Evaluation of genetic diversity of *Avicenniaceae* family in Indian sundarban by using RAPD and ISSR markers

Abdul Kader[1], Sankar Narayan Sinha[2], Parthadeb Ghosh[1*]

[1]Cytogenetics & Plant Biotechnology Research Unit, Department of Botany, University of Kalyani, Kalyani -741235, Nadia, West Bengal, India.
[2]Environmental Microbiology Research Laboratory, Department of Botany, University of Kalyani, Kalyani -741235, Nadia, West Bengal, India.
**Corresponding author, E-mail: pdggene@rediffmail.com. Mobile: +91 9163599264.*

Abstract
The present investigation was undertaken to describe the relationships among species of *Avicenniaceae* family which have both economic and medicinal activities, collected in Indian Sundarban by the help of RAPD and ISSR markers. In this study, three different species of *Avicennia* genus were collected. Frozen young leaves (-20^{0}C) were taken to isolate the genomic DNA using a slightly modified CTAB method. For this experiment, a set of 10 RAPD and 10 ISSR markers were used to analyse the genetic diversity. The study showed that ISSR markers were more efficient than RAPD markers for polymorphism detection, polymorphic bands content per primer and total no of loci detection per primer as they were 75.53%, 11.9 and 15.6, for ISSR and 69.04%, 9.6 and 13.7 for RAPD, respectively. However, the observed no of alleles, effective no of alleles, Nei's (1973) gene diversity and Shannon's information index (I) were higher for RAPD (1.6087, 1.6087, 0.3043 and 0.4219, respectively) than for ISSR (1.5357, 1.4356, 0.2345 and 0.3357, respectively). However, no such reports on genetic diversity using ISSR markers were available in the genus *Avicennia*. The results of this study can be seen as a starting point for future researches aimed to develop phylogenetic tree using more samples and more molecular markers.

***Keywords*:** *Avicenniaceae*, ISSR, Mangrove, RAPD, Sundarban.

INTRODUCTION

Sundarban is the largest deltaic complex of the world constitutes and an important coastal ecosystem, having many important plants both in terms of diversity in physiological characters and in biochemical constituents. These plants are gradually recorded as threatened or endangered in some recent surveys due to traditional anthropogenic uses and over exploitation (Gopal and Chauhan, 2006). In recent times, some reports appear in analysis and cataloguing the diversity of biochemical constituents and both for their antimicrobial and antitumerogenic activities. However, rarely systematic studies have been carried out towards the assessment of genetic diversity, their *ex situ* conservation or cataloguing the biochemical constituent of those threatened plants, especially in Sundarban delta of Indian territory.

The Black Mangrove family, *Avicenniaceae* Endl., includes a single genus, *Avicennia* L., with eight species worldwide and in Sundarban. The genus is represented by *Avicennia marina* (Forsk.) Vierh., *Avicennia officinalis* L., and *Avicennia alba* Blume. Plants of *Avicennia* are trees and woody shrubs distributed in coastal and estuarine habitats in tropical and subtropical areas, worldwide (Duke, 1991; Schwarzbach and McDade,

2002). They are characterized by high salt concentrations, low aeration of waterlogged soil, and frequently changing water levels due to tidal cycles that have been clearly evolved several times independently, within angiosperms (Ricklefs and Latham, 1993). The species of *Avicenniaceae* are highly tolerant to adverse environmental conditions (Tomlinson, 1994) and among them *A. marina* is the most frost resistant species (Chapman, 1977). This family can also provide systems for the production of valuable secondary metabolites, such as antibacterial and antiviral substances, commonly found in some mangroves (Al-Bahrany and Al-Khayri, 2003). The genus is uniform in its gross morphology and anatomy. There are often useful diagnostic characters seen in the field which are rarely transmitted to the labels of herbarium specimens (Tomlinson, 1986). The identification of these species is difficult due to many reasons including extensive intraspecific morphological variation, ecological adaptation, clinal differentiation, hybridization, gene flow and introgression.

