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Computer-aided RAPD fingerprinting of accessions from the ryegrass–fescue complex

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SUMMARY

RAPD (Randomly Amplified Polymorphic DNA) assay of 32 cultivar accessions from the ryegrass–fescue (*Lolium–Festuca*) complex was accomplished using ten decamer primers to assess (i) the power of RAPD technology to discriminate between individual commercial accessions and to produce cultivar fingerprinting, (ii) the degree of relatedness of accessions based on RAPD profiles in comparison with other existing classifications, and (iii) the possibility of automation of RAPD technology.

The variation of the correlation coefficient *r* as the primary output from the automated RAPD-profile processing summarizes variability derived from DNA isolation, the RAPD reaction, and final computer-image processing of RAPD profiles. The *AII* (Accession Identity Interval) of *r* for accession *Festuca arundinacea* cv. Lekora was determined experimentally and the value obtained was accepted as a valid interval for all the other accessions studied. In order to evaluate the discrimination potential of all ten primers together, a pooled-similarity matrix was computed. Employing this approach, we achieved 100% discrimination between all 35 accessions when using all ten primers. A dendrogram for all 35 accessions was obtained using average linkage cluster analysis (UPGMA – Unweighted Pair Group Method with Arithmetic Means). This procedure successfully produced smaller groups of higher taxonomic homogeneity. The relationships between the *Lolium–Festuca* accessions were also revealed by principal coordinate analysis (PCO) based on absorbance profiles from the RAPD assay. Again, all accessions were well separated, recognising even subspecies relationships. In general, PCO analysis confirmed the inferences made from the UPGMA method.

We successfully applied the computer-aided system of RAPD assay, based on an IBM PC computer, for discrimination of cultivars as well as for description of DNA-based relationships of accessions from various taxonomic groups of the *Lolium–Festuca* complex.

INTRODUCTION

There are good reasons for the incorporation of molecular markers into ryegrass–fescue (*Lolium–Festuca*) breeding programmes: (i) The construction of new fescue or ryegrass cultivars depends upon the selection and exploitation of the natural genetic variation of closely related grass genomes (Thomas & Humphreys 1991). Conventional plant improvement depends to a large extent upon the availability and possibility of monitoring existing natural variation, for which molecular markers are an excellent tool (Virk *et al*. 1995); (ii) In the development of core collections of genetic resources (Brown 1989), and in order to ensure accessibility of genetic resources for grass breeders, the analysis of molecular markers will help to identify the most promising accessions for breeding programmes; (iii) A candidate cultivar of fescue or ryegrass can be included in the 'List of Varieties' only when it complies with certain standards for distinctness, uniformity and stability. The corresponding testing programme may, therefore, have to be very comprehensive and, with an increasing number of registered cultivars, distinctness may be difficult to establish using morphological characters only. Molecular, especially DNA-based, markers are very powerful tools for solving this problem (Phillips & Vasil 1994); (iv) The *Lolium–Festuca* complex of related species provides a wide range of variability in traits for the development of versatile grasses adapted

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Table 1. *Accessions from* Lolium–Festuca *complex used in RAPD assay*

