GENETICS IDENTIFICATION OF SEA HOLLY (*Acanthus ilicifolius*) THROUGH DNA BARCODING FROM COASTAL CILACAP, CENTRAL JAVA, INDONESIA**

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ABSTRACT

Sea holly (*Acanthus ilicifolius*) is an important true mangrove species commonly growing on wetlands at the river mouths and coastal areas. Very limited information is available on the molecular taxonomy of sea holly growing along the coasts of Cilacap, Central Java, Indonesia. The present study aimed to identify the sea holly in coastal Cilacap and to produce a reference library on the molecular characteristics of the species. The recently recorded species were utilized for the barcoding investigation. Genetic identification was evaluated through the rbcL and matK gene. Young leaf samples of *A. ilicifolius* were collected for DNA extraction, isolation and amplification using the rbcL and matK primer. The length of rbcL gene was 608 bp and the matK gene was 970 bp. The evolutionary history was built using the Neighbor-Joining Method. The barcode sequences rbcL and matK were analyzed using BLAST and MULTALIN. The sequences were also submitted to NCBI. Genus *Acanthus* (Acanthaceae) and other genera were clustered in the same clade with high bootstrap value. The results indicated that locus of rbcL and matK gene cannot be used for species differentiation in *Acanthus*, however, these genes can be used for distinguishing the genus level within Acanthaceae.

Keywords: *A. ilicifolius*, DNA barcode, matK, rbcL, sea holly

INTRODUCTION

Sea holly (*Acanthus ilicifolius*) is an important true mangrove species that commonly grows on wetlands at the river mouths and coastal areas (Ragavan *et al.* 2015). It is often distinguished from the related genera by its spiny leaves, spicate terminal inflorescences, two bracteoles and uniform anthers (Duke 2006). In several countries, this plant functions as an efficacious medicianal plant (Ganesh & Vennila 2010; Simlai & Roy 2013; Paul & Seenivasan 2017).

Identification based on the morphological characteristics, such as leaf shape, shape of flowers, branching patterns and root shape is extremely prone to error (Sahu & Kathiresan 2012), as the morphology of *A. ilicifolius* can be affected by geography. Therefore, molecular identification is the key to discriminating different species. In this study, DNA barcoding used the short fragment of nucleotide sequence for fast, precise species identification (Dong *et al.* 2012; Li *et al.* 2012; Li *et al.* 2015; Vivas *et al.* 2014). DNA barcoding technology is still the ideal method for fast identification due to its convenience and low cost (Xu *et al.* 2017).

Molecular identification using DNA barcoding is often needed to obtain fast, low cost and accurate results. DNA barcoding uses mitochondrial DNA to identity up to the species level. The rbcL and matK genes from the chloroplast genome were used as the core barcode in the consortium for the Barcode of Life (CBOL Plant Working Group 2009). The rbcL gene is the large subunit of Ribulose-

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bisphosphate carboxylase gene. The matK gene is located with trnK gene and encodes the tRNA (Lys) (UUU). Substitution rate of the matK gene is the highest among the plastid genes (Radulovici *et al.* 2010). DNA barcoding using matK can discriminate more than 90% of species in the Orchidaceae but less than 49% in the nutmeg family (Kress *et al.* 2010). Another research on genetic identification using rbcL and matK genes revealed a 93% success in species identification (Burgess *et al.* 2011). The method can achieve discrimination up to 95% with the addition of the trnH-psbA intergenic spacer. This study aimed to evaluate the efficiency of the matK and rbcL gene for the identification of sea holly (*Acanthus ilifolicius*) from the coastal area of Cilacap.

MATERIALS AND METHODS

Collection of the Sample

A total of 7 leaves from 7 plants of *A. ilicifolius* were collected from different individual plants taken from the west coast of Cilacap, Central Java, Indonesia with geographical latitude of 8°35'S-8°48'S and longitude of 108°46'E-109°03'E.

