BIOLOGIA PLANTARUM 37 (2): 185-195, 1995

Identification and relationships of cultivated accessions from *Lolium-Festuca* complex based on RAPD fingerprinting

I. WIESNER, P. SAMEC and V. NAŠINEC

The Institute of Plant Molecular Biology, Czech Academy of Sciences, Branišovská 31, České Budějovice, CZ - 370 05 Czech Republic

Abstract

Randomly Amplified Polymorphic DNA (RAPD) technique with 15 arbitrary primers was used to identify and reveal relationships of the accessions comprising 4 species of *Lolium-Festuca* complex. Altogether 252 RAPD markers were considered in statistical treatment, 60 of which could be identified as potentially taxon-specific. All analyzed taxa were fully distinguishable using RAPD markers. *Lolium-Festuca* relationships based on RAPD data were evaluated using cluster analysis (UPGMA) and principle coordinate analysis (PCO). The results of UPGMA as well as PCO performed on data pooled from all RAPD profiles support separation of the two genera *Lolium* and *Festuca*.

Key words: cluster analysis, fescue, molecular markers, *Poaceae*, principal coordinate analysis, ryegrass.

Introduction

Molecular markers applied to plant systematics and evolution represent a powerful tool for solving ambiguities in the classification based mainly on morphological traits, which are usually strongly influenced by environment and are not selectively neutral (Crawford 1985). For genomes of *Lolium* and *Festuca*, molecular markers have been used to distinguish among species and cultivars or to study population dynamics (Ostergaard *et al.* 1985, Hayward *et al.* 1990, Murphy *et al.* 1990, Livesey and Norrington-Davies 1991, Dinelli and Bonetti 1992, Griffith and Banowetz 1992, Perez-Vicente *et al.* 1992, Charmet *et al.* 1993, Moller and Spoor 1993). Molecular markers have also been used in taxonomic studies of the *Lolium-Festuca* complex (Bulińska-Radomska and Lester 1985a,b 1988, Darbyshire and Warwick 1992).

The two genera *Lolium* and *Festuca* (fam. *Poacea*, subfam. *Festucoideae*) are believed to have monophyletic origin. Darbyshire and Warwick (1992) used

Received 22 June 1994, *accepted* 15 November 1994.
Acknowledgments: This research was granted as the research project Z-660-03 by the Ministry of Economy, Czech Republic.

chloroplast DNA restriction site variation to distinguish two evolutionary lines within *Festuca*, one comprising the majority of *Festuca* taxa and the second composed of subgenus *Schedonorus* and, alternatively, genus *Lolium*. *Lolium* and *Festuca* are considered to be very closely related based on morphological, biochemical, and genetic data: the occurrence of natural *Lolium* × *Festuca* hybrids, homology between chromosomes, similarities in seed starch grains, in the composition of oligosaccharides and water-soluble polysaccharides, in seed protein patterns, chloroplast DNA (cpDNA) restriction site patterns, cpDNA reassociation, thermal denaturation of genomic DNA or according to serology (for review see Bulińska-Radomska and Lester 1988, Darbyshire and Warwick 1992). Recently, sequences of the chloroplast *rps4* gene were obtained from *L. perenne*, *F. pratensis* and *F. gigantea* to study *Poaceae* phylogeny (Lejeune, unpublished).

At present the discrepancies still persist between the classification based on morphological and/or molecular characters. Inflorescence characters and cytogenetic data support the division of these two genera contrary to molecular data, which support the integration of *Lolium* into the genus *Festuca* (Bulińska-Radomska and Lester 1988, Darbyshire and Warwick 1992). The close molecular relationship of *Festuca* subg. *Schedonorus* to *Lolium* and the distant position of *Festuca* subg. *Festuca* has resulted in the new taxonomic combinations: *Lolium* subg. *Schedonorus*, *Lolium arundinacea*, *Lolium pratensis* to make the classification consistent with the phylogeny (Darbyshire 1993).

Recently, new very effective experimental approach has been widely adopted to the generation of DNA molecular markers called Randomly Amplified Polymorphic DNA (RAPD) based on PCR reaction primed with a short arbitrary primer (Munthali *et al.* 1992, Samec 1993).

