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Characterization and Identification of F_1 Hybrids in Eucalyptus by PCR Study using Random Primers

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A differential pattern in the RAPD profile of Eucalyptus F_1 natural hybrids FRI-4 (*E. terticornis* x *E. camaldulensis*), FRI-5 (*E. camaldulensis* X *E. tereticornis*) and controlled reciprocal crosses FRI-14 (*E. torelliana* X *E. citriodora*) along with their parental combinations was assessed through Polymerase Chain Reaction using commercially available primers. After electrophoresis of PCR product the RAPD banding pattern generated DNA fragments with 0.2 ~2.0 kbp as molecular weight. Out of the 17 primers used six were found to be polymorphic. Out of the 6 polymorphic primers, the primer M-122 was found to be the most polymorphic, generating 24 bands in three genotypes of FRI-14 and 15 in the parental combinations involved in its hybridization. The banding profile generated male specific loci confirming the hybrid status of the F1 genotypes. Further few hybrid specific loci were also observed when the amplification was carried out using the six polymorphic primers that can be used as a marker for the identification of hybrids at nursery stage.

Key words: *Eucalyptus*, hybrids, Polymerase chain reaction, Random Amplified Polymorphic DNA.

The genus *Eucalyptus* L. Herit. belongs to the family Myrtaceae and comprises about 700 species. These species show a lot of genetic diversity both with regard to qualitative and quantitative traits. Eucalyptus is of increasing commercial importance to the developing world for their multipurpose uses like pulpwood, sawn timber, fencing poles, extraction of essential oils for the use in pharmaceutical industries and bark of certain species yield fatty acids like oxalic acid and tannins.

The development of improved cultivars through hybridization has made a major contribution to increased productivity and quality of *Eucalyptus* species for which the hybridization of genetically different parents is carried out for the generation of F_1 hybrid cultivars. The importance of hybrids in tree improvement programs is well documented[1]. Based on the crossability pattern in the genus *Eucalyptus*, several inter-specific controlled as well as spontaneous hybrid combinations have been produced at the campus of Forest Research Institute, Dehradun, India. In the present context, differential heterotic reciprocal crosses have been made and F_1 hybrids produced using four parental species i.e. *Eucalyptus tereticornis*, *E. camaldulensis, E. citriodora* and *E. torelliana*. Out of these F_1 hybrids belonging to the 1970 reciprocal species crosses, are natural hybrids FRI-4 (*E. terticornis* x *E. camaldulensis*), FRI-5 (*E. camaldulensis* X *E. tereticornis*) and controlled reciprocal crosses FRI-14 (*E. torelliana* X *E. citriodora)*, FRI-15 (*E.*

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citriodora X *E. torelliana*). These hybrids have displayed pronounced degree of hybrid vigour both with respect to height and diameter, the two main parameters contributing towards the volume yield. Hence these hybrids appear to be an ideal system for clonal multiplication and for enhancing the productivity of the biomass in the limited land use.

The tree improvement work in India, which is based on phenotypic selection, propagation (sexually or asexually), progeny testing, followed by selection of superior progeny and repeating the cycle, is time consuming and at present could not produce desired results. The DNA based molecular markers has become an increasingly popular tool in genetic studies and in making genetic linkage maps for forest tree and plant species[2-5]. Random amplified polymorphic DNA (RAPD) assay have been used by several groups as efficient tools for identification of markers linked to important traits. The applications of RAPD and their related modified markers in variability analysis and individual specific genotyping has largely been carried out. This technique can be used to determine taxonomic identity, assess kinship relationship, detect interspecific gene flow, analyze hybrid speciation and create specific probes. Advantages of RAPD includes suitability for work on anonymous genomes, applicability to work where limited DNA is available, efficiency and low expenses[5]. It is also useful in distinguishing individual, cultivars or accessions[5]. The analysis of the fragment polymorphism in total genomic DNA through Polymerase chain reaction (PCR) would provide a valuable tool for resolving the relationship between the population and in the conformation of the hybrid status of the said F_1 hybrids through molecular genetic markers[6].