DNA Isolation

Total DNA were extracted from the leaf tissue of sea holly (*A. ilicifolius*) using Cetyl-trimethyl ammonium bromide (CTAB) (Sahu *et al.* 2016). The CTAB buffer was prepared from 20 mM EDTA; 1.4 M NaCl; 2% PVP-30; 1% β-mercaptoethanol; 10% SDS and 10 mg/mL proteinase K and mixed with leaf sample. The suspension was incubated at 60 °C for 60 min and then centrifuged at 14,000 rpm for 10 min at room temperature with equal volume of CIAA (24:1). The aqueous phase was transferred to a new tube and precipitated with 0.6 volume of cold isopropanol (-20 °C) and chilled with 7.5 M ammonium acetate and then stored at -20 °C for 1 h. The precipitated DNA was centrifuged at 14,000 rpm for 10 min at 4 °C then washed with 70% ethanol. DNA was finally dissolved in TE buffer (10 mM Tris–HCl, 1 mM Na2EDTA, pH 8.0). The DNA quantity was evaluated using agarose gel electrophoresis and the quality of total DNA was evaluated using nanodrop (Thermo Scientific, USA).

PCR and DNA Sequencing

The DNA samples were amplified using rbcL and matK plastid primers rbcLa_f: 5'-ATGTCACCACAAACZAGAG ACTAAAGC-3', rbcL724-r: 5'-"GTAAA ATCAAGTCCACCRCG"-3', matK_390-f: 5'-"CGATCTATTCATTCAATATTTC"-3' and matK_1326-r: 5'-TCTAGCACACGAAAGTC GAAGT-3' (CBOL Plant Working Group 2009). The total mixture was 50-µL containing 10-20 ng of template DNA, 200 µM of dNTPs, 0.1 µM primers and 1 unit of Taq DNA polymerase (Thermo Scientific, USA). The temperature profile of the PCR cycle for rbcL was 94 °C for 4 min; 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; repeated for 35 cycles, and final extension 72 °C for 10 min. For the amplification of the matK gene, the temperatures used were 94 °C for 1 min; 35 cycles of 94 °C for 30 s, 50 °C for 40 s, 72 °C for 40 s; repeated for 37 cycles, and final extension 72 °C for 5 min. The amplified products were separated by agarose gel (1.2%) electrophoresis and stained with ethidium bromide (Saddhe *et al.* 2016). PCR was conducted at the Research Laboratory of Universitas Jenderal Soedirman, Indonesia and sequencing analysis were performed at the 1st Base DNA Sequencing Service, Malaysia.

Phylogenetic Analysis

A phylogenetic tree was constructed using a free web service dedicated to reconstructing and analyzing phylogenetic relationships between molecular sequences (http://www.phylogeny.fr) to identify the genetic similarity and differences. Samples of rbcL and matK genes were compared to sequences from 8 species, retrieved from NCBI GenBank. Relationship in the rbcL and matK genes was determined using CLUSTAL W, Treeview version 1.5.2. and was used to generate the scoring method percent and the unrooted tree. The rbcL sample sequence was compared with *Acanthus spinosus (*MF349678.1), *Acanthus montanus* (L12592.1), *Sclerochiton kirkii* (JX572958.1), *Acanthopsis spatularis* (KF724239.1), *Acanthus ilicifolius* (KM255065.1), *Acanthus ilicifolius* (KX231351.1), *Acanthus ebracteatus* (KX231352.1), and *Acanthus ilicifolius* (KP697342.1) (Fig. 2). The matK sequence was compared with *Acanthus ilicifolius*

(KX231339.1), *Acanthus ilicifolius* (KM255080.1), *Acanthus ebracteatus* (KX231340.1), *Acanthus longifolius* (AJ429326.1), *Acanthus spinosus* (MF350143.1), *Acanthus mollis* (HE967332.1), *Acanthus montanus* (HQ384511.1) and *Aphelandra scabra* (JQ586377.1) (Fig. 3).

