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Research Article

Genetic Variation of Three Bruguiera Species from Karimunjawa Islands Detected by Using RAPD Molecular Markers

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Abstract

Background and Objective: *Bruguiera* is a mangrove species which commonly lives in the mangrove forest of Kemujan Island, Karimunjawa, Indonesia. The aim of this study was to evaluate the genetics of three morphologically similar brugueira and to determine the genetic relationship of the species. **Methodology:** Three morphologically similar species of *Bruguiera cylindrica*, *Bruguiera gymnorrhiza* and *Bruguiera lyliadica* were analyzed by RAPD (Random Amplified Polymorphic DNA) markers. DNA amplification was performed using five specific primers (OPA-01, OPA-02, OPA-03, OPA-04, OPA-05), PCR products were then analyzed using a Gel Analyzer and a dendogram generated numerically by the SIMQUAL association (Similarity for Qualitative) coefficient grouping method covered by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and computed with the NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) programme. **Results:** Fifteen DNA polymorphism types were obtained, ranging from 100-400 bp, with primers OPA-01 and OPA-03 generating the most RAPD products, ranging in size from 154-384 bp. **Conclusion:** This study makes the first attempt to broaden existing knowledge of the three *Bruguiera* on the island of Kemujan are genetically different. The dendogram patterns of the three species could be grouped into two clusters, with *B. cylindrica* and *B. gymnorrhiza* being more closely related than *B. lyliadica*.

Key words: *Bruguiera cylindrica*, *Bruguiera gymnorrhiza*, *Bruguiera lyliadica*, mangrove species, genetic relationship, RAPD and dendogram patterns

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Karimunjawa is a National Park in Indonesia which has an area of mangrove forest of approximately[1] 222.20 ha. Geographically, Karimunjawa is located at coordinates of 5°42'-6°00' SL, 110°07'-110°37'EL. This natural mangrove vegetation grows on almost all the small islands around Karimunjawa and the mangroves in Karimunjawa National Park are highly diverse[1,2], with 44 mangrove species identified in 25 families[1]. One of the most abundant areas is on the island of Kemujan, which has the largest mangrove forest[1,3].

The RAPD (random amplified polymorphic DNA) technique[1] has been used to study the genetic diversity of *Bruguiera* in Sri Lanka. A study of the effect of climate on the genetic diversity of *Bruguiera* species that grow along the Sri Lankan river showed that there was not a clear relationship between genetic differences and macrogeographic variations along the western and southern coastal areas of Sri Lanka, but that the small population of *Bruguiera seksangula* is genetically different. RAPD and RFLP (Restriction Fragment Length Polymorphism) molecular analyses have been used to analyze genetic variation of 31 mangrove species in India[4,5], confirming that the intra family classification and relation between genera in the Rhizophoraceae family. Genetic analysis of 20 mangrove species in Penjarangan Island, Banten, Indonesia using 10 randomized RAPD primers generated relatively good polymorphism bands and the genetic diversity was found to be relatively high[6]. These studies indicated that RAPD molecular analysis is sufficiently feasible to study the genetic diversity of a species.

To the best of author's knowledge, no molecular studies regarding the diversity of mangrove in Karimunjawa have been conducted. A molecular mangrove data collection in Indonesia would be of value as a source of the nation's biodiversity. Molecular data could be used to determine the taxonomic status and genetic relationships between mangroves, assisting morphological classification[7]. The morphological classification has the potential to be deficient because it is limited and directly influenced by the environment[8]. This is certainly a limitation when plants classified are still evolving[9]. On that basis, molecular data is more useful, because it has a more accurate data rate in the evolutionary process compared with morphological data.

Various molecular techniques have been used for the study of genetic diversity and relationships among plants. The latest development of synthetic propagation methods, PCR-RAPD has been used to find diversity among plants with ease[7,8,10,11]. RAPD is a DNA marker consisting of 10 base arbitrary sequences, containing at least 60% of guanine and cytosine bases[10]. The RAPD marker technique allows DNA analysis to be conducted quickly and easily, with a minimal amount of DNA using non-radioactive laboratory equipment and existing universal primers and does not require sequence preliminary information[7]. DNA sequences provide many character states because of different rates of change of nucleotide bases within different loci are more accurate and provide more natural kinship[10,12,13]. Molecular markers characteristic of DNA sequences in plants can be drawn from the genomes of nDNA, cpDNA and mtDNA[5,14]. Indeed, the RAPD method was used to show that the mangrove species, *Avicennia schaueriana* typically found in salt marsh and *Laguncularia racemose* found beside rivers have been genetic differences influenced by environmental or salinity factors[15].