Previous studies on the said hybrids have focused on the use of morphological markers for the identification and confirmation of the hybrid status. These morphological traits are governed by the multigene family and are altered by the environmental deviations. On the other hand the molecular markers are not deviated by environmental factors and reflect the actual pattern of genetic status. Thus, the objective of the study was to characterize the said F1 hybrids along with their parental combinations to analyze the male specific loci that could be used for the confirmation of the hybrid status at the nursery stages.

MATERIAL AND METHODS

Plant material

The study was conducted on the natural as well as spontaneous inter-specific F_1 hybrids as well as the parental combinations of Eucalyptus, situated in the campus of Forest Research Institute, Dehradun, India (altitude 610, latitude 30 N and longitude 78 E and annual rain fall 216 cm). These hybrids were produced in 1986 based on the crossability pattern in the genus *Eucalyptus*. The combinations of the species involved in crossing are given in Table 1. Foliage samples from the three genotypes each of FRI-4, FRI-5, FRI-14 and FRI-15 along with their parental combinations were used to analyze the RAPD amplification. The selection of the genotypes was based on the vigour of the hybrids reflecting the best height and mass.

Methods

Extraction of total DNA

Genomic DNA was extracted from young needles using the protocol described by Doyle and Doyle (1987)[7] with slight modification. Leaf material (250 mg) was ground using a mortar and pestle to fine powder by liquid N2 and suspended in 1 ml of extraction buffer (2% CTAB, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris HCl, pH 8.0, 2% w/v PVP40, 5 mM ascorbic acid, and freshly added 3 µl β-mercaptoethanol) added to 2 ml centrifuge tubes. The mixture was incubated at 60°C for about 30 min. Following incubation, 500 µl of chloroform: isoamyl alcohol (24: 1) was added, mixed to form an emulsion and centrifuged at 14,000 rpm for 10 min. The top aqueous phase was taken out in a fresh autoclaved centrifuge tube, and DNA was precipitated by addition of equal volume of cold iso-propanol. The precipitated DNA was precipitated by centrifugation at 10,000 rpm for 10 min. The DNA pellet was washed with wash buffer (76% ethanol, 10 mM ammonium acetate) and dried. The pellet was further treated with 5 µl RNase A (10 mg/l). The pellet was incubated and washed with cold 70% ethanol, dried and resuspended in 100 µl of TE buffer (10 mM Tris HCl; 1 mM EDTA; pH 8.0). The isolated DNA was quantified and all the samples were brought down to a uniform concentration of 5 ng/µl to be used as template DNA for Polymerase Chain reaction.

Determining the concentration of DNA

The DNA concentration and purity of

the samples was determined by measuring the absorbance ratio at 260/ 280 nm using Eppendroff Bio Photometer. Making different dilutions of DNA solution made the optimum concentrations of DNA for RAPD analysis.

RAPD Analysis

Primer Sequences Used for PCR Amplification

A total of 20 decamer primers were used for PCR amplification of F_1 hybrids and their parental combinations. The primer sequences used for the amplification are shown in Table 2.

Optimization of PCR components

Amplification was checked using 1 mg, 1.5 mg, 2 mg, 3mg and 4mg concentrations of DNA samples. The concentration was optimized in order to get the maximum product yield of the amplified intergenic spacer.

PCR Amplification conditions

PCR amplification was carried out in 25 ml of reaction mixture in PCR tubes, in MyCycler Thermalcycler (Bio Rad). The reaction mixture contained 2.5 mM of each dNTP (Sigma), 1X *Taq* polymerase buffer (Fermentas), 25 mM $MgCl_2$, 25 mm primer, 5 ng DNA template and 1 unit of *Taq* DNA polymerase (Fermentas). The reaction was overlaid with mineral oil and amplification was performed. The amplification was performed by initial denaturation of template DNA at 94°C for 1min followed by 41 cycles of 1min, 94°C; 1min, 34°C; 0.45 min, 72°C and final cycle having the final extension time of 8min at 72°C, using the fastest possible transmission between each temperature.

Electrophoresis

Amplified fragments generated following RADP amplification were separated on 1.5% agarose gel using 1X TBE buffer and ethidium bromide at the concentration of 5mg/ml. The gel was visualized under UV light using UVP Gel Documentation system.

