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CROSS-SPECIES AMPLIFICATION OF MICROSATELLITE LOCI IN THREE MANGROVE SPECIES OF INDIA

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ABSTRACT

This study analysed genetic relationship in three species of Rhizophora growing in southeast coast of India. Eight microsatellite loci in three mangrove species (*Rhizophora annamalayana, R. apiculata* and *R. mucronata*) were successfully amplified by cross priming. All the loci were polymorphic and ideal to use as markers in identification of population. However, the optimum annealing temperature to get scorable band in Rhizophora species varied from that reported in literature for the respective primer pair. Sixteen alleles were observed in eight loci. The number of alleles for overall species was 0.3333 per locus and for overall locus was 0.2619 per species. *Rhizophora annamalayana* and *R. mucronata* were found genetially much closer (0.006) than *R. apiculata* (0.008) as revealed by microsatellite data.

Key words: Mangrove, Rhizopora, Microsatellite, Genetic variation

INTRODUCTION

Mangrove forests are among the world's most productive ecosystems, situated at the interface between land and sea in the tropical and subtropical latitudes [1, 2]. *Rhizophora* species are generally important component of the mangrove vegetation. There are several natural hybrids of Rhizophora species, but their parental species are not clearly understood [3, 4]. There is significant inter- and intraspecific variability among the mangrove species, as revealed by using molecular markers such as AFLP and/or RAPD [5, 6, 7, 8]. Similarly there are inter- and intra-generic relations among the mangroves, as evident by using RAPD alone or with other markers [9]. Microsatellite markers are the very recently used tool for genetic variations of mangroves [10, 11]. In southeast coast of India, there are two Rhizophora species along with a natural hybrid species, but their parentage is not clear. But, there has been no report to study genetic relations between *Rhizophora* species in India using microsatellite and hence the present work fills the knowledge gap.

MATERIALS AND METHODS

Leaf samples were collected from three species of the botanical family Rhizophoraceae namely *R. apiculata* Blume, *R. mucoronata* Poir, and *R. annamalayana* Kathir., from the mangrove forests lying on the Pichavaram (Lat.11° 27'N; Long. 79° 47'E) in east coast of India. Genomic DNA was isolated from freshly collected leaves using the CTAB (cetyl trimethyl ammonium bromide) method [9, 12] with some modifications. About 5 g of leaf tissue was ground in ice cold condition and suspended in 10 ml of 2% CTAB buffer (2% CTAB; 0.1M Tris (pH 8.0); 20 mM EDTA; 1.4M NaCl; 2% PVP-40; 1% β mercaptoethanol). The suspension was incubated at 60^0C for 15 min with gentle mixing and extracted with equal volume of chloroform: isoamyl alcohol (24:1). To the aqueous phase, 0.2 ml of 5% CTAB was added and extracted with equal volume of chloroform: isoamyl alcohol

(24:1). The aqueous phase was transferred to a 20 ml tube and the DNA was precipitated with 0.6 ml of cold isopropanol and stored at -20 ^{0}C for 1 h. The sample was centrifuged and the precipitate was dissolved in TE buffer (10 mM Tris-HCl, 1 mM Na EDTA, pH 8.0). The solution was subsequently treated with RNase A. Finally 0.1 ml of 3 M sodium acetate (pH 5.2) and 2 ml of ice-cold ethanol were added to the aqueous phase to precipitate the DNA. The DNA was pelleted by centrifugation at 8,000g for 10 min at room temperature and the pellet was washed with 70% ethanol. The pellet was air-dried and dissolved in TE buffer. The genomic DNA concentration was estimated by 0.8% agarose gel electrophoresis.

Amplification of the microsatellite was carried out in 25 µl reaction mixture containing 50 ng of template DNA, 2.5 µl assay buffer, ($MgCl_2$), 2.0 µl of dNTPs mix, 0.5 µl of primer mix and 0.5 µl of *Taq* DNA polymerase, and amplified in a Thermal cycler (Tech Gene). The temperature profile consisted of a total of 25 cycles with 30 sec (5 min for the first cycle) at 94°C for template denaturation, 30 sec for primer annealing (Ta for specific primers are given in Table 1, and 1 min (2 min for the final extension) at 72°C for primer extension. The amplified product was checked in 10% polyacrylamide gel electrophoresis by silver staining. The primer details are given in Table. 1.

The allele frequencies - observed and expected heterozygosities (H_{ob} and H_{ex}), percentage of polymorphic loci for overall species, each population and the genetic distance between the three species was calculated by GENETIX Software, Version 4.0 [13].

