Establishing taxonomic identity and selecting genetically diverse populations for conservation of threatened plants using molecular markers

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The extent of genetic diversity within a species is an important determinant of successful adaptation to adverse environmental conditions. Assessment of extent of genetic diversity/variability is also important to monitor genetic erosion within a species. In threatened plant species, genetic diversity assessment helps in selection of genetically diverse populations to enrich the genetically impoverished populations, thus minimizing the probability of genetic drift. Confirming taxonomic identity of threatened species, particularly those belonging to species complexes with dispute identity, is another essential task in the conservation of threatened species, which is best resolved through molecular approaches. The present study estimated the genetic variability within and among the populations of four threatened species, viz. *Justicia beddomei* (C.B. Clarke) Bennet (Acanthaceae), *Embelia ribes* Burm. f. (Myrsinaceae), *Madhuca insignis* (Radlk.) H.J. Lam (Sapotaceae) and *Cycas beddomei* Dyer (Cycadaceae) using Inter Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR) markers for selecting the genetically diverse populations. The phylogeny was analysed through ITS (nrDNA) and *mat*K (cpDNA) sequences to confirm the species identity.

The phylogenetic analyses confirmed four distinct species of *Justicia,* which also revealed that *J. beddomei* and *J. adhatoda* were sister groups with a common ancestor showing rapid parallel speciation with *J. gendarussa* in one clade and *J. betonica* in another. *Madhuca insignis* with extremely small population in the Western Ghats (Karnataka to Kerala) might have undergone either extensive hybridization or incipient speciation. In case of *Embelia* species, a greater evolutionary closeness between *E. subcoraceae* and *E. floribunda* was revealed, while *E. ribes* had a distinct clad. Both ISSR and SSR markers distinguished various genotypes of *Cycas beddomei*.

Keywords: Conservation, genetic variability, molecular markers, phylogeny, threatened plants.

Introduction

CORRECT taxonomic identification of threatened species is fundamental to any conservation research and action. This is particularly true for the species complexes where inter-breeding is a frequent phenomenon and phenotypic plasticity is common[1]. Morphological characters may not be sufficiently informative to evaluate cryptic species that are morphologically similar but genetically divergent. In such situations, molecular marker-based phylogeny analyses help in segregating the species despite morphological similarities[2]. Phylogenetic studies have been helpful in estimating the evolutionary relationship, gene flow, genetic drift and degree of out-breeding in different taxa. DNA sequence-based character and distance analysis of various loci in closely related species reflect the fundamental relationships and genetic divergence, thus helping to segregate them into different species.

One of the most important intrinsic drivers of species extinction is genetic drift caused due to narrow genetic base within the population[3]. Given small population size of threatened species, the extinction probability due to genetic drift is very high. Therefore, prior to any conservation action, genetically diverse populations need to be identified, multiplied and introduced in nature for avoiding possible genetic drift.

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Genetic variations do play a crucial role in species adaptation and the subsequent evolutionary processes. Thus, patterns of genetic diversity within and among the populations of a species give an insight into the evolutionary history, and help predict the future risk of genetic erosion[4]. The genetic diversity of a plant species changes in time and space, and its distribution depends on its evolutionary history, breeding system, and several ecological, geographical and anthropogenic factors. A better understanding of diversity and its distribution within and among the populations is essential for identifying the population with wide genetic base that can be used as a source for enriching the genetically impoverished populations[5]. Assessment of such variation is particularly important in threatened species as the genetically diverse population can be used for expanding the extant population of a species, thus minimizing the probability of genetic drift thereby ensuring its conservation.

Utilization of nuclear as well as chloroplast markers for phylogeny such as Internal Transcribed Spacer (ITS), *rbc*L, *trn*L–*psb*H, *trn*L-F, *mat*K has been successful in establishing phylogenetic relationship among different taxa[2]. For genetic diversity analysis, simple sequence repeats (SSRs), intersimple sequence repeats (ISSRs), Single Nucleotide Polymorphism (SNP) have been proved to be extremely useful in several species. Therefore, analyses for population genetic structure taking the above two approaches can address the twin problems in conservation biology, i.e. establishing taxonomic identity of disputed taxa, as well as identification of genetically diverse populations for using them as source germplasm for species conservation.

In this article, we demonstrate the successful application of molecular marker tool in resolving the taxonomic dispute for establishing the species identity. Further, we hypothesized that appropriate species-specific molecular markers can delineate populations with greater genetic diversity. Both these aspects are essential for conserving the threatened species because of their small population size, narrow niche, high phenotypic plasticity and limited gene flow. Four threatened species were selected, viz. *Justicia beddomei* (C.B. Clarke) Bennet, *Embeli aribes* Burm f., *Madhuca insignis* (Radlk.) H.J. Lam and *Cycas beddomei* Dyer; each of them faces one or both the above-mentioned specific conservation problem.

Materials and methods

Justicia beddomei

The leaves of *J. bedommei* are used in preparation of Ayurvedic medicines for cold, cough, bronchitis, asthma and also used as antihelminthic[6]. The species is closely related to *Justicia adhatoda* (syn. *Adhatoda vasica* or *A. zeylanica*), commonly called as 'Vasa'; therefore, *J. beddomei* is often misidentified as *J. adhatoda*. Leaf and inflorescence size are considered as the distinguishing characters of these two species. *J. adhatoda* has bigger leaf and inflorescence while *J. beddomei* has relatively smaller leaf and inflorescence. However, this difference in morphology is considered as an ecotypic variation (http://envis.frlht.org/tds/1255_1.htm). Taxonomic characters of *J. adhatoda*, *J. beddomei* and two of the allied species, viz. *J. gendarussa* and *J. bettonica* are almost similar (Figure 1). It is, therefore, difficult to distinguish *J. beddomei* from the rest of the species based on phenotypic attributes. Thus, correct identification based on molecular phylogeny is an important aspect of the present study. Nuclear ribosomal ITS region was selected for molecular phylogeny. In addition to the sequence variability, PCR-RFLP (restriction fragment length polymorphism) of ITS amplicon was also used.

Madhuca insignis

M. insignis is a riparian species that is classified as 'Extinct' by the IUCN. It was rediscovered after a gap of 120 years from the Udupi district, Karnataka with only two surviving individuals[7], followed by other reports of its existence in Dakshina Kannada district, Karnataka and the Kasaragod district, Kerala, India[8–12]. In the present study, five species of the genus *Madhuca*, viz. *M. insignis*, *M. neriifolia*, *M. latifolia*, *M. longofolia* and *M. berdollimi*, were analysed using ITS sequences for establishing species identity.

Embelia ribes

E. ribes is a medicinally important threatened plant species. However, other species of the genus are often

Figure 1. Photographs showing leaf and inflorescence of: ***a***, *Justicia adhatoda*; ***b***, *Justica beddomei*; ***c***, *Justica betonica*; ***d***, *Justica gendarussa*.

Table 1. *Madhuca* species and out-group with their location and GeneBank accession numbers

