Assessment of Genetic Diversity in 31 Species of Mangroves and their Associates through RAPD and AFLP Markers

Arup Kumar Mukherjeea,*, Laxmikanta Acharyab, Pratap Chandra Pandab, and Trilochan Mohapatrac

a DNA Fingerprinting Laboratory, Division of Plant Biotechnology, Regional Plant Resource Centre, Nayapalli, Bhubaneswar 751 015, Orissa, India. Fax: 91-674-2550274. E-mail: arupmukherjee@yahoo.com

b Division of Taxonomy and Conservation, Regional Plant Resource Centre, Nayapalli, Bhubaneswar 751 015, Orissa, India
c DNA Fingerprinting and Rice Genome Mapping Laboratory, NRC on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi 110 012, India

* Author for correspondence and reprint requests

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Random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) markers were used to assess the genetic diversity in 31 species of mangroves and mangrove associates. Four AFLP primer combinations resulted in the amplification of 840 bands with an average of 210 bands per primer combination and 11 RAPD primers yielded 319 bands with an average of 29 bands per primer. The percentage of polymorphism detected was too high indicating the high degree of genetic variability in mangrove taxa both at inter- and intra-generic levels. In the dendrogram, species belonging to a particular family/genus, taxa inhabiting similar habitats or having similar adaptations tended to be together. There were exceptions too; as many unrelated species of mangroves form clusters. The intra-familial classification and inter-relationships of genera in the family Rhizophoraceae could be confirmed by molecular analysis. Both the markers RAPD and AFLP were found equally informative and useful for a better understanding of the genetic variability and genome relationships among mangroves and their associated species.

Key words: Mangroves, Molecular Characterization, Genetic Diversity

Introduction

Mangroves, the characteristic complex plant-communities of tropical and sub-tropical sheltered coastlines, have attracted considerable scientific attention during the last few decades. Like the terrestrial tropical rain forests, mangals have played a significant role in the economy of tropical societies for thousands of years, providing a wide variety of goods and services including wood production, support for commercial and subsistence fisheries, aquaculture, salt production and shoreline and coastal erosion control. Being ecologically interesting, strictly habitat-specific, highly resourceful, inhabiting vulnerable ecosystem and exhibiting peculiar morphological and anatomical adaptations such as vivipary and pneumatophores, mangrove plants have aroused considerable curiosity and have been the subject of taxonomic, phytogeographical, ecological, cytological, physiological and molecular studies (Schwarzbach and Ricklefs, 2000, 2001; Duke et al., 2002; McCoy and Heck, 1976; Lakshmi et al., 1997, 2000; Parani et al., 1997a,b, 1998).

The mangrove forests and coastal wetlands are being destroyed worldwide at an alarming rate due to human interference of various kinds and magnitudes resulting in loss of genetic diversity. Recently, molecular data concerning phylogenetic relationships and population structure have been obtained from macro-molecules like proteins and DNA and used for identifying populations with high genetic diversity that could be important sources for restoration and re-vegetation of these unique and important habitats (Parani et al., 1997a; Schwarzbach and Ricklefs, 2001).

The use of molecular markers in mangrove research has been reviewed by Schwarzbach and Ricklefs (2001), who described at length how protein and DNA markers have been helpful in the assessment of genetic diversity and in establishing inter-relationships and phylogeny among a number of mangrove taxa. As rightly pointed out then,
indirect detection methods of DNA sequence differences like AFLPs (amplified fragment length polymorphisms) and SSRs (simple sequence repeats) were not applied in mangrove plant research till 2001 and there was a single report on the use of ISSR (inter simple sequence repeat) for estimation of genetic diversity in a mangrove species e.g. Aegiceras corniculatum. Parani et al. (1998) used RAPD and RFLP data to analyze genomic relationships of 16 mangrove species of India. Recently, Maguire et al. (2002) have used AFLP and SSR markers for studying the intra-specific variability in Avicennia marina (Forsk.) Vierh., which is the only published account on application of an AFLP marker to mangroves. However, no report is yet available on the use of RAPDs and AFLPs in combination for establishing the genomic relationships among species of mangroves and their associates, in general. In the present study, the AFLP technique has been used for the first time for assessment of genetic diversity in 31 species of mangroves and mangrove associates in conjunction with RAPD.

**Materials and Methods**

**Plant material**

For the present study, 31 species of mangroves and their associates were included. Their correct botanical names with synonyms, if any, families to which each of them belong to, their ecological status, and locality of collection are given in Table I. Ten individuals of each species except R. stylosa (5 individuals) were randomly selected and fresh and young leaf samples were collected for isolation of genomic DNA.