RESULTS AND DISCUSSION

All the eight amplified microsatellite loci were polymorphic (100%) in three species of Rhizophora. In the locus RHSTCP 02, two alleles with size of 103 and 102 were observed in all the species. In the RHSTCP 05 locus, two alleles were found (155 and 156). In the RHSTCP 06 and RHSTCP 07 loci also, two alleles were 92, 93 and 66, 67 respectively. The software GENETIX could not recognize and provide the allelic frequency in the loci with allele size less than 100. The loci RHSTCP 08 and KACACP 07 showed two alleles with size of 149, 150 and 162,163 respectively in each species and the loci KACACP 11 and KACACP 17 also exhibited two alleles (200,201 and 181,182).

Among the three species, 16 alleles were observed in eight loci. The mean observed number of alleles for overall species was 0.3333 per locus. The mean observed number of alleles for overall locus was 0.2619 per species (Table 2). Based on the six polymorphic microsatellite loci, the genetic relatedness was calculated and displayed in Table 3. *Rhizophora annamalayana* was genetically closer to *Rhizophora mucronata* rather than to *Rhizophora apiculata*.

Microsatellites are valuable tool for examining genetic variation among the mangrove species. Because of their extremely high levels of polymorphism, they are widely used in population studies of mangrove species [10, 12, 14, 15, 16]. Despite their extremely fast rates of repeats, evolution of many microsatellite loci is quite conservative in their flanking regions and hence they can persist for long evolutionary time and they are much unchanged. Due to this, primers developed for a species from the flanking regions of a microsatellite locus can be used to amplify the same locus in other related species. Generally, the designing of microsatellite primers specific to new species

is expensive and time consuming, but the cross priming can be alternative and attractive option as it is cheap and fast.

Islam et al. [10] have developed primars for two mangrove species, *Kandelia candel* and Rhizophora *stylosa*, belonging to the botanical family Rhizophoraceae. These primars (Table 1) were used in this study for three species of Rhizophora, belonging to the same family for evaluation of cross species amplication of microsatellite loci. This cross-priming revealed polymorphic nature of all the loci and hence the microsatellite markers are ideal for population studies. However, the optimum annealing temperature to get scorable band in Rhizophora species varied from that reported in the literature. The optimization of PCR conditions is a matter of necessity for cross-amplification tests.

CONCLUSION

In this study, all the microsatellite loci were polymorphic and they differ in only one base between alleles. The genetic distance revealed that *Rhizophora annamalayana* and *R. mucronata* were more closely related than *R. apiculata*. Our laboratory discovered *Rhizophora annamalayana* as a new hybrid species, naturally formed between the two species, namely *R. apiculata* and *R. mucronata* [3, 4].

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REFERENCES

1. Kathiresan, K. and B. L. Bingham. 2001. Biology of mangrove and mangrove ecosystems. Advances in Marine Biology, 40 : 81-251
2. Kathiresan, K. and S.Z. Qasim. 2005. Biodiversity of mangrove ecosystems. Hindustan Publishing Carporation, India. 251p
3. Kathiresan, K. 1995. *Rhizophora annamalayana:* A new species of mangrove. Environment and Ecology, 13 (1) : 240-241
4. Kathiresan, K. 1999. *Rhizopora annamalayana* Kathir (Rhizophoraceae), a new Nothospecies from Pichavaram mangrove forest in Southeastern penisular India. Environment and Ecology, 17 (2): 500-5001
5. Parani, M., M. Lakshmi, S. Elngo, N. Ram, C.S. Anurhata, and A. Parida. 1997. Molecular phylogeny of mangroves II. Intra and inter-specific variation in *Avicennia* revealed by RAPD andRFLP markers. Genome, 40 : 487-495.
6. Parani, M., M. Lakshm, P. Senthilkumar, N. Ram, and A. Parida. 1998. Molecular phylogeny of mangroves V. Analysis of genomerelationships in mangrove species using RAPD and RFLP markers. Theor. Appl. Genet., 97 : 617-625
7. Parani, M., M.Lakshmi, B. Ziegenhagen, M. Fladung, P. Senthilkumar, and A. Parida. 2000. Molecular phylogeny of mangroves VII. PCR-RFLP of trnS-psbC and rbcL gene regions in 24 mangrove and mangrove-associate species. Theor. Appl. Genet., 100 : 454-460
8. Mukherjee, A,K., L.K. Acharya, I. Mattagajasingh, T. Mo-hapatra, P.C. Panda, and P. Das. 2003. Molecular characterization of three *Heritiera* species using AFLP markers. Biol. Plant., 47: 445- 448
9. Lakshmi, M., M. Parani, P. Senthilkumar, and A. Parida. 2002. Molecular phylogeny of mangroves VIII: Analysis of mitochondrial DNA variation for species identification and relationships in Indian mangrove Rhizophoraceae. Wetlands Ecology and Management, 10 : 355-362
10. Islam, M, S., C. Lian, Q. Geng, N. Kameyama, and T. Hogetsu. 2008. Chloroplast microsatellite markers for the mangrove tree species *Bruguiera gymnorrhiza, Kandelia candel*, and *Rhizophora stylosa*, and cross-amplification in other mangrove species. Conserv. Genet., 9: 989–993
11. Nettel, A., R.S. Dodd, J.A. Cid-Becerra, and J.D.L. Rosa-Velez. 2008. Development of microsatellite markers for the white mangrove (*Laguncularia recemosa* C.F