Data Analysis

Sequence alignment for rbcL and matK sequences was generated using the Multalin V.5.4.1 (http://multalin.toulouse.inra.fr/multalin/). All known sea holly sequences were searched using the 'BLASTn' tool and verified against the NCBI database and the highest-scoring hit from each query was taken as the mangrove identification (https://www.ebi.ac.uk/services and https://www.ncbi.nlm.nih.gov). Similarity percentage matrix was calculated based on Clustal 2.1 (www.ebi.ac.uk). The similarity percentage for the rbcL sample sequence was compared with *Acanthus ilicifolius* (KP697342.1), *Acanthus ilicifolius* (KM255065.1), *Acanthus ilicifolius* (KX231351.1), *Acanthus ebracteatus* (KX231352.1), *Acanthopsis spathularis* (KF724239.1), *Acanthus montanus* (L12592.1), *Sclerochiton kirkii* (JX572958.1) and *Acanthus spinosus* (MF349678.1) (Table 1), whereas the number of different nucleotides was compared with *Acanthus ilicifolius* (KP697342.1), *Acanthus ebracteatus* (KX231352.1), *Acanthopsis spathularis* (KF724239.1), *Acanthus montanus* (L12592.1), *Sclerochiton kirkii* (JX572958.1), *Acanthus spinosus* (MF349678.1), *Aphelandra aurantiaca* (MF349506.1), *Aphelandra scabra* (JQ590024.1), *Sclerochiton harveyanus* (JX572957.1), *Crossandra infundibuliformis* (JQ933287.1), *Rhinacanthus nasutus* (KF381120.1), *Aphelandra sinclairana* (L01884.1), *Avicennia officinalis* (KP697352.1), *Avicennia marina* (KP697350.1), *Ruellia blechum* (GU135168.1) and *Stachytarpheta jamaicensis* (JQ618493.1) (Table 2).

The Similarity percentage for the matK sample sequence was compared with *Acanthus ilicifolius* (KX231339.1), *Acanthus ebracteatus* (KX231340.1), *Acanthus ilicifolius* (KM255080.1), *Acanthus montanus* (HQ384511.1), *Acanthus spinosus* (MF350143.1), *Acanthus longifolius* (AJ429326.1), *Acanthus mollis* (HE967332.1) and *Aphelandra scabra* (JQ586377.1) (Table 3), whereas the number of different nucleotides was compared with *Acanthus ilicifolius* (KX231339.1), *Acanthus ebracteatus* (KX231340.1), *Acanthus ilicifolius* (KM255080.1), *Acanthus montanus* (HQ384511.1), *Acanthus spinosus* (MF350143.1), *Acanthus longifolius* (AJ429326.1), *Acanthus mollis* (HE967332.1), *Aphelandra scabra* (JQ586377.1), *Aphelandra aurantiaca* (JQ589891.1), *Aphelandra sinclairiana* (GQ981937.1), *Kudoacanthus albonervosus* (KX526470.1), *Sclerochiton harveyanus* (JX517343.1), *Sclerochiton kirkii* (JX518192.1), *Proboscidea altheifolia* (MF963699.1) and *Schlegelia parviflora* (AJ429345.1) (Table 4).

RESULTS AND DISCUSSION

Identification of Sea Holly (*A. ilicifolius*) using rbcL and matK Genes

The currently-used morphology-based identification and the declining group of taxonomists resulted in the weakening identification of species. One possible solution to this problem is the use of molecular method particularly for the mangrove species. DNA barcoding is designed to provide accurate and automated species identifications through the use of molecular species tags based on short, standard gene regions (Sadde *et al.* 2016; Harisam *et al.* 2019). In this research, the plastid core markers rbcL and matK cannot be used as DNA barcoding method for the based assessment of sea holly from the coastal Cilacap, however, the partial rbcL and matK genes from the chloroplast genome of sea holly were successfully amplified (Fig. 1).