Environmental heterogeneity in Karimunjawa Island can influence the genotype of vegetation, being well distributed and in great abundance[16]. In addition, the abundance of genotypes can also occur as a result of gene flow and natural selection, as over time the number of genotypes could be reduced due to pressure from other vegetation[17,18]. In mangrove habitats, salinity plays an important role in the distribution pattern because each species has a specific tolerance range for salinity[19,20]. Furthermore, environmental changes might lead to the emergence of genetic variation between species[4], with only those best suited to the environmental conditions surviving to reproduce[21]. Consequently, genetic diversity might correlate with morphological differences and mangrove survival[22]. The same species is likely to evolve genetically into new species.

Bruguiera widely spread on the island of Kemujan. Of the many species there are three *Bruguiera* that have morphological similarities, but data from local managers give different names to the species. The best assumption of three *Bruguiera* is one species that has evolved due to environmental factors. The focus of this study was to establish genetic linkages among three species of *Bruguiera* on Kemujan Island, Karimunjawa. The results of this study are expected to be used as an evaluation material for naming existing species or can actually support the naming is true.

This study aimed to witness the diversity of three species of *Bruguiera* found on Kemujan Island, Karimunjawa, *Bruguiera cylindrica* (BC), *Bruguiera gymnorrhiza* (BG) and *Bruguiera lyliadica* (BL) by a RAPD method.

MATERIALS AND METHODS

Plant material: *Bruguiera* mangrove samples were provided by Iwan Setiawan, B.L. (Karimunjawa National Park Conservation, Jepara, Indonesia). This study was started in

Table 1: Types of mangroves and location of samples

Code	Mangrove species	Coordinates
BC	*Bruguiera cylindrica*	5°49'23.5"S 110°28'01.1"E
BG	*Bruguiera gymnorrhiza*	5°49'33.1"S 110°27'35.0"E
BL	*Bruguiera lyliadica*	5°49'32.3"S 110°27'38.1"E

February, 2017 and spent a duration of three months. The sampling was performed in Kemujan, Karimunjawa Island as shown in Table 1. Young and fresh leaves of the three *Bruguiera* species were collected into silica-gel for DNA sequencing by the RAPD technique, conducted at the Cell and Molecular Laboratory of Biogen Centre, Bogor, Indonesia.

Genomic DNA isolation: Isolation of mangrove leaf genomic DNA was performed with a modified CTAB (cetyl trimethyl ammonium bromide) protocol. In brief, 100 mg of mangrove leaf samples were cut into small pieces and crushed using liquid nitrogen in a mortar until smooth, then 600 μL of 2% CTAB buffer (20 mM EDTA; 1.4 M NaCl; 2% PVP-40; 0.1 M Tris (pH 8.0); 1% β-mercaptoethanol) was added. The mixed suspension was placed in an Eppendorf and homogenised using a MPS-1 vortex before incubation in a water bath at 60°C for 30 min. The mixed suspension (dissolved at the bottom) was aliquoted into 500 μL aliquots and 500 μL of chloroform:isoamyl alcohol (24:1) was added to separate contaminants, such as proteins, from the DNA, followed by centrifugation (15000 rpm for 20 min) to separate the chloroform-isoamyl alcohol (IAA) and CTAB phases[23,24]. The 600 μL water phase solution was then transferred to a 1.5 mL microtube containing 20 μL NaOAc, 600 μL of cold isopropanol (-20°C) was added as a DNA binder and centrifuged at 15,000 rpm for 10 min before incubation for 1 h in the freezer (-20°C) pellet the DNA. The ethanol was discarded and pellets were washed twice with 500 μL of 70% ethanol for 20 min. The pellet was then dissolved in 100 μL TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and stored[25,26] at -20°C.

Genomic DNA quantification and purity measurements: DNA quality and quantity was assessed by 1% agarose gel electrophoresis and Nanodrop ND-2000 Spectrophotometry (Thermo Electron North America LLC). The concentration and purity of the DNA genome was determined by measuring the absorbance (A) at 260 and 280 nm and calculating the absorbance[8,11,27,28] ratio, A_{260}:A_{280}. The absorbance at 280 nm provides a measure of the protein contamination, while the 260 nm measurement is used to calculate the concentration of DNA in a sample[25].

