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Genetic Diversity Assessment of a Critically Endangered Medicinal Plant Populations from *Shorea tumbuggaia* Roxb.

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Abstract

A successful assessment of genetic diversity is consider vital for formulating conservation of the rare, endangered and threatened (RET) species. Our aim was to assess the genetic diversity in *Shorea tumbuggaia* Roxb. population (*Dipterocarpaceae* family)—a critically endangered medicinal plant of conservative interest using Random Amplified Polymorphic DNA (RAPD) technique. According to International Union for Conservation of Nature (IUCN) and Conservation Assessment and Management Planning (CAMP) *S. tumbuggaia* is considered as an endemic and globally endangered tree species. RAPD markers were used to estimate the genetic variability and similarity among the *S. tumbuggaia*. Eighteen (18) primers of OPU (1-20) series generated a total of 137 polymorphic bands out of 735 total bands (19.86% polymorphism), with an average of 40.83 amplified bands per primers and 598 bands showed monomorphic banding pattern with an average of 33.22 per primers. Genetic similarity coefficients calculated from RAPD data ranged from 0.82 to 0.95, with the highest value of 0.95 in ST10 and ST11 and the lowest value of 0.82 between accessions ST2 and ST9, ST3 and ST4, ST4 and ST10 and ST5 and ST8.

1. Introduction

The genus *Shorea* of Dipterocarpaceae family is represented by 196 rainforest tree species (Raju *et al*., 2009). Four species of *Shorea* i.e; *Shorea assamica*, *S. robusta*, *S. roxburghii* and *S. tumbuggaia* are documented (Janardhanan, 1993). Of these, *Shorea tumbuggaia* Roxb. commonly known as green dammer tree, is an endemic, globally endangered, semi-evergreen tree, restricted to the Southern Eastern Ghats parts of Chittor, Cuddapah and Nellore districts of Andhra Pradesh and North Arcot and Chengalpattu districts of Tamil Nadu in India (Rani and Pullaiah, 2002). These large sporadic resinous trees attain the maximum height of about 20-30m with maximum width of 150-190 cm. In addition to its timber-yielding potential, the tree is also known for its medicinal properties as an external stimulant and a substitute for Abietis; Resina and Pix Burgundica of European pharmacopoeias (Watt, 1889). The trunk of the plant is used as flag poles for temples. The plant extract is used as a cure for ear-aches and leaf juice as ear drops for children (Rao, 1998). Methanolic extract of leaves of this plant reported to have antinociceptive and anti-inflammatory activity (Jyothi *et al.,* 2008). The bark is reported as having anti ulcer activity (Patil *et al*., 2004) and leaves are used in the treatment of dysentery. The stem resin is also used for making incense sticks. The oleoresin which is exuded from the stem bark of *Shorea* can be used to cure hydrosis and alexiteric (Sumy *et al*., 2000). Ragini *et al*. (2011) reported antidiabetic and antioxidant activity of *S. tumbuggaia* in alloxan-induced diabetic rats and revealed that this plant had antihyperglycemic and hypolipidemic agent.

This species has become critically endangered due to over-exploitation (Savithramma and Sudarsanamma, 2006), habitat degradation and other biotic interferences. In addition, low rate of seedling establishment, inability of seedlings to compete with other plants, higher rates of the abortion of seeds and flowers, seed predation by insect pest at the pre-or post-dispersal stage, less availability of viable seeds and non-annual ephemeral flowering are collectively responsible for its 'endangered' status (Raju *et al*., 2009). According to International Union for Conservation of Nature (IUCN) Red List of Threatened Species, *S. tumbuggaia* is considered as an endemic, Data Deficient and globally endangered tree species (Ashton, 1998; Reddy *et al*., 2003). This species was assessed to be endangered at Conservation Assessment and Management Planning (CAMP) workshop in 2001

in India (Jadhav *et al.*, 2001). But due to the high level of threat *S. tumbuggaia* has categories to "critically endangered" plant group (Prasad *et al.*, 2006) requiring immediate attention towards its conservation.

