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COMPUTER-AIDED FINGERPRINTING OF ACCESSIONS FROM RYEGRASS-FESCUE COMPLEX

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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 among individual commercial accessions and to produce cultivar fingerprinting, (ii) the relatedness of accessions based on RAPD profiles in comparison with other existing classifications, and (iii) the possibility of automation of the RAPD technology.

The variation of correlation coefficient *r* as the primary output from the automated RAPD-profile processing summarizes variability coming 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 to be a valid interval for all the other accessions studied.

To evaluate the discrimination potential of all ten primers together, the pooled-similarity matrix was computed. Employing this approach, we achieved 100% discrimination of all 35 accessions when using all ten primers.

A dendrogram for all 35 accessions was obtained using the UPGMA clustering procedure. The clustering method successfully produced smaller groups of higher taxonomic homogeneity.

The relationships between *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, respecting even subspecies relations. 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.

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INTRODUCTION

There are good reasons to incorporate molecular markers into ryegrass-fescue (*Lolium-Festuca*) breeding programmes:

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).

In developing core collections of genetic resources (Brown 1989) 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.

A candidate cultivar of fescue or ryegrass can be included in the "List of Varieties" only when it complies with standards for distinctness, uniformity, and stability. The corresponding testing programme may be very comprehensive and with increasing number of registered cultivars, distinctness may be difficult to establish solely with morphological characters. The molecular, especially DNA-based, markers are very potent tools to solve this problem (Phillips & Vasil 1994).

The *Lolium-Festuca* complex of related species provides a wide range of variability in traits for the development of versatile grasses adapted to meet requirements 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. Abundant interspecific hybrids between *Lolium* and *Festuca* may demonstrate useful combinations of parental traits (Thomas & Humphreys 1991), but identification of grass cultivars, parental genomes, and their hybrids may be quite ambiguous without using molecular markers.

For *Lolium* and *Festuca* genomes, molecular (mostly protein, isozyme, and DNA-based) markers have been used to distinguish 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 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 accomplishable technique 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 *Lolium-Festuca* complex using three decamer primers (Stammers *et al.* 1995).

Our previous experiments showed that classification of culture germplasm from *Festuca-Lolium* complex could be successfully accomplished using RAPD fingerprinting (Wiesner *et.al.* 1995) with the technological modification, abbreviated sometimes as AFLP (Caetano-Anollés 1994). Moreover, RAPD profiles were shown to provide enough information to identify *Lolium x*

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 time-consuming AFLP with rapid conventional RAPD assay and performed RAPD assay of 32 cultivar accessions from *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, the relatedness of accessions based on RAPD profiles in comparison with those achieved by existing classification methods and possible automation of the RAPD technology.

MATERIALS AND METHODS

Plant material

The test array included 35 entries (Tab.1) representing 32 accessions from the *Lolium-Festuca* complex and three *Medicago* species, i.e. *M.sativa ssp.coerulea, M.s.ssp.quasifalcata,* and *M.truncatula* (which are model genomes used in the construction of genetic map of diploid alfalfa (Kiss *et al.* 1993; Sagan *et al.* 1995)) in order to analyse and compare jointly RAPD similarities of monocot and dicot genomes.

DNA isolation

Total DNA samples were extracted from etiolated 10-day-old leaves. DNA sample representative for each accession was prepared by pooling equal volumes of ten individual DNA samples each diluted to the same DNA concentration and originated from one individual plantlet derived from different seed of a named cultivar. We modified ultrafast NaOH method of Wang *et al.* (1993) for DNA isolation. Briefly, leaf tissue (50mg) was ground for 5min in 1.5mL Eppendorf tube in 200µL 0.5M NaOH on ice. After brief spinning 5µL of recovered supernatant was mixed with 95µL 0.1M TRIS-HCl (pH 8.0) in a 0.5mL Eppendorf tube. For longer storage, samples were frozen in liquid nitrogen in aliquots and stored at -70^{o}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 (25µL) contained 10mM TRIS-HCl (pH 8.3), 4mM $MgCl_2$, 240µM of each dNTP, 0.2µM of primer (see Tab. 2 for sequences), 25ng of total DNA (pooled sample representative for an accession, see above), and 1U *Taq* polymerase (Fermentas) overlaid with mineral oil. Amplification was conducted in the DNA thermal cycler 480 (Perkin Elmer Cetus). The amplification conditions were as follow: 35 cycles each consisting of a denaturation step of 20s at 94^{o}C followed by annealing step of 1min. at a 36^{o}C and extension step of 2min. at 72^{o}C. The last 15 extension steps were progressively extended by 5s/cycle. The last cycle was followed by 10min at 72^{o}C to ensure that primer extension reactions proceeded to completion. RAPD

profiles were generated in 2% ethidium bromide agarose gel with λ/PstI DNA marker as internal standard.

