RAPD based assessment of genetic diversity of *Butea monosperma* from different agro-ecological regions of India

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*Butea monosperma* (Lam.) Taub. (English. Flame of the forest) belonging to the family Fabaceae, is an anthropogenic tree of several castes and also a very useful tree for both local people and pharmaceutical industry. This valuable tree needs attention for the characterization of its genetic diversity, protection and cultivation. The present work describes randomly amplified polymorphic DNA (RAPD) analysis to assess the genetic divergence among 16 *Butea* accessions collected from four agro-ecological regions of India (nine agro-climatic sub zones), covering five states (Uttarakhand, Uttar Pradesh, Rajasthan, Madhya Pradesh and Karnataka). Out of the 30 ten mer random primers used for studying genetic divergence, 12 were polymorphic, generating a total of 145 amplification products with an average of 12 products per polymorphic primer and an estimated mean gene diversity of 0.43. Genetic relationships among accessions were evaluated by generating a similarity matrix based on Jaccard’s coefficient ranging from 0.53 to 0.79. The phenetic dendrogram generated by UPGMA analysis grouped accessions into four clusters. Primer 5, 12 and 16 were found most informative based on their resolving power and their potential to differentiate all the accessions. The degree of genetic variation detected among the 16 accessions with RAPD analysis suggested that RAPD could be used for studying genetic diversity in *Butea*. The study also demonstrated that *Butea* germplasm collected from different agro-ecological regions showed no isolation based on sub-climatic zones as the accessions collected from different sub-climatic zones grouped together in the genetic tree.

**Keywords:** DNA, *Butea monosperma*, molecular profiling, RAPD, agro-ecological regions

**Introduction**

Various types of molecular markers are utilized to evaluate DNA polymorphism. Random amplified polymorphic DNA (RAPD)¹, a PCR based technique is simple, cost-effective and a powerful tool in the analysis of plant genome characterization. Although, RAPD is criticized for its low reproducibility, but it is overcome by optimization of the RAPD reaction and maintenance of stringent conditions. RAPD has, therefore, been extensively used in assessing genetic diversity and relationship measures in various plant species.²⁻⁶

Large-scale characterization of plant species in varying geoclimatic conditions can be performed using various parameters such as seed morphometric traits and isozymes. However, environmental factors as well as the developmental stage of the plants influence such traits. DNA based markers provide new tool for ecological and genetic studies of evolutionary processes. Newer markers such as microsatellites and RAPD provide more detailed genetic information due to either the increased variability of loci or the greater numbers of the available loci.⁸⁻⁹ These markers have successfully been used to estimate levels of relatedness among the individuals, studies of mating systems, and of seed dispersal and seedling establishment in natural populations.¹⁰

*Butea monosperma* (Lam.) Taub. belonging to family Fabaceae, is commonly known as Flame of the forest. It is a medium sized deciduous tree, which attains a height of 10 to 15 m and a diameter of 20-40 cm at maturity. The plant is widely spread in naturally hilly ecosystems of India and Myanmar.¹¹,¹²

*B. monosperma* is an anthropogenic tree of several castes. It is considered as a sacred tree, referred to as a treasure of the Gods and finds references in Vedic literature. It is also a very useful tree for both local people and pharmaceutical industry. All parts of the tree are useful; chemical compounds from its bark, leaves, flowers and seeds have many ethno-medicinal applications. These compounds are used in pharmaceutical products for the treatment of diarrhoea, diabetes, liver problems, skin diseases, cancer and for use as aphrodisiacs.¹⁴⁻¹⁸ Its wood is

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used as firewood and for construction. The large leaves are used for making baskets and little bowls. The ecological importance of this species for habitat stabilization, afforestation and wasteland reclamation has been mentioned.