Species	Geographical location	Accession number
Madhuca insignis	Karnataka, India	KT958785, KT958786, KX545262, KT958784, KT958783, KT958778, KT958779, KT958780, KT958781, KX545264, KT958777, KX545263, KT958782, KT958776, KT958775, KX545261, KX545260, KX545259, KX545258
M. insignis	Kerala, India	KX545265, KX545266
M. neerifolia	Karnataka, India	KX545255
M. longifolia	Karnataka, India	KX545254
M. latifolia	Karnataka, India	KX545256
M. berdilloni	Karnataka, India	KX545257
M. microphylla	Sri Lanka	KF686226
M. korthalsii	Malaysia, Sarawak	KF686218
M. malaccensis	Malaysia, Peninsular	KF686225
M. laurifolia	Malaysia, Peninsular	KF686222
M. fulva	Sri Lanka	KF686214
M. oblongifolia	Malaysia, Sarawak	KF686229
M. palembanica	Indonesia, Sumatra	KF686231
M. sericea	Malaysia, Sarawak	KF686235
M. sarawakensis	Malaysia, Sarawak	KF686234
M. elmeri	Indonesia, Kalmantan	KF686212
M. leucodermis	Papua New Guinea	KF686223
M. pallida	Malaysia, Sarawak	KF686232
M. curtisii	Malaysia, Peninsular	KF686211
M. lancifolia	Malaysia, Peninsular	KF686221
M. erythrophylla	Malaysia, Sarawak	KF686213
M. prolixa	Malaysia, Sarawak	KF686233
M. kunstleri	Malaysia, Sarawak	KF686220
M. kingiana	Singapore	KF686217
M. motleyana	Malaysia, Peninsular	KF686228
M. barbata	Malaysia, Sarawak	KF686207
M. kuchingensis	Malaysia, Sarawak	KF686219
M. hainanensis	China	KF686215
M. crassipes	Malaysia, Sarawak	KF686208
M. utilis	Malaysia, Peninsular	KF686236
Manilkara zapota	Trinidad, South America	KF686242

indistinguishable due to extensive morphological variation[13]. It grows in semi-evergreen and deciduous forests occurring in the elevation range of 500 to 2500 m amsl in the Central Himalaya, Arunachal Pradesh, Assam, Meghalaya, Maharashtra, Andhra Pradesh, Karnataka, Kerala and Tamil Nadu[14,15]. It shows anti-fertility[16], antibacterial[17], antitumour, analgesic, anti-inflammatory[18,19], anti-spermatogenic[20]and chemo preventive activities[21]. *E. ribes* has close similarities with *Embelia tsjeriam-cottam*, *Embelia floribunda* Wall. and *Embelia subcoraceae* (Clarke) Mez. and poses a serious challenge in the identification of species based on morphological attributes. The genus has undergone significant genetic erosion and is at the threshhold of extinction because of low seed viability, poor seed germination and fragmentation in populations which have resulted in inbreeding in natural populations.

Cycas beddomei

C. beddomei, a gymnosperm is known only from the Kadapa–Tirupati Hills of the southeastern Ghats in Andhra Pradesh, northwest of Chennai in Eastern Peninsular India and some parts of China. It grows on open hill slopes and grassy woodland. In the present study, assessment of genetic diversity in *C. beddomei* was undertaken using ISSR and SSRs.

Sample collection

Fresh leaf samples of *J. beddomei* along with three other species, viz. *J. adhatoda*, *J. bettonica* and *J. gendarussa* were collected from the herbal garden of Trans-Disciplinary University, Foundation for Revitalisation of Local Health Traditions (TDU, FRLHT), Bengaluru. The leaf samples of five species of *Madhuca* (from 21 sample plants of *M. insignis* and one sample plant each from *M. neriifolia*, *M. latifolia*, *M. longifolia* and *M. berdollimi*) were collected from the field gene bank of TDU (Table 1). Leaves of plants of *E. ribes* from 12 populations, *E. floribunda* from 4 populations and *E. subcoraceae* from 4 populations located at elevation ranging from 1345 to 1912 m amsl in Meghalaya and Arunachal Pradesh (Table 2) were harvested and frozen in liquid nitrogen after thorough washing with tap water and rinsing with distilled

Table 2. Study sites, their geographical coordinates and population structure of *Embelia ribes*, *Embelia subcoraceae* and *Embelia floribunda* at the respective sites

Species	Location	Code	Elevation (m)	No. of individuals present	No. of individuals collected
E. ribes	Bomdila Road	ErBr	1345	1	1
	Lad Mawphlang	ErLM	1732	1	1
	Langkyrdem	ErLk	1536	5	1
	Mawmluh	ErMh	1484	4	1
	Mawsynram	ErMs	1602	5	1
	Pynursla	ErP	1584	3	1
	Saibakon	ErSk	1584	1	1
	Sohra rim	ErSR	1491	1	1
	Swer	ErS	1825	2	1
	Umdengpoh	ErUd	1554	2	1
	Upper Shillong	ErUS	1912	2	1
E. subcuraceae	Jarain	EsJ	1137	5	3
	Lad Mawphlang	EsLM	1732	5	1
	Mawmluh	EsMh	1484	10	6
E. floribunda	Bomdila Road	EfBR	1345	1	1
	Lad Mawphlang	EfLM	1732	1	1
	Laitryngew	EfLr	1491	1	1
	Mawkajem	EfMk	1567	1	1

Bomdila Road site is in Arunachal Pradesh and all other sites are in Meghalaya.

Table 3. Sampling details of *Cycas beddomei* in the Eastern Ghats

Code no.	Region	Altitude (m)	Sample size
Accession (1–13)	Talakona	811	13
Accession (14–19)	Tirumala hills	875	06
Accession (20)	Nellore	872	01

water. The leaf samples of *C. beddomei* representing three natural populations from reserved forest of Andhra Pradesh (Table 3) were collected during September 2014 to February 2015.

Genomic DNA isolation and PCR for ITS, matK, ISSR and SSR

Genomic DNA was extracted from young leaves using the *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide (CTAB) method described by Doyle and Doyle[22] with some modifications to each species. The purity and yield of DNA was checked using a UV spectrophotometer (GeneQuant Pro, Amersham Biosciences, USA).

PCR amplification of ITS and *mat*K regions from DNA of each sample was carried out with primer pairs P4–P5 (refs 23 and 24) and AF–8R (ref. 25) respectively. The reaction mixture for amplification of the target regions was optimized with 10× reaction buffer (Merck, India), 1 mM $MgCl_2$ (Merck, India), 0.4 mM dNTPs mixture (Merck, India), 0.03U *Taq* polymerase (Merck, India), 0.5 pmol primers (Metabion GmbH, Germany) and 50 ng of template DNA. Amplification reactions were carried out in an Applied Biosystems thermal cycler (Gene Amp 9700) with the reaction cycle comprising one cycle of 'hot start' (94°C, 5 min), 35 cycles of denaturation (94°C, 1 min), annealing (60°C for ITS and 50°C for *mat*K), extension (72°C, 1 min) and one cycle of chain elongation at 72°C, for 10 min. Polymerase chain reactions for ISSR amplification were carried out with genomic DNA of *E. ribes*, *E. floribunda* and *E. subcoraceae* in a final reaction volume of 25 µl containing 30 ng of DNA, 5U of *Taq* polymerase (Promega), 2 µl of 10× reaction buffer (Promega), 1.5 mM $MgCl_2$ (Promega), 0.2 mM of each dNTP (Promega) and 10 µM primer. A total of 20 ISSR primers were screened for *Embelia* spp. (Table 4). Amplification reactions were performed in an Applied Biosystem thermal cycler with the reaction cycle comprising one cycle of 'hot start' (94°C, 5 min), 40 cycles of denaturation (94°C, 30 s), annealing for 30 s at the respective temperatures, chain extension at 72°C for 30 s and one cycle of chain elongation at 72°C for 5 min. In case of SSR, the primers were synthesized commercially and used for PCR reaction as reported earlier for *Cycas* species[26,27]. The basic PCR programme to amplify DNA was as follows: an initial hot start and denaturing step at 94°C for 3 min followed by 40 cycles of 1 min denaturation at 94°C, 1 min annealing at the appropriate temperature (42°C or 62°C) depending on the primer; and 1 min primer elongation at 72°C. A final extension step at 72°C for 5 min was performed. Table 5 provides details of ISSR and SSR primers used for *Cycas* spp.

