*. This is the only locus for which different alleles have been fixed in *L. temulentum* and *L. remotum*.

Shikimic dehydrogenase (SDH). Four alleles are present at the locus for SDH with relatively few differences in frequencies among species. The slowest very rare, allele, seems to be specific to *L. rigidum* and *F. pratensis*.

Phosphoglucomutase (PGM). Only three alleles of the locus for PGM occur in the outbreeders, the slowest being specific for *L. rigidum*. All inbreeders, except *L. canariense*, are fixed for the fastest allele *Pgm_a*.

Malate dehydrogenase (MDH). The locus for this enzyme is fixed in all the inbreeding species and in *F. pratensis* and is monomorphic for the same allele *Mdh_a* in *L. perenne*. The highest amount of polymorphism is found in the group of *L. multiflorum* and *L. rigidum*.

Clear differences do exist between species for the frequency of certain alleles, which can thus be used as species markers: *Pgi_d* and *Sod_a* (rare in *L. perenne*, frequent in *L. rigidum* and *L. multiflorum*, as illustrated in Fig. 1).

There are relatively few "specific" alleles (occurring in only one species): *Pgi_a** (between *a* and *b*) in *L. persicum*, *Sod_a*$^+$ (faster than *a*) in some populations of *L. multiflorum*. *L. multiflorum* and *L. rigidum* show by far the highest number of alleles per locus.

A summary of the population genetics statistics is given in Table 3. Average gene diversity H ranges from 0 for the true inbreeders (*L. temulentum*, *L. remotum*, *L. persicum*, *L. subulatum*) to 0.3–0.45 for the outbreeders *L. perenne*, *L. multiflorum* and *L. rigidum*.

L. canariense populations show intermediate values, ranging from 0.05 to 0.16.

Wright's fixation index measures the deviation from panmixia: it is less than 0.15 for the self-incompatible outbreeders (due to crosses between related neighbours, known as Wahlund's effect) and reaches 1 for the true inbreeders. Again, *L. canariense* populations have intermediate values of 0.60 to 0.71. *L. canariense* is thus partially cross-pollinated with outcrossing rates, estimated from F_{is}, ranging from 0.17 to 0.25.

The dendogram of Fig. 2 displays the taxonomic relationships between the 32 *Lolium* populations. Four main clusters can be identified.

Fig. 1a–i. Illustration of isozyme allele frequency differences between species: **a**: PGM and PGI *L. perenne* CSK1 (*lanes 1–25*), *L. rigidum* TN17 (*lanes 26–50*); **b**: PGM and PGI *L. persicum* PER1 (*lanes 1–10*), *L. canariense* CA01 (*lanes 11–22*), *L. perenne* SP37 (*lanes 23–46*); **c**: ACP (two loci) *L. perenne* HUN1; **d**: SOD *L. rigidum* SP212 (*lanes 1–24*), *L. perenne* SP32 (*lanes 25–48*); **e**: GOT (three loci) *L. perenne* SP37; **f**: GOT (three loci) *L. perenne* HUN1; **g**: MDH *L. rigidum* IR01; **h**: IDH *L. perenne* GRE1 (*lanes 1–21*), *F. pratensis* (*lane 45*); **i**: SDH *L. perenne* DE27 (*lanes 1–18*), *L. rigidum* FR57 (*lanes 19–42*)

