

**RAPD AND SSR ANALYSIS OF AFFORESTED *SONNERATIA APETALA*
BUCH.-HAM. POPULATION FROM THE COASTAL AREAS
OF BANGLADESH**

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Abstract

Random amplification of polymorphic DNA (RAPD) and simple sequence repeats (SSR) primers were used to estimate and compare the genetic diversity within and among populations of *Sonneratia apetala* Buch.-Ham. from the mangrove forests in the coastal islands of Bangladesh. A total of 15 individuals were studied which produced a total of 32 bands from 05 random RAPD primers and 11 allele fragments from 05 SSR primers. At the species level, SSR primers detected relatively less polymorphic loci (40%) than RAPD primers (56.25%). Both primers revealed a moderate level of genetic diversity (average $I = 0.310$ for RAPD and average $H_o = 0.239$ for SSR). At the population level, Nijhum Dwip population contained a low level of genetic diversity compared to the other populations that might be due to selfing within population. Therefore, seed collection from Nijhum Dwip for further afforestation should be avoided.

Introduction

Bangladesh has 436570 hectares of mangrove forest representing about 3.2% of global mangroves (Giri *et al.* 2011). *Sonneratia apetala* Buch.-Ham. is the most abundant mangrove species with high adaptability and seed production in the natural mangrove forest in Bangladesh (Iftekher and Saenger 2008). It is a pioneer, true woody, and crypto-viviparous mangrove species (Tomlinson 1986). This species has been widely planted and the most successful in the mangrove afforestation program in the coastal regions and offshore islands, including Chittagong, Noakhali, Bhola, Barisal and Patuakhali in Bangladesh since 1966 (Saenger and Siddiqi 1993). The core objectives of the mangrove afforestation program involve to the protection of the residents of shoreline and their property against natural disasters. Monospecific stands of *S. apetala* dominated about 80% areas of the early plantations due to high survival and growth rate (Saenger and Siddiqi 1993). Seedlings grown in the nursery bed from the collected seeds are planted from July to August (Saenger and Siddiqi 1993). Afforestation strategies, including suitable plantation sites, the quality of planting materials (i.e., seeds and seedlings), post-establishment are not carefully maintained yet (Rahman *et al.* 2010). The baseline information on the status of planting materials is required to improve the understanding of mangrove species conservation (Rao and Hodgkin 2001).

Genetic diversity is a crucial component for the long-term survival, adaptation, and evolution of species (Maguire *et al.* 2002). Various evolutionary forces such as mutation, natural selection, genetic drift, and gene flow affect the genetic diversity of the population, thereby enhancing or reducing the adaptive ability of populations to an environment (Ratnam *et al.* 2014). Besides, demographic factors, including the quantity of pollen, female flower, flowering synchronicity and geographical distributions along with demographic processes such as mating systems, bottlenecks and long distance dispersal contribute to shaping the genetic diversity and structure of a forest population (Vekemans and Hardy 2004). Development of PCR-based DNA marker methods

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opens up a new window to address those intricate issues in the population genetics studies. Random amplification of polymorphic DNA (RAPD) is dominant marker decoded by the presence-absence coding of a distinct locus. RAPD marker method is easy, cost effective and does not require any prior genome sequencing knowledge (Triest 2008). Simple sequence repeats (SSR) or microsatellites gives the exact designation of a known locus whether it is heterozygous or homozygous, and infers some discerning attributes, including hyper-variability, multiallelic nature, codominant inheritance, reproducibility etc. making them the best codominant markers in population genetic studies (Parida *et al.* 2009). Cross-species amplification nature of SSR marker enables to use in the genetic analysis among closely related species (Lakshmi *et al.* 2002). Therefore, both SSR and RAPD marker are widely used in the study of population genetics, including phylogenetic relationship, gene mapping, linkage mapping, and marker assisted selections, backcrosses etc. (Triest 2008). Despite the great concern in conservation and management of mangrove forest genetic resources, a very limited research have been conducted in which most of the studies was confined to the genus *Avicennia* (Triest 2008). *Sonneratia* sp. is the least studied mangrove genus in population genetics, however, a few studies on phylogenetic study (Shi *et al.* 2000), natural hybridization (Qui *et al.* 2008), and speciation (Zhou *et al.* 2007) using DNA primer have been documented in the literature. The baseline information on the status of the genetic composition of this species across different natural and afforested mangrove forest remains undocumented in Bangladesh till date.

Although afforestation of mangrove species has been conducted in Bangladesh, there is no baseline information regarding genetic diversity of these species available. Therefore, in this study, it is aimed to determine and compare the genetic diversity of *S. apetala* using both RAPD and SSR primers in the afforested mangrove forests in Bangladesh.

