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Random amplified polymorphic DNA (RAPD) analysis for genetic diversity in *Terminalia bellirica* species (Roxb.), an important medicinal tree

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ABSTRACT

Terminalia bellirica Roxb. (family Combretaceae) also known as Bahera in hindi, is one of the important constituents of India preparation Triphala. It is found in all deciduous forest of India, the present study was formulated to acess the genetic diversity within each population, among the populations and also in micro propagated plantlets of T.bellirica using random amplification amplified polymorphic DNA (RAPD) marker. Plants were collected from four different provenances *of Chhattisgarh State, India, and ten from in vitro produced plantlets. Twenty primers were used for RAPD analysis. RAPD analysis yielded 168 total bands of which 152 polymorphic bands (90.0 percent polymorphism), with an average of 8.4 amplified bands per primer. The dendrogram was constructed using UPGMA method. Our results reveal very high level of genetic diversity in the species. These results also indicate that RAPD is a good molecular marker to study the genetic diversity of these species.*

Key words: *Terminalia bellirica*, genetic diversity, RAPD, Dendrogram, Molecular marker.

INTRODUCTION

Terminalia bellirica is well known medicinal tree. *Terminalia bellirica* is found in all deciduous forest of India up to an altitude of 1000 m. It is found in abundance in Madhya Pradesh, Uttar Pradesh, Punjab, Maharashtra States of India and also in Sri Lanka and Malaya. It also occurs in different parts of India excepting the dry region of Western India [1].

Its several parts are used to cure variety of diseases. The fruit of *Terminalia bellirica* contain about 17% tannins and β sitosterol, gallic acid, ellagic acid, ethyl gallate, galloyl glucose and chebulagic acid [1]. The fruit are useful in cough, hoarseness, eye disease, scorpionsting etc. .Bahara is also used as an astringent and in the treatment of dyspepsia and diarrhoea. *T. bellirica* has demonstrated antimicrobial activity in vitro, including activity against methicill in-resistant *S. Aureus* [2], certain *Terminalia* species demonstrate in vitro antiviral activity [3]. An extract of *Terminalia bellirica* showed significant inhibitory activity on human immunodeficiency virus-1 reverse transcriptase, with IC50 < or = 50 micrograms/ [4]. Four lignans (termilignan, thannilignan, hydroxy-3,4'-(methylenedioxy) flavan, anolignan B) possessed demonstrable anti-HIV-1 in vitro.

Molecular markers such as AFLP, RAPD, and microsatellite (SSR), provide powerful tool for the construction of genetic and physical maps. They are also utilized in marker assisted breeding programs, including those for hardwood species. Genetic maps are useful in determining the relationship between genes and QTLs [5]. In addition, markers have application for assessment of genetic diversity in populations and in paternity analysis. Markers have

been utilized in hardwood tree species and useful for comparative mapping of related taxa. Grattapaglia et al. [6] have utilized microsatellite markers in breeding programs for Eucalyptus. Moran et al. [7] generated a genetic linkage map containing RFLP and microsatellite loci. RAPD have been successfully applied to detect the genetic similarities or dissimilarities in micro propagated material in various plants [8, 9]. The power of RAPD is that it is a fast technique, easy to perform and comparatively cheap. It is immediately applicable to the analysis of most organisms because universal sets of primers are used without any need for prior sequence information [10]. RAPD was used in many different applications involving the detection of DNA sequence polymorphisms, mapping in different types of populations [11, 12], isolation of markers linked to various traits or specific targeted intervals [13,14] and applications such as variety identification and analysis of parentage [15, 16]. The present paper reports for the first time genetic variation in the populations of *T. bellirica*. Random amplified polymorphic DNA (RAPD) markers were used to assess genetic variation in different populations of *T. bellirica*.

MATERIALS AND METHODS

Plant material

The seeds of different provenances were grown in a nursery bed. Total DNA was extracted from leaf samples collected from individual plants in nursery. The sample size was 5 to 20 plants per provenance. There were 20 samples from 20 plants of Raipur, 5 samples from 5 plants of Bilaspur, 5 samples from 5 plants of Jagdalpur, 5 samples from 5 plants of Kawardha, 5 samples from 5 plants of Raigarh and 10 samples of tissue culture plants derived from different seedlings. Total 50 plants from five provenances and tissue culture plant were included in the study.