Molecular markers, which are being widely used to quantify genetic diversity within and between populations, are not prone to environmental influences and portray genetic relationships between plant groups (Arif *et al.*, 2010; Oliveira *et al.*, 2010). Therefore, the improvement of this family is needed through the utilization of available genetic diversity. The evaluation of genetic diversity and the construction of linkage maps would promote the efficient use of genetic variations in the breeding programs (Paterson *et al*., 1991). DNA markers provide an opportunity to characterize genotypes and to measure genetic relationships more precisely than other markers (Soller and Beckmann, 1983; Vijay Rana *et al.*, 2012) such as RAPD and ISSR markers which are PCR based and used in the large scale genetic diversity studies (Saiki *et al.*, 1988; Moreno *et al.*, 1998; Wolfe *et al.*, 1998; Blair *et al.*, 1999; Martín and Sánchez-Yélamo, 2000). The research work on genetic variation among *Avicenniaceae* family using molecular or biochemical methods is scant. However, Parani *et al*., (1997) reported an intra and inter-specific variation in Avicennia genus, revealed by RAPD and RFLP markers from different location of India, except Sundarban. No studies have been reported on the genetic identities and diversity of *Avicenniaceae* family on Sundarban forest. Thus, this report will be the first on the genetic diversity using ISSR markers of the species of *Avicenniaceae* family.

Therefore, the objectives of this project were (1) to study and compare genetic diversity among 3 species, using RAPD and ISSR markers, and (2) to detect interspecific genetic variations at the DNA level and (3) to evaluate the degree of polymorphism generated from each technique as a pre-requisite for their applicability to population genetics studies.

MATERIALS AND METHODS

Plant materials

The samples were collected from Gosaba region of Indian Sundarban forest in the month of June, 2011. The voucher specimens (170/BOT/ KUH) were deposited and preserved in the Department of Botany, University of Kalyani, Kalyani West Bengal, India, for reference.

Genomic DNA isolation and PCR amplification

Genomic DNA of 3 genotypes was extracted from frozen leaves (-20°C) following a CTAB method (Doyle and Doyle, 1987) with slight modifications. After purification, it was quantitified spectroscopically and visualized under a UV light after electrophoresis on a 0.8% (w/v) agarose gel stained by 0.5 µg/ml ethidium bromide. The resuspended DNA was stored in autoclaved ddH_2O. A total of 10 RAPD (Table 1) and 10 ISSR primers (Table 2) (Bangalore Genei Pvt. Ltd., Bangalore, India) were screened. PCR amplifications were carried out in a thermal cycler (Perkin Elmer, Gene Amp thermal cycler 2400) in a final volume of 25 µl, containing 25 ng template DNA, 200 µM each of the four dNTPs, 10 picomoles of primers, 10 mM $MgCl_2$, 2.5 µl Taq buffer (10 mM Tris HCl pH 9.0, 50 mM KCl) and 0.2 Unit Taq DNA polymerase (Bangalore Genei Pvt., Ltd., Bangalore India). The samples were subjected to initial denaturation for 3 min at 94°C, followed by 40 cycles (for RAPD) or 35 cycles (for ISSR) of 1 min at 94°C, 1 min at 36°C for RAPD and 36-64°C for ISSR (for different primers different annealing temperatures were used), and an extension for 2 min at 72°C with a final extension of 7 min at 72°C. Ten µl of amplified PCR product was separated by gel electrophoresis on a 1.8% agarose gel stained by 0.5 µg/ml ethidium bromide and photographed with a gel documentation system (Uvi Tec, UK).

Analysis of RAPD and ISSR profiles

DNA fragment sizes on agarose gel were estimated by comparing with 1 kb λDNA size markers. The banding patterns obtained from RAPD and ISSR were scored as present (1) or absent (0), each of which was treated as an independent character regardless of its intensity, to create the similarity matrix. Jaccard's similarity coef-

Table 1. List of random amplified polymorphic DNA (RAPD) primers.

Primer name	Sequence (5'-3')	Total No. of bands	No. of polymorphic bands	% of polymorphic bands	PIC value
RAPD 01	AAATCGGAGC	15	12	80.00	0.26
RAPD 02	GTCCTACTCG	16	10	62.50	0.15
RAPD 03	TGCGCGATCG	09	06	66.67	0.18
RAPD 04	GCACGCCGGA	11	08	72.73	0.22
RAPD 05	CTATCGCCGC	18	15	83.33	0.31
RAPD 06	CGGGATCCGC	10	06	60.00	0.19
RAPD 07	GCGAATTCCG	14	08	57.14	0.27
RAPD 08	GCACGCCGGA	17	13	76.47	0.18
RAPD 09	GTCCTTAGCG	12	07	58.33	0.12
RAPD 10	AACGTACGCG	15	11	73.33	0.14
Total		137	96	690.47	2.01
Avarage		13.7	9.6	69.04	0.20

Table 2. List of integrated simple sequence repeat (ISSR) markers primers.

