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European Journal of Experimental Biology, 2013, 3(6):103-110

ISSN: 2248 –9215
CODEN (USA): EJEBAU

Molecular genetic analysis of *Eucalyptus tereticornis* by using RAPD markers

Suman Tiwari*[1], Rakhi Tiwari[1], Richa Srivastava[2], Pratibha Kumari[2] and Sujeet Kr. Singh[1]

[1]*Division of Biotechnology, CytoGene Research & Development, Lucknow*
[2]*Department of Bioengineering, Integral University, Lucknow*

ABSTRACT

Random Amplified Polymorphic DNA (RAPD) polymorphism was employed to assess the genetic variations in the germplasm of Eucalyptus tereticornis. 20 trees under cultivation were analyzed with 10 RAPD primers of which all shows polymorphisms. Total genetic diversity (H) varied between 0.18 and 0.42 with an average of 0.35. Shannon's Information index (I) varied between 0.50 and 0.61, with an average of 0.52and overall genetic variation is 35%. Marker attributes like Polymorphism Information Content (PIC), Effective Multiplex Ratio (EMR), Marker Index (MI) and Resolving power (Rp)values were calculated to assess the discriminatory power of 10RAPD primers used. The PIC values ranged from 0.24 to 0.43 with an average of 0.34 per primer and the EMR ranged from 25 to 121 with a mean of 60.40 across all genotypes and the MI value ranged from 6.01 to 42.30 with an average of 19.55 per primer. The Rp values ranged from 20 to 140 with an average of 74 per primer. The highest value of similarity coefficient of 0.84 was found between the e-9 and e-1 while the lowest value of similarity coefficient of 0.20 was observed between the genotypes e-14 and e-11. The UPGMA-phenogram categorized the 20 trees into two major clusters based on genetic similarity and dissimilarity.

Key words: *Eucalyptus tereticornis*, Genetic variations, Polymerase Chain Reaction, RAPD, UPGMA-phenogram.

INTRODUCTION

Eucalyptus is a large genus of the Myrtaceae family which includes about 900 species and subspecies (Brooker and Kleingi, 2004). *Eucalyptus tereticornis* is a species oftreenative to eastern Australia. *Eucalyptus* trees were first introduced in India, as early as 1790, at Nandi Hills, in Karnataka. In 1843, it was successfully introduced in Nilgiri Hills. However, regular plantations were taken up only in 1851. Later on extensive plantation were undertaken in Punjab and Haryana, where the forest area is very small, to meet the demand of fuelwood, small timber and pulp wood. *E. tereticornis* is a fast growing tree that can reach 30-45 m in height and 1-2 m in diameter. The species grow in open forests or as scattered trees in alluvial plains and along streams, including brackish waters. It grows better in deep, well drained, light-textured, neutral or slightly acid soils (Eldridge *et al*.1994). The plants have become source of important fast-growing hardwood trees and *Eucalyptus* oil. Genetic diversity through random amplified polymorphic DNA (RAPD) markers has been studied in *E. tereticornis*. The use of RAPD markers to identify genetic variations is preferred over conventional morphological and biochemical markers since these are completely devoid of any interference from environment effect and growth stages of experimental material thus making them highly reliable. In present study 20 leaf sources were assessed for the existing genetic variability among them. These leaf sources proves to be an ideal system for assessing the genetic variability through the use of

DNA based markers. In the same context the variability among these leaf sources was assessed using Random Amplified Polymorphic DNA (RAPD).

MATERIALS AND METHODS

Plant Material:
Fresh leaves of *Eucalyptus tereticornis* species were collected.

DNA Extraction:
Total genomic DNA was extracted from fresh leaf samples which were collected separately from 20 trees of the same species. Plant tissues were ground under liquid nitrogen to a fine powder, and then bulked DNA extraction was performed by using modified protocol of Stange *etal.*(1998). DNA quality was estimated by measuring the 260:280 UV absorbance ratios (Csaikl *et al*., 1998).

