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MOLECULAR DIVERSITY ANALYSIS OF FLOWER COLOUR VARIANTS OF *BUTEA MONOSPERMA* (LAM.) TAUB. USING INTER SIMPLE SEQUENCE REPEATS

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ABSTRACT
Butea monosperma is the most important host tree for the *rangeeni* strain of lac insect, *Kerria lacca,* exploited commercially in India for lac cultivation. Six flower colour variants of *B. monosperma viz*., scarlet, yellow, golden yellow, chrome yellow, white and mustard colour were collected from different regions of Jharkhand. Inter Simple Sequence Repeat (ISSR) marker system was employed to investigate the genetic structure and diversity among these flower colour variants. Out of 45 ISSR primers, 12 were found to be informative generating 127 scorable bands. A maximum of 19 and a minimum of 7 scorable bands per primer were obtained. The resolving power of the primers ranged between 2 and 11.3. Average polymorphic band percentage for all the primers was 85%. The marker index ranged from 2.9 to 15.85. The estimated Jaccard similarity coefficient ranged from 0.35 to 0.52. The clustering analysis using Jaccard's coefficient showed 2 clusters; first - yellow and golden yellow and second - mustard, scarlet, chrome yellow and white flower colour variants.

INTRODUCTION

Butea monosperma (Lam.) Taubert is a medium sized deciduous tree native to tropical and subtropical climate. It is common throughout India, Burma and Sri Lanka. It belongs to the family *Fabaceae* and known as bastard teak, *dhak* and *palas* (Roonwal *et al*., 1958). It has beautiful flowers adorning leafless canopies during early summer and is aptly described as 'flame of the forest'. It is an economically important multipurpose tree species. Almost all parts of the tree namely roots, stem, bark, leaves, flowers, fruits, seeds and gum are used in Ayurvedic, Unani and Homeopathic medicines. *B. monosperma* showed potential hepatoprotective, anti-tumorigenic properties especially in breast, anti-osteoporotic, thyroid inhibitory, anti-viral, anti-inflammatory, anti-diabetic, anti-fungal, antioxidant, anti-diarrheal, anticonvulsant, anti-stress, memory and behavior stimulant, anti-microbial, anthelmintic, anti-asthmatic, anti-ulcer and astringent activities (Nadkarni, 2002; Burli and Khade 2007; Gupta *et al*., 2008; Mazumder *et al*., 2011). It not only provides timber, but also yields several non-timber forest products, such as a water soluble dye, fodder and leaves for platters (Pal and Bose, 2011). Besides all these applications, *B. monosperma* has been extensively used for *rangeeni* lac cultivation by tribals of India since time immemorial. Lac, a natural resin secreted by the lac insect, *Kerria lacca* (Kerr), is used in various industries such as food, pharmaceuticals, electrical, cosmetics, paints and varnishes (Ramani, 2010). Besides being a source of livelihood to millions of economically backward population especially tribals in Jharkhand, Madhya Pradesh, Chhattisgarh, Maharashtra and West Bengal *etc*., lac is now gaining national importance as a potential export commodity (Ramani *et al*., 2010). *B. monosperma* is the widely and commonly grown lac host plant for *rangeeni* strain of lac insect (Kapur, 1962). About 50% of lac produced in India is obtained from the *rangeeni* strain of lac insect (Pal *et al*., 2011).

In case of plant species, a lot of variations occur in leaf shape, leaf arrangement, flower colour, seed shape *etc*. *B. monosperma* is also known for the variations in flower colour, number of leaflets, leaf shape and arrangement. The flower colour of *B. monosperma* has been variously described as scarlet, red and orange-red (Anon., 1983, 1988, 1994). Scarlet coloured flower is the most commonly available flower colour in the nature. The rare yellow and white flowering trees have also been reported from different parts of the country (Sanjappa, 1987). White variety of *B. monosperma* which bears white flowers with yellowish central portion was reported by Kamran (1989) from Madhya Pradesh. Singh *et al*. (1983) has reported a yellow colour variant. Three rare variants of *B. monosperma*, with unifoliate leaves, yellow flowers and white flowers have been identified from Jharkhand (Kumar *et al*., 2006). Apart from these variants, some more flower colour variants *viz*., golden yellow, chrome yellow and mustard colour were also reported from Jharkhand (IINRG, Annual Report 2011-12). An understanding of the level of genetic

diversity, ascertaining the relationships and genetic structure among the flower colour variants of *B. monosperma* is extremely important for genetic improvement with respect to sustainable lac production and conservation in light of rampant deforestation. Knowledge about polymorphism and genetic relationships among variants could be an invaluable aid in crop improvement strategies to augment lac production, thereby uplifting the economic status of the tribal people who are involved in lac cultivation.

