International Journal of Biotech Trends and Technology
ISSN: 2249 – 0183 /doi:10.14445/22490183/IJBTT-V11I3P604

Volume 11 Issue 3, 23-30, July-Sep 2021

Diversity Analysis and Polymorphism Through RAPD Markers in *Eucalyptus Tereticornis* Sm

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Received Date: 05 June 2021
Revised Date: 12 July 2021
Accepted Date: 22 July 2021

Abstract

The genetic diversity of 25 genotypes of Eucalyptus tereticornis Sm. was analyzed using randomly amplified polymorphic DNA (RAPD) with 10 decamer primers. The number of scorable bands for each primer varied from 6(OPA-2) to 14 (M-131), with an average of 10 bands per primer. A total of 96 distinct DNA fragments (bands) were amplified, of which 80 were polymorphic, with172 to 1353 base pairs. The number of amplified bands per genotype varied from 5 to 13 and percentage polymorphism, from 73% to 93% with an average of 83.32%. The mean polymorphic information content (PIC) of RAPD primers was 0.34 and ranged from 0.19 to 0.44. Although genotypes originating from a single provenance tended to fall into the same cluster, those from the same location were dispersed across different clusters

Keywords - *Eucalyptus tereticornis, RAPD, genetic diversity, clustering*

I. INTRODUCTION

The global population is projected to be about 9.60 billion by 2050; as a result, the demand for energy and wood products from the industrial and the domestic sectors taken together is likely to increase by 40% over the next 20 years [1]. Raising large-scale plantations of fast-growing forest tree species is one of the means to meet part of that demand, especially for paper, plywood, and solid-wood products [2]. *Eucalyptus tereticornis* Sm. is one of the most important fast-growing tree species for agroforestry systems and a major source of raw material for the paper industry. The ever-increasing demand for pulp is typically met from plantations grown specifically for the purpose of farmers, forest departments, forest corporations, and paper mills.

E. tereticornis Sm., commonly known as forest red gum, has an extensive natural distribution from southern Papua New Guinea (PNG) to southern Victoria of Australia ($5^0$20'-38^0 08'S). In India, it is the predominant species in the plains of the southern part, with average productivity of 12–25 m^3 ha^{-1} $year^{-1}$. However, as a species, *E. tereticornis* lacks sufficient genetic variability, and this limitation has a cascading impact on its productivity and also restricts the choice for future breeding and other tree-improvement programs [3]. Moreover, the existing variability in the species has largely been exploited through field selection based on morphology, and the selected plants multiplied through clonal propagation to raise commercial plantations [4].

Genetic diversity analysis is essential in developing breeding strategies and has the scientific basis for enhancing forest tree species management [5]. Since Eucalyptus is prone to inbreeding depression in plantation settings, it is necessary to assess the genetic diversity of the individuals [6]. Molecular markers have proven to be a valuable tool to understand the genetic make-up of tree species to develop integrated tree-improvement programs that combine both conventional and non-conventional methods. Genetic diversity has traditionally been assessed through morphological or biochemical markers, which have some limitations in that they are subject to environmental influences on gene expression. Misidentification of genetic material due to the non-expression of morphological markers has led to the loss of valuable genetic information with serious economic implications.

Estimates of genetic relationships based on RAPD are in good agreement with those based on pedigree, RFLP, and isozyme data [7], [8], and RAPD markers have commonly been used in analyzing various parameters of populations as well as the diversity of the various clones of many commercial tree species [9], [10], [11] and [12]. These RAPD markers are known to use arbitrary primers because some of them amplify the DNA in highly conserved regions, leading to polymorphism at high levels of classification, and some even amplify the DNA in highly variable regions appropriately used for classification and analyses at and below the level of species [13]. RAPD markers offer significant advantages, including reduced DNA

Requirements and faster processing [14]. *E. tereticornis* plus trees representing provenances from the Australian state of Queensland, New South Wales, and Papua New Guinea were evaluated for genetic diversity using the RAPD marker method.

