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RAPD fingerprinting of diploid *Lolium perenne* × hexaploid *Festuca arundinacea* hybrid genomes

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Abstract

We tested the application of RAPD technology for identification of hybrid genomes originated from a maternal clone of *Lolium perenne* L. (2n = 2x = 14) bearing cytoplasmic male sterility, which was pollinated separately by five clones of *Festuca arundinacea* Schreb. cv. Barocco (2n = 6x = 42). Six classes of RAPD markers were recognized, specific to: *1*) *Festuca* genome and inherited into F1 hybrid genomes, *2*) *Lolium* genome inherited into F1 hybrid genomes, *3*) *Lolium*-specific bands not found in F1 progeny, *4*) *Festuca*-specific bands not found in F1 progeny, *5*) new bands found only in F1 hybrid profiles, *6*) bands common to all parental and F1 hybrid genotypes. RAPD data were shown to have full potential *a*) to serve as an unequivocal proof of genome recombination in perennial ryegrass × tall fescue hybrids, *b*) to identify hybrid genomes, *c*) to reveal phenetic relationships of the accessions from crossing families, *d*) to enhance, by fingerprinting, the selection of superior hybrid material for further breeding. RAPD data were found to be consistent with the *festucoid* phenotype of F1 hybrids.

Additional key words: cluster analysis, DNA typing, fescue, ryegrass.

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Abbreviations: CMS - cytoplasmic male sterility, RAPD - randomly amplified polymorphic DNA, RFLP - restriction fragment length polymorphism, SCAR - sequence characterized amplified regions, UPGMA - unweighted pair group method with arithmetic means.

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Introduction

The occurrence of natural intergeneric *Lolium-Festuca* hybrids has been known for a long time (Gymer and Whittington 1974). Taxonomic problems are caused when there is a continuous transition between natural parental phenotypes (Nitzsche 1974). Intergeneric crosses have also been widely generated experimentally (for review and references on synthetic hybrids, see Janeček 1984). Protein and isozyme electrophoretic profiles have been successfully used for identification of either somatic or sexual hybrid genomes (Eizenga and Buckner 1986, Humphreys 1989). Also DNA markers based on repetitive sequences have already been generated from *Festulolium* hybrids (Perez-Vincente *et al.* 1992). Cloned taxon-specific genomic regions represent a powerful approach for discrimination but the generation of a high number of markers routinely is too time-consuming to meet all the demands of a breeding programme. For its versatility, speed, possible automation, and capability to continue up to cloned (RFLP) or even sequence-characterized (SCAR) specific markers, RAPD technology is still the current method of choice for routine fingerprinting of breeding germplasm or cultivar lots.

The RAPD technique has already been successfully applied in the identification of species from *Lolium-Festuca* complex (Stammers *et al.* 1994, Wiesner *et al.* 1995). Hybrid genomes of *Festuca mairei* St.Yves and *Lolium perenne* L. were successfully monitored recently using RFLP technology to evaluate distant genomic relationships (Chen *et al.* 1995).

In present paper we tested the application of RAPD technology for identification of hybrid genomes of *L. perenne* × *F. arundinacea* cv. Barocco, and addressed three aims: *1*) to test the potential for generating genome-specific DNA markers for identification of hybrid genomes, *2*) to assess the consistency of *festucoid* phenotypes of F1 hybrids with RAPD molecular data and *3*) to reveal the phenetic relationships of all tested accessions from five perennial ryegrass × tall fescue experimental families using RAPD data.

Materials and methods

Plants: The breeding experiment was set up in using the maternal clone No. 2 of *L. perenne* (2n = 2x = 14) bearing CMS which was pollinated separately by five clones of *F. arundinacea,* cv. Barocco (2n = 6x = 42) (crossing families No. 1, 3, 4, 6, 8). Another perennial ryegrass clone (No. 3) was brought into the fingerprinting study as the reference clone for *Lolium* genome. Both *Lolium* clones were obtained from the Institut für Futterpflanzenzüchtung, Malchow, Germany.