Materials and Methods

The plant materials used in this study were collected from the three geographically distant afforested mangrove sites (population), namely Sonadia Island (21°30'43" N and 91°52'40" E) of Cox's bazar, Nijhum Dwip (21°35'0" N and 92°01'0" E) and Tomuruddin (22°20'31.41" N and 90°59'12.92" E) of Hatia in Noakhali District facing to the Bay of Bengal, Bangladesh. Young leaves from 10 individuals of *S. apetala* were randomly collected from each population and stored at -80°C in a refrigerator.

Genomic DNA from fresh leaves of each individual was extracted by soaking with liquid nitrogen in a mortar pestle in aseptic condition with CTAB methods (Doyle and Doyle 1987). For both RAPD and SSR analysis, 50 ng/μl of genomic DNA was used for PCR amplification. 5 RAPD and 5 SSR primers were selected for assessing the genetic diversity of the plant species (Table 1). Each 25 μl of polymerase chain reaction (PCR) mixture consisted of 2.0 μl template DNA (50 ng/μl) followed by 2.5 μl of *Taq* buffer A 10X (Tris with 15 mM MgCl₂), 1.0 μl of the primer, 0.5 μl of dNTPs 2.5 mM, 0.2 μl of 5 Unit/μl *Taq*DNA polymerase enzyme and 18.8 μl deionized sterile distilled water. The PCR amplification was performed in an oil free thermal cycler (Biometra, UNO II). For random RAPD primers, the DNA was subjected to initial denaturation at 95°C for 5 min. In the following 35 cycles, the denaturation was at 94°C for 1 min, RAPD primers annealing period 30 sec at 34°C and primer extension at 72°C for 3 min. The temperature for each primer annealing period was adjusted based on the melting temperature of the individual primers. Last cycle of final extension at 72°C for 7 min was performed. For SSR primers, initial denaturation was at 94°C for 2 min followed by 35 cycles of further denaturation, annealing, and extension were at 94, 55 and 72°C for 30 sec, respectively. In addition, one final extension was performed at 72°C for 8 min. After completion of cycling program, the reactions

were kept at 4°C. The amplified PCR products were separated by electrophoresis using 1% agarose gel containing ethidium bromide (0.5 µg/ml) and 100 ml 1×TAE buffer. Agarose gel electrophoresis was conducted in 1× TAE buffer at 50 volts and 100 mA for 1.5 hrs. One molecular 1.0 kb plus DNA ladder was electrophoresed alongside the RAPD and SSR reaction samples. DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system (CSL-Microdoc System, Cleaver Scientific Ltd.).

RAPD phenotypes for each locus were scored as present (1) or absent (0) for each individual as a binary data matrix. While SSR primers were scored as homozygous or heterozygous alleles. The band size was scored by comparison with 1 Kb plus DNA ladder (E-Gel®, Life Technologies). Genetic diversity indices were calculated after deleting 06 individuals, which had unsuccessful amplified bands or missing bands from RAPD data and 05 duplicate multilocus genotypes (MLGs) from SSR data. Therefore, a total of 9 individuals from RAPD data and 10 individuals from SSR data were selected for genetic diversity analysis. Both data sets were analyzed using software program Gen Alex version 6.0 (Peakell and Smouse 2006) as a dominant and codominant mode of inheritance in a diploid organism for RAPD and SSR primers, respectively, making the assumption that the populations were in Hardy-Weinberg equilibrium. The following parameters were analyzed to determine genetic diversity within and among populations: the number of polymorphic bands (NPB), percentage of polymorphic bands (PPB), number of alleles (N_a) per locus per population, effective number of alleles (N_e), Shannon's information index (I), observed heterozygosity (H_o), and expected heterozygosity (H_e).

Results and Discussion

A total of 30 individuals were studied of which 15 were discarded after the DNA extraction procedure due to the absence of DNA. Therefore, a total of 15 individuals, *i.e.* 05 individuals from each of the three populations were considered for further analysis. All the studied primers (05 SSR and 05 RAPD) produced amplified bands but the reproducibility of each primer varied from individual to individual. For RAPD primers, a total of 06 individuals of Sonadia Island (SON4 and SON5), Nijhum Dwip (NIJ2, NIJ3, NIJ4) and Tomuruddin (TOM5) had no amplified bands of any of five RAPD primers. Whereas, all individuals showed amplified bands of at least one SSR primer. An example for the banding pattern for the selected RAPD (OPK-08) primer and SSR (Sc_SSR_01) primer is shown in Fig. 1.

