

Species diversity and phenetic relationship among accessions of api-api (*Avicennia* spp.) in Java based on morphological characters and ISSR markers

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Abstract. Sabdanawaty FP, Purnomo, Daryono BS. 2021. Species diversity and phenetic relationship among accessions of api-api (*Avicennia* spp.) in Java based on morphological characters and ISSR markers. *Biodiversitas* 22: 193-198. Api-api (*Avicennia* spp.) from mangrove groups is rich in economic and ecological benefits. Research on the potential of api-api has been extensively done, but no research is specifically focused on the identification of api-api. This study aims to identify species diversity and phenetic relationships of api-api in Java based on morphological and molecular characters. The morphological analysis was based on 35 characters and referred to the descriptor. The PCA analysis on the morphological characters of *Avicennia* spp. showed a high diversity. Morphological characters have a high effect on grouping patterns such as habitat, leaf shape, leaf tip, leaf base, and petal color. Molecular observations were done on the polymorphism of DNA bands. The ISSR primers used were ISSR02, ISSR04, and ISSR10. PCR amplification of DNA was separated and visualized using a doc gel electrophoresis. The results showed a high diversity based on the percentage of DNA polymorphism. The research resulted in three species of *Avicennia* spp. in Java, namely *Avicennia officinalis*, *A. alba*, and *A. marina*. Phenetic relationships between *Avicennia* spp. form was based on similarity in character, not based on the area of origin. The environmental conditions in this study slightly opposed the character of *Avicennia* spp., which presumably due to the similarity in various marine areas of Java such as temperature, pH, and the required salinity.

Keywords: Api-api mangroves, *Avicennia*, ISSR marker, molecular characters, morphological characters

INTRODUCTION

Indonesia has mangrove forests in all major islands from Sumatra, Java, Kalimantan, Sulawesi to Papua. These mangrove forests harbor varying degrees of species diversity depending on physical conditions, substrate composition, hydrological conditions, and climatic types on these islands. A mangrove is a unique form of the forest ecosystem, located in the tidal areas of coastal areas, beaches, and small islands and a very potential natural resource (Said and Ehsan 2010). Api-api (*Avicennia* spp.) is one type of mangrove which is rich in benefits such as alternative food ingredients, wound treatment, high-quality paper raw material and can be used as a contraceptive material (Handayani 2018). The study results of Rinto (2011) showed that *Avicennia* spp. leaves are high in fiber and carbohydrate content. The bioactive components detected in the leaves of the *Avicennia* spp. plant are flavonoids, steroids, and reducing sugars. Active antioxidants contained in *Avicennia* spp. leaves can serve as a hepatoprotector because it can reduce levels of liver MDA, AST, and ALT enzymes in the blood, as well as histopathology of liver tissue lesions (Hardiningtyas et al. 2014).

Avicennia spp. also has many ecological benefits. The huge number, strong and dense aerial roots of *Avicennia* spp. are very effective for capturing and holding mud and

various rubbish that are swept away in the waters (Halidah et al. 2014). *Avicennia* spp. is commonly found downstream to intermediate estuarine zones throughout the intertidal region. Diversity occurs because the appearance of a character or phenotype is determined by genetic factors and environmental factors, and interactions between the two factors (Sobir and Syukur 2015). Molecular markers are DNA sequences tightly linked to a specific character/gene in the genome and can be passed from one generation to the next by following the law of inheritance (Purnomo 2016). *Avicennia* represents the largest polymorphic genus of mangroves and is known ecologically, systematically, morphologically, and genetically compared to other taxa. Research has been carried out on mangrove species to assess genetic diversity using genetic markers. Various genetic diversity studies had been done in *Avicennia* spp. using RAPD and ISSR molecular markers (Dasgupta 2017).