Table 1: Locations of *Terminalia bellirica* collections

S. N.	Location	No. of Plants	Sample Number
1.	Raipur, Chhattisgarh	20	Tb 1-Tb 20
2.	Kawardha, Chhattisgarh	5	Tb 21- Tb 25
3.	Raigarh, Chhattisgarh	5	Tb 26- Tb 30
4.	Bilaspur, Chhattisgarh	5	Tb 31- Tb 35
5.	Jagdalpur, Chhattisgarh	5	Tb 36- Tb 40
6.	Tissue culture plants	10	Tb 41- Tb 50

Genomic DNA extraction

Genomic DNA was extracted from fresh tender leaf samples using CTAB (cetyl trimethyl ammonium bromide) method with modifications as described by Khanuja et al. [17]. 4 g of fresh leaf of each 50 samples were ground to a fine powder in a mortar in liquid nitrogen and then transferred to Tarson tubes filled with 15 ml of freshly prepared and preheated 2.5 x CTAB extraction buffer. Extraction buffer consisted of 100 mM Tris - Cl (pH 8.0), 25 mm EDTA (pH 8.0), 1.5 M NaCl, 1% PVP and 0.2% _ - merceptoethanol. The suspensions of samples were incubated at 65^0C for 1 h. After cooling at room temperature an equal volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 10 K rpm for 10 min at room temperature. DNA from aqueous layer was precipitated by adding 5 M NaCl (30% of supernatant) and 0.6 volumes of chilled isopropanol. The mixture was centrifuged at 10 K rpm for 10 min. Pellets were washed with 80% alcohol and let the pellet dry. Pellets were dissolved in high salt TE buffer then RNase A were added and incubated at 370 C for 30 min. Again, it was extracted with equal volume of chloroform - isoamylalcohal (24:1). 2 volumes of cold ethanol were added in aqueous layer and centrifuged at 10 K rpm for 10 min. Pellets were washed with 70% alcohol and dissolved in TE (Tris- Cl- EDTA) buffer after drying.

RAPD analysis

The RAPD technique cosistes of peferential amplification of random sequences by PCR. In this assay. 20 different primer were used. A PCR tube contained 20 ng DNA, Taq DNA polymerase (0.6 unit), 100 mM dNTPs, 2.5 µl 10x polymerase buffer and 5 pmol decanucleotide primers. Samples were subjected to following thermal profile: 94 for 1 min, 35 for 1 min and 72^0C for 2 min for 44 cycles and final extension at 72^0C for 5 min. Separation of the amplified fragments was performed on 1.2% agarose gels, TAE 1% at 80 V during 2 h. The gels were stained with 0.5 µl-1 ethidium bromide for visualizing the RAPD fragments.

Screening of specific RAPD amplicon

Eighty Random primers (OPA 1-10, OPB 1-10, OPH 1-10, OPM 1-10, OPD 1-10, OPO 1-10 and MAP 1-20) were screened by RAPD for identification of specific marker. The screening of primers resulted in 20 MAP decamer primers [18] which showed polymorphisms with all 50 samples.

Data analysis

RAPD bands sizes were designated as amplified bands, and bands were shared as diallelic characters (present = 1 and absent = 0). The number of polymorphic bands is calculated for each population. Similarity matrices were computed based on Jaccard's similarity coefficient, using the SPSS. Calculate the SI between each sample and use UPGMA algorithm to construct the dendrogram.

Table 2: Sequences of MAP 1 to MAP 20 primers

S. N.	Primer	Sequence of primer	S. N.	Primer	Sequence of primer
1.	MAP 1	AAATCGGAGC	11.	MAP 11	CCCTGCAGGC
2.	MAP 2	GTCCTACTCG	12.	MAP 12	CCAAGCTTGC
3.	MAP 3	GTCCTTAGCG	13.	MAP 13	GTGCAATGAG
4.	MAP 4	TGCGCGATCG	14.	MAP 14	AGGATACGTG
5.	MAP 5	AACGTACGCG	15.	MAP 15	AAGATAGCGG
6.	MAP 6	GCACGCCGGA	16.	MAP 16	GGATCTGAAC
7.	MAP 7	CACCCTGCGC	17.	MAP 17	TTGTCTCAGG
8.	MAP 8	CTATCGCCGC	18.	MAP 18	CATCCCGAAC
9.	MAP 9	CGGGATCCGC	19.	MAP 19	GGACTCCACG
10.	MAP 10	GCGAATTCCG	20.	MAP 20	AGCCTGACGC