Primer screening and RAPD-PCR: Amplification of the DNA was performed the following primers from Genetika Science Indonesia (JR Scientific Inc.): GGATGCCACT (OPA-01), GGTGAACGCT (OPA-02), GTGTGCCCCA (OPA-03), CAGCGACTGT (OPA-04) and CTTCACCCGA (OPA-05). PCR amplification was performed using 5 μL DNA template, 5 μL master mix, 1 μL primer and 3.5 μL ddH$_2$O with the following cycling conditions: initial denaturation at 95°C for 10 min, then 45 cycles of denaturation at 94°C for 1 min, annealing 36°C for 1 min and extension at 72°C for 2 min, with a final elongation step at 72°C for 10 min. The PCR products were separated using horizontal 1.8% agarose gel electrophoresis in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide, before visualization using a Gel Doc™ XR+System[25].

Data analysis: RAPD results data are presented in binary data based on the presence or absence of DNA bands. The emerging band is the result of RAPD electrophoresis, which is separated by its molecular weight, with the thickness of the bands indicating the number of proteins that have the same molecular weight. The DNA profile was detected using UV$_{254}$ nm and sprayed with general reagent (cerium IV sulphate). The RAPD bands were discerned from the agarose gel and recorded as present (1) or absent (0) and assembled into a data matrix. A DNA fragment of the same size (same movement) was considered to have originated from the same locus. A dendogram was created numerically by associative coefficient grouping methods, where the association coefficient was determined by SIMQUAL (Similarity for Qualitative) procedures in UPGMA (Unweighted Pair Group Method with Arithmetic Mean), computed in the Numerical Taxonomy and Multivariate Analysis System[24,29,30] (NTSYS) ver. 2.1.

RESULTS

Three species of mangrove from the *Bruguiera* genus, namely *B. cylindrical* (BC), *B. gymnorrhiza* (BG) and *B. lyliadica* (BL) from Kemujan Island, Karimunjawa were successfully analyzed for genetic kinship. The RAPD method was used to detect possible genetic variations that occurred using five specific primers. Preliminary PCR assessment of the purity and concentration of the mangrove DNA showed that there was no differences in DNA purity and concentration. However, further testing conducted using the Nanodrop technique demonstrated that the *B. gymnorrhiza* sample had the highest purity quantity of 1.98 μg μL^{-1}, with a concentration of 178.2 nm, while the lowest purity was in BC samples with purity of 1.63 μg μL^{-1} and concentration of 110.9 nm. The results of DNA quantity test by using Nanodrop were listed in Table 2.

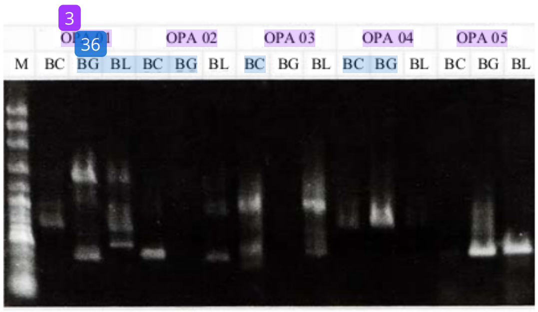

Fig. 1: PCR-RAPD visibility results from three *Bruguiera* species in five primers

M: Ledder, BC: *Bruguiera cylindrical*, BG: *Bruguiera gymnorrhiza* and BL: *Bruguiera lyliadica*

Table 2: DNA quantity test results using nanodrop 2010

Code	Species	Purity (μg μL^{-1})	Concentration (nm)
BC	*Bruguiera cylindrical*	1.63	110.9
BG	*Bruguiera gymnorrhiza*	1.98	178.2
BL	*Bruguiera lyliadica*	1.90	180.4

Table 3: Primers and base sequences used in RAPD analysis

Primer code	5'-3' DNA sequence	Characters
OPA-01	GGATGCCACT	Polymorphic
OPA-02	GGTGAACGCT	Polymorphic
OPA-03	GTGTGCCCCA	Polymorphic
OPA-04	CAGCGACTGT	Polymorphic
OPA-05	CTTCACCCGA	Polymorphic

Table 4: Scoring results from the DNA fragment examination profile

	Sample			
Primer	BC	BG	BL	MV
OPA-01	0	1	0	384
	1	0	0	241
	0	0	1	181
	0	1	0	154
OPA-02	0	0	1	273
	1	0	0	160
	0	0	1	148
OPA-03	0	0	1	288
	1	0	0	285
	1	0	0	166
	0	0	1	163
OPA-04	0	1	0	262
OPA-05	0	0	1	163
	0	1	0	160

BC: *Bruguiera cylindrical*, BG: *Bruguiera gymnorrhiza* and BL: *Bruguiera lyliadica*

The five RAPD primers (Table 3) amplified all samples and all had polymorphism properties, yielding different quality and quantity of DNA band patterns.