Agarose gel elution and sequencing

The amplicons of ITS region and *mat*K were resolved on a 0.8% agarose gel, which was pre-stained with ethidium

Table 4. List of primers and their nucleotide sequences used for amplification of DNA from different individuals of *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* from Meghalaya and Arunachal Pradesh

Primer	Sequence (5′–3′)	Reference	Annealing temperature (°C)
P4	TCC TCC GCT TAT TGA TAT GC	23	60
P5	GGA AGT AAA AGT CGT AAC AAG G	24	
AF	CTA TAT CCA CTT ATC TTT CAG GAG T	25	50
8R	AAA GTT CTA GCA CAA GAA AGT CGA		
ISSR809	$(AG)_8G$	UBC Primer	51
ISSR810	$(GA)_8T$	UBC Primer	48
ISSR819	$(GT)_8A$	UBC Primer	50
ISSR826	$(AC)_8C$	UBC Primer	50
ISSR834	$(AG)_8YT$	UBC Primer	52
ISSR842	$(GA)_8YG$	UBC Primer	51
ISSR857	$(AC)_8YG$	UBC Primer	60
ISSR859	$(TG)_8RC$	UBC Primer	50
ISSR801	(AT)8T	UBC Primer	45
ISSR807	(AG)8T	UBC Primer	47
ISSR811	(GA)8C	UBC Primer	50
ISSR815	(CT)8G	UBC Primer	46.8
ISSR824	(TC)8G	UBC Primer	48.5
ISSR825	(AC)8T	UBC Primer	51.4
ISSR861	(ACC)6	UBC Primer	60.6
ISSR864	(ATG)6	UBC Primer	47
ISSR868	(GAA)6	UBC Primer	45
ISSR872	GAT AGA TAG ATA GAT A	UBC Primer	47
ISSR882	VBV ATATATATATATAT	UBC Primer	45

bromide (0.5 µg/ml). The gel was documented in a gel documentation unit (Bio-rad, USA). Each experiment was repeated at least three times for each leaf sample to confirm its reproducibility and repeatability. Amplified ITS region and *mat*K were gel-purified using QIAquick gel extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The purified products of ITS and *mat*K were utilized in sequencing on a ABI 373 automatic sequencer (Applied Biosystems, Inc.) at Bangalore Genei Pvt Ltd, Bengaluru for *Justicia* species and Bioserve Pvt Ltd, Hyderabad for *Madhuca* and *Embelia* species.

PCR-RFLP of ITS region

The purified PCR products of ITS for four species of *Justicia* were subjected to restriction digestion with *Sfo*I and *Eco*RI (New England Biolab, UK) at 37°C for 1 h following the manufacturers' instructions. The digested PCR products were then resolved on 2% agarose, 0.5× TBE gel prestained with ethidium bromide (0.5 µg/ml) along with undigested amplicons.

ISSR/SSR profiling

The PCR amplicons of ISSRs and SSRs for *Embelia* and *Cycas* were electrophoresed on 3.5% agarose gel and the amplicons were visualized under UV light in ChemiDoc XRS + system with Quantity One® 1-D analysis software version 4.6.9 (Bio-rad, USA) after staining the gels with ethidium bromide. All amplifications were carried out in triplicate with five samples from each accession and only well-defined and reproducible bands were scored. Bands with the same migration were considered homologous fragments, independent of their intensity.

Analysis of sequencing data for ITS and matK

The obtained DNA sequences were subjected to homology analysis using CLUSTALW (http://www.ebci.ac.uk.clustalw/) with default parameters. Phylogenetic tree of ITS region was constructed following the maximum likelihood (ML) method using MEGA 6.0 (ref. 28) and Bayesian analysis using MrBayes v3.12 software[29]. Best-fit-model was determined using ML calculations in MEGA5.2. For the analysis, Kimura 2-parameter (K2)[30] and 1000 bootstrap replicates were used. Multiple alignment of ITS region of four species (*J. beddomei*, *J. adhatoda*, *J. gendarussa* and *J. betonica*) was performed. The homology score for ITS1, 5.8S and ITS2 was manually computed. All the ITS and *mat*K sequences generated have been deposited in the NCBI GenBank database. UPGMA (unweighted pair group mean analysis) was used to generate the dendrogram based on Nei's genetic distance values[31] for *Madhuca* species. Also the genetic distances between various individuals were calculated using pairwise deletion. In order to verify the length of the

Table 5. Total number of amplified fragments and the number of polymorphic fragments generated by PCR using selected SSR and ISSR markers in *Cycas*

Primer code	Sequence (5′----------3′)	Annealing temperature (°C)	Total no. of bands	No. of polymorphic bands	Polymorphism (%)
CycasSSR1	F:TGCTAAAGTGAACGACGAA; R:CATACTCCTTATCCCACGAAT	55–53	04	04	100
CycasSSR2	F:GTACCCCAGTCATTTGAGAC; R:AGCCAGCAGTAACCATTGCC	56–52	04	04	100
CycasSSR3	F:CAAAACTATGCTGTCAATCC; R:TTAGCATCACCAGTAATCCC	52–49	06	06	100
CycasSSR4	F:ACAGGGCATCGGAACACTAC; R:CTACTCTTCGGCTTCCAACG	55–53	05	04	80.0
CycasSSR5	F:TCTTGCTTACCCGTTTGCTT; R:CTCCTCGACGTTCAATCACA	54–52	05	05	100
CycasSSR6	F:TGCCCATTGATTTTGGTTTT; R:AAATTTGCTGATTCGGCTTC	53–51	05	05	100
CycasSSR7	F:ATTGCGGAACGAATATCGAC; R:TATCGCGAGGCCATAGGTAG	56–53	04	04	100
CycasSSR8	F:TCACAATGCCTTCCAGATCA; R:TGTGAAGGAAGTTGGCTGTG	53–51	04	03	75.0
CycasSSR9	F:ATGAACAAGCGGCTGAGTCT; R:CCCACCCTCTTTCTCTCTCC	57–55	05	03	60.0
CycasSSR10	F:CAGAGACTATTCGGGCCAAG; R:TCAAACCCTTCCACACATCA	55–53	06	05	83.3
CycasSSR11	F:CTCACCCATTTCTTAGTT; R:ATCATAGGGAAAGACCAT	51–49	04	04	100
CycasSSR12	F:CTTACGCATACGCCACTTC; R:GGACACAACCCCAATCAAA	57–55	05	05	100
CycasSSR13	F:CCTCCCAATCCTTCACAAA; R:CACCCACAGACAGACCAAT	57–55	03	03	100
CycasSSR14	F:ATGATTCCCATCCAAGC; R:TTCCACCCACCACCTATT	53–50	04	03	75.0
CycasSSR15	F:CACCCATTTCTTAGTTAAT R:GAATCATAGGGAAAGACCA	53–49	05	05	100
USB 807	(AG)8T	42.5	05	05	100.0
USB 808	(AG)8C	46.8	10	10	100.0
USB 810	(GA)8T	42.9	20	19	95.0
USB 811	(GA)8C	43.3	09	07	77.8
USB 815	(CT)8G	45.0	07	06	85.7
USB 835	(AG)8TC	42.7	10	09	90.0
USB 838	(GA)8AT	42.9	07	06	85.7
USB 839	(GA)8AC	43.3	08	08	100
USB 840	(CT)8T	43.5	06	06	100
USB 841	(AG)8GC	42.7	05	04	80.0
USB 843	(GA)8CT	52.9	07	07	100
USB 844	(GA)8AG	50.0	13	13	100
AM 2	(AAG)5GC	45.8	11	10	90.9
AM 4	(AAG)5CC	45.8	08	08	100
AM 6	(AGC)5GG	62.3	06	05	83.3
UBC 889	(AGT)2CGT(AC)7	56.6	10	08	80.0
UBC 818	(CA)9G	51.0	11	11	100
UBC 825	(AC)8T	51.4	08	07	87.5
UBC 827	(AC)8G	53.0	05	05	100
UBC 855	(AC)8CTT	54.6	08	08	100
UBC 813	(CT)8T	45.7	06	04	66.7
UBC 864	(ATG)6	43.6	13	13	100.0

