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Short Communication: RAPD fingerprinting key and phylogenetic of nine seagrass species from Sanur coastal water, Bali, Indonesia using *matK* sequences

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Abstract. *Pharmawati M, Nurkamila US, Stevanus. 2016. RAPD fingerprinting key and phylogenetic of nine seagrass species from Sanur coastal water, Bali, Indonesia using* matK *sequence. Biodiversitas 17: 687-693.* In Bali, there are nine species of seagrass identified based on morphological characteristics. Development of molecular markers assist identificaton and evolutionary studies of many species including seagrass species. This study aimed to develop a fingerprinting key of nine seagrass species found at Sanur (Sanur Beach and Sindhu Beach), Bali, based on RAPD markers and to analyse their phylogenetic relationships using the *matK* region. Seagrass samples were collected at low tide and DNA was extracted using CTAB buffer. Six RAPD primers were used in the study. Sequences of *matK* were analyzed using MEGA 5.2. The phylogenetic tree was constructed by Maximum Likelihood method with 1000 bootstrap replicates. Based on RAPD banding pattern, a DNA fingerprinting key was successfully developed using only one primer — OPB12. Phylogenetic analysis of *matK* sequences grouped seagrass species by genera. There were five clades identified and the tree recognised that Cymodoceaceae was paraphyletic. This result is in disagreement with a previous study using combined *rbcL* and *matK* sequences which discussed the monophyly of Cymodoceaceae. Recent published paper using ITS sequence showed that cymodoceaceae might not be monophyletic group. This result supported our finding that Cymodoceaceae is not in the monophyletic group. Combined DNA sequences of chloroplast, nuclear and published paper using ITS sequencing showed that Cymodoceaceae mitochondrial DNA will further resolve phylogeny of seagrass species.

Keywords: DNA fingerprinting key, *matK*, phylogenetic, RAPD, seagrass

INTRODUCTION

Seagrasses are flowering plants (Anthophyta) and grow in shallow water of coastal regions. Seagrass vegetation may consist of one species or a mix of two or more species. The seagrass ecosystem has an important role as a primary producer, stabilizer of the sea bed, and a habitat for animals such as the sea cucumber (Holothuridae), shrimp and dugong (*Dugong dugon*) (McKenzie 2003; Short et al. 2007). Seagrass meadows also support coral reef ecosystems by filtering and precipitating pollutants (Larkum et al. 2006).

There are 12 seagrass species in seven genera and two families reported in Indonesia (Kuriandewa et al. 2003). In Bali, seagrass beds are distributed along the south and southeast coast including Nusa Dua, Geger, Serangan and Sanur beaches (Arthana 2004). In other parts of Bali, seagrass grows at Candidasa (Sudiarta and Sudiarta 2011), Menjangan, Nusa Penida, Nusa Lembongan, Nusa Ceningan (Yusup and Asy'ari 2010) and Teluk Gilimanuk (Al Hakim and Wahyuni 2009).

Arthana (2004) reported seven seagrass species found in Sanur coastal waters, from Sanur Beach to Mertasari Beach. Those species are *Enhalus acoroides, Cymodocea rotundata, Cymodocea serrulata, Halophila ovalis, Halodule uninervis, Halodule pinifolia* and *Syringodium isoetifolium.* Another study by Yusup and Asy'ari (2010), recorded eight species in the same region but their descriptions were somewhat different.

Those different results may be because of an identification problem in taxonomy, because several seagrasses have a similar morphological character. Therefore, accurate and more reliable methods of identification are needed. Confirmation of identifications based on morphology can be done using DNA barcoding technique. DNA barcodes employ short DNA fragments (400-800 bp) that accommodate species diversity in wide taxa (Lucas et al. 2012; Selvaraj et al. 2013). Several genes in chloroplast DNA that have been used for identification, biodiversity detection and phylogenetic analysis are *rbc*L, *matK, trnH-psbA* dan *rps16-trnQ* (Lucas et al. 2012).

Random Amplified Polymorphic DNA is a common molecular marker applied to detect genetic diversity at the interspecific level (Pharmawati et al. 2004; Arif et al. 2010) and at the intraspecific level (Pharmawati and Candra 2015; Priya et al. 2015). This genetic marker has been used for species and cultivar identification in numerous plant species (Zhao et al. 2011; Saengprajak and Saensouk 2012).