**Genomic DNA isolation**

The genomic DNA was isolated from freshly collected leaves using the CTAB method (Saghai-Marooof et al., 1984) with some modifications. The crude DNA was purified by giving RNAseA treatment (@ 60 μg RNaseA for 1 ml of crude DNA solution) followed by three washes with phenol/chloroform/iso-amyl-alcohol (25:24:1 v/v/v) and subsequently three washes with chloroform/iso-amyl-alcohol (24:1 v/v). The upper aqueous phase was separated after centrifugation and mixed with 1/10 volume of 3 M sodium acetate. DNA was precipitated by adding 2.5 volumes of chilled absolute ethanol, pelleted, dried in vacuum and dissolved in T_{10}E_{1} buffer. Quantification of DNA was accomplished by analyzing the purified DNA on 0.8% agarose gels alongside diluted uncut lambda DNA as standard. DNA was diluted in T_{10}E_{1} buffer to the required concentration for different PCR analyses.

**RAPD analysis**

For RAPD analysis, PCR amplification of 25 ng of genomic DNA was carried out using 11 standard decamer oligonucleotide primers, i.e. OPM01, OPM06, OPM09, OPN05, OP01, OP01, OPQ20, OPS07, OPT04, OPT07 and OPT08 (Operon Tech. Alameda, USA). The RAPD analysis was performed as per the standard method of Williams et al. (1990). Each amplification reaction mixture of 25 μl contained 20 ng of template DNA, 2.5 μl of 10X assay buffer (100 mM Tris-HCl, pH 8.3, 0.5 mM KCl and 0.01% gelatin), 1.5 mM MgCl₂, 200 μM each of dNTPs, 20 ng of primer and 0.5 U TaqDNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, India). The amplification was carried out in a thermal cycler (Perkin Elmer, Model 9600, USA). The first cycle consisted of denaturation of template DNA at 94 °C for 5 min, primer annealing at 37 °C for 1 min and primer extension at 72 °C for 2 min. In the subsequent 42 cycles, the period of denaturation was reduced to 1 min while the primer annealing and primer extension time were maintained same as in the first cycle. The last cycle consisted of only primer extension at 72 °C for 7 min. PCR products were separated on a 1.5% agarose gel containing ethidium bromide (@ 0.5 μg/ml of gel solution). The size of the amplicons was determined using size standards (100 bp DNA ladder plus or DNA ladder mix, MBI Fermentas, Graiciuno, Vilnius, Lithuania). DNA fragments were visualized under UV light and photographed using a polaroid photographic system (FOTODYNE Incorporated 950 Walnut Ridge Drive Hartland, WI, USA).

