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Assessing genetic diversity and DNA barcodes efficiency of *Salacia* and related species from Western Ghats, Karnataka

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ABSTRACT

The genetic diversity of 10 genera belonging family Celastraceae (four *Salacia* species and nine other genera) was analyzed using 11 intersimple sequence repeat (ISSR) primers. Species discriminating ability of DNA barcodes *rbcL*, *matK,* and internal transcribed spacer (*ITS*) was evaluated for *Salacia* species sequences originating from Western Ghats of Karnataka and Kerala. For ISSR analysis, a total of 46 samples were collected from Western Ghats of Karnataka. ISSR primers revealed a mean polymorphism of 29.21 ± 7.89%. The DNA barcodes showed a high level of universality for polymerase chain reaction and sequencing. The ITS had the highest discriminate ability of 67.13% in the neighbor-joining tree-based method and in TaxonDNA software's, Best match (BM) and Best close match (BCM) had 59.59% and 58.58% correct identification success, respectively. Consortium for the Barcode of Life Plant Working Group's recommended barcodes *matK*, *rbcL*, and combined *matK* + *rbcL*, had lower correct identification success in both the tree-based method and TaxonDNA's BM and BCM.

1. INTRODUCTION

The *Salacia* genus belongs to the family Celastraceae. Several species belonging to this genus have been used in herbal medicine to treat primarily diabetes, obesity, and rheumatism. *Salacia* contains bioactive compounds that are effective antidiabetic, antiobesity, hepatoprotective, and antioxidant agents of tremendous medicinal value [1]. *Salacia* species have been widely traded as raw herbal medicines in India, Japan, China, and Korea due to their use in traditional medicine [2]. There are many plants in the family Celastraceae that have medicinal properties. Some plants that are used in herbal medicine in India and other parts of the world are *Cassine glauca, Celastrus paniculatus, Euonymus indica, Lophopetalum wallichianum, Gymnosporia montana, Maytenus heyneana, Microtropis wallichiana,* and *Pleurostylia opposita.*

The Western Ghats is one of the world's major biodiversity hotspots [3], and due to high biodiversity and endemism, areas of South India have attracted attention [4]. With high endemism and species richness in the Western Ghats, taxonomists have had a problem with proper identification, and this problem is profound when the samples are collected in their vegetative state. Eight species of the genus *Salacia* have been described in the "Flora of Karnataka" by Saldanha and Larsen [5], namely, *Salacia beddomei, Salacia chinensis, Salacia fruticosa, Salacia macrosperma, Salacia malabarica, Salacia oblonga, Salacia reticulata,* and *Salacia talbotii*.

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The status of conservation for *S. beddomei*, *S. macrosperma,* and *S. malabarica* has been labeled vulnerable, rare, and endangered respectively (http://florakarnataka.ces.iisc.ac.in). The status "Vulnerable" is given to *S. oblonga*, which is endemic to the Western Ghats of India and Sri Lanka, by the International Union for Nature Conservation. Habitat destruction by human activities through land clearing, for agriculture, for construction of the residence, and expansion of roads and railways, along with indiscriminate collection for its use in various medicinal applications, is the primary cause of the decline of *S. oblonga*. Species of *Salacia* are rarely found in deciduous forests. In coastal forests, *S. chinensis* and *S. fruticosa* are usually found *S. macrosperma* and *S. oblonga* inhabit evergreen forests. *Euonymus* species such as *Euonymus angulatus, Euonymus assamicus, Euonymus paniculatus, Euonymus serratifolius,* and *Cassine viburnifolia* native to India have also been documented as endangered and vulnerable.

Due to the high level of morphological similarities between species, the genus *Salacia* is often difficult to identify [5]. Dev and Anoop [6], and Devipriya and Devipriya [7], have used internal transcribed spacer (ITS) sequences to carry out phylogenetic studies for eight species and one variety of the genus *Salacia* collected from Kerala. In addition to ITS sequences, Dev and Anoop [6] have used *rbcL*, *matK*, and *trnH-psbA* sequences in their research.

Using traditional taxonomy, the task of proper identification becomes arduous for the very closely related species. DNA barcoding, irrespective of environmental factors affecting the growth, development, and morphology of an organism, is a complementary method for quick and accurate species identification. Inter-simple sequence repeat (ISSR) is a fast and very sensitive technique in detecting polymorphisms

at intra- and interspecific levels of variation using oligonucleotide primers [8]. As the method utilizes longer semi-arbitrary simple sequence repeats primers in high-stringency polymerase chain reaction (PCR) conditions, ISSRs are highly reproducible and polymorphic [9]. The ITS sequence is a popular choice for phylogenetic analyses [10] since it is amplified by a universal set of primers, and also ITS region is a simple sequence which is biparentally inherited, intragenomically uniform, and intergenomically variable with low functional constraints having a length of 500–700 base pairs [11].