RESULTS AND DISCUSSION

Genomic DNA was isolated from fresh leaf tissues of all the 50 plants of *T. bellirica* collected from different locations (Table 1). The procedure yielded 200-300 ng of DNA per 4.0 gm of tissue in 0.8% agarose gel electrophoresis (Figure 1). Of the 80 primers screened, MAP 1-20 primers (Table 2) produced distinct, highly reproducible amplification profile for all the screened samples (Figure 1 and Figure 2). These 20 primers were selected for further analysis of the plant materials.

Table 3: Table shows MAP1-20 primers, total amplified bands (AB), polymorphic bands (PB) in five provenances and in total samples of *T. bellirica*

S.N.	Primer	Polymorphism in provenance						Total AB	Total PB in *T.bellirica*
		Raipur AB/PB	Kawardha AB/PB	Raigarh AB/PB	Bilaspur AB/PB	Jagdalpur AB/PB	TCP AB/PB		
1	MAP 1	7/2	7/5	4/3	3/3	2/2	3/1	7	7 (1.0)
2.	MAP 2	5/4	5/2	6/2	5/2	6/4	9/6	9	9 (1.0)
3	MAP 3	8/6	3/2	7/4	6/4	7//4	6/2	8	8 (1.0)
4.	MAP 4	6/4	12/6	5/3	4/2	5/3	6/3	12	11 (0.9)
5.	MAP 5	10/4	7/4	8/4	8/5	8/4	8/2	10	9 (0.9)
6.	MAP 6	8/6	5/3	3/1	4/2	6/3	10/4	10	8 (0.8)
7.	MAP 7	6/3	4/2	4/3	3/1	3/2	7/5	7	7 (1.0)
8.	MAP 8	6/3	5/1	4/2	3/1	3/1	5/3	6	5 (0.8)
9.	MAP 9	6/3	4/3	5/4	7/1	7/1	4/1	9	9 (1.0)
10.	MAP 10	7/5	7/4	5/4	5/1	5/3	12/7	12	10 (0.8)
11.	MAP 11	6/4	3/2	3/1	3/1	4/1	3/1	6	6 (1.0)
12.	MAP 12	4/4	2/1	3/1	6/3	3/2	4/1	6	6(1.0)
13.	MAP 13	5/3	7/4	3/2	4/3	4/3	6/2	7	7 (1.0)
14.	MAP 14	4/2	3/1	4/2	9/6	5/2	4/1	9	9 (1.0)
15.	MAP 15	4/3	3/2	2/1	2/1	6/4	4/2	6	6 (1.0)
16.	MAP 16	4/3	4/2	7/4	3/2	4/3	5/3	7	7 (1.0)
17.	MAP 17	4/4	5/1	4/2	3/1	4/2	5/3	4	3 (0.7)
18.	MAP 18	8/2	11/6	9/1	8/1	8/2	7/3	11	8 (0.7)
19.	MAP 19	9/3	6/4	4/1	6/2	5/3	4/1	9	6 (0.6)
20.	MAP 20	13/11	6/2	6/2	5/1	6/1	5/2	13	11 (0.8)
Total % PB		130/81 62%	109/57 52%	96/47 48%	97/43 44%	101/50 49%	117/53 45%	168	152 90%

The MAP1 - 20 primers produced total 168 bands; the number of bands ranged 04 to 13 bands, with an average of 8.4 bands per primer; 152 bands (90.0%) of these were polymorphic (Table 3). The bands amplified by different primers showed diversity within and among the provenances. The size of the amplified bands varied with different primers and with DNA sample of different provenances. The MAP 4, MAP 12 and MAP 18 showed maximum size of band with 4268 base pairs and minimum size of band 831 base pairs. The *T. bellirica* samples from different provenances showed intra provenance variation. Raipur provenance exhibited highest (62%) polymorphism followed Kawardha (52%), Jagdalpur (49%) and Raigarh (48%) provenances. Lowest (44%) polymorphism occurred in Bilaspur provenance.