II. MATERIALS AND METHODS

Selection of genotypes

The seeds of the 13 provenances were obtained from the Australian Tree Seed Centre, CSIRO, Australia. Later, a progeny cum provenance trial of *E. tereticornis* was established in 2002 by Forest Research Institute, Dehradun, India (30°N, 78° E; 610 amsl altitude). The progeny trial consisted of open-pollinated progenies of 13 provenances (Table 1), belonging to Queensland (QLD) (8 provenances), New South Wales (NSW)(3 provenances), and Papua New Guinea (1 provenance). Open-pollinated seeds from the F_1 generation of an interspecific eucalyptus hybrid were used as control. Although provenances from North Queensland had been graded superior, in the trial and the top rank was claimed by Walsh River and Burdekin River, both from QLD in the previous study by [15]. More plus trees were selected from this population by comprehensive indexing [16] to choose the most valuable germplasm. From the initial shortlist, 49 genotypes, each of which was then assigned a due weightage score based on morphological traits [17], and finally, 25 plus trees were screened, which were also had resistant to diseases and insect pests for genetic diversity analysis using RAPD markers.

Table 1: Origin and provenance of 25 genotypes characterized through RAPD

Clone no.	Seed lot no.	Location within Queensland, Australia	Latitude	Longitude
FRI/ET/001	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/004	20468	Cardwell	18°10′ N	145°58′ E
FRI/ET/010	20472	Walsh River	17°20′ N	145°18′ E
FRI/ET/012	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/014	20470	Mill Stream Archer Creek	17°39′ N	145°21′ E
FRI/ET/016	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/017	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/029	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/031	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/032	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/100	20471	Helenvale	15°48′ N	145°15′ E
FRI/ET/101	20472	Walsh River	17°20′ N	145°18′ E
FRI/ET/102	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/103	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/104	20471	Helenvale	15°48′ N	145°15′ E
FRI/ET/105	20468	Cardwell	18°10′ N	145°58′ E
FRI/ET/107	FRI-4	Control/Check	30°10′ N	76°00′ E
FRI/ET/108	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/110	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/112	20469	Mitchell River MT Molloy	16°44′ N	145°20′ E
FRI/ET/119	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/120	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/121	20474	Burdekin River	19°48′ N	146°04′ E
FRI/ET/124	20468	Cardwell	18°10′ N	145°58′ E
FRI/ET/126	20474	Burdekin River	19°48′ N	146°04′ E

Extraction of DNA

Genomic DNA was isolated from juvenile leaves of 25 plus trees (Table 1) by Doyle and Doyle [18] and by Stange, Prehn, and Johnson [19]. Analytical-grade reagents and chemicals were used, obtained from Sigma (Sigma-Aldrich, St. Louis, MO, USA) 200–600 ng/µL of DNA from each 500 mg sample of leaves were quantified using a bio-photometer (Eppendorf) and by comparing band intensities with known standards of lambda DNA (Bangalore Genei Ltd, India). The quantified DNA was then diluted to a concentration of 5ng/µL using ultrapure autoclaved water.

Screening of primers

Initially, 24 decamer primers Operon (Qiagen Operon, Almeda, CA, USA) [20] were screened, based on the results of the amplification, for their ability to detect distinctly, clearly resolved, and polymorphic amplified products from five randomly selected plus trees of *E. tereticornis*. For greater efficacy, only those primers with high polymorphism were chosen. Ten most informative RAPD primers were finally used for evaluation.

Amplification using a polymerase chain reaction

The conditions RAPD-PCR amplification includes the concentration of $MgCl_2$, dNTPs, the primer, *Taq* DNA polymerase, and template DNA. The amplification reaction was performed in a total volume of 25 µL (reaction mixture) containing 1 µL template DNA (10 ng/µL), dNTPs (2.5 mM), decanucleotide primer (20 µM), $MgCl_2$ (2.5 mM), *Taq* buffer (10×), *Taq* DNA polymerase (5U), and autoclaved distilled water. The amplification was carried out in a thermal cycler (Mycycler, Bio-Rad, Hercules, USA) with the following condition: initial denaturation at 94°C for 2 min, followed by 41 cycles of denaturation at 94°C for 45 sec, annealing at 37°C for 1 min, extension at 72°C for 1 min, and the final extension for 10 min at 72°C. The amplification products were resolved on 1.5% (w/v) agarose gel using 1× TBE buffer (Tris HCl (pH 8.0), boric acid, and ethylenediamine-tetra acetic acid). Amplification with each of the selected primers was repeated at least twice, and only those bands that occurred consistently and were reproducible were considered for further analysis.