Numerous methods are currently available for analysis of genetic diversity. These methods have relied on pedigree data, morphological data, agronomic performance data, biochemical data and more recently molecular (DNA-based) data (Mohammadi and Prasanna, 2003). Molecular markers are one of the best suitable methods to characterize morphological variations. Among various molecular markers, PCR based markers are widely used because of their simplicity and ease to handle. PCR based molecular markers such as Random Amplification of Polymorphic DNA (RAPD), Inter-Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR) and Amplified Fragment Length Polymorphism (AFLP) have been successfully used to assess the genetic diversity in cultivars of many plant species. ISSR markers overcome the shortcomings of the low reproducibility of RAPD; the high cost of AFLP and the complexity of SSR. ISSR, a powerful molecular marker was first developed by Zietkiewicz *et al*. (1994). It uses single anchored repeat primers to amplify inter repeat region. ISSR provides a novel fingerprinting approach applicable for taxonomic and phylogenetic comparisons and as a mapping tool too. ISSR has been used as a tool for genetic diversity in common bean (Galvan *et al*., 2003), rice bean land races (Muthusamy *et al*., 2008), *Jatropha* (Grativol *et al*., 2011), bamboo (Yang *et al*., 2012), *Pongamia* (Kesari *et al*., 2010) *etc*. Therefore, this PCR-based marker was selected for the present study; as no prior information is available, whether these variants are different species or mutants or variants arise due to environmental factors.

The present study is the first attempt to assess the molecular diversity of flower colour variants of *B. monosperma* collected from different regions of Jharkhand. In this study, ISSR has been chosen as a molecular tool which would serve as a starting material for molecular breeding experiments in *B. monosperma* in the future and ultimately aid in sustainable lac production.

MATERIALS AND METHODS

Sample Collection

Young and healthy leaves of six flower colour variants of *B. monosperma* (1. Golden yellow, 2. Yellow, 3. Mustard, 4. Chrome yellow, 5. White and 6. Scarlet) were collected from different regions of Jharkhand. They were stored at -80ºC till DNA isolation.

DNA Isolation

DNA was isolated following CTAB (Cetyl Trimethyl Ammonium Bromide) method. One and half gram of young leaves was powdered separately in liquid nitrogen using mortar and pestle. The powdered leaf samples were extracted in 15mL CTAB buffer (2% CTAB, 100 mM TrisHCl, 20mM EDTA, 1.5 M NaCl, 2% PVP and 0.2% β-mercaptoethanol) containing 0.1 mg/mL proteinase K. The mixture was then incubated for 1.5 hours at 55ºC in water bath. The incubation was followed by centrifugation of the mixture at 12000 rpm for 15 minutes to spin the cell debris. Equal volume of chloroform:isoamyl alcohol (24:1) was added to the supernatant. The mixture was then centrifuged at 12000 rpm for 15 minutes. The DNA was precipitated by adding 0.7 volume of isopropanol to the aqueous phase centrifuged at 12000 rpm for 15 minutes. The DNA pellet obtained was washed with 70% ethanol. Ethanol was completely evaporated by air drying. The DNA pellet was re-suspended in 500 μL of TE buffer (10 mM Tris (pH 8.0) and 1mM EDTA). Purification of DNA was done by passing the