The Plant Working Group (2009) of the Consortium for the Barcode of Life (CBOL) proposed multilocus barcoding as an efficient method for barcoding and species discrimination in plants. For plant barcoding, CBOL recommends *matK* and *rbcL* regions to be the most effective universal barcodes. In the current study, using ISSR marker, we have assessed the diversity of four *Salacia* species along with eight genera (*C. glauca, C paniculatus, E. indica, L. wallichianum, G. montana, M. heyneana, M. wallichiana,* and *P. opposita*) belonging to the family Celastraceae sampled from Western Ghats of Karnataka and Kerala. Furthermore, three DNA barcodes (*matK*, *rbcL*, and ITS) were used to evaluate their ability for species discrimination within *Salacia* species.

2. MATERIALS AND METHODS

2.1. Collection of Plant Material and DNA Extraction

From different parts of the Western Ghats, leaves of *Salacia* species and related genera were collected [Table 1]. DNA was extracted by following a modified Stange *et al*. method [12].

2.2. PCR Amplification of ISSR Marker

Eleven primers were used in the current study [Table 2]. PCR was performed in a 20 ul volume reaction mixture contained 100 uM of dNTPs; 0.5 unit of Taq DNA polymerase; 10× Taq buffer-A (Merck Biosciences), 5 uM primer; and sterile water. For amplification of ISSR following program was used, initial denaturation at 94°C for 300 s, followed by 45 cycle of 94°C for the 60 s, the primer specific annealing temperature for 60 s, and 72°C for 120 s, and a final extension at 72°C for 300 s in Veriti™ 96-Well Thermal Cycler (Applied Biosystems). The amplified products were separated in 1.8% agarose gel stained with ethidium bromide using a 1× Tris-Borate-EDTA buffer. Using Gel Doc™ XR (Bio-Rad), the band patterns on the gel were photographed. Amplified fragments were score visually for the presence (1) or absence (0) of homologous bands. The polymorphism information content (PIC) value was calculated as suggested by Roldàn-Ruiz and Dendauw [13], and the marker index was calculated as defined by Varshney and Chabane [14]. Genetic relatedness among *Salacia* species and related genus was studied by Unweighted Pair Group Method with Arithmetic (UPGMA) averages cluster analysis using NTSYSpc software 2.02e [15]. For parametric calculation, software POPGENE 1.31 [16] was used. Principal Coordinates Analysis (PCoA) was done using GenAlEx 6.51b2 [17].

2.3. Barcode Region PCR

For the PCR amplification of the ITS1-5.8s-ITS2 sequence primers, ITS4 and ITS5 were used [18]. For the amplification of *matK* region primers, 3F_KIM f and 1R_KIM r designed by Ki-Joong Kim (unpublished) were used, and for *rbcL* region, the primers rbcLa-f and rbcLa-rev were used with PCR protocol as described by the authors.

2.4. Sequence Alignment and Data Analysis

The amplified PCR products were sent to Chromous Biotech, Bengaluru, for sequencing. The sequences were deposited in the NCBI database and the database assigned accession numbers were to the individual samples. The sequences were aligned using the Clustal W option in MEGA 5 software [19]. The sequences were concatenated using the software Sequence Matrix [20]. To estimate the barcoding gap while comparing the distributions of the pairwise intra- and interspecific distance for each barcode candidate with an interval distance of 0.05, software TaxonDNA was used [21], with "pairwise summary function." Each barcode candidate was measured for the correct identification proportion using TaxonDNA with "best match" (BM) and the "best close match" (BCM) test the species discrimination rates.

3. RESULTS

3.1. Homology Search and Sample Validation

Thirty-two sequences were generated for the ITS region and 18 and 15 sequences were generated for *matK* and *rbcL* barcoding regions, respectively. BLASTn was employed for homology search. For ITS sequence, all the samples sequenced in the current study, except *S. chinensis* samples SC6, SC7, SC8, SC9, SC10, and SC11 showed similarity identity >90% with the *Salacia* sequence in the database. The sample SC6 to SC10 had a similarity identity ≤85% to *Pristimera preussii*, and SC11 had a similarity of 85% to *Tristemonanthus nigrisilvae,* >98% homology with their respective genera. For the *matK* sequence except for samples SC6 and SC11 which had 99% homology to *Apodostigma pallens,* all other sequences had 99% homology with *Salacia cf. cochinchinensis*. For the *rbcL* sequence, SC10 had 99% homology with *Reissantia* species. Species *P. preussii*, *T. nigrisilvae, A. pallens,* and *Reissantia* species belong to subfamily Hippocrateoideae which is closely related to subfamily Salacioideae and have most of the morphological characters to be similar. Henceforth, the samples SC6-SC11 were been labeled Hippocrateaceae for the ISSR analysis and these samples were not included in the further analysis.