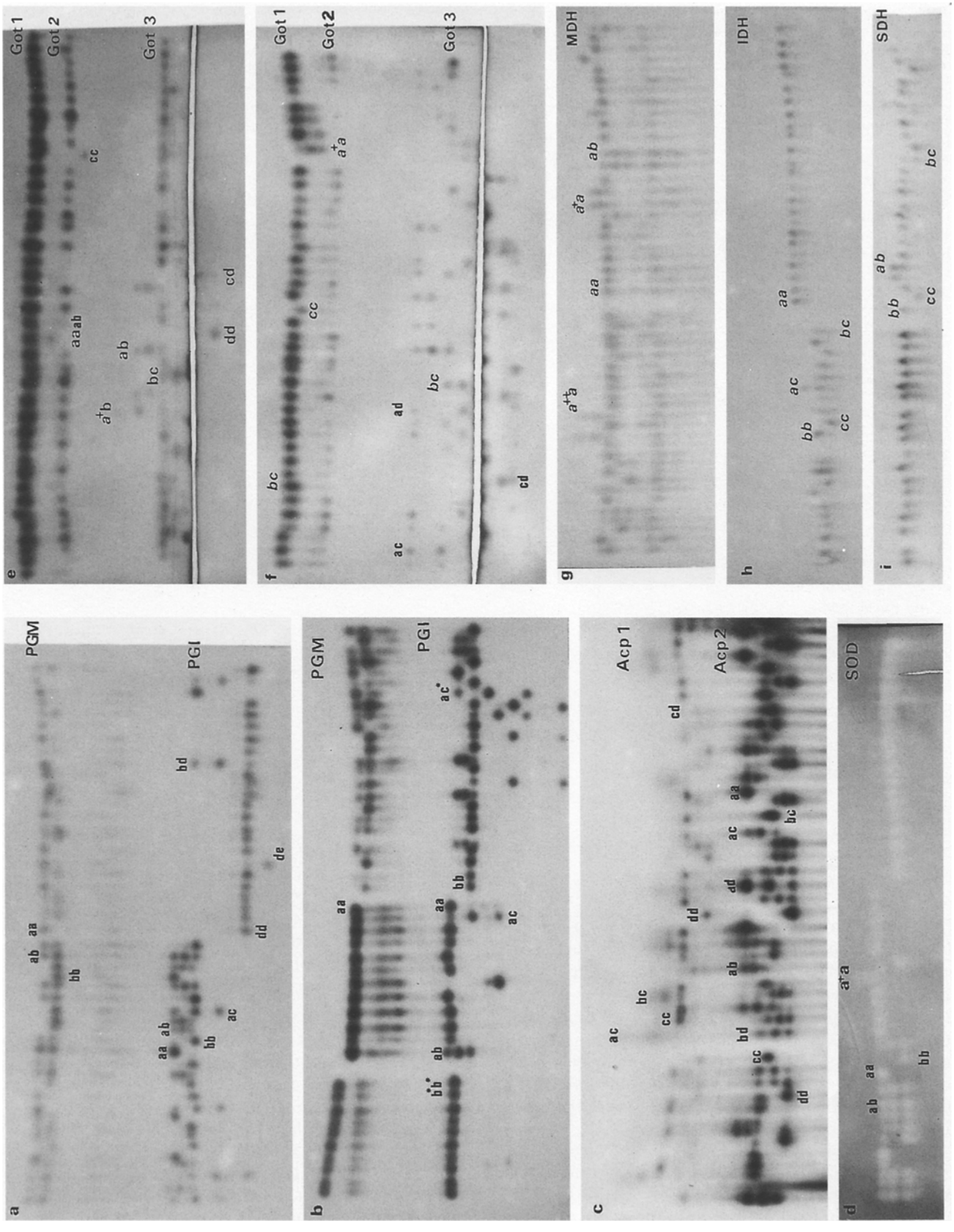
a
PGM
PGI
b
PGM
PGI
c
Acp 1
Acp2
d
SOD
e
Got 1
Got 2
Got 3
f
Got 1
Got2
Got 3
g
MDH
h
IDH
i
SDH

Fig. 1a–i

Table 3. Summary of the population statistics of the nine species

Item	Species								
	L. per.	*L. multi.*	*L. rigid.*	*L. tem.*	*L. rem.*	*L.pers.*	*L. subul.*	*L. canar.*	*F. prat.*
P: %polymorphic loci	76 (57–93)	91 (87–93)	86 (79–93)	0 –	7 –	3 (0–7)	14 –	35 (28–43)	6.0 57–64
N: mean no. of alleles/locus	2.13 (2.00–2.79)	2.83 (2.71–3.00)	2.77 (2.43–3.14)	1.0 –	1.07 –	1.03 (1–1.07)	1.14 –	1.40 (1.28–1.57)	1.96 (1.79–2.14)
H: mean heterozygosity	0.273 (0.226–0.335)	0.392 (0.383–0.406)	0.350 (0.243–0.407)	0 –	0.014	0.015 (0–0.03)	0.05 –	0.104 (0.09–0.11)	0.15 (0.10–0.20)
F_{is}: mean fixation index	0.044 (0.03–0.09)	0.12 (0.09–0.15)	0.15 (0.07–0.20)	1 –	1 –	1 –	0.9 –	0.645 (0.60–0.71)	0.02 (0.04–0.08)
t: estimated outcrossing	(0.92)	(0.78)	(0.74)	0	0	0	0.05	0.21	(0.96)