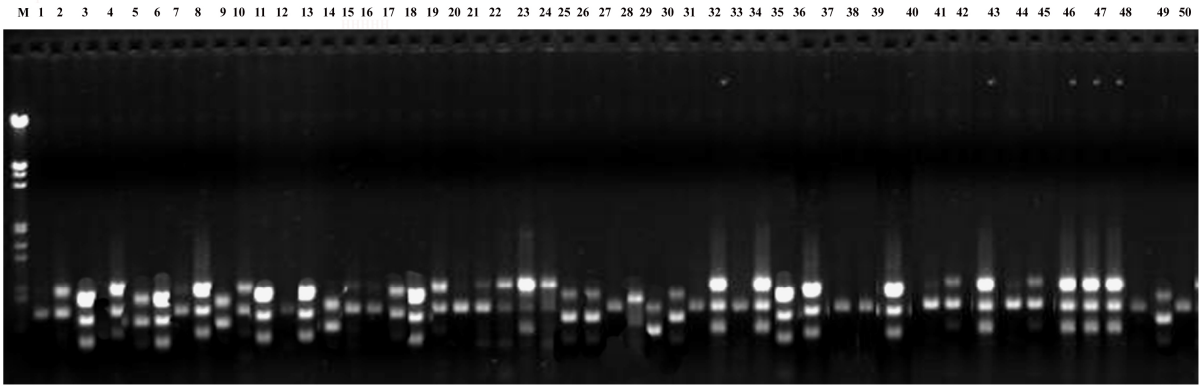

Figure 1- RAPD patterns of a 1-50 samples of *T.bellirica* using primer MAP 9. M – λ DNA/ Hind III double digest, Lane 1 to 20- Raipur samples, Lane 21 to 25- Kawardha samples, Lane 26 to 30- Raigarh samples, Lane 31 to 35- Bilaspur samples, Lane 36 to 40- Jagdalpur samples, Lane 41 to 50- Tissue culture plants

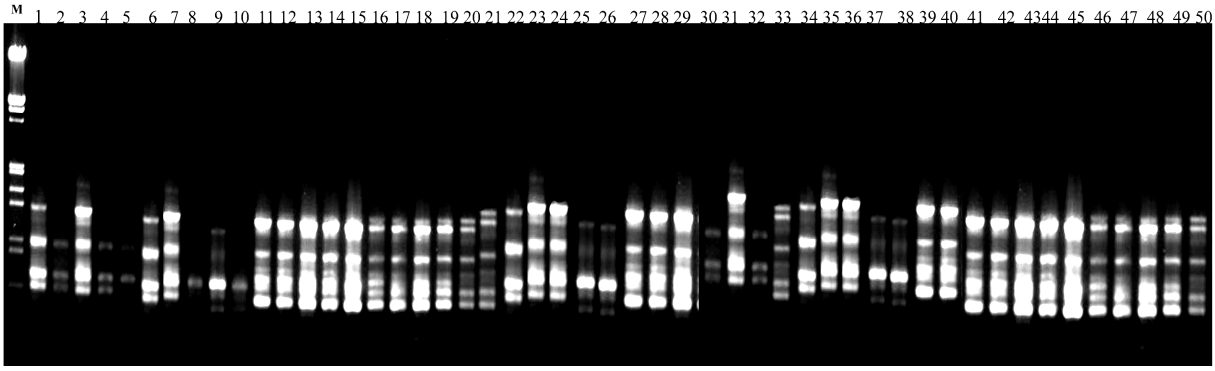

Figure 2- RAPD patterns of a 1-50 samples of *T.bellirica* using primer MAP 15. M – λ DNA/ Hind III double digest, Lane 1 to 20- Raipur samples, Lane 21 to 25- Kawardha samples, Lane 26 to 30- Raigarh samples, Lane 31 to 35- Bilaspur samples, Lane 36 to 40- Jagdalpur samples, Lane 41 to 50- Tissue culture plants

