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CASE STUDY

Mangrove plants using deoxyribonucleic acid barcodes for enhancing biodiversity and conservation

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ABSTRACT

BACKGROUND AND OBJECTIVES: Mangrove forests in North Sumatra and Aceh are concentrated on the east coast of Sumatra Island. Mangrove habitats are highly productive, diversified, and ecologically and commercially significant ecosystems. However, they are vulnerable to both anthropogenic and natural hazards. The identification of coastal ecosystem species, such as mangrove and coastal forests, is very important in conserving and using the biodiversity of coastal ecosystems, which appears to be hindered by a lack of taxonomic and molecular expertise. This study aimed to address the lack of reference deoxyribonucleic acid barcodes from mangroves in North Sumatra and Aceh and assess the effectiveness of four deoxyribonucleic acid barcoding methods in terms of primer universality, successful identification rate, barcoding gap and species-tree inference, and then phylogenetic tree construction.

METHODS: This study focused on selecting the main regions where mangroves are predominantly distributed in the provinces of North Sumatra and Aceh: Percut Sei Tuan and Deli Serdang mangrove areas, Pulau Sembilan and Lubuk Kertang of Langkat mangrove areas in North Sumatra, and Langsa mangrove areas in Aceh. The genomic deoxyribonucleic acid of mangrove plants was isolated from fresh leaf material using the Geneaid genomic deoxyribonucleic acid mini kit. Based on the guidance provided by the International Union for Biological Barcoding with four molecular sequences, deoxyribonucleic acid barcodes were chosen for amplification: chloroplast ribulose 1,5-bisphosphate carboxylase/oxygenase, maturase-K, transfer ribonucleic acid for histidine–photosystem II reaction center protein A, and nuclear genome internal transcribed spacer. The Tamura 3-parameter + Gamma method in molecular evolutionary genetics analysis X software was used to measure and describe the genetic distances between different species and within the same species. The construction of phylogenetic trees was carried out using the molecular evolutionary genetics analysis X from ribulose 1,5-bisphosphate carboxylase/oxygenase, transfer ribonucleic acid for histidine–photosystem II reaction center protein A, Internal transcribed spacer, and maturase-K barcodes based on the bootstrap analysis conducted using 100 permutations.

FINDINGS: This study showed that the primers ribulose 1,5-bisphosphate carboxylase/oxygenase, transfer ribonucleic acid for histidine–photosystem II reaction center protein A, internal transcribed spacer, and maturase-K had the highest success rates during amplification, which could be strong barcodes for enhancing taxonomic clarification and gaining insights into phylogenetic relationships. The primers ribulose 1,5-bisphosphate carboxylase/oxygenase, transfer ribonucleic acid for histidine–photosystem II reaction center protein A, internal transcribed spacer, and maturase-K had the highest success rates during amplification. The success rate for the ribulose 1,5-bisphosphate carboxylase/oxygenase gene was the highest (90% percent), followed by (86 percent), transfer ribonucleic acid for histidine–photosystem II react percent ion center protein Ainternal transcribed spacer (75 percent), and maturase-K (57 Percent). The significant differences were as follows: inter- and intraspecific genetic distance (probability (p) <0.001), maturase-K (probability = 0.0001), combination maturase-K + photosystem II reaction center protein A (probability = 0.0008), maturase-K + ribulose 1,5-bisphosphate carboxylase/oxygenase (probability = 0.0008), maturase-K + internal transcribed spacer (probability = 0.0003), ribulose 1,5-bisphosphate carboxylase/oxygenase + internal transcribed spacer (probability = 0.0002), photosystem II reaction center protein A + internal transcribed spacer (probability = 7.051e-05), and three combined markers maturase-K + photosystem II reaction center protein A + internal transcribed spacer (probability = 0.0007). It is noteworthy that the maturase-K barcode could construct the clustering and differentiate the mangrove species based on family and not from sites. The ribulose 1,5-bisphosphate carboxylase/oxygenase barcode showed that members of Rhizophoraceae (*Bruguiera parviflora, Rhizophora apiculata*, and *Rhizophora stylosa*), Ptiredeacea (*Acrostichum aureum*), and *Scyphiphora hydrophyllaceae* from Rubiaceae existed in one branch.

CONCLUSION: This study provided a reference database both molecularly and taxonomically to strengthen biodiversity assessment and monitor mangrove forests. This database can be used to clarify the results of deoxyribonucleic acid barcodes for morphological and biochemical identification in the eastern coast of Sumatra.

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INTRODUCTION

Human activities and changes in climate are the main causes of the degradation of coastal vegetation, such as mangroves (Wang and Gu, 2021). Anthropogenic activities, such as land-use alteration, habitat loss, mangrove tree cutting, industry, alien species invasion, and overuse of biological resources, have the potential to alter the structure of landscapes (Cahyaningsih *et al.,* 2022). There are 69 mangrove species worldwide, and deforestation is driving species extinction (Hutchison *et al*., 2014). Mangroves that have been restored are those that have undergone plantation, regeneration, reforestation, restoration, and rehabilitation. Mangrove restoration can potentially support a number of policy goals pertaining to sustainable development, climate change mitigation, and biodiversity conservation (Su *et al.,* 2021). Mangrove forest conservation and restoration initiatives are widely supported worldwide. Rapid species identification, biodiversity assessment, and ecosystem dynamics monitoring are required for these actions (Mao *et al.,* 2021). The global distribution of mangroves is divided into two hemispheres: the Atlantic East Pacific (12 species) and the Indo West Pacific (58 species) (Michel, 2014). Distribution of mangrove species encompasses 34 major species and 20 minor species, which belong to 20 genera and 11 families (Tomlinson, 2016). The World Mangrove Atlas database reported that 73 identified mangrove species and a few hybrids are dispersed in 123 nations, covering an area of 150,000 km^2 of global geography (Spalding *et al.,* 2010). The evolutionary relationship between mangrove species and traditional classification systems is difficult to understand. A novel method for identifying unidentified organisms is deoxyribonucleic acid (DNA) barcoding, which uses the species' DNA region as a guide (Li *et al.,* 2015). DNA barcodes are significantly successful for very rapid animal identification (Klippel *et al*., 2022), insects (Pfeiler, 2018), tropical and subtropical plants (Jin *et al*., 2023), and microorganisms (Chakraborty *et al*., 2014). DNA barcodes have effectively reconstructed evolutionary relationships, accurately classified species within the same genus, and uncovered novel species or cryptic variations (Kang *et al.,* 2017; Amandita *et al.,* 2019). In this regard, the study is focused on investigating the universality of DNA barcodes in mangroves and coastal plants (associated mangroves), situated at the interface between terrestrial and aquatic environments, toward proper identification of mangrove species, establishing a phylogenetic tree of mangrove and coastal flora, and establishing a scientific foundation for the preservation of mangrove and coastal biodiversity (Saddhe *et al.,* 2016). A claimed assessment of 14 mangrove species found in Goa, situated on the western coast of India, was performed using core DNA barcode markers: ribulose 1,5-bisphosphate carboxylase/ oxygenase (rbcL) and maturase-K (matK) (Saddhe *et al.,* 2016). According to the results of Saddhe *et al.* (2016), it was unequivocally established that the matK locus exhibited the highest identification rate as 72.09 percent (%) in terms of accurate assignment, followed by rbcL, transfer ribonucleic acid (RNA) for histidine–photosystem II reaction center protein A (trnH–psbA), internal transcribed spacer (ITS), and matK. The rbcL + matK locus, when combined, demonstrated adequate discriminatory power across mangrove genera and species, with the exception of Rhizophora, Sonneratia, and Avicennia (Saddhe *et al.,* 2016). Wu *et al.* (2019) reported that the phylogenetic tree constructed using the primer combination rbcL + matK + trnH–psbA + ITS for mangrove vegetation exhibited the highest rate of relationship support. Barcode DNA is currently an effective tool enabling fast and accurate plant identification (Li *et al.,* 2015). The studies from other mangroves connected to this study to attempt the first step toward the DNA barcodes of North Sumatran and Aceh mangrove and coastal forests based on plastid genes. Nonetheless, there remains a lack of research on DNA barcodes in mangrove and coastal forests in Indonesia, which is home to the world's biggest mangrove forest. Indonesia is host to the largest mangrove forest in the world, which is 2.7 million in 2020 (Basyuni *et al.,* 2022). Mangrove forests are predominantly located along the eastern coast of North Sumatra, spanning from Serdang Bedagai to Langkat Regency, Deli Serdang, Batu Bara, Tanjung Balai, Asahan, and Labuhanbatu (Basyuni and Sulistiyono, 2018). The urgency of this study is crucial in order to close the gap in a reference library of mangrove and coastal forests, both in terms of taxonomy and molecular data in North Sumatra and Aceh, Indonesia. This database can be used to strengthen DNA Meta barcoding studies of mangrove ecosystems. Research publications on DNA barcoding in the mangrove field

