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Assessment of *Avicennia* Species Using Leaf Morphology and Nuclear Ribosomal Internal Transcribed Spacer DNA Barcode

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ABSTRACT

The mangrove genus *Avicennia*, found in tropical and temperate regions, plays a crucial role in providing critical services such as habitat, shoreline stabilisation, and carbon sequestration. Given their ecological and economic significance, expanding knowledge by revising species recognition is essential for validating morphological characteristics and overlapping traits. This study reassessed *Avicennia* species using morphological and genetic analysis. Samples were collected from Pulau Bagan Pinang, Pulau Burong, Pulau Kamat, Pulau Merambong, and Sungai Kemasik, Peninsular Malaysia. Mature leaves were assessed for their morphological traits, whereas young leaves were used to extract DNA for internal transcribed spacer (ITS) sequences. Statistical and phylogenetic analyses were conducted to evaluate leaf morphology variations and genetic divergence. Leaf morphology and size ($p < 0.05$) varied significantly among *Avicennia* species across study sites. *Avicennia alba* and *A. rumphiana* from the islands exhibited shorter, narrower, and thicker leaves than Sungai Kemasik. *Avicennia marina* displayed consistent leaf sizes. Leaf hierarchical relationships showed three to four phenetic groups, with separations at Euclidean distances of 25.0 (length), 18.0 (width), and 25.0 (thickness). Phylogenetics of *Avicennia* revealed four clades with strong bootstrap supports (83-100%). The guanine-cytosine (%GC) content was consistent, ranging from 63.5% to 64.6%. *Avicennia rumphiana*

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displayed high intraspecific genetic variation (1.57%) and distinctness from other species, supported by morphological and genetic data. This integrated approach is crucial for species identification and effective biodiversity assessments.

Keywords: *Avicennia*, genetic, internal transcribed spacer (ITS), leaf morphology, leaf morphometric, mangroves

INTRODUCTION

The mangrove genus *Avicennia* L., locally known as Api-api, is monotypic and distributed across tropical and temperate regions (Tomlinson, 1986; Kavitha et al., 2010). Historically, the taxonomy of *Avicennia* has undergone several revisions, reflecting both morphological and genetic evidence that shaped the current classification (Thatoi et al., 2016). Based on the consensus in phylogenetic evolution, the latest updated checklist confirms that the genus *Avicennia* includes eight species: *Avicennia alba* Blume, *A. balanophora* Stapf & Moldenke., *A. bicolor* Standl., *A. germinans* (L.) L., *A. integra* N.C. Duke., *A. marina* (Forssk.) Vierh., *A. officinalis* L. and *A. schaueriana* Stapf & Leechm. ex Moldenke. The presence of species variance is in *A. alba*, *A. bicolor*, *A. germinans*, *A. marina*, *A. officinalis,* and *A. schaueriana* (Hassler, 2024). This genus is found across a range that extends from East Africa through the Indo-Malayan region to Australia and New Caledonia.

According to the botanical reports (Duke, 1991; Tomlinson, 2016; Watson, 1928), five *Avicennia* species (*Avicennia alba*, *A. officinalis*, *A, rumphiana*, *A. integra*, and *A. marina*) are confined to the area of Indo-western Pacific region. In contrast, the latest Catalogue of Life (Hassler, 2024) list recognises *A. rumphiana* as a variety of *A. marina*, according to Ridley (1923) and Bakhuizen (1921). Moldenke (1990) proposed that the morphological characteristics (for example, pale green leaf undersurface) distinguished *A. marina* var. *rumphiana* as a valid variety. However, as Duke (1991) noted, Moldenke formally made his new combination with *A. marina* but curiously never offered the correction in subsequent writings, preferring to use the Bakhuizen van den Brink name, leaving the change unresolved and controversial. Subsequent advances in phylogenetic research have been revisited to better understand the species relationships and introgressive hybridisation (Mori et al., 2015). Huang et al. (2014) found that *A. rumphiana* is genetically distinct from *A. marina*, and Li et al. (2016) still referred to *A. rumphiana* as a distinct species.

Classically, the method based on morphological characters was a traditional technique to name the species (Vy et al., 2017). Taxonomists also often depended on flowers to identify species (Borg & Schönenberger, 2011; Nadia et al., 2012). However, this approach is challenging when flowers are unavailable during non-flowering seasons. Consequently, researchers have explored alternative methods, such as using leaf morphology for species identification. For example, Duke (2012) used leaf morphology to identify specimens of

Avicennia. Leaf morphology is useful for field identification because it varies widely and is easy to observe in studies such as phytosociology, which needs to identify every tree, even when flowers and fruits are absent (Nascimento et al., 2021). In addition, Said and Ehsan (2010) emphasised that leaf characteristics such as shape, edge, colour, and base are important for distinguishing *Avicennia* species and classifying intraspecies variations. Studies by Abou Seedo et al. (2018), Farooqui and Dangi (2018), and Sabdanawaty et al. (2021) have effectively used leaf morphology for this purpose. However, relying only on morphological characteristics can be challenging, as environmental factors often influence them (Thatoi et al., 2016), including soil salinity and the seasonality of the local climate. Some species have evolved different leaf adaptations based on geographical location, leading to further confusion in species identification (Thatoi et al., 2016).

In recent years, there have been increasing efforts made by several authors, for example, Rani et al. (2018) and Ruang-areerate et al. (2022), on identification using various approaches among mangroves species, including *Avicennia* based on DNA barcoding (Bhadalkar et al., 2014). DNA barcoding is a technique that employs short, variable, and standardised DNA sequences to assess and classify species (Said & Bahnasy, 2023). Subsequently, molecular markers were utilised for various applications, from gene localisation to the genetic enhancement of plant varieties (Karuppaiya et al., 2020). The molecular markers used are Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeat (SSR), Sequence Tagged Site (STS), and Single Nucleotide Polymorphism (SNP) (Bhadalkar et al., 2014). Internal transcribed spacer (ITS) has been described as a primer for identifying mangrove plant species using leaves due to its effectiveness as a DNA barcode marker (Chen et al., 2015). Bast et al. (2016) noted that ITS regions are easily amplified even from small DNA samples, have moderate length (under 700 bp), and exhibit significant variation among closely related species. The evolution rate of ITS is appropriate at the species and generic levels, as it is phylogenetically explainable for phylogenetic reconstruction and flanked by highly conserved sequences within genera, making polymerase chain reaction (PCR) amplification and sequencing straightforward (Maguire & Saenger, 2000).

Since taxonomic confusion may arise from variations in morphology characteristics, the genetic approach was used as an important tool in this present study for species identification works. Although Tomlinson (2016) rarely used leaf shape in his identification key, he noted that it could be a distinguishing feature for some *Avicennia* species. He investigated the range of variation in leaf shape within each species. Therefore, this study reassessed *Avicennia* species using leaf morphology and genetic analysis of ITS sequences. Our findings are anticipated to enhance the accuracy of *Avicennia* species identification, contributing to more effective field observations and biodiversity assessments.

MATERIALS AND METHODS

Sampling Sites

The sample collection covered five mangrove sites in Peninsular Malaysia (Figure 1a). Pulau Bagan Pinang (2° 30' 28.29" N, 101° 49' 35.19" E) (Figure 1b) and Pulau Burong (2° 32' 46.58" N, 101° 47' 10.15" E) (Figure 1c), are mangrove islands located in the coastal areas of Port Dickson, Negeri Sembilan. Along the coast area of Pulau Kamat (2° 20' 50.83" N, 102° 2' 19.16" E) (Figure 1d) is in Teluk Gong, Malacca. These three islands are situated within the Strait of Malacca. Further south, Pulau Merambong (1° 18' 54.69" N, 103° 36' 36.58" E) (Figure 1e) is in Johor, directly exposed to marine conditions of the Johor Strait. Sungai Kemasik, an estuary located at Kemasik, Terengganu (4° 25' 6.17" N, 103° 27' 15.08" E) (Figure 1f), is influenced by a combination of saltwater from the South China Sea and freshwater inflow.

Plant Materials

For morphometric analysis, thirty mature leaves of *Avicennia* species were randomly collected from three individual trees, depending on their presence within the above mangrove sites. These leaf samples were placed in zip-lock plastic bags and transferred to the Aquatic Botany Laboratory, Department of Aquaculture, Universiti Putra Malaysia, for further leaf morphological analysis. The leaf morphological traits considered for physiognomic study are qualitative (leaf shape, margin, tip, base, upper surface, under surface, and colour) and quantitative (leaf length, width, and thickness). Five to eight young leaves were collected and placed in zip-lock plastic bags with silica gels for DNA analysis.

