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GENETIC DIVERSITY OF *EMBELIA* SPECIES, *MAESA INDICA* AND *ARDISIA SOLANACEA* SAMPLED FROM WESTERN GHATS OF KARNATAKA USING DNA MARKERS

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ABSTRACT: Genetic diversity of three *Embelia* species, *Maesa indica*, and *Ardisia solanacea* were assessed using RAPD and ISSR markers. Twenty-five sample was collected from various parts of Western Ghats of Karnataka. Nine RAPD primer and five ISSR primer produced a total of 48 and 40 loci respectively. Overall percentage polymorphism of RAPD and ISSR was 44.17%±13.13, 36.5±13.57% respectively. Polymorphic information content for RAPD and ISSR analysis had a range of 0.629-0.884, and 0.524-0.783 respectively. Resolving power of RAPD primers ranged from 1.6 to 6.24, while for ISSR primer the range was 4.64-10.16. UPGMA dendrogram of RAPD and ISSR revealed two and three clusters respectively. Within the population, *E. tsjeriam-cottam* had the highest polymorphism (>80%). Three principal coordinates accounted for 62.66% and 73.2% cumulative variation for RAPD and ISSR respectively. Marker index of ISSR primer (5.568) was higher than the RAPD primers (3.54), hence ISSR was the efficient marker in the current analysis.

KEYWORDS: Primulaceae, DNA Marker, Genetic Diversity, Polymorphism.

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1.INTRODUCTION

Genus *Embelia* are woody climbers [1] and *Ardisia solanacea* is a shrub belonging to the subfamily Myrsinoideae. *Maesa indica* belong to subfamily Maesoideae. Species of *Embelia, A. solanacea*, *M. indica* are found in semi-evergreen and deciduous forests of India. *E. ribes* is reported to be vulnerable in the southern state of peninsular India [2, 3]. It is vulnerable due to excessive harvesting,

because of its many uses in traditional medicine systems. Species of *Embelia* are a highly valuable medicinal plant with anthelmintic, carminative, antibacterial, antibiotic, hypoglycemic, and antifertility properties [4]. *Ardisia solanacea* and *Maesa indica* are also medicinal plants having a plethora of significant biological activity such as antidiabetic [5-7], antitumor [8, 9], antibiotic [10] larvicidal activity [11], Anticonvulsant [12], anxiolytic [13] to name a few. *Embelia* species and *Maesa indica* contains quinone derivative Embelin and Vilangin [14] and Kiritiquinone [15] respectively, and *Ardisia solanacea* contains α-amyrin and β-amyrin as one of the major chemical constituent [16]. The Western Ghats is one of the biodiversity hotspots of the world and contains several endemic flora [17]. There is highly varied heterogeneity of climate and topography which supports diverse vegetation types and distinct fauna. Therefore, it is necessary to know the diversity of flora within the localized region of Western Ghats to tailor effective conservation strategies. Randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) are quick and straightforward techniques which do not require prior knowledge of DNA sequence of the target organism. RAPD detects polymorphisms, using a single decamer nucleotide primer whereas ISSR identifies polymorphisms in identical inter microsatellite loci oriented in the opposite direction, using 12-24 base pair nucleotide primers which are simple sequence repeats [18]. RAPD and ISSR have been extensively used for the assessment of genetic diversity in a variety of cereal crops, commercial and medicinal plants. In previous studies of family Primulaceae phylogenetic analysis have been carried out by Martins, Oberprieler *et al.*, [19] using Inter transcribed spacers. For the *Embelia* species, Devaiah and Venkatasubramanian *et al.*, [20] designed sequence characterized amplified region (SCAR) markers for *Embelia ribes* to distinguish *Embelia ribes* from the adulterants, and Gowda, Chandrika *et al.*, [21] have used Amplified fragment length polymorphism (AFLP) to detect polymorphisms in *E. ribes* and *E. tsjeriam-cottam*. Using RAPD marker, Nagamani and Rani *et al.*, [22] were able to distinguish *E. ribes* from its substituents and adulterants. Chrungoo, Rout *et al.*, [23] have used ISSR primer and DNA barcode regions ITS and matK to check genetic diversity of *E.ribes, E. subcoraceae, E. floribunda* sampled from Meghalaya and Arunachal Pradesh regions of India. *Embelia* being a threatened and medicinally important plant, the present study is directed toward understanding the genetic variation at inter and intraspecies levels in *Embelia* species, also *Maesa indica* and *Ardisia solanacea* sampled from Western Ghats of Karnataka using DNA markers RAPD and ISSR.

