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Assessment of Genetic Diversity of Tectona grandis Linn. F. using SSR markers

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Abstract: Teak (*Tectona grandis* L.f.) is considered to be an extraordinarily durable building timber with a worldwide reputation. In molecular markers studies, DNA was extracted and amplified using standard protocols. Seven teak specific SSR markers were used for the study. All the primers amplification showed polymorphic variations between the clones and within seven primers CIRAD4 Teak Da12 showed maximum polymorphism among the fifteen clones. The result of SSR analysis generated a total 54 bands, which were scored as 1 (present) or 0 (absent). The binary data were used for calculating similarity matrix and to draw dendrogram. The Jaccard's similarity coefficient value was found to range from 0.45 to 0.91 with an average of 0.68. The clones were grouped into two major clusters I and II. Cluster I comprised of majority of clones numbering to thirteen and three for cluster II. The lowest similarity coefficient value of 0.45 was found between the sources of Andhra Pradesh and Maharashtra except for APJNB 1 and APMN 4. The highest similarity coefficient value of 0.91 was observed between TNT 17 and TNT 18. The result of clustering coefficient analysis showed the variations between the clones and this might be due to the source of the clones from where they are procured.

Keywords: Genetic diversity, SSR, *Tectona grandis* and Provenances

Introduction

Teak (*Tectona grandis* L.F.) is one of the major valuable timber trees worldwide. The teak belongs to the family Verbenaceae and it is a diploid species 2n = 36 (Gill *et al*. 1983). It is indigenous to India, Thailand, Myanmar and Laos. As teak has a wide geographic distribution in South East Asia, the natural populations develop heritable adaptations to local environmental factors in order to survive in different ecological conditions. In India, teak differs greatly in timber characteristics such as color, grain and texture as well as in physical, chemical, anatomical and mechanical properties (Bhat and Priya 2004).

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Characterization of diversity has long been based on morphological traits mainly. However, morphological variability is often restricted, characters may not be obvious at all stages of the plant development and appearance may be affected by environment. Nowadays, a variety of biochemical and genetic markers has been proposed to assess genetic variability as a complementary strategy to more traditional approaches in genetic resources management (Nybom and Bartish, 2000). DNA markers precisely characterize cultivars, provenances or genotypes and measure their genetic relationships. In population studies, they are used to estimate the degree of relatedness among individuals which is helpful for the determination of social behaviour, reproductive success and mating choice. Molecular markers complement phenological descriptors in the identification of similar accessions that need to be discarded to avoid inbreeding (Stoehr *et al*. 1998). The genetic diversity of few populations/ provenances of teak from India, Thailand and Indonesia has been assessed by RAPD (Changtragoon & Szmidt 2000, Nicodemus *et al*. 2005), AFLP (Shrestha *et al.* 2005) and microsatellite markers (Verhaegen *et al*. 2005, Fofana *et al*. 2009).

Microsatellites, alternatively known as simple sequence repeats (SSRs), short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs), are tandem repeats of sequence units generally less than 5 bp in length (Bruford and Wayne, 1993). These markers appear to be hypervariable, in addition to which their co dominance and reproducibility make them ideal for assessing genetic variation within and among populations.

Materials and methods

Basic plant material

The materials under present study constituted by fifteen clones of teak (*Tectona grandis* Linn. F.). The clones under study have been listed along with their sources in Table 1.

Table 1: Details of the clones under study

Sr. No.	*Name of clones*	*Source*
1.	APJNB -1	Andhra Pradesh
2.	APMN-4	
3.	APT-4	
4.	APT-17	
5.	APT-20	
6.	MHAL-A4	Maharashtra
7.	MHAL-A8	
8.	MHAL-P6	
9.	MHSC-J2	
10.	ORPB-15	Orissa
11.	ORANP-7	
12.	TNT-6	Tamil Nadu
13.	TNT-11	
14.	TNT-17	
15.	TNT-18	

Experimental site

The molecular analysis of data was done at Biotechnology Centre, Department of Botany, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola and the observations were taken from already established Teak Clonal Orchard at College of Forestry, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

Methodology for SSR Markers

Extraction of DNA

DNA was extracted from young leaf samples of each clone by modified CTAB (Cetyl Trimethyl Ammonium Bromide) method given by Doyle and Doyle (1990).