Computer-aided system of RAPD assay

Computer-aided comparison and statistical analysis of RAPD gel patterns were accomplished using GelManager ver.1.5 for Windows (BioSystematica, U.K.). Electronic RAPD images were obtained from photographs of gel patterns by scanning at 300dpi resolution with HP ScanJetIIP and stored as TIFF-format files for further processing. Automated digitization was compounded 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 only discrete selected bands. Absorbance profiles of RAPD patterns of individual accessions were computer-compared by correlation coefficient between each two absorbance profiles resulting in a similarity matrix. The correlation coefficient (r) was calculated according to the 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
$r = 0$ meaning absolute difference, while
$r = 1$ meaning 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 the similarity matrix of 91 correlation coefficients (r_i). Maximal and minimal values of r_i determined the upper (r_{max}) and lower limit (r_{min}) of accession-identity-interval (*AII*). For determination of error statistics of the accession-identity-interval (*AII*) correlation coefficient data were normalized according to formula:

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

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

For clustering of accessions average linkage cluster analysis (UPGMA) method was used (Jackman 1994). Principal coordinate analysis (PCO) was calculated using *MVSP Plus* ver. 2.1 software package for multivariate analysis (Kovach Computing Services, Pentraeth, Wales, UK).

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RESULTS AND DISCUSSION

Experimental determination of accession-identity interval (*AII*)

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

We estimated experimentally the *AII* for studied set of accessions on 14 sample genotypes of *Festuca arundinacea* cv. Lekora and hypothesised this to be valid identity-interval also for other accessions studied herein. A total of 14 independent DNA isolates from 14 plantlets of cv. Lekora were RAPD assayed using primer OPX-13 resulting in the similarity matrix. Absolute minimum of correlation coefficient (r_{min}) within this matrix was 0.74.

r_{min} is then interpreted as the lower limit of experimentally determined identity-interval (*AII*) within which any two accessions are considered as identical according to their RAPD profiles. Hence, a profile-comparison of any two accessions resulting in $r \geq \mathbf{0.74}$ means that those two accessions are declared as indistinguishable on the actual experimental background (P=0.054) because *AII* = **<0.74;0.99>**.

Discrimination among accessions

The computer-aided RAPD-assay system was adopted to analyze by each of ten RAPD primers separately the set of 35 accessions from the *Lolium-Festuca* complex (Tab. 1) and from the reference dicot genus *Medicago* (see Material and Methods), (Fig. 1). Ten primer-specific similarity matrixes were computed each comprising all 35 accessions. An accession to be distinguished from all others is required to have all its correlation coefficients *(r)* in primer-specific matrix $r \leq \mathbf{0.74}$ to be declared as distinguishable by that primer. This approach allows the evaluation of each primer for its potential to discriminate an accession from the remaining 34 accessions or for its potential to carry out the identification test (Fig. 2).

Applying this criterion, primer OPY-20 was revealed as the most potent because it resolved unambiguously 48.6% of all accessions. In contrast, primer OPY-18 gave such similar profiles that no accession could be identified at all. In average, one RAPD primer was able to resolve 24% of accessions unambiguously. Clearly, it is worth performing preliminary screening of primers for their discrimination potential before any large set of individuals is to be analyzed. Total variation coefficient (*V*) of number of accessions discriminated per primer was **67.9%.**

In order to evaluate the discrimination potential of all ten primers together, the 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 revealed that all *r* within the pooled matrix were $r \leq \mathbf{0.74}$ implying 100% discrimination of 35 accessions (P=0.054) when using all ten primers.