The remaining plant material (parental plants and F1 hybrid progeny) used in this study was produced at the Breeding Station of OSEVA UNI, Větrov, Czech Republic. The labeling of the accessions analyzed here remains the same as for the original raw breeding material.

DNA isolation and RAPD reaction: Total DNA samples were extracted from etiolated 10-d-old leaves following the modified ultra-fast NaOH method of Wang *et al.*

(1993) for DNA isolation. Briefly, leaf tissue (50 mg) was ground for exactly 5 min in a 1.5 cm^3 Eppendorf tube in 0.2 cm^3 0.5 M NaOH on ice. After brief spinning, 5×10^{-3} cm^3 of recovered supernatant was mixed with 95×10^{-3} cm^3 0.1 M TRIS-HCl (pH 8.0) in a 0.5 cm^3 Eppendorf tube. If longer storage required, 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). Each DNA sample was pooled from 10 individual plantlets.

Eighteen primers (*Operon Technologies*, Alameda, USA) were utilized for amplification of random DNA sequences in all accessions. RAPD reaction mixture (0.025 cm^3) contained 10 mM TRIS-HCl (pH 8.3), 4 mM $MgCl_2$, 240 mM of each dNTP, 0.2 mM of primer (see Table 1 for sequences), 25 ng of total DNA, and 1 U Taq polymerase (*Fermentas*, Vilnius, Lithuania) overlaid with mineral oil. Amplification was conducted in a DNA thermal cycler 480 (*Perkin Elmer Cetus*, Connecticut, USA). The amplification conditions were as follow: 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 (the only exception being for M13 primer with 48 °C) and an extension step of 2 min at 72 °C. The last 15 extension steps were progressively extended by 5 s per 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 λ/*PstI* DNA marker as internal standard.

Table 1. Sequences of primers used for RAPD fingerprinting.

Primer	Sequence	Primer	Sequence
OPY1	5'-GTGGCATCTC	OPY11	5'-AGACGATGGG
OPY2	5'-CATCGCCGCA	OPY13	5'-GGGTCTCGGT
OPY3	5'-ACAGCCTGCT	OPY15	5'-AGTCGCCCTT
OPY4	5'-GGCTGCAATG	OPY16	5'-GGGCCAATGT
OPY5	5'-GGCTGCGACA	OPY17	5'-GACGTGGTGA
OPY6	5'-AAGGCTCACC	OPY18	5'-GTGGAGTCAG
OPY7	5'-AGAGCCGTCA	OPY20	5'-AGCCGTGGAA
OPY9	5'-AGCAGCGCAC	P7	5'-TGCTCACTGA
OPY10	5'-CAAACGTGGG	M13	5'-GAGGGTGGCGGTTCT

Statistics: For phenetic clustering of accessions, the average linkage cluster analysis (UPGMA) method was used with the Nei-Li distance measure as previously reported (Wiesner *et al.* 1995). The calculations were performed using *MVSP Plus* ver. *2.1* software package for multivariate analysis (*Kovach Computing Services*, Pentraeth, UK).

Results

Genome-specific RAPD markers: The basic algorithm for identification of hybrid genomes is based on scoring RAPD bands which are present in one parental RAPD

profile with inheritance into F1 progeny and with absence in the second parental RAPD profile. Hence, we obtained a set of maternal and paternal hybrid markers. Using appropriate combinations of those markers the RAPD profiles of hybrid genomes can be identified.

We applied RAPD technology using a set of eighteen primers to generate fingerprints of six *Lolium-Festuca* genotypes comprising the parental and progeny members of the first family. Eighteen primers yielded altogether 167 markers taken into fingerprinting evaluations (9.3 informative markers per primer-profile were obtained on average). The RAPD profiles of members of family No.1 including the additional reference *Lolium* genotype (No. 3) are shown in Fig. 1.