Figure 1. (a) Sample collection at different sites; (b) Pulau Bagan Pinang; (c) Pulau Burong; (d) Pulau Kamat; (e) Pulau Merambong; and (f) Sungai Kemasikw
Source: Google Earth

DNA Extraction

The dried leaf samples were crushed and ground in a mortar to obtain fine powder. Approximately 25 mg of powder from each sample was transferred to a 1.5 µL Eppendorf tube. The total genomic DNA was extracted using a commercially available kit, DNeasy® Plant Mini Kit (Qiagen, Germany) derived from the manufacturer's instructions. Buffer API (400 µL) and RNase A (4 µL) were added to the 25 mg powder sample, vortexed, and incubated at 65°C for 15 minutes. After cooling, Buffer P3 (130 µL) was added, mixed, and incubated for 4 minutes on ice, followed by 1 minute at room temperature. After cooling, Buffer P3 (130 µL) was added, mixed, and incubated for 4 minutes on ice, followed by 1 minute at room temperature. The lysate was centrifuged, transferred to a QIAshredder column and centrifuged again. The flow-through was mixed with Buffer AW1 and processed through a DNeasy Mini spin column, followed by centrifugation. Buffer AW2 (500 µL) was added and centrifuged. Genomic DNA was eluted with Buffer AE (100 µL), incubated for 5 minutes, and centrifuged. The eluted DNA was quantified on a 0.6% agarose gel (1st BASE, Singapore) and visualised using runVIEW Gel Imager (Cleaver Scientific Ltd., UK) to obtain high-quality DNA.

Polymerase Chain Reaction (PCR) and Sequencing

The complete internal transcribed spacer (ITS) regions (including ITS-1, 5.8S rRNA gene, and ITS-2) were amplified using both universal primers (Integrated DNA Technologies, Singapore) of ITS-1 (forward: 5'-TCC GTA GGT GAA CCT GCG G-3') and ITS-4 (reverse: 5'- TCC TCC GCT TAT TGA TAT GC-3'). The amplification followed DreamTaq Green PCR Master Mix (2X) (Thermo Fisher Scientific™, USA) standard protocol with several modifications in the reaction mixture volume. The 35 µL reaction mixture contained 5 µL of template DNA, 16.5 µL of DreamTaq Green PCR Master Mix (2X), 1 µL of each primer, and 10.5 µL of nuclease-free water. The double-stranded fragments were employed with 35 cycles of the polymerase chain reaction (PCR; 94°C for 5 min; 94°C for 1 min, 53.3°C for 30 s, 72°C for 2 min; and final extension at 72°C for 10 min) in T100™ Thermal Cycler (Bio-Rad, Germany).

PCR products were verified through electrophoresis on 1% agarose gels, stained with Midori Green (Nippon Genetics Europe GmbH, Germany), and visualised under UV light. The size of the DNA fragments was determined using a GeneRuler 1 kb plus DNA ladder (Thermo Fisher Scientific™, USA) that had been premixed with a 6x loading buffer (1st BASE, Singapore). Gel electrophoresis was performed in TAE (Tris-Acetate-EDTA) buffer (1st BASE, Singapore) for 45 min at 87 V. Both forward and reverse strands were then sequenced bidirectionally using the Sanger method at 1st BASE Ltd., Malaysia.

Statistical Analysis

Quantitative data on leaf length, width, and thickness were subjected to statistical analysis using SPSS Statistics version 26 for Windows (IBM Corp., Armonk, NY, USA). Differences in mean values were compared using one-way ANOVA followed by Duncan's post hoc test at the $p < 0.05$ level (Zar, 2010).

The leaf morphometric variations among *Avicennia* species were determined from leaf length, width, and thickness means. A hierarchical cluster analysis was performed using Agglomerative hierarchical clustering (AHC), which was applied to the normalised data set by Ward's method, using Euclidean distances as a measure of dissimilarity. The dendrogram was generated in Microsoft Excel using the statistical software of XLSTAT 2021 (Addinsoft, Paris, France).

Clustering and Phylogenetic Analysis

PCR products with high-quality scores (> 20) were filtered and selected using FinchTV 1.4 (Geospiza, Seattle, USA; http://www.geospiza.com/finchtv) for sequence alignment analysis. Consensus sequences were assembled into contigs with BioEdit software, version 7.1.9 (Ibis Biosciences, USA; http://www.mbio.ncsu.edu/BioEdit/bioedit.html), and used for phylogenetic reconstruction (Hall, 1999). Twenty-seven ITS sequences of *Avicennia* from this study (Nos. 1 to 27), 28 sequences from other locations (Nos. 28 to 53), and two outgroups *Sonneratia* (Nos. 54 to 55) as shown in Table 1 were aligned using CLUSTALW version 1.83 (EMBL-EBI, Cambridge, UK) (Larkin et al., 2007). Sequences from other locations were retrieved from the National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/blast).

Phylogenetic analyses were performed using Maximum Likelihood (ML) in MEGA11 software (Tamura et al., 2021). Tamura 3-parameter model with gamma distributions (T92+G) was selected as the best-fitting substitution model. Model selection for ITS sequences identified the Tamura 3-parameter model with gamma distribution (T92+G) as the best fit using Akaike Information Criterion (AIC), Bayesian Information Criterion (BIC), and divergence estimation criterion (Kalyaanamoorthy et al. 2017). The analyses employed the bootstrapping method with 1000 replications using default parameters. A monophyletic clade comprising multiple individuals and exhibiting a bootstrap value greater than 60% in phylogenetic trees indicated successful species identification (Meier et al., 2006). Sequence divergence and pairwise distance estimations were also analysed in MEGA11 for their resolution inference. The guanine-cytosine (%GC) content was calculated for the ITS sequences of each species. The formula to calculate the %GC content, according to Altschul et al. (1990), is shown below.

$$\%\text{GC content} = \left(\frac{\text{Number of G bases} + \text{Number of C bases}}{\text{Total number of bases}}\right) \times 100\%$$

Table 1
List of Avicennia *species used in the molecular analysis in this study, including the length of its base pair sequences*

No.	Species	Geographic distributions	Accession number	ITS length (bp)
		Present study		
1.	*Avicennia alba*	Pulau Bagan Pinang	MY050304.1	695
2.	*Avicennia alba*	Pulau Bagan Pinang	MY050304.2	695
3.	*Avicennia alba*	Pulau Bagan Pinang	MY050304.3	695
4.	*Avicennia alba*	Pulau Burong	MY050304.10	695
5.	*Avicennia alba*	Sungai Kemasik	MY110307.1	695
6.	*Avicennia alba*	Sungai Kemasik	MY110307.2	695
7.	*Avicennia alba*	Sungai Kemasik	MY110307.3	695
8.	*Avicennia alba*	Pulau Merambong	MY010207.9	695
9.	*Avicennia alba*	Pulau Merambong	MY010207.10	695
10.	*Avicennia alba*	Pulau Merambong	MY010207.11	695
11.	*Avicennia alba*	Pulau Kamat	MY040310.1	695
12.	*Avicennia alba*	Pulau Kamat	MY040310.2	695
13.	*Avicennia marina*	Pulau Bagan Pinang	MY050304.4	694
14.	*Avicennia marina*	Pulau Bagan Pinang	MY050304.5	694
15.	*Avicennia marina*	Pulau Bagan Pinang	MY050304.6	694
16.	*Avicennia marina*	Pulau Burong	MY050304.11	694
17.	*Avicennia marina*	Pulau Burong	MY050304.12	694
18.	*Avicennia marina*	Pulau Burong	MY050304.13	694
19.	*Avicennia marina*	Pulau Merambong	MY010207.12	694
20.	*Avicennia marina*	Pulau Merambong	MY010207.13	694
21.	*Avicennia marina*	Pulau Merambong	MY010207.14	694
22.	*Avicennia rumphiana*	Pulau Merambong	MY010207.15	695
23.	*Avicennia rumphiana*	Pulau Merambong	MY010207.16	695
24.	*Avicennia rumphiana*	Pulau Merambong	MY010207.17	695
25.	*Avicennia rumphiana*	Sungai Kemasik	MY110307.4	695
26.	*Avicennia rumphiana*	Sungai Kemasik	MY110307.5	695
27.	*Avicennia rumphiana*	Sungai Kemasik	MY110307.6	695
		Other locations		
28.	*Avicennia alba*	India	MH243935.1	688
29.	*Avicennia. alba*	United States	EF540977.1	653
30.	*Avicennia alba*	Vietnam	MG880036.1	570
31.	*Avicennia alba*	Vietnam	MG880030.1	570
32.	*Avicennia alba*	China	KX641594.1	666
33.	*Avicennia marina*	India	MH243938.1	689
34.	*Avicennia marina*	United States	EF540978.1	652
35.	*Avicennia marina*	Saudi Arabia	MK027295.1	640
36.	*Avicennia marina*	United Kingdom	MN883387.1	647