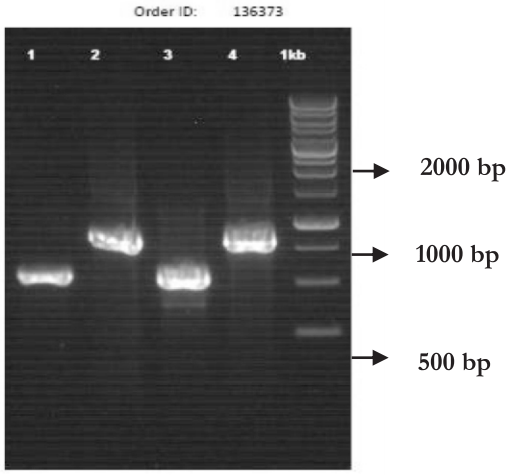

Figure 1 The amplification of rbcL gene (608 bp) and matK gene (970 bp) of sea holly (*A. ilicifolius*) using 1 kb marker

Table 1 Similarity percentage of sea holly (*A. ilicifolius*) based on rbcL gene with *Acanthus ebracteatus*, *Acanthopsis spathularis*, *Acanthus montanus*, *Sclerochiton kirkii* and *Acanthus spinosus* calculated using Clustal 2.1 (www.ebi.ac.uk)

Species	1	2	3	4	5	6	7
KP697342.1_*Acanthus ilicifolius*	100	100	100	100	100	100	100
KM255065.1_ *Acanthus ilicifolius*	100	100	100	100	100	100	100
KX231351.1_*Acanthus ilicifolius*	100	100	100	100	100	100	100
KX231352.1_*Acanthus ebracteatus*	100	99.29	98.3	98.3	98.3	98.3	98.4
KF724239.1_*Acanthopsis spathularis*	98.4	98.1	98.1	98.1	98.1	98.1	98
L12592.1_ *Acanthus montanus*	98.2	98.2	98.2	98.2	98.2	98.2	98.3
JX572958.1_*Sclerochiton kirkii*	98.2	98.2	98.2	98.2	98.2	98.2	98.2
MF349678.1_*Acanthus spinosus*	98.0	98.4	98.0	98.0	98.0	98.0	98.0

The DNA sequencing of rbcL and matK genes was performed in BLAST at NCBI to find similar sequences under the same or different genus within the family Acanthaceae and was calculated using Clustal 2.1 (www.ebi.ac.uk). The most highly significant similar identity sequences obtained from the GenBank based on rbcL gene are *Acanthus ilicifolius* (100%), *Acanthus ebracteatus* (100%), *Acanthopsis spathularis* (98.4%), *Acanthus montanus* (98.2%), *Sclerochiton kirkii* (98.2%), *Acanthus spinosus* (98.0%) (Table 1). The similarity was 98.4% for *Acanthopsis spathularis*, which has 9 different nucleotides with all *A. ilicifolius*. 98.02% similarity *Acanthus montanus which* has 10 different nucleotides with all *A. ilicifolius* (Table 2).

Table 2 Number of different nucleotides in the rbcL gene of sea holly (*A. ilicifolius*) and other species within Acanthaceae

Species	Accession number	Identity (%)	Number of different nucleotides
Acanthus ilicifolius	Sea holly	100	0
Acanthus ebracteatus	KX231352.1	100	0
Acanthopsis spathularis	KF724239.1	98.4	9
Acanthus montanus	L12592.1	98.2	10
Sclerochiton kirkii	JX572958.1	98.2	10
Acanthus spinosus	MF349678.1	98.0	11
Aphelandra aurantiaca	MF349506.1	98.0	11
Aphelandra scabra	JQ590024.1	98.0	11
Sclerochiton harveyanus	JX572957.1	98.0	11
Crossandra infundibuliformis	JQ933287.1	98.0	11
Rhinacanthus nasutus	KF381120.1	97.4	14
Aphelandra sinclairana	L01884.1	97.7	17
Avicennia officinalis	KP697352.1	96.4	20
Avicennia marina	KP697350.1	96.4	20
Ruellia blechum	GU135168.1	95.0	22
Stachytarpheta jamaicensis	JQ618493.1	95.0	22

Table 3 Similarity percentage of sea holly (*A. ilicifolius)* based on matK gene with *Acanthus ebracteatus*, *Acanthus longifolius, Acanthus montanus, Acanthus spinosus Acanthus longifolius, Acanthus mollis* and *Aphelandra scabra*