Relationships among accessions

In general, RAPD-phylogeny approach is acceptable, mostly for closely related genomes, due to the possibly occurrance of similar-sized RAPD bands from different regions of a genome referred to as the convergence and due to the possibly repetitive status of some RAPD bands (Stammers *et al.* 1995). Fortunately, genomes from within the *Lolium-Festuca* complex are very closely related as deduced from protein-marker data (Buliňska-Radomska & Lester 1988). 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, *Lolium-Festuca* complex matches the prerequisite for phylogenetic interpretations of RAPD data.

The increasing size of germplasm collections requires advanced statistical methods 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 the choice 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. There is sometimes reported the general objection to clustering procedure in that changing the input order of objects into clustering process may lead to objects differently joined to clusters (Lespérance 1990). In order to check and demonstrate eventual irreproducibility in clustering process, we repeated several-times our data clustering procedure on the same data matrix with randomize data input (procedure "randomize" in MVSP software). Comparison of alternative dendrograms (an example in Fig. 3*b*) demonstrated identical branching and creation of identical clusters of accessions. Branching identity of "randomized" dendrograms thus supported the general validity of dendrogram in Fig. 3*a* which is used for discusion below.

The RAPD-based clustering corresponded well to the expected greater phylogenetic distance between monocot and dicot genomes as all three *Medicago* accessions were clustered separately with respect to 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 may be substantially biased due to the convergence (homoplasy) phenomenon and possible presence of repetitive sequences passing concerted evolution (Stammers *et al.* 1995).

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 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 1-18, resp.19-35). Hence, observed separation of the *Festuca* accessions into two groups may reflect an imperfect correction function in 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, homogenous cluster of accessions no.6, 9, 13 demonstrates that genome of *F.pratensis* still keeps some of its specificity (Fig. 3). Both genomes of *F.pratensis* and *L.perenne* are involved in complex polyploid genome of *F.arundinacea* (Nitzche 1974; Borrill 1977). This statement is in part supported by cluster of accessions no. 29, 26, 23 (*F.arundinacea*) mixed with no.30, 31 (*F.pratensis*).

L.perenne and *L.multiflorum* are phylogenetically closely related (Jauhar 1975). That is supported by close positions of relevant homogenous clusters in dendrogram. However, the specificity of genomes is also reflected, cf. positions of no.17, 14, 11, 12, 5 (*L.perenne*) versus no.15, 16, 18 (*L.multiflorum*) in Fig. 3.

L.multiflorum var. *westerwoldicum* is probably mutated annual derivative of *L.multiflorum* (Mandy 1970). This statement is in agreement with positions of no.4, 10 and 21, 22 but clearly, specific genome features are conserved as well, cf. Clusters of no.15, 16, 18, also no.7, 8, and no.27, 28 in 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: no.15, 16, 18, and no.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 quite far from the remaining *Lolium-Festuca* complex as well as from one another. The reason for that remains unknown. With this exception we can conclude that our data from RAPD assay processed via UPGMA method are in a good agreement with classical taxonomic classification.

It is worth to compare results from various methods of multivariate analysis to verify conclusions. Therefore, association among *Lolium-Festuca* accessions was also revealed by principal coordinate analysis (PCO) based on absorbance profiles from 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 are well separated even with respect to subspecies relations. For *Lolium-Festuca* complex PCO analysis confirmed the inferences from UPGMA method.

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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	Vitessse	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 Palupín	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

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

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PRIMER	SEQUENCE
OPX-06	ACGCCAGAGG
OPX-13	ACGGGAGCAA
OPY-01	GTGGCATCTC
OPY-02	CATCGCCGCA
OPY-04	GGCTGCAATG
OPY-05	GGCTGCGACA
OPY-13	GGGTCTCGGT
OPY-17	GACGTGGTGA
OPY-18	GTGGAGTCAG
OPY-20	AGCCGTGGAA

Table 2 Sequences of OPERON primers used in RAPD assay.

