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Genetic relationships between *Lolium* (Poaceae) species revealed by RAPD markers

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ABSTRACT. The genus *Lolium* is one of the most important groupings of temperate forage grasses, including about eight recognized species that are native to some temperate and subtropical regions of the northern hemisphere. We examined genetic relationships among 18 accessions representing all *Lolium* species using RAPD markers. Among 50 random primers that we screened, 13 gave reproducible amplification banding patterns. Each of these 13 primers generated 19-43 scorable fragments. A total of 367 RAPD fragments were detected, of which 95.9% were polymorphic across all the *Lolium* accessions. Dice's coefficient of dissimilarity ranged from 0.016 to 0.622, which is indicative of substantial genetic variations in these *Lolium* accessions. A neighbor-joining cluster analysis, with bootstrap permutation, produced an unrooted dendrogram, which grouped 18 accessions into two main clades, supporting high bootstrap values (98 and 96%). The first clade included the self-pollinated species, *L. persicum*, *L. temulentum*, *L. remotum*, and *L. subulatum*. The cross-pollinated species, i.e., *L. multiflorum*, *L. perenne*, *L. rigidum*, and *L. canariense*, composed the second clade, in which *L. canariense* formed a distinct subclade, indicating its higher genetic separation from other allogamous species.

The value of r = 0.97 in the Mantel test for cophenetic correlation applied to the cluster analysis indicated the high degree of fit of the accessions to a group. A principal coordinate analysis, whose first three coordinates explained 72.6% of the variation, showed similar groupings as in the cluster analysis. The genetic relationships estimated by the polymorphism of RAPD markers are basically in agreement with those previously inferred with other genetic markers.

Key words: *Lolium*; RAPD markers; Taxonomy; Genetic relationship; Polymorphism

INTRODUCTION

The genus *Lolium* L. (Poaceae) is native to Europe, temperate Asia, and North Africa but has been widely distributed throughout temperate regions of the world (Charmet et al., 1996). It includes two of the most economically important forage grasses, namely Italian ryegrass (*L. multiflorum*) and perennial ryegrass (*L. perenne*), which are both widely grown as forage or turf grasses, especially in Europe, New Zealand, Australia, and other temperate/Mediterranean regions of the world. Generally, eight species are recognized: *L. perenne* L., *L. multiflorum* Lam., *L. rigidum* Gaud., *L. canariense* Steud., *L. persicum* Boiss. & Hohen. ex Boiss., *L. remotum* Schrank, *L. temulentum* L., and *L. subulatum* Vis. (= *L. loliaceum* Hand.-Mazz.), according to their reproduction manner in addition to morphological characters such as spike and leaf morphology and growth habit as well (Terrell, 1968). The first 4 species are wind-pollinated outbreeders, the latter 4 are self-pollinated species (Fearon et al., 1983; Jauhar, 1993), although *L. canariense* has been found to be partially cross-pollinated (Charmet and Balfourier, 1994). The *Lolium* species occur as diploid (2n = 14) (Terrell, 1968), but due to breeding activities, many tetraploid cultivars have been developed in the fodder crop species, perennial, and Italian ryegrass (Loos, 1993b). Besides, the outbreeding *Lolium* species are closely related to members of the genus *Festuca* of the section Bovinae, and they hybridize fairly easily with them (Borrill, 1976).

In spite of intensive breeding activities and advanced research aimed at improving the turf quality or forage yield, very little attention has been paid to the phylogenetic relationships within the genus *Lolium*. A better knowledge of the genetic relationships between *Lolium* species is of both theoretical and practical importance, which can serve as the basis of the taxonomic system and provide explicit information for a more efficient exploitation and utilization of genetic resources through hybridization by ryegrass breeders (Charmet and Balfourier, 1994).

Notwithstanding slight controversies, the previous results of some studies consistently support the notion that the genus *Lolium* can be divided into two groups based on reproduction mode: one for the cross-breeding species and another for the remaining inbreeding species (Charmet and Balfourier, 1994; Stammers et al., 1995; Charmet et al., 1997; Gaut et al., 2000; Polok et al., 2006). As *Lolium* is believed to be of extremely recent origin, its phylogeny has not been resolved with certainty (Gaut et al., 2000). This contributes greatly to the ambiguity of the taxonomic classification of the genus. A big challenge underlying the genus *Lolium* is in determining whether *L. perenne* (Bulinska-Radomska and Lester, 1988; Catalán, et al., 1997) or *L. rigidum* (Charmet and Balfourier, 1994) is the common ancestor of the genus. Another point at issue is that the phylogenetic placement of *L. canariense* and *L. subulatum* is delphic,