Polymerase chain reaction:
DNA amplification protocol was performed as described by Williams *et al.* (1990). PCR amplification was performed using ten random decamer arbitrary primers (OPA-02,OPA-05, OPA-11, OPA-12, OPG-09, M-31, M-147, M-33, M-29 and M-119).The sequences and molar concentration of primers are given in Table 1. The DNA amplifications were conducted in 25 µl reaction volumes containing the following reagents: 2.5 µl of dNTPs, 2.5 µl Mgcl2, and 2.5 µl of 10 × buffers, 3.0 µl of primer, 3.0 µl of template DNA, 1 µl of *Taq* polymerase and 10.5 µl of sterile dd H_2O. The reactions were carried out by the help of a Thermal Cycler. The first cycle was performed as denaturation for 1 min at 94°C, annealing for 1 min at 37°C and extension 1 min at 72°C and the final extension at the end of the cycle for 10 minutes at 72°C. A total of 41 cycles were carried out to obtain the amplification product. The amplification products were electrophoresed using 1.5 % Agarose gel with TBE Buffer for 1 h. Bands were visualized by Gel Documentation System. The similarity coefficient and the Unweighted Pair Group Method Based on Arithmetic Mean (UPGMA) clustering method were employed to construct the dendrogram.

Table-1: RAPD Primer sequences used for amplification

S. No.	Name of Primers	Base sequence (5'-3')	Molar conc.(µM)
1	OPA-02	TGCCGAGCTG	20
2	OPA-05	AGGGGTCTTG	20
3	OPA-11	CAATCGCCGT	20
4	OPA-12	TCGGCGATAG	20
5	OPG-09	CTGACGTCAC	20
6	M-29	CCGGCCTTAC	20
7	M-31	CCGGCCTTCC	20
8	M-33	CCGGCTGGAA	20
9	M-119	ATTGGGCGAA	20
10	M-147	GTGCGTCCTC	20

Statistical Analysis:
(a) Scoring
After the gel documentation done the photograph was opened in the software Adobe Photoshop and with the help of a ruler line the banding pattern was noted. Bands were scored as 1 for present band & 0 for the absent bands.

(b) Bootstrap analysis
Bootstrap values provide a confidence level for each branch point and indicate the actual number of times that the position of the branch point occurred during the iterative Bootstrap process. The higher the Bootstrap value, the more reliable is the measurement of the branch point value.

(c) Genotype Data
Genotype data obtained for the RAPD primers was used for assessing the discriminatory power of RAPD primer by evaluating three parameters:

i) Polymorphism information content (PIC)
The PIC value for each RAPD primer can be calculated as proposed by Roldan-Ruiz *et al.*

PIC = 2fi (1-2fi)

Where, PIC= Polymorphism information content of marker.

fi = Total no. of bands present in samples.

Average PIC value= Addition of total fi value/total number of Primer

ii)Marker index (MI):
The marker index was calculated as given in Varshney *et al.*

MI = PIC × EMR

where, EMR = Effective multiplex ratio (E) is defined as the product of the total number of fragments per primer (n) and the fraction of polymorphic fragments (β).

EMR = nβ

n= total no. of bands.
β= total no. of polymorphic bands.

iii) Resolving Power (Rp):
Resolving power of each primer was calculated according to Prevost and Wilkinson.

Rp = ΣIb
where,
Ib = Band informativeness

Ib can be calculated by formula:

Ib = 1- [2×(0.5-p)]

where, P = Total number of bands present.

RESULTS AND DISCUSSION

The isolated DNA was quantified and the concentration were optimize to 25ng/µl for RAPD -PCR reaction.