Gaertn, Combretaceae). Conserv. Genet., 9: 1037-1038
12. Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorsenson, and R.W Allard. 1984. Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA., 81:8014–8018
13. Belkhir, K., P. Borsa, J. Goudet, L. Chikhi, and F. Bonhomme. 1997. GENETIX logicielsous windows pour Ia ge'ne'tique des populations, http://www.univ-montp2.fr/~gentix/genetix/html
14. Kahrood, H.V., S.A.A .Karori, M. Pirseyedi Shirvany, and A. Danehkar. 2008. Genetic variation of mangrove species *Avicennia marina* in Iran revealed by microsatellite markers. *Af. J. Biotech. 7 (17)*: 3017-3021.
15. Takayama, K., T. Mariko, T. Yoichi, and K. Tadashi. 2008. Isolation and characterization of microsatellite loci in the red mangrove *Rhizophora mangle* (Rhizophoraceae) and its related species. Conserv Genet., 9 : 1323-1325
16. Li, L., Z.F. Wang, S.G. Jian, P. Zhu, M. Zhang, W.H. Ye, and H. Ren. 2008. Isolation and characterization of microsatellite loci in endangered *Cycas changjiangensis* (Cycadaceae). Consev. Genet., 10 (3): 793-795

Table 2 Overall heterozygosity (observed andexpected) through loci and species

Parameters	Mean overall loci	Mean overall species
H exp.	0.2262	0.2733
H nb.	0.2436	0.3037
Hobs.	0.2619	0.3333
P(0.95)	1.0000	1.0000
P(0.99)	1.0000	1.0000
A_n	2.0000	2.0000

H obs = Observed heterozygosity; Hexp = Expected heterozygisity; H nb = Heterozygosity (Non-biased) for small populations
$P_{(0.95)}$ = Polymorphism at 0.95 criteria; $P_{(0.99)}$ = Polymorphism at 0.99 criteria; A_n = Mean number of alleles per locus

Table 3 Genetic distance between three species of Rhizophora

Species	*Rhizophora annamalayana*	*Rhizophora mucronata*	*Rhizophora apiculata*
Rhizophora annamalayana	******		
Rhizophora mucronata	0.006	*****	
Rhizophora apiculata	0.010	0.008	*****

OBSERVATIONS:

Table 1 Details of the primers used in this study

Sl. No.	Locus Name	Donor Species	Base Sequences	Repeat Motif	Ta in recipient (^{0}C)	Reference
1	Rhstcp02	*Rhizophora stylosa*	CTTTCTTTTCTAATCCATTCG GTATGTGTCTCATATCTATCAC	T19	49	Islam et al. 2008
2	Rhstcp05	*Rhizophora stylosa*	CATAAATACTTTAACATAAATAAG GAAAAGAAGATATAGAAATTC	T10...A10	45	„
3	Rhstcp06	*Rhizophora stylosa*	GACTATCTCCACTATAGAAAG GGACGATCTATATGTAGAAAG	A12	47	„
4	Rhstcp07	*Rhizophora stylosa*	TTCGAACTGTTTCAAAGACCC AATCAGTTAATGAAAGAGCCC	A12	56	„
5	Rhstcp08	*Rhizophora stylosa*	ATCAACTTGCTATCGAACATC GAGCAATACGCCCCAAGTTC	A11	54	„
6	Kacacp07	*Kandelia candel*	TTAACTATTAACGTGTCATTC CCTCTCATGGTTCTCGGACAA	A8GA9	54	„
7	Kacacp11	*Kandelia candel*	TGCGACTATCTCCACTATA CCATAGAAGAAAGTATCTAGG	A9	51	„
8	Kacacp17	*Kandelia candel*	GTGAATCGTTAACGAAAAGAG CGACAACAAGTAAATTGATAG	T9GT10	52	„