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# PCR-RFLP and Sequencing of *trn*S/*trn*fM Fragment of *Enhalus acoroides* from Sanur Coastal Waters, Bali, Indonesia: A Preliminary Study

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## ABSTRACT

This study aimed to evaluate the resolution of PCR-RFLP and sequencing of *trn*S/*trn*fM in detecting genetic diversity of seagrass *Enhalus acoroides* at Sanur Beach, Sindhu Beach and Semawang Beach. This research used six samples *E. acoroides* from each location. DNA extraction method followed Doyle and Doyle. PCR amplifications were done using primers P672/P673, P674/P675 and ITS4/ITS5 followed by digestion using restriction enzymes *Eco*RI, *Pst*I, *Hind*III, *Bam*HI, *Rsa, Mva* and *Hin*fI. Sequencing analysis used PCR products of primers *trn*S/*trn*fM. Testing of DNA sequences of *E. acoroides* were conducted using BLAST (Basic Local Alignment Search Tool). DNA sequences were further analyzed using MEGA 5.2 software (Molecular Evolutionary Genetic Analysis) to evaluate variations of DNA. The sequence alignments were done using ClustalW software to determine the homology between the DNA sequences. The results showed that 18 samples of *E. acoroides* from Sanur, Sindhu and Semawang Beach have no polymorphism based on restriction enzyme analyses. Furthermore, sequencing of *trn*S/*trn*fM region of 18 samples *E. acoroides* showed that the sequences were identical.

**Keywords**: *Enhalus acoroides, PCR-RFLP, Sequencing trnS/trnfM, Sanur*

## INTRODUCTION

Seagrass meadow is one of coastal ecosystem with seagrass as dominant vegetation. Seagrass meadow may consist of single seagrass species or mix of two to 12 seagrass species on several types of substrates [1].

Seagrass ecosystem has high productivity through high $CO_2$ capture and nutrient trap [2]. Seagrass meadow has important functions both ecologically and economically. From ecological point of view, seagrass meadow acts as nursery ground and spawning area for marine biotas such as fish, molusc, sea urchin, sea star and other marine animals [3]. Besides that, seagrass itself performs as food resources for dugong (*Dugong dugon*) and green turtle (*Chelonia mydas*) [4]. Economically, beach with seagrass bed can be as recreational and educational areas.

Seagrass grows in shallow coastal waters at intertidal and sublittoral zones, in between high and low tide, and around rocky island in Indonesia [5]. There are 60 seagrass species identified in the world, and 13 of them are found in Indonesia [6].

In 2010, it was reported that there were eight species of seagrass in coastal waters of Denpasar, Bali [7]. A study in 2013 found that there were nine seagrass species in Sanur Beach and Sindu Beach, Sanur, Denpasar, Bali [8]. One of seagrass species is *Enhalus acoroides* which has the largest and longest leaf blade and can grow up to 100 cm. *Enhalus acoroides* is seagrass species that lives in all types of waters and grows well in shallow open waters [9].

At south east of Bali beaches, especially in Sanur areas, seagrass bed spreads from Sanur Beach to Mertasari Beach [10]. Semawang Beach is located adjacent to Mertasari Beach. Sindhu Beach is located 500 m from Sanur Beach while the distance of Sindhu Beach to Semawang Beach is approximately 4 km. Information on genetic diversity of *E. acoroides* at Sanur waters is useful for monitoring and determination of con-

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servation strategy.

Genetic diversity can be detected using molecular markers. One of molecular marker available is PCR-RFLP, which can detect diversity in intraspecific levels [11]. This marker is co-dominant marker and can detect high polymorphism [11]. PCR-RFLP technique has been used to discriminate Fagaceae species [12] as well as to detect variation in chloroplast DNA of wild brassica [13]. It also used to determine mode of inheritance of organelle DNA and phylogenetic relationships in plant [14]. One of chloroplast DNA fragment with high evolution rate is *trn*S/*trn*fM [15]. Beside chloroplast genome, nuclear genome also has high evolution level [16].

More sensitive method to detect DNA variation is DNA sequencing. DNA sequencing can be used to identify and determine the function of DNA fragment by comparing DNA sequence of samples to known DNA [17]. DNA sequencing of chloroplast regions has been employed to detect genetic diversity of *Shorea cutisii* [18], *Pistacia vera* L. [19], and to study phylogeny of Chrysanthemum [20].

In this study, PCR-RFLP of nuclear and chloroplast DNA were employed to analyse genetic diversity of *E. acoroides* from Sanur Beach, Sindhu Beach and Semawang Beach. Also, sequencing of *trn*S/*trn*fM fragment of chloroplast DNA was done to support the analyses.

## MATERIALS AND METHODS

### *Sample collection*

*Enhalus acoroides* samples were collected from three locations of Sanur waters (Figure 1) including Sanur Beach (S 8°40'16.52", E 115°15'91.33"), Sindhu Beach (S 8°68'51", E 115°26'53.8") and Semawang Beach (S 8°42'52.98", E 115°15'85.69"). Transect line of 200 m long was placed perpendicular to seashore. Two leaf samples were collected at seashore, two samples at the middle of transect line and two samples at the end of transect line. The distance between two samples was at least 5 m. At each sampling site, leaf blade was collected from one individual.