3.2. ISSR Analysis

A total of 89 loci were generated from 11 primers. Out of the 89 loci scored, 86 loci (96.63%) were found to be polymorphic. The PIC values of 11 ISSR primers ranged from 0.756 to 0.893. Four primers ISSR-02, ISSR-04, ISSR-05, and ISSR-07 showed 100% polymorphism. High RP scores were observed with the primer ISSR-13 (11.8) and ISSR-07 (11.2), and the low RP scores were seen in primers ISSR-12 (5.4), ISSR-15 (5.5) [Figure 1], and ISSR-10 (5.6) with average RP at 8.09 [Table 2]. Within species, polymorphism of *C. glauca* was 49.44%, *C. paniculatus* was 25.84%, *Hippocrateaceae* was 65.17%, *L. wallichianum* was 47.19%, *S. oblonga* was 31.46 %, *S. macrosperma* was 64.04%, *S. chinensis* was 28.09%, and *S. fruticosa* was 48.31%. Overall polymorphism for all the 46 samples was 47.42 ± 7.81%. Na, Ne, I, He, and percentage of polymorphic loci of each population are listed in Table 3.

The dendrogram generated from the UPGMA cluster analysis divided 46 samples into two clusters. Cluster 1 contained nine subclusters. Subcluster 1 was further divided into three groups. Group A contained all four samples of *S. oblonga* and 12 of 13 samples of *S. macrosperma.* Group B contained five samples of *S. chinensis*. Group C contained six samples of *S. fruticosa.*

Subclusters 7 and 9 contained single samples of *E. indica* and *M. wallichiana,* respectively. Subclusters 2 and 5 had only samples of

Table 1: Isolate code and sampling area in the Western Ghats.

Name	Sample code	Sampling area	Latitude (°N)	Longitude (°E)	Elevation (M)	Reference
Cassine glauca	CG1 CG2	Bisle Ghats, Hassan district	12° 43' 51.42''	75° 42' 41.18''	934	This study
Celastrus paniculatus	CP1 CP2	Hunsur, Mysore district	12° 7' 54.41''	76° 10' 7.30''	781	
	CP3	Mysore Zoo, Mysore district	12° 18' 9.95''	76° 39' 48.98''	748	
Euonymus indica	EI	Bisle Ghats	12° 41' 59.26''	75° 38' 25.12''	838	
Lophopetalum wallichianum	LW1	Mysore Zoo, Mysore district	12° 18' 9.95''	76° 39' 48.98''		
	LW2	Somwarpet, Kodagu district.	12° 40' 28.14''	75° 48' 7.30''	1106	
Gymnosporia montana	GC	Bisle Ghats, Hassan district	12° 42' 42.70''	75° 41' 40.62''	803	
Maytenus heyneana	ME	Virajpet, Kodagu district	12° 15' 20.28''	75° 45' 40.82''	907	
Microtropis wallichiana	MS	Sakleshpur, Hassan district	12° 55' 7.21''	75° 45' 4.7952''	914	
Pleurostylia opposita	P	Chamundi Hills, Mysore district	12° 16' 21.28'	76° 40' 15.0132''	1024	
Salacia chinensis	SC1, SC2, SC3, SC4, SC5	Udupi district	13° 21' 42.39''	74° 46' 2.0''	21	(Bajpe et at. 2018)
Hippocrateaceae	SC6, SC7, SC8, SC9, SC10, SC11	Makuta, Madikeri district	12° 4' 29.87''	75° 43' 31.10''	115	This study
Salacia macrosperma	SM1	Virajpet, Madikeri district	12° 11' 41.94''	75° 48' 14.40''	907	(Bajpe *et al*., 2018)
	SM2, SM3	Shanivarsanthe, Kodagu district.	12° 43' 35.96''	75° 53' 8.92''	965	This study
	SM4	Abbi Falls, Madikeri district	12° 27' 29.54''	775° 43' 10.38''	108	(Bajpe *et al*., 2018)
	SM5	Madikeri town, Madikeri district	12° 25' 27.91''	75° 44' 17.46''	1128	
	SM6	Bisle Ghats, Hassan district	12° 42' 33.46''	75° 40' 38.80''	614	
	SM7	Kigga, Chikkamagaluru district	13° 25' 1.53''	75° 11' 17.15''	664	
	SM8, SM9	Somwarpet	12° 43' 18.76''	75° 53' 55.7946''		This study
	SM10	Kigga, Chikkamagaluru District	13° 25' 1.53''	75° 11' 17.15''	664	(Bajpe *et al.*, 2018)
	SM12, SM13, SM14	Makuta, Madikeri district	12° 9' 37.99''	75° 47' 28.93''	755	
Salacia fruticosa	SF1, SF2, SF3, SF4, SF5, SF6	Makuta, Madikeri district	12° 6' 26.22''	75° 46' 47.21''	555	(Bajpe *et al*., 2018), This study
Salacia oblonga	SO1, SO2, SO3, SO4	Kigga, Chikkamagaluru district	13° 27' 7.60''	75° 11' 51.60''	657	(Bajpe *et al*., 2018), this study

C. glauca and *C. paniculatus.* Subcluster 3 contained *P. opposite* and *M. heyneana*. Subcluster 6 contained sample LW2 of *L. wallichianum* and *G. montana*. Subcluster 4 contained samples SC10, SC11, and LW1. Subcluster 8 contained samples SC6, SC7, and SC9. Finally, cluster 2 contained sample SC9 and SM1 [Figure 2].