probably because neither of these species has been included in many analyses (Gaut et al., 2000). It is a pity that most conclusions about *Lolium* phylogeny are based on morphology, cytogenetic analysis, or the number of isozyme loci. Morphology alone has failed to clearly resolve phylogenetic relationships owing to overlapping ranges of variation (Polok, 2007). The advent of molecular data has revolutionized the field of plant systematics and has led to new insights into phylogenetic relationships at all taxonomic levels. In previous molecular studies of *Lolium* phylogeny, most of the data employed were from the chloroplast genome or internal transcribed spacer (ITS) sequence (Darbyshire and Warwick, 1992; Catalán et al., 1997; Gaut et al., 2000; Balfourier et al., 2000; McGrath et al., 2007), whereas methods based on genome scanning molecular markers were rarely reported (Stammers et al., 1995; Polok et al., 2006). DNA marker-based fingerprinting is not typically influenced by environmental conditions, and therefore can be used to rapidly distinguish species using small amounts of DNA and to deduce reliable information on their phylogenetic relationships. Among available molecular markers, RAPD (random amplified polymorphic DNA) (Williams et al., 1990) is an inexpensive and rapid method not requiring any information regarding the genome of the plant, and has provided a powerful tool for the investigation of genetic variability in numerous plants. The aim of the present study was to use RAPD markers to estimate the level of genetic variation of *Lolium* and to detect phylogenetic relationships between 8 acknowledged ryegrass species.

MATERIAL AND METHODS

Plant material and RAPD amplification

Eighteen accessions belonging to the 8 species of *Lolium* were employed as the study material (Table 1). All were obtained from the National Plant Germplasm System, USA. Genomic DNA was extracted from fresh young leaves of ten individuals of a species using the CTAB protocol (Doyle and Doyle, 1990). DNA concentration was measured using a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Inc.) and 0.8% agarose gel electrophoresis. DNA was diluted in water to a final concentration of 10 ng/μL.

Table 1. Accessions of *Lolium* species used in the analysis.

No.	Voucher entry	Species	Origin
1	PI598452	*L. perenne* L.	Wales, United Kingdom
2	PI277847	*L. perenne* L.	Turkey
3	PI610828	*L. perenne* L.	Wales, United Kingdom
4	PI272118	*L. multiflorum* Lam.	Poland
5	PI376874	*L. multiflorum* Lam.	New Zealand
6	PI632537	*L. multiflorum* Lam.	Oregon, United States
7	PI239753	*L. rigidum* Gaud.	Algeria
8	PI239731	*L. rigidum* Gaud.	Fouka, Egypt
9	PI239795	*L. rigidum* Gaud.	Mosj.-Gol, Iran
10	PI422589	*L. temulentum* L.	Tetouan, Morocco
11	PI218085	*L. temulentum* L.	Peshawar, Pakistan
12	PI302664	*L. temulentum* L.	India
13	PI545637	*L. persicum* Boiss. & Hohen.	Golbasi, Turkey
14	PI545664	*L. persicum* Boiss. & Hohen.	Yesilova, Turkey
15	PI545676	*L. persicum* Boiss. & Hohen.	Agri, Turkey
16	PI197310	*L. subulatum* Vis.	Argentina
17	PI320544	*L. canariense* Steud.	Canary Islands, Spain
18	PI283611	*L. remotum* Schrank	France

Fifty random primers (Operon Technologies, Almeda, CA, USA) were initially screened on a sample of the accessions. Primers that produced reproducible, polymorphic bands were used to amplify the rest of the accessions. PCR amplifications were carried out in an MJ Research Thermal Cycler PTC-200, in a reaction volume of 20 µL containing 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.2 µM primer, 0.1 mM of each dNTP, 0.5 U Taq DNA polymerase (TaKaRa, Japan), and 20 ng template DNA. The amplification reaction profile was: an initial denaturation at 94°C for 5 min followed by 40 cycles at 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min, and a final 7-min extension at 72°C. The RAPD products were electrophoresed on a 1.5% agarose gel in 0.5X TBE (Tris-boric acid-EDTA) buffer at 100 V for 3.5 h. The gels were stained in 0.2 µg/mL ethidium bromide and photographed using a gel documentation system (Gel Doc BioRad 2000). Each assay was repeated twice, and only stable products were scored. A 100-bp DNA ladder was used as the molecular standard to confirm the appropriate RAPD markers.