Species	1	2	3	4	5	6	7
KX231339.1_*Acanthus ilicifolius*	99.3	99.3	99.3	99.3	99.3	99.3	99.3
KX231340.1_*Acanthus ebracteatus*	99.3	99.3	99.3	99.3	99.3	99.3	99.3
KM255080.1_ *Acanthus ilicifolius*	97.9	97.9	97.9	97.9	97.9	97.9	97.9
HQ384511.1_*Acanthus montanus*	96.5	96.5	96.5	96.5	96.5	96.5	96.5
MF350143.1_*Acanthus spinosus*	96.5	96.5	96.5	96.5	96.5	96.5	96.5
AJ429326.1_*Acanthus longifolius*	96.4	96.4	96.4	96.4	96.4	96.4	96.4
HE967332.1_*Acanthus mollis*	96.3	96.3	96.3	96.3	96.3	96.3	96.3
JQ586377.1_*Aphelandra scabra*	94.8	94.8	94.8	94.8	94.8	94.8	94.8

Table 4 Number of different nucleotides in the matK gene of sea holly (*A. ilicifolius*) and other species within Acanthaceae retrieved from GenBank

Species	Accession number	Identity (%)	Number of different nucleotides
Acanthus ilicifolius	KX231339.1	99.3	6
Acanthus ebracteatus	KX231340.1	99.3	6
Acanthus ilicifolius	KM255080.1	98.1	8
Acanthus montanus	HQ384511.1	96.5	19
Acanthus spinosus	MF350143.1	96.5	26
Acanthus longifolius	AJ429326.1	96.4	23
Acanthus mollis	HE967332.1	96.3	27
Aphelandra scabra	JQ586377.1	94.8	40
Aphelandra aurantiaca	JQ589891.1	94.5	41
Aphelandra sinclairiana	GQ981937.1	94.5	42
Kudoacanthus albonervosus	KX526470.1	94.4	47
Sclerochiton harveyanus	JX517343.1	93.8	48
Sclerochiton kirkii	JX518192.1	93.7	49
Proboscidea altheifolia	MF963699.1	91.4	75
Schlegelia parviflora	AJ429345.1	91.3	79

The matK gene showed poor performance at the species level identification, sea holly has 99.3% similarity with *Acanthus ebracteatus* (Table 3; Table 4). Species differentiation was also relatively weak for matK in the DNA barcoding of Poaceae (Saadullah *et al.* 2016). Single locus of DNA barcode could not provide significant level of differentiation. Therefore, a combination of 2 loci with 4 available markers is needed to determine the ability to differentiate at species level (Wu *et al.* 2019). Combination of rbcL + trnH-psbA genes for species differentiation in the mangrove species from the Guangdong Province (Wu *et al.* 2019). Lower discrimination was also reported on the complex and closest taxa of *Holcoglossum, Lysimachia*, *Curcuma* and *Ficus* using matK and rbcL genes (Xiang *et al.* 2011; Zhang *et al.* 2012; Li *et al.* 2012; Chen *et al.* 2015). Differentiation with 2-loci rbcL + trnH-psbA and matK + ITS had resulted in 100% differences between species (Purushosthaman *et al.* 2014). The combination between rbcL + matK markers showed better performance at the species and genus level identification (Sadde *et al.* 2016). Another study reported only 72% at species level resolution using the combined matK and rbcL genes (Saddhe *et al.* 2016).