The Inter Simple Sequence Repeat (ISSR) is a molecular marker that is generally used for the study of genetic diversity, gene tagging, genome mapping, and evolutionary biology in various plants. ISSR is currently used in many plant species (Bani et al. 2017). ISSR has been reported in several mangrove species such as *Excoecaria agallocha*, *Phoenix paludosa*, *Xylocarpus granatum* (Dasgupta et al. 2017), *Avicennia marina* (Forsk.) Vierh., *Avicennia officinalis*

L., and *Avicennia alba* Blume (Kader et al. 2012). Research on the potential of *Avicennia* spp. in Java is very diverse, but research that is specifically focusing on the diversity of the species is very limited. Thus, this research is focused on elucidating the diversity and phenetic relationships of *Avicennia* spp. in Java based on morphological and molecular characters using ISSR marker.

MATERIALS AND METHODS

Sample collection

Avicennia spp. accessions were collected from three locations in Java, Indonesia, i.e., Cilacap, Indramayu, and Probolinggo. There are three observation zones, firstly the zone closest to the coast, secondly the middle zone that is sometimes flooded by seawater, and the last zone is flooded by seawater.

Morphological identification

Observation of morphological characters of each species in the field included roots, stems, leaves, flowers, and fruits. Morphological identification was based on a descriptor from Guidebook For Southeast Asia with Modifications (Giesen et al. 2007; Said and Ehsan 2010; Spalding et al. 2010). Cluster analysis was done by using an Euclidean square with Unweighted Pair Group Method Using Arithmetic means (UPGMA) technique. The advantage of using the UPGMA technique is that grouping of all characters is weighted equally so that the similarity between samples is calculated based on the average character similarity (Surya and Hari 2017).

Molecular identification

Isolation of DNA from leaf samples of all accession was carried out manually by Grimm et al. (2017) method with some modifications. DNA isolation was started with the preparation of extraction buffer solution (100 mL Tris-HCl, 0.88 g NaCl and SDS 0.5 g), then one gram of each accession leaf was ground with a liquid Nitrogen and put in a 1.5 mL tube, added with 300 μ L 10% SDS, and then centrifuged at a speed of 13,000 rpm for 1 minute at 4 °C. A total of 300 μ L supernatant was transferred to a 1.5 mL

tube and then added with 300 μ L isopropyl alcohol and centrifuged again at a speed of 13,000 rpm for 20 minutes at 4°C. Supernatant was discarded and the DNA pellet was precipitated by adding 500 μ L 70% EtOH and centrifuged again at a speed of 13,000 rpm for 1 minute. DNA pellet was dried by opening the cap of the tube and turning it over for \pm 15 minutes, after being dry the tube containing DNA pellet was added with 50 μ L ddH₂O. After purification, the DNA was measured spectroscopically and visualized under UV light after electrophoresis at 0.8% agarose gel. DNA amplification was done by using a PCR method following My Taq HS Red Mix PCR protocol kit containing Bioline (12.5 μ L), ddH₂O (8.5 μ L), ISSR primers (2 μ L), and DNA samples (2 μ L) 2 μ L). The sample was subject to initial denaturation for 10 minutes at 95°C, followed by 35 cycles of denaturation for 1 minute at 95°C, annealing of for 1 minute at 36-64°C for ISSR (for different primers different temperature annealing was used), and extension for 2 minutes at 72°C with a final extension of 7 minutes at 72°C. 10 μ L of amplified PCR product was separated by 1.8% agarose gel electrophoresis. Band patterns obtained from ISSR results were scored as markers, it was scored (1) for presence or (0) for absence of DNA marker, each was treated as an independent character regardless of its intensity to make a similarity matrix (Kader et al. 2012).

RESULTS AND DISCUSSION

A total of 10 accessions were collected in the sampling location as shown in Table 1. Habitats at the three points of collection have different environmental conditions that interact directly with the accessions. This can affect variations in morphological characters (Purnomo 2017).

Field observation found no *Avicennia* spp. in the seawater inundated zone. These vegetation communities generally grow in intertidal and subtidal areas with adequate water flow and are protected from large waves and strong tidal currents. Therefore, many mangrove forests are found on shallow bay beaches, estuaries, deltas, and protected coastal areas, so that this study found in *Avicennia* spp. in zone I.