Data analysis

RAPD assays were performed in duplicate, and only those reproducible patterns clearly obtained were scored. It was assumed that bands of the same molecular weight in different samples were identical. The presence or absence of each amplified band was recorded as 1 (present) or 0 (absent) and treated as binary characters. For considering a marker as polymorphic, the absence of an amplified product in at least one species was used as a criterion. The polymorphism information content (PIC) for each RAPD marker was calculated with the formula: $PIC_i = 2f_i (1 - f_i)$, where PIC_i is the polymorphic information content of marker i, f_i is the frequency of the marker bands that were present, and $(1 - f_i)$ is the frequency of marker bands that were absent (Roldán-Ruiz et al., 2000). PIC values for dominant marker bands such as RAPD markers have a maximum of 0.5 for $f_i = 0.5$.

A binary matrix was used to estimate genetic distances between pairs by employing the Dice distance coefficient (Nei and Li, 1979). A dendrogram was constructed by the neighbor-joining (NJ) method, and the original binary matrix was bootstrapped 1000 times to measure the reliability of the branching patterns and the quality of the resulting phylogenetic groups. DARwin version 5.0 (Perrier et al., 1999) was used for the above calculations. The Mantel test (Mantel, 1967) was used to test the significance of the correlation coefficient between pairs of similarity matrices and cophenetic matrices and for determining cophenetic correlation values using NTSYS pc version 2.2 (Rohlf, 1998). Principal coordinate analysis (PCoA) was carried out by the NTSYS pc version 2.2 software to provide a graphical representation of the genetic relationships between the ryegrass accessions studied.

RESULTS

RAPD polymorphism and genetic variation

Fifty random decamer primers were used in this study, of which 13 were chosen for

analysis based on a reproducible and well-resolved RAPD pattern (Table 2). The size of the amplified products mainly ranged from 150 to 1800, with 19-43 bands per primer. A total of 367 RAPD polymorphic bands were generated by the 13 primers, at a rate of 28.2 bands per primer, of which 352 bands were found to be polymorphic. The percentage of polymorphic bands varied from 82.4 to 100% with an average of 95.9% for all primers, which could even be comparable with 98.8% polymorphism in characterizing some *Lolium* species by AFLP analysis (Polok et al., 2006). This result indicated that the genus *Lolium* possesses a high level of genetic diversity and abundant genetic variation. PIC scores per primer across the genus varied from 0.281 (OPB05) to 0.390 (OPAH08) with an average of 0.339, reflecting the same information to polymorphism ratio.

Table 2. Sequences of the primers and the results of amplification.

Primer	Sequence (5'→3')	Band size (bp)	Total bands	Polymorphism ratio (%)	PIC
OPA01	CAGGCCCTTC	170-2200	20	100.0	0.311
OPA07	GAAACGGGTG	180-1750	28	92.9	0.289
OPA16	AGCCAGCGAA	120-1360	43	97.7	0.371
OPB05	TGCGCCCTTC	210-2300	26	100.0	0.281
OPB18	CCACAGCAGT	140-1850	34	82.4	0.357
OPC04	CCGCATCTAC	190-1650	22	86.4	0.313
OPD15	CATCCGTGCT	130-1500	27	100.0	0.365
OPQ20	TCGCCCAGTC	250-1850	19	94.7	0.336
OPR10	CCATTCCCCA	130-1900	26	96.2	0.343
OPAH02	CACTTCCGCT	140-1850	32	100.0	0.377
OPAH06	GTAAGCCCCT	120-1650	33	97.0	0.357
OPAH08	TTCCCGTGCC	180-1580	29	100.0	0.390
OPAH15	CTACAGCGAG	140-1800	28	100.0	0.321
Total	-	-	367	-	-
Means	-	-	28.2	95.9	0.339

PIC = polymorphism information content.

Genetic relationships between species

The Nei-Li (Dice) distance matrix developed using the NTSYS software showed that distance index ranged from 0.016 to 0.622 with a mean of 0.458, thereby suggesting high levels of genetic variability in the species. The highest genetic distance value (0.622) was found between PI239753 (*L. rigidum*) and PI283611 (*L. remotum*), implying the farthest genetic relationship, and the lowest value (0.016) was found between PI218085 (*L. temulentum*), and PI302664 (*L. temulentum*), disclosing the closest genetic relationship among the 18 accessions tested. In addition, mean genetic distances among *Lolium* species was also calculated by averaging the values of the accessions within a given species. The highest interspecific genetic distance was 0.616 between *L. rigidum* and *L. remotum*, and the lowest interspecific distance was 0.318 between *L. temulentum* and *L. persicum*.