A study on seagrass species identification and phylogeny in Indonesia, based on molecular markers, has not been reported. This paper reports on the use of DNA barcoding based on *matK* fragments and RAPD markers to identify seagrass species from Sanur coastal waters and determine their genetic relationships. This paper also

demonstrates development of a DNA fingerprinting key for seagrass species based on RAPD markers. Furthermore, a phylogenetic tree of seagrass species from Sanur coastal waters based on *matK* sequences was constructed.

MATERIALS AND METHODS

Sample collection

Seagrass samples were collected from Sanur Beach and Sindhu Beach, Bali (Figure 1). Two samples for each species were collected in Sanur Beach (S 8° 67' 68.8" and E 115° 26' 52.2"), while one sample for each species was collected from Sindhu Beach (S 8° 68' 47.9" and E 115° 26' 65.1"). Morphological identification was conducted following den Hartog and Kuo (2006) and McKenzie and Yoshida (2009).

DNA extraction

DNA was extracted following Doyle and Doyle (1990). A leaf sample (0.1 g) was ground using a mortar and pestle and 1 ml of CTAB buffer (2% CTAB, 100 mM Tris/HCl, pH 8, 1,4 M NaCl, 50 mM EDTA, 2% mercaptoethanol) was added. The slurry was transferred to a microtube. The samples were incubated at 65°C for 30 min and centrifuged at 14,000 rpm for 10 min. Extraction with the same volume of chloroform:isoamyl alcohol (24:1) was done twice and DNA precipitation was conducted by adding 2/3 volume of cold isopropanol. The mixture was stored at -20°C for 16 hours. The DNA pellet was collected by centrifuging for 5 min at 8,000 rpm. The supernatant was discarded and the DNA pellet was washed with 70% ethanol followed by centrifuging for 5 min at 8,000 rpm and the DNA pellet was air dried. As much as 100 µl of sterile H_2O was added to dissolve DNA. RNase A (final concentration 10 µg/ul) was added, and incubated at 37°C for 30 min. Finally centrifuging at 8,000 rpm for 5 min was conducted to remove impurities, and the supernatant was transferred to a new tube.

DNA electrophoresis was done using 1% agarose gel in TAE buffer (Tris acetate-EDTA) stained with ethidium bromide (final concentration 0.5 µg/ml). DNA was visualised with a UV transilluminator. Lambda DNA (MBI Fermentas) at concentrations of 100 ng and 200 ng to compare the estimate of DNA concentration.

PCR-RAPD

PCR-RAPD was conducted in a 20 µl reaction mixture containing 1xPCR buffer (Taq Gold Applied Biosystem), 200 µM dNTP, 3 mM $MgCl_2$, 1.5 µM primer, 1U taq polymerase (Taq Gold Applied Biosystem), 25ng DNA and H_2O to reach 20 µl. The PCR cycles were as follows: initial activation step at 95°C for 1 min, followed by 38 cycles of 1 min at 94°C, 1 min at 37°C and 1.5 min at 72°C, with the final single cycle at 72°C for 10 min. Six RAPD primers (Operon Technology, USA) were used including OPA2 (5'- TGCCGAGCTG-3'), OPA4 (5'- AATCGGGCTG-3'). OPB12 (5'- CCTTGACGCA-3'), OPD11 (5'- TGCCCGTCGT-3'), OPH6 (5'- ACGCATCGCA-3') and UBC127 (5'-ATCTGGCAGC-3'from the University of British Columbia, Canada).

PCR of *matK*

The amplification of the *matK* region was conducted in a 25 µl reaction mixture. The mixture contained 1 x PCR buffer (Taq Gold Applied Biosystem), 200 µM dNTP, 2.5 mM $MgCl_2$, 1.5 µM of a forward primer and a reverse primer, 1U taq polymerase (Taq Gold Applied Biosystem), 25ng DNA and H_2O. For *matK* amplification, the primers used were P646 5'-TAATTTACGATCAATTCATTC-3' and P647 5′-GTTCTAGCACAAGAAAGTCG-3′ (Lucas et al. 2012).

The program of thermal cycling was as follows: initial activation step at 95°C for 5 min, followed by 35 x of denaturation at 95°C for 1 min, annealing at 54.°C for 2 min, elongation at 72°C for 2 min. Final elongation was conducted for one cycle at 72°C for 7 min.