In this paper we aimed to identify a set of RAPD molecular markers and compare the relationships based on those RAPD markers with the current view of *Lolium-Festuca* classification.

Materials and methods

Festuca arundinacea Schreb., cv. Kora (2n = 6x = 42) (FA_K), *F. pratensis* Huds., cv. Rožnovská (2n = 2x = 14) (FP_R), *F. pratensis* Huds., cv. Otava (2n = 2x = 14) (FP_O), (derived by breeding process from cv. Rožnovská) *Lolium perenne* L., cv. Tarpan (2n = 4x = 28) (LP_T), *L. perenne* L., cv. Sport (2n = 2x = 14) (LP_S), *L. multiflorum* Lamk. subsp. *italicum* A. Brl. Volkart, cv. Romul (2n = 2x = 14) (LM_R).

All analyzed taxa are certified by the Forage Breeding Institute, Troubsko u Brna, Czech Republic, from which the seeds were obtained.

The plantlets were grown up from seeds in greenhouse. For each accession, 5 g of leaves pooled from 10 individuals were harvested, immediately frozen in liquid nitrogen and stored at -70 °C.

DNA was extracted from frozen leaves according to Amasino *et al.* (1984). To prime RAPD reactions, single oligonucleotide primers were used from the *Molecular Medicine Unit*, London, UK (see Table 1 for primer sequences).

The reaction mixture (0.05 cm^3) contained 25 ng template DNA, 1.6 μM primer, 200 μM each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, and 1.25 U AmpliTaq polymerase (*Perkin Elmer Cetus*). Polymerase was added after the initial denaturation of template ("hot start" method).

Table 1. RAPD primers.

p1	5' - AGTCAGCCAC
p2	5' - GTGATCGCAG
p3	5' - GCTGGTGG
p4	5' - CGCGGCCA
p5	5' - ACGTATCTGC
p6	5' - TCGTCACTGA
p7	5' - TGCTCACTGA
p8	5' - CCGGCCGTCA
p9	5' - ACCGCGAAGG
p10	5' - GTGTGCCCCA
p11	5' - AGGGCGTAAG
p12	5' - AATCGGGCTG
p13	5' - TGCCGAGTCG
p14	5' - CCGTATACAC
p15	5' - GAGGCCTATA

The temperature profile started with preliminary denaturation for 5 min 95 °C followed by 35 cycles of 20 s at 95 °C, 20 s at 35 °C, and 1 min at 72 °C.

DNA fragments produced during RAPD reaction were separated on 5 % polyacrylamide gel (17:7, acrylamide:bis-acrylamide), alternatively gel contained 7 M urea, if higher resolution was required (Caetano-Anolles *et al.* 1991). Localization of fragments in the gel was accomplished by silver staining procedure according to Bassam *et al.* (1991).

The fingerprint profiles from the DNA-stained electrophoretic gels were visually scored by assigning a figure to each band (0 - band absent, 1 - band hardly visible, 2 - sharp band of medium intensity, 3 - diffusive band of extremely high intensity).

pUC18 cleaved by *Taq*I and *Sau*IIIA was used as DNA molecular mass marker in each gel. A RAPD band was considered polymorphic when its classification number differed among individual accessions by at least factor of 2. A RAPD band was considered taxon-specific, when its classification number for a particular taxon was higher by factor of 2 comparing with other taxa or, alternatively, by factor of 1, if the band present in a taxon was otherwise fully absent.

Estimates of the genetic similarity coefficient (GS) were calculated according to the formula (Nei and Li 1979):

$$GS = 2n_{xy}/(n_x + n_y)$$

where n_x and n_y are the total number of RAPD bands of the taxon X and Y, respectively, and n_{xy} is the number of bands shared by the two taxa.

Average linkage cluster analysis (UPGMA) and principal coordinate analysis (PCO) were performed using *MVSP Plus* ver. *2.1* software package for multivariate analysis (Kovach Computing Services, Pentraeth, UK).