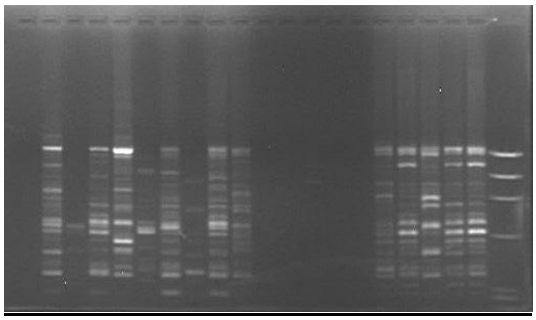

Figure-1: RAPD amplification products of primer M-31(CCGGCCTTCC)

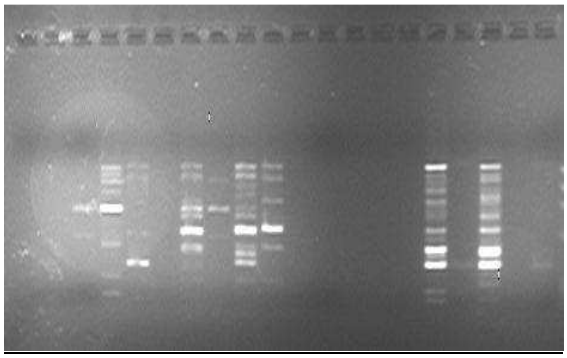

Figure-2: RAPD amplification products of primer M-119(ATTGGGCGAA)

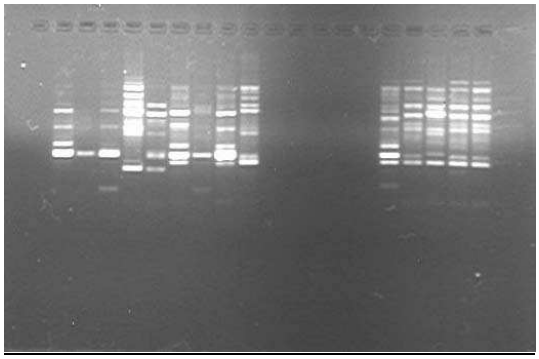

Figure-3: RAPD amplification products of primer OPA-02(TGCCGAGCTG)

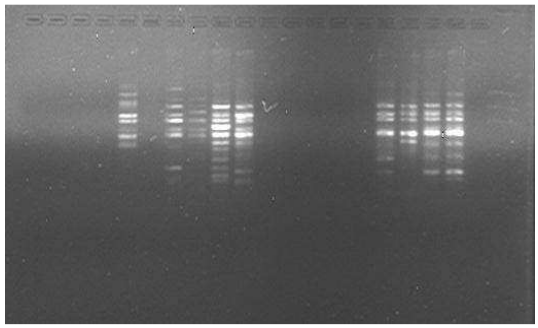

Figure-4: RAPD amplification products of primer OPA-05(AGGGGTCTTG)

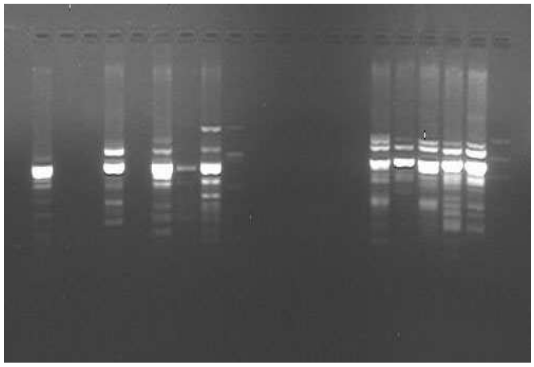

Figure-5: RAPD amplification products of primer M-29(CCGGCCTTAC)

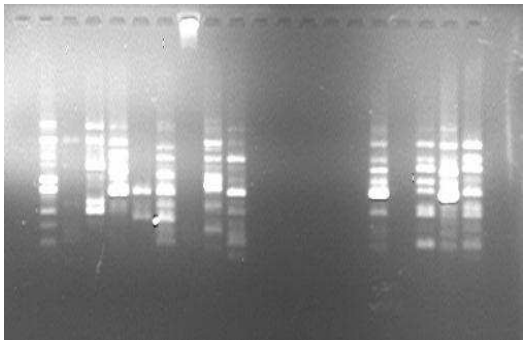

Figure-6: RAPD amplification products of primer M-33(CCGGCTGGAA)

