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ISSR and ITS analyses to assess genetic diversity and phylogeny to conserve an endemic and critically endangered tree, *Memecylon subcordatum,* in India

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ABSTRACT

Memecylon subcordatum (Melastomataceae) is an endemic and critically endangered species of the Kalakkad-Mundanthurai Tiger Reserve in India. Twenty five inter-simple sequence repeat primers were used to screen 86 individuals. Nine primers produced a total of 61 bands (6.77 bands primer^{-1}) ranging from 200 to 1900 bp wherein 48 bands were of polymorphic. Polymorphism was 78.69% within the species, 26.60% within the populations and 7.41%–48.15% among the populations. Genetic variation varied from 0.3069 (within species) to 0.1182 (within populations). The poor gene flow among the populations (N_m: 0.3133) could be due to the genetic barriers caused by the tributaries of Tambraparani river. The coefficient of genetic differentiation between the populations (G_{ST}) was 0.6148. The Bayesian analysis, multidimensional scaling analysis and principal coordinates analysis grouped all the 11 populations under 4 clades. The Mantel test showed significant correlation between the matrices of genetic and geographic distances ($r = 0.652$, $P < 0.001$) ranging from 8 to 44 km. The phylogram constructed by internal transcribed spacers to 13 *Memecylon* species showed 2 distinct clades wherein *Memecylon angustifolium*, an associate present along the river banks and at damp localities in the southern Tropical wet evergreen forests from 678 to 1432 m MSL, revealed close affinity. In addition to conserve all the existing natural populations in this protected area, the authors suggest to utilize genetic diversity-rich saplings and stem cuttings from 6 populations and to sow seed coat-removed seeds to enhance the percentage of germination.

1. Introduction

The genus *Memecylon* L. belongs to the family of Melastomataceae [1] and has about 300 species [2–4] or 300–400 species [5]. Members of the family are of shrubs and or trees. It is represented by 30 species in Sri Lanka [2], 43 in Borneo, Java, Malaya and Sumatra [3], 27 in Borneo [4], 40 in Guineo-Congolian Africa [5], 78 in Asia, Oceania and Madagascar [6], 70 in Africa [7], and 39 in India [8]. It is mostly distributed in the Western Ghats region of India and the Sri Lanka. It is known as 'one of the 36 Hot Spots in the world' [9]. A plethora of new species in *Memecylon* that have been published include *M. subramanii*, *M. balakrishnanii*, *M. manickamii*, *M. sivadasanii*, *M. tirunelvelicum*, *M. kollimalayana, M. bremeri*, *M. mundanthuraianum*, *M. agastyamalaianum*, *M. jadhavii*, *M. courtallense*, and *M. wayanadense*. Several new records such as *M. hookeri*, *M. scutellum*, *M. rivulare*, *M. sylvaticum*, *M. gracillimum, M. leucanthemum, M. rostratum* and *M. royenii*, *M. macrocarpum* and *M. clarkeanum* have been reported to India. The present study species, *M. subcordatum* was described by K.C. Jacob from Kannikatti in the Kalakkad-Mundanthurai Tiger Reserve (KMTR) on the basis of holotype specimen (MH Acc. No. 85417) collected on 22.9.1921.

Molecular markers were used as technical tools to assess affinity, origin and divergence time, and distinguish close relatives in Memecylaceae and Melastomataceae. On the basis of parsimony and maximum likelihood analysis of *cp*DNA sequences of *rbcL* and *ndhF* genes and *rpl16* intron, these two groups were treated as sister ones [10]. An *ndhF* analysis of 91 species belonging to 59 genera provided strength to this treatment and linked the Eurasian and North American fossils by molecular clock approach in biogeographical reconstruction [11]. The Bayesian analysis of combined chloroplast loci provided molecular estimates of divergence time to Memecylaceae and Melastomataceae in Africa and Madagascar [12]. Nine samples of *Memecylon* species were scrutinized by 20 RAPD primers using the unweighted pair group method with arithmetic mean (UPGMA). At the end, the process led to describe a new species of *M. wayanadense* and placed it along with its close relatives of *M. sivadasanii* and *M. rivulare* under cluster A. *M. angustifolium* was included under cluster B [8]. Population analyses reveal the relevance of molecular markers to prepare genetic linkage maps [13]. The ISSRs are found to be highly sensitive, reproducible and

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cost-effective [14] than randomly amplified polymorphic DNA – RAPD [15], and restriction fragment length polymorphism – RFLP [16] because of their applications which are very much useful in detecting diversity and biogeographic patterns among populations in closely related, or even clonal, individuals [17]. On the other hand, the internal transcribed spacers (ITS) in nuclear ribosomal region (nrDNA) are applied to generate a wealth of genetic information for extensive use to authenticate species and its populations [18]. It is clear that ISSR and ITS markers can play a major role to devise suitable management strategies in conservation research for implementation towards preserving the species for posterity and increasing the existing populations through in situ and ex situ conservation methods [19]. Further, the conservation strategies help to reduce the impact of habitat destruction and loss of genetic diversity, inbreeding and increased extinction risk due to shifts in distribution [20], habitat fragmentation, population isolation, and decline in population size [21]. On the basis of distribution patterns, the impact of geographical range, successional stages, and pollen and seed dispersal can be studied by assessing genetic variation within and between plant populations [22]. It is also possible to study changes arisen out of adaptation and survival to different environmental conditions in species biology, life history and evolutionary potential of the plant populations [23].

M. subcordatum is an endemic and critically endangered species and its distribution is restricted to the Agastyamalai Biosphere Reserve (2277.16 km^2) in India. The ISSR and ITS markers were used to measure genetic diversity at various levels and to study its affinity with close relatives. The results are presented here to facilitate implementation of suitable conservation management strategies in their natural habitats and to take up such research for conserving endemic and critically endangered plants of the world.