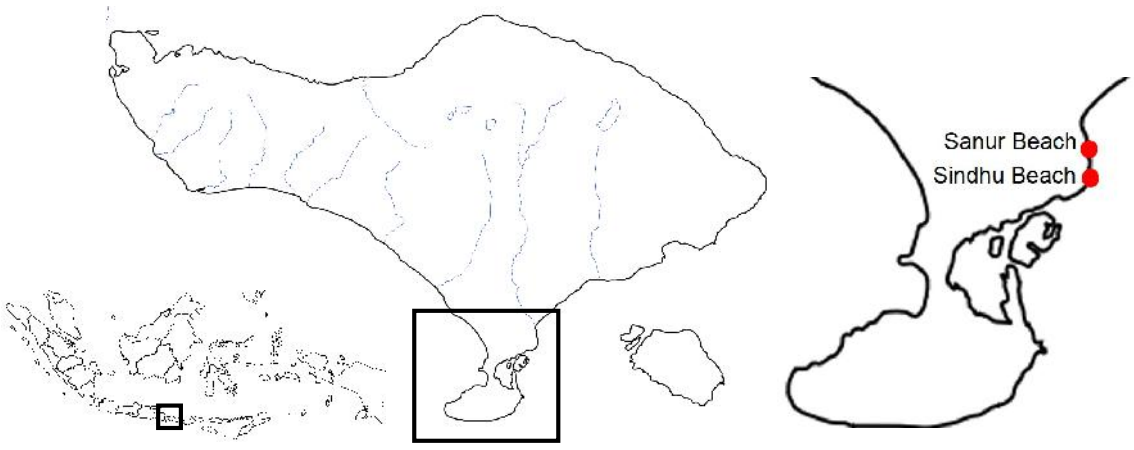

Figure 1. Sites of sample collection at Sanur Beach and Sindhu Beach, Bali, Indonesia

Electrophoresis of PCR products

PCR products of RAPD were visualised using 1.5 % agarose in TAE buffer stained with ethidium bromide, while for *matK* products, electrophoresis used 1% agarose gel. As a size marker, and 100bp DNA ladder (Promega) was included in each gel.

DNA sequencing

DNA sequencing of *matK* was done at forward strand using primer P646. The PCR products were sent to *Berkeley Sequencing Facility* in USA for sequencing,

Data analysis

A RAPD fingerprinting key was developed based on clear, major and reproducible PCR-RAPD bands from three samples in each seagrass species. Sequences of *matK* from all seagrass species were analysed using MEGA 5.2 *(Molecular Evolutionary Genetic Analysis*) and ClustalW was used to determine homology between sequences (Tamura et al. 2011). Phylogenetic reconstruction was conducted using a maximum likelihood General Time Reversible+ Gamma (GTR+G) model with 1000 bootstrap. *Pistia stratiotes* was used as an outgroup. Species determination used Basic Local Alignment Search Tool (BLAST).

RESULTS AND DISCUSSION

Based on morphological characteristics, nine seagrass species were identified from Sanur Beach and Sindhu Beach, Denpasar, Bali, Indonesia. The species were *E. acoroides, T. hemprichi, S. isoetifolium, Th. ciliatum, H. uninervis, H. pinifolia, Ha. ovalis, C. serrulata,* and *C. rotundata* (Figure 2; Table 1). Voucher specimens were deposited at the Herbarium Biologi Udayana (HBU), Universitas Udayana, Bali, Indonesia The morphological characters noted in the seagrass samples matched with those described in den Hartog and Kuo (2006) and McKenzie and Yoshida (2009). Previously, in Sanur water there were seven seagrass species reported (Arthana 2004), while in 2010, there were eight seagrass species found by Yusup and Asy'ari (2010). Other studies reported 10 seagrass species: *Zostera* sp., *H. pinifolia*, *H. uninervis*, *C. rotundata*, *C. serrulata*, *S. isoetifolium*, *Th. ciliatum*, *E. acoroides*, *Ha. ovalis* and *Th. hemprichii* (Sudiarta and Restu 2011). *Zostera* is distributed mainly in temperate coastal waters (den Hartog and Kuo 2006; Short et al. 2007). There are hardly any other reports available on the presence of *Zostera* in Indonesia. Careful identification is important to support a seagrass database for conservation strategies.