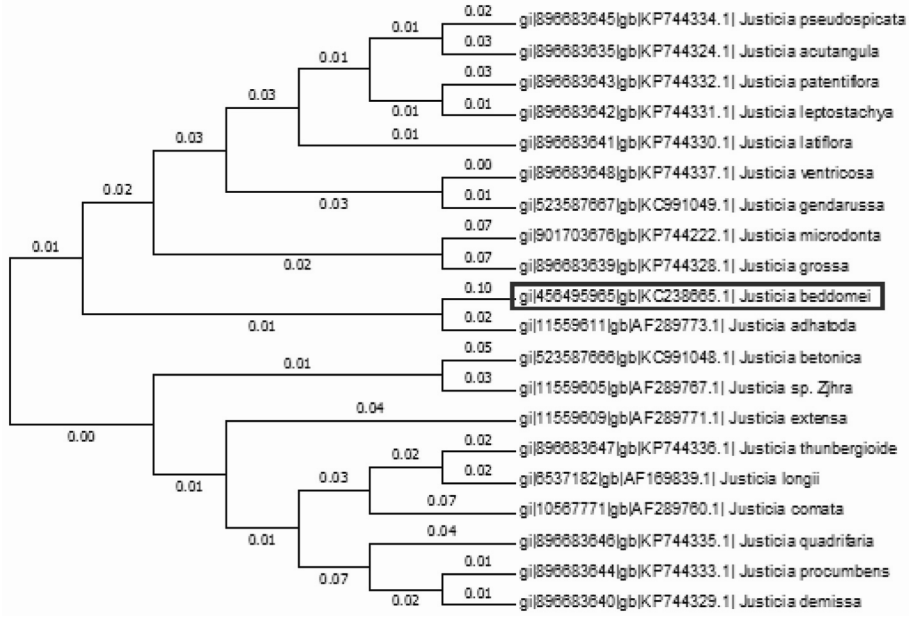

Figure 2. The Maximum Likelihood tree obtained by the phylogenetic analysis based on internal transcribed spacer sequences.

obtained tree branches, Neighbour–Joining (NJ) analysis of the ITS sequences was performed using MEGA 5.0; bootstrapping was carried out to obtain estimates of the nodes of the trees selected.

Data analysis for ISSR/SSR profiling

The ISSR and SSR profiles were scored for presence (1) or absence (0) of a band. The data matrix was converted into genetic similarity matrix using Jaccard coefficient with NTSYS-PC2.02j. The genetic relatedness amongst individuals in the tested populations was analysed using UPGMA based on Jaccard's coefficient. Computations were made using the program NTSYS pc 2.2 (Exeter Software, Stauket, NY, USA)[32]. The relationship between genetic similarity matrix of ISSR and SSR was analysed using the Mantel test[33]. Principal component analysis (PCA) of the data matrix was carried out with PAST ver. 1.89 (ref. 34).

Results and discussion

Resolving taxonomic dispute for Justicia beddomei

Results from the ITS sequence analysis and PCR-RFLP revealed clear distinction between *J. beddomei* and *J. adhatoda*. Direct sequencing of the gel-purified ITS amplicon yielded a 624 bp sequence for *J. beddomei*, 738 bp for *J. gendarussa*, and 736 bp sequence for *J. betonica*. Complete ITS sequence (687 bp) of *J. adhatoda* was used from NCBI. Maximum homology in the sequence was observed in the highly conserved region of 5.8S rRNA gene. Both the regions of ITS (ITS1 and ITS2) of the selected *Justicia* members showed adequate sequence variation among the species; however ITS2 region of *J. gendarussa* did not show any similarity with the other three species. Phylogenetic tree was constructed using the ITS sequences from NCBI database of 20 different species under the genus *Justicia*, which revealed two major clades. From the phylogenetic tree, it was evident that *J. beddomei* and *J. adhatoda* were sister groups and two distinct species with a common ancestor (Figure 2). In general, *Justicia* species were found to have rapid parallel speciation with *J. gendarussa* in one clade and *J. betonica* in another. The ITS sequences of *J. beddomei*, *J. adhatoda*, *J. gendarussa* and *J. betonica* contained unique recognition sites for specific restriction enzymes for which all the species were distinct in their PCR-RFLP patterns. When the ITS amplicons of the four selected *Justicia* species were subjected to restricted digestion with *Eco*RI or *Sfo*I, it yielded the expected restricted products (Table 6). Based on earlier reports, it is evident that the variation in *J. adhatoda* is very high along with a phenotypic plasticity[35,36]. However, *J. beddomei* seems to be more uniform than *J. adhatoda*. The ITS sequences and PCR-RFLP were successful in resolving the ambiguity that existed among the four species of *Justicia* in the present study.

Confirming taxonomic identity of Madhuca insignis

ITS sequences of all the five different species of *Madhuca* and various ITS sequences obtained from GenBank

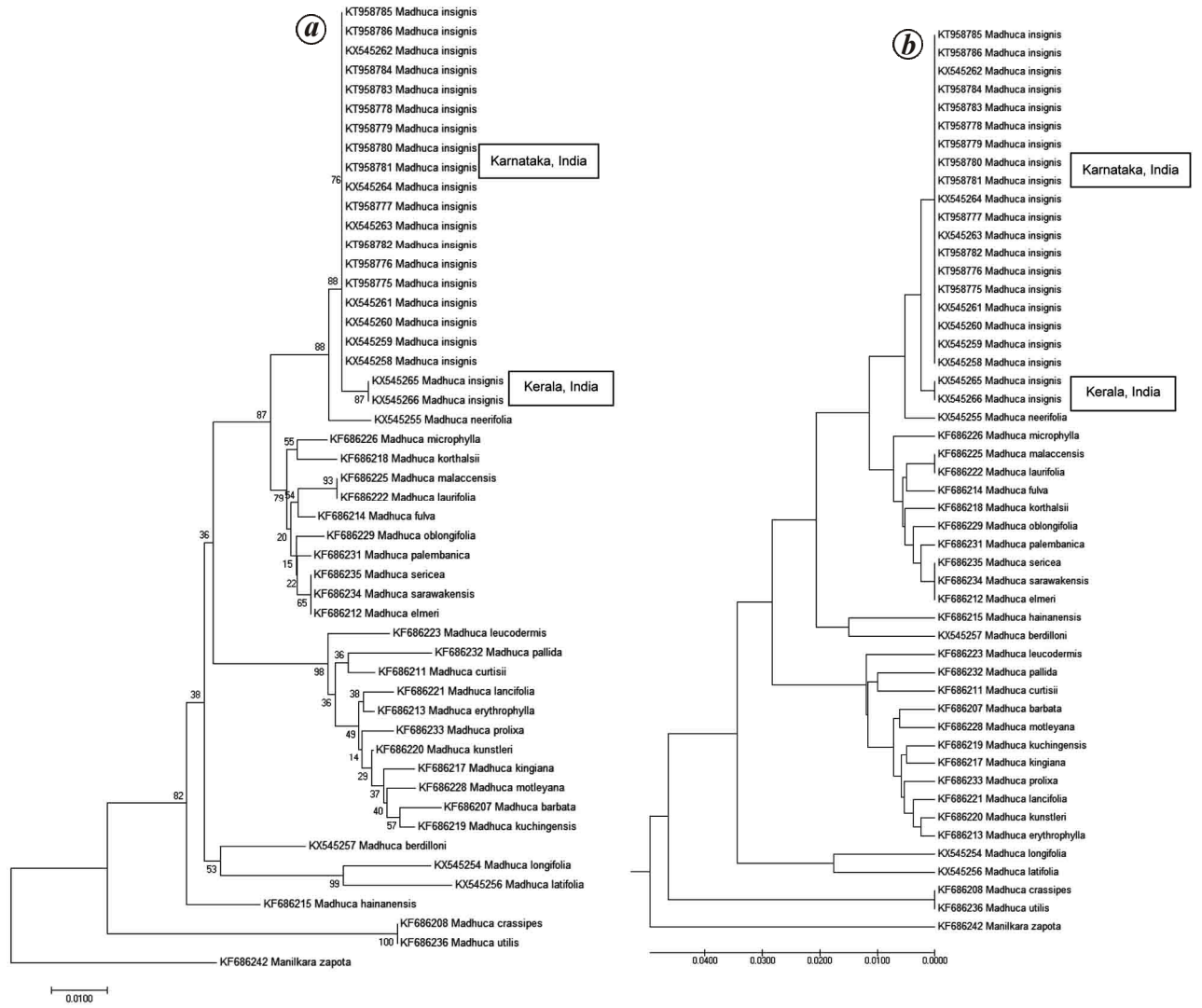

Figure 3. Phylogenetic tree of *Madhuca* species: ***a***, Neighbour–Joining analysis using Kimura 2-parameter method. ***b***, UPGMA analysis using Kimura 2-parameter method. *Manilkara zapota* has been used as outgroup.