Primer name	Sequence (5'-3')	Total No. of bands	No. of polymorphic bands	% of polymorphic bands	PIC value
ISSR 01	$(CT)_{10}$ GAC	16	12	75.00	0.16
ISSR 02	$(GA)_8T$	23	18	78.26	0.31
ISSR 03	$(TG)_8GA$	13	09	69.23	0.22
ISSR 04	$(CA)_8GT$	19	16	84.21	0.19
ISSR 05	$(GCA)_8AG$	12	09	75.00	0.23
ISSR 06	$(CTG)_8G$	15	10	66.67	0.15
ISSR 07	$(AG)_3GT$	18	13	72.22	0.14
ISSR 08	$(GACA)_4T$	09	06	66.67	0.03
ISSR 09	$(AT)_6C$	17	14	82.35	0.15
ISSR 10	$(GCA)_3GC$	14	12	85.71	0.14
Total		156	119	755.32	1.71
Avarage		15.6	11.9	75.53	0.17

ficient (J) was used to calculate the similarity matrix.

POPGENE 32 software was used to calculate Nei's unbiased genetic distance, the observed number of alleles (n_o), the effective number of alleles (n_e) and Shannon's information index (I). The polymorphic information content (PIC) was calculated by applying the following formula given by Powell *et al.,* (1996) and Smith *et al.,* (1997):

$PIC = 1 - fi^2$

$i = 1 – n$

Where, fi is the frequency of the i^{th} amplicon. The number of amplicons refers to the number of scored bands. The frequency of an amplicon was obtained by dividing the number of species, where it was found by the total number of species. The PIC value provides an estimate of the discriminating power of a marker.

RESULTS

A set of 10 RAPD with PIC values (Table 1) and 10 ISSR with PIC values (Table 2) primers were used for amplification and each primer gave the polymorphic banding pattern.

Table 3. Genetic variability as discerned through random amplified polymorphic DNA (RAPD) and integrated simple sequence repeat (ISSR) markers.

Parameter	Diversity value by RAPD marker	Diversity value by ISSR marker
Observed number of alleles (no)	1.61	1.54
Effective number of alleles (ne)	1.61	1.44
Nei's gene diversity (h)	0.30	0.24
Shannon's information index (I)	0.42	0.34

Table 4. Similarity matrix of random amplified polymorphic DNA (RAPD) and integrated simple sequence repeat (ISSR) markers profile.

	p1 RAPD	p1 ISSR	p2 RAPD	p2 ISSR	p3 RAPD	p3 ISSR
p1	1.00	1.00	0.38	0.63	0.39	0.67
p2	0.38	0.63	1.00	1.00	0.64	0.58
p3	0.39	0.67	0.64	0.58	1.00	1.00

Table 5. Similarity matrix for pooled random amplified polymorphic DNA (RAPD) and integrated simple sequence repeat (ISSR) markers data.

	p1	p2	p3
p1	1.00	0.51	0.54
p2	0.51	1.00	0.63
p3	0.54	0.63	1.00

The results showed that RAPD markers (Table 3) were more efficient than the ISSR markers. The observed number of alleles, the effective number of alleles, Nei's gene diversity and Shannon's information index (I) were higher for RAPD (1.6087, 1.6087, 0.3043 and 0.4219, respectively) than for ISSR (1.5357, 1.4356, 0.2345 and 0.3357, respectively). But, the average number of polymorphic bands per primer and the total number of loci detected per primer were higher for ISSR (11.9 and 15.6, respectively, Table 2) than for RAPD (9.6 and 13.7, respectively, Table 1). Also the degree of polymorphism was higher, 75.53% for ISSR (Table 2) than for RAPD markers, 69.04%, (Table 1).