2. MATERIALS AND METHODS

2.1. Sampling

Young leaves of *E. ribes, E. tsjeriam-cottam, E. basal, Maesa indica* and *Ardisia solanaceae* were collected from different locations of Western Ghats (Table 1) and brought to Department of studies in Biotechnology, Mysore of University, in ice bags.

Table 1: Isolates code, sampling area in the Western Ghats

SL. No	Name	Isolate Code	Origin
1	*E. ribes*	ER12	Bisli Ghat, Madekeri District
2	*E. ribes*	ER14	Kigga, Chikkamagaluru District
3	*E. Basal*	EB2	Somavarpete, Madikeri District
4	*E. Basal*	EB3	Madapura, Madikeri District
5	*E. Basal*	EB4	Somavarpete, Madikeri District
6	*E. Basal*	EB5	Madapura, Madikeri District
7	*E. Basal*	EB6	Kigga, Chikkamagaluru District
8	*E. tsjeriam-cottam*	ET1	Makuta, Madikeri District
9	*E. tsjeriam-cottam*	ET3	Makuta, Madikeri District
10	*E. tsjeriam-cottam*	ET4	Bisli Ghat, Madikeri District
11	*E. tsjeriam-cottam*	ET5	Somavarpete, Madikeri District
12	*E. tsjeriam-cottam*	ET6	Baghamandala Madikeri District
13	*E. tsjeriam-cottam*	ET7	Makuta, Madikeri District
14	*E. tsjeriam-cottam*	ET8	Bisli Ghat, Madikeri District
15	*E. tsjeriam-cottam*	ET9	Somavarpete, Madikeri District
16	*E. tsjeriam-cottam*	ET10	Makuta, Madikeri District
17	*E. tsjeriam-cottam*	ET11	Bisli Ghat, Madekeri District
18	*E. tsjeriam-cottam*	ET12	Makuta, Madikeri District
19	*E. tsjeriam-cottam*	ET13	Bisli Ghat, Madikeri District
20	*E. tsjeriam-cottam*	ET15	Makuta, Madikeri District
21	*Maesa indica*	MI2	Periyapatna, Mysore district
22	*Maesa indica*	MI3	Makuta, Madikeri District
23	*Maesa indica*	MI4	Bisli Ghat, Madikeri District
24	*Ardicia solanaceae*	AR1	Sakleshpura, Hassan District
25	*Ardicia solanaceae*	AR2	Sakleshpura, Hassan District

The leaves were cleaned with distilled water or alcohol and extracted on the same day. The remaining leaves were kept at -20°C. Plants were preliminarily identified from the competent taxonomist. The random decamer primers used were from “Kit G” and “Kit R” of Operon Technologies (Alameda, CA, USA) and Five ISSR primers are listed in Table 2.

Table 2: Details of RAPD and ISSR primers used for the amplification and Marker parameters

Marker	Primer code	Primer Sequences (5' to 3')	T_a°C	NL	PIC	RP	MR	MI
RAPD	OPG-09	CTGACGTCAC	36	7	0.815	4.24	5.33	3.54
	OPG-16	AGCGTCCTCC		10	0.878	5.92		
	OPG-17	ACGACCGACA		6	0.634	6.24		
	OPG-18	GGCTCATGTG		6	0.696	5.44		
	OPG-19	GTCAGGGCAA		5	0.787	3.92		
	OPR-2	CACAGCTGCC		5	0.629	5.12		
	OPR-3	ACACAGAGGG		3	0.663	2.88		
	OPR-7	ACTGGCCTGA		3	0.663	2.4		
	OPR-8	CCCGTTGCCT		3	0.884	1.6		
ISSR	ISSR02	CTCTCTCTCTCTCTCTAC	40	8	0.783	6.08	8	5.56
	ISSR03	CTCTCTCTCTCTCTCTGC	40	11	0.711	10.16		
	ISSR04	CACACACACACAAC	45	6	0.524	4.64		
	ISSR06	CACACACACACAAG	45	8	0.749	7.04		
	ISSR12	CACCACCACGC	32	7	0.713	6.72		

T_a =Annealing temperature; NL =Number of loci; PIC= Polymorphic Information Content; RP = Resolving Power; MR= Multiplex Ratio; MI= Marker Index