Ploidy level	Cultivar	Country	Breeding station	Number in array
		Lolium multiflorum Lam. ssp. *westerwoldicum* Wittm.		
2x	Rožnovský	Czechia	VST Rožnov	4
2x	Weldra	Holland	van der Have	22
2x	Vitesse	Holland	van der Have	3
2x	Limella	Germany	DSV	35
4x	Jivet	Czechia	ŠS Hladké Životice	7
4x	Andy	Holland	Limagrain Genetics	8
4x	Barspectra	Holland	Barenburg	2
4x	Kasjana	Poland	SHR Poturzyn	25
		Lolium multiflorum Lam. ssp. *italicum* (A. Braun) Volkart ex Schinz et Keller		
2x	Romul	Czechia	VÚZA Hrušovany	28
2x	Atalja	Denmark	Dansk Planteforaedling	16
2x	Bartolini	Holland	Barenburg	20
2x	Limulta	Germany	DSV	10
4x	Lolita	Czechia	ŠS Hladké Životice	18
4x	Jiskra	Czechia	ŠS Hladké Životice	15
4x	Bofur	Denmark	Dansk Planteforaedling	27
4x	Danergo	Denmark	Dansk Planteforaedling	21
		Lolium perenne L.		
2x	Bača	Czechia	ŠS Paluďn	24
2x	Sport	Czechia	ŠS Větrov	17
2x	Algol	Czechia	ŠS větrov	14
2x	Barlet	Holland	Barenburg	11
4x	Tarpan	Czechia	ŠS Hladké Životice	5
4x	Mustang	Czechia	ŠS Hladké Životice	19
4x	Basation	Holland	Mommersteeg	1
4x	Castle	Holland	Mommersteeg	12
		Festuca pratensis Huds.		
2x	Otava	Czechia	ŠS Větrov	6
2x	Rožnovská	Czechia	VST Rožnov	9
2x	Poseidon	Germany	NPZ H.-G. Lembke	13
2x	Capella	Germany	NPZ H.-G. Lembke	31
2x	BUF	Czechia	VST Zubří	30
		Festuca arundinacea Schreb.		
6x	Lekora	Slovakia	ŠS Levočské Lúky	26
6x	Kora	Czechia	ŠS Hladké Životice	23
6x	Stef	Poland	SHR Szelejevo	29

for turf or forage purposes. Moreover, combining the genomes of these species could be an effective means of producing hybrid derivatives of high agronomic potential. The ready availability of interspecific hybrids between *Lolium* and *Festuca* may permit the recognition of useful combinations of parental traits (Thomas & Humphreys 1991), but the identification of grass cultivars, parental genomes, and their hybrids may be quite ambiguous without using molecular markers or genomic *in situ* hybridization (GISH) on mitotic chromosome preparations (Humphreys & Pasakinskiene 1996).

Lolium and *Festuca* genomes, molecular (mostly protein, isozyme and DNA-based) markers have been used to distinguish between species and cultivars, to study population dynamics or to reveal taxonomic relatedness (for review of references, see Wiesner *et al.* (1995)). Within the DNA-based markers, effective RAPD technology based on polymerase chain reactions (PCR) primed with one short primer (Smith & Williams 1994; Virk *et al.* 1995) was widely adopted. The RAPD patterns may be regarded as empirical identifiers analogous to fingerprints. The RAPD technique is thus useful as a quick and readily utilizable technique in addition to the more traditional characteristics currently used for the assignment of Plant Variety Rights, seed certification and germplasm management. RAPD-based phylogenetic analysis was made on a set of 16 wild accessions from the *Lolium–Festuca* complex using three decamer primers (Stammers *et al.* 1995).

Previous experiments have shown that the classifi-

cation of germplasm from the *Festuca–Lolium* complex can be accomplished successfully using RAPD fingerprinting (Wiesner *et al*. 1995) with the technological modification, DNA amplification fingerprinting, sometimes abbreviated as DAF (Caetano-Anollés 1994). Moreover, RAPD profiles were shown to provide enough information to identify *Lolium* × *Festuca* hybrid genomes. Even a portion of both parental genomes (festucoid *v*. loloid hybrid nature) could be recognized (I. Wiesner *et al*., unpublished).

In the present study we replaced the time-consuming DAF method with a rapid conventional RAPD assay and performed a RAPD assay of 32 cultivar accessions from the *Lolium–Festuca* complex using ten decamer primers to assess the power of RAPD technology to discriminate between individual commercial accessions and to develop cultivar fingerprinting technology, to test the relatedness of accessions based on RAPD profiles in comparison with those achieved by existing classification methods and to consider the possible automation of RAPD technology.