Table 1: List of primers used along with their sequences

Primer	Primer Sequence	Length (mer)
ISSR-6	5′- AGA GAG AGA GAG AGA GC – 3′	18
ISSR-7	5′- CTC TCT CTC TCT CTC TG - 3′	18
ISSR-8	5′ - CAC ACA CAC ACA AC - 3′	14
ISSR-9	5′ - CTC TCT CTC TCT G - 3′	13
ISSR-10	5′ - CTC TCT CTC TCT A - 3′	14
ISSR-11	5′ - CAC ACA CAC ACA GG - 3′	14
ISSR-12	5′ - CTC TCT CTC TCT CTC TAC - 3′	18
ISSR-13	5′ - CTC TCT CTC TCT CTC TGC - 3′	18
ISSR-14	5′ - CAC ACA CAC ACA AG - 3′	14
ISSR-15	5′ - CAC ACA CAC ACA GT - 3′	14
ISSR-16	5′ - GAG AGA GAG AGA GG - 3′	14
ISSR-17	5′ - GTG TGT GTG TGT GTG TA - 3′	17
ISSR-18	5′ - CAC CAC CAC GC - 3′	11
ISSR-19	5′ - GAG GAG GAG GC- 3′	11
ISSR-20	5′ - CTC CTC CTC GC - 3′	11
ISSR-21	5′ - GTG GTG GTG GC - 3′	11
ISSR-22	5′ - CGC GCG CGC GCG A - 3′	13
ISSR-23	5′ - GAG AGA GAG AGA TC - 3′	14
ISSR-24	5′ - CGC GCG CGC GCG T - 3′	13
ISSR-25	5′ - TCT TCT TCT TCT GC - 3′	14
ISSR-26	5 ′- GAG AGA GAG AGA GAG AAT -3′	18
ISSR-27	5′- CAC ACA CAC ACA CAC AG -3′	17
ISSR-28	5′- CAC ACA CAC ACA CAC AT -3′	17
ISSR-29	5′- CTC TCT CTC TCT CTC TA -3′	17
ISSR-30	5′- GTG TGT GTG TGT GTG TC -3′	17
ISSR-31	5′- TCT CTC TCT CTC TCT CG -3′	17
ISSR-32	5′- ACA CAC ACA CAC ACA CC -3′	17
ISSR-33	5′- CAG CAG CAG CAG CAG A -3′	16
ISSR-34	5′- CAG CAG CAG CAG CAG C -3′	16
ISSR-35	5′- ATG ATG ATG ATG ATG ATG C -3′	19
ISSR-36	5′- CAA CAA CAA CAA CAA -3′	15
ISSR-37	5′- CAG CAG CAG CAG -3′	12
ISSR-38	5′- GAT AGA TAG ATA GAT AGA TAA -3′	21
ISSR-39	5′- GAC AGA CAG ACA GAC AC -3′	17
ISSR-40	5′- GAC AGA CAG ACA GAC AG -3′	17
ISSR-41	5′- GAC AGA CAG ACA GAC A -3′	16
ISSR-42	5′- GAT AGA TAG ATA GAT AGA TA -3′	20
ISSR-43	5′- ACA CAC ACA CAC ACA CYT -3′	18
ISSR-44	5′- ACA CAC ACA CAC ACA CYA -3′	18
ISSR-45	5′- ACA CAC ACA CAC ACA CYG -3′	18
ISSR-46	5′- TGT GTG TGT GTG TGT GRT -3′	18
ISSR-47	5′- TGT GTG TGT GTG TGT GRC -3′	18
ISSR-48	5′- TGT GTG TGT GTG TGT GRA -3′	18
ISSR-49	5′- GTG TGT GTG TGT GTG TYC -3′	18
ISSR-50	5′- GTG TGT GTG TGT GTG TYA -3′	18

*Note: for degenerate nucleotides Y = C, T; R = A, G

Table 2: Summary of results obtained from 12 ISSR profiles

Primer ID	Band size (bp)	Total no. of bands scored	No. of polymorphic bands	$I_b$av	EMR	% Polymorphism	DI	MI	Rp
ISSR-10	300-2000	7	7	0.50	7.00	100	0.82	5.77	3.52
ISSR-11	400-1500	12	11	0.55	10.08	92	0.89	8.98	6.66
ISSR-13	550-2000	7	5	0.28	3.57	71	0.81	2.9	2.00
ISSR-19	400 1500	12	9	0.38	6.75	75	0.89	6.03	4.66
ISSR-20	500-1500	11	8	0.33	5.82	73	0.86	5.01	4.00
ISSR-24	200-2000	19	19	0.52	17.05	100	0.92	15.85	11.33
ISSR-25	300-2000	10	6	0.26	3.60	60	0.86	3.12	2.66
ISSR-26	400-1200	8	7	0.54	6.13	88	0.86	5.26	4.33
ISSR-27	450-800	9	8	0.56	7.11	89	0.84	5.97	5.00
ISSR-29	400-1200	11	11	0.70	11.00	100	0.89	9.82	7.67
ISSR-32	400-1100	13	13	0.74	13.00	100	0.91	11.87	9.67
ISSR-42	400-2000	8	6	0.54	4.50	75	0.84	3.76	4.33

