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MOLECULAR CHARACTERIZATION OF AN ENDANGERED SPECIES-*EUGENIA CALCADENSIS*

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The RAPD technique was used to access the genetic diversity of *Eugenia calcadensis* Bedd. an IUCN red listed species collected from Agasthiarmalai Biosphere Reserve in the southern Western Ghats of India. Twenty primers produced 161 reproducible bands with 33 % polymorphism. The EC_4 population displayed the highest percentage of polymorphism (33.54) and highest level of genetic variability (0.15). The UPGMA phenetic dendrogram showed distinct separation of five locations into two major groups having 90 % similarity. Among the accession studied, the Mancholai (EC_4) accession is considered as the superior genotype because of highest level of genetic variability and percentage of polymorphism.

Keywords: *Eugenia calcadensis*, Myrtaceae, Western Ghats, Genetic diversity.

Eugenia calcadensis Bedd., locally known as Chennelli, is a vulnerable, small woody evergreen tree of the family Myrtaceae. It is narrow endemic in and around of Pabanasam and Singampatti hills of the southern Western Ghats of India (WCMC 1998). Due to habitat loss, the species is depleting at an alarming rate hence enlisted as threatened species (IUCN ver 2.3, 2016-3: Viswanath *et al.* 2014). Molecular markers are powerful tool for analysis of genetic diversity including gene flow, genetic distance, genetic identity (Williams *et al*. 1990, Zucchi *et al.* 2005). They are employed to identify superior genotype for future *in-vitro* conservation (Rameesur and Sanmukhiyai 2011). In the present study, an attempt has been made to find out genetic diversity of *Eugenia calcadensis*.

MATERIALS AND METHODS

Plant Materials

Plants growing at five different sites (Karaiyar, Kezhamailar, Sorimuthuaiyanarcoil, Mancholai and Mundanthurai were selected for present study. The voucher specimen (Collection No.76846) was deposited in JNTBGRI, Palode, Thiruvananthapuram.

Genomic DNA isolation and purification

Tender leaves were collected from different accessions and stored immediately at -70°C for DNA extraction. It was stored in deep freezer (REMI,India) Total genomic DNA was extracted using the modified CTAB method (Doyle and Doyle 1987) and purified according to the Sambrook and Russel (2000). Concentration of the purified genomic DNA was adjusted to 10 ng/ µl in different aliquots and stored at -20°C for PCR amplification.

PCR reaction

A total of thirty RAPD primers were used for 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 programmed PCR-amplification in a Thermocycler (Eppendorf). Amplification process included, initial denaturation of DNA at 95ºC for 5 minutes, denaturation 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 followed by storage at 4ºC till electrophoresed. The amplification 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 UV transilluminator was used

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to visualize the gels.

Data analysis

Amplification profiles of five populations were compared with each other. The standard coefficient method (Nei and Li 1979) was used to calculate the Genetic similarity matrix among populations of each samples. 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

The RAPD technique was used to characterize the populations of *Eugenia calcadensis* and establish the relationships between them. In the initial step, 30 arbitrary 10-mer primers were first screened, of which 20 primers produced reproducible, multiple band profiles with fragments that varied from 5 to 10. The same types of band were observed at different frequencies in all populations. Many additional bands were neglected which were not reproducible. The size of the RAPD fragments varied from 0.2 to 1.0 kbp (Fig 2.). Nei's overall genetic diversity or heterozygosity was 0.0765. Dharmar and John De Britto (2015) reported that 12 ISSR primers produced 70.59% of polymorphic band in *Plumbago zeylanica*. Ciarmiello *et al.* (2015) reported that 16 RAPD primers produced 278 reproducible bands of which 220 (79.13%) were polymorphic in fig tree. A great deal of genetic polymorphism has observed among the accessions of *Jatropha curcus.* Among 20 primers were reproduced averages of 7 bands with 57 % polymorphism (Dhakshanamoorthy *et al*. 2015). Our results showed 161 reproducible bands and 33 % polymorphism. The highest percentage of polymorphism was 33.54 in the EC_4 accession and the lowest percentage of polymorphism was 22.36 in the EC_2 accession (Table 1).

Genetic differentiation and relationships

Genetic and gene diversity measures were calculated and tabulated in Table 1. The EC_2 population was found to be least diverse (0.09). The EC_4 population displayed the highest level of variability (0.15) and the EC_1 population revealed intermediate diversity (0.14). The genetic distance between the population ranged from 0.0575 to 0.1636 and the genetic identity ranged from 0.8509 to 0.9441 (Table 2). The overall observed and effective numbers of alleles were 1.2050 and 1.1274 respectively.