Table 1 (*continue)*

No.	Species	Geographic distributions	Accession number	ITS length (bp)
37.	*Avicennia marina*	Thailand	KT004470.1	468
38.	*Avicennia marina*	China	MF063712.1	549
39.	*Avicennia marina* subsp. *australasica*	United States	AF365978.1	671
40.	*Avicennia marina* subsp. *australasica*	China	KX641591.1	666
41.	*Avicennia marina* subsp. *eucalyptifolia*	China	KX641592.1	666
42.	*Avicennia marina* subsp. *marina*	China	KX641593.1	666
43.	*Avicennia marina* var. *rumphiana*	China	KX641595.1	666
44.	*Avicennia bicolor*	United States	EF540989.1	652
45.	*Avicennia germinans*	United States	EF540985.1	652
46.	*Avicennia germinans*	China	KX641596.1	667
47.	*Avicennia germinans*	Brazil	AB861217.1	648
48.	*Avicennia officinalis*	India	MH243949.1	688
49.	*Avicennia officinalis*	India	KJ784553.1	669
50.	*Avicennia officinalis*	China	KX641597.1	665
51.	*Avicennia officinalis*	Vietnam	MG880054.1	569
52.	*Avicennia schaueriana*	United States	EF540986.1	652
53.	*Avicennia schaueriana*	Brazil	AB861406.1	646
54.	*Sonneratia alba*	China	KJ511914.1	626
55.	*Sonneratia caseolaris*	China	AF420219.1	631

RESULTS

Leaf Morphology and Morphometric Variation Among *Avicennia* Species

Leaf shapes among *Avicennia* species vary, even within the same species at different locations (Figure 2). *Avicennia alba* leaves from Pulau Bagan Pinang, Pulau Burong, Pulau Kamat, and Pulau Merambong are lanceolate with sharply pointed apices (Figure 2a). In contrast, *A. alba* leaves from Sungai Kemasik are elliptic (Figure 2b) or obovate (Figure 2c), with acute to nearly rounded apices. *Avicennia marina* leaves from Pulau Bagan Pinang, Pulau Burong, and Pulau Merambong exhibit variations, including leaf curling and blade shapes that range from elliptic with acute (Figure 2d) to cuspidate (Figure 2e) apices and ovate shapes with obtuse apices (Figure 2f). *Avicennia rumphiana* leaves from Pulau Merambong and Sungai Kemasik (Figure 2g-i) are elliptic to oblong with acute to rounded apices. Additional details on morphological and morphometric differences for each individual species are provided in Tables 2–4.

Leaf size and thickness vary among *Avicennia* species, with statistical analysis (one-way ANOVA) showing significant differences in the five collection sites ($p < 0.05$) (Tables 2-4). *Avicennia alba* on the islands of Pulau Bagan Pinang, Pulau Burong, Pulau Kamat, and Pulau Merambong had shorter leaf lengths, narrower widths, and thicker leaves compared

to those along the open coast riverine of Sungai Kemasik (Table 2). *Avicennia marina* showed slight variation, with leaf sizes and thickness similar to those of the islands (Pulau Bagan Pinang, Pulau Burong and Pulau Merambong) (Table 3). *Avicennia rumphiana* from Pulau Merambong had shorter leaf lengths, narrower widths, and thicker leaves than those growing along Sungai Kemasik (Table 4).

Based on a dendrogram, the hierarchical clustering (HC) analysis classified the 27 *Avicennia* samples into three major clusters (Figure 3, Table 5), with a Euclidean distance of 47.5%. Cluster 1 consisted of both *A. alba* and *A. marina*, encompassing most samples. Cluster 2 was exclusively comprised of *A. alba*, characterised by the longest leaf lengths among all samples, ranging from 8.66 cm to 9.13 cm. Cluster 3 represented *A. marina* and *A. rumphiana* mixed. These results highlight the variation in leaf morphometrics among *Avicennia* species, which could lead to overlapping species classifications when considering samples from different study sites, with *A. alba* displaying the longest leaf lengths.

Phylogenetic Position of *Avicennia* Species Based on ITS Sequences

The *Avicennia* species were classified into four strongly supported clades (Clades 1 to 4), with bootstrap supports ranging from 83% to 100%, yielded in the most suitable sequence alignment using Maximum Likelihood (ML) analysis (Figure 4). All *Avicennia* accessions occupied separate topological positions, indicating no overlap

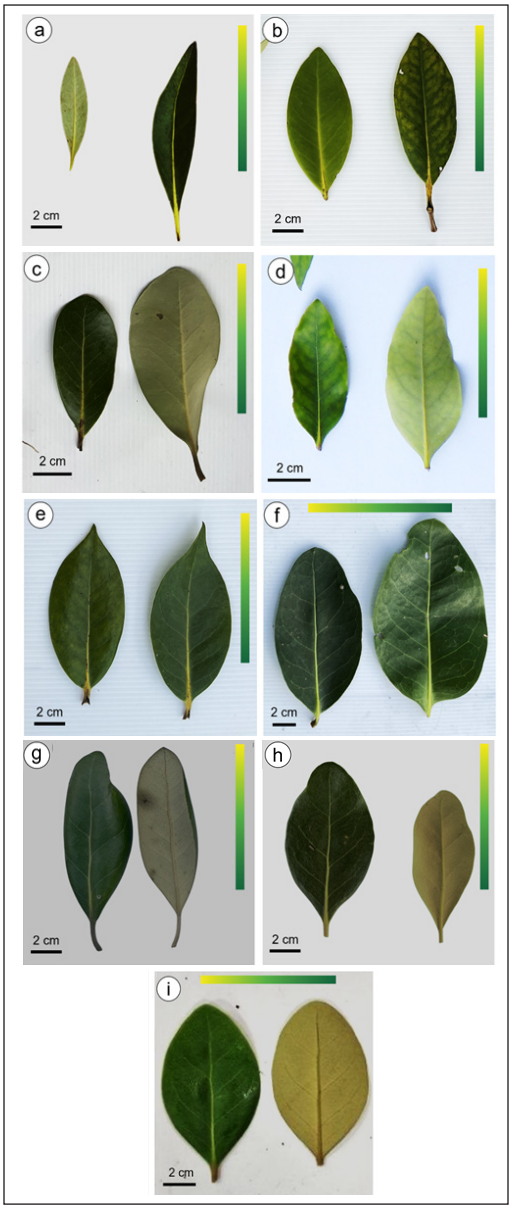

Figure 2. Leaf morphology variability of *Avicennia* species. (a) Lanceolate shape with sharply pointed apex; (b) elliptic shape with acute apex; (c) obovate shape with almost rounded apex of *Avicennia alba*; (d) Elliptic shape with acute to e) cuspidate apex; (f) ovate shape with obtuse apex of *A. marina*; and (g) oblong shape with acute apex, (h) elliptic shape with acute to (i) rounded apex of *A. rumphiana*. The scale bar (2 cm) indicates measurements, and the colour bar represents leaf colour variation from dark green to yellowish green

Table 2
Morphological leaf characteristics and ANOVA results (mean ± standard deviation and ranges) from morphometric measurements of Avicennia alba *in different sites*

	Morphology						Morphometric		
Study sites	**Blade shape**	**Margin**	**Apex**	**Base**	**Upper surface**	**Under surface**	**Length (cm)**	**Width (cm)**	**Thickness (mm)**
Pulau Bagan Pinang									
MY050304.1	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.95±1.06^{def} (6.0-9.8)	2.85±0.62^{ab} (1.5-4.0)	0.42±0.05^{b} (0.36-0.50)
MY050304.2	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.76±1.27^{def} (6.0-11.0)	2.57±0.61^{bc} (1.4-3.9)	0.42±0.05^{b} (0.36-0.50)
MY050304.3	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.99±1.44^{cde} (6.0-10.8)	2.58±0.55^{bc} (1.4-3.4)	0.42±0.06^{b} (0.36-0.54)
Pulau Burong									
MY050304.10	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	8.39±1.23^{bcd} (6.0-11.0)	2.54±0.63^{c} (1.5-4.0)	0.51±0.02^{a} (0.45-0.55)
Pulau Kamat									
MY040310.1	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.72±1.15^{def} (6.0-10.2)	2.49±0.58^{c} (2.0-5.0)	0.52±0.07^{a} (0.42-0.76)
MY040310.2	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.77±1.16^{def} (6.0-10.2)	2.46±0.58^{c} (2.0-5.0)	0.51±0.05^{a} (0.42-0.70)
Pulau Merambong									
MY010207.9	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.32±0.91^{ef} (5.7-9.0)	2.40±0.45^{cd} (1.7-3.1)	0.39±0.13^{bc} (0.24-0.75)
MY010207.10	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.24±0.73^{f} (5.0-8.6)	1.90±0.24^{e} (1.2-2.4)	0.37±0.09^{cd} (0.20-0.54)
MY010207.11	Lanceolate	Entire	Sharply pointed	Acute	Smooth dark green	Smooth silvery grey	7.39±0.88^{ef} (5.6-9.3)	2.16±0.41^{de} (1.4-3.0)	0.34±0.07^{d} (0.22-0.46)
Sungai Kemasik									
MY110307.1	Elliptic	Entire	Acute	Acute	Smooth dark green	Smooth silvery grey	8.66±1.51^{abc} (7.0–11.2)	3.02±0.41^{a} (2.1–4.0)	0.23±0.04^{e} (0.18–0.33)