Table 3: Similarity matrix of flower colour variants of *B. monosperma* obtained from ISSR analysis

	Golden Yellow	Yellow	Mustard	Chrome Yellow	White	Scarlet
Golden Yellow	1					
Yellow	0.43	1				
Mustard	0.38	0.46	1			
Chrome Yellow	0.41	0.36	0.41	1		
White	0.37	0.40	0.35	0.36	1	
Scarlet	0.36	0.39	0.52	0.48	0.47	1

isolated genomic DNA through the HiMedia columns (cat. no. MB507). The purified DNA samples were analyzed on 1.0% agarose gel using 0.5X TBE buffer. The DNA samples were quantified using spectrophotometer.

ISSR PCR Conditions

The thermo cycling reactions were carried out on a Gene-amplification PCR system (Sensoquest Lab Cycler). Fifty nanograms of DNA was amplified with respective ISSR primers (10 picomoles), 1.25 units *Taq* DNA polymerase, 0.1 mM dNTPs and 2.5 mM $MgCl_2$. The list of ISSR primers used in the study is listed in Table 1. The following conditions were used for all primers. Initial denaturation at 94ºC for 2 minutes, followed by 40 cycles of 94ºC (denaturation) for 30 seconds, 45ºC (annealing) for 45 seconds and 72ºC (extension) for 2 minutes and the final extension at 72ºC for 15 minutes. The PCR products were loaded on 1.5% agarose gel. The gel was run at 130 V for 1.5 hours. Documentation of the amplification products was done using the Syngene (Genesnap from Syngene, Bioimaging System) and analysis of bands was done using the analysis software (Syngene Genius). The DNA fragment sizes were estimated in comparison with 100 bp DNA ladder (MBI Fermentas, #SM0313).

Scoring of bands and data analysis

Only the clear, unambiguous and reproducible bands present across the DNA samples were used for scoring. In reference to molecular weight marker, at particular band size, the presence of bands was scored '1'; while the absence of bands or very faint bands was scored '0'. The smeared and fuzzy bands obtained with certain primers were excluded from the analysis. Information content of the ISSR primers was estimated through Diversity Index (DI), Marker Index (MI) and Resolving Power (RP). Marker Index was calculated using the following formula MI = Diversity index (DI) X Effective Multiplex Ratio (EMR). DI of a primer is defined as $1 - \Sigma P_i^2$, where P_i is the band frequency of the *i*th allele. EMR is the product of number of polymorphic bands (*i.e.* absence of band at least in one genotype at a particular locus) per primer and the fraction of polymorphic bands (Milbourne *et al*., 1997). Resolving power was calculated as per the following formula $Rp = \Sigma I_b$. Band informativeness $I_b = 1-[2 X |0.5-p|]$, where p is the proportion of the six genotypes containing the band (Prevost and Wilkinson, 1999).

The binary data were analyzed using NTSYSpc software version 2.02i (Rholf, 1998). The similarity matrix was constructed based on Jaccard's coefficient. Clustering was done by UPGMA using SAHN module and the dendrogram was created subsequently.

RESULTS AND DISCUSSION

In order to understand the extent of genetic diversity of flower colour variants in *B. monosperma* occurring in different regions of Jharkhand and its utilization in crop improvement programmes for sustainable lac production, a PCR-based marker system *i.e.*, Inter Simple Sequence Repeats (ISSR), was used. DNA fingerprinting based on different marker systems is done regularly to study the genetic diversity among the different genotypes or germplasm collections and group them into specific categories. ISSR is already known for its simplicity, rapidness, reliability and efficiency and is regularly applied in different plant and tree crops for studying genetic diversity, genetic relationship, germplasm analysis, seed purity evaluations, gene quantitative trait loci mapping, marker assisted selection, identification and confirmation of hybrids and parents and evolutionary study (Khajudparn *et al*., 2012). Earlier Vaishali *et al*. (2008) had studied genetic diversity of *B. monosperma* from different agro-ecological regions of India based on RAPD. Similar study, using RADP, ISSR and SRAP was also carried out by Vashishtha *et al*. (2013) in *B.*