2.2. DNA isolation, RAPD, and ISSR reaction

Genomic DNA was isolated according to Stange, Prehn *et al.*, [24] protocol. DNA was quantified using NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific) and diluted to 25 ng for use in polymerase chain reaction (PCR). The reaction mixture contained 100 uM of each dNTPs (Merck Biosciences), five uM of primer (Sigma, USA), 0.5 Unit of Taq DNA polymerase (Merck Biosciences) and 1x Taq buffer (Merck Biosciences) in a total volume of 20 ul. ISSR-PCR amplification was carried out for 40 cycles. An Initial denaturation for 5 minutes at 94°C, followed by a cyclic process of denaturation for 1 minute at 94ºC, annealing at temperature standardized for each primer for 1 minutes and extension at 72°C for 1 minutes, and a final extension at 72 °C for 5 minutes in Applied Biosystems Veriti Thermal Cycler. For RAPD-PCR, the protocol was similar to ISSR except for the annealing temperature which was 36°C for all the primers. Amplified products were separated in 1.8% agarose gel containing ethidium bromide using 1x TBE buffer. DNA fragments were visualized under UV light. The band patterns were photographed using Gel Doc™ XR (Bio-Rad).

2.3. Data collection and Analysis

The banding patterns obtained from RAPD and ISSR were scored as present (1) or absent (0) and to create binary matrix was for RAPD and ISSR primers. Multiplex ratio (total number

monomorphic and polymorphic loci/ number of assays) were calculated. The polymorphic information content (PIC) of each marker was calculated as proposed by Roldàn-Ruiz, Dendauw *et al.*, [25], marker index (MI) described by Varshney, Chabane *et al.*, [26] and resolving power (RP) by Prevost and Wilkinson *et al.*, [27]. Percentage of polymorphic band (PP), observed number of alleles (na), effective number of alleles (ne), Shannon's information index (I) and Nei's gene diversity (H) total heterozygosity (Ht), average heterozygosity (Hs), coefficient of gene differentiation (Gst) and gene flow (Nm) between the populations and among the individuals within each population was analysed using software POPGENE [28]. The similarity matrix was subjected to cluster analysis by unweighted pair group method for the arithmetic mean (UPGMA), and a dendrogram was generated. GenAlEx6 [29] was also used to calculate Principal Coordinates Analysis (PCoA) that plots the relationship between distance matrix elements based on their first two principal coordinates. The product-moment correlation (r) based on Mantel Z value was computed to measure the degree of relationship between similarity index matrices produced by two-marker systems. The RAPD and ISSR data were subjected to a hierarchical analysis of molecular variance (AMOVA), as described by Excoffier, Smouse *et al.*, [30]

3. RESULTS AND DISCUSSION

Forty RAPD primers (Operon primer kits OPG and OPR 20 primer from each kit) were used to detect genetic polymorphism. Out of the 40 RAPD primers, 9 primers i.e. OPG-09, -16, -17, -18, -19 and OPR-02, -03, -07, -08 showed reproducible amplified DNA polymorphism. Number of amplified fragments ranging from 3 to 10. A minimum number of bands were seen in the primer OPR -03, -07, -08 (3 bands) and maximum bands were observed in primer OPG16 (10 bands). From the nine primers, a total of 48 loci were generated. All the 48 loci of were polymorphic, making polymorphism generated by RAPD markers to be 100%.PIC range from 0.629-0.884 with OPR-8 and OPR-2 having the highest and lowest scores respectively. Likewise, the resolving power of primer OPG-17 (6.24) and OPR-8 (1.6) had the highest and lowest scores respectively. The MR of RAPD analysis was 5.33 and MI was 3.54. N, na, ne, I and H of All the 25 samples was 1.038, 1.285, 0.244, 0.165 respectively. N, na, ne, I and H of *E. ribes, E. tsjeriam-cottam, E. basal, Maesa indica* and *Ardisia solanaceae* population determined by RAPD analysis are listed in Table 3.

