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Genetic Analysis of *Eugenia singampattiana* Bedd.- A Critically Endangered Plant

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Abstract

The Molecular markers have been widely used in analysing genetic diversity of species for the conservation approach. *Eugenia singampattiana* Bedd. is an endemic and critically endangered species in the Southern Western Ghats of India due to the loss of habitat. There were 20 Random Amplified Polymorphic DNA (RAPD) markers applied to find out genetic diversity among the five existing populations. The Result showed that the genetic diversity (*H*) ranged from 0.13 to 0.28, the genetic diversity overall groups (H_T) on an average was 0.39, genetic diversity within populations (*Hs*) was 0.31, genetic differentiation (G_{ST}) between populations overall loci was 0.26. Inter population gene flow (*Nm*) was 4.89. Highest percentage of polymorphism showed only 37.5%. This low percentage of polymorphism within the populations is attributed to small population size and reduced gene flow. The result showed on evidence of the species become extinct due to loss of genetic richness among the population.

Keywords: Genetic diversity, *Eugenia singampattiana* Bedd., RAPD, Critically endangered.

Introduction

The Western Ghats of India is one of the ecologically sensitive zones in the World. It represents about 4000 species of flowering plants and out of this, nearly 38 percent are endemic (Nair and Daniel, 1986). This high level of diversity and endemism has conferred as the hot spots status (Johnsingh, 2001). Globally, there are 229 plant species are represented as threatened in the Western Ghats. Among these, 39 are Critically Endangered, 111 are Endangered, and 79 are Vulnerable. *Eugenia singampattiana* is one of the critically endangered endemic species based on population size reduction of ≥90% over the last 10 years or three generations due to loss of habitat. It belongs to the family Myrtaceae. It is a small evergreen medicinal tree found at the tail end of Southern Western Ghats regions of Tamil Nadu. The plant has potential activity like anticancerous, antitumerous, antioxidative, antimicrobial, antifungal, anti inflammatory, antihyperlipidaemic and antidiabetic agents (Viswanath *et al.*, 2014). Therefore, there is an urgent need to analyse genetic diversity for development of modern conservation system. Intra species variation is prerequisite for adaptive change for long term conservation of the species.

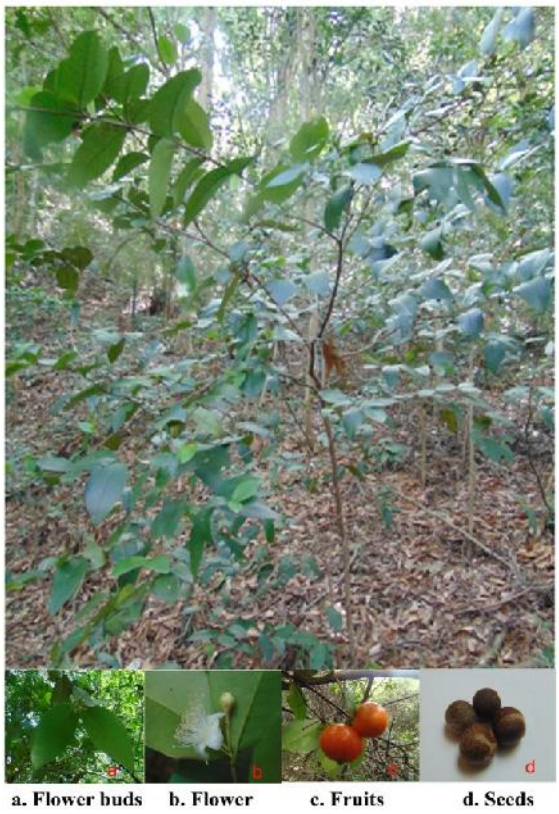
The Molecular markers have been widely used in analysing genetic diversity and to find out highly adaptive population (Warude *et al.*, 2003). Polymerase Chain Reaction (PCR)-based methods such as RAPD marker system has been used because of its wide usage of genetic diversity study and it reflects the variation of the whole genomic DNA. It would be a better parameter to measure the pattern of genetic diversity of the rare and endangered plants (Lal *et al.*, 2011). Hence, the present study deals with the analysis of genetic diversity of *Eugenia singampattiana* to find out highly adaptive population for future conservation approach.

Materials and Methods

Plant materials

The plant materials were collected from five existing populations such as Inchikuzhi (ES_1), Kannikatti (ES_2), Oothu (ES_3), Karaiyar (ES_4) and Kothaiyar (ES_5) . The plant was identified and checked with the Herbarium of Jawaharlal Nehru Tropical Botanical Garden and Research Institute (JNTBGRI) and the voucher specimen (Collection No.76845) was deposited in JNTBGRI.