MATERIALS AND METHODS

Plant material

The test array included 35 entries (Table 1) representing 32 accessions from the *Lolium–Festuca* complex and three *Medicago* species, i.e. *M. sativa* ssp. *coerulea*, *M. sativa* ssp. *quasifalcata* and *M. truncatula* (which are model genomes used in the construction of genetic maps of diploid lucerne (Kiss *et al*. 1993; Sagan *et al*. 1995) in order to analyse and compare the RAPD patterns of distant genomes.

DNA isolation

Total DNA samples were extracted from etiolated 10-day-old leaves. A DNA extract, representative of each accession, was prepared by pooling equal volumes of ten individual DNA samples. The samples were taken from ten individual plantlets each of which originated from seed. A modified procedure of the ultrafast NaOH method of Wang *et al*. (1993) was used for DNA isolation. Leaf tissue (50 mg) was ground for 5 min in a 1·5 ml Eppendorf tube in 200 µl 0·5 M NaOH on ice. After brief spinning, 5 µl of recovered supernatant was mixed with 95 µl 0·1 M TRIS-HCl (pH 8·0) in a 0·5 ml Eppendorf tube. For storage, samples were frozen in liquid nitrogen in aliquots and stored at −70 °C. DNA concentration was measured using Hoechst 33258 fluorescent dye (Cesarone *et al*. 1979).

RAPD reaction

Ten decamer oligonucleotides (Operon Technologies) were first scored for high complexity and maximum 'among-genomes' variation in previous adjustments of the RAPD assay. The best primers were then utilized for amplification of random DNA sequences of all accessions. RAPD reaction mixture (25 µl) contained 10 mM TRIS-HCl (pH 8·3), 4 mM $MgCl_2$, 240 µM of each dNTP, 0·2 µM of primer (see Table 2 for sequences), 25 ng of total DNA (pooled sample representative of each accession, see above), and 1 U *Taq* polymerase (Fermentas) overlaid with mineral oil. Amplification was conducted in a DNA thermal cycler 480 (Perkin Elmer Cetus). The amplification conditions were as follows: 35 cycles each consisting of a denaturation step of 20 s at 94 °C followed by an annealing step of 1 min at 36 °C and an extension step of 2 min at 72 °C. The last 15 extension steps were progressively extended by 5 s/cycle. The last cycle was followed by 10 min at 72 °C to ensure that primer extension reactions proceeded to completion. RAPD profiles were generated in 2% ethidium bromide agarose gel with $\lambda/PstI$ DNA marker as internal standard.

Computer-aided system of RAPD assay

Computer-aided comparisons and statistical analyses of RAPD gel patterns were carried out using GelManager version 1.5 for Windows (BioSystematica, UK). Electronic RAPD images were obtained from photographs of gel patterns by scanning at 300 dpi resolution with HP ScanJetIIP and stored as TIFF-format files for further processing. Automated digitization was complemented with the exploitation of a full-image information of the RAPD profiles by taking into account the continual interval of a gel track and not just discrete selected bands. Absorbance profiles of RAPD patterns of individual accessions were computer-compared by correlation coefficients between each two absorbance profiles, resulting in a similarity matrix. The correlation coefficient (r) was calculated according to the following formula (Jackman 1994):

$$r = \frac{\Sigma^n[(Y_{ij} - Y_{jav})(Y_{ik} - Y_{kav})]}{\sqrt{\{[\Sigma^n((Y_{ij} - Y_{jav})^2][\Sigma^n(Y_{ik} - Y_{kav})^2]\}}} \qquad (1)$$

where for two absorbance profiles j and k of n points, Y = profile height (absorbance value), Y_{av} = average profile height, $r = 0$ denotes absolute difference, while $r = 1$ denotes identity of accession profiles.