The average gene diversity within populations (*Hs)* was 0.31, the highest *Hs* was 0.7867 and the lowest *Hs* was 0.2009. The total diversity (H_T) ranged from 0.2956 to 0.4787 and the average was 0.3916. The mean genetic differentiation (G_{ST}) between populations over all loci was 0.28 and the G_{ST} ranged from 0.1477 to 0.3892. The gene frequency ranged from 0.7403 to 0.7478 and the average was 0.7434. The observed number of alleles (*Na*)

Table 1. Analysis of polymorphism obtained with RAPD primers in different accessions of *E. calcadensis*

Accession	*Na*	*Ne*	*H*	*I*	NPL	% of polymorphism
EC_1	1.2730	1.1976	0.1093	0.1592	44	27.31
EC_2	1.2236	1.1792	0.0978	0.1402	36	22.36
EC_3	1.2547	1.1912	0.1067	0.1546	41	25.47
EC_4	1.3354	1.2826	0.1510	0.2147	54	33.54
EC_5	1.2795	1.2407	0.1274	0.1807	45	27.95

EC_1 – Karaiyar; EC_2 – Kezhamailar EC_3 – Sorimuthuaiyanarcoil;
EC_4 – Mancholai; EC_5 – Mundanthurai. *Na* – Observed number of alleles;
Ne – Effective number of alleles; *H* – Gene diversity;
I – Shannon Information Index; NPL – Number of Polymorphic Loci

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

Accession	EC_1	EC_2	EC_3	EC_4	EC_5
EC_1	****	0.8509	0.8590	0.8538	0.8820
EC_2	0.1624	****	0.9379	0.9255	0.9441
EC_3	0.1612	0.0641	****	0.9379	0.9441
EC_4	0.1636	0.0775	0.0641	****	0.9193
EC_5	0.1256	0.0575	0.0575	0.0842	****

Table 3. Genetic and gene diversity within and between the populations of *E. calcadensis* 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	7	0.4787	0.3837	0.3240	7.7802	0.7256
2	OPA02	TGCCFAGCTG	9	0.2956	0.2587	0.2977	11.1778	0.7474
3	OPA03	AGTCAGCCAC	5	0.4039	0.3198	0.2397	5.4791	0.7403
4	OPA07	CTGCATCGTC	5	0.2956	0.2587	0.2077	11.1778	0.7403
5	OPA08	CAAACACCCC	9	0.3538	0.2751	0.2582	11.9325	0.7468
6	OPA10	GTGATCGCAG	8	0.4052	0.2675	0.3524	2.3432	0.7433
7	OPB08	GTCCACACGG	8	0.4747	0.7867	0.1650	2.8759	0.7478
8	OPB11	GTAGACCGGT	7	0.4239	0.3140	0.2673	1.4862	0.7474
9	OPB14	TCCGCTCTGG	8	0.3996	0.2588	0.3603	0.9203	0.7442
10	OPB17	AGGGAACGAG	9	0.3558	0.2209	0.3789	0.8378	0.7439
11	OPB20	GGACCCTTAC	10	0.4657	0.3810	0.1754	3.6734	0.7415
12	OPH01	GGTCGGAGAA	9	0.3224	0.2303	0.2860	1.9334	0.7427
13	OPH03	AGACGTCCAC	8	0.3412	0.2257	0.3598	2.0714	0.7475
14	OPH06	ACGCATCGCA	6	0.3314	0.2264	0.3098	0.9965	0.7435
15	OPH10	CCTACGTCAG	10	0.4787	0.4071	0.1477	5.9193	0.7414
16	OPX04	CCGCTACCGA	9	0.4461	0.3063	0.3007	7.2230	0.7408
17	OPX05	CCTTTCCTTC	7	0.4379	0.3567	0.1911	3.2890	0.7470
18	OPX09	GGTCTGGTTG	10	0.4234	0.2999	0.2886	2.2701	0.7461
19	OPX12	CAGACAAGCC	9	0.3756	0.2766	0.3891	1.4425	0.7453
20	OPX18	CAGAAATGGA	8	0.3234	0.2009	0.3892	0.7790	0.7461

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

ranged from 1.2236 to 1.3354. The EC_2 population found to be least diverse (1.22). The EC_4 population displayed the highest level of variability (1.33) and average was 1.2732. The mean effective number of alleles (*Ne*) was 1.2182, highest was 1.1976 in EC_1 and lowest was 1.2407 in EC_5. Shannon Information Index (*I*) ranged from 0.1402 to 0.2147 and the average was 0.16988. The mean number of polymorphic loci (NPL) was 44 and ranged

from 36 to 54 for all the accessions of *E. calcadensis* which were depicted in the table 3.