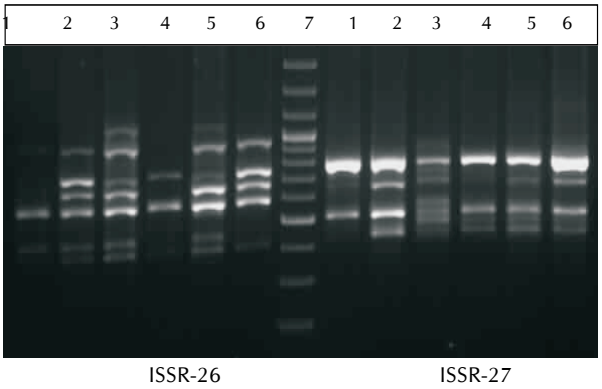

Figure 1: 1.5% agarose gel image of ISSR profile of flower colour variants obtained with ISSR-26 and ISSR-27 primers; lane1: golden yellow, 2: yellow, 3: mustard, 4: chrome yellow, 5: white, 6: scarlet, 7:100 bp ladder

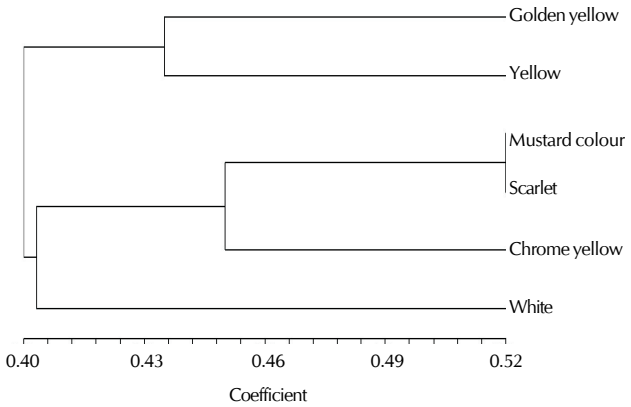

Figure 2: Dendrogram of flower colour variants of *B. monosperma* based on UPGMA cluster analysis of ISSR data

monosperma collected from different geographical regions of India. In this study, we employed ISSR to study the molecular diversity of flower colour variants of *B. monosperma* collected from different regions of Jharkhand. It uses a single SSR-containing primer to amplify regions between adjacent, inversely oriented SSRs in a PCR to generate multi locus markers (Reddy *et al*., 2002). ISSR markers are transferable, hyper variable, highly polymorphic, multi-allelic dominant markers, relatively simple to interpret and show high information content (Souframanien and Gopalakrishna, 2004).

ISSR analysis

Genomic DNA was isolated following CTAB method and purified by passing through HiMedia columns. The purity index of the DNA was ascertained by measuring (A260/A280). The values existed between 1.8 and 2.0. Out of 45 ISSR primers screened; 12 primers have been found to give satisfactory amplification in all the genotypes studied. The ISSR PCRs were repeated twice to assure the reproducibility. The product size for all the primers ranged from 200 bp to 2.0 Kb. A representative gel using primers ISSR-26 and 27 is given in Fig. 1. The total number of loci amplified in all the assays was 127 and the number of polymorphic loci was 110. The primer ISSR-24 recorded maximum scorable bands with 19, while ISSR-10 and 13 recorded minimum scorable bands with seven. The summary of the analysis revealed that the average number of bands per primer was 10.58, while average number of polymorphic bands per primer recorded was 9.16. Our results were comparable with other trees such as *Pongamia* (Kesari *et al*., 2010) and tung tree (Li *et al*., 2009). Our results showed more number of bands as compared to the study carried out by Vashishtha *et al*. (2013) in *B. monosperma* populations wherein they obtained 6.1 average numbers of bands per primer and 5.1 average number of polymorphic bands per primer with the ISSR markers. The distribution of different SSR regions in the genome determines the number of markers generated. The results obtained from different ISSR primers have been compiled in Table 2.

Polymorphism and Marker Performance

All the 12 selected primers produced polymorphic bands. Four primers (ISSR -10, 24, 29 and 32) gave 100% polymorphism. The range of polymorphism for rest of the primers was from 60% to 92%. The average polymorphism for all primers was 85.25% showing the robustness of the primers used in distinguishing the genotypes selected. It has been reported that the ability to resolve genetic variation may be more directly related to the number of polymorphisms detected by the marker system (Yee *et al*., 1999). Vaishali *et al*. (2008) reported 86% polymorphism with RAPD markers among *B. monosperma* populations from different agro-ecological regions of India. While studying *B. monosperma* populations from different geographical regions of India, Vashishtha *et al*. (2013) obtained 77.2% polymorphism with ISSR marker system. Even a very high polymorphism level of 100% was achieved in few ISSR studies as in the case of *Tectona grandis* (Ansari *et al*., 2012) and 96.02 % in *Albizzia* sp. (Aparajita *et al*., 2008). In a striking contrast, a very low level of polymorphism (10.08%) was obtained in *Pongamia* ISSR study (Kesari *et al*., 2010).