The 6 RAPD primers screened amplified multi-band patterns in each seagrass species. Among the six primers tested, OPB12 resulted in the clearest and most reproducible band patterns. PCR-RAPD products using primer OPB12 are shown in Figure 3. Using the PCR-RAPD data from primer OPB12, a DNA fingerprinting key for nine seagrass species was developed (Figure 4).

Identification of seagrass species relied on morphological characteristics. Seagrass has both sexual and asexual reproduction, however the flower, as a distinct morphological trait, is hardly ever found (Papenbrock 2012). This makes identification of seagrass species difficult. Genetic analysis provides a tool to clarify species identity, diversity and distribution (Short et al. 2007). A fingerprinting key based on PCR-RAPD banding pattern has now been generated to identify seagrass species. The success of RAPD as a molecular marker to develop a fingerprinting key has been reported in radish cultivars using five RAPD primers (Pradhan et al. 2004). The RAPD fingerprinting key for seagrass species could be developed using only one primer (OPB12). This is an ideal condition, because the low number of primers will reduce time and cost. Therefore, this finding provides guidance for seagrass identification for further purposes such as single species identification, and distribution and conservation studies.

Fragments of *matK* were amplified from 18 seagrass samples (nine species each from Sanur Beach and from Sindhu Beach). Electrophoresis of PCR products resulted in 950 bp fragments in all species. There was no length polymorphism detected. Analysis of each seagrass sequence using BLAST (*Basic Local Alignment Search Tool*) showed high similarities (92%-100%). BLAST analysis does not confirm all seagrass species that were identified morphologically Three out of nine seagrass species could not be confirmed (Table 2). This could be because there are limited sequence variations in *S. isoetifolium*, *Th. ciliatum* and *H. pinifolia* available at the NCBI database. In addition, according to Arif et al. (2010) the failure of sequence base analysis to differentiate between the species is caused by the high similarity between the DNA sequences of amplified regions.

Phylogenetic analysis based on a Maximum Likelihood GTR+R model is shown in Figure 5. The seagrass species were grouped based on their genera. Each seagrass species from Sanur Beach and Sindhu Beach was grouped at the same branch. This indicates a low variation of seagrass species from the two locations. Sindhu Beach is located next to Sanur Beach, it is predicted that there is gene flow between the two locations. The pollination system of seagrass is hydrophily (Papenbrock 2012) which supports the hypothesis of gene flow where pollen may be distributed by sea currents.

The tree recognised that the Cymodoceaceae were not monophyletic (Figure 5). The phylogenetic using the partial *matK* gene in this study is in disagreement with the phylogenetic of seagrass published previously (Lucas et al. 2012) which combined data of *rbcL* and *matK* sequences. According to Lucas et al. (2012) the concatenated *rbcL* and *matK* sequences resulted in major clades which represented Hydrocharitaceae, Zosteraceae and Cymodoceaceae families. The clade containing *Enhalus*, *Thalassia* and *Halophila* was well supported. Similarly, the clade with *Halodule, Sringodium* and *Cymodocea* had a high support value (Lucas et al. 2012).

The discordance of our results with published findings could be because of the use of partial *matK* fragments in our analysis. In our study, unidirectional sequencing was conducted for 18 seagrass samples representing nine species using forward primer. Complete *matK* sequence

from bidirectional sequencing and other DNA fragments may better resolve phylogenetic of seagrass species from Sanur Beach and Sindhu Beach. Sequence of *rbcL* gene is a major locus gene that is considered to be used in plant DNA barcoding (CBOL Plant Working Group 2009; Vijayan and Tsou 2010; Hollingsworth 2011; Lucas et al. 2012).

Recent study using ITS sequence reported that Cymodoceaceae might be a non-monophyletic group (Nguyen et al. 2015). Using ITS sequences, *Halodule* and *Cymodocea* were grouped in different clades. This seem to be in concordance with our finding. Peterson et al. (2014) also suggested that Cymodoceaceae is non monophyletic group. Sequences from nuclear, chloroplast and mitochondrial DNA need to be carefully combined to further clarify whether Cymodoceaceae is a monophyletic or non monophyletic group (Nguyen et al. 2015).