As the stability and the evolutionary potential of a species depend on its genetic variation, it is important to obtain knowledge of the amount of genetic diversity to provide information for the development of strategies for the conservation and sustainable utilization of a species. PCR derived markers with non-specific primers have been exceedingly popular since they are independent of sequence information from target species. RAPD is simple PCR based technique and used extensively in studying genetic relationships among accessions of various plant species (Williams et al., 1990). Knowledge on the genetic diversity and relationships among plant varieties is important to recognize gene pools, for intellectual property rights (IPR) purposes and to develop effective conservation and management strategies. The genetic diversity of a species has great implications for its long-term survival and evolution. Therefore, knowledge of the levels and patterns of genetic diversity is important for designing conservation strategies for threatened and endangered species. As per literature concerned only a scant work is reported on evaluation of genetic diversity in dipterocarps using molecular markers (Rath et al., 1998; Cao et al., 2006). In most dipterocarps, genetic variation within species is largely unknown.

At present, no study has been carried out to assess the genetic diversity and to correlate it with the existing endangered nature of *S. tumbuggaia*. Keeping this in mind, the present study was undertaken with the objectives of locating, collecting and generating species- specific fingerprints for *S. tumbuggaia* and to assess genetic diversity expressed as percentage polymorphism and evaluate significant variations within subpopulations using RAPD. The present paper deals with investigations related to genetic variability in *S. tumbuggaia* population, a critically endangered medicinal plant of conservative interest from Tirumala Hills (Tirupati) of Andhra Pradesh state, in India. According to the authors knowledge this is first description into field of genetic variability of *S. tumbuggaia* from this area.

2. Materials and Methods

2.1. Collection of plant materials

The study area was visited frequently during the collection tour and plant materials have been identified and verified with the help of taxonomists. Tender leaves of *S. tumbuggaia* were collected exclusively from 11 locations from the Tirumala Hills, Tirupati, Andhra Pradesh, India (Latitudes 13° 40' 59.7^{0}N, Longitude 79° 20' 49.9°E and altitude 853 M above sea level) (Table 1; Fig. 1) and brought to the laboratory in ice bags. Being a critically endangered plant species, the number of individuals from the natural population was reduced. If more than one sample was collected from a site, it was treated as a single accession for analysis. Care has been taken to maintain a geographical distance of about 3-6 kilometres between each collection site in order to reduce genetic similarity in accessions and to minimize sampling error while interpreting the data.

Table 1: Details of *Shorea tumbuggaia* accessions collected from different locations at Tirumala Hills, Tirupati, A.P., India

Accession code no.	Source
*ST 1	Papavinasanam
ST 2	Japali
ST 3	Alipiri
ST 4	Silathoranam
ST 5	Papavinasanam-II
ST 6	Narayangiri
ST 7	Deer Park
ST 8	Sri Venkateshwara Temple
ST 9	Akasganga
ST 10	Gogarbham Dam
ST 11	Japali Anajaney Swamy Temple

*ST-*Shorea tumbuggaia*

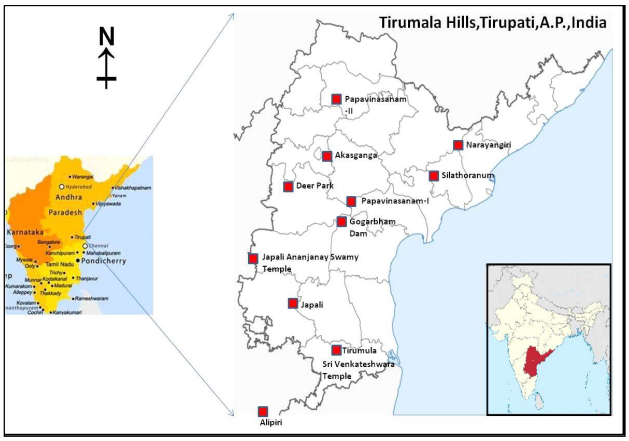

Fig. 1: Surveyed region: Tirumala Peaks and Theerthams

2.2. DNA isolation

About 3 gm of fresh leaf material was harvested from forest trees of approximately 20-30 years of age from eleven individual plants and bulked for each accession. The quality and quantity of genomic DNA