Zucchi *et al.* (2005) reported that the genetic variability of ten *Eugenia dysenterica* populations characterized had 27.03% and 72.97% genetic variability. These populations are differentiating with restricted and geographic distribution dependent gene flow. Present study reveals that mean genetic diversity (*H*) ranged from 0.0978 to 0.1510. The average gene flow from one population to the other generation (*Nm*) was 4.2804 while the lowest was 0.7790 and the highest was 1.9325. This comparison had made because of data obtained with other markers, which allow greater number of loci than our screened markers.

Correlation between populations using Similarity index.

In order to study the correlation between populations, UPGMA algorithm was used to draw a dendrogram for the five populations (Fig 1). Using *Jaccard*'s matrix a dendrogram was produced based on similarity index (SI), which showed distinct separation of five locations into two major groups having 90 % similarity. Between the two major clusters, the upper cluster (UC) possessed EC_1, EC_2 and EC_5 accessions. Further, the accessions of the UC had grouped into two major sub clusters having 93 % similarity. The upper sub cluster 2 (USC 2) was further sub divided into USC 2A and USC 2B while the upper sub cluster 1 (USC 1) contained a solitary collection of EC_1. The USC2A comprised of EC_2 and the USC2B comprised of EC_5 with 96 % similarity. The accession belonging to the lower cluster (LC) was divided into two sub clusters LSC1 collected from EC_3 and LSC2 from EC_4 having 92 % similarity.

Rashmi *et al.* (2004) suggested that the understanding of genetic variability within the species is an important input to determine the future *in vitro* conservation approach. Among the accessions studied, the Mancholai (EC_4) accession was considered as the superior genotype based on high level of genetic variability (0.15) and percentage of polymorphism (33.54%).

CONCLUSION

This study reveals that RAPD technique is very effective in determining the genetic diversity and important for identifying the superior genotypes of *E.calcadensis* based on the polymorphism and genetic variability among the five accessions. Overall percentage of