Analysis

A total of 20 primers were used to get clear reproducible results used in the study. All bands scored were scored as present or absent for each DNA sample and the molecular weight was estimated using Labworks 4.5 software. Further the RAPD profiles were analyzed for the F_1 hybrids and their parental combinations to confirm the hybridity and to detect the genotype specific markers. Estimating the amplification of parental loci in the F_1 hybrids did the comparison of parent and F_1 genotypes.

RESULTS AND DISCUSSION

Out of the 17 decamer primers used for the RAPD analysis, only 6 primers viz. M-188, M-119, M-31, M-33, M-122 and M-191 yielded the best amplification products. The amplified products were scored on the basis of presence or absence of bands. Consistent and reproducible results were considered for screening. Each RAPD band was considered as an independent character/locus in terms of base pair length. RAPD banding pattern among parents and their F_1 hybrid genotypes was compared to assess the hybridity at the DNA level.

Table 1. Details of Eucalyptus F_1 hybrids involved in the study

Hybrid	Parental Combinations	Experimental I.D
FRI-4	*E. tereticornis* X *E. camaldulensis*	FRI-4: 13
FRI-4	*E. tereticornis* X *E. camaldulensis*	FRI-4: 14
FRI-4	*E. tereticornis* X *E. camaldulensis*	FRI-4: 15
FRI-5	*E. camaldulensis* X *E. tereticornis*	FRI-5: 16
FRI-5	*E. camaldulensis* X *E. tereticornis*	FRI-5: 17
FRI-5	*E. camaldulensis* X *E. tereticornis*	FRI-5: 18
FRI-14	*E. torelliana* X *E. citriodora*	FRI-14: 4
FRI-14	*E. torelliana* X *E. citriodora*	FRI-14: 5
FRI-14	*E. torelliana* X *E. citriodora*	FRI-14: 12
FRI-15	*E. citriodora* X *E. torelliana*	FRI-15: 6
FRI-15	*E. citriodora* X *E. torelliana*	FRI-15: 7
FRI-15	*E. citriodora* X *E. torelliana*	FRI-15: 8
FRI-15	*E. citriodora* X *E. torelliana*	FRI-15: 9
FRI-15	*E. citriodora* X *E. torelliana*	FRI-15: 10

The RAPD profile generated through agarose gel electrophoresis was used to determine the male specific (pollen donor) band sharing in the F_1 hybrids, maternal loci in F_1 hybrids and in the identification of reproducible F_1 hybrid specific loci. The results showed that these primers were useful in generating at least one single band that was shared between the male parent (i.e. pollen donor in hybridization) and offspring. Though we observed considerable difference in the intensity of the bands, such qualitative differences were not considered in the present data analysis. Using 6 polymorphic primers a total of 159 and 78 bands were amplified in all F_1 genotypes and four parental species respectively. Table 3 shows the locus of the pollen donor that has been amplified in the F_1 genotypes. Out of the 6 polymorphic primers, the primer M-122 was found to be the most polymorphic, generating 24 bands in three genotypes of FRI-14 and 15 in the parental combinations involved in its hybridization Figure 1 and 2.

In all the F_1 genotypes non-parental bands were observed, few of which were hybrid between the maternal and paternal bands (Fig. 1-4). This might be the result of recombination during

Table 2. Sequence, hybrid specific and male specific PCR products amplification of RAPD primers.

S. No.	Primer	Hybrid experimental I.D.	No of hybrid specific bands	Mol.Wt (base pairs)	Shared paternal loci(base pairs)
1	MOSSELER-29-CCGGCCTTA C	-	-	-	
2	MOSSELER-31-CCGGCCTTC C	FRI-15	1	1167	657
		FRI-14	1	823	689
3	MOSSELER-33-CCGGCTGGA A	FRI-15	1	591	539
		FRI-14	-	-	541
4	MOSSELER-119-ATT GGG CGA T	FRI-5	2	1811, 800	949
		FRI-4	2	1775, 812	1536, 949
5	MOSSELER-66-GAG GGC GTG A	-	-	-	-
6	MOSSELER-82-GCG CCC GAG G	-	-	-	-
7	MOSSELER-83-GGG CTC GTG G	-	-	-	-
8	MOSSELER-110-TAG CCC GCT T	-	-	-	-
9	MOSSELER-116-TAC GAT GAC G	-	-	-	-
10	MOSSELER-122-GTAGACGAG C	FRI-14	2	962, 603	1065
11	MOSSELER-169-ACGACGTAG G	-	-	-	-
12	MOSSELER-186-GTGCGTCGC T	-	-	-	-
13	MOSSELER-188-GCTGGACAT C	FRI-5	2	1335, 810	1151
		FRI-4	1	625	896, 818
14	MOSSELER-191-CGATGGCTT T	FRI-15	-	-	842
		FRI-14	-	-	597
15	MOSSELER-131-GAA ACA GCG T	-	-	-	-
16	MOSSELER-132-AGG GAT CTC C	-	-	-	-
17	MOSSELER-147-GTG CGT CCT C	-	-	-	-