Scoring of bands and data analysis

The amplicons were scored manually for the presence or absence of bands as 1 or 0, respectively. Amplified products 172–1353 bp in length were considered for the analysis, and genetic similarities among sample pairs were measured, pair by pair, to obtain their similarity coefficients with the Jaccard coefficient [21]. The binary data generated from 10 primers were then subjected to cluster analysis using the sequential, agglomerative, hierarchical, and nested (SAHN) technique and the unweighted pair group method with arithmetic averages (UPGMA) using the software DARwin 5.0. A dissimilarity matrix was generated with Dice index, subjected to principal component analysis with unweighted NJ algorithm using the software NTSYS to visualize the genetic relationships among the provenances. The final dendrogram was then constructed using NTSYS (ver 2.0 e) [22].

The discriminatory power of all the markers was assessed by evaluating four parameters, namely polymorphic information content (PIC), effective multiplex ratio (EMR), marker index(MI), and resolving power (Rp).

1. Polymorphic information content was calculated for the dominant marker system (RAPD) using the following formula

$PIC_i = \{2f_i(1 - f_i)\}$

where PIC_i is the PIC of marker i, fi is the frequency of the marker fragments that were present, and $(1 - f_i)$ is the frequency of marker fragments that were absent; the PIC was averaged over the fragments for each primer combination.

2. Effective multiplex ratio was estimated as defined by Varshney [23] using the following formula

$EMR = n.\beta$

Where n is the total number of loci, and β is the fraction of polymorphic markers estimated after considering the polymorphic loci (np) and the non-polymorphic loci (nnp) as β= np / (np+ nnp).

3. Marker Index was calculated using the formula, as described by Tatikonda [24] as follows

$MI = PIC \times EMR$

4. The resolving power is a feature of the primer combination that indicates the discriminatory potential of primer combination; the Rp of each primer was calculated using the formula, as described by Prevost and Wilkinson [25], as follows:

$Rp = \sum I_b$

where I_b is band informativeness, which can be represented on a 0-1 scale by adopting the following formula

$I_b = 1 - [2 \times |0.5 - p|]$

Where p is the proportion of all accessions containing bands.