Table 2 *(continue)*

	Morphology						Morphometric		
Study sites	**Blade shape**	**Margin**	**Apex**	**Base**	**Upper surface**	**Under surface**	**Length (cm)**	**Width (cm)**	**Thickness (mm)**
MY110307.2	Elliptic and obovate	Entire	Acute and almost rounded	Acute	Smooth dark green	Smooth silvery grey	8.71±1.55^{ab} (6.0–11.9)	3.00±0.61^{a} (2.0-4.0)	0.23±0.05^{e} (0.13–0.31)
MY110307.3	Elliptic	Entire	Acute and sharply pointed	Acute	Smooth dark green	Smooth silvery grey	9.13±1.84^{a} (5.0–11.7)	3.03±0.65^{a} (2.0–4.5)	0.23±0.04^{e} (0.18–0.31)
						df	11	11	11
						MS	10.82	3.68	0.35
						F	6.77	12.48	79.06
						p-value	0.000	0.001	0.001

Note. The varying superscript alphabet in the same column demonstrates the contrast at $p < 0.05$ (ANOVA Duncan's post hoc test)

Table 3
Morphological leaf characteristics and ANOVA results (mean ± standard deviation and ranges) from morphometric measurements of Avicennia marina *in different sites*

	Morphology						Morphometric		
Study sites	**Blade shape**	**Margin**	**Apex**	**Base**	**Upper surface**	**Under surface**	**Length (cm)**	**Width (cm)**	**Thickness (mm)**
Pulau Bagan Pinang									
MY050304.4	Elliptic, curling	Entire	Acute	Acute	Smooth, green	Smooth, pale green	7.14±0.93^{ab} (4.0–8.9)	3.28±0.57^{a} (2.0–4.1)	0.43±0.11^{a} (0.28–0.56)
MY050304.5	Elliptic, curling	Entire	Acute, obtuse, cuspidate	Acute	Smooth, green	Smooth, pale green	7.52±1.26^{a} (4.5–10.0)	3.51±0.85^{a} (2.0–5.0)	0.46±0.09^{a} (0.28–0.56)
MY050304.6	Elliptic, curling	Entire	Acute, obtuse	Acute	Smooth, green	Smooth, pale green	7.60±1.32^{a} (4.9–9.8)	3.52±0.64^{a} (2.0–4.6)	0.43±0.10^{a} (0.28–0.56)
Pulau Burong									
MY050304.11	Elliptic, curling	Entire	Acute	Acute	Smooth, green	Smooth, pale green	7.35±0.93^{a} (5.6–9.0)	2.66±0.35^{c} (2.0–3.2)	0.35±0.04^{b} (0.25–0.40)

Table 3 *(continue)*

Study sites	Morphology						Morphometric		
	Blade shape	Margin	Apex	Base	Upper surface	Under surface	Length (cm)	Width (cm)	Thickness (mm)
MY050304.12	Elliptic, curling	Entire	Acute, obtuse	Acute	Smooth, green	Smooth, pale green	7.14±1.10^{ab} (4.5–9.1)	2.45±0.44^{c} (1.6–3.1)	0.32±0.05^{b} (0.22–0.40)
MY050304.13	Elliptic, curling	Entire	Acute, obtuse	Acute	Smooth, green	Smooth, pale green	7.04±0.95^{ab} (4.5–9.0)	2.71±0.37^{bc} (1.9–3.6)	0.30±0.05^{bc} (0.23–0.40)
Pulau Merambong									
MY010207.12	Elliptic, curling	Entire	Acute	Acute	Smooth, green	Smooth, pale green	7.06±0.81^{ab} (5.7–8.9)	2.97±0.56^{b} (2.0–4.0)	0.27±0.05^{c} (0.19–0.38)
MY010207.13	Elliptic, curling	Entire	Acute	Acute	Smooth, green	Smooth, pale green	6.60±0.89^{b} (5.0–8.9)	2.64±0.56^{c} (1.8–4.0)	0.27±0.04^{c} (0.20–0.34
MY010207.14	Elliptic and ovate, curling	Entire	Acute, obtuse	Acute	Smooth, dark green	Smooth, pale green	6.61±0.80^{b} (5.0–8.9)	3.57±0.64^{a} (2.0–3.3)	0.27±0.10^{c} (0.19–0.56)
						df	8	8	8
						MS	3.67	5.83	0.17
						F	3.57	17.83	39.06
						p-value	0.000	0.000	0.000

Note. The varying superscript alphabet in the same column demonstrates the contrast at $p < 0.05$ (ANOVA Duncan's post hoc test)

Table 4
Morphological leaf characteristics and ANOVA results (mean ± standard deviation and ranges) from morphometric measurements of Avicennia rumphiana *in different sites*

Study sites	Morphology						Morphometric		
	Blade shape	Margin	Apex	Base	Upper surface	Under surface	Length (cm)	Width (cm)	Thickness (mm)
Pulau Merambong									
MY010207.15	Elliptic	Entire	Acute, rounded	Acute, rounded	Smooth, green	Densely pubescent, yellow-green	7.18±0.64^{bc} (5.7–8.6)	3.13±0.45^{b} (2.0–4.0)	0.28±0.04^{a} (0.20–0.34)

Table 4 *(continue)*

	Morphology						Morphometric		
Study sites	**Blade shape**	**Margin**	**Apex**	**Base**	**Upper surface**	**Under surface**	**Length (cm)**	**Width (cm)**	**Thickness (mm)**
MY010207.16	Elliptic and oblong	Entire	Rounded	Acute, rounded	Smooth, dark green	Densely pubescent, yellow-green	7.14±0.71^{c} (5.0–8.0)	3.22±0.53^{b} (2.0–4.3)	0.28±0.04^{a} (0.20–0.34)
MY010207.17	Elliptic and oblong	Entire	Rounded	Acute, rounded	Smooth, dark green	Densely pubescent, yellow-green	7.13±0.66^{c} (5.7–8.0)	3.06±0.45^{b} (2.0–4.0)	0.28±0.04^{a} (0.18–0.34)
Sungai Kemasik									
MY110307.4	Elliptic	Entire	Acute, rounded	Acute, rounded	Smooth, green	Densely pubescent, yellow-green	7.74±0.66^{a} (6.0–9.0)	4.20±0.35^{a} (3.0–5.0)	0.21±0.07^{b} (0.09–0.34)
MY110307.5	Elliptic	Entire	Acute, rounded	Acute, rounded	Smooth, dark green	Densely pubescent, yellow-green	7.66±0.44^{a} (6.7–8.2)	4.28±0.34^{a} (3.8–5.0)	0.21±0.03^{b} (0.10–0.29)
MY110307.6	Elliptic	Entire	Acute, rounded	Acute, rounded	Smooth, dark green	Densely pubescent, yellow-green	7.48±0.46^{ab} (5.0–9.0)	4.32±0.34^{a} (4.0–5.0)	0.22±0.06^{b} (0.17–0.29)
						df	5	5	5
						MS	2.24	11.62	0.04
						F	6.21	66.76	21.78
						p-value	0.000	0.000	0.000

Note. The varying superscript alphabet in the same column demonstrates the contrast at $p < 0.05$ (ANOVA Duncan's post hoc test)

Table 5
Distribution of Avicennia *species based on leaf morphometrics in different clustering classes*

Clustering class	No. of species	Within-class variance	Minimum distance to the centroid	Average distance to the centroid	Maximum distance to the centroid	Species
1	13	0.292	0.214	0.464	0.933	*A. alba* and *A. marina*
2	3	0.067	0.124	0.198	0.297	*A. alba*
3	11	0.369	0.276	0.544	0.820	*A. marina* and *A. rumphiana*

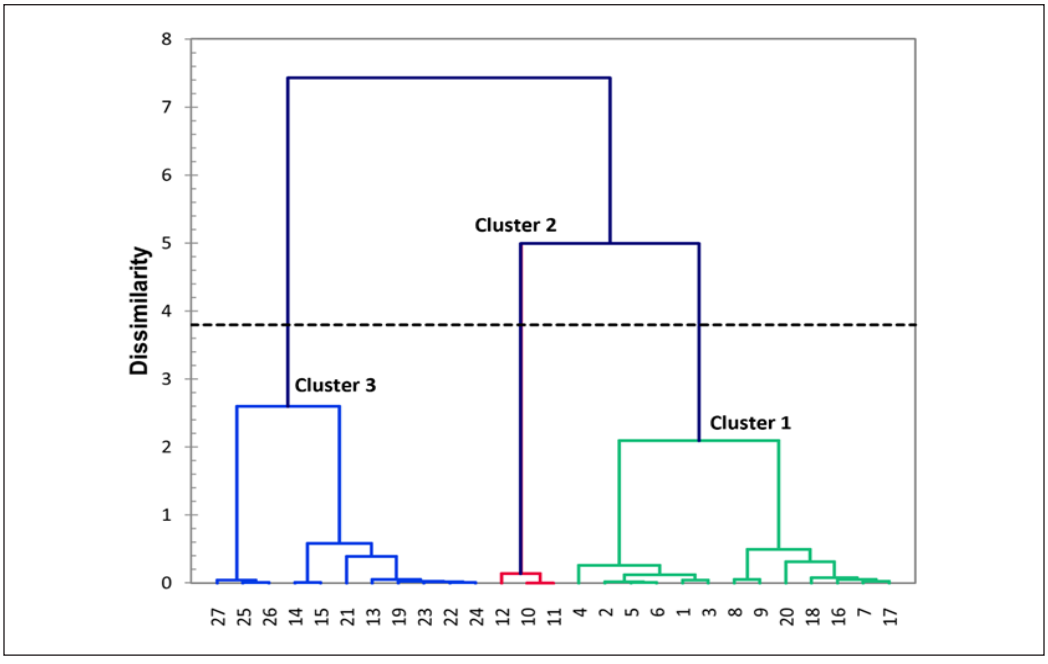

Figure 3. Dendrogram showing the classes of hierarchical clustering of the variability leaf morphometric (leaf lengths, widths and thickness) among 27 samples of *Avicennia* species