A total of 14 independent DNA isolates from 14 plantlets of cv. Lekora were RAPD-assayed using primer OPX–13, resulting in a similarity matrix of 91 correlation coefficients (r_i). Maximal and minimal values of r_i determined the upper (r_{max}) and lower limits (r_{min}) of the accession-identity interval (*AII*). For the determination of error statistics of the accession-identity interval (*AII*) correlation coefficient data were normalized according to the following formula:

$$r_i(\text{norm}) = \arcsin\sqrt{r_i} \qquad (2)$$

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Table 2. *Sequences of Operon primers used in RAPD assay*

Primer	Sequence
OPX–06	ACGCCAGAGG
OPX–13	ACGGGAGCAA
OPX–01	GTGGCATCTC
OPY–02	CATCGCCGCA
OPY–04	GGCTGCAATG
OPY–05	GGCTGCGACA
OPY–13	GGGTCTCGGT
OPY–17	GACGTGGTGA
OPY–18	GTGGAGTCAG
OPY–20	AGCCGTGGAA

Probability $P(r < r_{min})$ for two accessions of the same cultivar (error statistic of *AII*) was then calculated by the integration of the normal distribution curve using STATGRAPHICS version 7.0.

For clustering of accessions, the average linkage cluster analysis (UPGMA – Unweighted Pair Group Method with Arithmetic Means) method was used (Jackman 1994). Principal coordinate analysis (PCO) was calculated using the MVSP PLUS version 2.1 software package for multivariate analysis (Kovach Computing Services, Pentraeth, UK).

RESULTS AND DISCUSSION

Experimental determination of accession-identity interval (AII)

The variation in the correlation coefficient r as the primary output from computer-aided RAPD-profile processing (see Materials and Methods) summarizes the variability derived from DNA isolation, the variability of the RAPD reaction, and the variability of the final computer-image processing of the RAPD profile.

We estimated experimentally the *AII* for the set of accessions on 14 sample genotypes of *Festuca arundinacea* cv. Lekora assuming that the *AII* obtained is was also valid for the other accessions studied. A total of 14 independent DNA isolates from 14 plantlets of cv. Lekora were RAPD-assayed using primer OPX–13, resulting in a similarity matrix. The absolute minimum of the correlation coefficient (r_{min}) within this matrix was 0·74. r_{min} was then interpreted as the lower limit of the experimentally determined accession-identity interval (*AII*) within which any two accessions were considered to be identical according to their RAPD profiles. Hence, a profile comparison of any two accessions resulting in $r \geqslant 0{\cdot}74$ means that those two accessions are considered to be indistinguishable on the actual experimental background ($P = 0{\cdot}054$) because $AII = \langle 0{\cdot}74;\ 0{\cdot}99 \rangle$.

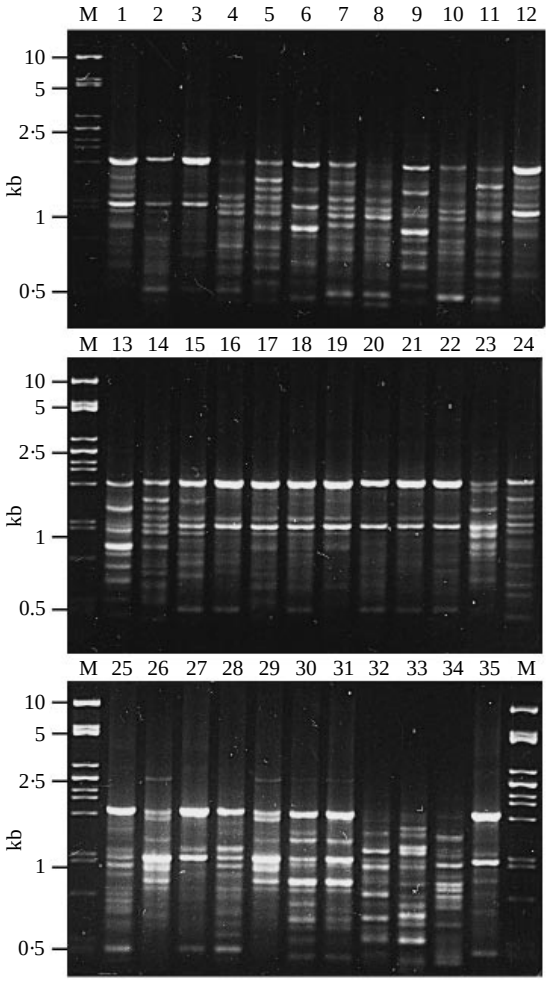

Fig. 1. RAPD polymorphism of 32 accessions from the *Lolium–Festuca* complex and 3 dicot *Medicago* accessions generated by primer OPX–13. Lane numbers correspond to the accession numbers presented in Table 1. M = DNA molecular weight marker ($\lambda/PstI$).