Only a few tree species like *Azadirachta indica* (neem), *Prospis* and *Acacia* have been characterized for their genetic diversity. This valuable tree also needs attention for the characterization of its genetic diversity, protection and cultivation. The present work describes RAPD analysis to assess the genetic divergence among its 16 accessions collected from four agro-ecological regions (nine agro-climatic sub-zones) of Uttarakhand, Uttar Pradesh, Rajasthan, Madhya Pradesh and Karnataka states.

**Materials and Methods**

**Plant Material**

Samples of mature leaves were collected from the *Butea monosperma* trees of approximately same age (based on trunk size, height, etc.) from various agro-ecological major zones and sub-climatic zones. They were shed dried at the site, and stored in a sealed plastic bag at room temperature for further experiments after assigning an individual alphanumeric code as mentioned in Table 1.

**DNA Extraction**

The total genomic DNA was isolated from various leaf samples following the method of Doyle and Doyle as modified by Weising. The extracted DNA was subjected to an additional step of purification with chloroform:isoamyl alcohol (24:1) treatment followed by precipitation with chilled solution of ethanol-NaOAc (1.5 mL of 3 M NaOAc in 30 mL ethanol) The pellet was washed with 70% ethanol, air dried and dissolved in TE buffer. The DNA was checked on 0.8% agarose and visualized under Mini-Transilluminator (Bio-Rad, India). The gel was photographed and analysed by a Kodak gel documentation system (Model EDAS 290) using Lambda DNA double digest (Bangalore Genei Pvt. Ltd.) as a standard (Fig. 1).

**RAPD Analysis**

A total of 30 random decamer primers (custom synthesized by Bangalore Genei Pvt. Ltd., GC content >50%) were used for RAPD analysis. DNA amplification reaction was performed in 25 µL reaction volume which contained an end concentration 2.5 mM each of the dNTPs, 1 U/µL Taq polymerase enzyme, 25 ng DNA template and 10 ng

![Fig. 1 — DNA of *B. monosperma* from different agro-ecological zones.](image)

<table>
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<th>Sub zones</th>
<th>Place</th>
<th>Sample code</th>
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primer in Taq polymerase assay buffer (1 X). Amplification reaction was carried out in a Bio-Rad Thermal cycler with the following thermal profile: one cycle of 4 min at 94°C (initial denaturation) followed by 45 cycles of 15 sec at 94°C (denaturation), 45 sec at 40°C (primer annealing) and 90 sec at 72°C (primer elongation), and finally one cycle of 4 min at 72°C (final extension). Amplified PCR products were separated on 1.5% (w/v) agarose gel in 1 X TBE (pH 8.3) with EtBr in a final concentration of 10 µg/mL. Electrophoresis was performed at 100 volt for 2 h. Then the gel was visualized, photographed and analysed as described earlier. A low range DNA ruler (Bangalore Genei Pvt. Ltd.) was used as a molecular size marker. The reproducibility of the amplification products was checked twice for each polymorphic primer.

Data Analysis

RAPD bands were scored for presence (1) and absence (0) across all Butea accessions for each primer. The pairwise genetic similarities among all pairs of samples were estimated with Jaccard’s coefficient. The statistical analysis was carried out using NTSYS-PC software (version 2.11s). In order to group genotypes into discrete clusters, a dendrogram was constructed by employing UPGMA. Resolving power (Rp) for each primer was calculated following Prevost and Wilkinson’s method for selecting primers that can distinguish maximal number of accessions. Rp of a primer was calculated as 1-[2 X (0.5-p)], p being the proportion of the 16 accessions containing the bands.