among species observed. In Clade 1, *A. alba* from this study was clustered with *A. alba* from other geographic distributions (Vietnam, India, China, and the United States of America supported by a high bootstrap value of 100%. Clade 2 was separated into two subclades supporting the 80% bootstrap. In this clade, *A. rumphiana* from this study formed a subclade with *A. marina* var. *rumphiana* from China with a 99% bootstrap value, whereas *A. officinalis* from other geographic distributions fell outside these subclades with an 80% bootstrap value. Clade 3 consisted of *A. marina* accessions with a moderately strong BS value of 83. Eleven subclades based on species similarities were formed in Clade 3, including *A. marina* and *A. marina* subsp. *australasica, A. marina* subsp. *marina* and *A. marina* subsp. *eucalyptifolia.* Only two subclades were clustered in the same group. In contrast, other accessions were well segregated into separated subclades based on their genetic sequences' dissimilarity, supported by BS values ranging from 61% to 95%. Clade 4 comprised three *Avicennia* accessions*:* (1) *A. bicolor*, (2) *A. germinans* and (3) *A. schaueriana*, which were retrieved from GenBank, with a bootstrap value of 99%.

Table 6 presents the final alignment of 27 nrITS sequences from *Avicennia* species with total nucleotides ranging from 643 to 695. The percentage of guanine-cytosine (%GC) content for *A. alba* ranges from 63.6% to 64.2% (695 base pairs). For *A. marina*, the %GC content ranges from 64.2% to 64.6% (694). Meanwhile, *A. rumphiana* exhibits a varied %GC content ranging from 63.5% to 64.6% across the 643 to 695 nucleotides.

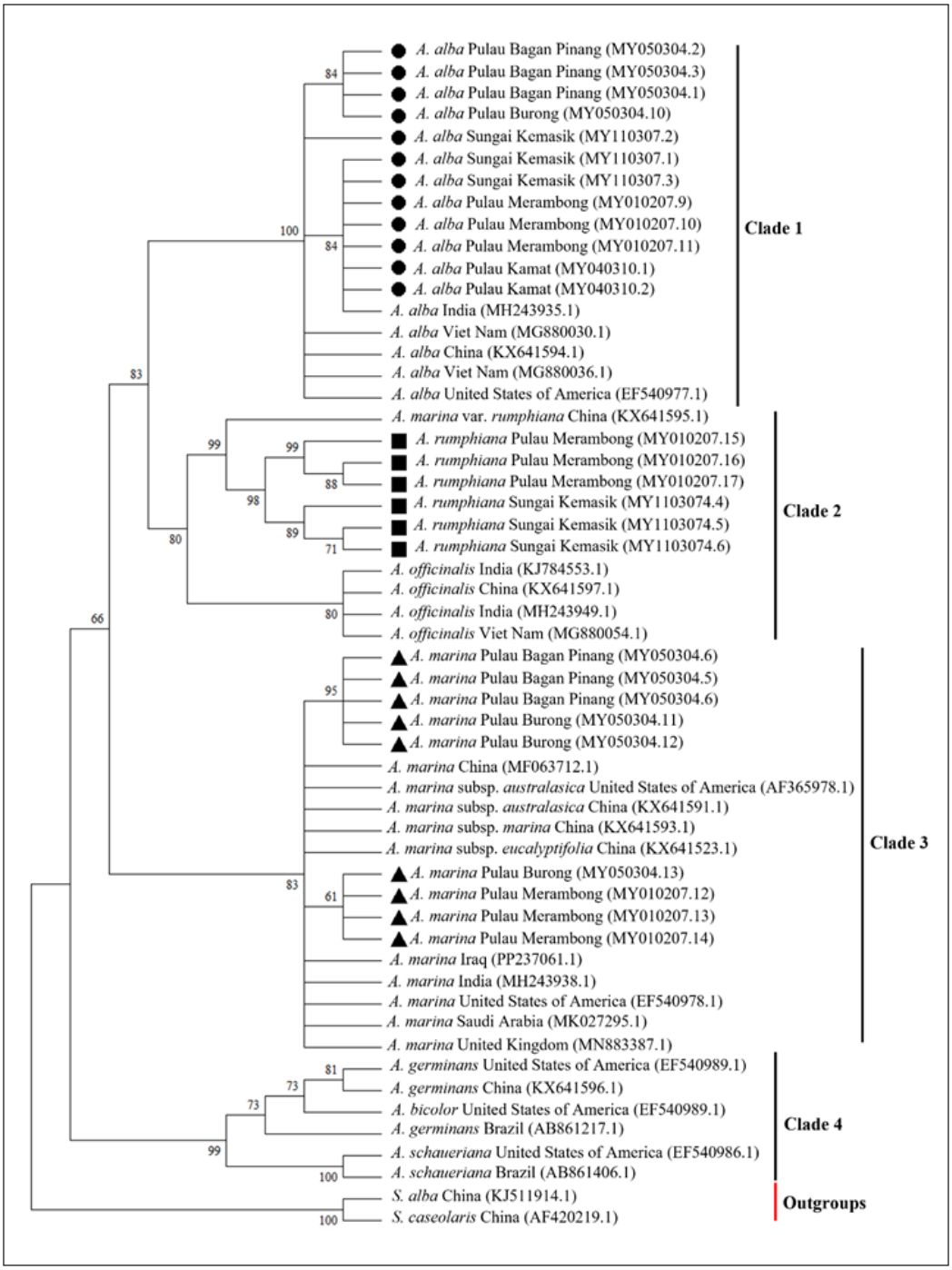

Figure 4. Phylogenetic tree of *Avicennia* species inferred from Maximum Likelihood (ML) analysis using 696 base pairs (bp) of 55 nucleotide sequences nrDNA ITS1, 5.8S rDNA, and ITS2. Bootstrap support values above 60% are shown on branches

Note. Shapes on the nodes of the phylogenetic tree indicated accessions obtained from this study

Table 6
Nucleotide composition (%) of the sequenced Avicennia *species from different sites*

Sites, species and accession number	Nucleotide composition (%)				Total number of nucleotides
	T(U)	C	A	G	
Pulau Bagan Pinang					
A. alba (MY050304.1)	16.0	33.2	20.4	30.4	695
A. alba (MY050304.2)	16.0	33.2	20.4	30.4	695
A. alba (MY050304.3)	16.0	33.2	20.4	30.4	695
Pulau Burong					
A. alba (MY050304.10)	16.0	33.2	20.4	30.4	695
Pulau Kamat					
A. alba (MY040310.1)	15.8	33.2	20.0	30.9	695
A. alba (MY040310.2)	15.8	33.2	20.0	30.9	695
Pulau Merambong					
A. alba (MY010207.9)	15.8	33.2	20.0	30.9	695
A. alba (MY010207.10)	15.8	33.2	20.0	30.9	695
A. alba (MY010207.11)	15.8	33.2	20.0	30.9	695
Sungai Kemasik					
A. alba (MY110307.1)	15.8	33.2	20.0	30.9	695
A. alba (MY110307.2)	15.8	33.2	20.3	30.6	695
A. alba (MY110307.3)	15.8	33.2	20.0	30.9	695
Pulau Bagan Pinang					
A. marina (MY050304.4)	16.3	33.1	19.5	31.1	694
A. marina (MY050304.5)	16.3	33.1	19.5	31.1	694
A. marina (MY050304.6)	16.3	33.1	19.5	31.1	694
Pulau Burong					
A. marina (MY050304.11)	16.3	33.1	19.5	31.1	694
A. marina (MY050304.12)	16.3	33.1	19.5	31.1	694
A. marina (MY050304.13)	16.0	33.1	19.3	31.4	694
Pulau Merambong					
A. marina (MY010207.12)	16.0	33.1	19.3	31.4	694
A. marina (MY010207.13)	16.0	33.1	19.3	31.4	694
A. marina (MY010207.14)	16.0	33.1	19.3	31.4	694
Pulau Merambong					
A. rumphiana (MY010207.15)	15.6	34.1	19.8	30.6	643
A. rumphiana (MY010207.16)	15.3	33.3	21.0	30.4	694
A. rumphiana (MY010207.17)	15.3	33.4	21.2	30.2	695
Sungai Kemasik					
A. rumphiana (MY110307.4)	15.4	33.4	20.9	30.4	695
A. rumphiana (MY110307.5)	15.4	33.4	20.9	30.4	695
A. rumphiana (MY110307.6)	15.4	33.4	20.9	30.4	695