Figure 1. The map of *Avicennia* spp. sampling in three locations in Java, Indonesia, i.e., Cilacap, Indramayu, and Probolinggo

The leaf is prominently varied in morphological characters and can be directly used to distinguish among species (Spalding et al. 2010). Figure 1 shows the variation of the leaf shape of the samples in this study. The oval leaf shape has a flat leaf edge. The tip is tapered but some are blunt. Tapered leaf base to slightly blunt. This leaf shape appearance belongs to the species *Avicennia marina*. Lancet leaf that is elongated and wavy like a ribbon was found in the P3A1 and I2A1 samples. They have flat curved leaf edges, tip, and base of the blunt leaf. These leaf characters belong to the species *Avicennia alba*. The shape of the ovate leaves with a flat leaf edge, rounded edges, and leaf base is an appearance of a species of *Avicennia officinalis* (Said and Ehsan 2010).

The use of cluster analysis employing the Euclidean distance matrix aims to group observational data into several groups with classification criteria based on the

dissimilarity level. The closer to the 0 Euclidean distances, the higher the similarity (Urbaniak et al. 2019). The results of the morphological cluster analysis of *Avicennia* spp. (Figure 2) shows the formation of two main groups. PCA analysis was performed to show the pattern of clustering accessions and the role of each character in the clustering process (Table 2).

Table 2. Morphological characters that have a high influence on grouping based on principal component analysis

Character	Cluster I	Cluster II
Petal color	Greenish-yellow	Greenish-yellow, Plain-yellow
Habitat	Mud, Mud sand	Beach sand, Mud, Mud sand
Leaf Shape	Oval	Lancet, Oval
Leaf tips	Rounded	Tapered, Blunt
Leaf base	Rounded	Spiky, Blunt