**AFLP analysis**

AFLP analysis was performed following the modified method of Vos et al. (1995) and as per the protocol supplied with the ‘AFLP Core Reagent Kit’ and the ‘AFLP Starter Primer Kit’ of Life Technologies (Grand Island, New York, USA). The amplified products were analyzed in prewarmed 5% acrylamide electrophoresis gels. Gels were run at 55 W for approx. 2 h, transferred to Whatman filter paper (No. 1) and dried under vac-
Table I. Details of the species taken for the present study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Family</th>
<th>Place(s) of collection</th>
<th>Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avicennia alba Bl.</td>
<td>Avicenniaceae</td>
<td>Dangamala*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Acanthus ilicifolius</td>
<td>Acanthaceae</td>
<td>Dhamara*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Aegiceras corniculatum (L.) Blanco</td>
<td>Myrsinaceae</td>
<td>Kajalpatia*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Aegialitis rotundifolia Roxb.</td>
<td>Plumbaginaceae</td>
<td>Thakurdian*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Caesalpinia bonduc (L.) Roxb.</td>
<td>Caesalpiniaceae</td>
<td>Dangamala*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Cryptocoryne ciliata (Roxb.) Schott</td>
<td>Araceae</td>
<td>Kola*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Crinum defixum Kar.-Gawl.</td>
<td>Amaryllidaceae</td>
<td>Kola*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Cynometra tripa Kostel.</td>
<td>Caesalpiniaceae</td>
<td>Dangamala*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Cerbera manghas L.</td>
<td>Apocynaceae</td>
<td>Kansaridian*</td>
<td>Back mangrove</td>
</tr>
<tr>
<td>Derris heterophylla (Willd.) Back. and Bakh.</td>
<td>Fabaceae</td>
<td>Musadiyan*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Derris scandens (Roxb.) Benth.</td>
<td>Fabaceae</td>
<td>Kanika*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Exceocaria agallocha L.</td>
<td>Fabaceae</td>
<td>Dangamala*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Heritiera fomes Buch.-Ham.</td>
<td>Sterculiaceae</td>
<td>Kharinasi*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Heritiera littoralis Dryand</td>
<td>Sterculiaceae</td>
<td>Kalibhanjadian*</td>
<td>Back mangrove</td>
</tr>
<tr>
<td>Heritiera macrophylla Wall.</td>
<td>Sterculiaceae</td>
<td>Botanic Garden, R.P.R.C.<em>, BBSR</em>, Orissa.</td>
<td>Main land</td>
</tr>
<tr>
<td>Pongamia pinnata (L.) Pierre</td>
<td>Fabaceae</td>
<td>Dangamala*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Lumnitzera racemosa Willd.</td>
<td>Combretaceae</td>
<td>Ek Kula*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Suada maritima Dumort.</td>
<td>Chenopodiaceae</td>
<td>Thakurdian*</td>
<td>Mangrove associate</td>
</tr>
<tr>
<td>Tylophora tenius (Bl.) Bijdr.</td>
<td>Asclepiadaceae</td>
<td>Kajalpatia*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Xylocarpus granatum Koenig</td>
<td>Meliaceae</td>
<td>Dangamala*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Bruguiera cylindrica (L.) Bl.</td>
<td>Rhizophoraceae</td>
<td>Dangamala*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>B. gymnorrhiza (L.) Lamk.</td>
<td>Rhizophoraceae</td>
<td>Ek Kula*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>B. parviflora Wt. and Arn. ex Griff.</td>
<td>Rhizophoraceae</td>
<td>Thakurdian*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>B. sexangula (Lour.) Poir.</td>
<td>Rhizophoraceae</td>
<td>Dangamala*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Cassipourea ceylanica (Garden) Aston</td>
<td>Rhizophoraceae</td>
<td>Badakuda Island, Chilika Lake, Orissa</td>
<td>Main land</td>
</tr>
<tr>
<td>Carallia brachiata (Lour.) Merr.</td>
<td>Rhizophoraceae</td>
<td>Botanic Garden, R.P.R.C.<em>, BBSR</em>, Orissa.</td>
<td>Main land</td>
</tr>
<tr>
<td>Ceriops tagal (Perr.) Robinson</td>
<td>Rhizophoraceae</td>
<td>Kanika*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Kandelia candel (L.) Druce</td>
<td>Rhizophoraceae</td>
<td>Suajore*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>Rhizophora apiculata Bl.</td>
<td>Rhizophoraceae</td>
<td>Mahisamara*</td>
<td>True mangrove</td>
</tr>
<tr>
<td>R. stylosa Griff.</td>
<td>Rhizophoraceae</td>
<td>Campbell Bay, Andaman Island</td>
<td>True mangrove</td>
</tr>
<tr>
<td>R. mucronata Lamk.</td>
<td>Rhizophoraceae</td>
<td>Dangamala*</td>
<td>True mangrove</td>
</tr>
</tbody>
</table>

* All these samples were collected from different sites of Bhitarkanika wildlife sanctuary, Orissa.
* R.P.R.C., Regional Plant Resource Centre.
+ BBSR, Bhubaneswar.

umo (BioRad Model 583). AFLP products were observed when exposed to X-ray films (Kodak-Bi-oMax MR) for 2–5d. For the present study, four best primer combinations (EACT/MCAC, EACT/ MCTA, EAGC/MCAA and EAGC/MCTT) were selected out of 9 primer combinations used in a previous study involving 3 species of Heritiera (Mukherjee et al., 2003).

Data analysis
The banding patterns obtained from RAPD gel and AFLP autoradiographs were scored as present (1) or absent (0). The data obtained from both the categories of markers were pooled for different analyses. Jaccard’s coefficient of similarity (Jaccard, 1908) was measured and a dendrogram based on similarity coefficients generated by the un-weighted pair group method using arithmetic averages (UPGMA) and SAHN (sequential, agglomerative, hierarchical, and nested) clustering methods (Sneath and Sokal, 1973). Similarity matrices obtained with RAPD and AFLP primers were compared with the combined similarity matrices accomplished by the RAPD and AFLP primers using the Mantel test (Mantel, 1967). Principal co-ordinate analysis (PCA) was also performed for confirmation of the grouping of the taxa. All analyses were done using the computer package NTSYS-PC (Rohlf, 1997).
Results

Molecular markers

In case of RAPD analysis, eleven random decamer primers amplified 319 bands with the fragment range of 4000 to 100 bp (Table II). None of the bands was monomorphis. The number of bands ranged from 22 to 42 in different primers.

The average number of bands amplified in the individual primer was 29. The RAPD patterns obtained using primers OPM01 and OPQ01 are shown in Fig. 1.