Genetic similarity was calculated by the Nei's similarity index value considering ISSR and RAPD approaches individually, as well as together. Based on the ISSR marker system, the similarity index values ranged from 0.577 to 1.00 (Table 4) while on the basis of RAPD markers it ranged from 0.381 to 1.00 (Table 4). Similarity indices values based on both the marker systems together ranged from 0.510 to 1.00 (Table 5) indicating more diversity in the case of RAPD. Though, the highest polymorphic information content (PIC) was the same for both the primers (RAPD 05 and ISSR 02 primers, Tables 1 and 2, respectively, Figure 1) i.e., 0.310 but it varied for RAPD and was from 0.123 to 0.310 and 0.031 to 0.310 for ISSR. Therefore, according to the PIC values ISSR primers seem to be better than RAPD ones. However, all these values were not significantly different from each other.

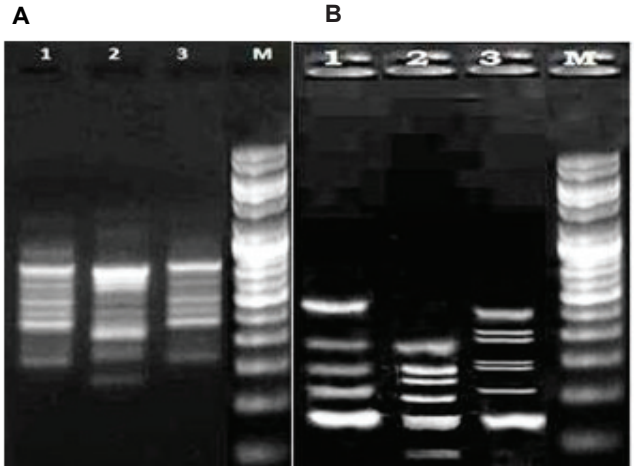

Figure 1. Genomic DNA amplification pattern in three species viz., *Avicennia marina*, *Avicennia officinalis* and *Avicennia alba* with **A:** ISSR primers on left side and **B:** RAPD primerson right side. Lane1: *Avicennia marina*; Lane2: *Avicennia oflcinalis*; Lane3: *Avicennia alba;* M: Marker.

DISCUSSION

The present study offers an optimization of primer screening for the evaluation of genetic relationships among *Avicenniaceae* species collected from Sundarban. Polymorphism in a given population is often due to the existence of genetic variants, represented by the number of alleles at a locus and their frequency of distribution, in a population. Heterozygosity corresponds to a probability that two alleles taken at random from a population can be distinguished, using the marker in

question. Thus, a convenient quantitative estimate of marker utility and the polymorphism detected can be given in terms of the Nei's genetic diversity (h) and Shannon's information index (I). In this work, we compared the applicability of ISSR and RAPD as genetic markers to characterize 3 species of Avicennia genus, *Avicenniaceae* family. However, no such reports on genetic diversity using ISSR markers were available in the genus *Avicennia.* Two marker systems, ISSR and RAPD used in the present study have also been used as effective tools to evaluate genetic diversity and in the future it will focus light on the phylogenetic relationships. The RAPD technique has been widely used both for studies on wild plants (Yeh *et al.*, 1995; Khasa and Dancik, 1996; Manica-Cattani *et al.*, 2009) and for studies on cultivated plants (Sharma and Dowsons, 1995; Yilmaz *et al.*, 2012). By contrast, researches employing the ISSR technique have mainly focused on cultivated species (Moreno *et al.*, 1998; Wang *et al.*, 1998; Blair *et al.*, 1999). In this study, it could be concluded that the ISSR is also an effective molecular tool to calculate the genetic diversity on wild plants like mangrove species. This method is also useful in providing the background necessary for the effective management and conservation of forest trees. Though RAPD has been mostly used for diversity analysis because it is simple and fast, but there are questions about reproducibility, utility in genetic diversity analyses, mapping and genotype identification. In the case of ISSR, certain studies indicated that it produces more reliable and reproducible bands because of its higher annealing temperatures and longer primer sequences which consider it to be superior to RAPD (Singh *et al.*, 2011). ISSR also proved to be useful in diversity studies, gene mapping, germplasm identification and the characterization of gene bank, as well as the identification of closely related species (Singh *et al.*, 2011). In addition, when geographical coordinates and genetic differentiation are combined, such markers can detect changes in the gene flow in forest trees (Lu *et al.,* 2005). Both ISSR and RAPD markers are equally efficient marker systems in *Avicenniaceae* family because of their capacity to reveal several informative bands in a single amplification. Such studies on genetic diversity, especially intraspecific and interspecific variation, can contribute to the development of conservation strategies by identifying units for conservation (Chokchaichamnankit *et al.*, 2008). The conservation strategy also leads to preserve the genetic variation and the evolutionary process in viable populations of ecologically and commercially valuable varieties (genotypes), in order to prevent potential extinction.