Figure 2. Seagrass species found at Sanur Beach and Sindhu Beach, Denpasar, Bali, Indonesia. Note: A. *Enhalus acoroides*, B. *Thalassia hemprichii*, C. *Syringodium isoetifolium*, D. *Thalassodendron ciliatum*, E. *Halodule uninervis*, F. *Halodule pinifolia*, G. *Halophila ovalis*, H. *Cymodocea serrulata*, I. *Cymodocea rotundata*. Bar = 5 cm

Table 1. Morphological characters of seagrass species from Sanur Beach and Sindhu Beach, Bali, Indonesia

Sample code	Morphology of rhizome	Leaf	Species
A_1, A_2, 1	Herbaceous, covered with bristles, diameter 1.5 cm-2.5 cm, short branches	Ribbon like, leaf tip rounded, L=30-60 cm, W=2-3 cm, margin rolled slightly, parallel longitudinal vein, vein number 15-20	*E. acoroides*
B_1, B_2, 9	Herbaceous, internode 2-5 mm, 1 short branch from each node	Ribbon like, round apex, L=3-10 cm, W= 05-1 cm, longitudinal vein, vein number=10-15	*T. hemprichii*
C_1, C_2, 2	Herbaceous, internode distance 1-1.5 cm, 1 branch from each node	Erect shoot, cylindrical, L=4-18 cm, W=1-2 mm	*S. isoetifolium*
D_1, D_2, 3	Strong/robust, internode 0.6-1.5 cm, 1 branch from each node	Ribbon like, linier, falcate, L=2-8 cm, W= 0.5-1 cm, leaf tip rounded, margin with teeth	*Th. ciliatum*
E_1, E_2, 5	Herbaceous, internode 5-9 mm, 1 branch from each node	Ribbon like, L=3-8 cm, W=3-5 mm, leaf apex tridentate	*H. uninervis*
F_1, F_2, 4	Herbaceous, internode 2-5 mm, 1 branch from each node	Ribbon like, L=5-14 cm, W=1-2 mm, rounded leaf tip with serrations	*H. pinifolia*
G_1, G_2, 6	Herbaceous, internode 1-1.5 cm, 2 short branches from each node	Leaf shape oblong, ovate, smooth leaf surface, L=0.5-1.5 cm, W=4-10 mm, branched cross vein, vein number=12-16	*Ha. ovalis*
H_1, H_2, 7	Strong/robust, internode 1-2 cm, 1 branch from each node	Ribbon-like, narrow at the base, serrate to dentate apex, L=4-8 cm, W= 0,5-1 cm, longitudinal vein, vein number=13-17	*C. serrulata*
I_1, I_2, 8	Herbaceous, internode 1,5-3 cm, 1 branch from each node	Ribbon like, apex rounded, L=2-10 cm, W=4 mm-0.8 cm, longitudinal vein, vein number=10-15	*C. rotundata*

Note: A-I: Seagrass species from Sanur Beach, 1-9: Seagrass species from Sindhu Beach, L: Length of leaf, W: Width of leaf