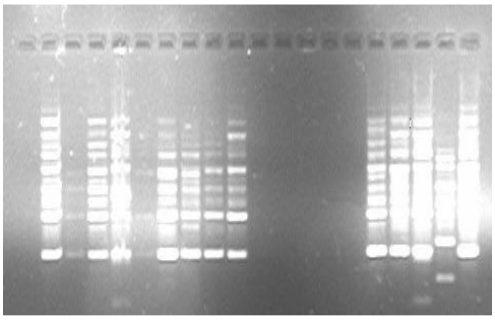

Figure-7: RAPD amplification products of primer M-147(GTGCGTCCTC)

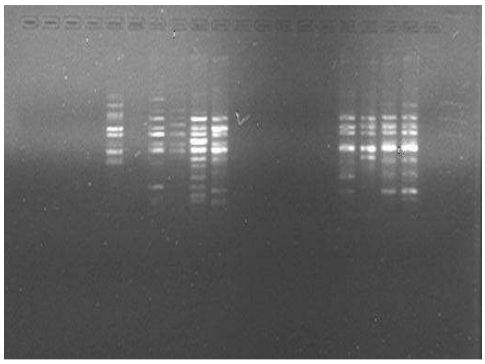

Figure-8: RAPD amplification products of primer OPA-09(CTGACGTCAC)

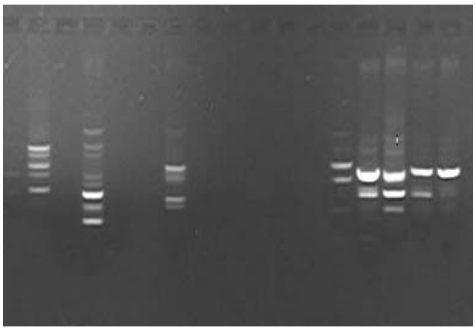

Figure-9: RAPD amplification products of primer OPA-11(CAATCGCCGT)

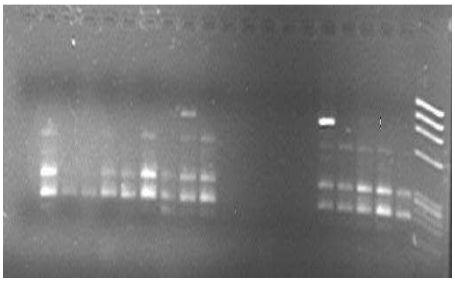

Figure-10: RAPD amplification products of primer OPA-12(TCGGCGATAG)

Table-2: RAPD Primer- PIC, EMR, MI &Rp values

S. No.	Primer Name	PIC	EMR	MI	Rp
1	M-29	0.38	36	13.89	56
2	M-31	0.27	121	33.44	64
3	M-33	0.42	64	27.34	114
4	M-119	0.24	25	6.01	20
5	M-147	0.43	90	8.21	140
6	OPA-02	0.43	100	42.30	116
7	OPA-05	0.37	36	13.34	68
8	OPA-11	0.30	64	19.59	48
9	OPA-12	0.29	49	14.35	62
10	OPG-09	0.34	49	17.06	52
11	Average	0.34	63.40	19.55	74

The PIC values for RAPD ranged from 0.24 to 0.43 with an average of 0.34 per primer combination. Highest value (0.43) was scored with the primer M-147 and the lowest value (0.24) was scored with the primer M-119.The EMR values for RAPD ranged from 25 to 121with an average of 63.40 per primer combination. Highest value (121) was scored with the primer M-147 and the lowest value (25) was scored with the primer M-119. The MI values for RAPD ranged from 6.01 to 42.30 with an average of 19.55 per primer combination. Highest values (42.30) were scored with the primer OPA-02 and the lowest value (6.01) was scored with the primer M-119. The Rp values for RAPD ranged from 20 to 140 with an average of 74 per primer combination. Highest value (140) was scored with the primer M-147 and the lowest value (20) was scored with the primer M-119.