isolated using traditional cetyltrimethyl-ammonium bromide (CTAB) (Doyle and Doyle, 1990) protocol was poor, as *S. tumbuggaia* is a latex yielding plant, rich in polyphenols and polysaccharides (Ankanna and Savithramma, 2011). Hence a modified protocol was followed, as described here. The leaf material was ground in liquid nitrogen, along with 1% polyvinylpyrrolidone (PVP; HIMEDIA, Mumbai), and then homogenized in 400 µl CTAB extraction buffer with 0.2% b-mercaptoethanol incubated at 65°C for 30 min. followed by two extractions with chloroform and isoamylalcohol (24:1, v/v) at 12,000 rpm for 10 min. Collected clear supernatant was mixed with same volume of chilled isopropanol. Precipitation was carried at –20°C for 30 min. and centrifuged at 10,000 rpm for 10 min. Pelleted DNA was washed with 70% ethanol, spin at 10,000 rpm for 10 min. Air dried the pellet and suspended in high salt TE or sterile milli Q and 5 µl of RNase (10 mg ml^{-1}) was added and kept at 4°C overnight.

Purification steps were carried with chloroform: isoamyl alcohol (24:1). Finally, DNA was pelleted with 0.5 volume of sodium acetate (3 M) and 2 volumes of absolute ethanol, followed by ethanol wash (70%), air dried and resuspended in appropriate volume of low salt TE. The quantity of the DNA was estimated spectrophotometrically and quality was checked on 0.8% agarose electrophoretically. The DNA was diluted to 30 ng μl^{-1} for RAPD analysis.

2.3. RAPD analysis

Twenty random decamer oligonucleotide primers from OPU (1-20) series were used for PCR reaction (Table 2). Preliminary tests identified eighteen primers of OPU series resulted in terms of number, spacing and intensity of bands that could be reliably scored. RAPD reaction and procedures were carried out as described by Williams *et al*. (1990). The reaction mixture (25µl) containing 25-50 ng of DNA, 2.5 µl Taq DNA polymerase enzyme (Fermentas, USA), 0.4µM each dNTPs (Fermentas, USA), 2.5Mm $MgCl_2$ (Fermentas, USA) and 0.4 µM decamer primer (Operon, USA).The thermocycler (PTC 200, MJ Research Inc., USA) was programmed for initial denaturation at 95°C for 10 min followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 35°C for 1.30 min and extension at 72°C for 1.30 min in each cycle. The last cycle allowed an additional incubation for final extension of 10 min at 72°C.

Table 2: Primers with their sequences used for RAPD analysis of *Shorea tumbuggaia*, and the total number of bands, polymorphic amplification products, percentage of polymorphism yielded by each primer

Primer	Sequence (5' to 3')	Mol. Wt. Range (bp)	Total bands	Monomorphic Bands	Polymorphic bands	% Polymorphism
OPU-01	ACGGACGTCA	1375-564	43	33	10	23.2
OPU-02	CTGAGGTCTC	831-564	30	22	8	26.6
OPU-03	CTATGCCGAC	947-564	35	27	8	22.8
OPU-04	ACCTTCGGAC	–	–	–	–	–
OPU-05	TTGGCGGCCT	1584-831	38	30	8	21.0
OPU-06	ACCTTTGCGG	1904-564	39	33	6	15.3
OPU-07	CCTGCTCATC	1904-564	40	33	7	17.5
OPU-08	GGCGAAGGTT	947-564	44	44	0	0
OPU-09	CCACATCGGT	–	–	–	–	–
OPU-10	ACCTCGGCAC	1375-831	31	22	7	22.5
OPU-11	AGACCCAGAG	1904-564	27	22	5	18.5
OPU-12	TCACCAGCCA	1584-564	42	33	9	21.4
OPU-13	GGCTGGTTCC	1584-564	41	33	8	19.5
OPU-14	TGGGTCCCTC	1904-564	52	44	8	15.3
OPU-15	ACGGGCCAGT	1584-564	35	22	13	37.1
OPU-16	CTGCGCTGGA	1375-564	36	22	14	38.8
OPU-17	ACCTGGGGAG	1374-564	29	22	7	24.1
OPU-18	GAGGTCCACA	947-564	50	44	6	12.0
OPU-19	GTCAGTGCGG	1584-564	72	66	6	8.3
OPU-20	ACAGCCCCCA	1374-564	51	46	7	13.7
	Total		**735**	**598**	**137**	
	Average/ primer		**40.83**	**33.22**	**7.61**	**19.86**