The results of the present study can be seen as a starting point for future researches aimed at defining the level of inter-specific genetic diversity to create a phylogenetic tree. These studies have given important clues in understanding genotype relationship, which may further assist in developing and planning breeding strategies and for conservation of genetic resources.

ACKNOWLEDGEMENTS

The authors are grateful to University Grant Commission (UGC), New Delhi, an organ of the government of India for providing financial assistance to the first author. They are extending their thanks to the head of the department of botany of University of Kalyani for providing DST-FIST central equipment facility. They are also expressing their thanks to Sinchan Adhikari a research scholar of the department of botany of University of Kalyani for his help in analysis the result.

REFERENCES

Al-Bahrany A. M., and Al-Khayri J. M. (2003). Micropropagation of grey mangrove *Avicennia marina*. *Plant Cell Tissue and Organ Culture*, 72: 87-93.

Arif M., Zaidi N. W., Singh Y. P., and Haq Q. M. R., (2010). A comparative analysis of ISSR and RAPD markers for study of genetic diversity in *shisham* (*Dalbergia sissoo*). *Plant Molecular Biology Reports*, 27: 488-495.

Blair M. W., Panaud O., and Mccouch S. R. (1999). Inter-simple sequence repeat (ISSR) amplification for analysis of microsatellite motif frequency and fingerprinting in rice (*Oryza sativa* L.*). Theoretical and Applied Genetics*, 98: 780-792.

Chapman V. J. (1977). Introduction In Ecosystems of the World. vol. 1. In V. J. Chapman (Eds), Wet coastal ecosystems (pp. 3-15). New York: *Elsevier*.

Chokchaichamnankit P., Chulalaksananukul W., Phengklai C., and Anamthawat-Jónsson K. (2008). Species and genetic diversity of *Fagaceae* in northern thailand based on ISSR markers. *Journal of Tropical Forest Science,* 20: 8-18.

Doyle J. J., and Doyle J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bulletin*, 19: 11-15.

Duke N. C. (1991). A systematic revision of the mangrove genus *Avicennia* (*Avicenniaceae*) in australasia. *Australia Systems Botanique,* 4: 299-324.

Gopal B., and Chauhan M. (2006). Biodiversity and its conservation in the sundarban mangrove ecosystem*. Aquatic Sciences*, 68: 338-354.

Khasa P. D., and Dancik B. P. (1996). Rapid identification of white-engelman spruce species by RAPD markers. *Theoretical and Applied Genetics*, 92: 46-52.

Lu D. P., Read R. L., Humphreys D. T., Battah F. M., Martin

D. I. K., and Rasko J. E. J. (2005). PCR-based expression analysis and identification of micro RNAs. *Journal RNAi Gene Silenc*, 1: 44-49.

Manica-Cattani M. F., Zacaria J., Pauletti G., Atti-Serafini L., and Echeverrigaray S. (2009). Genetic variation among south Brazilian accessions of *Lippia alba* Mill. (*Verbenaceae*) detected by ISSR and RAPD markers. *Brazilian Journal of Biology*, 69: 375-380.

Martín J. P., and Sánchez-Yélamo M. D. (2000). Genetic relationships among species of the genus *Diplotaxis* (*Brassicaceae*) using inter-simple sequence repeat markers. *Theoretical and Applied Genetics*, 101: 1234-1241.

Moreno S., Martin J. P., and Ortiz J. M. (1998). Inter-simple sequence repeat PCR for characterization of closely related grapevine germplasm. *Euphytica,* 101: 117-125.