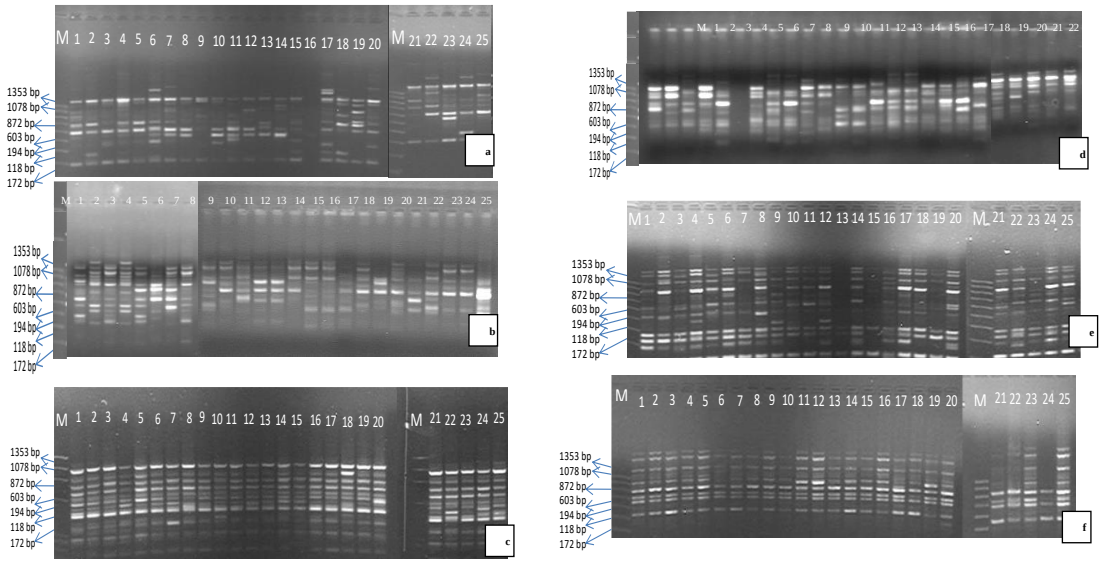

Fig. 1: Gel photographs for RAPDs (M-198, M184, M-131, OPA-2, OPA -12 and OPN-6) depicting gradient amplification for various genotype, Lane M is *Φ* × 174 DNA /*Hae* III Digest, a) M-198(PIC= 0.44), b) OPN-6(PIC= 0.42), c) OPA-2 (PIC= 0.38), d) OPA-12(PIC= 0.36), e) M-131 (PIC= 0.35) and f) M-184 (PIC= 0.29)

III. RESULTS AND DISCUSSION

Banding pattern

The RAPD primers showed that the amplification of total genomic DNA over the genotypes with polymorphism was 83.32%. The number of amplified products obtained per primer varied from 5 to 13, with an average of 8.0 bands per primer (Table 2 and Fig.1). Maximum polymorphic bands (13) were obtained using M-131 and minimum (5) using OPN-6. The values of PIC varied from 0.19 (M-169) to 0.44 (M-198), and a majority of 43 fragments showed the value to be between 0.4 and 0.5. In the remaining 67 fragments, the PIC value was less than 0.4. The overall PIC value per primer was 0.34. The values of MI, which indicate the overall efficiency in detecting polymorphism, ranged from 1.11 to 4.23, with an average of 2.32 per primer; markers M-132 and M-198 had higher a MI, 3.83 and 3.56, respectively. The values of Rp, which is a feature of the primer combination that indicates the discriminatory potential of a primer, ranged from 0.26 to 0.88 with an average of 0.56 per primer, although the highest Rp value (0.88) was recorded for primer M-184 and the lowest (0.26), for primer M-169.

Cluster analysis

The similarity coefficients of the 25 plus trees ranged from 0.11 to 1.00, indicating high genetic variability among the selected genotypes. The maximum similarity was observed between genotypes FRI/ET/124 (Cardwell, QLD) and

FRI/ET/126 (Burdekin River, QLD) and the minimum between genotypes FRI/ET/110 (Burdekin River, QLD) and FRI/ET/001 (Burdekin River, QLD). It is noteworthy that the majority of genotypes originating from the Burdekin River (QLD) provenance were distinct. Moreover, genotypes from the same location were well dispersed across different clusters. In total, the 25 genotypes were grouped into five clusters (Fig.3). Clusters IV was the largest, with13 genotypes, followed by Cluster I and Cluster III with 3 genotypes each (Table 3). Cluster IV was the most heterogeneous because it contained genotypes originating from different geographical regions. The genotypes derived from the Burdekin River (QLD) provenance were represented in all the clusters except Cluster VI, whereas cluster VII consisted of 2 genotypes, each from different provenance.