Fig 1. Habit of *Eugenia singampattiana* Bedd



DNA fingerprinting

Genomic DNA isolation and purification

The total genomic DNA was extracted using the modified CTAB method (Doyle and Doyle, 1987) from tender uninfected leaf samples and purified according to the Sambrook and Russel (2000). Concentration of the purified genomic DNA in each case was adjusted to 10 ng/ µl in different aliquots and stored at - 4° C for PCR amplification.

PCR reaction

Thirty RAPD primers were used for PCR amplification of the genomic DNA (Williams *et al.*, 1990). PCR reactions were carried out in a final volume of 25 µl, which contained 2.5 µl 10X taq polymerase buffer, 2.0 µl of deoxyribonucletides (dNTPs), 3.5 µl $MgCl_2$, 0.1 µl of taq DNA polymerase, 2.0 µl of deca oligonucleotide primer, 2.0 µl of template DNA and 12.9 µl of sterile dis.H_20. The reaction mixture was subjected to programme PCR-amplification in a Thermocycler (Eppendorf mastercycler nexus gradient). Amplification process contain, initial denaturation of DNA at 95ºC for 5 minutes, denaturation at 94ºC for 30 seconds, annealing at 35ºC for 1 minute and extension at 72ºC for 2 minutes followed by thirty five cycles and final extension at 72ºC for 5 minutes. The amplified products are stored at 4ºC till electrophoresis. The PCR products were resolved by electrophoresis on 1.5 % agarose gel containing ethidium bromide along with 1 Kb ladder DNA as a standard molecular weight size marker. The gels were visualized under UV transilluminator.

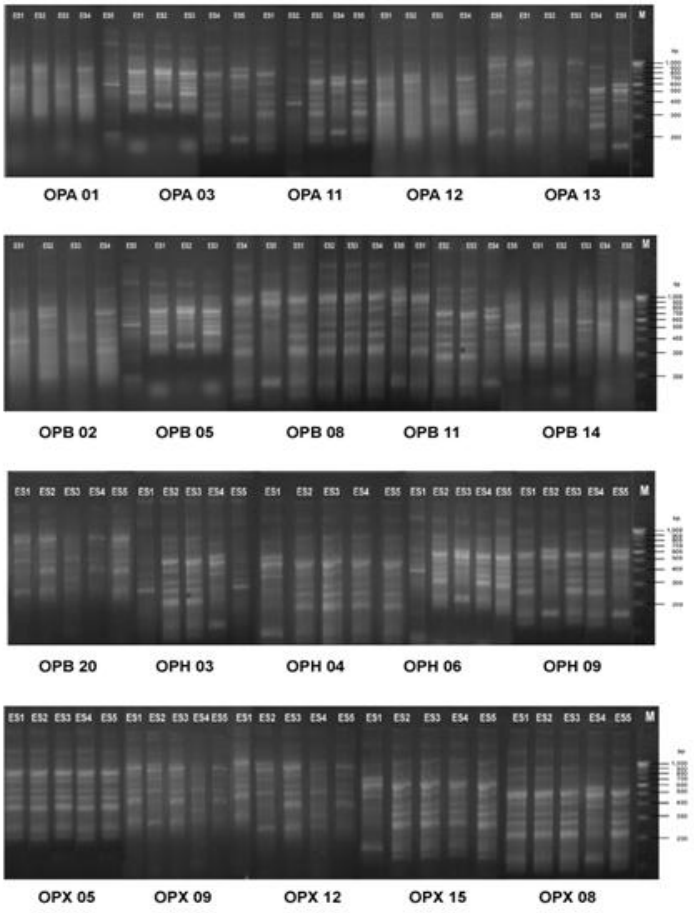
Data analysis

Amplification profiles of populations were compared with each other. Genetic similarity matrix among populations of each samples were calculated using the standard coefficient method (9). The dendrogram was constructed using the UPGMA (Unweighed Pair Group Method with Arithmetic Average) (Sneath and Sokal, 1973) algorithm in SHAN clustering module of NTSYS-pc software version 1.5 (Rohlf, 1989). The genetic diversity within and between populations according to Nei's formula (Nei, 1973) was calculated using POPGENE package version 1.31software (Yeh, *et al.*, 1999).

Results and Discussion

A total of 30 arbitrary 10-mer primers screened, of which 20 primers produced reproducible, multiple band profiles with a number of amplified DNA fragments that varied from 4 to 10. A sum of 152 polymorphic bands was observed. The size of the RAPD fragments varied from 0.2 to 1.0 kbp (Plate 2).