are still limited, both in terms of the use of markers and the number of sample collections. This study will facilitate future studies on the diversity of mangrove species still growing in North Sumatra and Aceh using four markers at four study sites. This study aims to analyze the lack of reference DNA barcodes from mangroves and to assess the effectiveness of four DNA barcoding methods in terms of primer universality, successful identification rate, barcoding gap, species-tree inference, and phylogenetic tree construction that were used to assess the efficacy of four DNA barcoding techniques. This study has been conducted in North Sumatra and Aceh, Indonesia, in 2021.

MATERIALS AND METHODS

Sample area and collection

The study area is in the provinces of North Sumatra and Aceh: Percut Sei Tuan 3°43'49" north (N) 98°46'14" east (E) 141,44 square meter (m^2) and Deli Serdang mangrove areas; Pulau Sembilan 4°08'33"N 98°14'34"E 122,41 m^2 and Lubuk Kertang 4°02'57" N 98°17'49" E 2.643,58 m^2 of Langkat mangrove areas in North Sumatra; and Langsa mangrove areas 4°31'20" N 98°00'59" E 837,69 m^2 in Aceh. Fig. 1 illustrates the locations gathered. All mangrove samples were identified at species level as described by Tomlinson (2016). The study focused on selecting the main regions where mangroves are predominantly distributed in the provinces of North Sumatra and Aceh. Table 1 shows the exhaustive details regarding the sampling locations. According to the DNA barcode sample collection standards, samples were collected from two to three individuals of each mangrove species. To simplify the extraction of DNA molecular components, fresh leaves were picked. A cumulative count of 253 individuals of mangrove vegetation from 31 different species of mangrove plants was gathered.

DNA extraction and sequence analysis

The genomic DNA of mangrove plants was isolated from fresh leaf material using the Geneaid genomic DNA mini kit. Based on the guidance provided by the International Union for Biological Barcoding (CBOL Working Plant Group, 2009) and prior researches (Saddhe *et al.*, 2016; Wu *et al.*, 2019; Mao *et al.*, 2021) on plant DNA barcoding, four molecular sequences, chloroplast rbcL, matK, trnH–psbA, and nuclear genome ITS, were used to amplify the extracted

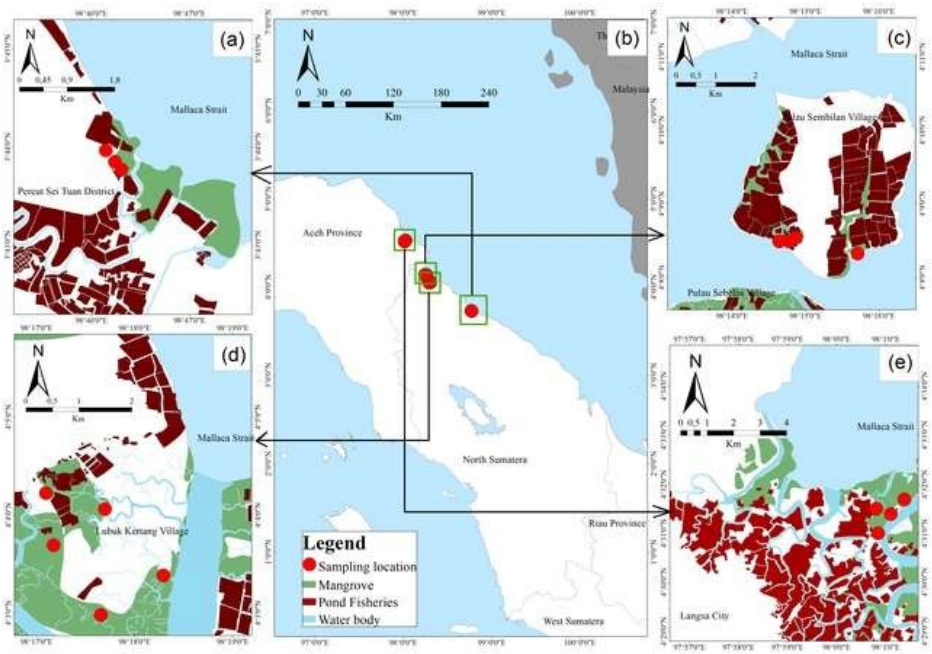

Fig. 1: Geographic location of the study area in sample collection from Percut Sei Tuan (a) of North Sumatra, and, North Sumatra and Aceh provinces (b) of Indonesia, Pulau Sembilan (c) of North Sumatra, Lubuk Kertang (d) of North Sumatera, Langsa (e) of Aceh

mangrove DNA from North Sumatra and Aceh (Table 2). The plant working group of the International DNA Barcode Alliance recommends referring to the polymerase chain reaction (PCR) system for life, optimization, and correction as previously reported (CBOL Working Plant Group, 2009).

The PCR contains the necessary information and processes for primer information and amplification.

Table 1: The data pertaining to mangrove samples gathered in the provinces of Aceh and North Sumatra

No	Species	Family	Status	Life form	Sites	Red List
1	*A. aureum*	Pteridaceae	MA	Shrub	Percut Sei Tuan	LC
2	*R. apiculate*	Rhizophoraceae	TM	Tree	Percut Sei Tuan	LC
3	*N. fruticans*	Arecaceae	TM	Palm	Percut Sei Tuan	LC
4	*A. alba*	Acanthaceae	TM	Tree	Percut Sei Tuan	LC
5	*A. ilicifolius*	Acanthaceae	TM	Shrub	Percut Sei Tuan	LC
6	*A. officinalis*	Acanthaceae	TM	Tree	Percut Sei Tuan	LC
7	*A. marina*	Acanthaceae	TM	Tree/shrub	Percut Sei Tuan	LC
8	*R. stylosa*	Rhizophoraceae	TM	Tree	PercutSei Tuan	LC
9	*E. agallocha*	Euphorbiaceae	MA	Tree/shrub	Percut Sei Tuan	LC
10	*X. granatum*	Meliaceae	TM	Tree	Pulau Sembilan	LC
11	*B. parviflora*	Rhizophoraceae	TM	Tree	Pulau Sembilan	LC
12	*N. fruticans*	Arecaceae	TM	Palm	Pulau Sembilan	LC
13	*A. auriculiformis*	Fabaceae	MA	Tree	Pulau Sembilan	LC
14	*E. agallocha*	Euphorbiaceae	MA	Tree/shrub	Pulau Sembilan	LC
15	*S. hydrophyllacea*	Rubiaceae	TM	Tree/shrub	Pulau Sembilan	LC
16	*A. marina*	Acanthaceae	TM	Tree/shrub	Pulau Sembilan	LC
17	*A. officinalis*	Acanthaceae	TM	Tree	Pulau Sembilan	LC
18	*A. aureum*	Pteridaceae	TM	Shrub	Pulau Sembilan	LC
19	*A. ilicifolius*	Acanthaceae	TM	Shrub	Pulau Sembilan	LC
20	*A. alba*	Acanthaceae	TM	Tree	Pulau Sembilan	LC
21	*S. hydrophyllacea*	Rubiaceae	TM	Tree/shrub	Lubuk Kertang	LC
22	*A. marina*	Acanthaceae	TM	Tree/shrub	Lubuk Kertang	LC
23	*A. alba*	Acanthaceae	TM	Tree	Lubuk Kertang	LC
24	*E. agallocha*	Euphorbiaceae	MA	Tree	Lubuk Kertang	LC
25	*S. alba*	Lythraceae	TM	Tree	Lubuk Kertang	LC
26	*A. officinalis*	Acanthaceae	TM	Tree	Lubuk Kertang	LC
27	*A. ilicifolius*	Acanthaceae	TM	Shrub	Lubuk Kertang	LC
28	*N. fruticans*	Arecaceae	TM	Palm	Lubuk Kertang	LC
29	*R. stylosa*	Rhizophoraceae	TM	Tree	Lubuk Kertang	LC
30	*A. ilicifolius*	Acanthaceae	TM	Shrub	Langsa	LC
31	*A. officinalis*	Acanthaceae	TM	Tree	Langsa	LC