The three axes PCoA accounted for a cumulative variation of 46.87% [Figure 3]. The first axis and the second axis accounted for 24.84% and 12.21%, respectively. From the AMOVA data, we found out that 36% of the molecular variance was found among the population while within the population, this value was found to be 64%.

3.3. Intra- and Interspecific Diversity and Barcoding Gap

The aligned sequence length of ITS was 870 bp, *matK* was 848 bp, and *rbcL* was 700 bp. Among the single barcode, ITS had the most variable sites of 588 bp and parsimony-informative characters of 265 bp [Table 4]. The pairwise intraspecific and interspecific distances in the seven barcode combinations ranged from 0.0% to a maximum of 58% and 0% to 63%, respectively. The mean intraspecific and interspecific distances were the lowest for *rbcL* and highest for ITS [Table 5]. Among seven barcodes, none had visible barcoding gaps. All barcodes had overlaps between their intra- and interspecific distances without distinct barcoding gaps [Figure 4].

Table 2: List of ISSR primers used for the amplification of the 46 samples of *Salacia* species and closely related members with in Celastraceae family and marker parameters calculated for each ISSR primer used.

Primer	Primer sequence	T_m	TNB	NPB	PIC	EMR	MI	RP
ISSR-02	$(CT)_8AC$	40	8	6	0.821	13.98	6.43	6.7
ISSR-03	$(CT)_8GC$	40	9	9	0.878	9.34	4.32	9.9
ISSR-04	$(CA)_6AC$	45	13	13	0.893	10.93	3.24	8.7
ISSR-05	$(CA)_6GT$	45	9	9	0.876	10.17	3.81	9.1
ISSR-06	$(CA)_6AG$	45	11	8	0.860	11.08	4.81	8.5
ISSR-07	$(CA)_6GC$	45	9	9	0.882	7.96	2.98	11.2
ISSR-10	$(GA)_6CC$	48	10	6	0.813	14.88	7.02	5.6
ISSR-12	$(CAC)_3GC$	37	10	6	0.769	16.92	6.62	5.4
ISSR-13	$(GAG)_3GC$	37	13	10	0.891	7.62	3.19	11.8
ISSR-15	$(GTG)_3GC$	38	7	5	0.756	15.76	4.64	5.5
ISSR-16	(GA)9T	48	8	5	0.791	14.23	6.68	6.6

Table 3: Genetic variability within the members of Celastraceae family collected from Western Ghats of Karnataka as discerned through ISSR.

Species	N	Na	Ne	I	He	PPL
Cassine glauca	2	0.899±0.083	1.183±0.033	0.156±0.028	0.107±0.019	49.44%
Celastrus paniculatus	3	1.225±0.090	1.336±0.041	0.284±0.032	0.193±0.022	25.84%
Lophopetalum wallichianum	2	1.213±0.088	1.334±0.038	0.285±0.032	0.195±0.022	47.19%
Salacia oblonga	4	1.124±0.075	1.214±0.037	0.181±0.029	0.123±0.020	31.46%
Salacia macrosperma	13	1.719±0.071	1.573±0.036	0.481±0.026	0.328±0.018	84.27%
Salacia fruticosa	6	1.225±0.089	1.333±0.042	0.162±0.029	0.188±0.022	48.31%
Salacia chinensis 1	5	1.337±0.098	1.200±0.038	0.276±0.032	0.111±0.020	28.09%
Hippocrateaceae	6	1.045±0.076	1.386±0.039	0.343±0.029	0.228±0.021	65.17%
All samples	46/3.538±0.097	0.914±0.024	1.197±0.010	0.167±0.008	0.113±0.005	29.21±7.89%

N: Number of samples, Na: No. of different alleles, Ne: No. of effective alleles, I: Shannon's Information Index, He: Expected heterozygosity, PPL: Percentage of polymorphic loci