Based on the data of RAPD amplified products, dendrogram for 50 samples of *T. bellirica* were constructed using UPGMA algorithm (Fig. 3). In the dendrogram the 50 samples were divided into two major groups based on the Genetic similarity. The first major group was again classified into 2 sub groups and shared 0.45 % similarity coefficient. The first subgroup was included all the 20 samples from Raipur provenance (Tb1 to Tb 20). The second subgroup included all tissue culture plants (Tb 41 to Tb 50). The tissue culture plants originated from nodes of different seedlings from Raipur provenance, because of this the tissue plant share group with Raipur provenance plant. The second major group was again classified into 4 sub groups and they shared 0.47 % similarity coefficient. First sub group included the samples collected from Kawardha provenance (Tb 21 to Tb 25). The secound sub group included the accession samples collected from Raigarh provenance (Tb 26 to Tb 30). The third sub group included the samples collected from Bilaspur provenance (Tb 31 to Tb 35). The fourth sub group included the samples collected from Jagdalpur provenance (Tb 36 to Tb 40). The similarity index based on the number of shared RAPD products. These plants exhibited 90% polymorphism. The percentage of polymorphism between samples i.e. 90.0% was higher in comparison to other plant eg. *Phyllanthus amarus* (65%), *Changium smyrniodes* (69%), *Lonicera*

(83.9%), *Pinus sylvestris* (60.3%). This shows that the species genetic diversity by itself is high compared to other species. The results showed that the genetic diversity of this samples is higher, possibly allowing it to more easily adapt to environmental variations. The genetic differentiation of samples of *T. bellirica* could brodally be explained as a results of aboitic (geographical e.g.- hydrographic connection or climatic differentiation) factors. In this study, we have developed RAPD marker for *T. bellirica*.