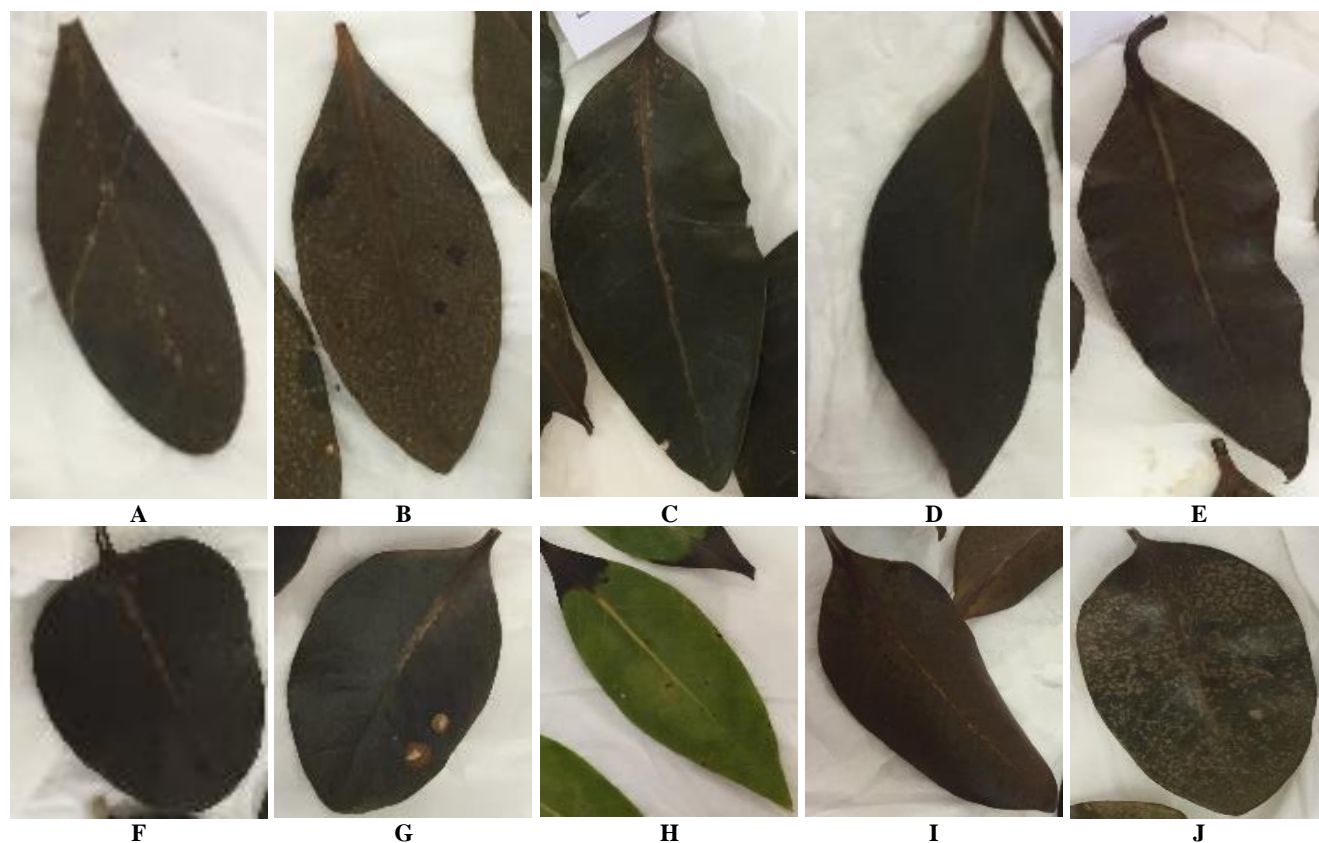


Figure 1. Morphological variation of 10 leaves of *Avicennia* spp. A. C2A1, B. C3A1, C. I2A1, D. I2A2, E. I3A1, F. I3A2, G. P2A1, H. P3A1, I. P3A2, J. P3A3

Table 1. List 10 accessions of *Avicennia* spp. Observed in the present study

Code	Origin	L	species
C ₂ A ₁	Cilacap	On the beach, very close to the sea, sandy.	<i>Avicennia marina</i>
C ₃ A ₁	Cilacap	On the mainland, muddy	<i>Avicennia marina</i>
I ₂ A ₁	Indramayu	On the beach, very close to the sea, sandy.	<i>Avicennia alba</i>
I ₂ A ₂	Indramayu	On the beach, very close to the sea, sandy.	<i>Avicennia officinale</i>
I ₃ A ₁	Indramayu	On the mainland, muddy	<i>Avicennia officinale</i>
I ₃ A ₂	Indramayu	On the mainland, muddy	<i>Avicennia marina</i>
P ₂ A ₁	Probolinggo	On the beach, very close to the sea, sandy.	<i>Avicennia marina</i>
P ₃ A ₁	Probolinggo	On the mainland, a little sandy muddy	<i>Avicennia alba</i>
P ₃ A ₂	Probolinggo	On the mainland, a little sandy muddy	<i>Avicennia marina</i>
P ₃ A ₃	Probolinggo	On the mainland, a little sandy muddy	<i>Avicennia officinale</i>

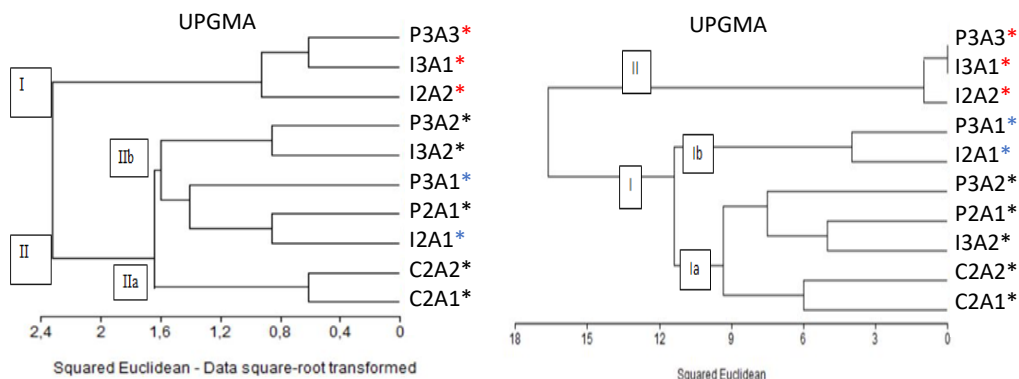


Figure 2. Dendrogram of the genetic relationship among 10 accessions of *Avicennia* spp. based on morphological characters (left side) and on molecular characters (right side)