Results

RAPD profiles: RAPD analysis was used to identify and reveal relationships of six accessions comprising four species within *Lolium-Festuca* complex (Fig. 1). The reproducibility of RAPD profiles was tested under various reaction conditions (data not shown). Under the same concentration of DNA template, *Taq* polymerase, primer and using the same temperature profile of the RAPD reaction no significant disturbances in RAPD profiles could be found.

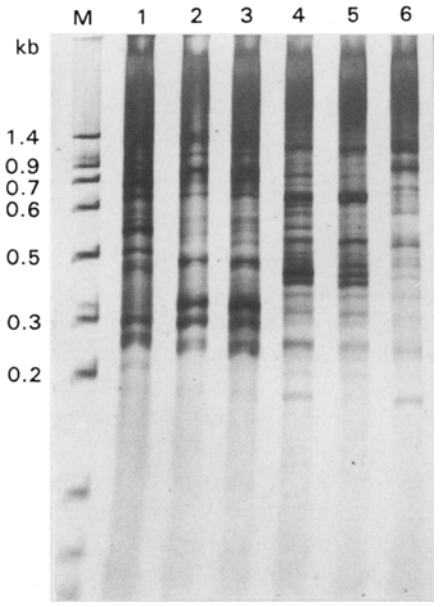

Fig. 1. Polyacrylamide gel of silver-stained RAPD products generated with primer p8. M - *Taq*I and *Sau*IIA cleaved pUC18; 1 - *Festuca arundinacea* Schreb., cv. Kora; 2 - *Festuca pratensis* Huds., cv. Rožnovská; 3 - *Festuca pratensis* Huds., cv. Otava; 4 - *Lolium perenne* L., cv. Tarpan; 5 - *Lolium perenne* L., cv. Sport; 6 - *Lolium multiflorum* Lamk., subsp. *italicum* A. Brl. Volkart, cv. Romul.

The number of bands in a profile varied depending mostly on the primer sequence (Fig. 2). The composition of a RAPD profile ranged from 30 bands (primer p10), 27 bands (primers p2 and p12) to none in profile of the primer p15. Altogether 252 RAPD bands could be considered for further statistical treatment. The important information is a number of polymorphic bands (markers) obtainable from a particular primer. Such a number defines information value of a primer. We obtained at maximum 17 polymorphic bands per primer (for primer p8) (Fig. 3).

Taxon identification: We revealed 60 potentially taxon-specific RAPD bands (markers) for genus *Festuca*, genus *Lolium*, species *F. arundinacea*, *F. pratensis*, *L. perenne*, and *L. multiflorum* (Fig. 4). The number of specific markers varied among primers. The most potent primer p2 yielded 12 specific markers (genus *Lolium* (1), *F. arundinacea* (7), *F. pratensis* (3), *L. perenne* (1)). Primer p7 gave four markers, all of them specific for *F. arundinacea*, and was thus assigned as "*F. arundinacea*-specific". For both genera and species level specific markers could be revealed.

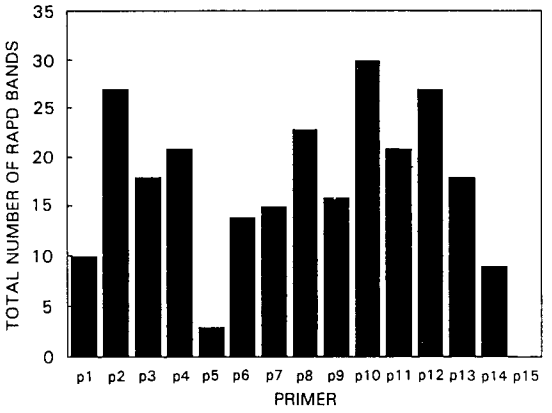

Fig. 2. Histogram of the complexity of RAPD profiles generated by individual primers.