The distance matrix based on the Dice genetic distance is graphically represented as a dendrogram using the NJ method, since this method is less sensitive to varying mutation rates (Figure 1). A cophenetic correlation coefficient of 0.97 was obtained between the Dice dissimilarity matrix and the derived NJ cophenetic matrix, revealing a good fit between the dendrogram clusters and the dissimilarity matrices. According to the dendrogram, all 18 accessions of the 8 species were separated into 2 distinct

clusters. The first cluster (Cluster I) was composed of the self-pollinated species *L. temulentum*, *L. persicum*, *L. remotum*, and *L. subulatum*, which was subdivided into 2 subgroups and strongly supported by a 98% bootstrap value. The first subclade included *L. persicum*, *L. temulentum*, and *L. remotum*, supported by a 100% bootstrap value; the second subclade was formed by *L. subulatum*, which showed the greatest distance from the other three inbreeders. The second cluster (Cluster II) comprised four cross-pollinated species, *L. perenne*, *L. multiflorum*, *L. rigidum*, and *L. canariense*, which was divided into 2 subclusters and was supported by a 96% bootstrap value. *L. canariense* formed a single subclade and it was clearly differentiated from the other 3 species in Cluster II. Furthermore, accessions within species always tended to first group together and then to related species.

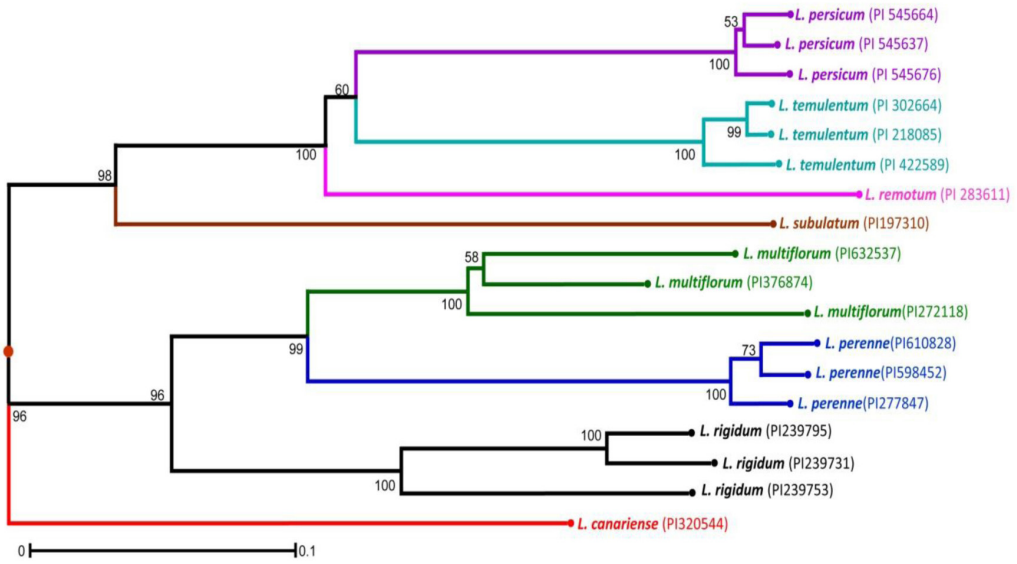

Figure 1. Unrooted dendrogram constructed using the neighbor-joining method from the RAPD data matrix calculated with the formula given by Nei and Li (1979). The numbers at the tree nodes are bootstrap values reported as percentage.

It is worth comparing results from various methods of multivariate analysis to verify conclusions. Therefore, besides the cluster analysis, we also performed PCoA, again based on the matrix derived from the Dice distance coefficients (Figure 2). The first three principal coordinates accounted for 46.85, 14.96, and 10.83% of total variation, respectively. The autogamous section and the allogamous section were clearly separated in the projection onto the first principal coordinate (axis 1). The interspecies and intraspecies relationships were well reflected according to the second and third coordinates (axes 2 and 3) (Figure 2). Overall, clustering results based on the NJ method were corroborated by the PCoA ordination method.