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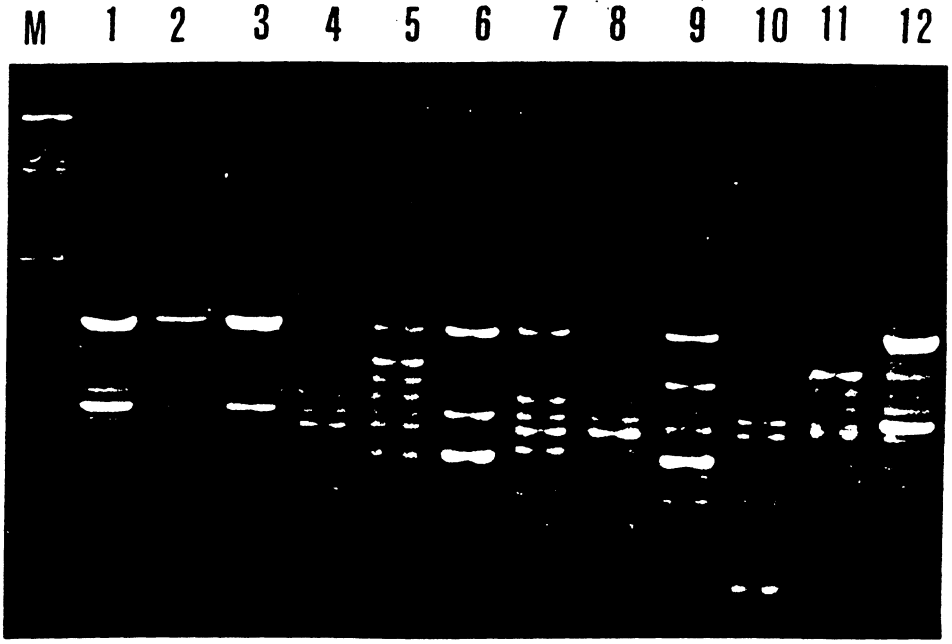

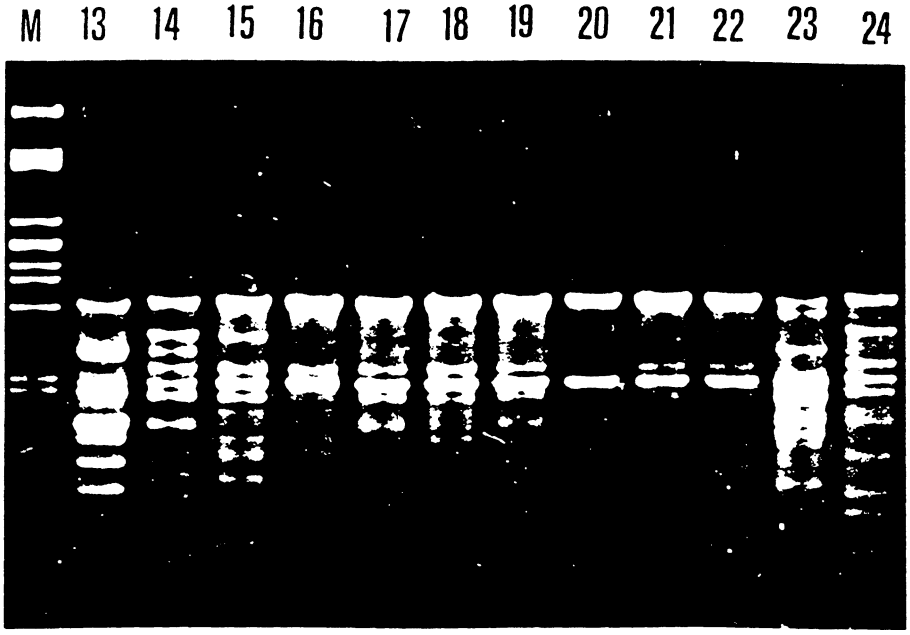

Fig. 1 RAPD polymorphism of 32 accessions from *Lolium-Festuca* complex and 3 dicot *Medicago* accessions generated by primer OPX-13 (see next page Fig. 1 (continuation)). Lane numbers correspond to the numbers of accessions presented in tab.1.