Table 6. Digestion pattern showing the restriction sites for *Eco*R1 and *Sfo*1 in *Justicia beddomei*, *Justicia adhatoda*, *Justicia gendarussa*, and *Justicia betonica*

Plant	*Eco*R1 (G/AATTC)	*Sfo*1 (GGC/GCC)
J. beddomei (624 bp)	–	566
J. adhatoda (687 bp)	–	544, 606
J. gendarussa (738 bp)	590/594	–
J. betonica (736 bp)	–	565, 627

for several additional *Madhuca* taxa were analysed using MEGA 5.0 for distance-based UPGMA and NJ analysis, which resulted into two well-resolved major clades (Figure 3 *a* and *b*). The first major clade comprised of *Madhuca* species from Malaysia and the second major clade included rest of the *Madhuca* species in a number of sister clades. However, it was noticed that the sections of Indian species like *M. insignis* and *M. neriifolia* clustered together as sister clades, whereas in case of the other subclades, *Madhuca* species from Malaysia, Indonesia, Sri Lanka, China and Papua New Guinea clustered together. This revealed that *Madhuca* species have a Pan-Asia Pacific distribution. All the accessions of *M. insignis* clustered together in a distinct clade from the other *Madhuca* species. The grouping of *M. insignis* with *M. neriifolia*, a closely related species suggests their common origin. This can also be adjudged from the similarity in their morphological traits, viz. young leaf shape, fruit and seed shape, and extremely sweet tasting edible fruit and flowers. The lowest genetic distance (1%) was found between *M. insignis* and *M. neriifolia*, whereas corresponding highest genetic difference (6.4%) was seen between *M. insignis* and *M. latifolia.* The present study suggests that the species within the genus *Madhuca*, particularly *M. insignis* might be undergoing either extensive hybridization

Table 7. Comparative nucleotide sequence analyses of ITS and *mat*K using population of *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* from Meghalaya and Arunachal Pradesh. Figures in parenthesis represent values in percentage

		E. ribes		*E. subcoraceae*	
Region	Parameters	Sohra region (East Khasi Hills, Meghalaya)	Pynursla region (East Khasi Hills, Meghalaya)	Mawmluh (East Khasi Hills, Meghalaya)	Jarain (Jaintia Hills, Meghalaya)
ITS	Length range (bp)	646–669	659–673	648–668	648–673
	No. of conserved sites	629 (93.3)	612 (90.8)	613 (91.6)	625 (92.9)
	No. of variable sites	34 (5.0)	59 (8.8)	52 (7.8)	35 (5.2)
	Sequence divergence (%)	2.74	4.26	4.19	3.42
	Transition/transversion (R)	0.73	0.66	0.51	0.51
*mat*K	Length range (bp)	863–863	863–863	863–863	863–863
	Aligned length (mean) (bp)	863	863	863	863
	No. of conserved sites	804 (93.2)	803 (93.0)	842 (97.6)	857 (99.3)
	No. of variable sites	59 (6.8)	60 (7.0)	21 (2.4)	6 (0.7)
	No. of informatives	4 (0.5)	6 (0.9)	5 (0.6)	0
	Sequence divergence (%)	3.66	3.66	0.98	0.00
	Transition/transversion (R)	0.83	0.84	0.26	0.20

Table 8. Nucleotide sequence analyses with respect to ITS region of *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* from populations located in Meghalaya and Arunachal Pradesh

					Sequence statistics				Nucleotide pair frequencies			
Region	Taxon	Indels	Sequence divergence (%)	G + C (%)	CS (%)	VS (%)	PIS (%)	SS (%)	ii	si	sv	R (si/sv)
ITS	*E. ribes* + *E. subcuraceae* + *E. floribunda*	15	13.59	56.43	67.28	32.71	31.01	1.69	563	38.0	47.0	0.8
	E. ribes	1	3.65	57.223	89.47	10.52	4.95	5.57	622	10	13	0.76
	E. subcoraceae	0	4.15	56.34	89.50	10.49	7.87	2.62	621	10	17	0.59
	E. floribunda	2	3.90	55.46	93.82	6.17	3.82	2.35	652	14.0	12.0	1.18
ITS1	*E. ribes* + *E. subcoraceae* + *E. floribunda*	12	11.67	57.25	56.17	40.07	34.08	5.61	203	14.0	16.0	0.91
	E. ribes	1	2.55	57.86	90.13	9.86	3.13	6.72	217	2.0	3.0	0.64
	E. subcoraceae	0	3.50	57.53	90.58	9.41	7.62	1.79	215	3.0	5.0	0.64
	E. floribunda	2	5.59	57.80	90.98	9.01	3.52	5.49	239	9.0	5.0	1.74
5.8S	*E. ribes* + *E. subcoraceae* + *E. floribunda*	0	0.00	52.69	100	0	0	0	167	0.0	0.0	0.0
	E. ribes	0	0	52.69	100	0	0	0	167	0.0	0.0	0.0
	E. subcoraceae	0	0	52.69	100	0	0	0	167	0.0	0.0	0.0
	E. floribunda	0	0.00	52.69	100	0	0	0	167	0.00	1.00	0.50
ITS2	*E. ribes* + *E. subcoraceae* + *E. floribunda*	1	23.13	53.135	46.89	53.10	51.93	1.16	198	25.00	34.00	0.75
	E. ribes	1	7.02	59.52	81.78	18.21	9.68	8.52	239	8.00	10.0	0.79
	E. subcoraceae	0	7.39	57.67	81.78	18.21	13.17	5.03	239	7.00	12.0	0.57
	E. floribunda	0	4.78	54.942	92.63	7.36	6.58	0.77	246	5.00	7.00	0.76

CS% Conserved sites; VS%, Variable sites; PIS%, Parsimony informative sites; SS% Singleton sites. ii, Identical pairs; si, transitional pairs; sv, transversional pairs.

or incipient speciation. In this case it can be more related to the process of incipient speciation, as the individuals of *M. insignis* have limited range of distribution with scattered or fragmented populations of very small sample size (mostly one or two), that occur in isolated patches throughout the Western Ghats (Karnataka to Kerala). It can be hypothesized that *M. insignis* species may be remnants of a former widespread and continuous population

in the whole of the Western Ghats, that may have been disturbed by various anthropogenic activities such as human settlements, clearance of forest lands for agriculture, sand mining, flooding, shortage of rainfall due to climate change and other natural calamities.

Inter- and intra-specific variations in the genus Embelia

The ITS region of different species of *Embelia* was sequenced in the present study and sequence variations in the ITS region were analysed to assess inter- and intra-specific relationship among *E. ribes*, *E. subcoraceae* and *E. floribunda.* The ITS region of *E. ribes* showed a highly conserved nature with 89.5% conserved sites (Table 7). More specifically, ITS1 and ITS2 showed 90.1% and 81.8% conserved sites respectively. The multiple sequence alignment of the complete ITS region from *E. ribes*, *E. subcoraceae* and *E. floribunda* revealed 67.3% conserved sites. In inter-specific level, ITS1 showed higher level of sequence conservation than ITS2. The 5.8S region did not show any inter-specific sequence variation (Table 8). The distance based NJ tree generated from the multiple sequence alignment of ITS region resolved various accessions of *E. ribes*, *E. floribunda* and *E. subcoraceae* into two distinct clades. The accessions of *E. ribes* clustered together in a single clade (cluster II) in the tree, while accessions of *E. floribunda* and *E. subcoraceae* segregated into two sub-clades within a clade (cluster I) (Figure 4). The clustering pattern in the NJ tree showed close evolutionary relationship between *E. floribunda* and *E. subcoraceae.* The *mat*K region of all the three species of *Embelia* studied in the present investigation showed a very high level of conservation with 92.4%, 96.8% and 99.8% conserved sites in *E. ribes*, *E. subcoraceae* and *E. floribunda* respectively (Table 9). The NJ tree constructed using *mat*K sequences clearly segregated all the accessions of *E. ribes*, *E. floribunda* and *E. subcoraceae* in three clusters. Cluster I grouped together accession of *E. subcoraceae* and *E. floribunda*, whereas cluster II grouped together all the accessions of *E. ribes* (Figure 5). Thus, the NJ tree generated for ITS and *mat*K sequences clearly differentiated three species of *Embelia* from one another. In addition, it also revealed a greater evolutionary closeness between *E. subcoraceae* and *E. floribunda*. However, the sequence variations in the ITS and *mat*K regions appeared to be less informative in determining intra-specific variations among the three species. Nevertheless, the ITS region showed a higher resolution than *mat*K.