Note. T = Thymine, C = Cytosine, A = Adenine, G = Guanine. The nucleotide composition (%) was analyzed using MEGA11

ITS Sequence Similarity Between *Avicennia* Species

The evolutionary divergence and pairwise distances of ITS sequences among species of *Avicennia* are detailed in Supplementary Tables 1, 2 and 3. *Avicennia alba* from various locations, including India, Vietnam, China, and the United States, are identical or exhibit 99.4% ITS sequence similarity (4-bp difference) (Supplementary Table 1). In Clade 3, *A. marina* exhibits a high sequence 99.2%–100% similarity (5-6-bp difference) in the ITS region with *A. marina* from other locations (Supplementary Table 2). In Clade 2, *A. rumphiana* from Pulau Merambong and Sungai Kemasik exhibit high ITS sequence 99%–100% similarity (7-bp difference) (Supplementary Table 3). *Avicennia rumphiana* and *A. marina* var. *rumphiana* share ITS sequences of 98.9% similarities (7-8-bp difference). However, *A. rumphiana* and *A. officinalis* show relatively lower ITS sequence 96.4%–96.7% similarity 22–25-bp difference). Thus, within this clade, the ITS sequences of *A. marina* var. *rumphiana* accession from China (KX641595.1) are similar to the *A. rumphiana* sequences in the present study.

The molecular distances between (interspecific variation) and within (intraspecific variation) species were analysed using the Tamura 3-parameter model (Table 7). Interspecific variation showed variation ranging from 3.56% to 6.61%. The value of the closest molecular distance (3.56%) was between *Avicennia alba* and *A. marina*, while the furthest is between *A. alba* and *A. rumphiana*. The intraspecific variation within *A. rumphiana* (1.57%) was the highest, while *A. alba* (0.29%) and *A. marina* (0.24%) share similarly low variation.

Table 7
Estimates of average evolutionary divergence over sequence pairs between species (interspecific variation) and within species (intraspecific variation)

Clades	**Interspecific variation**			**Intraspecific variation (%)**
	A. alba	*A. marina*	*A. rumphiana*	
A. alba		3.56%	6.61%	0.29%
A. marina	0.0356		6.50%	0.24%
A. rumphiana	0.0661	0.0650		1.57%

DISCUSSION

Variation in Leaf Morphology and Morphometric

The present study represents six leaf morphological characteristics (Tables 2–4) for consideration in identifying different species of *Avicennia*. Due to divergent evolution, *Avicennia* species exhibit varied taxonomic descriptions (Thatoi et al., 2016). Krauss et al. (2023) have previously reported that environmental conditions play a significant

role in shaping the leaf morphology variation of mangroves, including species within the *Avicennia*, as they adapt to the ecological challenges of mangrove habitats.

Studies in Malaysia by Rahman et al. (2023) at the mangrove estuarine wetland of Setiu in Terengganu and by Cheah (2016) at the riverine Linting wetlands describe *A. alba* leaves as having a silvery-grey or white colouration with a silvery upper surface, consistent with the findings of this study. *Avicennia alba* is also distinguished from other *Avicennia* species by their narrow and lanceolate leaves (An et al., 2022). These descriptions align with the characteristics of *A. alba* observed on the islands of Pulau Bagan Pinang, Pulau Burong, Pulau Merambong, and Pulau Kamat. Such leaf trait differences along the estuary of Sungai Kemasik are difficult to apply due to variations in individual trees, particularly in leaf blade shape and apex shape characteristics. In this context, the *A. alba* leaves from Sungai Kemasik exhibit a contrasting leaf shape, being elliptic to ovate with almost rounded and acute apex shapes instead of the sharply pointed characteristics observed in leaves from the islands.

However, previous studies have also described a range of leaf shape characteristics such as broadly elliptical leaves along the coastal areas of Northern Mindanao, Philippines (Osing et al., 2019), lanceolate at the estuary of East Kalimantan, Indonesia (Saptiani et al., 2018), and ovate at the sea fringe of Kien Giang, Vietnam (Duke, 2012). Hence, the leaf blade and apex shape were the leaf characteristics that further divided *A. alba* into two primary groups within the species in this study. It should be noted that the *A. alba* considered in the present study were exposed to different environmental conditions. Thus, the differences between the leaf characteristics observed would have been mainly due to the interaction with the particular environment of the location. This suggestion of leaf within *A. alba* differed and required comparable studies in different environmental conditions globally.

The leaf characteristics of *A. marina* have been extensively documented in previous studies. Leaves have been described as elliptical at the eastern coasts of Thailand (Huang et al., 2014) and on sheltered shores of Malaysia (Shin et al., 2015), ovate along the coastal areas of Indonesia (Noor et al., 2012), and occasionally lanceolate at the sea fringe of Kien Giang, Vietnam (Duke, 2012). They have also detailed the apex shape of *A. marina* as acute, obtuse, almost rounded, slightly acuminate, or variably pointed. These descriptions are consistent in this study's different sampling sites for *A. marina*, where both elliptic and ovate leaf shapes were reported, particularly in Pulau Merambong. The elliptic shape was consistently observed in other study sites (Pulau Bagan Pinang, Pulau Burong and Pulau Merambong). The elliptic and ovate shape of *A. marina* aligns more closely with the descriptions found in previous studies, particularly in Malaysia, Thailand and Indonesia, while the lanceolate shape is less frequently reported. It is important to differentiate *A. marina* from *A. alba* found in Sungai Kemasik, as their leaf characteristics can overlap,

potentially leading to confusion. Thus, a key identifier for *A. marina* is the presence of leaves with curled margins. The leaf feature differentiates it from *A. alba*. Win et al. (2021) and Primavera (2004) also described that the leaf blades range from flat to curly.

Further, the comparative assessment shows that the leaf of *A. rumphiana* can easily be distinguished from *A. alba* and *A. marina*, notably the presence of powdery hairs on *A. rumphiana* hairy undersurface leaves, a trait absent in the characteristics of the other two species. This characteristic was also identified in previous morphology assessments by Prakashamani et al. (2019) and Mariano et al. (2019). *Avicennia rumphiana,* primarily found in Pulau Merambong and Sungai Kemasik, was classified as vulnerable by the International Union for Conservation of Nature (IUCN, 2024). Previously, *A. rumphiana* was identified as *A. lanata*, with its distribution ranging from Peninsular Malaysia and the Philippines to New Guinea (Giesen et al., 2007). Blade shapes of *A. rumphiana* observed at Pulau Merambong and Sungai Kemasik include elliptic, oblong and obovate with both acute and rounded apexes. In the present study, the leaf morphology of *A. rumphiana* showed only slight distinctions compared to reports from other regions. The leaf blades in this study were elliptic to ovate with acute bases, aligning with descriptions of *A. rumphiana* along the east coast of Peninsular Malaysia (Shin et al., 2015), the mangrove estuarine areas of Setiu wetlands in Terengganu (Rahman et al., 2023), and in the Panay Islands, Philippines (Primavera et al., 2004).

However, this differs slightly from *A. rumphiana* in the Zamboanga del Sur coastal areas of the Philippines (Mariano et al., 2019) and at the sea fringe of Kien Giang, Vietnam (Duke, 2015), which described the species with elliptic to ovate leaves characterised by a rounded base. Primavera et al. (2004), Mariano et al. (2019) and Chan et al. (2022), along with the present study, reported a yellowish-green leaf undersurface covered with dense hairs. In contrast, Huang et al. (2014) reported a russet, tomentose, nearly brown undersurface on the eastern coasts of Thailand, while Duke (2012) noted similar characteristics at the sea fringe of Kien Giang, Vietnam. These variations highlight the distinct leaf characteristics of *A. rumphiana* species, reflecting their variability that may be attributed to geographic distribution or local environmental conditions.

Moreover, the dark green colouration on the upper surface and the presence of dense powdery hairs undersurface were noted as common traits in these studies. *Avicenna rumphiana* is often misidentified as *A. officinalis* due to their similarities in hairy leaves, as described by Selvam and Karunagaran (2004). However, as outlined by Primavera et al. (2004), *A. officinalis* exhibits shiny and glossy upper surface leaves, contrasting with the dull dark green surface of *A. rumphiana*, a similarity noted in the leaf surface observation of *A. rumphiana* in this study as well. Additional distinctions observed in various studies include those by Primavera et al. (2004), who highlighted differences such as the yellowish-to-russet tomentose undersurface of *A. rumphiana* leaves, as opposed to the yellowish-green

undersurfaces of *A. officinalis* (Cheah, 2016). Therefore, in morphological comparison, *A. rumphiana* demonstrates morphological similarities consistent with previous studies.