Table 3. Total number of amplification products using six polymorphic primers in parental species and F_1 hybrids

Hybrid-Primer combination	No. of bands (hybrids)	No. of bands (parental species)	No. of polymorphic loci
FRI-4-M-188	24	9	2
FRI-4-M-119	19	7	1
FRI-5-M-188	15	7	2
FRI-4-M-119	16	6	4
FRI-14-M-31	15	6	-
FRI-14-M-33	5	6	-
FRI-14-M-122	24	15	-
FRI-14-M-191	8	4	1
FRI-15-M-31	14	6	3
FRI-15-M-33	10	7	1
FRI-15-M-119	9	5	1

fertilization[8,9] or might be the result of crossing over during meiosis that may result in the loss of priming sites and thus amplification was seen in parents but not in the hybrids[10]. Eucalyptus being the highly open cross-pollinated and heterozygous species can be expected for high degree of segregation of characters in the progenies. Therefore it is expected to find all bands from each parent not present in the hybrid of Eucalyptus species. The RAPD fingerprints generated by polymorphic primers confirm the hybridity in the Eucalyptus species (Table 2). The similar studies have been reported by various other workers mentioning the usefulness of RAPD profiling in the identification, registration

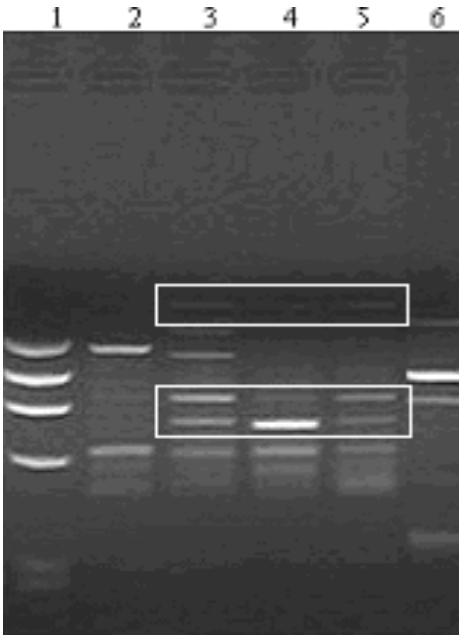

Fig. 1. RAPD profile of FRI-5 genotypes along with their parental combinations as amplified with M-119 primer. Lane**:** 1- DNA Ladder (*Phi X174* / *Hae III* digest), Lane: 2- *E. camaldulensis* (seed parent), Lane: 3- FRI-5: 16, Lane: 4- FRI-5: 17, Lane: 5- FRI-5: 17, Lane: 6- *E. tereticornis* (pollen donor)

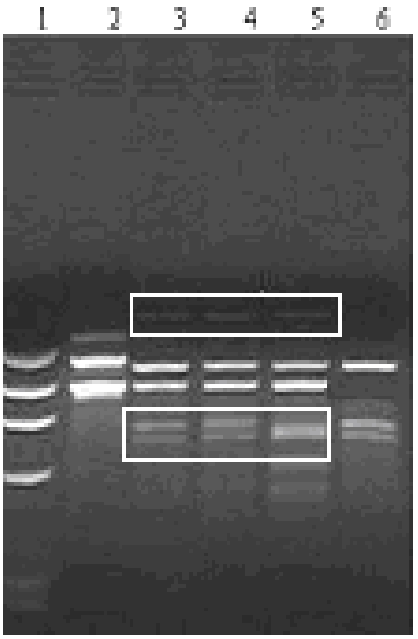

Fig. 2. RAPD profile of FRI-5 genotypes along with their parental combinations as amplified with M-188 primer. Lane: 1- DNA Ladder (*Phi X174* / *Hae III* digest), Lane: 2- *E. tereticornis* (pollen donor) Lane: 3- FRI-5: 16, Lane: 4- FRI-5: 17, Lane: 5- FRI-5: 18, Lane: 6- *E. camaldulensis* (seed parent)