Principal Component Analysis/PCA aims to classify the observed samples by reducing the character of most of the major components which are less and mutually independent (Bhartaya et al. 2011). In this study, the observations on the morphological characters of roots, stems and leaves were conducted, but only the morphological characters of the leaves are significantly diverse. So that the leaf morphological character is considered the main component while other characters were reduced after PCA analysis. The number of main components formed can be seen from the square value. The eigenvalue is valid in calculating the number of main components that are formed more than one so that values less than one not included (Bhartaya et al. 2011). The eigenvalue describes how much a character plays a role in forming a group. The higher the Eigenvalue of a character, the greater the character's influence in the grouping (Bro and Smilde 2014). The study results revealed 6 morphological characters that play a dominant role as a cluster differentiator, i.e., zone, habitat, leaf shape, leaf tip shape, leaf base shape, and petal color. In this study, leaf organs have an important role in determining the classification. Each species has a unique leaf characteristic. The present study showed that leaf organs are the most important characters in cluster formation. According to Said and Ehsan (2010), the leaf morphological characters such as leaf shape, leaf edge, leaf surface color, and leaf base are important characters used in distinguishing one species from another, and as a basis for classifying intraspecies *Avicennia* spp. in the Al-Sharm site, Egypt. Zone, as a cluster differentiator, includes morphological characters that play a dominant role as a distinguishing cluster but in reality, the field between the two clusters formed each can be found in all zones.

Therefore the zone character cannot be used as a distinguishing character.

Mangrove communities will be grouped based on their zoning, parallel to the coastline inland to the boundary of the mangrove ecosystem and terrestrial zone. The composition, density, frequency, and dominance of mangrove species as well as their classification patterns are largely influenced by environmental factors, such as soil texture, salinity, and physiography. One factor often interacts with another to produce a mangrove vegetation environment.

Morphological characters are not absolutely reliable indicators of genetic variation or taxonomic differences because they tended to be heavily influenced by environmental factors (Oktavianingsih et al. 2019). The results of checking the quality of DNA in elfogenomes (Figure 3) show satisfactory results because the DNA obtained looks intact in the absence of a smear. This is very important because intact DNA gives accurate results in the PCR process (Syafaruddin 2011).

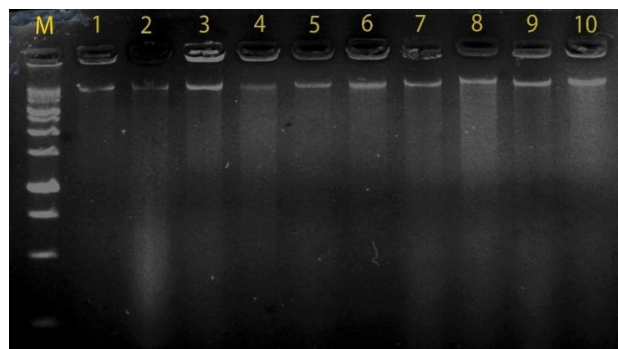


Figure 3. Elfogenom of 10 *Avicennia* spp.