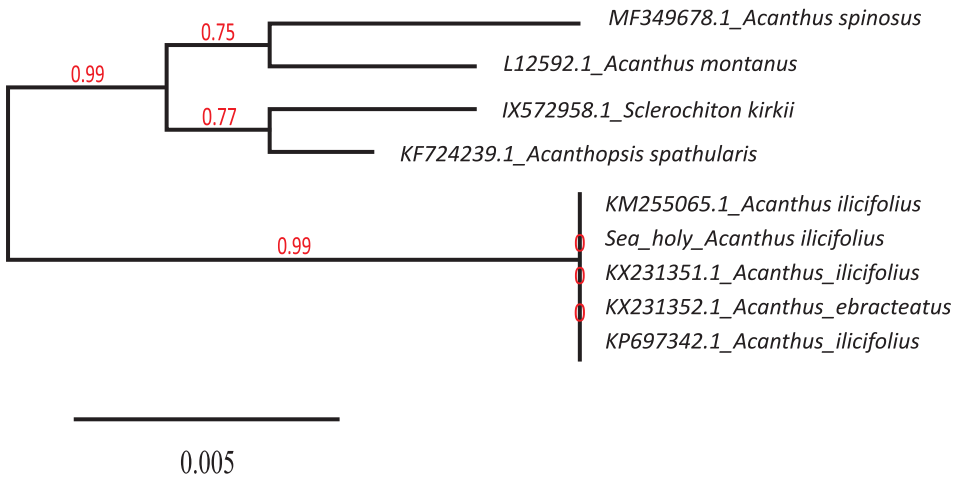

Figure 2 Phylogenetic tree of genus *Acanthus, Acanthopsis* and *Sclerochiton* constructed based on likelihood phylogeny of nucleotide sequences of rbcL gene

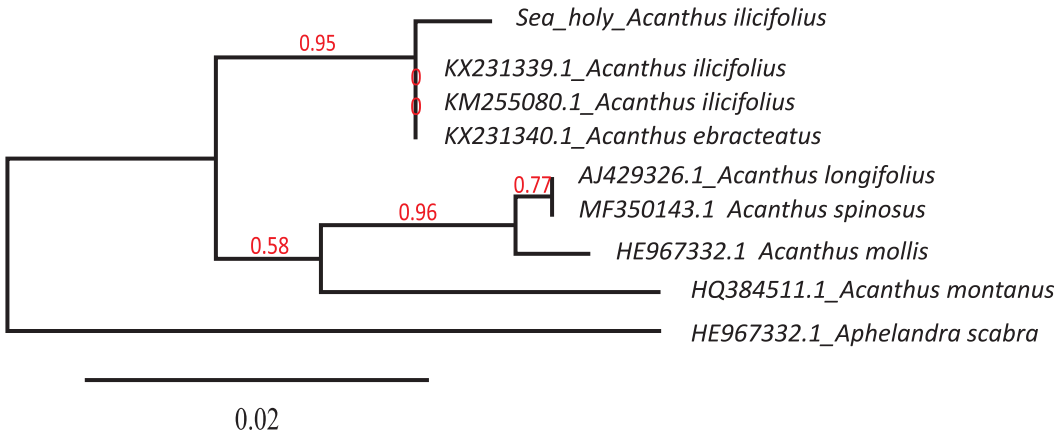

Figure 3 Phylogenetic tree of genus *Acanthus* and *Aplelandra* constructed based on likelihood phylogeny of nucleotide sequences of matK gene

The results showed that rbcL locus could not differentiate sea holly from *Acanthus ilicifolius* and *Acanthus ebrecteatus*, but can differentiate from *Acanthus montanus*, *Acanthus spinosus* and other genera (*Acanthopsis* and *Sclerochiton*) (Fig. 2). The results from phylogenetic tree also showed that matK locus could not differentiate sea holly from *Acanthus ebrecteatus*, however, matK locus can separate sea holly from *Acanthus longifolius, Acanthus spinosus, Acanthus mollis, acanthus montanus* and *Aphelandra scabra* (Fig. 3).

The phylogenetic tree was built from the highest likelihood in sequence alignment while the number of genetic change was built from horizontal dimension of the phylogenetic tree. Values of 0.005 and 0.002 explain the length of the branch representing the number of nucleotide substitution (number of substitution per 100 nucleotide site). There was no indel (insertion and deletion) found in rbcL and matK gene sequence of sea holly (*A. ilicifolius*).