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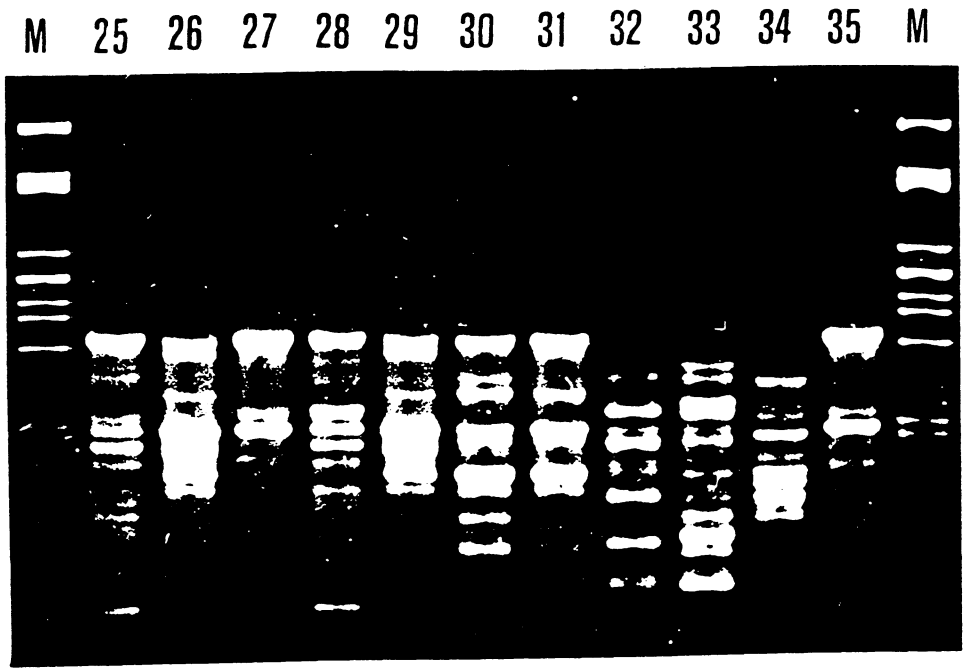

Fig. 1 (continuation)

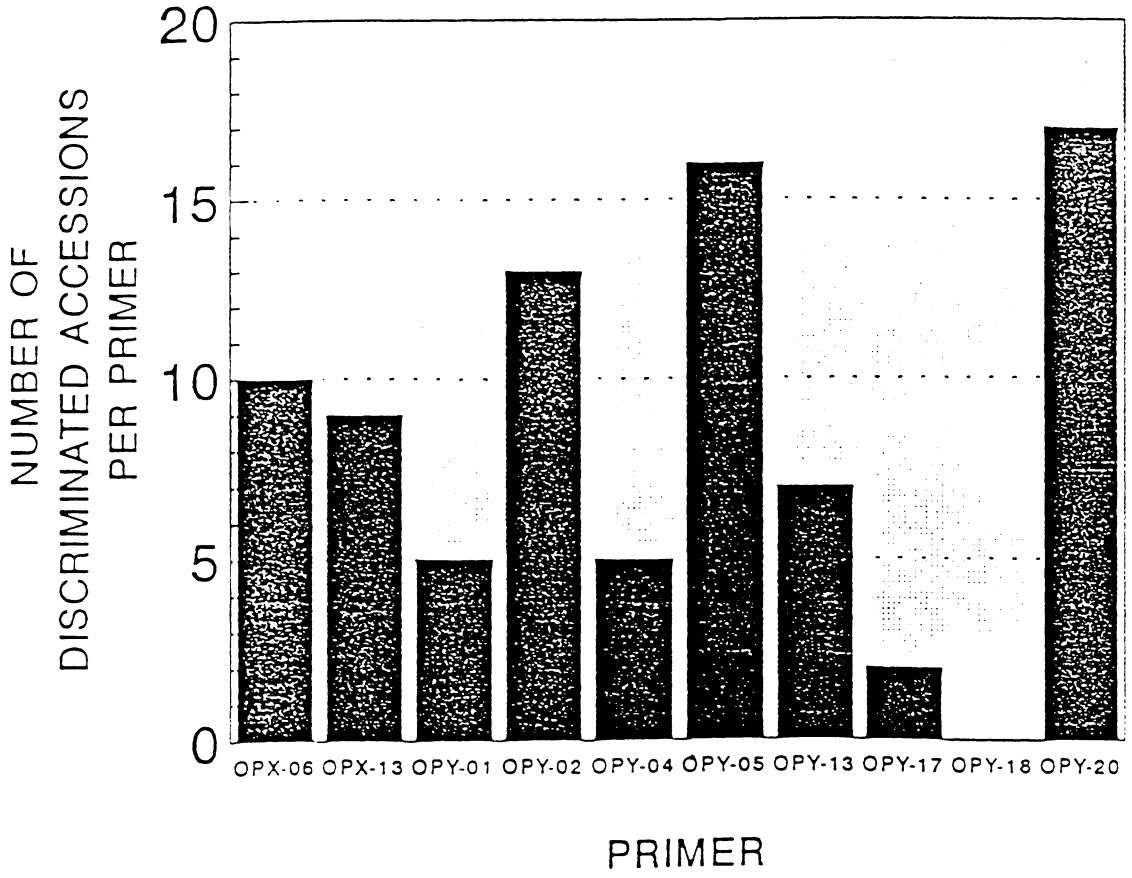

Fig. 2 Histogram of the discrimination potential of RAPD primers expressed as a number of fully discriminated accessions per primer.