Leaf characteristics, including length, width, thickness, perimeter, area, and mass, are crucial indicators of mangrove health, productivity, and overall condition (Dookie et al., 2023). These measurements serve as a basis for calculating additional leaf parameters, including leaf-specific area, leaf mass per area, density, relative water content, and sclerophylly indices (Liu et al., 2017). Primavera et al. (2004) summarised the leaf descriptions of the mangroves in the Philippines based on the leaf length and width. One-way ANOVA and Duncan's post hoc tests indicated significant differences ($p < 0.05$) in all leaf parameters among *Avicennia* species, indicating leaf size variation within the genus. According to Duncan's post hoc analysis, it is suggested that the leaf width of *A. alba* may reach widths of up to 4.0 cm, potentially overlapping with the broader leaf widths observed in *A. marina*. This similarity in leaf width between the two species could lead to confusion during field identification. Despite variations observed within species, particularly in leaf width between island populations and those near Sungai Kemasik, the range of width measurements for *A. alba* leaves remains consistent with previous studies. Primavera et al. (2004) reported widths ranging from 2 to 5 cm, while Duke (2012) noted widths between 2.0 and 4.6 cm.

Similarly, the average length and width of *A. marina* closely match Duke's (1990) findings, ranging from 7.3 to 8.2 cm in length and 5 to 8 cm in width. Saenger and Brooks (2008) and Ahmed et al. (2022) also reported similar widths, ranging from 1.9 to 4.3 cm. Our measurements for *A. rumphiana* also fall within the ranges identified by Primavera et al. (2004) and Mariano et al. (2019). Several studies, including those by Barhoumi et al. (2021), Mollick et al. (2021), and Alam and Hossain (2023), utilise leaf measurements to assess responses to various environmental factors, such as soil and water salinity in mangroves rather than for species identification. Therefore, this study highlights the need for further research on leaf measurements to distinguish between different species.

Genetic Diversity Within the *Avicennia* Species

A common problem in identifying the *Avicennia* species is the variation in morphology and divergence within the genus (Nguyen et al., 2014). This variability makes species identification based on morphology challenging, especially outside the flowering season. Genetic methods offer a more reliable approach, particularly internal transcribed spacer (ITS). ITS are advantageous due to their easy amplification from small DNA quantities and moderate size (below 700 bp), which facilitates amplification and sequencing of high-degree variation even among closely related species (Bast et al., 2016). This study uses the ITS locus to represent Malaysia's *Avicennia* species' first DNA barcode-based biodiversity assessment. The genetic structure of Malaysian *Avicennia* has been examined

using nuclear microsatellites (Wee et al., 2013, 2020) and chloroplast sequences (Triest et al., 2021). A clear East-West genetic division has been identified in *A. marina* and *A. alba* Peninsular Malaysian populations based on microsatellite markers (Wee et al., 2020; Triest et al., 2021). All *Avicennia* accessions in this study were separated into four distinct clades, each supported by high bootstrap (BS) values. Our findings align with those of Li et al. (2016), who observed that *A. marina* and *A. officinalis* form distinct clusters separate from *A. rumphiana* and *A. alba*. Similarly, Saddhe et al. (2017) demonstrated the genetic distinctness of *A. alba* from *A. marina* and *A. officinalis* or *A. integra*. Our findings, where *A. alba*, further support this genetic separation. *Avicennia alba* formed a separate clade from other *Avicennia* species with a BS value of 83.

In Clade 2, *A. marina* var. *rumphiana* from China and *A. rumphiana* in this study show genetic association but with varying support, forming a cluster with *A. officinalis*. *Avicennia rumphiana*'s taxonomic history often leads to confusion with *A. marina* var. *rumphiana* and *A. lanata* (Duke, 1991). Ridley (1923) and Moldenke (1960) initially treated *A. lanata* and *A. marina* var. *rumphiana* as separate species. However, Duke (1991) later found their descriptions identical, recognising *A. rumphiana* as distinct and categorising *A. lanata* and *A. marina* var. *rumphiana* as synonyms. Despite this, the Catalogue of Life (Hassler, 2024) still recognises *A. rumphiana* as an accepted variety of *A. marina* (*A. marina* var. *rumphiana*) and noted its distribution in Southeast Asia, including Malaysia. However, we follow the taxonomy of *A. rumphiana* H. Hallier (*A. lanata*) as per Tomlinson (1986), Duke (1991) and Japar Sidik (1994), distinguishing it from *A. marina* var. *rumphiana*. This distinction is based on our morphological details of the leaves, which align with the botanical descriptions of *A. rumphiana* provided by Duke (1991) and Giesen et al. (2007).

These descriptions characterise the leaves as ovate or elliptic, dark green above, and covered with dense, fawn-coloured, velvety hairs on the undersurface. Our ITS sequencing has proven essential in assessing genetic relationships, revealing significant divergence between *A. rumphiana* and *A. marina*, as indicated by their grouping in separate clades (Clade 2 and Clade 3). The molecular evidence obtained from our analysis complements morphological descriptions, enhancing our understanding of the evolutionary relationships among these species and supporting the rationale for the recognition of *A. rumphiana* as a separate entity.

Guanine-cytosine (%GC) content determines genetic and species diversity (Liu et al., 2023). We observed consistent %GC content within *Avicennia*, ranging from 63.5% to 64.6%. Given the widespread use of ITS as a marker in plant systematics, our finding of consistent %GC content in *Avicennia* significantly contributes to our evolutionary model. It suggests similar genomic stability and nucleotide composition among the species.

Genetic pairwise distance is the standard measurement of genetic heterogeneity and the raw material for evolutionary change (Ellegren & Galtier, 2016). In our analysis, the

pairwise distance between *A. alba* clade sequences was the smallest (0.006), followed by *A. marina* (0.008), while *A. rumphiana* sequences exhibited the greatest distance (0.010). Literature suggests it typically shows 0–3 bp differences within a species (Lidén et al., 1995; Sun et al., 1994) and more than 3 bp differences between species (Sun et al., 1994). Our nucleotide differences (0.6%–1% sequence dissimilarity; 4–7-bp difference) support this hypothesis. Despite being collected from different sites, no evidence of hybridisation among taxa was found based on pairwise distance. This finding is consistent with Duke (1992), who also noted the absence of hybrids in *Avicennia* compared to other widely distributed mangrove genera. The pairwise distance among *A. alba* in this study ranged from 0.000 to 0.006 (4-bp difference), consistent with Rani et al. (2021), who reported a pairwise distance of 0.007 between *A. alba* from Sajnekhali, Sundarbans Delta and Kerala, India, indicating the coexist evolution. Both *A. alba* and *A. marina* had a slight intraspecific variation of 0.29 and 0.24%, respectively.

Avicennia rumphiana forms a distinct subclade within Clade 2 with a strong bootstrap support value of 99. However, this species has notable genetic diversity, as evidenced by a significant pairwise distance of 0.010 among individuals (1% sequence dissimilarity; 7-bp difference). Their intraspecific variation was also found to be high, at 1.57%. *Avicennia rumphiana* accession MY010207.17 from Pulau Merambong exhibits notably lower ITS sequence (99.3% sequence similarity; 5-base pair difference) compared to the other two accessions from the same location (MY010207.15 and MY010207.16). In contrast, it (MY010207.17) shares a higher ITS sequence (99.7% sequence similarity; 2-bp difference) with accessions from Sungai Kemasik. Distinct differences in leaf morphological traits further indicate this diversity. For instance, morphologically, there are differences between *A. rumphiana* accessions from Pulau Merambong, particularly MY010207.16 and MY010207.17, and those from Sungai Kemasik. The former displayed darker green leaves and differences in apex shapes (rounded), whereas the latter had lighter green leaves with acute to rounded apex shapes.

Although the classification remains genetically controversial, this study has resolved it using leaf morphological characteristics. The variability in genetic distance observed in *A. rumphiana* aligns with the differences in leaf morphology, demonstrating a clear relationship between genetic diversity and leaf characteristics. Therefore, by referencing botanical descriptions, we have placed the taxon under the species name *A. rumphiana*, as accepted in this study. These sequences were compared with those of *A. marina* var. *rumphiana*, *A. officinalis*, and other *Avicennia* species available in NCBI GenBank to determine their taxonomic positions.

As indicated by pairwise distance, genetic diversity within the *A. marina* was low (0.000-0.008), which aligns with findings reported by Malekmohammadi et al. (2022) using ITS. Similarly, using microsatellite markers, low genetic variation was observed

in *A. marina* populations in northern Australia (Maguire et al., 2002) and along the east coast of India (Zolgharnein et al., 2002), on Zifaf and Sajid islands in Vietnam (Giang et al., 2003). However, these previous studies assessed the genetic diversity of *A. marina* using microsatellite markers, which differs from the approach employed in this study, which utilised ITS.

CONCLUSION

The present study reassessed and confirmed *Avicennia* species identification and provided an overview of individuals' morphological and genetic variation. Morphological analysis revealed significant differences in six leaf characteristics and three morphometric traits ($p < 0.05$) of *Avicennia* leaf morphology. This study also utilised ITS sequences to better understand relationships within *Avicennia*. The ITS sequences of 27 *Avicennia* accessions and 26 accessions from other locations revealed four distinct clades (BS = 83-100). The consistent %GC content within *Avicennia* also indicates the level of genomic stability and nucleotide composition among the species. Despite significant divergence found in pairwise genetic distance among *Avicennia* accessions in Clade 2, the recognition of *A. rumphiana*'s taxonomic position is resolved based on evidence involving morphology, confirming its acceptance as a distinct species in this study. Leaf morphological differences further support the phylogenetic position that separates *A. rumphiana* from *A. marina* and *A. officinalis*. Therefore, our findings show that leaf morphological and genetic analyses are reliable for determining relationships in *Avicennia* and can contribute to refining species classifications and updating mangrove species records. These advances are crucial for effective biodiversity assessments.