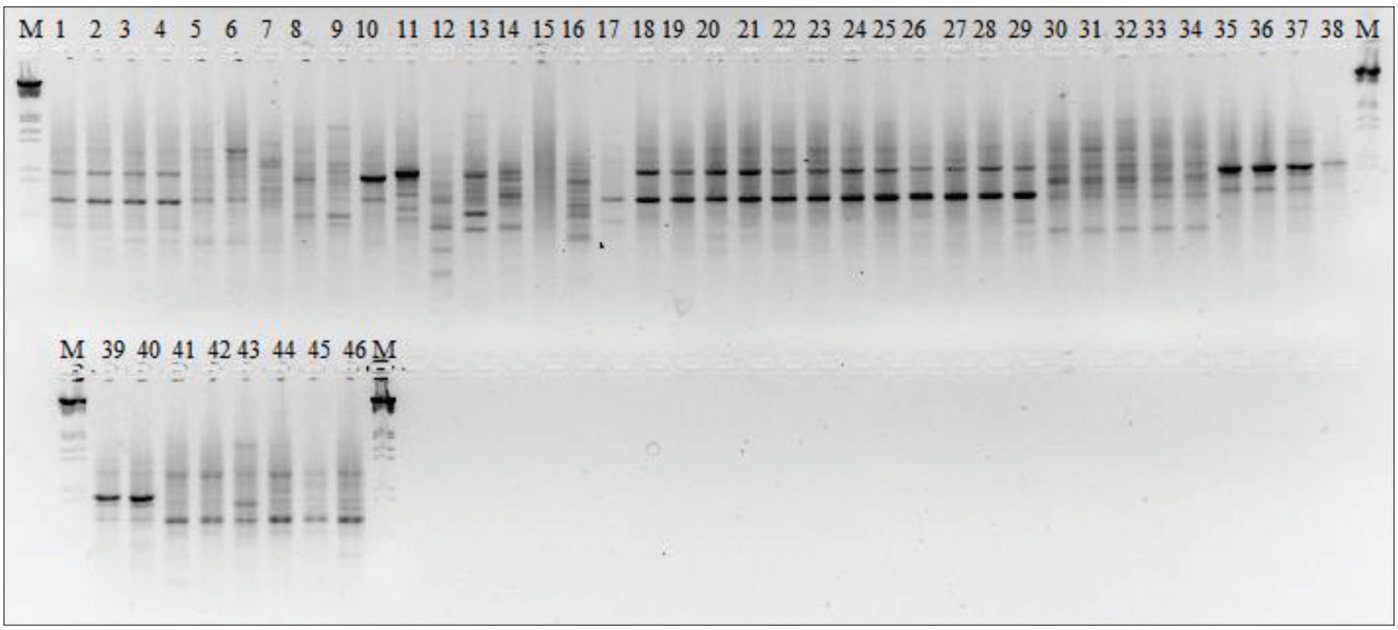

Figure 1: Inter-simple sequence repeat (ISSR) pattern of 46 samples of *Salacia* species and closely related members with in Celastraceae family amplified using primer ISSR-15. Lanes 1–4: *Salacia oblonga* (SO1-SO4), lanes 5–7: *Celastrus paniculatus* (CP1–CP3), lanes 8 and 9: *Cassine glauca* (CG1 and CG2), lanes 10 and 11: *Lophopetalum wightianum* (LW1 and LW2), lane 12: *Microtropis stocksii* (MS), lane 13: *Gymnosporia wallichiana* (GC), lane 14: *Pleurostylia opposita* (p), lane 14: *Euonymus indicus* (EI), lane 15: *Maytenus heyneana* (MH). Lanes 16–29: *Salacia macrosperma,* (SM1–SM14), lanes 30–34: *Salacia chinensis* (SC1–SC5). Lanes 35–40: Sample belonging to the subfamily Hippocrateoideae (H1–H6), lanes 41–46: *Salacia fruticosa* (SF1–SF6). M: Molecular weight marker (λ DNA digested with *Eco* RI and *Hind* III).