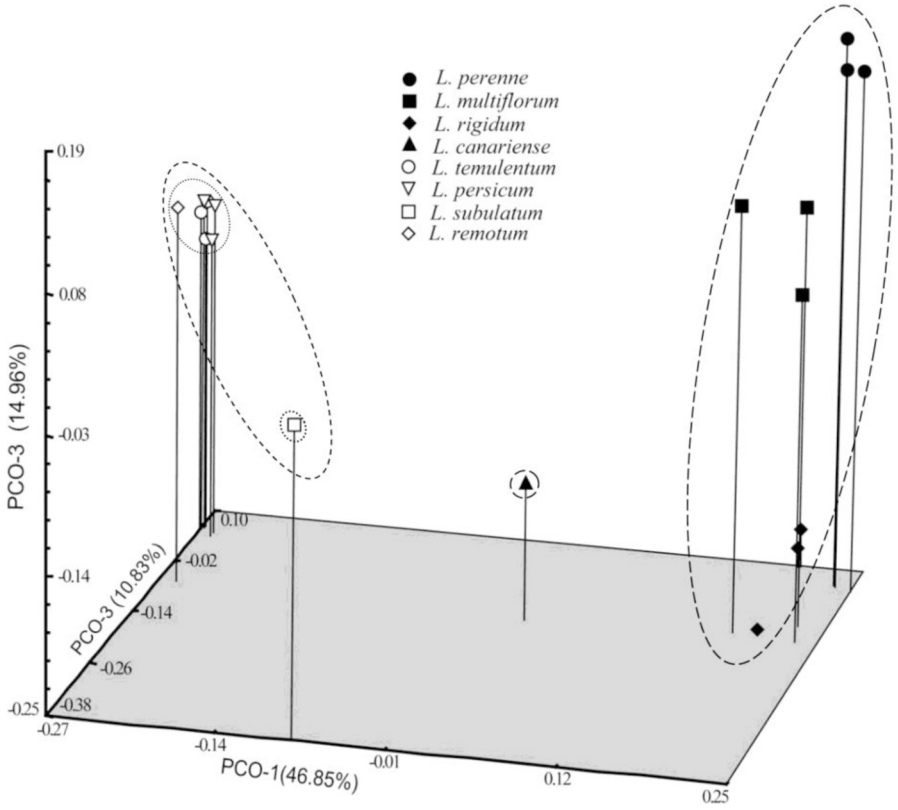

Figure 2. Principle coordinate plot for the first three principal coordinates estimated for RAPD markers of the eight *Lolium* species.

DISCUSSION

The RAPD technique has been successfully employed in taxonomic and genetic diversity studies of some *Lolium* species due to the simplicity, low cost and non-requirement of DNA sequence information prior to application (Stammers et al., 1995; Vieira et al., 2004; Bolaric et al., 2005). The genetic validity of RAPD markers has been questioned, because co-migrating bands may correspond to non-homologous DNA sequences. However, several authors (Stammers et al., 1995; Lannér et al., 1996) have checked the homology of RAPD bands by hybridization with RAPD fragments used as probes and have found low error rates, which are not likely to significantly affect estimates of genetic relatedness. Moreover, another problem of low reproduction (stability) of RAPD assay could be well solved using high-quality DNA samples, an optimized reaction system and program in a standard operation. In the present study, RAPD on bulked total DNA (Yu and Pauls, 1993; Charmet et al., 1997) proved to be an efficient tool in estimating overall genetic similarities between ryegrass accessions, and gave highly correlated estimates of genetic distances. Furthermore, bootstrap re-sampling

from the combination of RAPD bands provided fairly good coefficients of variation.

Owing to the difficulty of sampling accessions within some *Lolium* species, the taxa under study may not adequately represent the breadth of phylogenetic diversity. Nonetheless, this study of 18 accessions covered, to our knowledge, all of the eight recognized *Lolium* species studied with various methods to date. Although this sample is insufficient to illustrate fine details about the evolution of the genus *Lolium*, the sample is sufficient to yield new insights into species' broad phylogenetic trends. In the present investigation, phylogenetic analysis of the dissimilarity matrix based on RAPD markers could readily divide the *Lolium* species into a self-pollinated section and another cross-pollinated one. This segregation coincides strongly with previous studies using isozyme (Charmet and Balfourier, 1994), ITS sequence (Charmet et al., 1997; Gaut et al., 2000), RFLP (Charmet et al., 1997), RAPD (Stammers et al., 1995), and morphological data (Mirjalili et al., 2008). In general, the autogamous group can be well separated from the allogamous group by botanical characters. For instance, the inbreeders are on average smaller, contain fewer florets, and have ears emerging earlier (Loos, 1993a). This strong differentiation or distinction between self- and cross-pollinated species may result from their different divergence time. The *Lolium* species appeared to be of recent origin (Stammers et al., 1995), and the self-fertilizing species diverged first from the common ancestor and the cross-pollinated species last (Charmet et al., 1997). Last but not least, it should be pointed out that UPGMA clustering was not utilized on RAPD data, because the assumption of a "molecular clock" can hardly be made for RAPD (Charmet et al., 1997).