Protocol for DNA isolation

2g fresh leaf sample was grinded in liquid nitrogen to obtain the fine powder. The powder was immediately homogenized by adding prewarmed (60°C) extraction buffer and transferred to 2 ml Eppendorf tubes and was mixed by inversion. The mixture was incubated for 60 min at 65°C in hot water bath with intermittent shaking after every 10 min. After incubation, the digest was allowed to cool at room temperature. The tubes were centrifuged at 12,000 rpm for 10 min at room temperature. About 80 per cent of the supernatant obtained was transferred immediately to another 2 ml tubes with wide bore pipette without disturbing the pellet of cell debris. Equal volume of Chloroform: Iso-amyl alcohol (24:1) mixture was added to the supernatant and mixed by 15-20 gentle inversions to emulsify both the components. The tubes were allowed for phase separation for 10 min and centrifuged at 12,000 rpm for 10 min at room temperature. About 70 per cent of the upper aqueous phase obtained was transferred immediately to another 2 ml tube. Care was taken not to disturb the interphase. The above step was followed immediately by slow addition of 0.8 volume (of the combined volume of aqueous phase and sodium acetate) of ice-cold isopropanol and mixed by gentle inversion. The tubes were then kept at -20°C for 30 min. The samples were then centrifuged at 12,000 rpm at room temperature for 10 min. After centrifugation a pellet was formed at the bottom of the eppendorf tubes. The supernatant was removed and the pellet was washed with 70 per cent ethanol twice. The pellet was air-dried for 30-60 minutes and then dissolved in 0.5 ml of TE buffer. The pellets were allowed to dissolve completely overnight at 4°C without agitation.

Ribonuclease A treatment

RNA was removed by giving Ribonuclease treatment. RNase A (2.5 mg/ml) was added to the DNA sample @ 100 mg/ml and incubated at 37^0C for 1 hour.

DNA quantification

a) Gel method

(i) Confirmation of DNA in the sample was carried out on agarose gel (0.8%) containing ethidium bromide @ 0.5 mg/ml in a horizontal gel electrophoresis system.

(ii) 2 µl of genomic DNA of each genotype + 1.6 µl 6X loading dye + 6.4 µl sterile water (*i.e.*, 10 µl concentration) was loaded in each well.

(iii) After completion of 5 cm run the gel was observed under UV light and the DNA yield and quality was confirmed.

(b) Spectrophotometric method

(i) 1 ml of TE buffer was taken in a cuvette and the spectrophotometer was calibrated at 260 nm as well as 280 nm wavelengths as control.

(ii) DNA quantification was done to 5µl of DNA sample in 995 µl distilled water. The sample was mixed properly and the optical density (OD) was recorded at both 260 and 280 nm.

(iii) The DNA concentration was estimated employing the following formula.

$$Amount\ of\ DNA(\mu g/\mu l) = \frac{(OD)260 \times 50 \times dilution\ factor}{1000}$$

(iv) The quality of DNA was judged from the ratio of the OD values recorded at 260 and 280 nm.

(v) DNA concentration was finally adjusted to 25 ng/µl by addition of TE 10:1, pH 8.0.

PCR Amplification

SSR primer pairs were used for the present investigation. The list of forward and reverse primers used for amplification of the genomic DNA with their sequences, is given in Table 2. The synthesis of following primers was done from Eurofins Genomics India Pvt. Ltd., India.