Discrimination between accessions

The computer-aided RAPD-assay system was used to analyse, using each of ten RAPD primers separately, a set of 35 accessions from the *Lolium–Festuca* complex (Table 1) and from the reference dicot genus *Medicago* (see Materials and Methods; Fig. 1). Ten primer-specific similarity matrices were computed, each of which comprised all 35 accessions. In order to distinguish one accession from the others, all its correlation coefficients (r) in the primer-specific matrix should be $r \leqslant 0{\cdot}74$ to be considered as a separate genome by that particular primer. This approach allows the evaluation of each primer for its potential to distinguish an accession from the remaining 34

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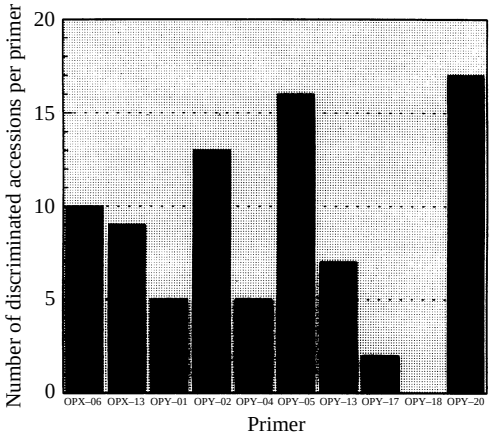

Fig. 2. Histogram of the discrimination potential of ten different RAPD primers expressed as the number of fully discriminated accessions per primer.

accessions or for its potential to carry out the identification test (Fig. 2).

Applying this criterion, primer OPY–20 was found to be the most powerful because it resolved unambiguously 48·6 % of all accessions. In contrast, primer OPY–18 produced such similar profiles that no accession could be identified at all. On average, one RAPD primer was able to resolve 24 % of accessions unambiguously. Clearly, it is worth performing a preliminary screening of primers for their discrimination potential before any large set of individuals is to be analysed. The total variation coefficient (V) of the number of accessions discriminated per primer was 67·9 %.

In order to evaluate the discrimination potential of all ten primers together, a pooled-similarity matrix was computed, collecting in each position the absolute minimum value of r from screening all ten primer-specific matrixes. Applying this approach, we found that all r within the pooled matrix were 0·74, implying 100 % discrimination between all 35 accessions ($P = 0{\cdot}054$) when using all ten primers.

Relationships among accessions

In general, the RAPD-phylogeny approach is appropriate for closely evolutionary related genomes, due to the possible occurrence of similar-sized RAPD bands from different regions of a genome, referred to as the convergence, and also due to the possible repetitive status of some RAPD bands (Stammers *et al.* 1995). Fortunately, genomes from within the *Lolium–Festuca* complex are very closely related, as deduced both from protein-marker data (Buliňska-Radomska & Lester 1988) and from genomic *in situ* hybridization (Humphreys *et al.* 1995, Humphreys & Pasakinskiene 1996). DNA-base composition distributions appear to be very useful in distinguishing taxa below tribal rank within the *Poaceae* and provide an effective means of identifying species (Wei & Wang 1995). The close relationship of *Lolium* and *Festuca*, indicated by the ease with which they form intergeneric hybrids, is confirmed by their nearly identical DNA-base composition distributions (King & Ingrouille 1987). Thus, the *Lolium–Festuca* complex matches the criteria for phylogenetic interpretations of RAPD data.