TM, true mangrove; MA, mangrove associate (both classification to Wang *et al.*, (2021)); LC, least concern (Hutchison *et al.*, 2014)

Table 2: The primers used to amplify DNA barcodes from North Sumatran and Aceh mangroves

DNA barcode	Primers	Sequences (5′–3′)	Amplification procedure	Sources
rbcl	rbcl F	ATGTCACCACAAACAGAGACTAAAGC	72°C 1 min, 35 cycles; 72°C 7 min, 72°C 1 min, 35 cycles; 72°C 7 min	Kress *et al.*, 2009
	rbcl R	GTAAAATCAAGTCCACCRCG		
matK	Matk F	ACCCAGTCCATCTGGAAATCTTGG TTC	94°C 3 min; 94°C 45 s, 51°C 45 s, 72°C 1 min, 35 cycles; 72°C 7 min	Wu *et al.*, 2019
	Matk R	ACCCAGTCCATCTGGAAATCTTGG TTC		
psbA-trnH	PsbA F	GTTATGCATGAACGTAATGCTC	94°C 3 min; 94°C 30 s, 55°C 1 min, 72◦C 1 min, 35 cycles; 72◦C 7 min	Sang *et al.*, 1997
	trnH R	CGCGCATGGTGGATTCACAATCC		Tate and Simpson, 2003
ITS	ITS 1	GTCCACTGAACCTTATCATTTAG	94◦C 3 min; 94◦C 30 s, 55◦C 1 min, 72◦C 1 min, 35 cycles; 72◦C 7 min	White *et al.*, 1990
	ITS 4	TCCTCCGCTTATTGATATGC		

Following gel electrophoresis detection, all amplicons were forwarded to Macrogen (Macrogen Asia Pacific Pte. Ltd., Singapore) for Sanger sequencing. GenBank was used to conduct basic local alignment search tool (BLAST) searches for the sequences that resulted from the bidirectional sequencing of the four fragments of DNA barcodes. If significant discrepancies were identified between the sequences and the original species, explanations were sought and confirmed by consulting experts until the BLAST results of the sequences and the original species were consistent in terms of the same genus or family. The software UGENE v. 40.1 (Salinas *et al.*, 2024) was used to sequence plasmids from DNA samples that were sequenced twice to align. The nucleotide sequence data from DNA barcode collections have been stored in the GenBank/European Molecular Biology Laboratory (EMBL)/DNA Data Bank of Japan (DDBJ) nucleotide sequence database, together with the corresponding accession codes: R1-R167 (rbcL), OQ695790-OQ695870; M7-M167 (matK), OQ695871-OQ695909; P1-P167 (trnH–psbA), OQ695910-OQ695977; and T4-T167 (ITS), OQ695979-OQ696041.

Barcoding Gap and Species-Tree Inference

The Tamura 3-parameter + Gamma method in molecular evolutionary genetics analysis (MEGA) X (Kumar *et al.,* 2018) was used to measure and describe the genetic distances between different species and within the same species. This was done for each individual barcode area (matK, rbcL, psbA, ITS, matK + rbcL, matK + ITS, rbcL + ITS, and matK + rbcL + psbA + ITS) and for all the barcode regions combined. The Tamura 3-parameter model was determined to be the optimal substitution model for the matK, rbcL, and ITS sequences using MEGA X. This model takes into consideration the variations in transitional and transversional alterations and the biased guanine (G) + cystosine (C) content (Tamura, 1992); the gamma (G) distribution is typically fitted to the available data of symptom onset and thus represents the rate of evolution between them assimilating the generation times to the serial interval (Park *et al.*, 2020). Sequences obtained from each individual of the respective species, classified based on their morphology, were organized to calculate the genetic distances between different species and within the same species. The tool grammar of graphics plot-2 (GGPLOT2) in R programming was used to calculate and plot boxplots to display the percentage distribution of inter- and intraspecific divergences for each marker (R Core Team, 2022). The divergence at intra- and inter-population levels of the samples was assessed by DNA sequences. Saddhe *et al.* (2016) reported that the highest intraspecific distances are found along the diagonal, whereas minimum interspecific distances are found below it.

Phylogenetic tree

The construction of phylogenetic trees was carried out using the MEGA X software (Kumar *et al.,* 2018) from rbcL, matK, trnH–psbA, and ITS barcodes based on the bootstrap analysis conducted using 100 permutations. The neighbor-joining (NJ) approach was employed, and the Kimura'2-parameter model was used as the basis for the analysis (Tamura *et al.,* 1992). Phylogenetic trees were created by using the unique sequence of barcodes for each individual.

Data analysis

The ranking of the success rate of PCR amplification was consistent with that of sequencing (Gong *et al.,* 2009). The efficiency of the species identification approach was assessed using BLAST. Initially, a regional database was created for the four DNA fragments in Ugene v. 40.1 (ugene.net), and all sequence comparisons were stored as *.fasta files to align the sequence orientation and remove any gaps between sequences. The BLAST software from the National Center for Biotechnology Information (NCBI) in the United States was used to conduct a comprehensive comparison of each sequence with all sequences included in the database. The quantification standard was determined based on the percentage of identical sites. If the lowest value of the identical sites within a particular species is higher than the value found between individuals of all other species, it was determined that the sequence of this species has been correctly recognized and award it a match to the reference sequences. The statistical analyses and data visualizations were performed using R version 4.1.3 (R Core Team, 2022), together with the Tidyverse package (which includes read rectangular (readr), tidy data (tidyr), dplyr program, and GGPLOT2) (Wickham *et al.,* 2019) and hrbrthemes (Rudis, 2020) packages. The percentage distribution of the divergences within and between species for each marker was computed and subsequently

displayed in boxplots using the GGPLOT2 software. Wilcoxon signed-rank tests were performed to assess the significance of intra- and interspecific genetic distances (α = 0.001).