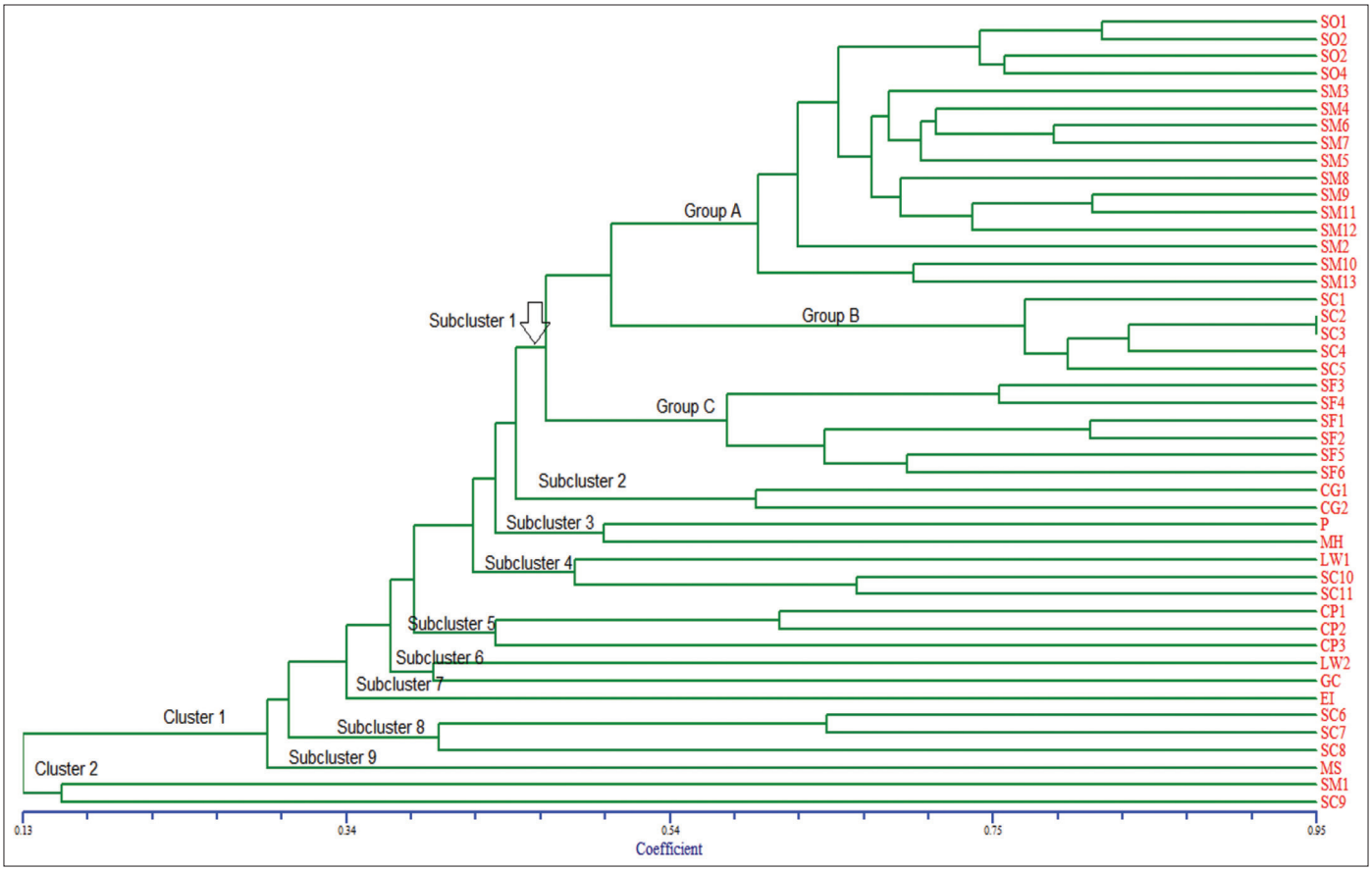

Figure 2: UPGMA dendrogram of 46 samples of *Salacia* species and closely related members with in Celastraceae family based on inter-simple sequence repeat marker.

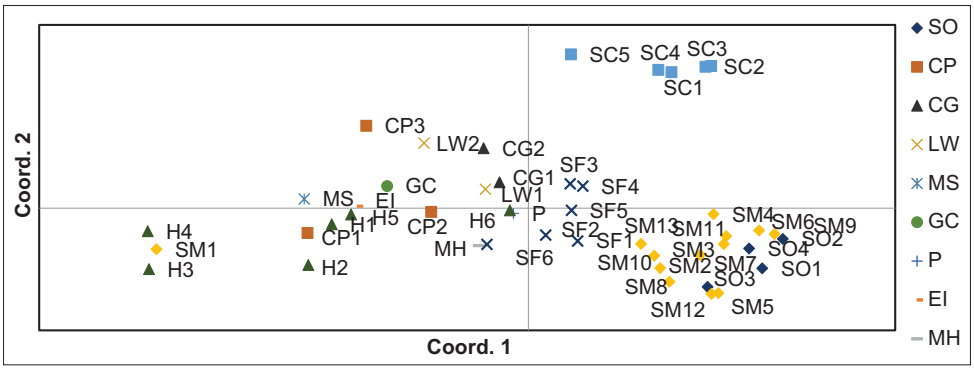

Figure 3: Inter-simple sequence repeat data principal coordinates analysis (PCoA) of 46 samples of *Salacia* species and closely related members with in Celastraceae family. PCoA accounting for 46.87% of cumulative variation, with the first, second, and third component accounting for 24.84%, 12.21%, and 9.82%, respectively.

Table 4: Evaluation of three DNA barcodes and combinations of the barcodes.

Barcode	Number of samples	No. of species	Length of aligned sequence (bp)	No. of parsimony informative sites/variable sites	Ability to discriminate (tree-based method-NJ)
ITS	99	11	870	240/380	67.13%
matK	94	9	848	37/56	8.59%
rbcL	91	9	700	14/37	10.39%
ITS+matK	99	12	1718	275/419	36.60%
ITS+rbcL	99	12	1570	253/407	41.98%
matK+rbcL	72	9	1545	15/30	5.09%
ITS+matK+rbcl	99	12	2418	288/446	31.30%

3.4. Species Discrimination

Analysis using TaxonDNA's BM and BCM and neighbor-joining (NJ) TBM to evaluate the discriminatory power of the three barcodes in *Salacia* species provided varied results. In both methods, the highest species discrimination success was obtained by ITS (BC and BCM – 59.59% and TBM – 67%) among the three single barcodes. The other two plastid DNA barcodes, that is, *matK* and *rbcL* had 41.48% (BC and BCM) and 8.59% (TBM), 30.76% (BC and BCM), and 10.39% (TBM) species identification rate, respectively [Tables 4 and 6]. When barcodes were combined, the highest discrimination rate of 41.98% was observed in ITS + *rbcL* (TBM) and 49.55% in ITS + *matK* (BC and BCM) [Tables 4 and 6]. The bootstrap support values of single barcodes were higher than the barcode combinations. Both the BM and BCM methods provided the same species discrimination success [Table 2].