Table-3: Genetic dissimilarity matrix of various genotypes of *Eucalyptus tereticornis* based on RAPD analysis

Pop ID	e-1	e-2	e-3	e-4	e-5	e-6	e-7	e-8	e-9	e-10	e-11	e-12	e-13	e-14
e-1	1													
e-2	0.452	1												
e-3	0.512	0.606	1											
e-4	0.452	0.788	0.392	1										
e-5	0.452	0.452	0.318	0.537	1									
e-6	0.336	0.630	0.431	0.493	0.412	1								
e-7	0.373	0.493	0.472	0.630	0.493	0.373	1							
e-8	0.452	0.452	0.431	0.452	0.452	0.300	0.300	1						
e-9	0.847	0.680	0.559	0.493	0.493	0.582	0.452	0.336	1					
e-10	0.493	0.493	0.606	0.412	0.582	0.582	0.412	0.493	0.412	1				
e-11	0.493	0.683	0.606	0.493	0.582	0.493	0.452	0.537	0.493	0.336	1			
e-12	0.515	0.559	0.680	0.392	0.606	0.515	0.515	0.515	0.392	0.392	0.249	1		
e-13	0.537	0.630	0.654	0.537	0.493	0.493	0.412	0.412	0.493	0.582	0.232	0.392	1	
e-14	0.537	0.537	0.654	0.53	0.493	0.680	0.630	0.537	0.452	0.412	0.200	0.283	0.373	1

The genetic distances between the analysed individuals were calculated by simple matching method to obtain a matrix of genetic distance (Table-3). The similarity coefficient values ranged from 0.20 to 0.84. The highest value of similarity coefficient of 0.84 was found between the e-9 and e-1 which was followed by similarity coefficient of 0.78 between genotypes e-4 and e-2. The lowest value of similarity coefficient that is 0.20 was observed between the genotypes e-14 and e-11.

Table-4: Genetic variation statistics of RAPD Primers

Locus	na*	ne*	H*	I*
OPA-05	1.50	1.30	0.18	0.56
OPA-11	1.87	1.62	0.35	0.51
OPA-12	1.87	1.25	0.28	0.43
OPG-09	2.00	1.65	0.37	0.55
M-29	2.00	1.54	0.33	0.50
M-31	2.00	1.71	0.39	0.57
M-33	2.00	1.66	0.37	0.58
M-119	2.00	1.77	0.42	0.60
M-147	1.89	1.59	0.34	0.51
OPA-02	2.00	1.78	0.42	0.61
Mean	1.92	1.62	0.35	0.52
Standard deviation	0.26	0.30	0.14	0.19

According to Nei's (1987) Molecular Evolutionary Genetics, we obtained (Table-4) no. of alleles (na) to be 1.92, effective no. of alleles (ne) to be 1.62, Nei's gene diversity (h) is 0.35 & Shannon's information index (I) is 0.52. And the standard deviation value of no. of alleles (na) to be 0.26, effective no. of alleles (ne) to be 0.30, Nei's gene diversity (h) is 0.14& Shannon's information index (I) is 0.19 and overall genetic variation is 35%.