Average band informativeness ($I_b$av) is a measure of closeness of a band to be present in 50% of the genotypes under study. The average band informativeness for the primers ranged from 0.26 to 0.74 with a mean value of 0.49. Resolving power of a primer is the capability of a primer to distinguish the given genotypes. Resolving power for the primers used in the study ranged from 2.00 to 11.33 with an average of 5.48 per primer. Out of the 12 primers used four had resolving power above five. Wide range of resolving power with ISSR primers has been reported by earlier workers. Resolving power of 0.92 has been reported for barley (Guasmi *et al*., 2012) and 19.87 for *Jatropha curcas* (Grativol *et al*., 2011). It is reasonable to say that primers with higher Rp values have a greater capacity to separate different genotypes. The primers viz., ISSR-24, ISSR-32 and ISSR-29 having higher Rp values were able to distinguish at least 50% of the colour variants used in the study.

Diversity index ranged between 0.81 and 0.92 with a mean value of 0.86. Diversity index value permits to evaluate the discriminative ability of each marker. Higher the diversity index, higher is the marker informativeness. The Marker Index (MI)

can be considered to be an overall utility of the marker system. It varied between 2.9 and 15.85 with an average of 7. A near MI value of 8.86 was observed in *Canna generalis* (Patra *et al*., 2008). Marker index depends and directly proportional to effective multiplex ratio and diversity index.

Similarity Matrix

The binary data were used to make pair wise comparisons of the flower colour variants based on shared and unique amplicons to generate a similarity matrix following Jaccard's coefficient with NTSYSpc software. The similarity coefficient value ranged from 0.35 between mustard and white flower colour variants to 0.52 between mustard and scarlet colour variants with a mean value of 0.41 (Table 3). The similarity coefficient of common flower colour scarlet was found to be the highest with mustard (0.52), followed by chrome yellow (0.48), white (0.47), yellow (0.39) and golden yellow (0.36) flower colour variants. However, a higher range of similarity coefficient (0.53 to 0.79) was obtained in RAPD based genetic diversity assessment of *B. monosperma* (Vaishali *et al*., 2008).

Clustering Analysis

Based on the similarity matrix, the dendrogram was constructed following UPGMA algorithm (Fig. 2). The UPGMA dendrogram based on ISSR data depicted two principle clusters: I and II. Cluster I comprised of two flower colour variants: golden yellow and yellow. The Cluster II comprised of four flower colour variants: mustard colour, scarlet, chrome yellow and white. However, white formed a separate sub cluster in cluster II, revealing its genetic makeup would be different from other variants. In this study, mustard coloured flower variant was very closely related to the most common scarlet coloured *B. monosperma*. Yellow and golden yellow variants were clustered together. Similar result was obtained in RAPD study of these colour variants (unpublished data from our lab). The appearance of golden yellow variant may be due to the crossing between the scarlet and yellow coloured variant since the distance between yellow and golden yellow variants is less than a km in natural habitat. Vashishtha and co-workers (2013) observed that ISSR marker was not able to cluster the *B. monosperma* populations collected from relatively closer geographic distances at all instances. Since we are using different flower colour variants from different parts of Jharkhand our ISSR maker system was able to cluster populations from relatively closer geographic distances. In *Butea,* cross pollination is a rare event which may lead to some variations in flower colour. It is reasonable to assume that mustard, golden yellow and chrome yellow flower colour variants may be the outcomes of this rare out-crossing phenomenon.

It can be concluded that ISSR marker system is found to be very informative and efficient in analyzing the genetic diversity of the flower colour variants of *B. monosperma*. This study has provided some reliable fingerprinting profiles which can be used for developing SCAR (Sequence Characterized Amplified Region) markers and also in marker assisted selection for genetic improvement of this economically important tree species. There is a possibility of losing the rare variants of this tree species in the near future because of over exploitation due to its medicinal properties. Some more informative markers can be employed for analyzing the variations of *Butea* occurring in the nature and use them in conservation programmes.

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