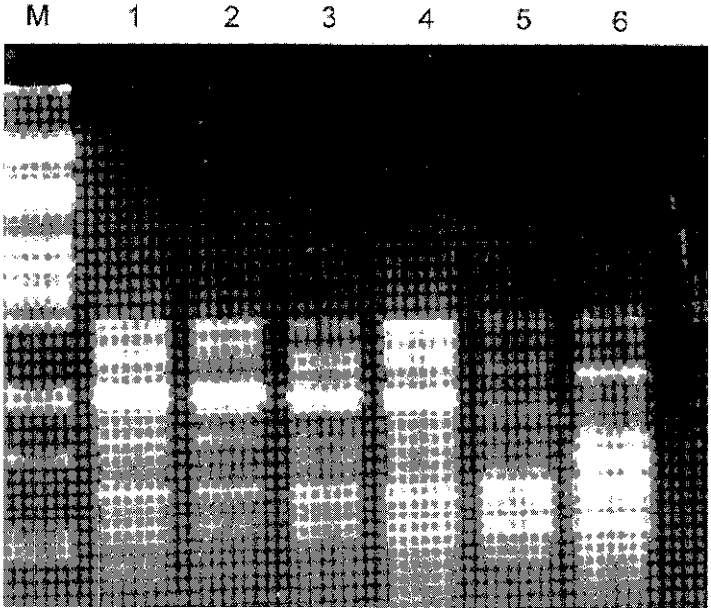

Fig. 1. RAPD polymorphism of parental and hybrid clones from *Lolium perenne* × *Festuca arundinacea* crossing family No.1 generated by primer OPY-17. *M* - molecular mass marker *λ/PstI*, *1* - *F. arundinacea* clone 1, *2* - F1 progeny of *L. perenne* × *F. arundinacea* clone 1/1, *3* - F1 progeny of *L. perenne* × *F. arundinacea* clone 1/2, *4* - F1 progeny of *L. perenne* × *F. arundinacea,* clone 1/3, *5* - reference *L. perenne* clone, *6* - maternal *L. perenne* clone.

The RAPD profiles were scored for genome-specific markers. Six classes of markers could be recognized (Fig. 2): *1*) markers specific to *Festuca* genome and inherited into F1 hybrid genomes; *2*) markers specific to *Lolium* genome inherited into F1 hybrid genomes; *3*) *Lolium*-specific bands not found in F1 progeny; *4*) *Festuca*-specific bands not found in F1 progeny; *5*) new bands found only in F1 hybrid profiles; *6*) bands common to all parental and F1 hybrid genotypes. For the routine identification of hybrid genotypes each primer was analyzed for its potential to generate hybrid-distinctive RAPD bands (Fig. 3). A total of 60 genome-specific markers were obtained (42 fescue-specific and 18 ryegrass-specific) with the average

production of 3.3 RAPD markers per primer. The among-primer coefficient of variation of marker-production (Vc) was 95.14 %. Such a high Vc value indicates the insertion of a preliminary screening step into RAPD technology when dealing with