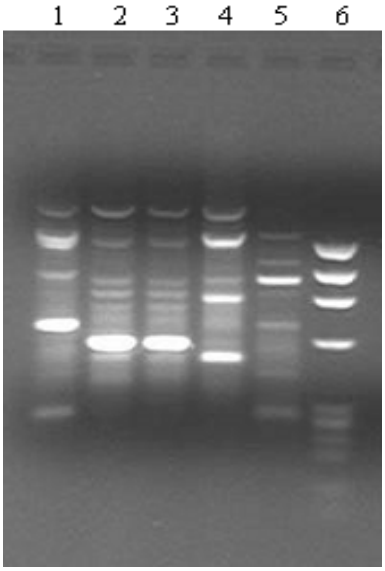

Fig. 3. RAPD profile of FRI-14 genotypes along with their parental combinations as amplified with M-122 primer. Lane**:** 1- *E. torelliana* (seed parent), Lane: 2- FRI-14: 4, Lane: 3- FRI-14: 5, Lane: 4- FRI-14: 12, Lane: 5- *E. citriodora* (pollen donor), Lane: 6- DNA Ladder (*Phi X174* / *Hae III* digest)

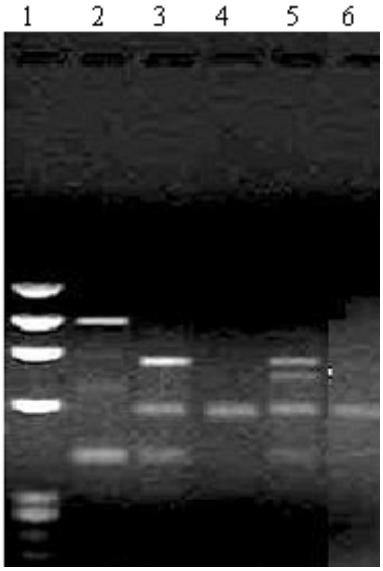

Fig. 4. RAPD profile of FRI-14 genotypes along with their parental combinations as amplified with M-191 primer. Lane**:** 1- DNA Ladder (*Phi X174* / *Hae III* digest), Lane: 2- *E. torelliana* (seed parent), Lane: 3- FRI-14: 4, Lane: 4- FRI-14: 5, Lane: 5- FRI-14: 12, Lane: 6- *E. citriodora* (pollen donor)

and protection of plant species[11-13]. Molecular characterization of the Eucalyptus F_1 hybrids using RAPD markers has also been reported by Masako *et al*. (1997)[14] in analysis of *E. globus*, *E. citriodora* and *E. gunni* and in other plants also[15].

The RAPD fingerprinting of the F_1 hybrids showed few bands that were specific for the F_1 hybrids and were not amplified in the parental combinations used. In few combinations as FRI-4, FRI-5 and FRI-14 there were two hybrid specific loci amplified using M-119, M-118 and M-122 respectively, while in the rest combinations only one hybrid specific loci per primer was amplified (Table 3). In all the cases there was no overlapping of the base-pair loci within the hybrid crosses or within the primers used. Therefore the information obtained through characterization using RAPD will be useful in the identification of hybrid for the above-mentioned species at nursery stage. In contrast to the molecular markers, qualitative pattern of isoenzymes was also used for the confirmation of the hybridity in the said hybrids but was not found suitable as it generated very low level of polymorphism and more maternal inheritance was observed for the peroxidase loci. But in present study, it was evident that the percentage of shared loci was more of seed parent but the effect was minimized due high level of polymorphism and dominant expression of RAPD markers.

CONCLUSION

This is evident from the results of present study that this technique can be adopted for large scale screening of hybrids in heterogeneous systems like Eucalyptus and other open pollinated forest tree species having a long rotation period which is in accordance with the previous studies[16]. Also, confirmation of the hybrids through markers diagnostic of male parent and production of hybrid specific markers could be practical, economical and time saving in case of forest tree species.

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