For AFLP analysis, four primer combinations resulted in the amplification of 840 bands with an average of 210 bands per primer. The number of bands amplified varied from 195 to 217 (Table II).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>No. of loci</th>
<th>Primer combination</th>
<th>No. of loci</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPM01</td>
<td>GTTGGTGCGCT</td>
<td>23</td>
<td>EACT/MCAC</td>
<td>195</td>
</tr>
<tr>
<td>OPM06</td>
<td>CTGGGCAACT</td>
<td>27</td>
<td>EACT/MCTA</td>
<td>212</td>
</tr>
<tr>
<td>OPM09</td>
<td>GTCTTGCGGA</td>
<td>23</td>
<td>EAGC/MCAA</td>
<td>217</td>
</tr>
<tr>
<td>OPN05</td>
<td>ACTGAACGCC</td>
<td>22</td>
<td>EAGC/MCTT</td>
<td>216</td>
</tr>
<tr>
<td>OPP01</td>
<td>GTAGCAGTCCA</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPQ01</td>
<td>GGGACGATGG</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPQ20</td>
<td>TCGCCCCAGTC</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPS07</td>
<td>TCCGATGCTC</td>
<td>33</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPT04</td>
<td>CACAGGCGGA</td>
<td>38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPT07</td>
<td>GGCAGGCTGT</td>
<td>32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OPT08</td>
<td>AACGGCGGACA</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL</td>
<td></td>
<td>319</td>
<td></td>
<td>840</td>
</tr>
</tbody>
</table>

Table II. Details of molecular analysis in 31 species using AFLPs and RAPDs.

Fig. 1. RAPD pattern of 31 species using (a) OPM01 and (b) OPQ01 primer. M, 100 bp ladder mix (MBI Fermantas); lanes 1 to 31, different species arranged according to Table I.
and none of the bands was found to be monomorphic. A representation of AFLP banding pattern of 31 species of mangroves and mangrove associates is shown in the Fig. 2.

**Genomic relationships**

The RAPD and AFLP data were pooled and the genetic similarity was calculated using Jaccard's coefficient (Jaccard, 1908). The highest similarity was observed between *Heritiera littoralis* and *H. macrophylla* (0.9083) and the lowest similarity was observed between *Tylophora tenius* and *Aegiceras corniculatum* (0.1201). A similar result was obtained when a cluster diagram was constructed using the UPGMA method and SAHN clustering. The dendrogram showed two major clusters; one with 8 species and the other with rest 23 species of mangroves (Fig. 3). All the members of Rhizophoraceae formed a single sub-cluster and a similar result was observed in case of three species of *Heritiera* (Sterculiaceae). Within the family Rhizophoraceae, the two non-mangrove elements...
got separated from the nine other mangrove Rhi-
zophoraceae, both sharing a common node and
making separate sub-clusters. Similarly, two spe-
cies of Derris (Fabaceae) formed a sub-cluster in
the dendrogram. Both the monocotyledonous taxa
Cryptocoryne ciliata and Crinum defixum came
under one sub-cluster justifying their taxonomic
alienation. The result was also confirmed using
principal coordinate analysis (Fig. 4).

Fig. 4. Principal co-ordinate analysis showing grouping
of different taxa of mangroves and their associates (the
abbreviations are as used in Fig. 3).

**Discussion**

Thirty-one species of mangroves and their asso-
ciates were fingerprinted using RAPD and AFLP
markers. Eleven RAPD markers produced 319
bands at an average of 29 bands per primer in con-
trast to 19.80 bands per primer reported by Parani
et al. (1998). This difference in banding pattern
could be due to the selection of primers as well as
the plant materials. In the present investigation,
we observed that four AFLP primer sets detected
a total of 840 bands, all of which were polymorphic in
nature. Eleven RAPD primers also produced 319 poly-
morphic bands. Russell et al. (1997) and Garcia-
Mas et al. (2000) reported similar results with
RAPD and AFLP markers while evaluating the
genetic diversity in barley and melon respectively.