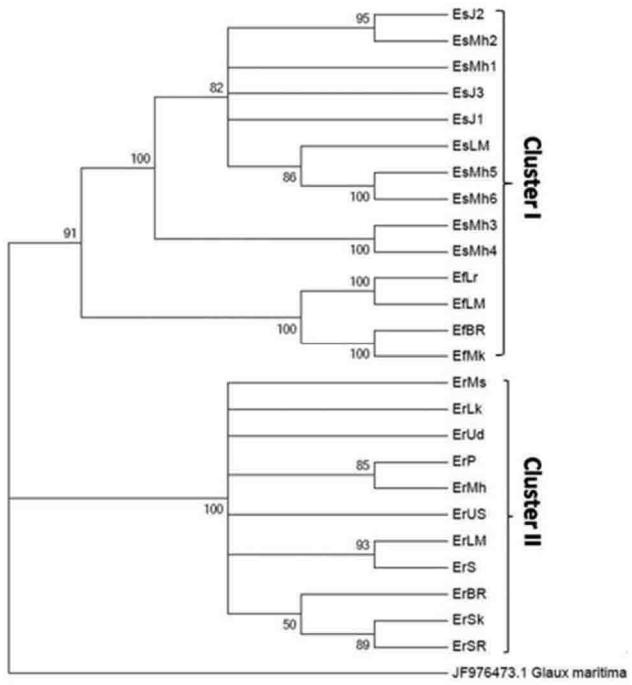

Figure 4. Neighbour–Joining phylogenetic tree constructed using Kimura 2-parameter method on MEGA6 sequences representing nuclear ITS region of *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* from Meghalaya and Arunachal Pradesh. Numbers at each node represent boot-strap values. Tree is scaled to the number of nucleotide substitutions per site. The tree is rooted with *Glaux maritima.*

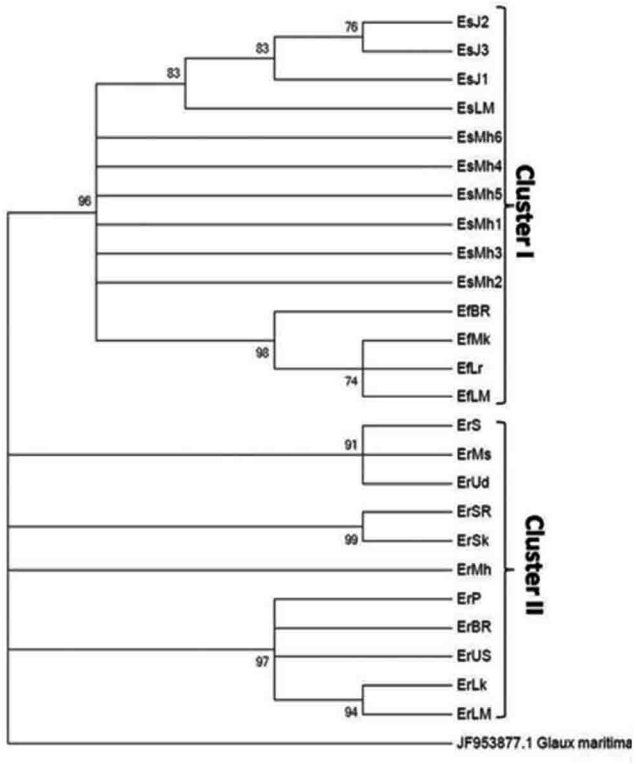

Figure 5. Neighbour–Joining phylogenetic tree constructed using Kimura 2-parameter method on MEGA6 of nucleotide sequences of *mat*K gene of *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* from Meghalaya and Arunachal Pradesh. Numbers at each node represent bootstrap values. Tree is scaled to the number of nucleotide substitutions per site. The tree is rooted with *Glaux maritima.*

Table 9. Nucleotide sequence analysis with respect to the *mat*K region of *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* from populations located in Meghalaya and Arunachal Pradesh

*mat*K	Taxon	Indels	Sequence divergence (%)	CS (%)	VS (%)	PIS (%)	SS (%)	ii	si	sv	R (si/sv)
	E. ribes + *E. subcoraceae* + *E. floribunda*	0	4.09	86.67	13.32	11.23	2.08	828	15.00	21.0	0.70
	E. ribes	0	2.56	92.35	7.64	5.90	1.73	841	9.00	13.0	0.75
	E. subcoraceae	0	1.22	96.75	3.24	1.96	1.27	852	3.00	7.0	0.43
	E. floribunda	0	0.11	99.76	0.23	0.00	0.23	563	38.00	47.0	0.80

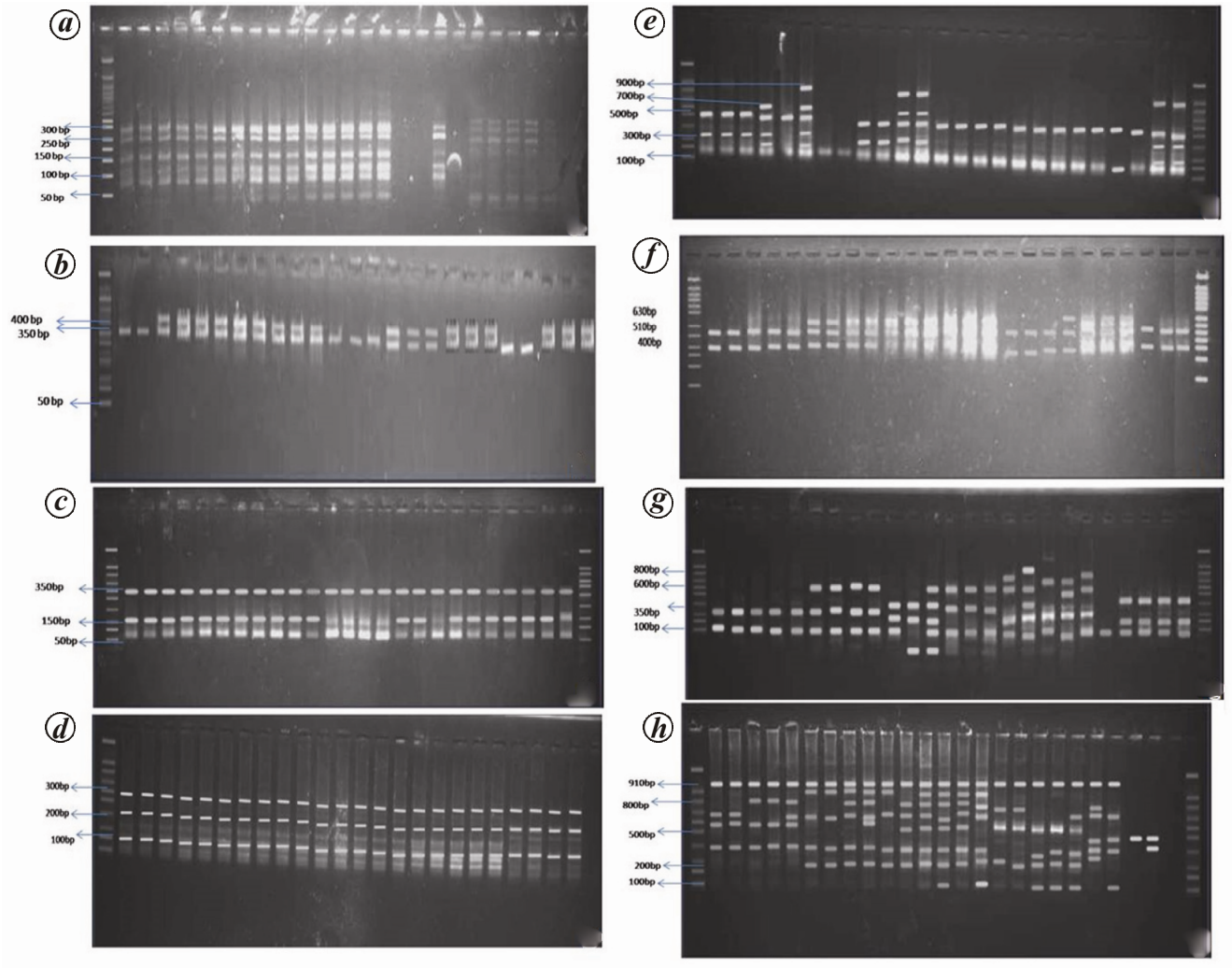

Figure 6. Agarose gel electrophoresis profile of ISSR amplification in *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* from Meghalaya and Arunachal Pradesh. Amplification profile with (***a***–***h***) primers: ***a***, ISSR809; ***b***, ISSR810; ***c***, ISSR819; ***d***, ISSR826; ***e***, ISSR834; ***f***, ISSR842; ***g***, ISSR857; ***h***, ISSR859. Ladder M, 50 bp/100 bp represented in left side and 1st well of all gel pictures.