Preparation of reaction mixture for PCR

The PCR was carried out in small reaction tubes, containing a reaction volume typically of 25 µl that was inserted into a thermal cycler (Applied Biosystems Gene Amp PCR System 2700) that heated and cooled the reaction tubes within it to the precise temperature required for each step of the reaction. The thermal cyclers had heated lid to prevent condensation on the inner wall of the reaction tube caps. For PCR reaction, master mix was prepared first.

PCR Amplification

The PCR was performed in a Thermal Cycler (Applied Biosystems Gene Amp PCR System 2700) using a programme for the SSR primer. In this study the following programme was set up and used for amplification as mention in Table 3.

Table 2. List of SSR markers used for amplification of the genomic DNA

Sr. No.	*Primers*	*Primer sequences*
1	CIRAD2TeakB07	F: 52 -GGGTGCTGATGATTTTGAGTT-32
		R: 52 -CTAAGGAGTGAGTGGAGTTTT-32
2	CIRAD3TeakDa09	F: 52 -CTCGCTTCTTTCCACATT-32
		R: 52 -ATCATCGCGCATCGTCAA-32
3	CIRAD1TeakG02	F: 52 -TTAACGCCAAATCCCAAAG-32
		R: 52 -CACAAAGAGAACCGACGAG-32
4	CIRAD3TeakB02	F: 52 -ATGAAGACAAGCCTGGTAGCC-32
		R: 52 -GGAAGACTGGGGAATAACACG-32
5	CIRAD3TeakF01	F: 52 -GCTCTCCACCAACCTAAACAA-32
		R: 52 -AAAACGTCTCACCTTCTCACT-32
6	CIRAD4TeakDa12	F: 52 -CGCACACCAGTAGCAGTAGCC-32
		R: 52 -GCCGGAAAAAGAAAAACCAAA-32
7	CIRAD1TeakB03	F: 52 -ATGAAGACAAGCCTGGTAGCC-32
		R: 52 -CACTACCACTCATCATCAACACA-32

*All primers were developed by Verhaegen et al. (2005).

Table 3: Programme for SSR-PCR

Step	*Temp. °C*	*Duration*	*Cycles*	*Function*
1	96	4 min	1	Initial Denaturation
2	95	30 sec	38	Denaturation
	51*	45 sec		Annealing
	72	2 min		Extension
3	72	8 min	1	Final Extension
4	4	"		Storage

*Annealing temperature varied from primer to primer.

After completion of the cycles keep the samples at 4°C till electrophoresis.

Separation of amplified products by Polyacrylamide gel electrophoresis

a) The amplified products were resolved on 10% PAGE on a Genei's Vertical Gel Electrophoresis System (glass plate size 24 cm x 19 cm).

b) Preparation of gels involved the following steps:

   1) The glass plates were first washed with liquid detergent then rinsed thoroughly with tap water and washed with distilled water and air-dried. The dried plates were rinsed with methanol and again air-dried.

   2) The plates with spacers were assembled together with the help of clamps and the bottom of the plates was sealed using a small volume of PAGE solution with APS and TEMED.

3) Required volume of APS (0.075 per cent) and TEMED (0.0875 per cent) was added at the end, and the PAGE solution was quickly poured between the glass plates (taking precaution not to introduce any air bubble). Immediately the comb was inserted and the gel was allowed to polymerize for about half an hour.
4) After polymerization the comb was removed carefully and the wells of gel were carefully cleaned with the distilled water.
5) The gel plates were mounted on the vertical gel electrophoresis system with the help of clamps and the upper and lower buffer chambers were filled to the required volume with 1x TBE.
6) After completing all the assembly, gel was pre-run for 30 min at constant voltage.
7) After pre-run, the denatured 25 ìl of the amplified PCR product with the loading buffer was loaded with the help of loading tip in each well and the one gel was electrophoresed at constant voltage of 90 to 100 V for 15 min.
8) Loading in second plate was done after period of 15 minutes. Finally, both the gels were run at constant voltage of 90 to 100 V for 12 – 14 hours
9) After electrophoresis the gels were stained using silver staining.