The increasing size of germplasm collections means that advanced statistical methods are needed to classify and measure the variability involved. The multivariate UPGMA clustering method generally yields results which are the most accurate for classification purposes (Rohlf & Wooten 1988). This method was also that used in GelManager software.

Applying the UPGMA clustering procedure, the dendrogram for all 35 accessions was computed (Fig. 3*a*) to display their relationships. The method successfully produced smaller groups of higher taxonomic homogeneity. A general objection to the clustering procedure is that changing the input order of objects into the clustering process may lead to objects being differently placed within clusters (Lespérance 1990). In order to check and demonstrate reproducibility in the clustering process, the data clustering procedure was repeated several times on the same data matrix with randomized data input (procedure 'randomize' in the mvsp software). Comparison of alternative dendrograms (for example Fig. 3*b*) demonstrated identical branching and the creation of identical clusters of accessions. The branching identity of 'randomized' dendrograms thus supported the general validity of the dendrogram presented in Fig. 3*a*, which is discussed below.

The RAPD-based clustering corresponded well with the expected greater phylogenetic distance between monocot and dicot genomes, as all three *Medicago* accessions were clustered separately from the *Lolium–Festuca* complex and classified by the lowest correlation coefficient. Even the subspecies of *M. sativa* were grouped together. However, the actual distance of monocot versus dicot genomes shown here, based on the similarity of RAPD patterns, may be affected by the possibility of evolutionary convergence (homoplasy) or concerted evolution (Stammers *et al.* 1995). These evolutionary phenomena could well occur during the history of genomic sequences, which are amplified in the RAPD reaction and which subsequently create RAPD patterns.

The dendrogram of the accessions from the *Lolium–Festuca* complex agreed with classical taxonomic clustering in that fully homogenous taxonomic groups in the dendrogram may be recognized by matching the level of genus and species. For technical reasons, all 35 accessions had to be split into two groups and analysed on two separate gels (accessions