Table 3: Genetic variability within *E. ribes, E. tsjeriam-cottam, E. basal, Maesa indica, Ardisia Solanaceae* discerned through RAPD and ISSR

Marker	Species	N	na	ne	h	I	NPL	PP
RAPD	*E. ribes*	2	1.1250	1.0884	0.0756	0.0518	6	12.50 %
	E. tsjeriam-cottam	13	1.8958	1.6384	0.5146	0.3534	43	89.58 %
	E. basal	5	1.4583	1.2155	0.2137	0.1366	22	45.83 %
	Maesa indica	3	1.4792	1.3035	0.2667	0.1789	23	47.92 %
	Ardisia Solanaceae	2	1.2500	1.1768	0.1512	0.1036	12	25.00 %

ISSR	*E. ribes*	2	1.1500	1.1061	0.0621	0.0907	6	15 %
	E. tsjeriam-cottam	13	1.8250	1.5655	0.3180	0.4660	33	82.50
	E. basal	5	1.5000	1.1551	0.1134	0.1913	20	50%
	Maesa indica	3	1.2750	1.2227	0.1201	0.1720	11	27.50 %
	Ardisia Solanaceae	2	1.0750	1.0530	0.0311	0.0454	3	7.50%

N=Number of samples; na=Observed number of alleles; ne= Effective number of alleles; h=Nei's gene diversity; I=Shannon's information index; NPL=Number of polymorphic loci; PP=Percentage of polymorphic.

RAPD dendrogram revealed the presence of two major clusters 1 and 2. The cluster is further divided into four sub-cluster A, B, C, D. Sub-cluster A contained all samples of *E. ribes* and a sample of *E. tsjeriam-cottam* ET4. Sub-cluster B included all samples of *E. basal* and two samples *E. tsjeriam-cottam* ET1 and ET6. Sub-cluster C and D contained two (ET5, ET12) and three (ET3, ET7, ET8) samples of *E. tsjeriam-cottam.* Cluster 2 contained two sub-cluster I and II. Sub-cluster I included five samples *E. tsjeriam-cottam* (ET9, ET10, ET11, ET13, ET15) and two samples of *Maesa indica* MI2, MI3. Sub-cluster II contained all samples of *Ardisia solanaceae* and a sample of *Maesa indica* MI4 (Figure 1). From, UPGMA dendrogram it was clear that *E. tsjeriam-cottam* ambiguously grouped with *E. ribes, E. basal,* and *Maesa indica*. Even in the PCoA unclear cluster group of *E. tsjeriam-cottam* was observed. Three principle co-ordinates accounted for 62.66% of cumulative variation with first and second principle co-ordinates accounting for 30.77%, 20.10% of variation respectively (Figure 2).

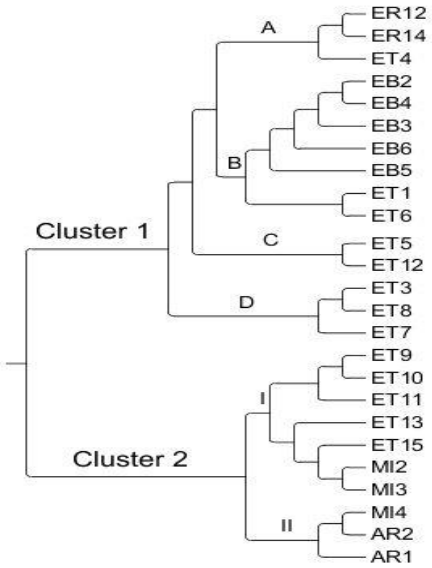

Figure 1: Dendrogram of RAPD UPGMA analysis of *E. ribes, E. tsjeriam-cottam, E.basaal, Maesa indica,* and *Ardisia Solanaceae* samples