RESULTS AND DISCUSSION

Sequence analysis

Sequence statistics were calculated for 253 individuals that successfully amplified and sequenced from 31 different species of mangrove plants (Table 3). A total of 75 sequences of mangrove plants was obtained from the Percut Sei Tuan region. The rbcL gene had the highest rate of successful amplification, followed by trnH–psbA, ITS, and matK. The rbcl gene exhibited the most favorable outcome in terms of sequencing, achieving a success rate of 82%. The trnH–psbA sequence had the highest success rate (72%), followed by ITS (66%) and matK (60%). A total of 89 sequences of mangrove trees were gathered from the Pulau Sembilan region. The primers rbcL, trnH–psbA, ITS, and matK showed the highest percentages of successful amplification. The rbcL gene exhibited the highest success rate (90%), followed by trnH–psbA (86%), ITS (75%), and matk (57%). A total of 65 sequences of mangrove plants were obtained in the Lubuk Kertang area. The amplification success rate for these sequences was as follows: rbcL, followed by ITS, trnH–psbA, and matK. The rbcL and ITS sequencing had the highest success rate (83%), followed by trnH–psbA (80%) and matK (60%). A total of 24 sequences of mangrove plants were collected from the Langsa area. The rbcL, trnH–psbA, ITS, and matK genes had the highest rates of amplification success. The efficacy of the sequence of the four barcode primers varied depending on the locations (Table 3).

Mangroves collected in this study belonged to 14 species (*Acrostichum aureum, Avicennia alba, A. marina, A. officinalis, Acacia auriculiformis, Acanthus ilicifolius, Bruguiera parviflora, Excoecaria agallocha, Nypa fruticans, Rhizophora apiculata, R. Stylosa, Sonneratia alba, Scyphiphora hydrophyllacea, Xylocarpus granatum*), 11 genera (*Acrostichum, Acacia, Acanthus, Avicennia, Bruguiera, Excoecaria, Nypa, Rhizophora, Sonneratia, Scyphiphora, Xylocarpus*), and 9 families (Acanthaceae, Arecaceae, Euphorbiaceae, Fabaceae, Lythaceae, Meliaceae, Pteridaceae, Rubiaceae, Rhizophoraceae). High-quality DNA barcodes for 120 specimens of the aforementioned species were obtained, which were subjected to sequencing for rbcL and matK. The rbcL sequencing yielded an average of 516 base pairs (bp) from 81 sequences, with no insertions, deletions, or stop codons. Conversely, the matK sequencing resulted in 702 bp from 39 sequences, with no insertions, deletions, or gaps in the form of start/stop codons. Furthermore, psbA-trnh consisted of an average of 464 bp from 69 sequences, and ITS produced an average of 638 bp from 63 sequences. This study represents the inaugural endeavor to

Table 3: The four barcoding fragments success percentages for PCR amplification and sequencing at each of the four mangrove sites

Sites	Barcode	Amplicon success rate (%)	Sequence success rate (%)	Individual
Percut Sei Tuan	rbcL	100	82	25
	matK	71	60	12
	trnh–psbA	89	88	22
	ITS	85	66	16
Pulau Sembilan	rbcL	100	90	30
	matK	63	57	12
	Trnh–psbA	87	86	25
	ITS	87	75	22
Lubuk kertang	rbcL	100	83	20
	matK	62	60	9
	Trnh–psbA	83	80	16
	ITS	100	83	20
Langsa	rbcL	100	50	6
	matK	75	50	6
	Trnh–psbA	100	50	6
	ITS	100	50	6
Total				253

assess the variety of Indonesian mangroves, namely, those from North Sumatra and Aceh, using four DNA barcodes: rbcL, matK, psbA-trnh, and ITS.

Previous report on DNA barcode investigation (Table 4) using rbcL and matK on *A. ilicifolius* in the coast of Cilacap, Central Java (Harisam *et al.,* 2018; Harisam *et al.,* 2020). However, several studies of mangrove identification were based on molecular markers such as microsatellite on *A. marina* from Cilegon, West Java (Manurung *et al.,* 2017); *A. alba*, *A. marina*, and *A. officinalis* from Java Island (Sabdanawaty and Daryono, 2021); *R. apiculata* from Sunda Island (Yahya *et al.,* 2015); *R. mucronata* from Sumatra Island (Wee *et al.,* 2014); RAPD marker on *R. mucronata* from Timor Island (Ihwan and Hakim, 2019) and western coast of Timor Island (Ihwan *et al.,* 2020); *Bruguiera* species from Karimunjawa Island, Central Java (Susilo and Meitiyani, 2018); SRAP marker on *R. mucronata* (Senjaya, 2021); and morphological characters on *A. alba*, *A. marina*, and *A. officinalis* (Sabdanawaty and Daryono, 2021). Recent identification of two *Lumnitzera* species, *L. littorea* and *L. racemosa*, was done using double digest restriction site-associated DNA sequencing (ddRADseq) approach (Manurung *et al.,* 2023). These studies provided various markers in mangrove identification. DNA barcoding technology may help protect mangrove ecosystems by allowing for fast species identification (Mao *et al.,* 2021) (Table 4). The utilization of DNA barcoding has become a potent method for addressing the lack of reference information on mangroves and enhancing the understanding of mangrove ecosystems. This study indicated that rbcL and psbA had a high sequence rate, in addition to the significant inter- and intraspecificity of matK, and the combination of barcodes led to their application to other mangroves. The low success sequence rate of ITS in this study was supported by previous barcode studies of mangrove in Guangdong Province, China (Wu *et al.,* 2019), and tropical cloud forest in Jianfengling, Bawangling, and Limushan, China (Kang *et al.,* 2017). Accurate species identification through DNA barcoding relies on comparing the obtained sequences with existing databases. In regions with limited genetic data for mangroves, reference databases may need to be expanded. Further DNA barcodes from other Indonesian mangrove regions are required to clarify this issue. DNA barcoding involves the use of specific regions of the genome as molecular markers to identify and classify species. In the context of mangroves, DNA barcoding can be applied to study their genetic diversity, identify species, and improve taxonomic knowledge. This method is especially valuable when the morphological identification of species is difficult due to the intricate and overlapping characteristics of mangrove plants (Mao *et al.,* 2021).

Table 4: Comparison studies on molecular methods for mangrove identification

Mangrove species	Methods	Sources
A. ilicifolius	DNA barcode (rbcl and matK)	Harisam *et al.,* 2018; Harisam *et al.,* 2020
A. marina	Microsatellite	Manurung *et al.,* 2017
A. alba, *A. marina*, *A. officinalis*	Microsatellite	Sabdanawaty and Daryono, 2021
R. apiculata	Microsatellite	Yahya *et al.,* 2015
R. mucronata	Microsatellite	Wee *et al.,* 2014
R. mucronata	Randomly amplified polymorphic DNA (RAPD) marker	Ihwan and Hakim, 2019; Ihwan *et al.,* 2020
Bruguiera	RAPD marker	Susilo and Meitiyani, 2018
R. mucronata	Sequence-related amplified polymorphism (SRAP) marker	Senjaya, 2021
A. alba, *A.marina*, *A. officinalis*	Morphological characters	Sabdanawaty and Daryono, 2021
Lumnitzera species, *L. littorea* and *L. racemosa*	DNA sequencing (ddRADseq)	Manurung *et al.,* 2023
Acrostichum aureum, Avicennia alba, A. marina, A. officinalis, Acacia auriculiformis, Acanthus ilicifolius, Bruguiera parviflora, Excoecaria agallocha, Nypa fruticans, Rhizophora apiculata, R. Stylosa, Sonneratia alba, Scyphiphora hydrophyllacea, Xylocarpus granatum	DNA barcodes (matK, rbcL, trnh–psbA, ITS)	This study

The gaps in mangrove DNA barcodes may vary based on the geographical environment (sites) and the specific research emphasis. It is important to take note of this variability. As a consequence, accurate species identification and assessment of genetic relationships for those particular mangrove species or regions may be challenging (Saddhe *et al.,* 2016; Wu *et al.,*2016).