Table 4. The DNA fragment amplification results of 10 *Avicennia* spp. using three ISSR primers

Primer	Sequence	Annealing	Number of bands	Number of polymorphic bands	% polymorphic Bands
ISSR 02	(GA) ₈ T	45 °C	12	10	83.3 %
ISSR 04	(CA) ₈ GT	50 °C	11	10	90.9 %
ISSR 10	(GCA) ₃ GC	45 °C	8	4	50.0 %
Total fragment			31	24	

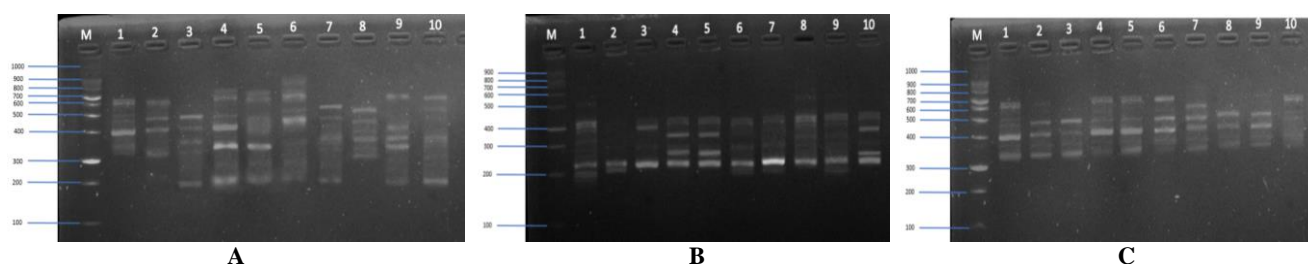


Figure 4. DNA banding pattern of 10 accessions of *Avicennia* spp. amplified using three primers: (A) ISSR 02, (B) ISSR 04, and (C) ISSR 10

PCR results show that 3 ISSR primers can amplify DNA from *Avicennia* spp.. The PCR amplification using ISSR primers produced 31 DNA fragments, 24 of which are polymorphic (Table 4). The lowest percentage of polymorphic bands was 50% and the highest was 90,9%, indicating a high genetic diversity.

Analysis of DNA fragments is based on the presence (code 1) or absence (code 0) of DNA bands (Arif et al. 2010). Dendrogram generated from cluster analysis using MVSP version 3.2 of the 10 accessions of *Avicennia* spp. is shown in Figure 2.

The results of the dendrogram formed based on molecular characters are different from the dendrogram produced by morphological characters. Dendrogram of 10 accessions *Avicennia* spp. based on molecular characters was divided into two main clusters. The first main cluster consists of three species which are the group of *Avicennia officinalis*. The second cluster is divided into two sub-clusters, the first sub-cluster consists of two species which are the *Avicennia alba* group, and the next sub-cluster consists of 5 species which are the *Avicennia marina* group.

The formed grouping pattern does not depend on the similarity in the geographic location of the area from which the accession was taken. The results of the dendrogram show grouping based on the similarity of molecular characters that each accession has. Accessions in one location do not necessarily have the same character. In general, there was almost no difference between the morphological characters of the accessions. However, molecular differences in characters can be seen from the DNA banding pattern formed by each accession. It is the variation in the pattern of DNA bands that characterizes an accession.

The use ISSR molecular markers requires no information about genome sequences of the organism to be tested (Dasgupta et al. 2017). ISSR can detect high polymorphisms, and has the potential to detect variations between populations that are geographically separated, as well as individual variations within populations. The manual DNA isolation method by Grimm (2017) can be applied to the ten accessions (Figure 3). PCR results show that 3 ISSR primers could amplify DNA from *Avicennia* spp (Figure 4). The three primers are ISSR 02, ISSR 04, and ISSR 10, thus these three primers are specific for *Avicennia* spp. The results of this study indicate a high level of diversity of *Avicennia* spp. based on molecular

characters. The diversity of molecular characters can be seen from the pattern of DNA bands produced (Sakpere 2011). The more similar the banding pattern produced by the samples, the closer the relationship among the samples.

Identification of species of *Avicennia* spp. in Java Island revealed three species, namely, *Avicennia marina*, *Avicennia alba*, and *Avicennia officinalis*. Morphological diversity of leaf shape, leaf tip, and leaf base is a character that can be used as a reference for determining each *Avicennia* species. High level of genetic diversity based on the assessment of observed DNA polymorphisms. Phenetic relationship among *Avicennia* spp. on the island of Java quite close. Both morphological and molecular observations, grouping occurs based on the similarity of characters, not based on geographic points of origin.

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