Plate 2. RAPD-PCR fingerprinting of *Eugenia singampattiana*



ES1 – Inchikuzhi; ES2 – Kannikatti; ES3 – Oothu; ES4 – Karaiyar ES5 – Kothyar; M - Marker

Genetic and gene diversity measures were calculated according to Nei's index using POPGENE software and results were depicted in the Table1. The mean genetic heterozygosity or diversity (*H*) ranged from 0.1320 to 0.2863. The ES_1 population was found to be least diverse (0.13). The ES_4 population displayed the highest level of variability (0.28) and the ES_3 population revealed intermediate diversity (0.14). The observed number of alleles (*Na*) ranged from 1.3224 to 1.6250. The ES_2 population was found to be least diverse (1.32). The ES_4 population displayed the highest level of variability (1.62) and average was 1.4. The mean effective number of alleles (*Ne*) was 1.3103. The highest was 1.5433 in ES_4 population and the lowest was 1.2375 in ES_1 population. Shannon Information Index (*I*) ranged from 0.1928 to 0.4052 and the average was 0.2431. The mean number of polymorphic loci (NPL) was 53 and ranged from 49 to 57 for all the accessions. The highest percentage of polymorphism was 37.50 in ES_4 accession.

Table1. Analysis of polymorphism in different accessions of *E. singampattiana*

Accession	*Na*	*Ne*	*H*	*I*	NPL	% of polymorphism
ES_1	1.3355	1.2375	0.1320	0.1928	51	33.55
ES_2	1.3224	1.2468	0.1351	0.1949	49	32.24
ES_3	1.3421	1.2541	0.1400	0.2031	52	34.21
ES_4	1.6250	1.5433	0.2863	0.4052	57	37.50
ES_5	1.3750	1.2698	0.1506	0.2196	56	36.50

ES_1 – Inchikuzhi; ES_2 – Kannikatti; ES_3 – Oothu; ES_4 – Karaiyar; ES_5 – Kothyar
Na – Observed number of alleles; *Ne* – Effective number of alleles; *H* – Gene diversity; *I* – Shannon Information Index; NPL – Number of Polymorphic Loci

The overall observed and effective number of alleles was about 1.500 and 1.3365 respectively and the overall percentage of polymorphic loci was 50. Nei's overall genetic diversity or heterozygosity was 0.1958. The genetic distance between the population ranged from 0.2117 to 0.4389 and the genetic identity ranged from 0.6447 to 0.8092 (Table 2). The average gene diversity within populations (*Hs)* was 0.31, the highest *Hs* was 0.35826 and the lowest *Hs* was 0.1453. The total diversity (H_T) ranged from 0.2179 to 0.4835 and the average was 0.3924. The mean genetic differentiation (G_{ST}) between populations over all loci was 0.26 and the G_{ST} ranged from 0.1532 to 0.4620. The gene frequency ranged from 0.7394 to 0.7527 and the average was 0.7464. The average gene flow from one population to other population (*Nm*) was 4.8989 while the lowest was 0.6677 and the highest were 21.88 (Table 3).

Table 2. Nei's unbiased measures of Genetic distance and Genetic identity of *E. singampattiana*

Accession	ES_1	ES_2	ES_3	ES_4	ES_5
ES_1	****	0.7697	0.7632	0.7697	0.6447
ES_2	0.2617	****	0.8092	0.7763	0.7303
ES_3	0.2617	0.2117	****	0.7829	0.7237
ES_4	0.2617	0.2532	0.2448	****	0.7829
ES_5	0.4389	0.3144	0.3234	0.2448	****

Nei's genetic identity (above diagonal) and genetic distance (below diagonal)

In order to study the correlation between populations, UPGMA algorithm was used to draw a dendrogram for the five populations of *E. singampattiana* (Fig 2). A Jaccard's matrix was used to produce a dendrogram based on SI, which showed distinct separation of the collected accessions from five locations into two major groups having 77 % similarity. Among the two major clusters, the accession belonging to the lower cluster (LC) was collected from ES_3, while accessions belonging to the upper cluster (UC) were collected from ES_1, ES_2, ES_4 and ES_5. Further the accessions of the UC was grouped into two major sub clusters (USC1 &USC2) having 79 % similarity. The upper sub cluster 1 (USC 1) was sub divided into two sub clusters USC1A which was collected from ES_1 and USC1B collected from ES_2 having 83% similarity. USC2 was sub divided into two sub cluster USC2A collected from ES_4 and USC2B collected from ES_5 and they showed 93 % similarity.