Intra- and Interspecific Relationship

A genetic distance analysis was conducted using 253 sequences from the datasets. Regardless of the marker employed, there were significant differences in intra- and interspecific genetic distances ($p < 0.001$, Wilcoxon signed-rank test) (Fig. 2), except for matK + rbcL + ITS ($p = 0.001$) or ($p < 0.005$). There is no significant difference for rbcL ($p = 0.1715$), psbA ($p = 0.2265$), and ITS ($p = 0.1802$), because these barcodes have no interspecific distance data. For inter- and intraspecific genetic distance, significant difference ($p < 0.001$) for matK ($p = 0.0001$), combination matK + psbA ($p = 0.0008$), matK + rbcL ($p = 0.0008$), matK + ITS ($p = 0.0003$), rbcL + ITS ($p = 0.0002$), psbA + ITS ($p =$ 7.051exponent (e)-05), and three combined markers matK + psbA + ITS had $p = 0.0007$. Figure 2 showed that matK, rblc, and psbA could be strong barcodes for enhancing taxonomic clarification and gaining insights into phylogenetic relationships.

Multiple lines of research have been documented to substantiate this study, for example, at Goa, India, based on rbcL and matK (Saddhe *et al.,* 2016). Furthermore, Wu *et al.* (2019) showed that the

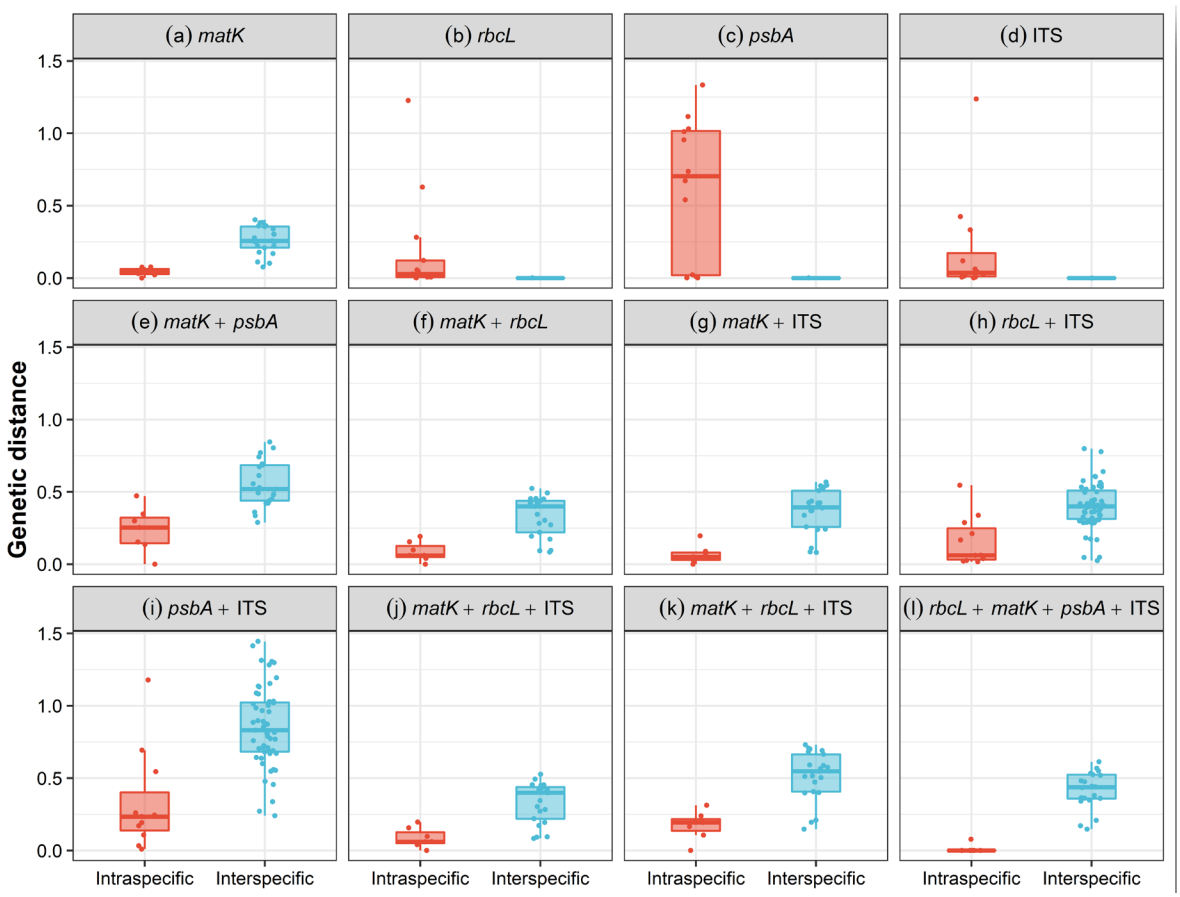

Fig. 2: Interspecific (red) and intraspecific (blue) genetic distances for 253 mangrove samples from Sumatra using barcode markers matK (a), rbcL (b), psbA (c), ITS (d), and their combinations (e–l). Significant variations in inter- and intraspecific distances were observed for all markers or combinations, as determined by a Wilcoxon signed-rank test ($p < 0.001$), except for no significant difference for rbcL ($p = 0.1715$), psbA ($p = 0.2265$), and ITS ($p = 0.1802$), due to the lack of interspecific distance information in these barcodes

primer combination rbcL + matK + trnH–psbA + ITS had the highest relationship support rate. Mao *et al.* (2021) concluded that the use of ITS is adequate for barcoding mangrove species and coastal plants in South China. Phylogenetic trees showed that all the DNA barcodes revealed that matK, rbcL, and psbA could be strong barcodes for enhancing taxonomic clarification and gaining insights into phylogenetic relationships. matK was distinguished from other barcodes to differentiate mangrove associates from true mangroves due to the low successful rate of sequence and number of individuals sampled. The gaps in mangrove DNA barcodes may vary based on the geographical environment (sites) and the specific research emphasis (Table 3, Fig. 2). It is vital to take note of this variability. As a result, accurate species identification and assessment of genetic relationships for those particular mangrove species or regions may be challenging or incomplete. The variation in the performances of each barcode was noted and their complement each other showing the significant combinations of barcodes. Furthermore, the rbcL marker in this study supported previous studies on Rhizophoraceae tribe formed two branches with *R. apiculate*, *R. mucronate*, *R. stylosa*, and *R. lamarkii* and another branch of *X. granatum* and *C. decandra* (Saddhe *et al.*, 2017). Minimum interspecific distances are found below the diagonal, while maximum intraspecific distances are found along it (Saddhe *et al.*, 2016). Low level of interspecific genetic diversity generally occurred in the mangroves of North Sumatra and Aceh, due to the DNA barcodes' lack of genetic distance, except for matK, indicate species with a barcode gap. This finding disagreed with previous results that a low level of intraspecific genetic diversity occurred in the mangroves of China (Mao *et al.*, 2021). This disparity is a result of the mangroves' geomorphology setting, oceanographic connectivity, and environmental factors (Saddhe *et al.*, 2016; Gouvea *et al.*, 2023). It has been shown that the land processes of mangroves and the ocean are dominated by the geomorphology of the coast (Gouvea *et al.*, 2023).