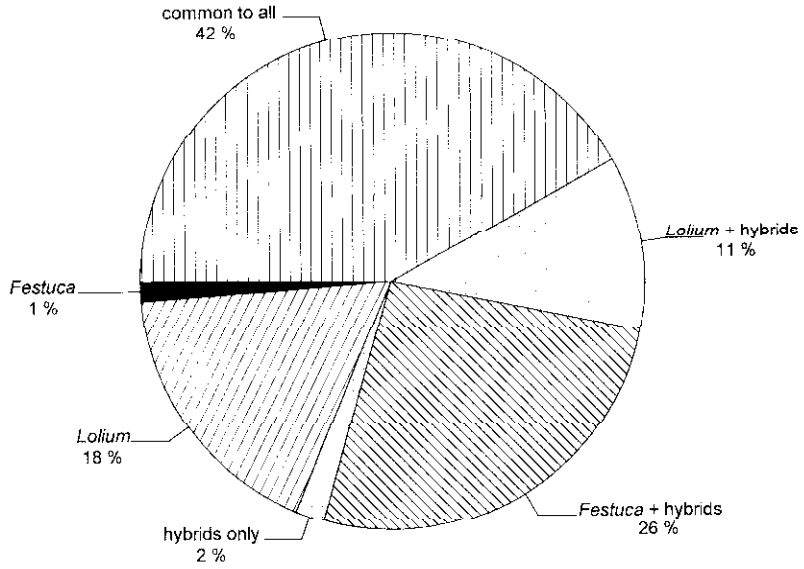

Fig. 2. Pie chart showing relative specificity of RAPD markers.

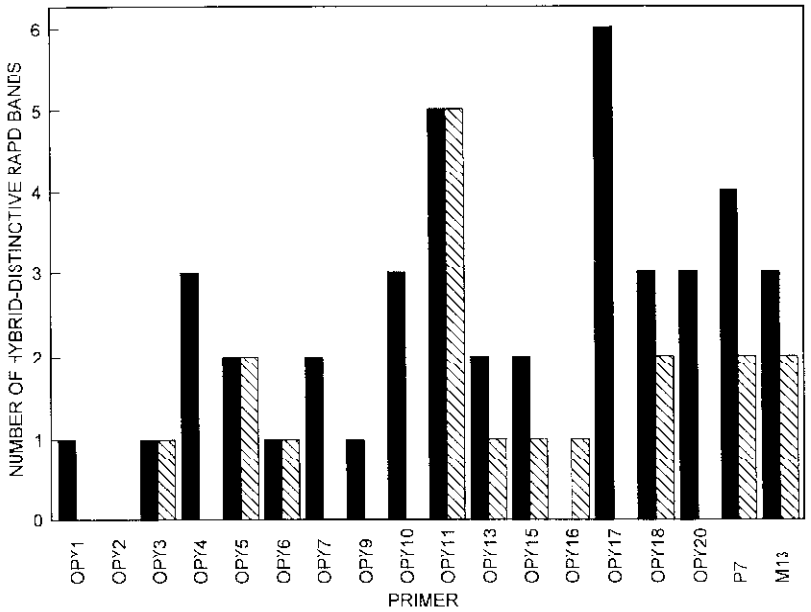

Fig. 3. Histogram showing the potential of individual RAPD primers to provide hybrid-distinctive markers (RAPD bands). *Full columns* - *Festuca*-specific bands, *hatched columns* - *Lolium*-specific bands.

new hybrid material in order to define the most potent primers and to achieve better economy of scale in hybrid identification analyses.

OPY17 was found to be the most potent primer for the identification of the *Festuca* genome in hybrids. OPY11 was the most versatile primer in its ability to generate both *Lolium*- and *Festuca*-specific bands. Primers OPY1, OPY4, OPY9, OPY10, OPY17 and OPY20 targeted sequences specific exclusively to the *Festuca* genome, while only one primer, OPY16, yielded exclusively *Lolium*-specific markers. Primer OPY2 was unable to give any marker useable in identification of hybrid genomes.

Relationships of crossing families: RAPD technology using 10 primers (OPY1, OPY2, OPY3, OPY4, OPY5, OPY6, OPY7, OPY13, OPY20, P7) was applied to generate fingerprints of 25 *Lolium-Festuca* genotypes comprising the members of the five hybrid families (family No. 1, 3, 4, 6, 8) selected as the most promising breeding germplasm. Each of the five families consisted of a clone of *L. perenne* used as female parent (identical genotype in all crosses) pollinated independently in each family by a different clone of *F. arundinacea.* Within each family three F1 progeny genotypes were taken for further analysis. An additional *L. perenne* genotype (No. 3) was included in each analysis as the reference *Lolium* genotype.