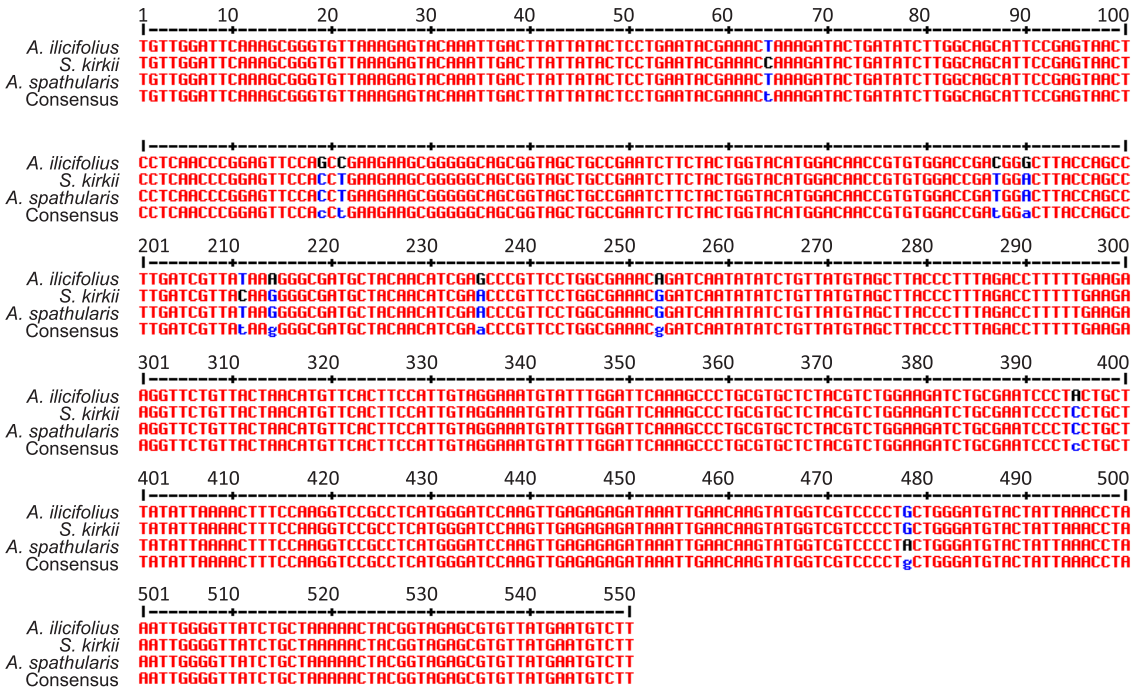

Figure 4 Multi alignment of rbcL gene sequence of *Acanthus ilicifolius, Acanthopsis spathularis* and *Sclerochiton kirkii*