Phylogenetic analysis

Various segments were used to build phylogenetic trees, and the average rate of support for each node was calculated. The phylogenetic trees displayed a radial pattern, with a solitary branch comprising species that were either identical or closely related, as shown in Figs. 3–5 for matK, rbcl, psbA, and ITS, respectively. In this matK barcode, Fig. 3 shows the species relationships from mangrove leaves from 39 individual mangrove species. These data revealed that mangrove species fell into four major groups of representative family: Acanthaceae (two clusters), Arecaceae, and Rubiaceae (Fig. 3). The first branch of Acanthaceae consisted of *A. officinalis, A. alba*, and *A. ilicifolius* from only two sites: Percut Sei Tuan and Pulau Sembilan. The second branch of Acanthaceae is comprised of *A. alba, A. marina*, and *A. ilicifolius* with diverse all sites: Percut Sei Tuan, Pulau Sembilan, Lubuk Kertang, and Langsa. Alternatively, *N. fruticans* belongs to Arecacea derived from Percut Sei Tuan and Pulau Sembilan (Fig. 3). The fourth cluster of Rubiaceae consisted of *S. hydrophyllaceae* from Percut Sei Tuan nad Pulau Sembilan (Fig. 3). It is noteworthy that the matK barcode could construct the clustering and differentiate the mangrove species based on family and not from sites. Figure 4 shows the rbcL barcode for the species relationship between mangrove leaves from 81 individual mangrove species. The data revealed six branches: Arecaceae, Acanthaceae, Euphorbiaceae, Pteridaceae, Rubiaceae, and Rhizophoraceae. Arecaceae comprises of *N. fruticans* from Percut Sei Tuan and Pulau Sembilan, while another branch of *A. ilicifolius, A. alba, A. marina,* and *A. officinalis* belongs to Acanthaceae from Percut Sei Tuan, Pulau Sembilan, and Langsa. Furthermore, species of *E. agallocha* belong to Euphorbiaceae from Pulau Sembilan, R67 was out grouped from R68 and R69 and formed another branch sit to cluster *Sonneratia alba* and *A. aureum* (Fig. 4). Another *E. agallocha remained in one* branch. It is noteworthy that members of Rhizophoraceae (*B. parviflora, R. apiculata*, and *R. stylosa*), Ptiredeacea (*A. aureum*), and *S. hydrophyllaceae* from Rubiaceae existed in one branch (Fig. 4). Unexpected results from psbA-trnh barcode led to 12 clusters. Acanthaceae had four branches, Euphorbiaceae, Pteridaceae, and Rubiaceae consisted of two clusters, while Rhizophoraceae and Rubiaceae had one branch (Fig. 5). It is worth noting that this psbA-trnh prime for Acanthaceae differentiated the phylogenetic tree into four branches: the first branch comprised of *A. alba* and *A. marina*; the second branch consisted of *A. ilicifolius* only; the third branch had *A. officinalis*; and the fourth branch consisted of *A. alba, A. marina*, and

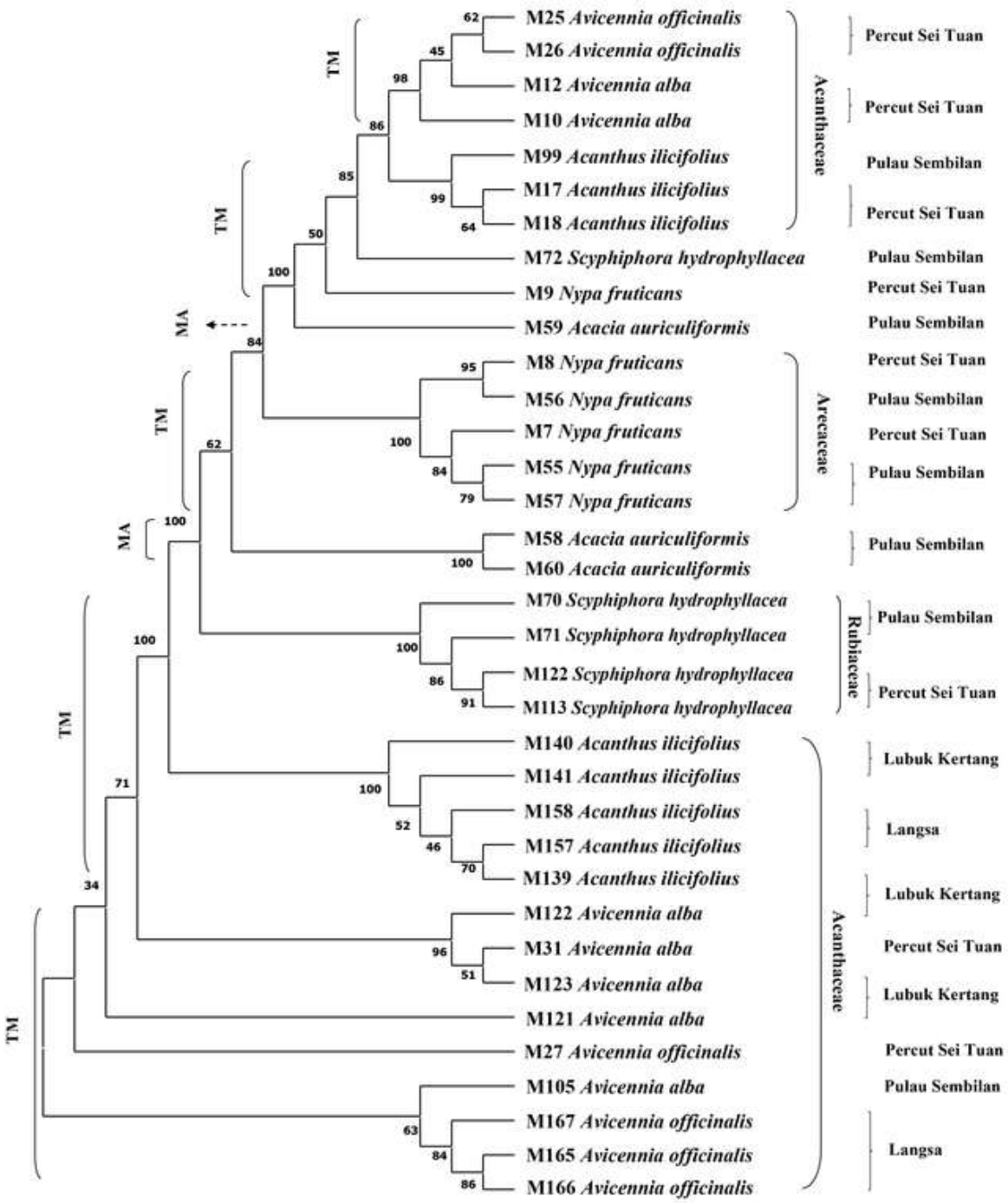

Fig. 3: The phylogenetic tree of mangroves in North Sumatra and Aceh, constructed using a fragment of the matK gene