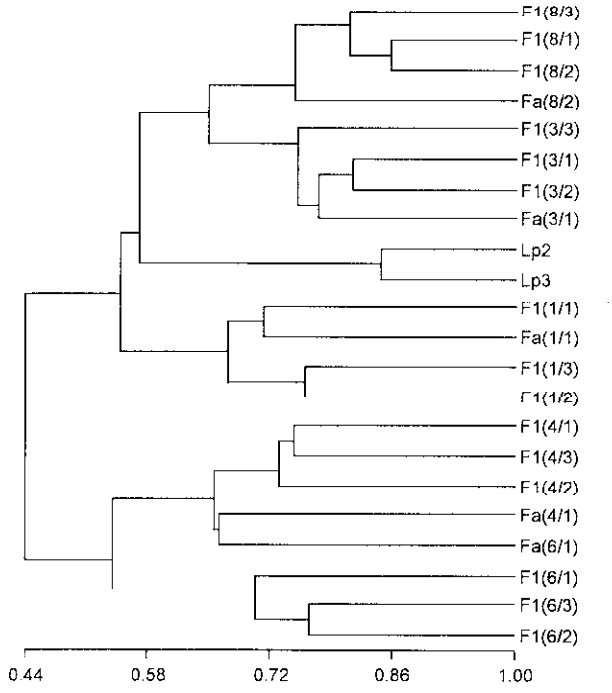

Fig. 4. Dendrogram constructed by UPGMA cluster analysis showing phenetic clustering of accessions from crossing families

Table 2. Genetic similarity index

Fa(4/1)	F1(8/2)	F1(4/2)	F1(4/3)	Fa(6/1)	F1(6/1)	F1(6/2)	F1(6/3)	Fa(1/1)	F1(1/1)	F1(1/2)	F1(1/3(	Fa(3/1)	F1(3/1)	F1(3/2)	F1(3/3)	Fa(8/1)	F1(8/1)	F1(8/2)	F1(8/3)	LP3
1.000																				
0.676	1.000																			
0.636	0.728	1.000																		
0.669	0.746	0.731	1.000																	
0.661	0.636	0.640	0.676	1.000																
0.514	0.626	0.624	0.654	0.668	1.000															
0.411	0.530	0.534	0.571	0.615	0.745	1.000														
0.378	0.471	0.475	0.493	0.539	0.660	0.765	1.000													
0.486	0.416	0.454	0.463	0.515	0.504	0.528	0.598	1.000												
0.431	0.435	0.419	0.444	0.421	0.448	0.394	0.465	0.711	1.000											
0.451	0.479	0.465	0.489	0.443	0.553	0.576	0.581	0.676	0.631	1.000										
0.464	0.503	0.461	0.466	0.506	0.509	0.513	0.564	0.666	0.706	0.759	1.000									
0.464	0.459	0.445	0.501	0.484	0.460	0.418	0.508	0.575	0.656	0.591	0.759	1.000								
0.439	0.471	0.446	0.405	0.494	0.508	0.429	0.499	0.571	0.610	0.694	0.783	0.768	1.000							
0.450	0.451	0.449	0.400	0.440	0.481	0.389	0.450	0.543	0.625	0.653	0.691	0.780	0.813	1.000						
0.450	0.480	0.464	0.421	0.411	0.400	0.294	0.359	0.438	0.581	0.548	0.596	0.725	0.713	0.811	1.000					
0.388	0.398	0.384	0.414	0.423	0.468	0.404	0.440	0.545	0.441	0.604	0.605	0.604	0.673	0.705	0.633	1.000				
0.369	0.458	0.459	0.408	0.363	0.488	0.389	0.431	0.456	0.506	0.589	0.595	0.621	0.689	0.776	0.729	0.798	1.000			
0.366	0.443	0.413	0.386	0.340	0.428	0.385	0.430	0.444	0.500	0.573	0.514	0.543	0.626	0.719	0.698	0.754	0.856	1.000		
0.396	0.449	0.413	0.433	0.304	0.436	0.371	0.388	0.471	0.461	0.529	0.445	0.504	0.551	0.676	0.618	0.686	0.783	0.834	1.000	
0.455	0.450	0.428	0.421	0.360	0.433	0.373	0.418	0.453	0.455	0.511	0.543	0.491	0.555	0.550	0.568	0.585	0.651	0.626	0.590	1.000
0.405	0.419	0.384	0.384	0.328	0.385	0.371	0.419	0.379	0.398	0.464	0.521	0.499	0.539	0.556	0.545	0.546	0.633	0.610	0.541	0.845