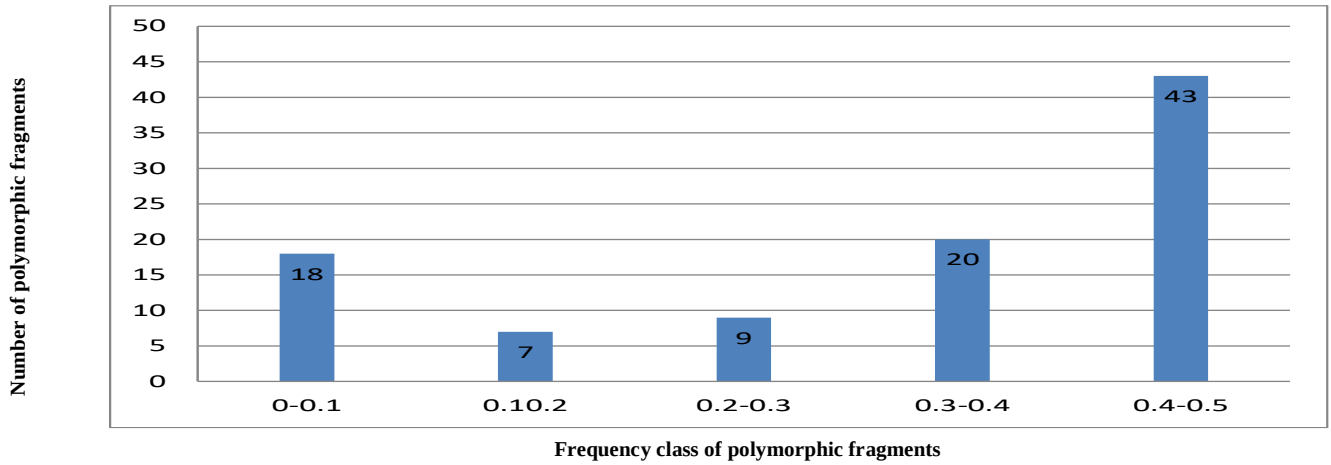

Fig. 2: **Frequency distribution for polymorphic fragments from pooled data in *Eucalypts tereticornis* genotypes**

Principal coordinate analysis (PCoA), based on the genetic similarity matrix, showed that the results arrived at through PCoA were almost similar to those arrived at through UPGMA. A three-dimensional scatter plot of genotypes showed the geometrical distances within the genotypes with minimal distortion, and the UPGMA-based clustering of the genotypes was well supported by that based on PCoA in allocating the genotypes to different clusters without overlaps (Fig. 4).

The magnitude of genetic diversity as ascertained using RAPD markers is likely to be a milestone in devising future conservation and improvement programs for E. tereticornis. The number of amplified products per primer varied from 5 to 13, with an average of 8 bands per primer. The maximum polymorphic bands (13) were obtained using M-131 and the minimum (5) with OPN-6. The values of PIC varied from 0.19 (M-169) to 0.44 (M-198); the highest PIC (0.44) was with the primer M-198 and the lowest (0.19), with primer M-169. The values of Rp ranged from 0.26 (M-169) to 0.88 (M-188), with an average of 0.56. The values of MI ranged from 1.11 (M-169) to 4.23 (M-131). As can be seen in Fig. 1, of the total 80 polymorphic bands, 43 polymorphic were in the group that showed frequencies between 0.40 and 0.50, and 18 were found in the frequencies between 0 and 0.1 (Fig. 2). An earlier investigation on selected primers involving an interspecific F1 population of E urophylla × E tereticornis, including 2 parents and 212 sibs 30 had also reported a high level of polymorphism and heterozygosity at RAPD loci between the two parents.

Genetic diversity is an essential component of any effective tree improvement program involving the synthesis of hybrids. The RAPD primers used in the present study resulted in a high level of polymorphism (83.32%) and, similarly, a high value of the PIC (0.53), indicating the presence of a dominant marker 31. The high PIC value for the RAPD primers showed that the primers used in the present study were informative enough for assessing genetic diversity. In fact, the PIC values not only helped in narrowing down the candidate primers from 23 to 10 but also identified 7 of them, namely M-131, M-132, M-156, M-198, OPA-2, OPAF-12, and OPN-6 (Table 2).

It is expected that these primers with high values of PIC, Rp, and polymorphism could potentially be used for large-scale screening of E. tereticornis germplasm at an early age. Using a similar methodology, the genetic diversity analysis of 49 genotypes of Dalbergia sissoo Roxb. had been analyzed using10 decamer RAPD, the mean values of PIC ranged from 0.24 (M-198) to 0.33 (OPA-07). Both UPGMA and PCoA used in the present study also showed that the

relationships among the genotypes were complementary to each other.

The clustering pattern of the genotypes proved highly informative, and the information can be used for devising an effective tree improvement program for the species.

Among the different clusters, Clusters I and IV, with genotypes FRI/ET/001 and FRI/ET/110, respectively, were found to be the most divergent members of these two clusters, in different combinations, can be exploited easily for setting up a hybridization program and for establishing seed orchards. The Jaccard similarity coefficient varied from 0.11 to 1.0 and helped in understanding the genetic diversity and the clustering pattern for commercial deployment of the clonal stock.

The extent of genetic diversity between genotypes resolved several issues of individual identity despite the high level of relatedness. These divergent genotypes will be particularly suitable as a base material for locating resistance to shoot infection of eucalypts, a devastating disease caused by Cylindrocladium quinqueseptatum.