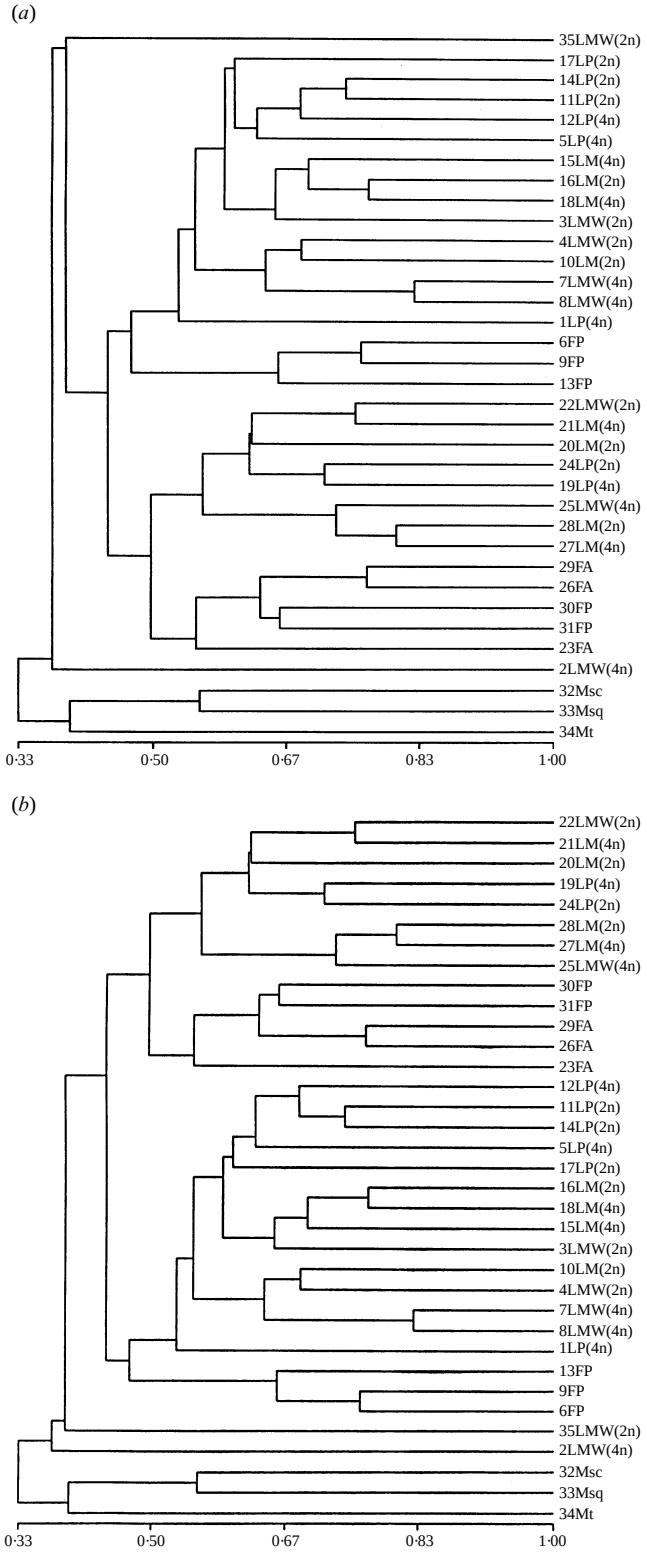

Fig. 3. For legend see opposite.

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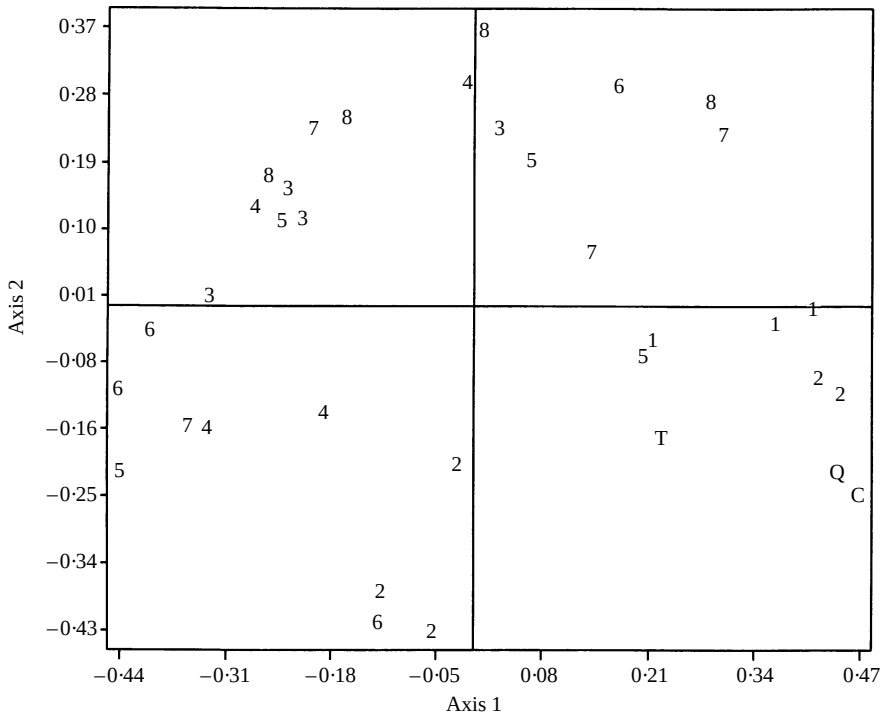