In the present investigation, it was not possible
to segregate the mangroves, mangrove associates
and non-mangroves from the species studied un-
like the findings of Parani et al. (1998). This could
be due to the selection of more taxa from a single
genus/family irrespective of their habitat condi-
tions and the use of a different marker system i.e.
AFLP instead of RFLP where random primer sets
were selected without any prior information of
their amplification pattern. While analyzing the
genomic relationships of 16 mangrove species us-
ing RAPD and RFLP data, Parani et al. (1998)
used the probes originating from mangroves only
and also targeted the chloroplast DNA (cpDNA)
regions which are inherited maternally and are
highly conserved (Harris and Ingram, 1991;
observed that the results of Parani et al. (1998)
differ strikingly from previously published large-
scale phylogenetic studies using molecular and
non-molecular data. They attributed the lack of
congruence, in this particular case, to the small
number of taxa studied, use of extremely variable
and homoplastic characters of RAPD and RFLP
and identification of lineages by cluster analysis
(UPGMA), which does not allow for unequal
rates of evolution. In another work, Parani et al.
(2000) studied the molecular phylogeny of 24 spe-
cies of mangroves and mangrove associates by
analysis of $trnS$-$psbC$ and $rbcL$ gene regions
through RAPD and RFLP markers. However, in
the present study, we have taken entire genomic
DNA and also four AFLP primer combinations
from the ones used in our earlier study on phylog-
eny of 11 members of Rhizophoraceae (Mukher-
jee et al., 2004).

The dendrogram showed grouping of taxa be-
longing to a particular genus or family under the
same cluster. Eleven members of the family Rhi-
zophoraceae comprising of nine mangroves and
two terrestrial non-mangrove representatives (Ta-
ble I) formed a single sub-cluster justifying their systematic position. The non-mangroves were further segregated from the mangrove Rhizophoraceae (tribe Rhizophoraceae) in the sub-cluster supporting the intra-familial division into tribes (Tobe and Raven, 1988). The genus Bruguiera (4 species) and Rhizophora (3 species) also formed separate clades comprising species of their own. The members of the genus Rhizophora, Ceriops and Kandelia showed closer affinity than Bruguiera which maintained a distant place in the tribe. The isolated position of Bruguiera from other Rhizophoraceae was also evident in the phylogenetic tree constructed by Schwarzbach and Ricklefs (2001) on the basis of chloroplast DNA, nuclear ribosomal DNA and morphology. Of the Rhizophoraceae, the genera Ceriops and Kandelia had closer relationship between them and shared a common node in the dendrogram. Three species of Heritiera also came under a single cluster and H. littoralis showed more similarity with H. macrophylla, an observation made earlier by Mukherjee et al. (2003). Similar observations were made with respect to the two species of Derris (D. heterophylla and D. scandens) and between the two monocotyledonous taxa Cryptocoryne ciliata and Crinum defixum.

Though taxonomically distantly placed, Avicennia alba, Aegiceras corniculatum and Acanthus ilicifolius shared a common node. The affinity of Aegiceras and Avicennia has been earlier reported by Parani et al. (1998) using RAPD and RFLP markers. All the three species viz. Acanthus ilicifolius, Aegiceras corniculatum and Avicennia alba have salt excreting glands on their leaves in common and it is likely that all of them might have evolved some genetic similarity to adapt to the stressful mangrove environment. However, non-inclusion of Aegialitis rotundifolia, another prominent salt excreting species, in this group is not understandable. There is also no suitable explanation for the relatedness observed between Xylocarpus granatum and Tylophora tenius.

It was interesting to note that all the species inhabiting less salinity regimes like Pongamia pinnata, Xylocarpus granatum, Lumnitzera racemosa, Suaeda maritima formed a cluster in the dendrogram. Once again, inclusion of the three species of Heritiera in the above clade does not appear justifiable.

A fairly high ‘r’ value (0.81) obtained on comparison of AFLP and RAPD data indicated that both the markers are comparable in the present investigation. This was in conformity with the earlier result of Garcia-Mas et al. (2000). The comparison of individual AFLP primers with that of the pooled data showed that only two primers namely EACT/MCTA and EAGC/MCAT were sufficient enough for genetic characterization of all the 31 species now investigated.

As commonly conceived, mangroves are a heterogeneous assemblage of diverse groups of plants with independently derived lineages and the species are defined ecologically by their occurrence in tidal swamp forests and physiologically by their ability to withstand high salt concentrations and low soil aeration (Schwarzbach and Ricklefs, 2001). Even with the knowledge that mangroves are polyphyletic in origin, the inter-relationships of many constituent taxa have been the matter of controversy over years. According to Schwarzbach and McDade (2002), strongly convergent evolution of many characters to the stressful mangrove environment may be the most important factor contributing to this confusion. As a matter of fact, a number of common morphological characters is observed among unrelated mangrove species but such similarities are not to be found between mangroves and their closest terrestrial relatives.

The limitations of RAPD and AFLP data in the present study emphasize the need for phylogenetic work based on conserved DNA sequences involving more numbers of mangrove species. Though the work is of preliminary nature, the molecular data shall provide information on genetic diversity of the mangroves and have impact on conservation and management of this important group of plants.

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