Using phenetic cluster analysis we revealed mutual relationships among crossing families. The similarity matrix was the first output from the cluster analysis (Table 2). The relationships of all members of five crossing families are given in the dendrogram (Fig. 4).

Discussion

F. arundinacea is often hybridized with *Lolium* to achieve incorporation of *Lolium* genes responsible for higher digestibility and nutritive value while retaining useful *Festuca* traits (plasticity, growing dynamics, resistance, *etc.*) (Eizenga *et al.* 1991).

The success of such a grass breeding program depends on the choice of a proper germplasm. In order to make good choices, there is a requirement for a proper tool to classify the extent of genetic variation and genetic relationships. This substantiates the call for efficient (DNA) fingerprinting systems developed especially to recognize hybrid genomes.

In general, the close phylogenetic relationships of the *F. arundinacea* genome (reported as PPG1G1G2G2, Jauhar 1975) with a *Lolium perenne* genome (reported as LL) was demonstrated by successful generation of RFLPs on perennial ryegrass by heterologous probes derived from tall fescue genome (Xu *et al.* 1992). In accordance with these findings we were able to generate, besides *Festuca*-specific and *Lolium*-specific RAPD bands, also bands common to both parental genomes as well as to hybrids.

Further, we found the prevalence of *Festuca*-specific RAPD markers over *Lolium*-specific ones in our hybrids, which is in accordance with the morphological *festucoid* type of the hybrid plants we selected. Badoux (1973), who studied an effect of parental genome on the F1 generation of *L. multiflorum* × *F. arundinacea* hybrids, also obtained F1 plants of *festucoid* habit, *i.e.* in morphology and anatomy close to the pollinator *F. pratensis* while crude protein content was intermediate. Contrary to the *festucoid* habit of sexual hybrids, somatic hybrids resulting from different processes expressed leaf and inflorescence morphology intermediate in phenotype between both parents (Takamizo *et al.* 1991). Patroclinal inheritance of habit in the F1 generation was also referred by Zwierzykowski (1980) for the *L. multiflorum* genome.

Surprisingly, we also found quite new bands in hybrids which were not present in any parental genome. The explanation for these hybrid-specific bands may arise from the rearrangements of hybrid genomes due to recombination of parental chromosome regions. In relation with this the creation of new amplicons may be assumed (Caetano-Anolles *et al.* 1991). The frequency of markers belonging to class 3, resp. 4 (see Results) may be overestimated due to only three F1 hybrid genomes being studied in each family.

Using phenetic cluster analysis we revealed mutual relationships between crossing families. Clearly, families No. 3 and 8 are closest to the *Lolium* female genotype (being not interspersed into other families within the dendrogram). Families 3, 8, and 1 represent a *loloid* group while families 4 and 6 a *festucoid* group on the

dendrogram. The parental *Festuca* clones of families 4 and 6 were classified close together with longer distances for progenies reflecting probably the segregation in F1 generation. Heterogeneity of the *festucoid* group is evident as the *F. arundinacea* pollinator of family 6 is mapped closer to family 4 than to its own progeny (Fig. 4). This is in accordance with Ceccarelli *et al.* (1992, 1993) who refers to high polymorphism and plasticity of hexaploid *F. arundinacea*, namely about significant differences in genome size and organization which probably improves the genome fitness.

We can conclude that the RAPD technique has high fingerprinting potential suitable for rapid generation of *Festuca-Lolium* genome-specific markers and is suitable for rapid quantification of genotype similarities.

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