In the self-fertilized cluster on the RAPD-based dendrogram, *L. persicum* was closer to *L. temulentum* than either of these 2 species was to *L. remotum*. This observation is confirmed by previous results based upon analysis of plant morphology (Loos, 1993a), isozyme (Loos, 1993b), AFLP (Polok et al., 2006), and ITS sequence (Charmet et al., 1997). *L. subulatum* has been viewed as intermediate between the cross-breeding and the inbreeding group (Charmet et al., 1997). According to the present investigation, *L. subulatum* is a species distinct from the other three inbreeders. However, there is no strong consensus on the phylogenetic position of *L. subulatum*, not only because this species can be closely related to allogamous *Lolium* species in some studies (Gaut et al., 2000; Polok, 2007), but because this species has been rarely included in analyses. To date, *L. temulentum* and *L. remotum* are known only as weeds of crops, and have been progressively disappearing by widespread use of farm chemicals. Since they possess the valuable sources of self compatibility genes to transfer to outbreeders, their conservation for germplasm resources must be considered (Thorogood and Hayward, 1992).

In the cross-pollinated group on the RAPD-based dendrogram, *L. multiflorum* and *L. perenne* are the closest related species, and *L. rigidum* is relatively close to both of them, while *L. canariense* formed the most distant subclade. Cytological (Naylor, 1960) and electrophoretic (Bulinska-Radomska and Lester, 1985) evidence suggests that *L. perenne*, *L. multiflorum*, and *L. rigidum* should be regarded as a subspecies of one biological species, as they are all interfertile, providing that flowering dates are compatible. However, the present observation indicated that these three outbreeding species can be well separated in spite of the overlap of distribution range, morphological variation, and limited natural hybridization and introgression (Loos, 1993a,b; Charmet and Balfourier, 1994; Charmet et al., 1996; Bennett et al., 2000). A high level of intraspecific variation of *L. multiflorum* inferred from branch length of the NJ tree was found, which is common to morphological studies (Bennett et al., 2000). This may correspond to its multifold form life cycle, since *L. multiflorum* is not a true annual and may

behave as a biennial or short-lived perennial depending on environmental conditions (Terrell, 1968). Close association between these three species and possible recent evolutionary divergence (Stammers et al., 1995; Charmet et al., 1997) have resulted in varying degrees of similarity between them. Interestingly, according to RAPD (Stammers et al., 1995) or AFLP data (Polok et al., 2006), *L. multiflorum* has a position closer to *L. perenne* than to *L. rigidum,* while according to the isozyme data, *L. multiflorum* is closer to *L. rigidum* than to *L. perenne* (Charmet and Balfourier, 1994; Bennett et al., 2002). Different clustering methods and/or characteristics of genetic markers may be responsible for the above controversy.

According to our investigation, *L. canariense* has a distant relationship with other typical allogamous species despite forming the same cluster. This could be confirmed by analyses of isozymes (Charmet and Balfourier, 1994), ITS sequence (Gaut et al., 2000), and RFLP marker of cpDNA (Charmet et al., 1997). *L. canariense* is generally considered an autogamous species but is also supposed to be intermediate with respect to the mode of reproduction due to partial cross-pollination (Charmet et al., 1994). Furthermore, strictly annual *L. canariense* is endemic to the Canary Islands in the Atlantic Ocean, which may suffer severe bottleneck effects in speciation (Francisco-Ortega et al., 2000). This may lead to the formation of a distinct gene pool of *L. canariense* relative to other cross-pollinated *Lolium* species.

In conclusion, the RAPD marker-based study of genetic variations facilitates the delineation of *Lolium* species. The use of molecular markers with good stability and high polymorphism, such as SSR or AFLP, and DNA sequence-based analyses in future studies that include additional accessions representing a broader geographic distribution, should allow us to elucidate the systematic positions and origins of *Lolium* species.

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