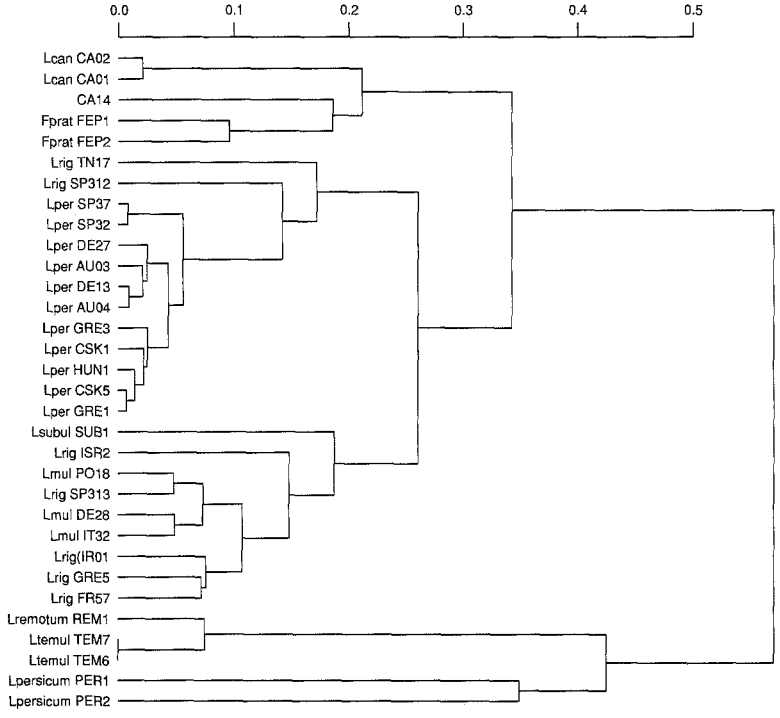

Fig. 2. Dendogram of UPGMA clustering of the 32 population from Nei's genetic distance

The first cluster groups all the weedy, strictly-inbreeding *L. persicum*, *L. remotum* and *L. temulentum*, the last two species being more related to each other than to *L. persicum*. It should be noted that the two *L. temulentum* populations, although from very distant origins, are very similar. This small group is the most differentiated one, the average distance with the other three clusters being 0.60.

The second cluster comprises five *Lolium rigidum* accessions, three *L. multiflorum* accessions and the *L. subulatum* sample. Like *L. temulentum*, the three *L. multiflorum* populations, although from distant geographic locations, are very close to each other. All the *L. perenne* populations, one *L. rigidum* from southern Spain, and one *L. rigidum* from the Canaries belong to the third cluster. The genetic distances within the *L. perenne* group do not exceed 0.1 and are well related to geographic distance: the two populations from Galicia (northern Spain) are clustered together, as are the two populations from Austria, while the two from Germany and the populations from Czechoslovakia are grouped with those from Hungary and Greece. The two *L. rigidum* populations are more distant from *L. perenne* and their clustering with it may be an artefact.

Surprisingly, at the other end of the dendrogram, the subtropical *L. canariense* and the only samples

from the genus *Festuca* are grouped together in the fourth cluster. This group is less differentiated from the group of outbreeders than are the true weedy self-pollinated *L. remotum*, *L. temulentum* and *L. persicum*.