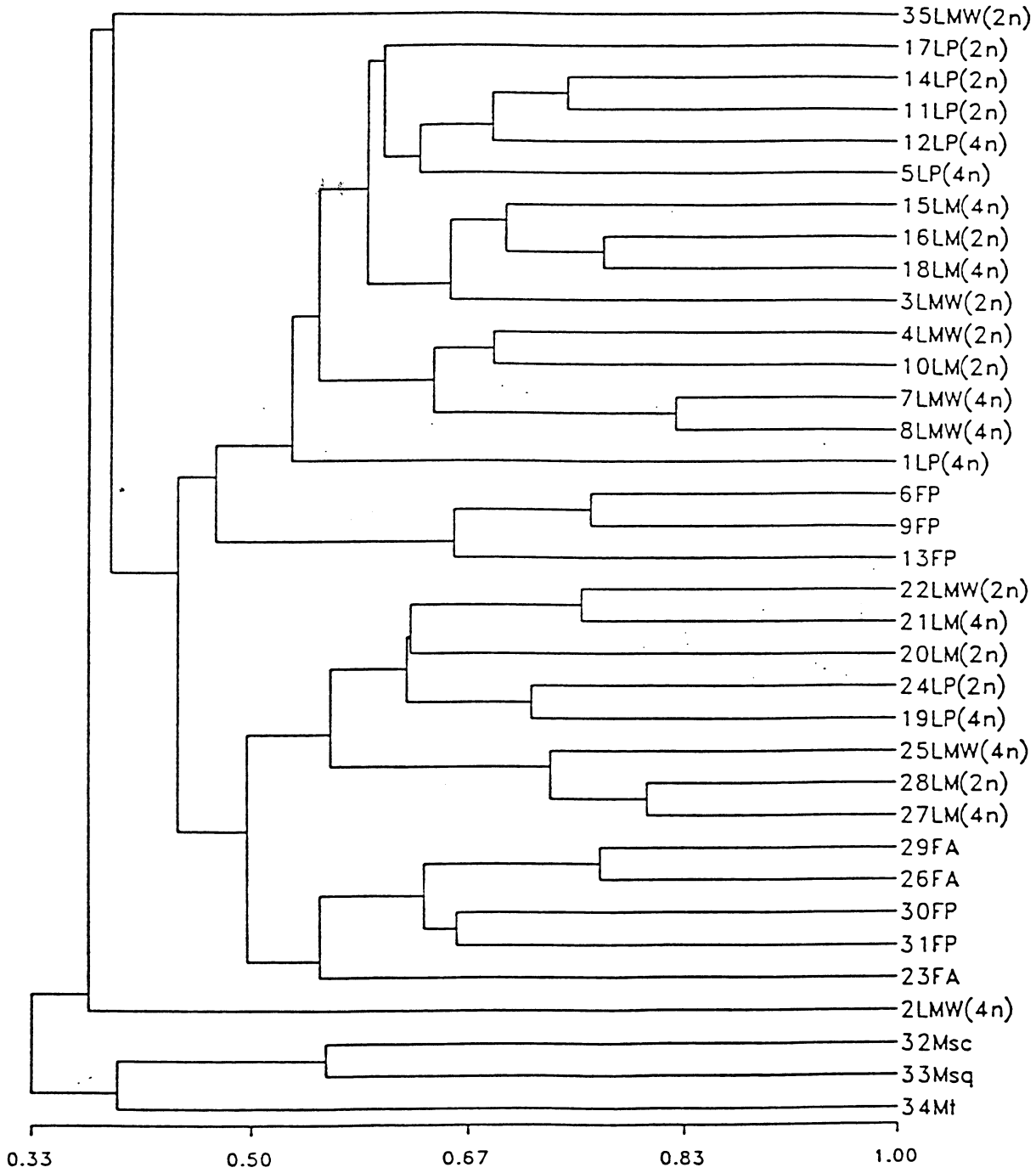

Fig. 3a Two "randomized" variants of dendrogram (with identical branching) of accessions from *Lolium-Festuca* complex and dicot reference genus *Medicago* constructed by the UPGMA cluster analysis based on the correlation coefficient (r) between computer-generated absorbance profiles pooled from all 10 primer-profiles (see also Fig. 3b on the next page).