Estimation of genetic variability in Embelia species from North East India

Out of the 20 primers used for amplification of ISSRs, eight yielded robust and reproducible polymorphic amplification patterns (Figure 6). A total of 42 bands, with an average of 5.25 products per primer, were generated. Among these, 33 bands (68.02%) were polymorphic and 9 (1.12%) were monomorphic (Table 10). The number of bands ranged from 2 (in the case of primer-810) to 10 (in the case of primer-857). While the primers-809, 834 and 859 generated the highest number of polymorphic bands, primer-859 showed highest Rp (10.16) and MI (5.98) values (Table 10). The UPGMA dendrogram

Table 10. ISSR amplification from DNA isolated from *E. ribes*, *E. floribunda* and *E. subcoraceae* from populations in Meghalaya and Arunachal Pradesh

Primers	Total individuals	TB	PB	MB	PPB	PIC	Rp	MI	Jaccard's similarity
ISSR809	25	6	6	0	100	0.09	7.52	4.64	
ISSR810	25	2	1	1	50	0.2	3.36	2.07	
ISSR819	25	3	1	2	33.33	0.09	5.68	3.50	
ISSR826	25	5	2	3	40	0.37	6.96	4.29	
ISSR834	25	5	5	0	100	0.67	4.48	2.76	0.58–1.00
ISSR842	25	3	1	2	33.3	0.25	4.96	3.06	
ISSR857	25	8	7	1	87.5	0.74	7.2	4.44	
ISSR859	25	10	10	0	100	0.66	10.16	5.98	
Total		42	33	9					
Average number of bands per primer		5.25	4.12	1.12	68.02	0.38	6.29	3.84	0.79

TB, Total number of bands; PB, Number of polymorphic bands; MB, Number of monomorphic bands; PPB, Percentage of polymorphic bands; PIC, Polymorphic content; Rp, Resolving power; MI, Marker index.

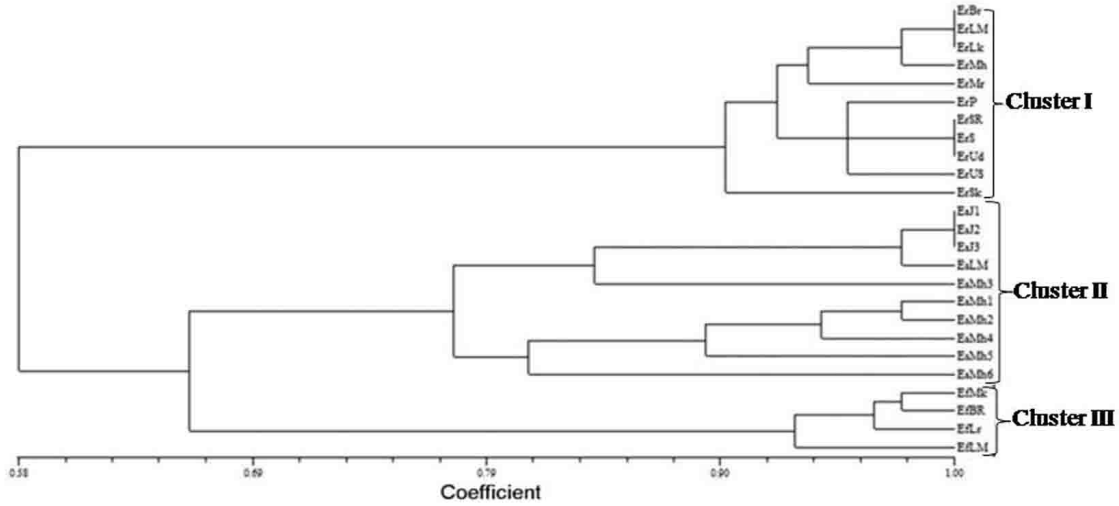

Figure 7. Dendrogram based on UPGMA cluster analysis using ISSR markers for 25 accessions of *Embelia ribes*, *Embelia floribunda* and *Embelia subcoraceae* collected from Meghalaya and Arunachal Pradesh. The three species clearly segregated into three clusters.

generated from the binary matrix, clustered the plants into two broad clades with Jaccard's coefficient of similarity ranging from 0.58 to 1.0. All the accessions belonging to *E. ribes* clustered together into one group; the accessions belonging to *E. floribunda* and *E. subcoraceae* clustered in two subgroups in the other cluster (Figure 7). The plants of *E. ribes* from Bomdila Road (Arunachal Pradesh), Lad Mawphlang (Meghalaya) and Langkyrdem (Meghalaya) showed the highest similarity coefficient of 1.0. Analysis of genetic variation within the three species using ISSR markers revealed that the intra-specific genetic variation was relatively high in *E. ribes* than that in *E. floribunda* and *E. subcoraceae* (Figure 7). It is possible that the low genetic diversity may be one of the factors contributing towards the current threat status of the species.

Assessment of genetic variability in Cycas beddomei

A total of 22 ISSRs and 15 SSRs were selected to generate clear and scorable bands with considerable polymorphism (Table 5; Figure 8) with 20 accessions of *C. beddomei* collected from natural forest of Andhra Pradesh. Using 22 ISSRs, a total of 193 bands were produced with an average of ~8.77 bands per ISSR marker. Among these 193 bands, 179 were polymorphic (92.74%). The size of the ISSR fragments ranged from 100 to 3000 bp (Table 5). ISSR-810 amplified a maximum of 20 fragments, whereas ISSR-807 and ISSR-841 produced the lowest number (5) of bands. Similarly, 69 amplified SSR products were scored across 20 accessions of *C. beddomei* by 15 selected custom-synthesized SSR primers with 91.30% polymorphism. The number of fragments

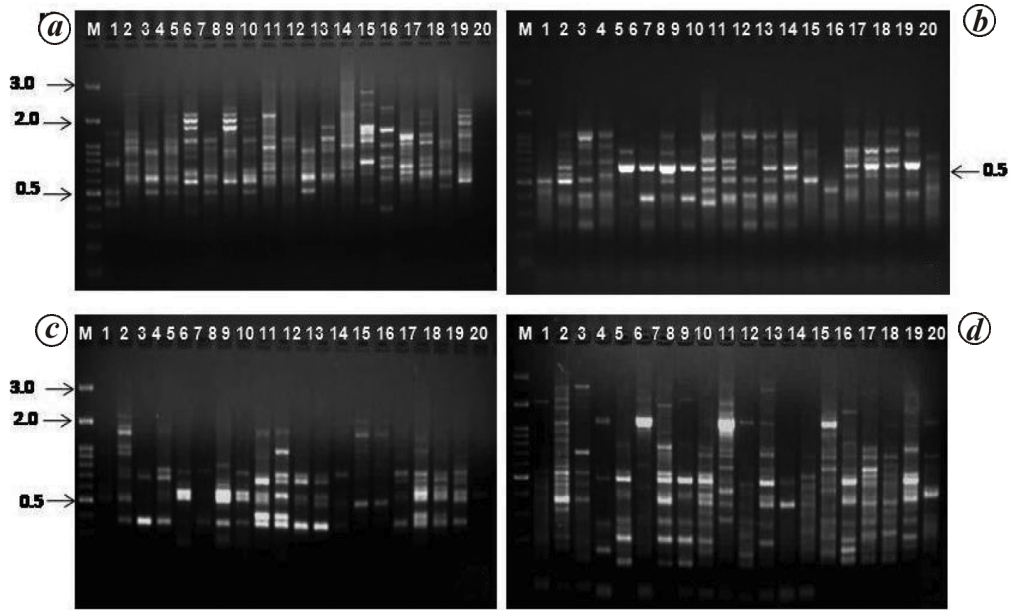

Figure 8. ISSR banding patterns of *Cycas beddomei* generated by the primers. ***a***, UBC-889; ***b***, USB-808; ***c***, UBC-864; ***d***, USB-810. M, Molecular weight ladder (kb). Lanes 1–20 indicates accessions (arrows indicate some of the band scores).