4. DISCUSSION

ISSR motifs GA, CA, CT, GTG, CAC, and GAG were used in the present study, of which CA and GAG motifs produced maximum scorable loci. The overall mean percentage of polymorphism of only *Salacia* species (28 samples) was 48.03 ± 12.86%. This could be due to the addition of seven more samples in the current study. Similarly, higher polymorphism for medicinal plants sampled at a particular geographical region within India is observed in *Oroxylum indicum* (49.61%) [13]. ISSR has proved to be useful in species discrimination as it can detect very low variation with closely related species [9,14].

From our sample survey, we found out that the identification of *Salacia* species is indeed difficult as *S. oblonga* and *S. macrosperma* had very similar morphological characters. *Salacia* species can be easily misidentified and incorrect species can be used in the medicinal preparations, especially when two or more species are present in the area of sampling. Have used the random amplified polymorphic DNA marker of identifying genetic similarity in seven *Salacia* species samples from Wayanad, Kerala.

From Nei genetic distance and identity, *S. oblonga* is closely associated with *S. macrosperma* than *S. chinensis* and *S. fruticosa*. Within *Salacia* species, *S. macrosperma* has the highest percentage of polymorphic of 84.27% followed by *S. fruticosa* (48.31%) and *S. oblonga.* Since some of the species of *Salacia* are endangered, the effort to conserve germplasm by micropropagation of *S. chinensis* [15], *S. reticulata* [16], and *S. oblonga* [17] has been carried out. Molecular analysis suggests that *S. chinensis* has the lowest variation within the species, making it a suitable candidate for large-scale micropropagation, conservation, and alternative for other endangered species of *Salacia*. The use of *S. reticulata* in traditional medicine in Sri Lanka has created pressure for the supply of this plant from the wild. Therefore, the use of *S. chinensis* as a substituent has been suggested by Keeragalaarachchi and Dharmadasa [18]. *Salacia* species considered in the current study have been known to contain catechins, quinones, friedo-oleanones, quinone methide, and triterpenoids in them, which may be responsible for their pharmacological and medicinal properties.

Dev and Anoop [6] analyzing *Salacia* sampled from Kerala by NJ tree-based method observed that ITS2 and trnH-psbA region had 100% and 40% efficiency in species discrimination, while *matK* and *rbcL* failed in species discrimination. However, our present study using TaxonDNA software indicated that *matK* and *rbcL* barcodes are also useful in the correct identification of *Salacia* species sampled from Western Ghats of Karnataka and Kerala.

CBOL has recommended a combination of *matK*+*rbcL* as a universal barcode for plants. However, in the present study, the single barcode had higher discriminatory success than the combination of two or three barcodes. A combination of *matK*+*rbcL* had the lower species resolution of 32.69% in "BM" and lowest in the NJ tree-based method (5.09%) among all the combinations. This may be due to the low substitution

Table 5: Summary of the pairwise intraspecific and interspecific distances in the barcode loci of *Salacia* species of Western Ghats (Karnataka and Kerala).

Barcode locus	Intraspecific distance	Mean*	Interspecific distance	Mean*	Threshold**
	Maximum		Maximum		
ITS	59%	1.64	63%	5.42	33.08%
matK	4%	0.11	6%	0.56	0.61%
rbcL	2.5%	0.07	3.5%	0.38	1.71%
ITS+matK	58.0%	0.72	63%	3.34	24.52%
ITS+rbcL	59%	0.88	63%	3.59	25.32%
matK+rbcL	4%	0.11	6%	0.53	1.16%
ITS+matK+rbcL	58%	0.58	63%	2.93	23.65%

*Minimum interspecific distance is 0% for all the barcodes. **Threshold as computed for the pairwise summary

Table 6: Identification success based on the "best match" and "best close match" function of the program TaxonDNA.

Barcode	Best match			Best close match				T	CI
	C	A	I	C	A	I	No match		
ITS	59 (59.59%)	32 (32.32%)	8 (8.08%)	58 (58.58%)	32 (32.32%)	8 (8.08%)	1 (1.01%)	3	4
matK	39 (41.48%)	53 (56.38%)	2 (2.12%)	39 (41.48%)	53 (56.38%)	2 (2.12%)	0 (0.0%)	0.61	2
rbcL	28 (30.76%)	57 (62.63%)	6 (6.59%)	28 (30.76%)	57 (62.63%)	6 (6.59%)	0 (0.0%)	1.71	1
ITS+matK	56 (49.55%)	45 (39.82%)	12 (10.61%)	56 (49.55%)	45 (39.82%)	11 (9.73%)	1 (0.88%)	3	3
ITS+rbcL	49 (42.6%)	58 (50.43%)	8 (6.95%)	48 (41.73%)	58 (50.43%)	8 (6.95%)	1 (0.86%)	3	2
matK+rbcL	34 (32.69%)	64 (61.53%)	6 (5.76%)	34 (32.69%)	64 (61.53%)	5 (4.8%)	1 (0.96%)	1.16	3
ITS+matK+rbcl	42 (35.59%)	64 (54.23%)	12 (10.16%)	42 (35.59%)	64 (54.23%)	11 (9.32%)	1 (0.84%)	3	2