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APPENDIX

Supplementary Table 1
Evolutionary divergence and pairwise distances among Avicennia *species*

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	*A. alba* PBP (MY050304.2)													
2	*A. alba* PBP (MY050304.3)	0.000												
3	*A. alba* PBP (MY050304.1)	0.000	0.000											
4	*A. alba* PB (MY050304.10)	0.000	0.000	0.000										
5	*A. alba* SKEM (MY110307.1)	0.006	0.006	0.006	0.006									
6	*A. alba* SKEM (MY110307.2)	0.003	0.003	0.003	0.003	0.003								
7	*A. alba* SKEM (MY110307.3)	0.006	0.006	0.006	0.006	0.000	0.003							
8	*A. alba* PM (MY010207.9)	0.006	0.006	0.006	0.006	0.000	0.003	0.000						
9	*A. alba* PM (MY010207.10)	0.006	0.006	0.006	0.006	0.000	0.003	0.000	0.000					
10	*A. alba* PM (MY010207.11)	0.006	0.006	0.006	0.006	0.000	0.003	0.000	0.000	0.000				
11	*A. alba* PKA (MY040310.1)	0.006	0.006	0.006	0.006	0.000	0.003	0.000	0.000	0.000	0.000			
12	*A. alba* PKA (MY040310.2)	0.006	0.006	0.006	0.006	0.000	0.003	0.000	0.000	0.000	0.000	0.000		
13	*A. marina* PBP (MY050304.4)	0.037	0.037	0.037	0.037	0.036	0.037	0.036	0.036	0.036	0.036	0.036	0.036	0.027
14	*A. marina* PBP (MY050304.5)	0.037	0.037	0.037	0.037	0.036	0.037	0.036	0.036	0.036	0.036	0.036	0.036	0.027
15	*A. marina* PBP (MY050304.6)	0.037	0.037	0.037	0.037	0.036	0.037	0.036	0.036	0.036	0.036	0.036	0.036	0.027
16	*A. marina* PB (MY050304.11)	0.037	0.037	0.037	0.037	0.036	0.037	0.036	0.036	0.036	0.036	0.036	0.036	0.027
17	*A. marina* PB (MY050304.12)	0.037	0.037	0.037	0.037	0.036	0.037	0.036	0.036	0.036	0.036	0.036	0.036	0.027
18	*A. marina* PB (MY050304.13)	0.039	0.039	0.039	0.039	0.033	0.036	0.033	0.033	0.033	0.033	0.033	0.033	0.027
19	*A. marina* PM (MY010207.12)	0.039	0.039	0.039	0.039	0.033	0.036	0.033	0.033	0.033	0.033	0.033	0.033	0.027
20	*A. marina* PM (MY010207.13)	0.039	0.039	0.039	0.039	0.033	0.036	0.033	0.033	0.033	0.033	0.033	0.033	0.027
21	*A. marina* PM (MY010207.14)	0.039	0.039	0.039	0.039	0.033	0.036	0.033	0.033	0.033	0.033	0.033	0.033	0.027
22	*A. marina* var. *rumphiana* China (KX641595.1)	0.022	0.022	0.022	0.022	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020	0.020
23	*A. rumphiana* PM (MY010207.15)	0.067	0.067	0.067	0.067	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.064	0.055
24	*A. rumphiana* PM (MY010207.16)	0.068	0.068	0.068	0.068	0.070	0.068	0.070	0.070	0.070	0.070	0.070	0.070	0.055

Supplementary Table 1 *(continue)*

		1	2	3	4	5	6	7	8	9	10	11	12	13
25	*A. rumphiana* PM (MY010207.17)	0.068	0.068	0.068	0.068	0.070	0.068	0.070	0.070	0.070	0.070	0.070	0.070	0.055
26	*A. rumphiana* SKEM (MY110307.4)	0.063	0.063	0.063	0.063	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.054
27	*A. rumphiana* SKEM (MY110307.5)	0.063	0.063	0.063	0.063	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.054
28	*A. rumphiana* SKEM (MY110307.6)	0.063	0.063	0.063	0.063	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.060	0.054
29	*A. officinalis* India (MH243949.1)	0.024	0.024	0.024	0.024	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022
30	*A. officinalis* China (KX641597.1)	0.024	0.024	0.024	0.024	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022
31	*A. officinalis* VietNam (MG880054.1)	0.025	0.025	0.025	0.025	0.024	0.024	0.024	0.024	0.024	0.024	0.024	0.024	0.024
32	*A. officinalis* India (KJ784553.1)	0.024	0.024	0.024	0.024	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022	0.022

Note. PBP = Pulau Bagan Pinang, PB = Pulau Burong, PKA = Pulau Kamat, PM = Pulau Merambong, SKEM = Sungai Kemasik

Supplementary Table 2
Evolutionary divergence and pairwise distances among Avicennia *species*

		1	2	3	4	5	6	7	8	9
1	*A. marina* PBP (MY050304.4)									
2	*A. marina* PBP (MY050304.5)	0.000								
3	*A. marina* PBP (MY050304.6)	0.000	0.000							
4	*A. marina* PB (MY050304.11)	0.000	0.000	0.000						
5	*A. marina* PB (MY050304.12)	0.000	0.000	0.000	0.000					
6	*A. marina* PB (MY050304.13)	0.004	0.004	0.004	0.004	0.004				
7	*A. marina* PM (MY010207.12)	0.004	0.004	0.004	0.004	0.004	0.000			
8	*A. marina* PM (MY010207.13)	0.004	0.004	0.004	0.004	0.004	0.000	0.000		
9	*A. marina* PM (MY010207.14)	0.004	0.004	0.004	0.004	0.004	0.000	0.000	0.000	
10	*A. marina* var. *rumphiana* China (KX641595.1)	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021	0.021
11	*A. rumphiana* PM (MY010207.15)	0.067	0.067	0.067	0.067	0.067	0.065	0.065	0.065	0.065
12	*A. rumphiana* PM (MY010207.16)	0.070	0.070	0.070	0.070	0.070	0.070	0.070	0.070	0.070
13	*A. rumphiana* PM (MY010207.17)	0.070	0.070	0.070	0.070	0.070	0.070	0.070	0.070	0.070

Supplementary Table 2 *(continue)*

		1	2	3	4	5	6	7	8	9
14	*A. rumphiana* SKEM (MY110307.4)	0.064	0.064	0.064	0.064	0.064	0.062	0.062	0.062	0.062
15	*A. rumphiana* SKEM (MY110307.5)	0.064	0.064	0.064	0.064	0.064	0.062	0.062	0.062	0.062
16	*A. rumphiana* SKEM (MY110307.6)	0.064	0.064	0.064	0.064	0.064	0.062	0.062	0.062	0.062
17	*A. officinalis* India (MH243949.1)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
18	*A. officinalis* China (KX641597.1)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
19	*A. officinalis* VietNam (MG880054.1)	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027	0.027
20	*A. officinalis* India (KJ784553.1)	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025

Note. PBP = Pulau Bagan Pinang, PB = Pulau Burong, PKA = Pulau Kamat, PM = Pulau Merambong, SKEM = Sungai Kemasik

Supplementary Table 3
Evolutionary divergence and pairwise distances among Avicennia *species*

		1	2	3	4	5	6	7	8	9	10	11
1	*A. marina* var. *rumphiana* China (KX641595.1)											
2	*A. rumphiana* PM (MY010207.15)	0.011										
3	*A. rumphiana* PM (MY010207.16)	0.011	0.007									
4	*A. rumphiana* PM (MY010207.17)	0.011	0.007	0.000								
5	*A. rumphiana* SKEM (MY110307.4)	0.011	0.003	0.010	0.010							
6	*A. rumphiana* SKEM (MY110307.5)	0.011	0.003	0.010	0.010	0.000						
7	*A. rumphiana* SKEM (MY110307.6)	0.011	0.003	0.010	0.010	0.000	0.000					
8	*A. officinalis* India (MH243949.1)	0.011	0.035	0.035	0.035	0.033	0.033	0.033				
9	*A. officinalis* China (KX641597.1)	0.011	0.035	0.035	0.035	0.033	0.033	0.033	0.000			
10	*A. officinalis* VietNam (MG880054.1)	0.013	0.036	0.036	0.036	0.035	0.035	0.035	0.001	0.001		
11	*A. officinalis* India (KJ784553.1)	0.011	0.035	0.035	0.035	0.033	0.033	0.033	0.000	0.000	0.001	

Note. PM = Pulau Merambong, SKEM = Sungai Kemasik