2.4. Agarose gel electrophoresis

Amplification products were separated by gel electrophoresis (at a constant current of 50 m A) through 1.5% agarose gels in 0.5 x TBE buffer, with a marker (Lambda DNA/Eco RI+ Hind III (low range DNA ruler, Bangalore Genei, India, Fig. 2). After staining in ethidium bromide the gels were visualized and imaged using gel documentation system (Nighthawk ™, pdi Inc., New York, USA).

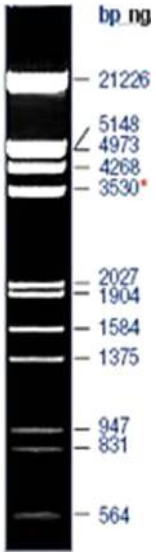

Fig. 2: DNA marker Lambda DNA/Eco RI+HindIII,3

2.5. Data analysis

Data was scored as '1' for the presence and '0' for the absence of a DNA band of each accession. The bands resulting from different sets of RAPD primers for different populations of *S. tumbuggaia* were scored on agarose gel photograph in Gel documentation system for its presence and absence across the populations collected. The image profiles of banding patterns were recorded and molecular weight of each bands were determined by Alpha View Software. The results were analysed based on the principle that a band is considered to be polymorphic if it is absent in at least one individuals or accessions. Similarity index of bands which were common between two accessions was estimated by Nei and Li (1979). The final RAPD data generated with 18 primers were used to calculate pair wise similarity coefficients using Jaccard's coefficient of similarity (Jaccard, 1908). The cluster analysis was performed by Tree Con TM (Scanalytics Inc, USA) software for generating phylogenetic tree using the unweighted pair group method with arithmetic average (UPGMA) method (Nei and Li, 1979).

3. Results

3.1. RAPD analysis

A total of 20 decamer random primers were tested in eleven accessions of *S. tumbuggaia* species for examining RAPD pattern, of these 18 primers of OPU series amplified in all the accessions. In all, 735 amplified bands were obtained of which 137 bands were polymorphic, while 598 bands were monomorphic. On average 40.83 bands per primer were scored. The average polymorphism was 19.86%. Out of 18 primers, OPU-19 primer produced maximum 72 bands, while primer OPU-11, OPU-17 and OPU-2 produced minimum 27, 29 and 30 bands, respectively.

RAPD analysis was used to evaluate the degree of polymorphism and genetic variability among 11 accessions of *S. tumbuggaia* was further analysed according to the resultant RAPD markers. The PCR reactions were repeated 3 times for each of the 11 accessions and the resultant DNA bands were highly reproducible. Results show that each accession collected from different localities showed genetic variability and similarity in RAPD profiles by using different primers.

Of the 20 random primers screened, OPU series (1-18) primers produced distinct, highly reproducible amplification profile for all the screened samples (Fig. 3a,b). A wide variation in the number of polymorphic bands ranging from 5 to 14 and monomorphic bands from 22 to 66 was observed. The highest 72 bands were observed from primer OPU-19 and 27 bands for primer OPU-11. The average number of polymorphic (7.61), monomorphic (33.22) per primer and the percentage of polymorphism ranged from 8.3 (OPU-19) to 38.8 (OPU-16). Primer OPU-16 amplified the highest number of polymorphic bands (14), while the lowest number (5) was observed with primer OPU-11. The size of amplified fragments varied with the different primers, ranging from 564 to 1904 bp. From RAPD profiles, the presence and absence of bands were scored and converted into similarity index (SI) value using the coefficient of similarity (Nei and Li, 1979). Using averaged similarity matrix (Table 3), a dendrogram was generated based on UPGMA method (Fig. 4). As evident from the dendrogram, accession number ST4 was completely outgrouped from the rest. The genetic similarity matrix between accessions ranged from 0.82 to 0.95.