2. Materials and methods

2.1. Study species and sampling

A total of 429 individuals, 113 seedlings (26.34%), 108 saplings (25.17%), 208 reproductive trees (48.49%) belonging to 11 natural populations of *M. subcordatum* was recorded using global positioning system (GPS) from the study area of KMTR between 2009 and 2012 (Table 1; Fig. 1). Approximately, at the rate of one for every five plants, 86 samples of tender leaves were collected in an air zip-lock polyethylene bags at regular intervals of about 10 m and stored at −20 °C until DNA isolation. The authentic voucher specimens of MBV, CR & PSK 1412–1416 and 1420–1425 were deposited in the Herbarium of the Centre for Research and Development of Siddha-Ayurveda Medicines (CRDSAM), Department of Plant Science, Bharathidasan University, Tiruchirappalli, India, for reference.

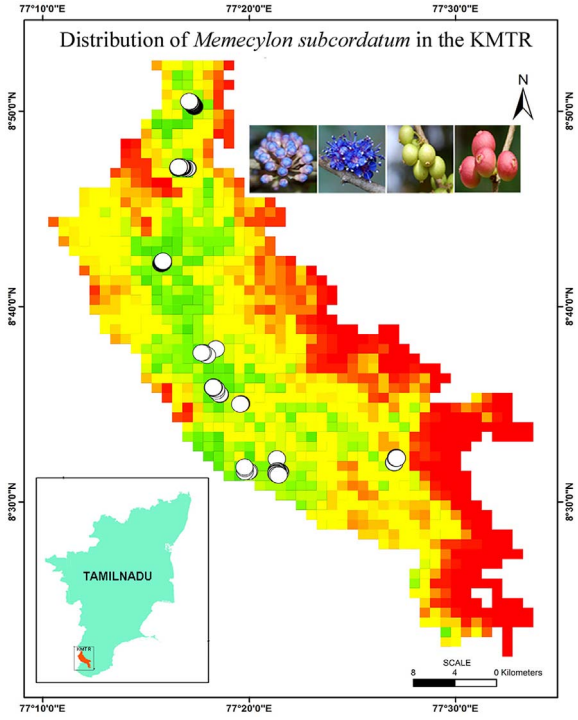

Fig. 1. Geographic locations of 11 populations of *M. subcordatum* in the KMTR.

2.2. DNA extraction and ISSR amplification

A modified Cetyl-trimethyl ammonium bromide (CTAB) method [24] was used to extract genomic DNA. The polymerase chain reaction (PCR) amplification was performed with some modifications [17]. Out of 25 primers screened, 9 ISSR primers that produced the clearest and most reproducible bands were selected to study samples. The primers were of Memecy-1 (AATGAATAATAATGAAAAATGGATT), Memecy-2 (ATTTGGTTGTTGTTTGGGTCTTTGA), Memecy-3 (TTGGGTGGTTTTTG TGTTGGCTAC), Memecy-4 (AATGAATAATAATGAAAAATGGATT), Memecy-5 (GATAGAAAGAGAGAAAAAAATATCA), Memecy-6 (AATG AATAATAATGAAAAATGGATT), Memecy-7 (ATGTGTTTTTATTATTTT TGCTTTG T), Memecy-8 (TTTATTTTATTTGTGTTTTTCTGA) and Memecy-9 (ATATTTTTTTCTCTCTTTCTATC AA). Amplification reactions were carried out in 20 µl (10 µl *Taq* premix; 4 µl of molecular biology grade water; and 3 µl each of primer and template DNA). The PCR was performed with 96-well Eppendorf Mastercycler Pro S, Hamburg, Germany, under the following conditions: initial denaturation for 2 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, annealing at 52–55 °C for 45 s, extension at 72 °C for 3 min and a final extension at 72 °C for 7 min. The PCR products were separated on a 1.5% agarose gel

Table 1
Locations of 11 sampled natural populations of *M. subcordatum* in the KMTR.

Populations ID	Location	SD	SP	RT	Latitude, Longitude	Altitude (m)	Voucher number	ITS GenBank number
Pop.1	Mundanthurai	10	8	22	8°35′N, 77° 18′E	998	BDUT 1412	KC662179
Pop.2	Mundanthurai-1	4	12	18	8° 35′N, 77° 18′E	1036	BDUT 1413	KC662180
Pop.3	Upper Kodayar	10	8	16	8° 35′N, 77° 19′E	1304	BDUT 1414	KC662183
Pop.4	Nondimangadu	5	6	15	8° 47′N, 77° 16′E	971	BDUT 1415	KC686603
Pop.5	Kannikatti	7	8	21	8° 37′N, 77° 16′E	1032	BDUT 1416	KC686604
Pop.6	Muthukuzhivayal	10	3	12	8° 31′N, 77° 21′E	1329	BDUT 1420	KC662178
Pop.7	Vaniyankalpudavu	12	9	15	8° 50′N, 77° 17′E	1006	BDUT 1421	KC686605
Pop.8	Devarpudavu	10	6	12	8° 52′N, 77° 16′E	1040	BDUT 1422	KC686606
Pop.9	Ingikuzhi	6	8	16	8° 37′N, 77° 16′E	678	BDUT 1423	KC686607
Pop.10	Kodamadi	15	14	23	8° 42′N, 77° 15′E	1432	BDUT 1424	KC662182
Pop.11	Shengaltheri	24	26	38	8° 32′N, 77° 27′E	1430	BDUT 1425	KC662181

RT reproductive trees, SD seedlings, SP saplings.