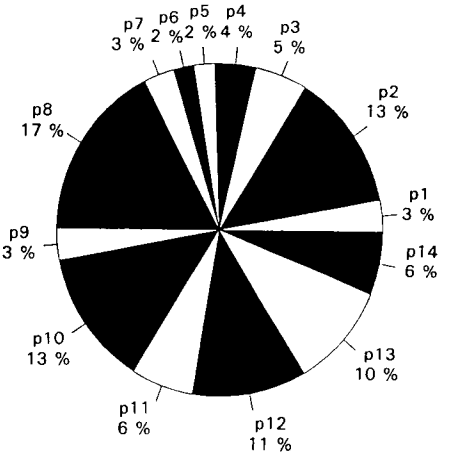

Fig. 3. Pie chart depicting the proportions in production of polymorphic RAPD bands generated by individual RAPD primers. The higher the percentage of polymorphic bands, the higher is the information (marker) value of a primer.

All studied accessions could be identified and distinguished based on their RAPD profiles.

Taxon relationships: The pairwise similarity matrix was computed between all the accessions considering RAPD bands as individual characters using the MVSP software package (Table 2). This matrix was then taken for both cluster analysis (UPGMA) and principal coordinate analysis (PCO). The average similarity coefficient found for studied *Lolium-Festuca* complex was GS = 0.666.

Table 2. Genetic similarity matrix.

	FA_K	FP_R	FP_O	LP_T	LP_S	LM_R
FA_K	1					
FP_R	0.714	1				
FP_O	0.705	0.888	1			
LP_T	0.600	0.588	0.606	1		
LP_S	0.610	0.577	0.609	0.907	1	
LM_R	0.630	0.532	0.577	0.699	0.751	1

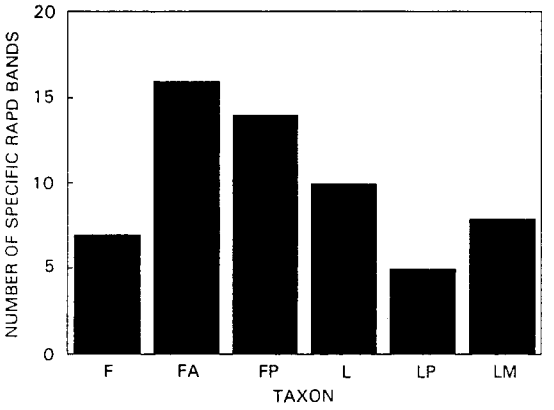

Fig. 4. Histogram of taxon-specific RAPD bands for individual taxa. F - genus *Festuca*; FA - *Festuca arundinacea* Schreb.; FP - *Festuca* pratensis Huds.; L - genus *Lolium*; LP - *Lolium perenne* L.; LM - *Lolium multiflorum* Lamk, subsp. *italicum* A. Brl. Volkart.

Dendrogram from all RAPD profiles, *i.e.* the graphic output of UPGMA cluster analysis (Fig. 5) showed full separation of the two genera *Lolium* and *Festuca* (GS = 0.592).

Inter-species relationships were reflected as expected: for *F. arundinacea* - *F. pratensis* GS = 0.710 and very close to this value was the similarity of *L. perenne* and *L. multiflorum* (GS = 0.725).

Intra-species relationships did not reflect the distances expected from the breeding relationships. For *F. pratensis* cv. Rožnovská vs. Rožnovská-related cv. Otava we found lower GS = 0.888 than for the cultivars of different ploidy level with no reported breeding relationships, *i.e.* 4x versus 2x, *L. perenne* cv. Tarpan *versus* cv. Sport, respectively (GS = 0.907).

As various RAPD primers may prime DNA amplification in distant regions of a genome it was interesting to analyze profile of each primer individually. Three groups of primer-related profiles could be distinguished (dendrograms not shown), which fully, in-part or at no level corresponded with morphological classification supporting *Lolium-Festuca* generic separation. Dendrograms derived from RAPD

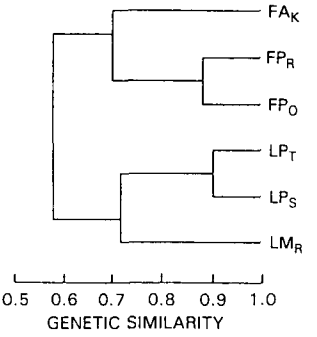

Fig. 5. Dendrogram of *Lolium* and *Festuca* accessions constructed by the UPGMA cluster analysis based on genetic similarity coefficient (GS) calculated from RAPD data pooled from 15 primer-profiles.