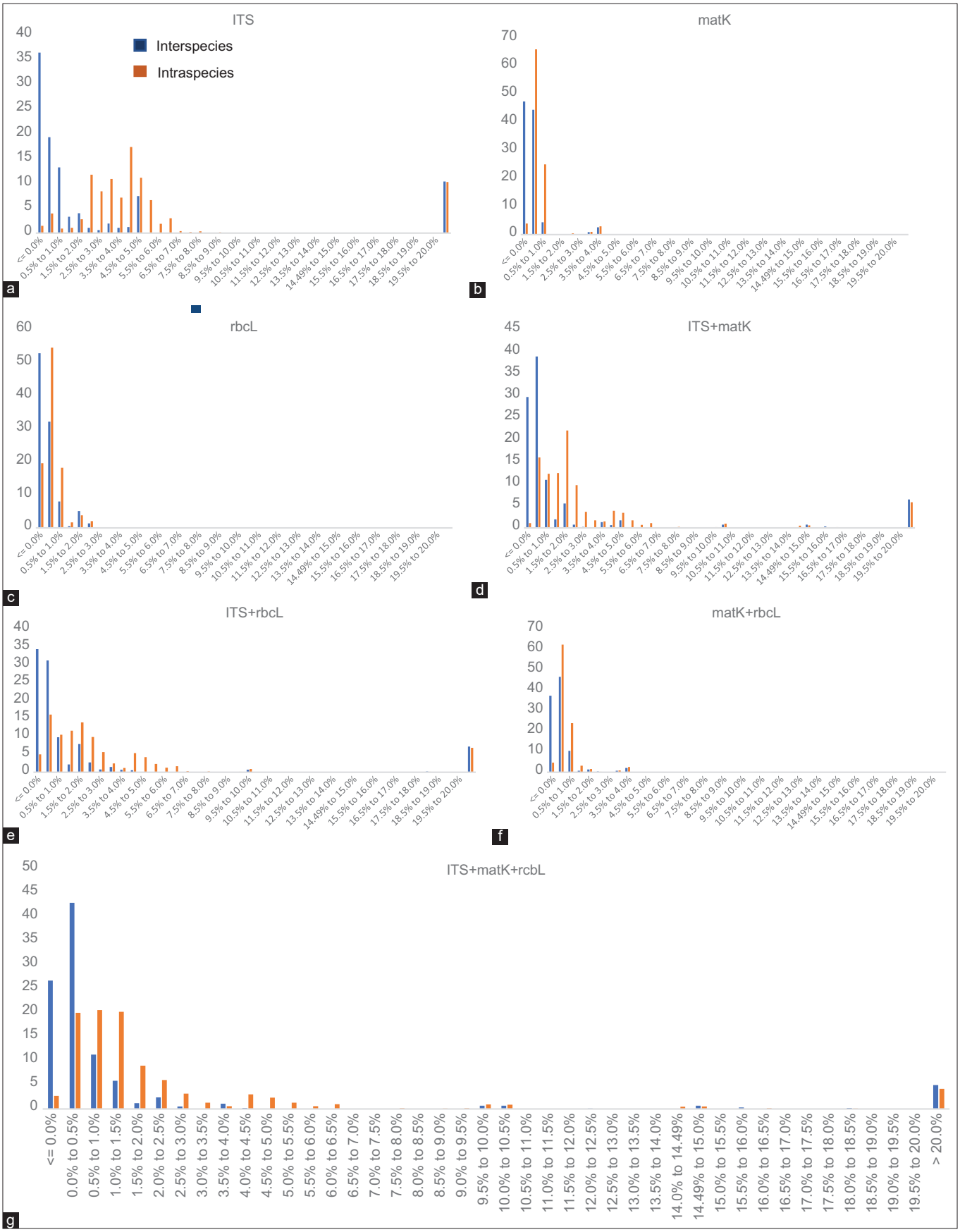

Figure 4: (a-g) Relative distribution of intraspecific and interspecific Kimura 2-parameter distance for three DNA barcodes and its combination in genus *Salacia* of Western Ghats of Karnataka and Kerala.

rates of these coding genes. A very low species delineation was also observed in genus *Nepenthes* concerning *rbcL+matK* barcodes [19].

5. CONCLUSION

The ISSR DNA markers and barcodes (ITS, *matK*, and *rbcL*) are useful tools for the molecular validation of taxonomically described plant organisms. No single DNA marker is sufficient for the proper authentication of *Salacia* species and its associated species. It is essential to identify the most effective marker and its efficiency of species discrimination, for proper molecular identification. This study shows that *Salacia* ISSR marker is useful for the assessment of genetic diversity and that the ITS sequence can also be used for identification in a satisfactory manner.

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7. CONFLICTS OF INTEREST

All the authors declare that there are no conflicts of interest regarding the publication of this paper.

8. FINANCIAL SUPPORT

None.

9. AUTHORS' CONTRIBUTIONS STATEMENT

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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