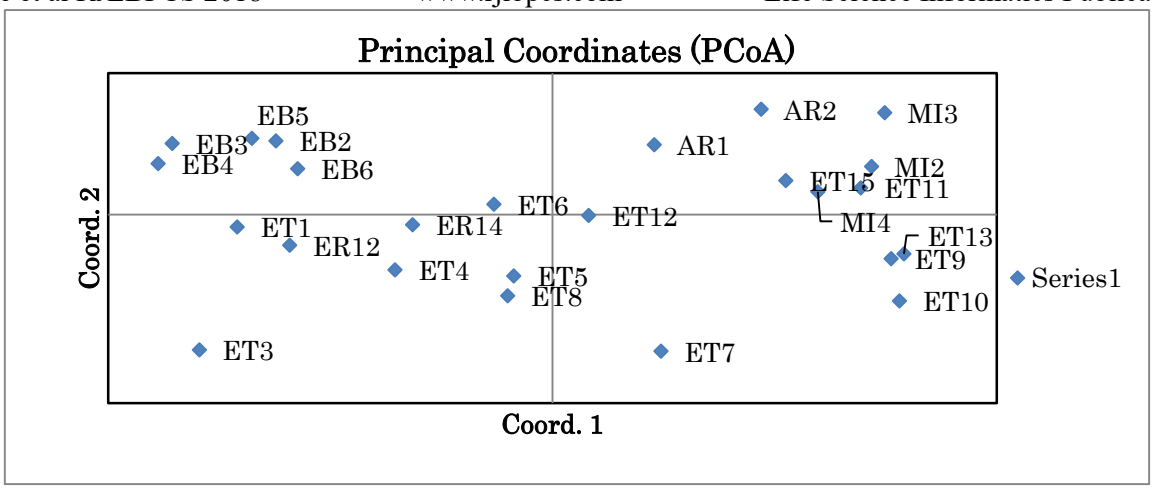

Figure 2: RAPD, the principal coordinate study of *E. ribes, E. tsjeriam-cottam, E. basal, Maesa indica* and *Ardisia Solanaceae* samples

Five ISSR primers produced 40 polymorphic loci. Maximum loci were generated by primer ISSR-3 (11 loci), and minimum loci were produced by ISSR-4 (6 loci). The PIC ranged from 0.524-0.783 with ISSR-4 had the lowest score, and ISSR-02 had the highest score. Primer ISSR-4 had lowest RP of 4.64 while ISSR-3 had the highest resolving power of 10.16. MR and MI for ISSR data were found to be 8 and 5.56 respectively. Overall N, na, ne, I and H of twenty-five sample was 0.975,1.220,0.193,0.129. Values of N, na, ne, I and H of *E. ribes, E. tsjeriam-cottam, E. basal, Maesa indica* and *Ardisia solanaceae* population determined by ISSR analysis are listed in Table 2. ISSR dendrogram revealed three clusters, cluster I contained *E. ribes* and *E. basal* samples; Cluster 2 contained only *E. tsjeriam-cottam* samples and cluster three included *Maesa indica* and *Ardisia Solanaceae* samples (Figure 3). PCoA plot was in accordance with dendrogram. Three clusters of *E. basal*, *E. tsjeriam-cottam,* and *Maesa indica* could be seen in PCoA plot. However, *E. ribes*, samples ER12, ER14, *E. basal* sample -EB2, *E. tsjeriam-cottam*-samples ET-1, ET-5, ET-6, ET-11 did not confirm for the clear cluster, and ET12 was in close affinity with *Ardisia Solanaceae* AR1, AR2 samples. Three principal co-ordinate axes accounted for cumulative variation of 73.2% with first, second and third axis accounting for variation of 43.1%,18.21%, and 11.88% respectively (Figure 4).

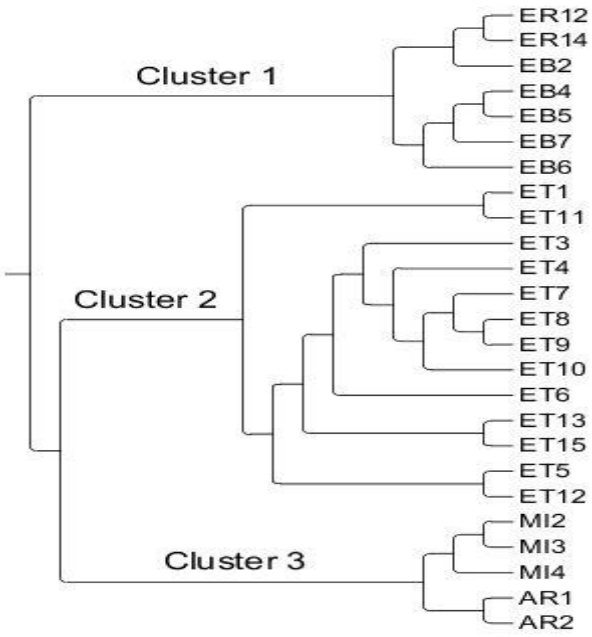

Figure 3: Dendrogram of ISSR UPGMA analysis of *E. ribes, E. tsjeriam-cottam, E. basal, Maesa indica* and *Ardisia Solanaceae* samples