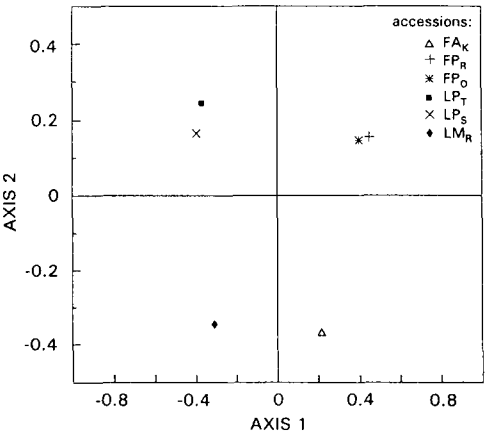

Fig. 6. Associations between *Lolium* and *Festuca* accessions revealed by principal coordinate analysis (PCO) based on genetic similarity coefficients (GS) calculated from RAPD data pooled from 15 primer-profiles. FA$_K$ - *Festuca arundinacea* Schreb., cv. Kora; FP$_R$ - *Festuca pratensis* Huds., cv. Rožnovská; FP$_O$ - *Festuca pratensis* Huds., Otava; LP$_T$ - *Lolium perenne* L., cv. Tarpan; LP$_S$ - *Lolium perenne* L., cv. Sport; LM$_R$ - *Lolium multiflorum* Lamk, subsp. *italicum* A.Brl. Volkart, cv. Romul.

profiles of primers p1, p2, and p12 fully corresponded to the classical separation of genera and species and reflected intra-species relationships as expected according to the breeding history. Primers p8, p10, and p13 separated genera and species accordingly (positions of species in dendrograms not intermixed), while intra-specific relationships were contrary to breeding relationships. In-part correspondence was found for primer p14, where only genera were separated (species were intermixed in

dendrogram). Primer p7 reflected only the close intra-species relationships. Dendrograms derived from profiles of primers p3 and p5 did not express any consistency with the existing classification of *Lolium-Festuca* complex.

It is worth to compare results from various methods of multivariate analysis to verify conclusions. Therefore, besides the cluster analysis we applied also the principal coordinate analysis (PCO) based again on the matrix derived from genetic similarity coefficients (GS). Using PCO we processed the data pooled from all RAPD profiles (Fig. 6). The first three principal coordinates accounted for 48 %, 23 %, and 17 % of total variation, respectively. The genera *Lolium* and *Festuca* were clearly separated in the projection onto the first principal coordinate (axis 1). The inter-species and intra-species relationships were well reflected according to the second coordinate (axis 2) (Fig. 6).

The results of PCO fully corresponded with the established systematics based mainly on morphological traits.

Discussion

At present, DNA sequence data reported for the taxa of *Lolium-Festuca* complex are very limited according to the search of the GenBank (Benson *et al.* 1993), last release 83.00 (June 1994). The only sequences available are that of allergen *LolpI*, *LolpII*, *rps4* gen (*L. perenne*), 26S rRNA gene (*F. arundinacea*), *rps4* gene (*F. pratensis*), and *rps4* gene (*F. gigantea*). *rps4* gene cluster represents the start of sequence-phylogenetic study with yet no published results (Lejeune, unpublished). Sequence data were still published by Perez-Vicente *et al.* (1992), who reported two species-specific sequences for *Lolium multiflorum* (173 nucleotides long fragment) and for *Festuca arundinacea* (102 nucleotides long fragment).

Traditional RFLP and recently developed DNA typing using repetitive sequences require to have a knowledge of sequence or "polymorphic" probe in hand to monitor and exploit DNA polymorphism while RAPD technique is well applicable even on the analysis of fully unknown genomes without need for specific probe (Munthali *et al.* 1992). Clearly, RAPD technique is for its speed and simplicity the method of choice for taxonomic and phylogenetic studies on the molecular level (Arnold *et al.* 1991).