RAPD primers produced a total of 32 reliable bands from 15 individuals (Table 1). The number of bands per primer ranged from 3 to 10 with an average of 6.4. The size of amplified fragments ranged from 200 to 1850 bp (Table 1). The average number of band was relatively low when compared to the average band locus per primer of 17.2 in the genus *Rhizophora* using the same RAPD primers (Jagadeesh *et al.* 2012). On the other hand, from five SSR primers, a total of 11 alleles were produced ranged from 1 to 3, with an average of 2.2, which ranged between 75 and 230 bp (Table 1). These results are consistent with the previous cross-species amplification study on both *Sonneratia caseolaris* (L.) Engl. and *S. alba* Sm. (Ouyang *et al.* 2011).

At the population level, the highest polymorphism regarding RAPD primers (93.75%) with 30 polymorphic bands found in Sonadia Island population followed by Tomuruddin (50%) with 16 polymorphic bands and Nijhum Dwip population (25%) with 11 polymorphic bands (Table 2). The numbers of observed and effective alleles were found to be higher in the Sonadia Island population (1.875 and 1.680, respectively) (Table 2) and lower in the Nijhum Dwip population (0.594 and 1.177, respectively) (Table 2). The mean Shannon's information index (I) for RAPDs was likely to be relatively higher in the Sonadia Island (0.555) population followed by Tomuruddin (0.230) and Nijhum Dwip population (0.151) (Table 2).

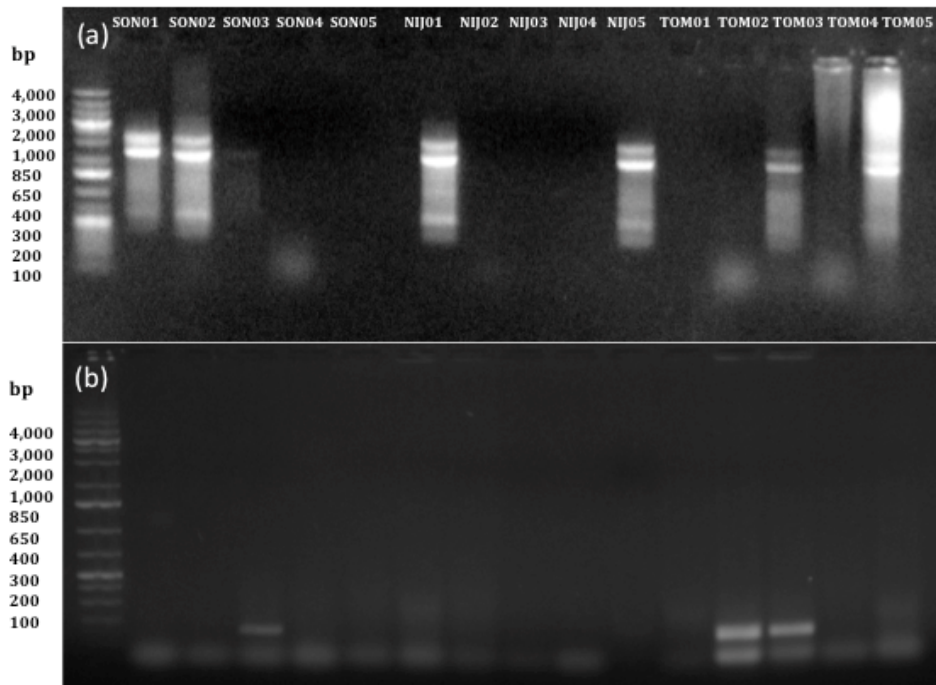


Fig. 1. Overview of the amplified bands of the selected primers: (a) OPK-8 (RAPD primer) and (b) Sc_SSR_01 (SSR primer).

Table 1. Repeat motifs, ranges of amplicons, number and ranges of bands with 5 RAPD and 5 SSR primers used on *Sonneratia apetala*.

Primers	Repeat motifs	Range of amplicons (bp)	Number of bands scored	Range of bands with size (bp)	References
OPK-08	GAACACTGGG	2500 - 480	3	1850 - 450	Jagadeesh <i>et al</i> (2012)
OPL-05	ACGCAGGCAC	2020 - 490	6	900 - 300	"
OPL-18	ACCACCCACC	5000 - 480	10	1800 - 200	"
OPM-20	AGGTCTTGGG	3000 - 200	6	1000 - 400	"
OPQ-09	GGCTAACCGA	3800 - 280	7	750 - 300	"
Sc_SSR_1	(AAG) ₈	250	3	98 - 200	Ouyang <i>et al.</i> (2011)
Sc_SSR_2	(GAT) ₅ (GCC) ₅	187	3	77 - 161	"
Sc_SSR_3	(AGGGA) ₆	250	1	230	"
Sc_SSR_4	(TGAGCG) ₆	161	2	75 - 79	"
Sc_SSR_5	(CT) ₁₈	169	2	95 - 99	"