Figure 1 UPGMA dendrogram of *E. calcadensis*

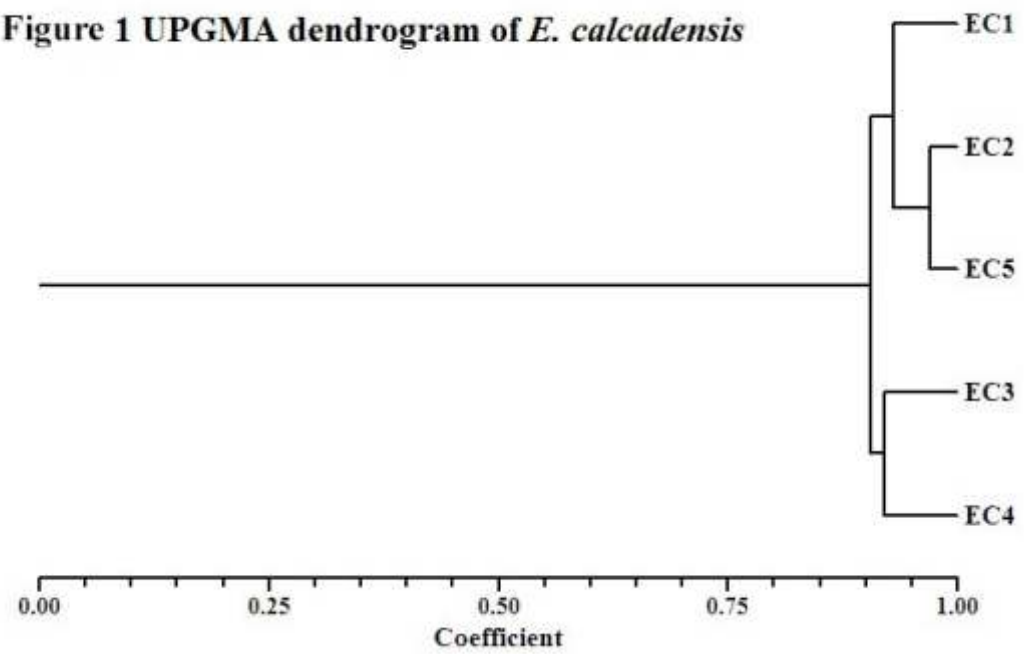
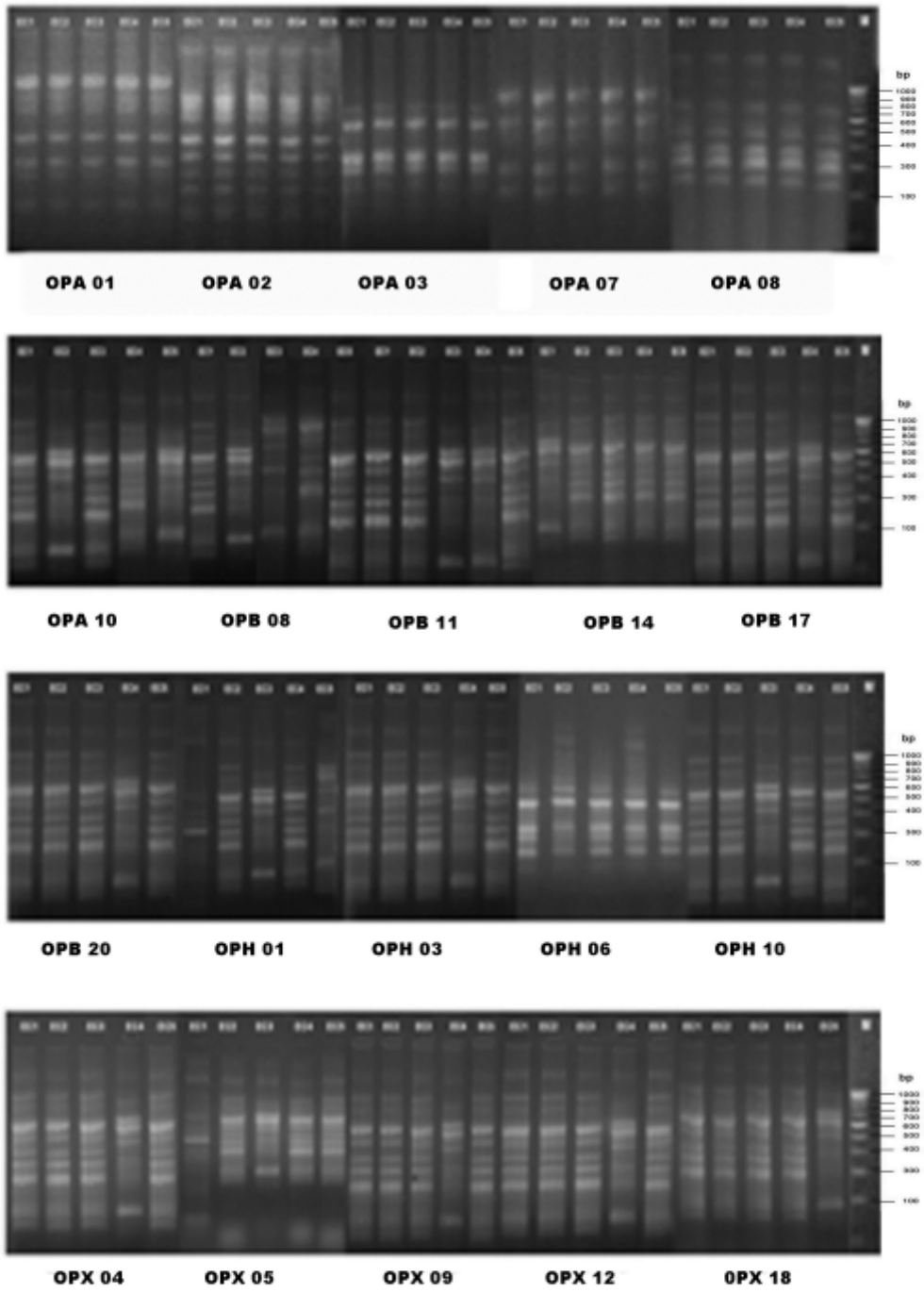


Figure 2. RAPD-PCR Fingerprinting of *Eugenia calcadensis*



EC1 - Karaiyar, EC2 - Kezhamailar, EC3 - Sorimuthaiyanarcoil, EC4 - Mancholai EC5 - Mundanthurai, M - Marker.

polymorphism is low due to high narrow endemic nature of this species. Among the accessions studied, the Mancholai (EC_4) accession has highest level of genetic variability and percentage of polymorphism and consider as the superior genotype. Hence there is chance to long term viability of this population and it is a valuable part of strategies for ex situ conservation of this important endangered plant.

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