In this paper we applied RAPD technique on the genotype identification and on the systematics of *Lolium-Festuca* complex. The total number of RAPD bands in profiles generated by various primers differed substantially. This is in accordance with the model, where a primer may direct the amplification of a quite different part of genome (Tingey and Tufo 1993). The reason remains unclear why we found no extreme for primers p3 and p4 (shorter octamers in comparison with the rest of decamer primers), which should match, by a simple statistics, in much higher frequency within genome.

The average frequency of polymorphic RAPD bands was reported to be 0.3 per primer in *Arabidopsis thaliana*, 0.5 per primer in *Glycine max*, 1 per primer in *Zea mays*, and 2.5 per primer in *Neurospora crassa* (Tingey and Tufo 1993). We

obtained the average frequency of 6.5 polymorphic bands per primer within six genotypes of *Lolium-Festuca* complex. We assume two reasons are responsible for such a high frequency. First, reported frequencies correspond to intra-specific polymorphism while we investigated polymorphism up to the inter-genera level. Second, mostly agarose gel electrophoresis with ethidium bromide staining of RAPD bands was used instead of more informative polyacrylamide gel with highly sensitive silver staining we used for *Lolium-Festuca*.

In general, UPGMA cluster analysis together with principal coordinate analysis (PCO) done on pooled RAPD data showed sharp separation of the genera *Lolium* (represented by the cross-pollinated species) and *Festuca* (represented by two species of sect. *Bovinae*). This dendrogram expressed very similar relationships to dendrogram based on protein data (Bulińska-Radomska and Lester 1988) with the exception that by protein profiles *F. pratensis* was classified closer to *Lolium* giving thus contrary to the current RAPD data support of common generic status for species of *Festuca* sect. *Bovinae* together with cross-pollinated species of *Lolium*.

Dendrogram derived from chloroplast DNA (cpDNA) restriction site variation classified *F. arundinacea* closer to *L. perenne* than to *F. pratensis*, what again supports the realignment of *Lolium* and *Festuca* (Darbyshire and Warwick 1992). Contrary to these results our dendrogram constructed from the pooled RAPD data (Fig. 5) supports separation of these two genera in accordance to the morphological characters. Interestingly, after cpDNA data, *L. perenne* has position closer to *F. arundinacea* than to *F. pratensis* while according to the seed protein data *L. perenne* is closer to *F. pratensis* than to *F. arundinacea*. Different clustering using seed protein polymorphism and/or cpDNA polymorphism may reflect evolutionary differences of these two genomes.

The separation of *L. perenne* and *L. multiflorum* is common for both protein (Bulińska-Radomska and Lester 1988) and our RAPD data supporting the species status according to both methods of multivariate analysis.

Morphological characters separate the genera *Lolium* and *Festuca* from each other while molecular markers tend to combine *Lolium* and *Festuca* sect. *Bovinae* into probably monophyletic new genus *Lolium* comb. nova (Darbyshire 1993). The explanation for this controversion may be accepted that genes coding for morphological and/or molecular markers are located at distant regions of a genome or even originate from different genomes (cpDNA). As various sequence regions may be of a different phylogenetic history, the inconsistent distances are often obtained when comparing the taxa by different types of markers. Individual RAPD markers amplify together various regions of nuclear, plastide, and mitochondrial genomes and hence some dendrograms constructed from individual single-primer RAPD profiles support classification based on morphological traits while others are consistent with already reported molecular data or even completely inconsistent with any existing classification. Interestingly, dendrogram after the profile of primer p11 gives relationships quite similar to dendrogram based on cpDNA data (Darbyshire and Warwick 1992).

In general, herein presented RAPD data support classification system of *Lolium-Festuca* complex based mainly on morphological characters.

We can conclude, that RAPD technique was shown to be powerful tool for displaying DNA polymorphism within *Lolium-Festuca* complex and that RAPD constitutes promising strategy toward generation of taxon-specific markers.

RAPD markers thus represent new type of molecular characters useful in investigation of taxonomic and phylogenetic relationships within *Lolium-Festuca* complex.

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