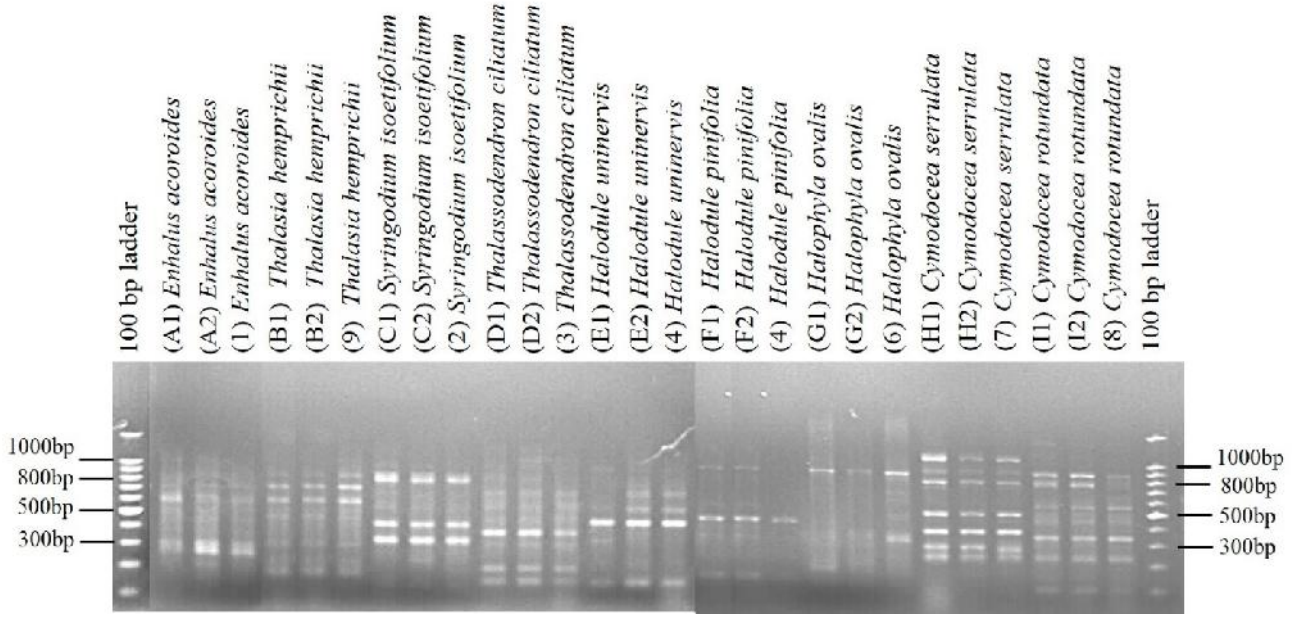

Figure 3. PCR-RAPD amplification of nine seagrass species using primer OPB12. A-I: Seagrass species from Sanur Beach, 1-9: Seagrass species from Sindhu Beach, Bali, Indonesia

1. OPB12$_{(800)}$	P	*T. hemprichii*, *S. isoetifolium*, *C. serrulata*, *C. rotundata*
1.1. OPB12$_{(1160)}$	P	***C. serrulata***
1.2. OPB12$_{(1160)}$	A	*T. hemprichii*, *S. isoetifolium*, *C. rotundata*
1.2.1. OPB12$_{(310)}$	P	***S. isoetifolium***
1.2.2 OPB12$_{(310)}$	A	*T. hemprichii*, *C. rotundata*
1.2.2.1..OPB12$_{(695)}$	P	***T. hemprichii***
1.2.2.2. OPB12$_{(695)}$	A	***C. rotundata***
2. OPB12$_{(260)}$	P	*E. acoroides*, *C. serrulata*
2.1. OPB12$_{(590)}$	P	***E. acoroides***
2.2. OPB12$_{(590)}$	A	***C. Serrulata***
3. OPB12(355)	P	*Th. ciliatum*, *C. rotundata*
3.1. OPB12$_{(172)}$	P	***Th. ciliatum***
3.2. OPB12$_{(172)}$	A	***C. rotundata***
4. OPB12$_{(900)}$	P	*H. pinifolia*, *Ha. ovalis*, *C. serrulata*
4.1. OPB12$_{(400)}$	P	***H. pinifolia***
4.1.1. OPB12$_{(378)}$	P	***C. serrulata***
4.1.2. OPB12$_{(378)}$	A	***Ha. ovalis***
4.2. OPB12$_{(400)}$	A	*Ha. ovalis*, *C. serrulata*
5. OPB12$_{(122)}$	P	*Th. ciliatum*, *H. uninervis*
5.1. OPB12$_{(400)}$	P	***H. uninervis***
5.2. OPB12$_{(400)}$	A	***Th. ciliatum***

Figure 4. PCR-RAPD fingerprinting key for seagrass species from amplification products using primer OPB12. RAPD bands are designed as primer name followed by the fragment size in bp. The presence (P) and absence (A) of bands resulted in identification of seagrass species listed in Table 1 (bold text)