PCR-RAPD amplification of the total DNA of the three mangrove genomes using five primers produced 15 DNA bands that could be well-scored. The marker used to calculate the molecular weights was a 1 kb DNA ladder (BioLabs). The molecular weight of the PCR products was between 100-400 bp. Analysis using the Gel Analyzer revealed that most DNA bands were generated by the OPA-01 and OPA-03 primers, with band sizes ranging from 154-384 bp in Fig. 1.

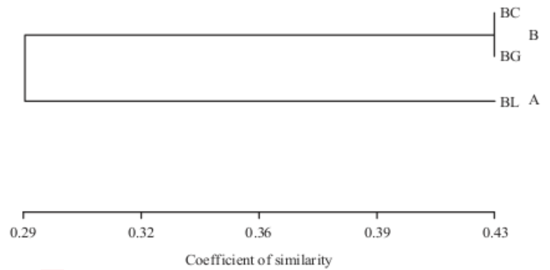

Fig. 2: UPGMA dendogram based on 15 RAPD polymorphic markers showing similarity relationships between *B. lyliadica* (cluster A) and *B. cylindrical* and *B. gymnorrhiza* (cluster B)

PCR-RAPD amplification produced multiple polymorphic bands of varying size. The band patterns were scored using the NTSYSpc programme to create a dendogram. The largest number of bands in primary OPA-01 and OPA-03 was 8 polymorphism bands, with molecular weights ranging from 100-400 bp. The scoring of the amplification results of the RAPD technique on *Bruguiera* was conducted by assigning the number "1" to the emerging band and the value "0" to the non-emerging band. The scores of all three samples were shown in Table 4.

From the scores of DNA bands shown in Table 4, only the OPA-4 primer produced one DNA band in the *B. gymnorrhiza* sample with a molecular weight (MV) of 262 bp, while OPA-01, OPA-02, OPA-03 and OPA-05 primers produced bands of various sizes. The coefficient value of genetic similarity between *Bruguiera* accession was between 25-100%. From the results of dendogram analysis of the NTSYSpc application shown in Fig. 2, two clusters (A and B) were obtained, cluster A was *B. lyliadica* and cluster B consisted of *B. cylindrical* and *B. gymnorrhiza*.

DISCUSSION

The use of RAPD molecular markers to analyze the DNA variation of the three *Bruguiera* species on Kemujan Island was relatively successful, as it produced a clear polymorphic locus, hence detected genetic variations. PCR-RAPD visualization with five specific primers amplified the three *Bruguiera* samples, generating a total of

15 polymorphic bands. Furthermore, the RAPD technique is useful due to its shorter processing and lower cost[6,31].

DNA analysis using agarose medium is associated with errors, particularly in the DNA isolation[25,32,33], so the DNA was also analyzed using the Nanodrop in this study. Acceptable values for DNA purity[11] are 1.7-2, values outside this range indicate contamination which could potentially impact the subsequent DNA analysis[27]. The Nanodrop results showed that the *B. gymnorrhiza* sample had the highest purity, with the *B. cylindrical* sample being the least pure. All five RAPD primers generated DNA for each sample ranging in size from 100-500 bp, showing their polymorphic properties, that is, the position of the DNA band in a straight line or parallel[6]. Monomorphic properties are very likely to occur in similar studies, indicating that the primers are unable to distinguish between sample[23], but this was not the case in this study.

With regards to the relationship between *B. cylindrical*, *B. gymnorrhiza* and *B. lyliadica*, scoring of the emerging DNA bands using the NTSYSpc, SAHN, SIM and NTED programmes presented in the form of dendogram showed that *B. lyliadica* is different from other samples, whereas *B. cylindrical* and *B. gymnorrhiza* are similar. The sample can be divided into two groups, namely cluster A containing, *B. lyliadica* and cluster B containing *B. cylindrical* and *B. gymnorrhiza*. Morphologically, the three species are similar, but they are genetically different.

CONCLUSION

High intraspecific variability based on RAPD molecular markers occurs independently which shows population-specific adaptation to their local environment. This results provide relevant information for effective and efficient practices for conservation of the *Bruguiera* population and the results of this study could be made as recommendations for further species conservation.

SIGNIFICANCE STATEMENT

This study discover the effectiveness of five primers (OPA-01, OPA-02, OPA-03, OPA-04 and OPA-05) which can work well in producing *Bruguiera* polymorphic bands that can be beneficial for further study of other mangrove study in Indonesia. This study will help researchers to uncover the critical areas of the diversity of *Bruguiera* species that many researchers were not able to explore.

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