Results

Fingerprint Profile

There are seven major agro-ecological regions in India. For the present work, leaf samples of B. monosperma were collected from 16 sites representing four major agro-ecological zones viz. arid, semi-arid, dry sub-humid and moist sub-humid belonging to 9 agro-climatic sub zones distributed in the five states (Uttarakhand, Uttar Pradesh, Madhya Pradesh, Rajasthan and Karnataka). Except Karnataka, the other states covered the northwest part of India. Further, to study the variation between the samples from the plants located within 10 m distance, two samples were collected from Jodhpur (arid zone). Then, to know about the genetic relationship between the accessions located at the same site but at a distance of more than 10 m but less than 200 m, two samples were collected from Bangalore (semi-arid). Hence, a total of 18 samples were collected; 16 from different accessions and two each from the same location. The samples were analyzed for genetic diversity using 30 random primers. Of the 30 primers, 20 primers (66%) resulted in amplification, of which 12 primers (60%) gave reproducible and scorable results. The pictures of RAPD PCR gel amplified by primer G5, G12 and G16 are shown in Fig. 2. Out of a total of 145 fragments generated by random decamer primers, 126 (86%) were polymorphic with an average of 10 polymorphic products per primer.

The number of markers detected by each primer depends on primer sequence and the extent of variation is genotype specific. The number of products amplified by the polymorphic primers varied from 8-17. The primers 5, 12 and 16 detected maximum products. Gene diversity was calculated for each primer, which varied from 0.58-0.79 values with a mean diversity of 0.63 (Table 2).

Fig. 2— Gel picture showing the RAPD pattern of B. monosperma from different agro-ecological zones generated by 3 RAPD primer (a-G 5, b- G 12, c- G 16).
Rp of the 12 RAPD primers ranged from 0.34-15.07 with 10.84 as the average value. Based on Rp and the ability of primers to differentiate all the accessions, the primers 5, 12 and 16 were found most informative with Rp of 15.07, 14.97 and 14.33, respectively.

**Genetic Similarity Matrix and Cluster Analysis**

RAPD data were used to make pairwise comparison of the accessions based on shared and unique amplification products to generate a similarity matrix with NTSYS-PC (version 2.11s). Similarity value for all the 16 accessions ranged from 0.53 to 0.79 (Fig. 3). Of the 16 samples analyzed, two accessions, BD-9 and BD-15, collected from Kanpur and Singoli displayed the maximum genetic similarity, with a 0.79 similarity coefficient value.

The similarity matrix representing Jaccard’s coefficient was used to cluster the data following the UPGMA algorithm. As per Fig. 4 one genotype (Jodhpur) is not grouped. Rest of the genotypes were grouped into three clusters. The two samples from Jodhpur where the plants were less than 10 m apart showed the similarity coefficient value 0.98 and did not group with other genotypes. The cluster one was represented by seven samples from Bhilwara (D-4.2), Kota (D-4.2), Jhalawar (D-4.2), Meerut (D-4.3), Banasthali (D-3.3), Pratapgarh (D-4.2) and Chittorgarh (D-4.2). All samples of this group belonged to semi-arid agro-ecological major zone and three agro-climatic sub zones. The maximum similarity within group (0.77) was shown by the samples collected from Meerut and Banasthali despite
belonging to different agro-climatic sub zones, D-4.3 and D-3.3, respectively. It was followed by the similarity coefficient value 0.76 represented by the samples collected from Pratapgarh and Chittorgarh, both belonging to the same agro-climatic sub zone, D-4.2. This sub cluster showed least similarity with the other members of this group though they belonged to same agro-climatic sub zone. The cluster two was represented by four accessions from Pantnagar (CM-6.2), Saharanpur (CD-5.4), Lucknow (CD-5.4) and Roorkee (CD-6.1). Out of these four accessions, Pantnagar and Roorkee belonged to moist sub-humid zone and were related with each other with a similarity coefficient value 0.68. The other two members of this group from Saharanpur and Lucknow belong to dry sub-humid agro-ecological zone and showed the similarity with moist agro ecological zones with a coefficient value 0.66. The cluster three was represented by three accessions of Banaras (CD-4.1), Singoli (D-4.2), Kanpur (CD-5.4) and one accession from Bangalore (D-4.4) with two samples more than 10 m apart. This cluster represented the dry sub-humid agro-ecological zone with the exception of accession from Bangalore and Singoli, which belonged to semi-arid agro-ecological zone. Two samples collected from within Bangalore region showed maximum similarity (0.98) within the cluster. The accessions from semi-arid and dry sub-humid zones showed similarity coefficient value of 0.66. Overall, the accessions from Singoli and Kanpur belonged to semi arid dry sub-humid agro-ecological zone respectively, showed the maximum similarity. The minimum similarity was exhibited by the accessions from Jodhpur and Bangalore with a similarity value of 0.57 as these belonged to different agro-ecological zones and are geographically far apart from each other.