Analyzing genetic diversity is the most important component of any breeding and genetic improvement program aimed at ecosystem stability and forest sustainability [26]. This analysis makes it possible to choose divergent parents for hybridization either to

Maximize the gains from heterosis or synthesize new recombinants for subsequent generations. Incidentally, it was also found that many RAPD variations, up to 0.59, in clonal identification of micro-propagated Eucalyptus have been attributed to mislabelling [27].

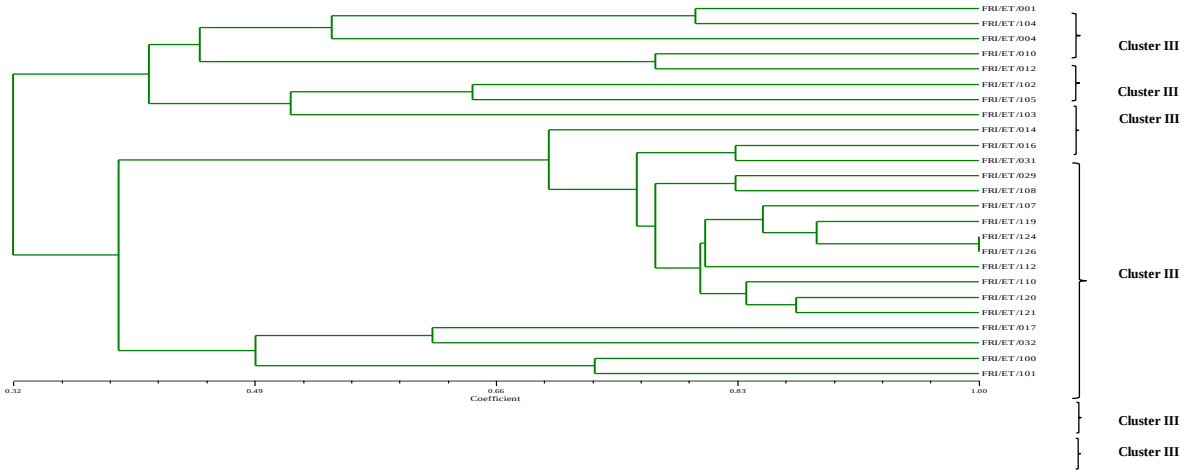

Fig.3: Dendrogram obtained from 25 genotypes of Eucalyptus tereticornis Sm. with UPGMA based Jaccard'

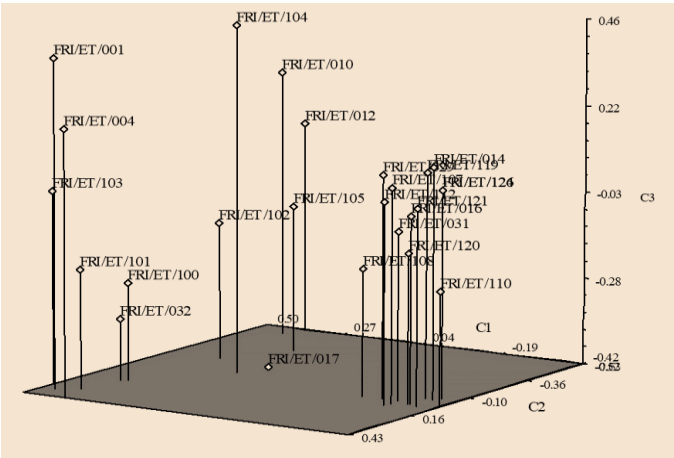

Fig.4: **Principle Coordinate Analysis Map Based On Rapd Markers of 25 Genotypes of *E. Tereticornis* Sm.**

Table 2: Details of ten RAPD primers used in analyzing genetic diversity in *Eucalyptus tereticornis* Sm