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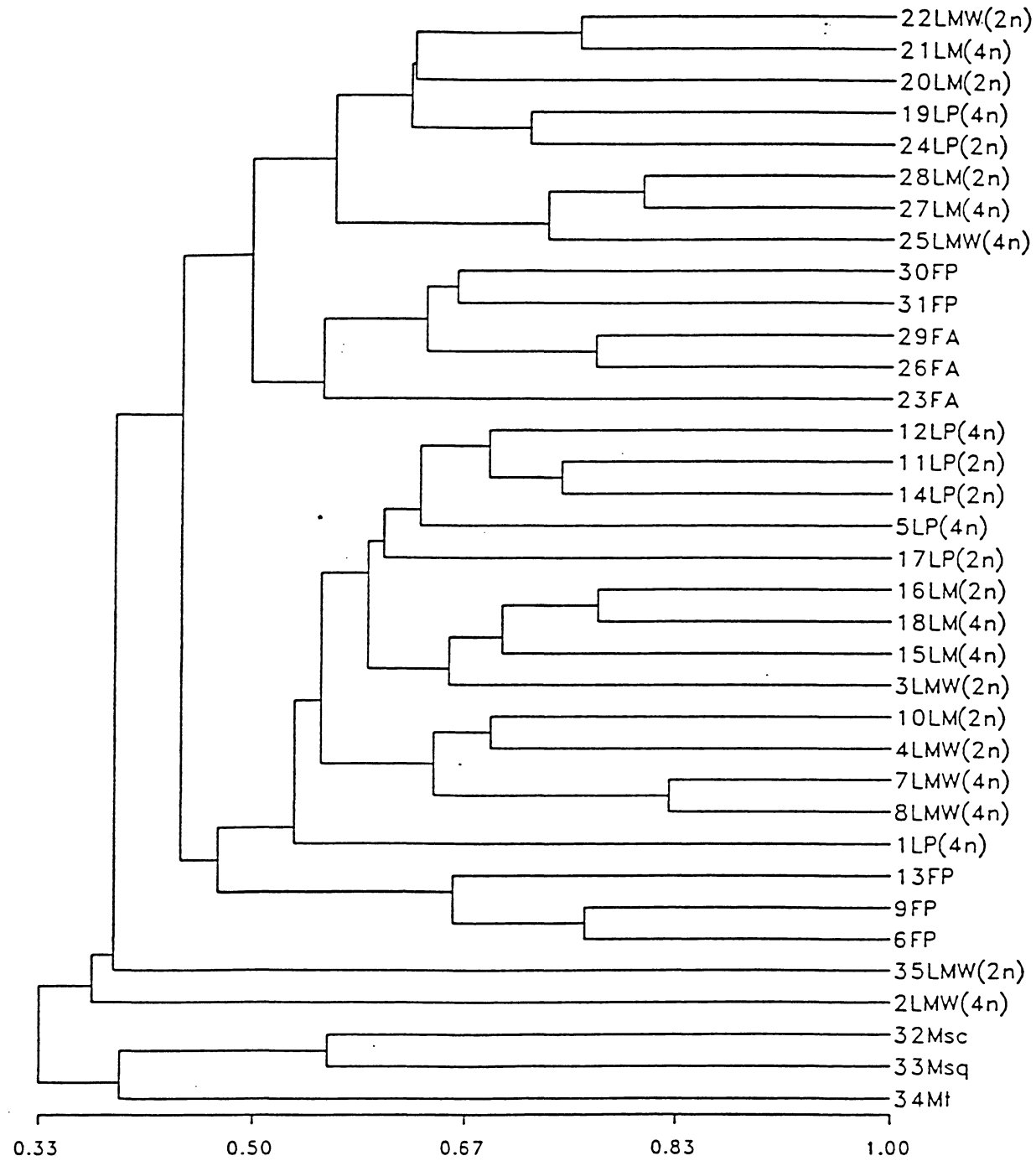