Discussion and conclusion

As mentioned in the introduction, most of our basic knowledge on the taxonomy of the genus *Lolium* comes from the work of Terrell (1966, 1968) which is based on the collection and investigation of nearly 5000 herbarium specimens, as well as on an exhaustive review of the literature in the fields of botany and cytogenetics. Based on compatibility and hybrid-fertility data (Essad 1954; Jenkin 1954), Terrell (1968) recognized *L. temulentum*, *L. remotum* and *L. persicum* as one group. He was of the opinion that *L. temulentum* and *L. remotum* originated from the same basic stock in Southwest Asia. Since they are known only as weeds of cultivated crops, they probably evolved in close association with primitive agriculture. *L. persicum*, restricted to Southwest Asia, could be a derivative of the same basic stock or part of a prototype stock from which the other two taxa were derived. Our results from 13 isozyme loci largely confirm this view. These three species are clustered together, the weedy *L. temulentum* and *L. remotum* being the most closely related. Moreover, if we accept this hypothesis, it may allow us to calibrate the molecular clock based on Nei's genetic distance: assuming that cereal agriculture is about 10,000 years old gives a value of $\alpha = 0.42/2 \times 10000 = 2 \times 10^{-5}$, 0.42 being the mean distance between *L. persicum* and the two weedy species; using this value for α allows us to estimate $t = 0.08/(2 \times 2 \times 10^{-5}) = 2000$ years as the divergence time between *L. temulentum* and *L. remotum*. This value seems compatible with the beginning of flax culture (*L. remotum* is a specific weed of flax, *L. temulentum* a weed of cereals). However, the average mutation rate obtained seems too high. The term I_0 due to founder effect probably cannot be discarded because the ability for self-pollination may have appeared in a limited number of plants. Therefore the relative distances should be considered only as relative times of divergence. Moreover, this founder effect is less likely to occur in the outbreeding species which generally have a high effective population size. The Nei's distance in their case would reflect only the cumulated mutations since the time of divergence, and thus the relative distances are not directly comparable between the outbreeding and the self-pollinating species.

The fact that the two *L. temulentum* accessions, although from very distant origins (Iran and Portugal), are similar, would imply that the species has remained fixed since the beginning of its evolution as a weed of cereals, probably because of its strict autogamy.

This group of "true self-pollinating" species is clearly separated from the other three clusters. This distinction has already been supported by Essad (1954) through the use of discriminant functions, and subsequently by Bulinska-Randomska and Lester (1985) using similarity coefficients from seed protein electrophoretic diagrams. The results of isozyme electrophoresis on phosphoglucose isomerase (Emoto 1985) are also in agreement with this conclusion.

Terrell stated that *L. perenne* and *L. multiflorum* form another group, while *L. rigidum* is a polymorphic complex made up of several elements. This holds true in our results for *L. perenne*, the 11 populations of which are much more similar to each other than to any other species. On the other hand, the three *L. multiflorum* accessions are very close to each other within a group including *L. rigidum*. This similarity has already been described by Bulinska-Randomska and Lester (1985). This supports the hypothesis that *L. multiflorum* is derived recently from a common source of *L. rigidum*.

L. rigidum populations can be found either in cluster 2 with *L. multiflorum* or in cluster 3 with *L. perenne*. The two *L. rigidum* populations from Andalusia (southern Spain) are classified in separate clusters. This, and the fact that *L. rigidum* is the species with the highest diversity indices (number of alleles, average heterozygosity), leads us to postulate that *L. rigidum* is the most likely common ancestor of the genus *Lolium* at least for the group of the outbreeders.

The three *L. canariense* accessions are grouped together, the two populations from the same Island (Tenerife) being the closest. Using the previous value $\alpha = 2 \times 10^{-5}$ leads to an estimation of about 9000 years as the time of divergence between the group of *L. canariense* populations and the two groups of outbreeding species. Surprisingly *Festuca pratensis* groups in the same cluster as *L. canariense*.

These results are somewhat contradictory with the previous assumptions of Terrell. He supposed that *L. canariense* was perhaps more similar to the *L. perenne* group. If *L. canariense* appears to be the result of isolation after chance dispersal of continental *Lolium* populations, its most plausible ancestor is *L. rigidum*, probably from Morocco or southern Spain (possibly imported by the first human settlement 9000 years ago). Terrell interpreted *L. subulatum* as an offshoot of the *strictum-rottbollioides* element of *L. rigidum*. The present study appears to confirm this hypothesis. The only *L. subulatum* sample studied is placed in cluster 2 with *L. rigidum* and *L. multiflorum*, although slightly distinct from these two species.

Several previous studies (Terrell 1966; Bulinska-Randomska 1985) based on morphological traits show

an intergradation among *L. multiflorum*, *L. perenne*, and the polymorphic *L. rigidum*. Terrell reported that repeated hybridizations and introgressions may have occurred during their evolution, especially over the past thousand years of man's disturbance of habitats in the Mediterranean. Our findings do not entirely support this hypothesis, as the *L. perenne* populations are distinctly grouped together. There are, however, indications that introgressions have occurred in some populations from southern Europe, as for example the relatively high frequency of some alleles (*Pgi_d*, *Pgi_e*, *Sod_a*) usually more frequent in *L. rigidum* and *L. multiflorum*. Even so, these introgressions were not sufficient to hide a clear-cut divergence between the two species which might have occurred around 6000 years ago.