Fig. 4. Associations of accessions from the *Lolium–Festuca* complex and dicot reference genus *Medicago* generated by principal coordinate analysis (PCO) based on the correlation coefficient *r* between computer-generated absorbance profiles pooled from ten primer profiles: **1**, *Festuca arundinacea* Schreb. (2n = 6x); **2**, *Festuca pratensis* Huds. (2n = 2x); **3**, *Lolium perenne* L. (2n = 2x); **4**, *Lolium perenne* L. (2n = 4x); **5**, *Lolium multiflorum* Lam. var. *westerwoldicum* Wittm. (2n = 2x); **6**, *Lolium multiflorum* Lam. var. *westerwoldicum* Wittm. (2n = 4x); **7**, *Lolium multiflorum* Lam. ssp. *italicum* (A.BRAUN) Volkart ex Schinz et Keller (2n = 2x); **8**, *Lolium multiflorum* Lam. ssp. *italicum* (A.BRAUN) Volkart ex Schinz et Keller (2n = 4x); **T**, *Medicago truncatula*; **C**, *M. sativa* ssp. *coerulea* Ledeb.; **Q**, *M. sativa* ssp. *quasifalcata* Sinsk.

1–18 and 19–35 respectively). Hence, the observed separation of the *Festuca* accessions into two groups may reflect an imperfect correction function in the GelManager software designed to remove 'among-gel-error' during image processing.

It is believed that various species of *Festuca* as well as *Lolium* originated phylogenetically from *F. pratensis* (Jauhar 1975). From this point of view, the homogenous cluster of accession nos. 6, 9 and 13 demonstrates that the genome of *F. pratensis* still retains some of its specificity (Fig. 3). Both genomes of *F. pratensis* and *L. perenne* are involved in the complex polyploid genome of *F. arundinacea* (Borrill 1972; Nitzsche 1974; Humphreys *et al.* 1995; Humphreys & Pasakinskiene 1996). This statement is in part supported by the cluster of accession nos. 29, 26 and 23 (*F. arundinacea*) mixed with nos. 30 and 31 (*F. pratensis*).

Lolium perenne and *L. multiflorum* are phylogenetically closely related (Jauhar 1975). This is supported by the close proximity of the relevant homogenous clusters in the dendrogram. However, the specificity of genomes is also reflected (cf. positions of nos. 17, 14, 11, 12 and 5 (*L. perenne*) *v.* nos. 15, 16 and 18 (*L. multiflorum*) in Fig. 3).

L. multiflorum var. *westerwoldicum* is probably a mutated annual derivative of *L. multiflorum* (Mandy 1970). This statement is in agreement with the positions of nos. 4, 10 and nos. 21, 22 but, clearly, specific genome features are conserved as well (cf.

Fig. 3*a*, *b*. Two 'randomized' variants of a dendrogram (with identical branching) of accessions from the *Lolium–Festuca* complex and dicot reference genus *Medicago* constructed by the UPGMA cluster analysis based on the correlation coefficient (*r*) between computer-generated absorbance profiles pooled from all ten primer profiles. See Table 1 for names of accessions.

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clusters of nos. 15, 16 and 18, also nos. 7, 8, and nos. 27, 28 in the dendrogram).

Tetraploid accessions of *L. perenne* and *L. multiflorum* are synthetic derivatives of the original diploid *Lolium* species (Fojtík 1975). Close relations of *Lolium* accessions of different ploidy levels are demonstrated by two clusters: nos. 15, 16 and 18 and nos. 27, 28, respectively.

The dendrogram positions of accessions no. 2 and no. 35 (tetraploid and diploid *Lolium multiflorum* Lam. var. *westerwoldicum* Wittm.) are unexpectedly located at some distance from the remaining *Lolium–Festuca* complex as well as from one another. The reason for this remains unknown. With this one exception, we conclude that our data from the RAPD assay processed via the UPGMA method are in a good agreement with classical taxonomic classification methods.

It is worthwhile to compare results from various methods of multivariate analysis to verify these conclusions. Therefore, the association among *Lolium–Festuca* accessions was also tested by principal coordinate analysis (PCO) based on absorbance profiles from the RAPD assay (Fig. 4). The first three principal coordinates accounted for 15·1, 8·7 and 7·2% of the total variation, respectively. Again, dicot accessions were well separated even with respect to subspecies relations. For the *Lolium–Festuca* complex, PCO analysis confirmed the results of the UPGMA method.

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