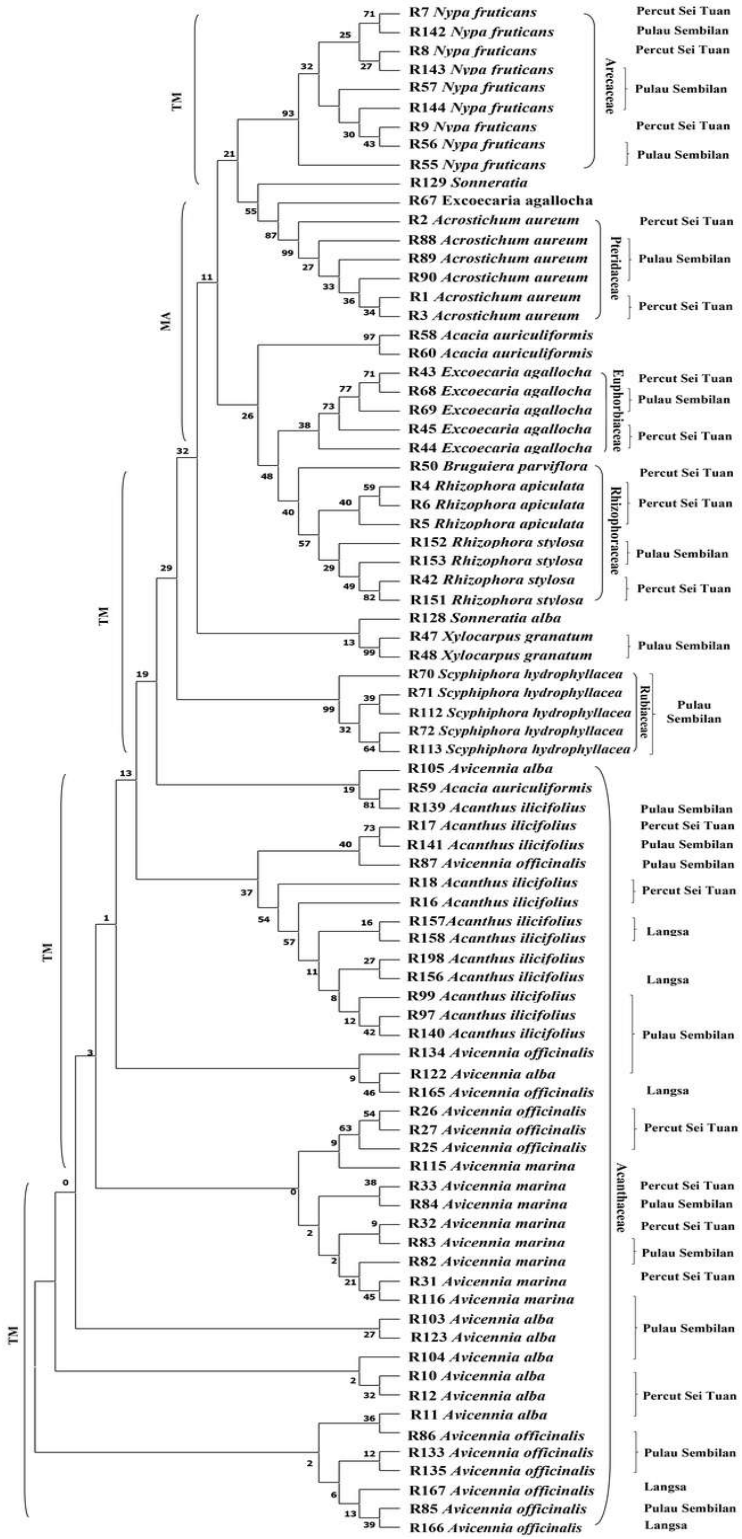

Fig. 4: The phylogenetic tree of mangroves in North Sumatra and Aceh, constructed using a fragment of the rbcL gene

Fig. 5: The phylogenetic tree of mangroves in North Sumatra and Aceh, constructed using a fragment of the trnH–psbA gene

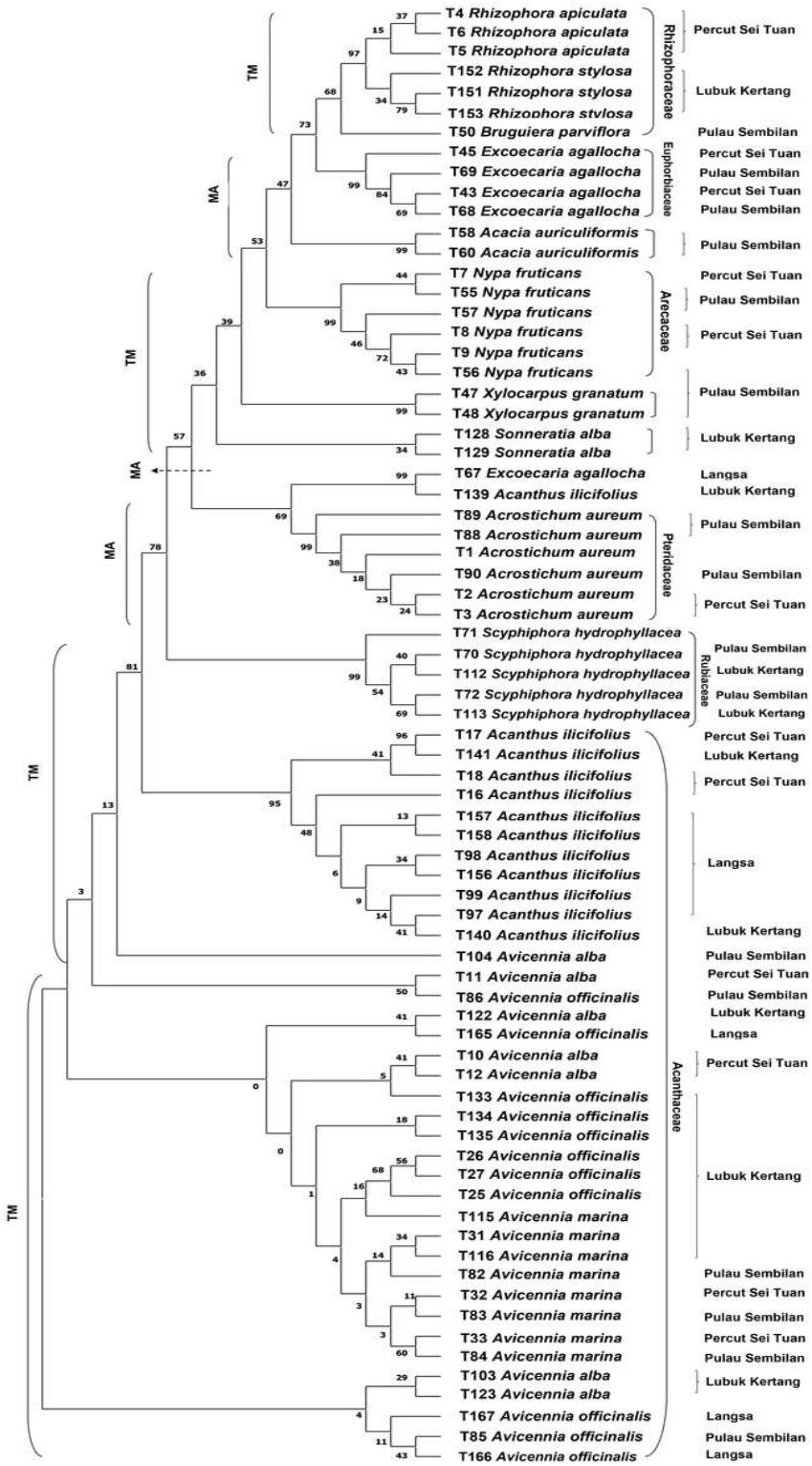

Fig. 6: The phylogenetic tree of mangroves in North Sumatra and Aceh, constructed using a fragment of the ITS gene

A. officinalis (Fig. 5). The cluster of Euphorbiaceae had two branches, the first consisted of *E. agallocha* from Lubuk Kertang and Percut Sei Tuan, and the second one, *E. agallocha*, belong to Lubuk Kertang, Percut Sei Tuan, and Pulau Sembilan (Fig. 5). The first cluster of Rubiaceae comprised of *S. hydrophyllaceae* from Lubuk Kertang and the second cluster of Rubiaceae belongs to *S. hydrophyllaceae* from Pulau Sembilan. In contrast, both clusters of Pteridacea comprised of *A. aureum* from Pulau Sembilan and Percut Sei Tuan. Fig. 6 shows ITS barcode consisting of six branches, which the most was Acanthaceae, from *A. ilicifoilus, A. alba, A. marina*, and *A. officinalis* sit on one cluster. Furthermore, Arecaceae comprised of *N. fruticans*; Pteridaceae comprised of *A. aureum*; Rhizophoracea comprised of *R. apiculata, R. stylosa,* and *B. parviflora*; and Rubiaceae for *S. hydrophyllaceae* also formed one cluster for all samples. In contrast, Euphorbiaceae formed one cluster except for *E. agallocha* from Langsa (Fig. 6).