Shannon's information index (I) derived from RAPD data is to be considered as an effective measure for analyzing genetic diversity because this can preclude the bias into data by the inability to detect the intra-locus variation (Parani and Parida 1997). Shannon's information index of diversity in *S. apetala* (I = 0.312) was found to be higher when compared to the other species like *Bruguiera gymnorrhiza* L. (I = 0.1192) and *Heritiera fomes* Buch.-Ham. (I = 0.0874) using 13

different RAPD primers in Indian mangrove swamps obtained by Dasgupta *et al.* (2015). The mean Shannon's information index was also higher than the average expected diversity ($H_e = 0.210$). Unlike RAPD analysis, the highest polymorphism (80%) was detected in Tomuruddin population for SSR primers followed by 40% polymorphic loci in Sonadia Island population (Table 2). Surprisingly, all SSR loci detected from Nijhum Dwip population were monomorphic with the same number of observed and effective allele (0.400) and nearly fixed inferring to high level of inbreeding. Such homozygous condition was observed in the natural population of *S. alba* of Hainan (China) and Queensland (Australia) (Ouyang *et al.* 2011). Except Nijhum Dwip population, the genetic diversity indices varied between the other two populations. Such as observed number of alleles, the effective number of alleles and Shannon's information index of diversity were 1.80, 1.53 and 0.549, respectively for Tomuruddin, and 1.20, 0.976 and 0.188, respectively for Sonadia Island (Table 2). The mean observed and expected heterozygosity was comparatively higher in Tomuruddin ($H_o = 0.567$ and $H_e = 0.356$) than that of Sonadia Island population ($H_o = 0.150$ and $H_e = 0.119$) (Table 2).

Table 2. Genetic diversity statistics of *Sonneratia apetala* (Mean \pm SE): NPB, number of polymorphic band; PPB, percentage of polymorphic band; N_a , observed number of alleles; N_e , effective number of alleles; I, Shannon's information index; H_e , expected heterozygosity; H_o , observed heterozygosity.

Population	NPB	PPB	N_a	N_e	I	H_e	H_o
RAPDs							
Sonadia Island	30	93.75	1.875(0.087)	1.680(0.055)	0.555(0.031)	0.381(0.024)	-
NijhumDwip	11	25	0.594 (0.155)	1.177 (0.055)	0.151 (0.047)	0.104 (0.032)	-
Tomurruddin	16	50	1.00(0.180)	1.214 (0.045)	0.230 (0.043)	0.144 (0.028)	-
Mean	19	56.25	1.156 (1.00)	1.357 (0.038)	0.312 (0.030)	0.210 (0.020)	-
SSRs							
Sonadia Island	2	40	1.200 (0.374)	0.976 (0.268)	0.188(0.119)	0.119(0.077)	0.150(0.100)
Nijhum Dwip	0	0	0.400 (0.245)	0.400 (0.245)	0	0	0
Tomurruddin	4	80	1.80 (0.490)	1.530(0.440)	0.549 (0.169)	0.356 (0.106)	0.567(0.194)
Mean	3	40	1.133(0.256)	0.969 (0.215)	0.246 (0.088)	0.158 (0.057)	0.239(0.093)

However, at the species level, the mean observed heterozygosity (0.239) was higher than the mean expected heterozygosity ($H_o = 0.158$) revealing a considerable level of genetic diversity that might result in random mating within populations. The observed genetic diversity of *S. apetala* indicated a moderate level of genetic variation (average $H_o = 0.239$), compared with the observed heterozygosity detected in the other mangrove species in the natural forest such as Colombian *Avicennia germinans* L. population (average $H_o = 0.390$) (Salas-Leiva *et al.* 2009) and *Rhizophora mangle* L. (average $H_o = 0.494$) (Arbelaez-Cortes *et al.* 2007) using microsatellite loci attributed to random mating populations. Frankham *et al.* (2007) reported that the survival rate of heterozygotes with an ageing of individual plant enable woody plants to retain historic genetic variations. Therefore, a moderate level of genetic diversity in both Sonadia Island and Tomurruddin population might result in random mating within and among population along with high survival rate of heterozygotes of the mother tress. While least or no genetic diversity in Nijhum Dwip population might result in selfing within populations. Selfing may reduce the effective number of population and thereby, favoring local bi-parenting inbreeding and genetic drift (Loveless and Hamrick 1984). There are two possible reasons for the attainment of least or no genetic diversity of Nijhum Dwip population: (i) the planting materials collected from plus trees or a limited number of mother trees that accumulated similar alleles in the planted trees, and (ii) the sampling effect i.e., individuals with low allelic variation might be sampled. Since the forest

department usually collect seeds for new plantation program along the coastal areas, care should be taken to avoid seed collection from the Nijhum Dwip island which might further aggravated the inbreeding depression and enervate further regeneration of *S. apetala* in the long run.

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