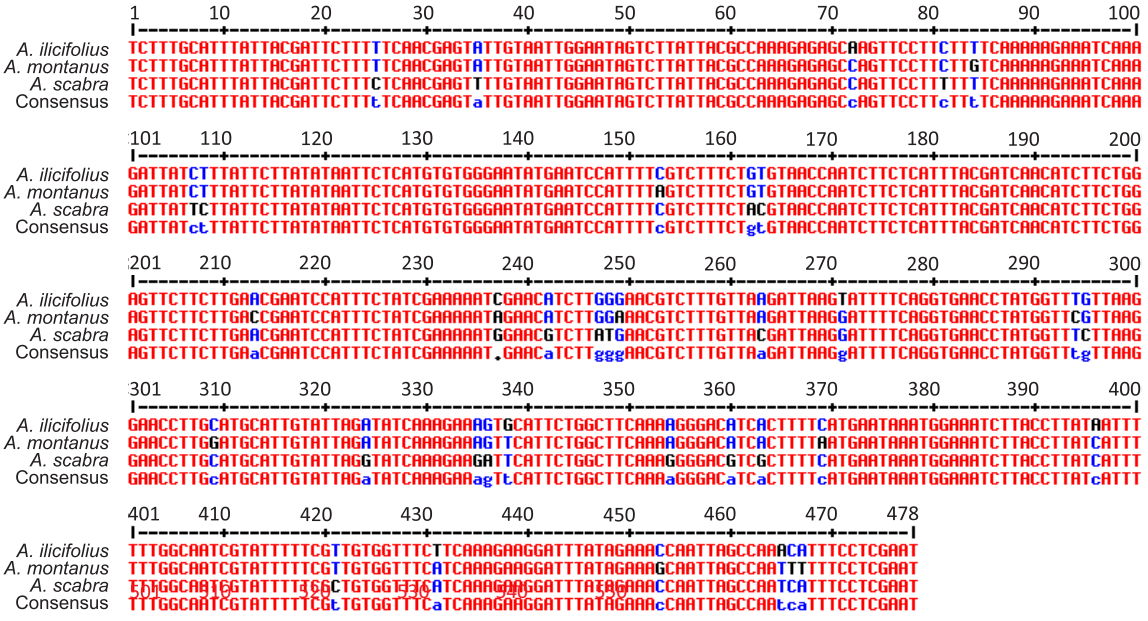

Figure 5 Multi alignment of matK gene sequence of *Acanthus ilicifolius, Acanthus montanus* and *Aphelandra scabra*

The position of different nucleotides in species with the same or different number of nucleotides was not the same in rbcL and matK. The alignment results showed that *Acanthus ilicifolius*, *Acanthopsis spathularis* and *Sclerochiton kirkii* has 11 point of different nucleotide (Fig. 4), while *Acanthus ilicifolius, Acanthus montanus* and *Aphelandra scabra* has 36 points of different nucleotide (Fig. 5). The multi alignment of rbcL sequences showed little variations compared with the multi alignment of matK sequences. These results confirmed that matK gene is more sensitive than rbcL gene (Saddhe *et al.* 2016; Harisam *et al.* 2018).

Many molecular marker techniques have been used to identify tropical mangrove species. Several studies, however, have suggested that one marker alone was not accurate enough for identification (Dong *et al.* 2012). Hence, the rbcL and matK gene can be considered as a

barcode for mangrove species. The highest rate of sequencing was observed in rbcL at 98.7%, while amplification as well as sequencing rate of matK was at 99%.

DNA barcoding was primarily essential in mangrove species identification in as much genetic diversity assessment is essential in endemic, endangered and rare species. Therefore, this information on species identification performed using rbcL and matK sequence is very important for mangrove conservation. The results showed that rbcL and matK genes sequence cannot separate *A. ilicifolius* from *A. ebracteatus*. However, other researches on terrestrial plants were successful in species identification using rbcL and matK sequences (Kress *et al.* 2010; Kuzmina *et al.* 2012; Sadde *et al.* 2016). The species were assigned to their taxa based on two methods, the similarity-based method using BLAST score based on single linkage (BLASTClust) and the tree-based method (NJ). In India, the combined rbcL and matK gene sequencing revealed significant variations and was used to identify *Acanthus ilicifolius* and other mangrove species (Sadde *et al.* 2016). However, considering that sea holly has 100% similarity with two other mentioned species, it is suggested that the rbcL gene cannot be used to differentiate *Acanthus* species (Table 2) although species identification success rate using the rbcL seemed to be higher at rbcL recovery (ranging from 90% to 100%) (Burgess *et al.* 2011). Even though the nine different nucleotides in rbcL gene can already place *Acanthopsis spathularis* as a different species from *Acanthus ilicifolius*, the ideal minimum range for discrimination of species is from 0.0-0.27% or 99.74-100% similarity in order to place organisms in the same species level (Purushothaman *et al.* 2014). Moreover, since matK gene displayed significant variations; therefore, it can be used for DNA barcoding in Acanthaceae family (Sadde *et al.* 2016). The present coding regions of matK and rbcL, which are often recommended in DNA barcode researches only showed moderate variability in *Populus* genus and was inefficient for use in species differentiation. This study results suggested that one marker alone, such as matK and rbcL genes, was not helpful in identifying sea holly (*Acanthus ilicifolius*) at the species level.

CONCLUSION

The identification of sea holly (*Acanthus ilicifolius)* based on matK and rbcL gene was not successful at the species level. However, sea holly was successfully differentiated at the genus level**.** A single locus of DNA barcode could not provide an adequate level of differentiating; therefore, the combination of more than two loci is suggested.

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