The finding on the different groups between true mangrove and mangrove associates, particularly rbcL, psbA, and ITS barcodes, was supported by other studies on polyisoprenoid composition (Basyuni *et al.,* 2017), leaf traits and salt contents (Kang *et al.,* 2017), and leaf litter decomposition (Chanda *et al.,* 2016). To fill the reference database for mangrove identification, DNA barcoding is one of the most important and significant scientific visions as an effective tool for species-level identification (Harisam *et al.,* 2019). In North Sumatra and Aceh, DNA barcoding can be used to identify mangrove species. This is crucial in areas where there might be limited taxonomic expertise or where morphological traits are not easily distinguishable. The use of DNA barcodes enhances the accuracy and efficiency of biodiversity assessment in mangrove ecosystems compared to traditional methods, such as morphological characters (Saddhe *et al.,* 2016; Trivedi *et al.,* 2016). This study also implied to enhance the understanding of the genetic diversity and distribution of mangrove plant species, for example, by increasing the number of mangrove populations and status of species conservation (Wu *et al.,* 2019). By creating a comprehensive DNA barcode library of mangrove species, conservationists can track changes in species composition and detect potential threats to specific species or populations (Habib *et al.,* 2021). DNA barcoding can be used for biodiversity monitoring and conservation efforts (Trivedi *et al.,* 2016). Filling reference gaps in mangroves using DNA barcodes from North Sumatra and Aceh, Indonesia, can lead to valuable insights into mangrove biodiversity assessment, support conservation efforts, and contribute to sustainable management practices and strategies in these ecologically significant regions.

CONCLUSION

It is challenging to comprehend the evolutionary link between mangrove species and conventional classification schemes. DNA barcoding is a unique technique for identifying unknown organisms; it employs a species' DNA region as a guide. However, there is a lack of study on DNA barcodes in mangrove and coastal forests in Indonesia, which is home to the largest mangrove forest in the world. This study concentrated on selecting the main regions where mangroves are predominantly distributed in the provinces of North Sumatra and Aceh, Indonesia. DNA barcoding has emerged as an effective tool for improving the knowledge of mangrove ecosystems and solving the lack of reference data on mangroves. This study of genetic distance was performed using 253 sequences from the datasets. With the exception of matK + rbcL + ITS ($p = 0.001$) or ($p < 0.005$), there were significant variations in intra- and interspecific genetic distances ($p < 0.001$, Wilcoxon signed-rank test) for all markers used. Due to the lack of interspecific distance data for rbcL ($p = 0.1715$), psbA ($p = 0.2265$), and ITS ($p = 0.1802$) barcodes, there is no significant difference. There was a significant difference ($p < 0.001$) in the inter- and intraspecific genetic distance for matK ($p = 0.0001$), matK + psbA ($p = 0.0008$), matK + rbcL ($p = 0.0008$), matK + ITS ($p = 0.0003$), rbcL + ITS ($p = 0.0002$), psbA + ITS ($p = 7.051e-05$), and three combined markers matK + psbA + ITS. In addition to the great inter- and intraspecificity of matK, this study has shown that rbcL and psbA had a high sequencing rate. This combination of barcodes has allowed the use of these barcodes to other mangroves. Using particular genomic areas as molecular markers to identify and categorize species is known as DNA barcoding. DNA barcoding can be used in the context of mangroves to investigate their genetic diversity, recognize species, and advance taxonomic understanding. Filling reference gaps of mangroves using DNA barcodes might significantly enhance the understanding of mangrove biodiversity

in North Sumatra and Aceh. This method helped to identify previously unknown or misidentified species, contributing to the overall knowledge of the site's mangrove vegetation. DNA barcoding provided the aid in identifying rare or endangered mangrove species, enabling better conservation prioritization and management strategies to protect these ecologically significant habitats. The phylogenetic trees showed a radial structure, with a single branch made up of closely related or identical species. It is worth noting that the mangrove species could be distinguished based on family rather than location attributable to the matK barcode's ability to create the clustering. rbcL revealed six branches in the tree: Arecaceae, Acanthaceae, Euphorbiaceae, Pteridaceae, Rubiaceae, and Rhizophoraceae. Present work filled a gap of reference databases both molecularly and taxonomically to strengthen the biodiversity assessment and monitoring in mangrove and coastal forests of North Sumatra and Aceh, Indonesia. DNA barcoding has emerged as an effective technique to mitigate the deficiency of reference data on mangroves and augment the comprehension of mangrove ecosystems. In addition to the great inter- and intraspecificity of matK, this study has shown that rbcL and psbA had a high sequencing rate. This combination of barcodes has allowed the use of these barcodes to other mangroves. Using DNA barcoding, mangrove species in North Sumatra and Aceh may be accurately identified. This is especially important in situations where taxonomic knowledge may be scarce or physical characteristics are difficult to differentiate. Conservation and biodiversity monitoring can both benefit from the use of DNA barcoding. Conservationists are able to monitor shifts in the species composition of mangroves and identify possible hazards to individual species or populations by building an extensive DNA barcode library of these species. By employing DNA barcodes from North Sumatra and Aceh, Indonesia, to fill the reference gaps in mangroves, conservation efforts can be supported by the government, in line with the government's ambition to restore 600,000 hectares of mangrove forests by 2021–2024. Sustainable management can be implemented in ecologically important areas to provide insights into mangrove diversity while providing insights into improving the socioeconomic conditions of neighboring communities in the context of environmentally sound development by protecting and conserving species and providing attractions (ecotourism).

AUTHOR CONTRIBUTIONS

M. Basyuni conceived and designed the experiments, analyzed and interpreted the data, contributed reagents and materials, wrote the manuscript draft, and reviewed and finalized the final draft. R. Syahbana performed the experiments and analyzed and interpreted the data. A.B. Rangkuti analyzed the data and reviewed the manuscript text. NA Pradisty analyzed the data and reviewed the manuscript text. A. Susilowati analyzed and interpreted the data and reviewed the manuscript text. LAM Siregar interpreted the data and reviewed the manuscript draft. S.S. Al Mustaniroh analyzed the data and reviewed the manuscript draft. A.A. Aznawi analyzed and interpreted the data and organized the text. A. Mubaraq collected references, prepared the manuscript, and organized the text. E.R. Ardli, S.H. Larekeng, V. Leopardas, Y. Isowa, and T. Kajita reviewed the manuscript draft. T. Kajita contributed materials. All authors read and approved the final manuscript.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest. In addition, the ethical issues, such as plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy, were observed by the authors.

ABBREVIATIONS

%	Percent
α	Alpha
BLAST	Basic local alignment search tool
bp	Base pairs
C	Cystosine
CBOL	Consortium for the Barcode of Life
DDBJ	DNA Data Bank of Japan
ddRADseq	Double digest restriction site-associated DNA sequencing
DNA	Deoxyribonucleic acid
E	East
e	Exponent
EMBL	European molecular biology laboratory
G	Guanine
Ga	Gamma
GGPLOT2	Grammar of graphics plot 2
ITS	Internal transcribed spacer
IUCN	International Union for Conservation of Nature
km²	Square kilometers
LC	Least concern
MA	Mangrove associate
matK	Maturase-K
MEGA	Molecular evolutionary genetics analysis
m²	Square meter
NCBI	National Center for Biotechnology
NJ	Neighbor-joining
N	North
P	Probability
PCR	Polymerase chain reaction
psbA	Photosystem II reaction center protein A
RAPD	Randomly amplified polymorphic DNA
rbcl	Ribulose 1,5-bisphosphate carboxylase/oxygenase
readR	Read rectangular
RNA	Ribonucleic acid
SRAP	Sequence-related amplified polymorphism
tidyr	Tidy data
TM	True mangrove
trnH–psbA	Transfer RNA for histidine–photosystem II reaction center protein A
UGENE	Unified genome browser and editor

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