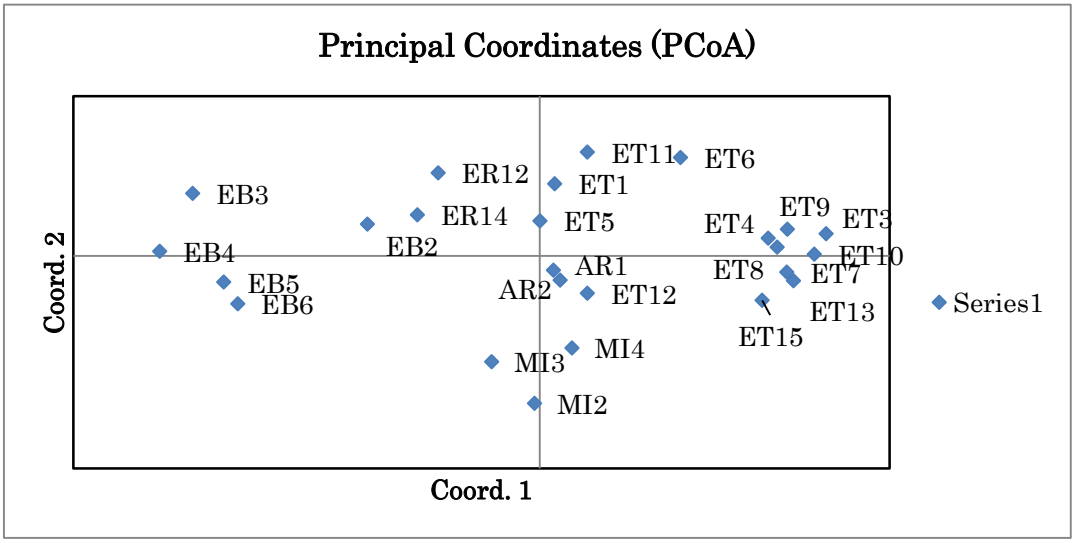

Figure 4: ISSR principal coordinate analysis of *E. ribes, E. tsjeriam-cottam, E. basal, Maesa indica* and *Ardisia Solanaceae* samples.

Mantel test was employed to determine the coefficient of correlation between the genetic matrices generated by RAPD and ISSR primers. The coefficient of correlation between RAPD and ISSR was r=0.403 which means that there is no strong correlation between the two analysis. For RAPD AMOVA estimated the variance of 2.877 which accounted for 27% of the variation among the population and conversely 73% of variation was within the population. For ISSR AMOVA estimated variances of 5.186 and 4.572 which correlated to 53% and 47% of the variation among and within population respectively. Overall PP for RAPD was 44.17%±13.13, while for ISSR overall PP was 36.5±13.57%. *E. tsjeriam-cottam* exhibited highest PP within the species (RAPD-89.85%, ISSR-

82.5%) followed by *E. basal* (RAPD-45.83%, ISSR-50%) and *Maesa indica* (RAPD-47.92%, ISSR-27.50%) and low PP within the species seen *E. ribes* (RAPD-12.5, ISSR-15%) *and Ardisia Solanaceae* (RAPD-25%, ISSR-7.5%). Gowda, Chandrika *et al.*, [21] in AFLP analysis observed about 88% of polymorphism in both *E. ribes* and *E. tsjeriam-cottam*. In the current study, *E. tsjeriam-cottam* had similar PP. MI which a measure of the overall efficiency of a marker and the average RP which indicates the discriminatory potential of the DNA marker was highest for ISSR, making it the efficient marker for the genetic diversity studies of *Embelia* species, *Maesa indica, and Ardisia Solanaceae*. In the RAPD analysis by Nagamani and Rani *et al.*, [22] High PIC of > 0.9 was in primers OPF7 and OPD5 primers. In comparison, average PIC from our study was 0.665. While Chrungoo, Rout *et al.*, [23] used eight ISSR primers which yielded 42 bands of which 33 (68%) bands were polymorphic. The average RP was 5.25 in their study which is very similar to average RP of the current study.

4. CONCLUSION

DNA marker RAPD and ISSR are an instrumental technique in understanding the genetic diversity of sampled species. Among the two methods, time and time again ISSR has emerged as a robust marker than RAPD. From both marker technique, we got an overview of the genetic diversity of *Embelia* species, *Maesa indica and Ardisia Solanaceae* samples from Western Ghats of Karnataka.

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CONFLICT OF INTEREST

The authors declare that have no conflict of interest

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