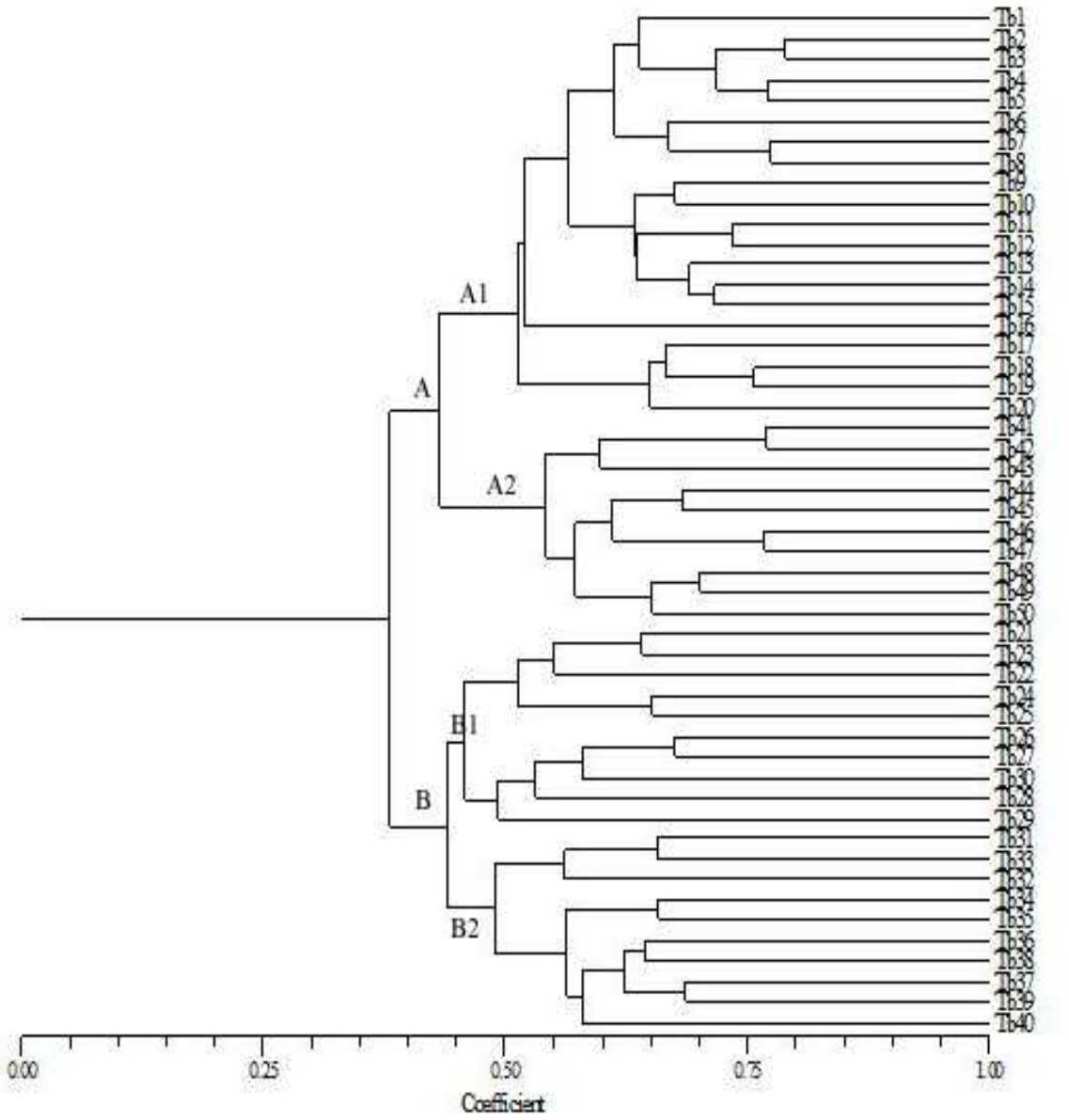

Figure 3. Dendrogram showing clusters, Cluster A (Tb1 to Tb 20 and Tb 41 to Tb 50) and Cluster B (Tb 21 to Tb 40). Sub cluster A_1 – Tb 1 to Tb 20, A_2 – Tb 41 to Tb 50. Sub cluster B_1 – Tb 21 to Tb 30, B_2 – Tb 31 to Tb 40

Several reports are available to demonstrate the use of RAPD markers for determination of genetic variation in plants. Jain et al. [19] studied molecular diversity in *Phyllanthus amarus* by RAPD profiling of 33 collections from different location using MAP primers. Mathur et al. [20] studied genetic fidelity of micro-cloned progeny of *Chlorophytum borivilianum*. They scored 79 amplified reproducible monomorphic bands with three different sets of 24 decamer primers (14 MAP, 3 OPO, 7 OPA primers). Khanuja et al. [18] used 60 random primers to analyze 11 accessions from six taxa of *Mentha*. Nanda et al. [21] used forty primers and selected 17 primers on the basis of their ability to detect distinct, clearly resolved and polymorphic amplified product for the analysis of six species of

Acacia. They found high degree of diversity (70%) within the six tree species of *Acacia*. Abd-El-Haleem et al. [22] reported genetic analysis and RAPD polymorphism in Wheat Genotypes. Naugzemys et al. [23] reported genetic variation and relationship among 39 accessions of *Lonicera caerulea* and one accession of *L. xylosteum*. Batitini et al. [24] evaluated the genetic diversity of seven populations of *Anemopaegma arvense*, using random amplified polymorphic DNA markers. Ponnuswami et al. [25] used RAPD markers for identify the desirable traits in Palmyrah palm. Maia et al. [26] used random amplified polymorphic DNA (RAPD) markers to detect polymorphism and to examine relationships among four table grape clones from NorthWestern Paraná, in Southern Brazil. Khurana et al. [27] evaluated the genetic variation in the *Jatropa curcas* using RAPD and ISSR marker. Suwanchaikasem et al. [28] demonstrated that *T. laurifolia* was successfully distinguished from its related species based on their molecular signatures. They show that RAPD analysis as a technique that is able to examine the phylogenetic relationship of different plant species. Osman et al. [29] used RAPD marker for the identify *Eucalyptus* species genome.

CONCLUSION

The present study is the first report that provides genetic information of *T. bellirica* populations. The level of RAPD variation in population of *T. bellirica* has been investigated. The majority of RAPD variations in this species were between the populations rather than within population. It is suggested that RAPD markers could be successfully applied for detecting genetic variability in natural population of *T. bellirica*. Moreover RAPD marker will have a major impact on the conservation and improvement of a tree species *T. bellirica*.

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