The high level of similarity between *Lolium* and *Festuca* has been reported by many authors (Stebbins 1956; Terrel 1966; Borril 1976). Cytogenetic studies (Essad 1954; Jenkin 1954) showed that the *Lolium* genome seems to be homoeologous with one genome of the polyploid tall fescue (*F. arundinacea*). Immunochemical studies of seed storage proteins confirm the similarity between *Lolium* and *F. arundinacea*, *F. altissima*, *F. gigantea* and *F. pratensis* (Butkute and Konarev 1980). The chloroplastic DNAs of *L. multiflorum*, *F. pratensis* and *F. arundinacea* are all very similar and quite different from that of *F. rubra* (Lehvaslaiho et al. 1987). Additional botanical and biochemical studies (Bulinska-Radomska and Lester 1988) confirm these phylogenetic relationships and suggest that the putative ancestor should be close to *F. pratensis* and *L. perenne*. Recently Xu et al. (1992), found similar patterns of cross-hybridization of tall fescue DNA probes with both *F. pratensis* and *L. perenne*. They concluded that these two species are closely related.

The present results from isozymes suggest that *F. pratensis* is related to *L. rigidum*, although most hybrids found in nature are with *L. perenne*. Again it would be necessary to assess other accessions of *F. pratensis* to clarify this point. Moreover, Xu et al. (1992) did not compare *F. pratensis* with *L. rigidum*. Probably *F. pratensis* is very close to the group of outbred *Lolium*, compared to other *Festuca* species, but the relationships at a finer scale remain to be clarified.

In conclusion, what inferences can be drawn from our results about the evolutionary origin of the species of the genus *Lolium*?

Thomas (1981) postulated that speciation in the genus *Lolium* might have involved isolates of *L. perenne*, since the inbreeding species appear the most differentiated. His assumption was based on the respective content in total nuclear DNA (euchromatin and heterochromatin) of the different species, which increases from *L. perenne* (4.16 pg) to *L. rigidum* (4.33 pg), *L. subulatum* (5.49 pg) and *L. temulentum* (6.23 pg), the same figure being found for the length of heterochromatic bands in all four species. It is known that evolutionary processes mostly lead to an increase of some repetitive DNA sequences. Evidence of chromosome segment duplication between *L. perenne* and *L. temulentum* has been reported by Naylor and Rees (1958). It should be noted, however, that the difference in DNA content between *L. perenne* and *L. rigidum* is small and probably not significant.

Alternatively, most authors (Malik 1967; Borrill 1976) agree that the Mediterranean basin is the center of origin of the genus, and that the common ancestor should have its closest affinity with *L. rigidum*. Our results support the latter hypothesis, since *L. rigidum* and *L. multiflorum* appear to be much more polymorphic, both within and between populations, than is *L. perenne*. If the value of $\alpha = 2 \times 10^{-5}$ is correct, then the tree of the differentiation of *Lolium* species would fit historical processes such as the emergence of primitive agriculture in the Middle East and its expansion towards Europe. Further studies are needed to test this hypothesis, such as those developed for human populations by Rendine et al. (1986) or Sokal et al. (1989).

The present results are, however, limited by the relatively low number of loci assayed. The use of molecular markers, such as RFLP or RAPD, on forage grasses, currently in progress in several laboratories (Hayward, personnal communication), should allow us to make more precise and definite inferences about *Lolium* origins and taxonomy.

Acknowledgements. The authors wish to thank the following scientists who have kindly provided us some of the accessions used in this study: Dr. I. D. Thomas (WPBS, Aberystwyth, UK), Dr. J. A. Oliveira (INIA Mabegondo, Spain), Dr. B. Loos (CPRO-DLO Wageningen, NL), Dr. Horvath (Gene bank Tapioszele, Hungary), Dr. Cagas (Inst. Fodder Crops, Zubri, Czeskoslovakia), Dr. A. Dovrat (Faculty of Agriculture, Rehovot, Israêl), Dr. Vaitsis (Central Greece Research Center, Larissa, Greece).