3.2. Genetic distance and UPGMA analysis

In the cluster analysis all accessions were grouped under 3 major clusters. Cluster 1 contains five

Table 3: Similarity matrix for Jaccard's coefficient for *Shorea tumbuggaia*: range of values from 0 to 1.0 indicating increasing similarity. Numbers (1-11) in the table represent location listed in Table 1

	ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	ST10	ST11
ST1	1.00										
ST2	0.94	1.00									
ST3	0.93	0.88	1.00								
ST4	0.85	0.83	0.82	1.00							
ST5	0.88	0.86	0.88	0.87	1.00						
ST6	0.89	0.88	0.88	0.87	0.90	1.00					
ST7	0.89	0.84	0.89	0.84	0.85	0.89	1.00				
ST8	0.89	0.83	0.87	0.85	0.82	0.89	0.94	1.00			
ST9	0.85	0.82	0.85	0.85	0.86	0.87	0.89	0.92	1.00		
ST10	0.90	0.87	0.89	0.82	0.90	0.88	0.91	0.89	0.91	1.00	
ST11	0.91	0.88	0.90	0.88	0.89	0.92	0.92	0.92	0.88	0.95	1.00

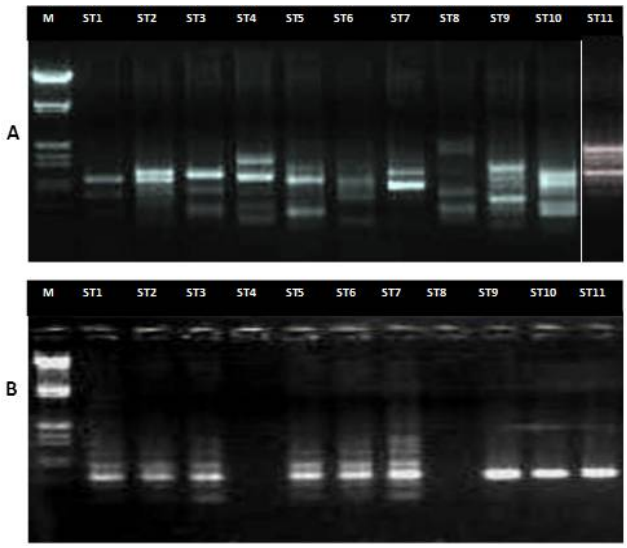

Fig. 3: RAPD profiles of eleven *Shorea tumbuggaia* accessions with two primers: *(A)* OPU-1, (B) OPU-3. M-represents 1 Kb molecular size marker. See Table 1 for accession codes

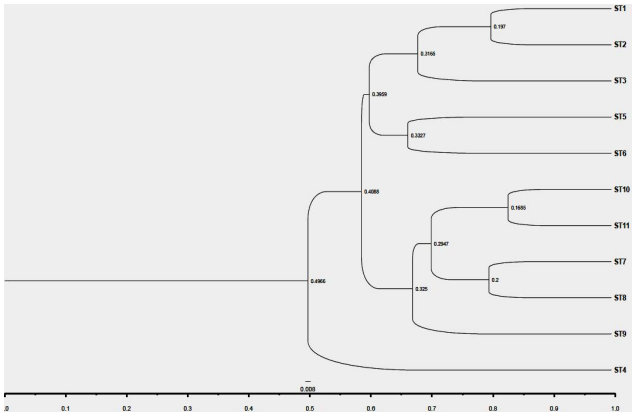

Fig. 4: UPGMA dendrogram showing relationship (variation) among eleven *S. tumbuggaia* accessions using 18 RAPD markers