**Discussion**

Ecological and geographical differentiations are important factors influencing strategies for breeding and sampling the tree species. In this background, various accessions of *B. monosperma*, were collected from various locations as described earlier. Major regions and sub zones have been classified according to Sehgal.

To access the genetic diversity of *B. monosperma* samples from different agro-ecological zones in India, 30 random primers were used of which 20 primers gave amplification and of them, 12 (60%) resulted in polymorphic, scorable and reproducible results. These primers generated 145 fragments of which 126 (86%) were polymorphic. Deshwal et al. reported 14 (58%) polymorphic primers with 73 amplification products.
in neem accessions. In contrast, Goswami and Ranade\textsuperscript{22} reported only 29% polymorphic primers with 357 bands in \textit{Prosopis} species. Thus, the number of polymorphic primers and fragments generated were not of similar range for tree species. They can vary significantly in different plant species. This is understandable as product amplification depends upon the sequence of random primers and their compatibility within genomic DNA. The number of markers detected by each primer depends on primer sequence and the extent of genetic variation, which is genotype specific\textsuperscript{6}.

On comparison with other tree species, the gene diversity in \textit{B. monosperma} was comparatively of narrow range, but with relatively higher individual gene diversity values (0.58-0.79) as well as higher mean gene diversity value (0.65). For example, Deshwal \textit{et al}.\textsuperscript{21} analyzed 29 samples of neem, an open pollinated tree, for its gene diversity and found it to vary in the range of 0.2-0.88 with 0.49 mean gene diversity. The higher mean gene diversity could be explained since the samples were collected form four agro-ecological regions in contrast to the samples of neem collected from two agro-ecological zones.

\(R_p\) has been found to correlate strongly with genotype diagnosis and so has potential for a number of applications\textsuperscript{30}. It is possible that several primers included in a preliminary study are able to distinguish between all of the genotypes used. Such primers are the most likely to be selected for larger applications although there is currently no basis for comparing between them. In the present study, primers 7, 15 and 16 were able to recognize all the 16 accessions used and so could not be segregated on the basis of their ability to diagnose genotypes. Nevertheless, these could be ranked according to their \(R_p\) values under the reasonable premise that primers with higher \(R_p\) value have a greater capacity to separate different accessions\textsuperscript{30}. The random primers used for estimating gene diversity were found most suitable for the purpose. As they showed much higher value (6.2-15.07) than reported in neem (0.21 to 2.62) with RAPD primers\textsuperscript{3} and (2.86 to 9.46) in date palm with ISSR primer. The higher value of \(R_p\) revealed that the primers used in the present study are most suitable for assaying gene diversity in \textit{B. monosperma} collected from different agro-ecological zones of India.