Nei M. (1973). Analysis of gene diversity in subdivided populations*, Proceedings of the National Academy of Sciences of the United States of America*, 70: 3321-3323.

Oliveira E. C., Junior A. T., Goncalves L. S., and Pena G. F. (2010). Optimizing the efficiency of the touchdown technique for detecting inter-simple sequence repeat markers in corn (*Zea mays*). *Genetics and Molecular Research*, 9: 835-842.

Parani M., Lakshmi M., Elango S., Ram N., Anuratha C. S., and Parida A. (1997). Molecular phylogeny of mangroves 11. Intra- and inter-specific variation in *Avicennia* revealed by RAPD and RFLP markers. *Genome*, 40: 487-495.

Paterson A. H., Tanksley S. D., and Sorrells M. E. (1991). DNA markers in plant improvement. *Advances in Agronomy,* 46: 39-90.

Powell W., Morgante M., Andre C., Hanfey M., Vogel J., Tingey S., and Rafalski A. (1996). The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding*, 2: 225-228.

Rana V., Thakur K., Sood R., Sharma V., and Sharma T. R. (2012). Genetic diversity analysis of *Tinospora cordifolia* germplasm collected from northwestern Himalayan region of India. *Journal of Genetics*, 91: 99-103.

Ricklefs R. E., and Latham R. E. (1993). Global patterns of diversity in mangrove floras. In R. E. Ricklefs and D. Schluter (Eds), *Species diversity in ecological communities*: historical and geographical perspectives (Pp. 215-229). Chicago: University of Chicago Press.

Saiki R. K., Gelfond D. H., Stoffel S., Scharf S. J., and Higuchi R. (1988). Primer-directed enzymatic amplification of DNA with a thermo-stable DNA polymerase*. Science*, 239: 487-499.

Schwarzbach A. E., and McDade L. A. (2002). Phylogenetic relationships of the mangrove family *Avicenniaceae* based on chloroplast and nuclear ribosomal DNA sequences. *System Botanique*, 27: 84-98.

Sharma S. K., Dowsons I. K., and Waugh R. (1995). Relationships among cultivated and wild lentils revealed by RAPD analysis. *Theoretical and Applied Genetics*, 91: 647-654.

Singh D. R., Srivastava A. K., Srivastava A., and Srivastava R. C. (2011). Genetic diversity among three *Morinda* species using RAPD and ISSR markers. *Industrial Journal of Biotechnology,* 10: 285-293.

Smith J. S. C., Hin E. C. L., Shu H., Smith O. S., Wall S. J., Senior M. L., Mitchell S.E., Kresovich S., and Ziegle J. (1997). An evaluation of the utility of SSR loci as molecular markers in maize (*Zea mays* L.): Comparisons with data from AFLPs and pedigree. *Theoretical and Applied Genetics,* 95: 163-173.

Soller M., and Beckmann J. S. (1983). Genetic polymorphism in varietal identification and genetic improvement. *Theoretical and Applied Genetics*, 67: 25-33.

Tomlinson P. B. (1986). The botany of mangroves. Cambridge: Cambridge University Press.

Tomlinson P. B. (1994). The botany of mangroves. Cambridge: Cambridge University Press.

Wang G., Mahalingam R., and Knap H. T. (1998). (C-A) and (G-A) anchored simple sequence repeats (ASSRs) generated polymorphism in soybean, *Glycine max* (L.). *Merr Theoretical and Applied Genetics*, 96: 1086-1096.

Wolfe A. D., Xiang Q. Y., and Kephart S. R. (1998). Assessing hybridization in natural populations of Penstemon (*Scrophulariaceae*) using hypervariable intersimple sequence repeat (ISSR) bands. *Molecular Ecology*, 7: 1107-1125.

Yeh F. C., Chong D. K. X., and Yang R. C. (1995). RAPD variation within and among natural populations of *Trembling aspen* (*Populus tremuloides* Michx.) from alberta*. Journal of Heredity*, 86: 454-460.

Yilmaz K. U., Paydas-Kargi S., Dogan Y., and Kafkas S. (2012). Genetic diversity analysis based on ISSR, RAPD and SSR among Turkish apricot germplasms in Iran Caucasian eco-geographical group. *Scientia Horticulturae*, 138: 138-143.