accessions (ST1, ST2, ST3, ST5 and ST6) collected from Papavinasanam, Japali, Alipiri, Papavinasanam-II and Narayangiri, respectively. This was further sub-divided into two sub-clusters, 1 A and 1B. Subcluster 1A contains three accessions (ST1, ST2 and ST3) belonging to different locations such as, Papavinasanam, Japali and Alipiri, clearly shows that accessions collected from Papavinasanam and Japali are closely related with a similarity indices of 0.94. Cluster 1B contained two accessions, namely; ST5 and ST6 collected from Papavinasanam-II and Narayangiri, respectively showing 90% similarity between them. Cluster 2 was further divided in two sub-clusters (2C & 2D). Sub-clusters 2C have two accessions ST10 and ST11 collected from Gogarbham Dam and Japali Anajanay Swamy Temple, respectively and showed a similarity of 95%. The sub-clusters 2D had three accessions (ST7, ST8 and ST9) collected from Deerpark, Sri Venkateshwara Temple and Akasganga, respectively. Cluster 3 with a single accession ST4 collected from Silathoranum outgrouped from the rest. There was no correlation between genetic distance and geographical distance in the present study.

4. Discussion and Conclusion

Assessment of genetic diversity in wild endangered tree species is very important because of low diversity and long life-cycle. In addition these trees produce different levels of secondary metabolites interfering in the analysis. When no or very less information is available on genetic diversity it is necessary to work on a simple, rapid and dependable analysis. Hence for this tree, in this investigation RAPD procedure was undertaken to interpret the level of genetic diversity. RAPDs polymorphism revealed a low level of genetic diversity in this critically endangered plant, *S. tumbuggaia*, with an average of 19.86% polymorphic RAPD fragments. It was observed that the

accessions ST1 and ST2 shared a similarity of 94%. Similarly, ST10 and 11 are 95% similar. Interestingly these accessions showing higher similarity are from nearby places in both the cases. But overall the analysis indicates a low level of difference i.e., low polymorphism among the accessions tested in this investigation, though the accessions are from distant places. The reason for low polymorphism in different accessions of *S. tumbuggaia* might be due to short-distance seed dispersal, and self-compatibility nature. As cluster 2 having accessions, ST10, ST11, ST7, ST8 and ST9 with high similarity are from different locations of Tirumala Hills of Andhra Pradesh substantiates the observation of low polymorphism. Again this species have restricted distribution over a relatively small area with high level of self-pollination (post-zygotic incompatibility).This indicates restricted gene flow which ultimately cause less variability (Raju *et al*., 2009).

In general, Shorea species are predominantly outcrossing and strongly self-incompatible (Chan, 1981; Sakai *et al*., 1999; Lee *et al*., 2000; Nagamitsu *et al*., 2001; Obayashi *et al*., 2002). But *S. tumbuggaia* is described to be self-incompatible species exhibiting weak protogyny which interns inhibits out-crossing and consequently also reduced to selfing (Bertin and Newman, 1993). Outcrossing plants in general exhibit higher levels of genetic diversity within populations. Also endemic tree species and species with a narrow geographic distribution harbour less genetic variation than widespread species (Hamrick *et al*., 1992). Present investigation supports this line of evidences. Similarly, a high level of genetic similarity has been reported among three natural populations of *S. robusta* using 12 loci from 8 isozyme systems (Suoheimo and Luukkanen, 1999). Similar results were also obtained in *Shorea* (*S. leprosula*, *S. acuminata* and *S. cursitii*) through RAPD analysis (Harada *et al*., 1994). Compared to other dipterocarps studied using different markers (Harada *et al*., 1994; Murawski *et al*., 1994; Rath *et al*., 1998; Lee *et al*., 2000), the 11 accessions of *S. tumbuggaia* species investigated through RAPD in this investigation showed low levels of genetic diversity.

Analysis of genetic diversity of rare and endangered species might be efficacious measurement and strategy for conservation of these species. Based on the results of this investigation and the information available for *S. tumbuggaia*, two alternative conservation strategies may be proposed. Either the population of this species can be increased through tissue culture or vegetative propagation or proper crossing methodology may be devised after study the floral biology in a detailed manner.

Though the study includes only 11 accessions of the species, still it provides a lot of information about the existing polymorphism as this accession were representative of population for this area. As this species is medicinally very important and endemic in nature with availability in a specific area. Conservation strategies should be evolved with more detailed analysis in future.

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