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Primer code	Primer sequence	NSB [1]	NMB [2]	NPB [3]	PPB [4]	PIC [5]	Rp [6]	MRP [7]	MI [8]
M-131	GTT CTC GTG T	14	2	13	92.86	0.35	0.43	0.03	4.23
M-132	CGA TGG CTT T	12	1	11	91.67	0.38	0.54	0.05	3.83
M-156	GCA GGA CTG C	7	1	6	85.71	0.34	0.53	0.09	1.75
M-170	GAA ACG GGT G	11	3	8	72.73	0.26	0.34	0.04	1.51
M-184	CTG ACG TCA C	11	3	8	72.73	0.29	0.88	0.11	1.69
M-198	TCG GCG ATA G	10	1	9	90.00	0.44	0.72	0.08	3.56
OPA-2	CCT GCG ACA G	7	1	6	85.71	0.38	0.49	0.08	1.95
OPA-12	TCC CGG TGA G	7	1	6	85.71	0.36	0.62	0.10	1.85
OPN-6	CCG GCT GGA A	6	1	5	83.33	0.42	0.82	0.16	1.75
M-169	GTA GAC GAG C	11	3	8	72.73	0.19	0.26	0.03	1.11
Total		96	17	80	-	-	-	-	-
Maximum		14	3	13	93	0.44	0.88	0.16	4.23
Minimum		6	1	5	73	0.19	0.26	0.03	1.11
Average		9.60	1.70	8.00	83.32	0.34	0.56	0.08	2.32

[1]No. of scored bands, [2]No. of monomorphic bands, [3]No. of polymorphic bands, [4]Percentage of polymorphic bands, [5]Polymorphic information content, [6]Marker index, [7]Resolving power, [8]Mean resolving power

Table 3: Details of genotypes grouped into the different clusters

Clusters (No. of genotypes)	Clone No.	Geographical origin
I (3)	FRI/ET/001	Burdekin River
	FRI/ET/104	Helenvale
	FRI/ET/004	Cardwell
II (2)	FRI/ET/010	Walsh River
	FRI/ET/012	Burdekin River
III (3)	FRI/ET/102	Burdekin River
	FRI/ET/105	Cardwell
	FRI/ET/103	Burdekin River
IV (13)	FRI/ET/124	Cardwell
	FRI/ET/126	Burdekin River
	FRI/ET/029	Burdekin River
	FRI/ET/119	Burdekin River
	FRI/ET/121	Burdekin River
	FRI/ET/110	Burdekin River
	FRI/ET/031	Burdekin River
	FRI/ET/016	Burdekin River
	FRI/ET/107	Forest Research Institute, Dehra Dun, Uttrakhand, India
	FRI/ET/108	Burdekin River
	FRI/ET/112	Mitchell River MT Molloy

	FRI/ET/114	Cardwell
	FRI/ET/120	Burdekin River
V (2)	FRI/ET/032	Burdekin River
	FRI/ET/017	Burdekin River
VI (3)	FRI/ET/100	Helenvale
	FRI/ET/101	Walsh River

Conclusion

The study aimed at estimating the genetic variation in the selected plus trees of Eucalyptus tereticornis species based on the 10 RAPD primers. Using RAPD primers for the 25 plus trees, 13 primers showed a polymorphic reaction, of which 10 were highly polymorphic. Deploying RAPD proved highly informative and powerful in assessing genetic variability in E. tereticornis. The analysis of genetic diversity is particularly valuable in tree improvement programs because trees live much longer than annuals or herbs. Nonetheless, genetic diversity analyzed using DNA-based markers allows direct assessment of variation and offers a highly efficient and informative means of characterizing diversity at the level of both population and the genotype. Similarly, another study on E. tereticornis also showed genetic variation in the selected E.tereticornis species based on the 10 RAPD primers (Nishad et al., 2014). Analyzing genetic diversity is an extremely component of planning appropriate breeding strategies and also provides a scientific basis for managing forest genetic resources, especially tree species. Schemes based on DNA markers have the advantage of being readily deployable because they require only small amounts of genomic DNA and provide markers in regions of genomes that have been inaccessible to analysis so far. The techniques of molecular approaches to breeding should therefore be applied more widely not only to identify, conserve, and exploit the existing variation but also to screen or to develop various genotypes for different end uses and to add to the diversity

Acknowledgment

The authors are thankful to the Director, Forest Research Institute, Dehra Dun, Uttarakhand, India, for providing the necessary facilities to carry out the investigation. Thanks are also due to Dr. H S Ginwal, Senior Scientist, Division of Genetics and Tree Improvement, Forest Research Institute, for his inputs and timely suggestions. The authors are also thankful to Hon'ble Vice-Chancellor, CAU (Imphal), for support in the successful completion of the work.

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