References

Borrill M (1976) Temperate grasses. In: Simmonds N (ed) Evolution of Crop plants Longman, London, pp 137–142

Brown AHD (1979) Enzyme polymorphism in plant populations. Theor Pop Biol 15:1–42

Bulinska-Radomska Z Lester RN (1985) Relationships between five species of *Lolium*. Pl Syst Evol 148:169–175

Bulinska-Radomska Z Lester RN (1988) Intergeneric relationships of *Lolium*, *Festuca* and *Vulpia*. Pl Syst Evol 159: 217–227

Butkute BL, Konarev AU (1980) Immunochemical study of *Lolium* in relation to the phylogeny of the genus. Bot Zhurnal 65:1453–58

Charmet G, Balfourier F, Oliveira JA *Lolium canariense*: an endemic ryegrass from Canary Islands. Proc XVII Int Grassland Congress, Palmerston-North, New Zealand (in press)

Cornish MA, Hayward MD, Lawrence MJ (1980) Self incompatibility in ryegrass. V. Genetic control in diploid *Lolium perenne* L. Heredity 43:95–106

Emoto T (1985) Isozyme variation in the genus *Lolium*. I. Phylogenetic relationships of *Lolium* species. J Jap Soc Grassland Sci 30:327–334

Essad S (1954) Contribution à la systématique du genre *Lolium*. Annales INRA Paris, série B4:325–351

Fearon CH, Hayward MD, Lawrence MJ (1983) Self incompatibility in ryegrass. V. Genetic control in diploid *Lolium multiflorum*. Heredity 50:35–45

Greneche M, Lallemand J, Michaud O (1991) Comparison of different enzyme loci as a means of distinguishing ryegrass varieties by electrophoresis. Seed Sci Technol 19: 147–158

Hayward MD, Mc Adam NJ (1977) Isozyme polymorphism as a measure of distinctiveness and stability in cultivars of *Lolium perenne* L. Z. Pflanzenzücht 79:59–68

Jenkin J (1954) Interspecific and integeneric hybrids in herbage grasses. VIII. *Lolium*. J Genet 52:318–331

Kimura M, Crow JF (1964) The number of alleles that can be maintained in a finite population. Genetics 49:725–738

Kloot PM (1983) The genus *Lolium* in Australia. Aust J Bot 31:421–435

Lehvaslaiho H, Saura A, Lokki J (1987) Chloroplast DNA variation in the grass tribe *Festucae*. Theor Appl Genet 74:298–302

Malik CP (1967) Cytogenetic studies of the F_2 hybrid of *L. multiflorum* × *L. rigidum* and the species relationships in the genus *Lolium*. Der Züchter 37:261–274

Naylor B (1960) Species differentiation in the genus *Lolium*. Heredity 15:219–233

Naylor B, Rees H (1958) Chromosome size in *L. perenne* and *L. temulentum*. Nature 181:854–855

Nei M (1972) Genetic distance between populations. Am. Nat 106:283–292

Nei M (1978) Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89:583–590

Ostergaard H, Nielsen G, Johansen H (1985) Genetic variation in cultivars of diploid ryegrass, *Lolium perenne* and *Lolium multiflorum*, at five enzyme systems. Theor Appl Genet 69:409–421

Pollans NO, Allard RW (1985) Inheritance of electrophoretically-detectable variants in ryegrass. J. Hered 76:61–62

Rendine S, Piazza A, Cavalli-Sforza LL (1986) Simulation and separation by principal components of multiple demic expansion in Europe. Am Nat 128:681–706

Sokal RR, Jacquez GM, Wooten MC (1989) Spatial autocorrelation analysis of migration and selection. Genetics 121:845–855

Stebbins GL (1956) Taxonomy and evolution of genera, with special reference to the family Graminae. Evolution 10:235–245

Swofford DL, Selander RB (1981) Biosys I: a Fortran Program for the comprehensive analysis of electrophoretic data in population genetics and systematics. J Hered 72:281–283

Terrell EE (1966) Taxonomic implications of genetics in ryegrasses. Bot Rev 66:138–164

Terrell EE (1968) A taxonomic revision of the genus *Lolium*. Tech Bull US Dept Agric 1392:1–65

Thomas HM (1981) The Giemsa C-band kariotypes of six *Lolium* species. Heredity 46:263–267

Thorogood D, Hayward MD (1992) Self compatibility in *Lolium temulentum* L.: its genetic control and transfer into *L. perenne* L., and *L. multiflorum* Lam. Heredity 68:71–78

Xu WW, Sleper DA, Chao S, (1992) Detection of RFLPs in perennial ryegrass, using heterologous probes from tall fescue. Crop Sci 32:1366–1370