Fig. 3b See legend of Fig. 3a

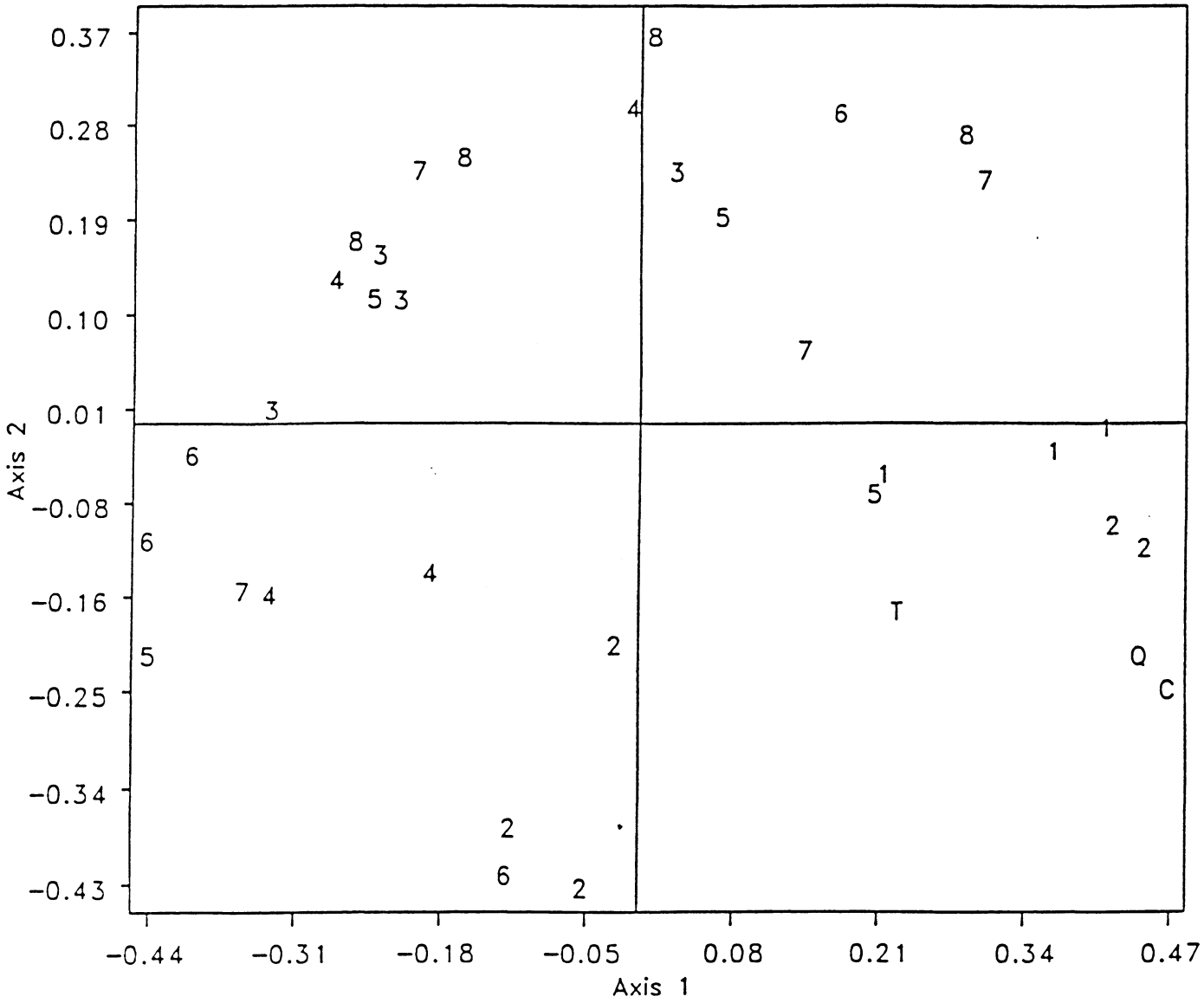

Fig. 4 Associations of accessions from *Lolium-Festuca* complex and dicot reference genus *Medicago* generated by principal coordinate analysis (PCO) based on the correlation coefficient *r* between computer-generated absorbance profiles pooled from 10 primer-profiles.

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.

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