Silver staining

To resolve the SSR products the silver staining was carried out. Following steps were involved:

a) After electrophoresis the gels were carefully removed from the glass plates and transferred to a tray containing double distilled water and kept for 5 min for gentle shaking.
b) The distilled water in the above tray was replaced with fixing solution containing 135 ml DD H_2O + 15 ml Methanol + 750 ìl glacial acetic acid and kept for another five min with gentle shaking.
c) The above fixing solution was removed from the tray and retained for further use.
d) silver solution i.e., staining solution was now poured in the tray. The silver solution was prepared by dissolving 0.3g Silver nitrate, 15ml of methanol and 750 ìl glacial acetic acid in 150ml of distilled water.
e) The staining was done for 5 min with gentle shaking.
f) The silver solution was removed from the tray and gel was rinsed for a while in distilled water.
g) The gel was transferred to a developing solution (prepared by dissolving 4.5g Sodium hydroxide pellets and 450ìl Formaldehyde in 150ml distilled water).
h) The solution in the tray was shaken gently for 5 to 10 min to appear the DNA bands.

i) The staining was stopped by rinsing the gel for 5 min in the fixing solution retained after step c.
j) The gel was placed on the platform of gel documentation system and photographed under EPI white light.

Analysis of SSR data

The bands were scored '1' for presence and '0' for absence in DNA samples amplified to create a binary data matrix. The data obtained by scoring the profiles with different primers were then subjected to the construction of similarly matrices using Jaccard's coefficients (Jaccard, 1908) using NTSYS-pc software, version 2.02 (Rohlf, 1998). The similarity values were then used for cluster analysis. Sequential Hierarchical Agglomerative Non over lapping (SHAN) clustering was done using UPGMA method (Unweighted Pair Group Method Analysis; Sokal and Michener 1958).

Results and discussion

Diversity at Genetic Level

The results of the SSR amplification showed that, all the seven primers showed pure amplification for the entire selected clonal genomic DNA. Further, all the primers amplification showed polymorphic variations between the clones. Of all the primers used, CIRAD4 Teak Da12 showed maximum polymorphism among the fifteen clones.

Clustering of clones on the basis of SSR profile

The result of SSR analysis generated a total 54 bands, which were scored as 1 (present) or 0 (absent). The binary data were subjected to analysis using NTSYS-pc (Numerical Taxonomy System, version-2.02, Rohlf 1998) for calculating similarity matrix. The SIMQUAL programme was used to calculate Jaccard's coefficients and clustering was done using UPGMA method.

The Jaccard's similarity coefficient value was found to range from 0.45 to 0.91 with an average of 0.68. The clones were grouped into two major clusters I and II Table 4).

Cluster I comprised of majority of clones numbering to thirteen and three for cluster II. The lowest similarity coefficient value of 0.45 was found between the sources of Andhra Pradesh and Maharashtra with exception to APJNB 1 and APMN 4. The highest similarity coefficient value of 0.91 was observed between TNT 17 and TNT 18.

The major cluster, Cluster I resolved into two sub-clusters at a coefficient level of 0.50 viz., A and B. All the population from Maharashtra (MHAL A4, MHAL A8, MHAL P6 and MHSC J2), Tamil Nadu (TNT 6, TNT 11, TNT 17 and TNT 18) and APJNB 1 from Andhra Pradesh grouped into sub-cluster A. Sub-cluster B consists of population from Orissa (ORANP 7 and ORPB 15) and APMN 4 from Andhra Pradesh. At a coefficient level of 0.63, the major cluster, Cluster II resolved into two sub-clusters viz., A and B. Sub-

Table 4: Jaccard's similarity matrix of the fifteen Tectona grandis Linn. f. clones