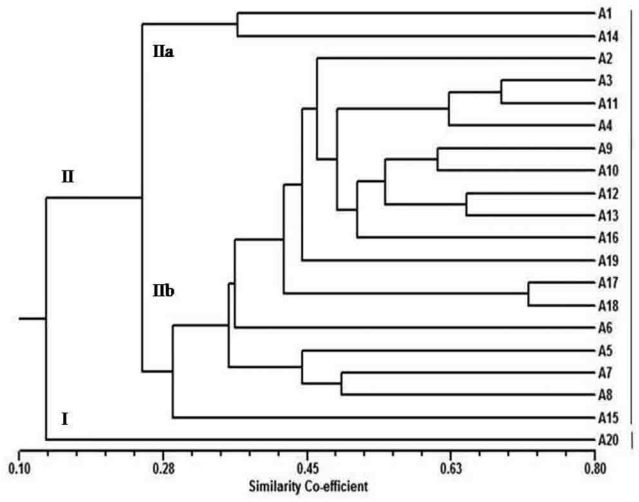

Figure 9. Dendrogram based on the UPGMA cluster analysis of genetic similarity using ISSR markers for 20 accessions of *Cycas beddomei* collected from three forest regions.

ranged from 3 to 5, and size ranged from 100 to 500 bp. Nine Cycas SSRs exhibited 100% polymorphism and CycasSSR13 generated least number of fragments. CycasSSR3 generated maximum number (6) of polymorphic markers. The genetic similarities between the accessions revealed by SSR analysis ranged from 0.09 to 0.72. The average number of amplification products per SSR primer was ~4.93. The genetic variations through molecular markers have been worked out in a number of medicinal plants[37–39]. The results show that both ISSR and SSR marker systems are efficient to distinguish 20 accessions of *C. beddomei*. The ISSR markers revealed higher polymorphism (92.74%) in comparison to SSR markers (91.30%) (Table 5). The similarity matrix obtained in the present study was used to construct a dendrogram by the UPGMA method for both SSR and ISSR data (Figure 9). The dendrograms generated by both marker systems (SSR and ISSR) were in broad agreement with each other. The UPGMA tree showed three major groups and most of the related accessions were grouped together in their respective clusters. On the basis of ISSR analysis the UPGM tree has two major clusters, i.e. accession-20 formed a single major cluster-I, while the other 19 accessions formed the other major cluster-II. Both the major clusters have only 15% similarity between them. The cluster II with 19 accessions were again differentiated into two sub-clusters, i.e. sub-clusters IIa and IIb. Sub-cluster IIa had two accessions (accession-1 and accession-14) and they showed 36% similarity with each other. Sub-cluster IIb had 17 accessions exhibiting 30% similarity among themselves. Accessions-17 and 18 had 73% similarity with each other. On the basis of SSR markers, the 20 accessions were divided into two major clusters (cluster-I and cluster-II) with 3% similarity among the clusters. The first cluster had only one accession (accession 20) which was similar to the cluster generated through ISSR marker. The other 19 accessions made one group with 20% similarity with each other. Further, cluster-II was divided into two minor clades, i.e. cluster-IIa having five accessions and cluster-IIb with 14 accessions. Among the all accessions, accession-20 was placed under a separate cluster by both the approaches (SSR and ISSR). PCA also revealed that accession-20

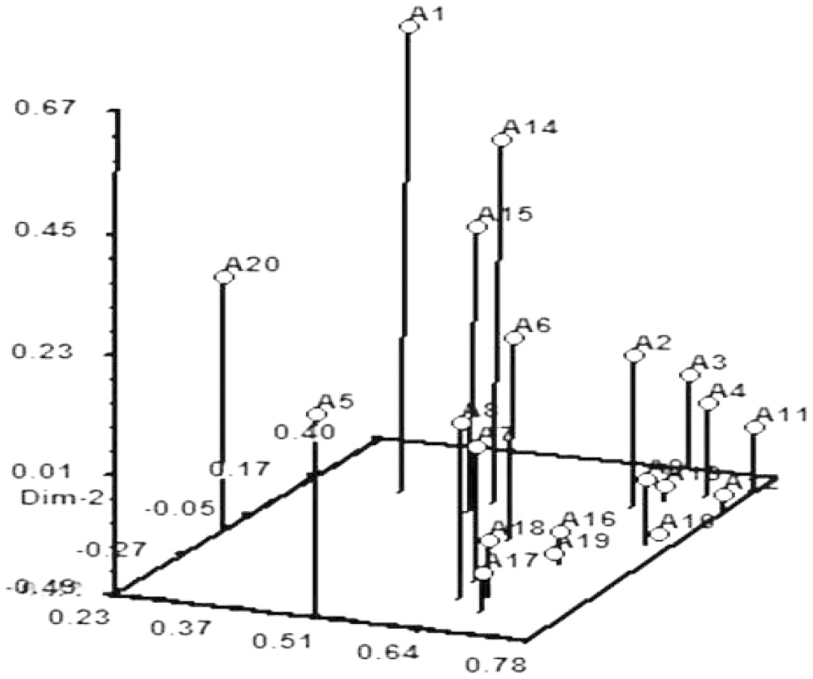

Figure 10. Principal component analysis of 20 accessions of *Cycas beddomei* collected from three forest regions based on ISSR markers.

was placed separately among all the accessions (Figure 10). The above analyses clearly indicate the existence of intra-specific genetic diversity in *C. beddomei*, with Nellore population (accession 20) being genetically distinct from the rest of 19 accessions. The differences in the number of accessions estimated by molecular markers in this study are similar to the results obtained by Rajaseger *et al.*[40] in *Ixora coccinea* and *Ixora javanica*. Taxa-specific molecular markers could be utilized for development of specific markers for taxa identification. Due to slow reproduction rates and absence of long-distance dispersal of seeds as reported by Bank *et al.*[41], it seems reasonable to assume that the species, *C. beddomei* has been derived from a common ancestor through multiple vicariate events, which arose from geographical isolation resulting from the collision of the Indian plate with the Eurasian plate and from glaciations in the Pleistocene[42]. Duminil *et al.*[43] reported that population genetic structure and species traits in seeded plants were key variables in evolutionary biology. Kaushik *et al.*[44] reported that the gene flow of *C. beddomei* is limited on the basis of morphological characteristics such as fruit and seed traits. In general, there was a negative correlation between geographical and genetic distance within each region that was confirmed by the Mantel test ($r = 0.472$, $P/0.001$). Moderate to low genetic diversity and high inbreeding rate[45] lead to the short-term survival of tree species, pushing them to the verge of extinction. Thus, priority conservation actions must be taken to conserve these species in their own habitats by checking anthropogenic destruction, enriching the genetically impoverished populations with diverse germplasms, and allowing them to propagate and increase in number through natural regeneration.

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ACKNOWLEDGEMENTS. We thank Department of Biotechnology, New Delhi for financial assistance (Project No. BT/Env/BC/01/2010). We also thank Prof. C. R. Babu, Prof. R. Umashaanker, Prof. L. M. S. Palni, Dr M. Aslam, Dr K. S. Charak and Dr Omkar Tiwari for critical comments during the course of this research and the anonymous reviewers for their constructive comments that helped improve the manuscript.

doi: 10.18520/cs/v114/i03/539-553