Fig 2. UPGMA dendrogram showing the genetic relationship of *five populations of E. Singampattiana*

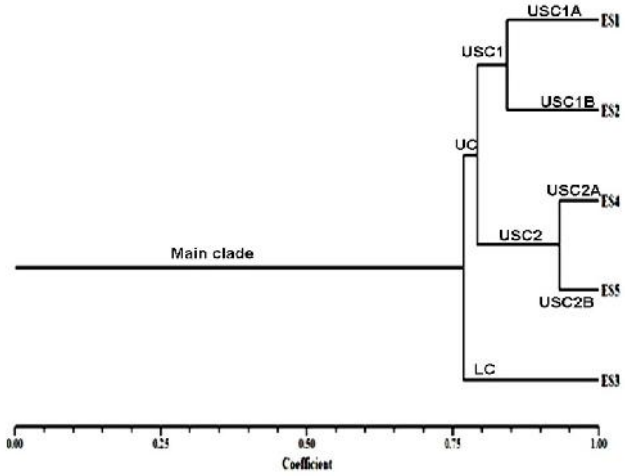


Table.3 Genetic and gene diversity within and between the populations of *E. singampattiana* for RAPD markers

S.No	Primers	Sequence 5'-3'	No. of polymorphic fragments	H_T	*Hs*	G_{ST}	*Nm*	Band Frequency
1.	OPA 01	CAGGCCCTTC	4	0.4051	0.4987	0.3305	0.7166	0.7457
2	OPA03	AGTCAGCCAC	9	0.35758	0.2389	0.3615	1.1006	0.7471
3	OPA11	CAATCGCCGT	8	0.2179	0.1453	0.2494	3.5093	0.7436
4	OPA12	TCGGCGATAG	7	0.3026	0.2304	0.2455	2.1221	0.7468
5	OPA13	CAGCACCCAC	10	0.3518	0.3047	0.1532	6.3651	0.7476
6	OPB02	TGATCCCTGG	5	0.3314	0.2595	0.2500	3.4576	0.7447
7	OPB05	TGCGCCCTTC	7	0.4565	0.3692	0.1807	4.0933	0.7498
8	OPB08	GTCCACACGG	7	0.4130	0.2945	0.3420	2.8021	0.7481
9	OPB11	GTAGACCGGT	8	0.3684	0.2093	0.4538	2.5829	0.7452
10	OPB14	TCCGCTCTGG	6	0.4067	0.2152	0.4620	0.6677	0.7452
11	OPB20	GGACCCTTAC	9	0.4572	0.3402	0.2495	1.881	0.7453
12	OPH03	AGACGTCCAC	8	0.4189	0.3251	0.2196	2.9665	0.7464
13	OPH04	GGAAGTCGCC	8	0.3150	0.6898	0.3170	4.4826	0.7394
14	OPH06	ACGCATCGCA	9	0.4767	0.3751	0.2121	1.9300	0.7481
15	OPH09	TGTAGCTGGG	7	0.4424	0.3179	0.2747	1.8631	0.7441
16	OPX05	CCTTTCCTTC	7	0.4283	0.0226	0.0226	21.5831	0.7527
17	OPX08	CAGGGGTGGA	10	0.4492	0.3633	0.1909	17.5125	0.7471
18	OPX09	GGTCTGGTTG	7	0.4236	0.5826	0.2421	4.0929	0.7466
19	OPX12	CAGACAAGCC	8	0.4835	0.3275	0.3213	2.1254	0.7503
20	OPX15	CAGACAAGCC	8	0.3436	0.2588	0.2809	12.1254	0.7449

***H_T* - Total diversity; *Hs* – Gene diversity within populations; *G_{ST}* - Genetic differentiation; *Nm* – Gene flow**

The polymorphism percentage are low due to small population size because geographically restricted species showing lower levels of genetic variation than widely distributed species (Hamrick and Godt 1996,). *Eugenia singampattiana* is geographically distributed on only lat. l8°33'N to 8°42'46''N and between long. 77°17'55''E to77°21'37''E with two fragmented population (Gobalan and Henry, 2000). These narrowly distributed nature causes lower polymorphism. Similarly low rate of polymorphism was also reported (Catana *et al.*, 2013, Bantawa *et al.*, 2011) in endangered plants. The habitat type is another important factor shaping the degree of genetic diversity and differentiation (Shikano *et al.*, 2010). Because of the poor knowledge of the historical distribution, it is difficult to explain the historical factors that may have contributed to the low genetic variation in this species. This species grows on sandy

clay loam with a pH between 6 and 6.6 at slopes in different altitudes from 300 m to 900 m (Sarcar *et al.*, 2006). The average gene flow from one population to other population (*Nm*) was 4.8989 and gene flow can occur through seed dispersal (Ellstrand, 1992). In field observation, number of seedlings were very less because seed germination requires 65 to 85 days (Sarcar *et al.*, 1999). The low percentage of polymorphism, reduced gene flow due to small population size and loss of genetic richness among the population are molecular evident of this species become extinct.

Conclusion

Analysis of RAPD data can be used to detect genetic variation of *E. singampattiana*. It showed that the populations which exhibited low percentage of polymorphism, small population size, reduced gene flow and other historical factors disclose the alarm of species extinction. Among the natural populations, the Karaiyar (ES_4) accession has possessed the highest percentage of polymorphism. Hence, this population can be adapted for the long term conservation through *ex-situ* and *in-situ* approach.

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