1.0000000
0.4444444 1.0000000
0.3636364 0.5000000 1.0000000
0.4482759 0.5000000 0.6666667 1.0000000
0.5384615 0.4230769 0.5925926 0.8181818 1.0000000
0.5357143 0.5384615 0.3939394 0.3870968 0.4137931 1.0000000
0.6000000 0.5416667 0.3870968 0.3793103 0.4615385 0.8636364 1.0000000
0.5000000 0.5000000 0.4000000 0.3448276 0.3703704 0.6666667 0.7619048 1.0000000
0.4333333 0.4814815 0.4375000 0.4333333 0.4137931 0.6923077 0.5769231 0.6666667 1.0000000
0.4000000 0.6000000 0.4074074 0.4000000 0.3750000 0.4400000 0.5000000 0.5238095 0.4400000 1.0000000
0.4230769 0.6190476 0.3793103 0.3703704 0.3461538 0.5200000 0.5909091 0.6190476 0.5200000 0.8750000 1.0000000
0.4827586 0.4285714 0.5862069 0.4827586 0.5185185 0.5172414 0.5769231 0.6000000 0.5172414 0.4400000 0.5200000 1.0000000
0.4814815 0.4800000 0.5357143 0.4814815 0.4615385 0.5185185 0.5833333 0.6818182 0.5769231 0.5000000 0.5909091 0.8636364 1.0000000
0.5000000 0.4444444 0.5517241 0.5555556 0.5384615 0.4827586 0.5384615 0.5600000 0.4827586 0.4583333 0.5416667 0.8695652 0.8181818 1.0000000
0.5000000 0.4444444 0.5517241 0.5000000 0.4814815 0.4827586 0.5384615 0.6250000 0.5357143 0.4583333 0.5416667 0.8695652 0.9047619 0.9090909 1.0000000

cluster A consist of APT 4 from Andhra Pradesh and sub-cluster B contains APT 17 and APT 18 also from Andhra Pradesh. Apart from Andhra Pradesh population other three populations showed similarity in their clustering pattern. APJNB 1 clone showed similarity to Maharashtra and Tamil Nadu population while APMN 4 clustered with Orissa population. The principal component analysis estimated using genetic distance values through NTSYS-pc software, mostly supported the UPGMA cluster analysis and also provided resolution of teak populations according to their geographical locations from where they are obtained.

Table 5: Cluster analysis based on SSR data

Particulars	*Cluster*	*Sub-cluster*	*Sub-sub cluster*	*Sub-sub-sub cluster*	*No. of genotype*
	I	A	A1		1
			A2	I	4
				II	4
		B	B1		1
			B2	I	1
				II	1
	II	A			1
		B	B1		1
			B2		1
Total	2	-	-	-	15

The results of the present study showed that the genetic variability between the same species with different clones could be due do the reproductive strategies such as selfing and vegetative propagation (Waller *et al.*, 1987). Further, the result of clustering coefficient analysis showed the variations between the clones and this might be due to the source of

the clones from where they are procured. Dendrogram in the present study did not indicate very clear pattern of clustering for Andhra Pradesh clones. The genetic closeness among the clones within a population for others *i.e.*, Maharashtra, Orissa and Tamil Nadu can be explained by the high degree of commonness in their genotype. Similar results of clustering based on their habitat and location were obtained by in *Gliricidia spp* by Chalmers *et al*., 1992 using RAPD markers, Shreshta *et al*., 2005 in teak using AFLP markers and Fofana *et al*. (2009) on the basis of SSR marker analysis of teak.

Conclusion

The molecular data in addition to morphological data can be of great use in in understanding genotype relationship, which may further assist in developing and planning breeding strategies, genetic conservation and to insure tracking of future evolution of variability. The study can be used to evaluate the status of genetic background of the clonal orchard whether they contain correct clones and ramets and have sufficient genetic diversity as the representative of the species gene pool. They can also be applied to evaluate the status of genetic resources of those which never have been established but planned to establish as a guideline in collection of samples.

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