We report the genetic diversity values in the range of 0.57-0.79 in \textit{B. monosperma}. The range of genetic diversity values broadly indicate the degree of heterogeneity or homogeneity in different accessions of the plant species\textsuperscript{22}. There are few other tree species where the genetic diversity range has been determined. Deshwal \textit{et al}.\textsuperscript{21} reported a genetic diversity value of 0.72-0.96 on the basis of 24 RAPD primers in different accessions of neem collected from 2 agro-ecological and 12 sub-climatic zones of India. However, based on AFLP marker somewhat higher range (0.48-0.93) was reported\textsuperscript{32}. On the other hand, on the basis of 14 RAPD primers in various species of \textit{Prosopis} viz. \textit{P. glandulosa}, \textit{P. pallida} and \textit{P. juliflora}, the genetic diversity values were 0.28-0.62, 0.18-0.75 and 0.19-0.72, respectively. One possible reason cited for higher genetic diversity values for \textit{P. juliflora} was that in this case the accessions represented different agro-ecological regions while in the other species the accessions were from the same region. In the present study, the accessions represented 4 different agro-ecological zones from 9 sub-climatic regions in India. Despite this, \textit{B. monosperma} showed somewhat lower range of genetic diversity, which may imply conservation of germplasm and lower level of heterogeneity. One possible reason may be that this species has limited geographical distribution; predominantly only in naturally hilly ecosystem of India and Myanmar\textsuperscript{11,12}. In contrast, date palm\textsuperscript{33} has shown a much higher degree of genetic diversity, 0.02-0.77. However, \textit{Acacia} showed a genetic diversity range value similar to \textit{B. monosperma} 0.09-0.31\textsuperscript{22}.

To estimate the potential of individual primers for characterization of variability in \textit{B. monosperma}, data obtained from individual primers were processed separately (not shown). Different primers grouped the accessions in 2-6 clusters. However, none of the individual primers could cluster these plants into region specific or agro-climatic sub zone specific cluster. While studying the diversity between the samples collected within a short distance, the results revealed (not shown) that even the most polymorphic primer could not distinguish either the two individuals collected from Jodhpur within 10 m area (a similarity coefficient value of 0.97 for both) or collected beyond 10 m distance but less than 200 m from Bangalore (a similarity coefficient value of 0.92).

The samples from Jodhpur and Bangalore showed significant genetic diversity with a coefficient value of 0.57. This is understandable as these samples are located far apart (> 2500 km), at different altitudes and belong to two different agro-ecological major
zones, viz. arid (Jodhpur) and semi arid (Bangalore). This shows that climatic conditions and physical parameters may affect the plant genome as the plant is adapted and these changes are inherited through genome to the next generation.

Cluster analysis has clearly indicated that there is eco-geographical isolation between the samples collected from the four agro-ecological zones with some exceptions. The samples from Jodhpur belonged to arid agro-ecological zone did not group with other samples. This is understandable as this is the only germplasm belonging to arid zone hence, placed separately at one end of the dendrogram. The samples from semi-arid agro-ecological zones cluster separately (except one sample from Bangaore, which clustered with the samples belonging to dry sub-humid agro-ecological zone). The cluster two and three are represented by mixing of samples from two agro-ecological zones. The study did not show the separation of individuals from different agro-climatic sub zones of same agro-ecological region. Deshwal et al.21 also found the similar results in neem collected from two agro-ecological zones. Human intervention, which makes partitioning and distribution of variability complex is cited as reason for the grouping of samples to one cluster collected from different sub zones. It may be true in our study also. This assumption has been further supported by neem34. The same reason may be attributable to grouping of samples to one cluster in our present work on B. monosperma. Lack of clustering according to their geographic origin was observed previously in other studies based on allozyme35, SSRs36 and AFLPs37. In all these studies, a wide range of geographical origin with few accessions per origin were analysed. Conversely, in several other studies which involved numerous accessions per geographical origin (either regions or countries), genetic variation was observed to be correlated with geographical origins38,39.

The present study suggests that RAPD is appropriate for analysis of genetic variability in closely related genotypes. Moreover, RAPD could differentiate the plants collected from Rajasthan and Bangalore though belonging to same agro-climatic sub zones.

The high level of genetic diversity suggested the regional approach for conservation of B. monosperma. The species or at least a large part of its genetic diversity may be lost in the near future due to its medicinal and other uses and its consequent exploitation, if appropriate conservation measures are not adopted. Since single or even a few plants will not represent the whole genetic variability in Butea, there appears to be a need to maintain sufficiently large populations in natural habitats to conserve genetic diversity in Butea and avoid genetic erosion.

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