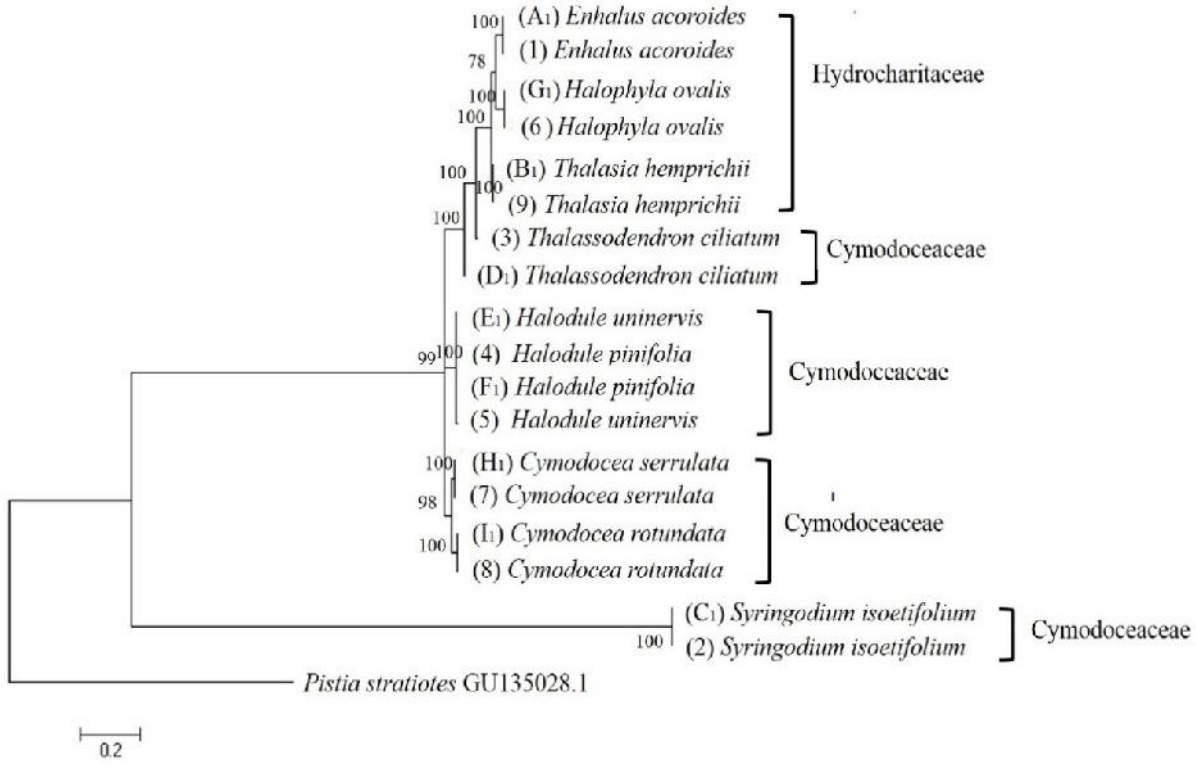

Figure 5. Phylogenetic analysis of seagrass species collected from Sanur Beach and Sindhu Beach, Bali, Indonesia using *matK*. Numbers at branch line demonstrate bootstrap support with 1000 replicates. A-I: Seagrass species from Sanur Beach, 1-9: Seagrass species from Sindhu Beach

Table 2. Characteristics of the *matK* sequences from seagrass species collected from Sanur Beach and Sindhu Beach, Bali, Indonesia

Sample code	Species name	Size* (bp)	Readable sequence (bp)	Accession number	Organism	Sequence identity	E-value
A_1	*E. acoroides*	950	856	AB002569.1	*E. acoroides*	100	99
B_1	*T. hemprichii*	950	856	AB002577.1	*T. hemprichii*	99	99
C_1	*S. isoetifolium*	950	849	KF488511.1	*S. filiforme*	99	95
D_1	*Th. ciliatum*	950	856	AB002577.1	*T. hemprichii*	92	99
E_1	*H. uninervis*	950	856	JN225379.1	*H. wrightii*	99	99
F_1	*H. pinifolia*	950	855	JN225379.1	*H. wrightii*	100	98
G_1	*Ha. ovalis*	950	856	AB002570.1	*Ha. ovalis*	99	99
H_1	*C. serrulata*	950	855	JN225359.1	*C. serrulata*	99	98
I_1	*C. rotundata*	950	556	KF488504.1	*C. rotundata*	100	97
1	*E. acoroides*	950	855	AB002569.1	*E. acoroides*	99	99
2	*S. isoetifolium*	950	846	KF488511.1	*S. filiforme*	99	96
3	*Th. ciliatum*	950	856	AB002577.1	*Th. hemprichii*	94	99
4	*H. pinifolia*	950	855	JN225379.1	*H. wrightii*	100	98
5	*H. uninervis*	950	856	JN225379.1	*H. wrightii*	98	99
6	*Ha. ovalis*	950	856	AB002570.1	*Ha. ovalis*	99	99
7	*C. serrulata*	950	856	JN225359.1	*C. serrulata*	99	98
8	*C. rotundata*	950	856	KF488504.1	*C. rotundata*	100	97
9	*T. hemprichii*	950	856	AB002577.1	*T. hemprichii*	99	99

Note: * = Determine by gel electrophoresis. A-I: Seagrass species from Sanur Beach, 1-9: Seagrass species from Sindhu Beach

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