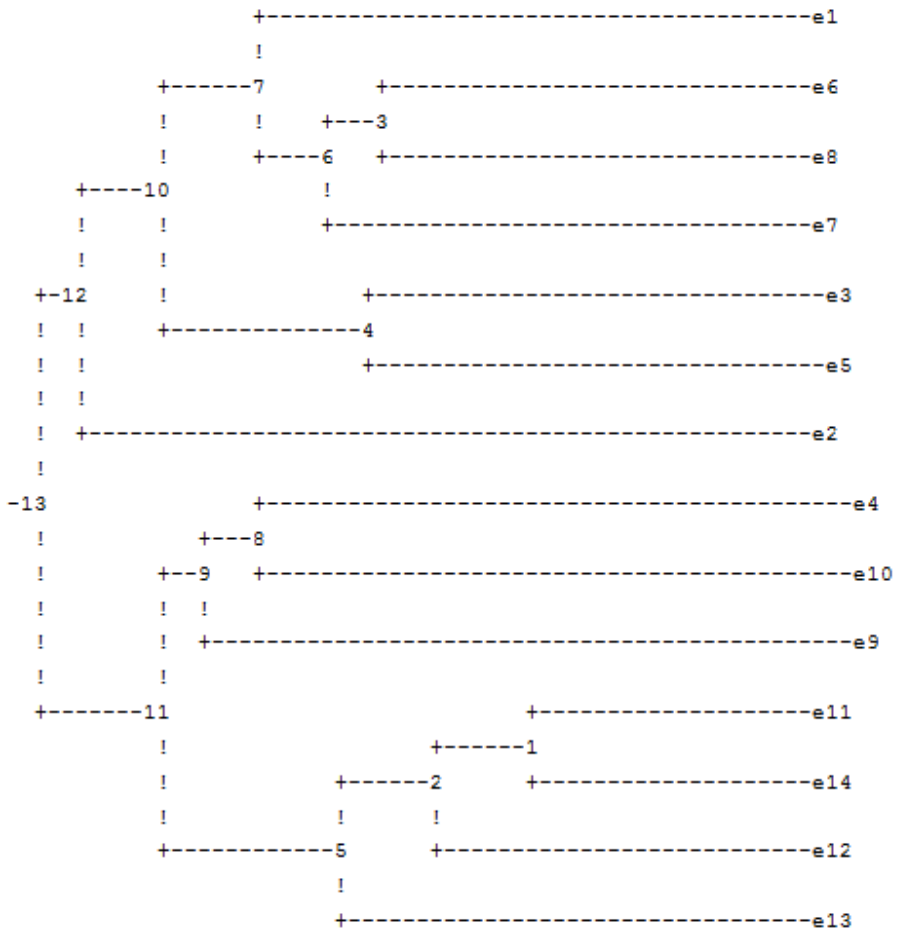

Figure-5: Dendrogram showing UPGMA clustering of *Eucalyptus tereticornis* population based on average genetic distance by using RAPD

The dendogram revealed two major clusters. The first major cluster was divided into 2 sub clusters. The first sub cluster contains e-1, e-6, e-8 and e-7 samples in which e-6 and e-8 were similar with a value of 1.0.The second sub

cluster contains e-3, e-5 samples in which both were similar with a value of 1. The second major cluster was divided into 2 sub cluster. The first sub cluster contains e-4, e-10 and e-9 in which e-4, e-10 were similar with the value of 1. And second sub cluster contains e-11, e-14, e-12 and e-13 in which e-11 and e-14 were similar with the value of 1.

CONCLUSION

In the present study the total genomic DNA was isolated using CTAB method (Stange *et al*., 1998) from the leaves of *Eucalyptus tereticornis* taken. The isolated DNA was quantified and the concentration were optimize to 25ng/µl for RAPD-PCR reaction. Optimized concentration of RAPD-PCR reaction Mixture was used is of 25 µl reaction volume containing the following reagents: 2.5 µl of dNTPs, 2.5 µl Mgcl2, and 2.5 µl of 10 × buffer, 3.0 µl of primer, 3.0 µl of template DNA, 1 µl of *Taq*polymerase and 10.5 µl of sterile dd H2O. PCR reaction condition used were denaturation at 94ºC for 1 min, annealing at 37ºC for 1 min., extension at 72ºC for 1 min. and final extension at end for 7 min at 72ºC. These were then used for RAPD assay. Tenprimers of RAPD were used in this study are useful in estimating genetic variation between genotypes. It is an important tool for genetic improvement of tree species. The similarity coefficient and the UPGMA clustering method were employed to construct the dendrogram. Keeping in view the markers which have wide ranging applications in the field of genetics including population studies will help in analyzing the genetic diversity & establish variation &relationship within species of Eucalyptus populations. The information will be useful in breeding & management program so that the population with high specific variability could be conserved which is better adapted to respond to selective pressure & ensure long term survival of the species. This work will contribute in future tree improvement & breeding work in the species.

Acknowledgement
We are thankful to the CytoGene Research & Development, Lucknow for providing the facilities and kind support throughout the research work, we are also thankful to the Almighty without whose consent nothing is possible.

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