### *DNA extraction*

DNA extraction was conducted using CTAB extraction buffer [21] with modification by increasing EDTA concentration to 50 mM. Leaf of *E. acoroides* (0.1 g) was ground using mortar and pestle and 1 ml extraction buffer (2% CTAB, 100 mM Tris/HCL pH 8, 1.4 M NaCl, 50 mM EDTA pH 8, 0.2% β-*mercaptoethanol*) was added. The extract was transfered to microtube and incubated at 65°C for 30 minutes, followed by centrifugation at 14.000 rpm for 10 minutes. Supernatant was transfered to a new tube and added by the same volume of chloroform : isoamylalcohol (24 : 1). After vortexing for 2 minutes, the tubes were centrifuged at 14.000 rpm for 10 minutes. Aquous layer was transfered to a new tube followed by the addition of the same volume of cold isopropanol.

After overnight incubation at -20°C, samples were centrifuged at 14.000 rpm for 5 minutes. DNA pellet was washed with 70% ethanol and sentrifuged for 3 minutes. DNA pellet was air dried and diluted with 100 µL sterile water. DNA solution was kept in -20°C.

DNA was visualised using 1% agarose gel electrophoresis stained with ethidium bromide [22]. To estimate DNA concentration, lambda DNA of known concentrations were included in the gel. Electrophoresis was run at 100 V for 30 minutes and visualised using UV transiluminator.

### *DNA amplification*

DNA amplifications were done for chloroplast DNA and nuclear DNA fragments. Table 1 shows the sequences of primer pairs used. Total volume of PCR reaction was 20 µL containing 75 ng DNA template, 1× PCR buffer, 2 µM $MgCl_2$ 200 µM dNTP, 1 unit Taq DNA Polymerase (MDBio) and 2 µM primer. PCR cycles for amplification are as follow: 1) initial denaturation at 95°C for 5 minutes, 2) denaturation at 95°C for 1 minute, 3) annealing at 56°C (depending on primer pair used) for 2 minutes, 4) extension at 72°C for 1 minute 30 seconds, 5) final extension at 72° for 10 minutes. Cycles 2 to 4 were repeated for 40 cycles.


Figure 1. Sample collection sites

Table 1. Primers used in this study, their sequences and annealing temperatures

| Primer | Location | Amplified region | Sequence | Annealing temperature | Reference |
|---|---|---|---|---|---|
| P672 | chloroplast | *trn*Q/*rp*S | 5′-GCGTGGCCAAGYGGTAAGGC-3′ | 56°C | Lucas *et al.* |
| P673 | | | 5′ GTTGCTTTYTACCACATCGTTT-3′ | | (2012) [23] |
| ITS4 | nucleus | ITS4/ITS5 | 5'-CCCGCCTGACCTGGGGTCGC-3' | 54°C | White *et al.* |
| ITS5 | | | 5'-TAGAGGAAGGAGAAGTCGTAACAA-3' | | (1990) [24] |
| P674 | nucleus | ITS5a/ITS4 | 5′-CCTTATCATTTAGAGGAAGGAG-3′ | 62°C | Lucas *et al.* |
| P675 | | | 5′-TCCTCCGCTTATTGATATGC-3′ | | (2012) [23] |
| *trn*S | chloroplast | *trn*S/*trn*fM | 5'-GAGAGAGAGGGATTCGAACC-3' | 62°C | Demesure *et al.* |
| *trn*fM | | | 5'-CATAACCTTGAGGTCACGGG-3' | | (1995) [25] |

Visualisation of PCR products was performed using 1% agarose gel electrophoresis at 100 V for 45 minutes and stained with ethidium bromide. Observation was done using UV transiluminator. As size marker, low DNA mass ladder (Invitrogen) was used.

### *Restriction enzymes digestion*

PCR product of primer pair P672/P673, ITS4/ITS5 and P674/P675 were cut with various restriction enzymes including *Eco*RI, *Pst*I, *Hind*III, *Bam*HI, *Rsa*, *Mva* and *Hin*fI. As many as 4 µL PCR product was included in the reaction together with 4.7 µL $H_2O$, 1× restriction buffer, and 1 unit restriction enzyme. The reaction was done at 37° C for 2 hours. The results were visualized using 1.5% agarose gel electhophoresis. Low DNA mass ladder (Invitrogen) was included in the gel as size marker.

### *DNA sequencing*

PCR products using primer pair *trn*S-*trn*fM of 18 *E. acoroides* samples were sent to sequencing facility at Berkeley Sequencing Facility, USA. The sequences were analysed using MEGA 5.2 (Molecular Evolutionary Genetic Analysis) for homolog determination between sequences.

## RESULTS AND DISCUSSION

The 18 *E. acoroides* samples were successfully amplified using primer pair P672/P673, ITS4/ITS5, P674/P67, and *trn*S/*trn*fM. No length polymorphism was detected in all PCR products. Therefore, restriction enzyme digestions were performed. PCR products of primer P672/P673, ITS4/ITS5 and P674/P675 were digested using various restriction ezymes. Restriction enzymes used were *Rsa*, *Hind*III, *Mva*, *Bam*HI, *Pst*I, *Hin*fI and *Eco*RI. The sizes of digested DNA fragments were shown in Table 2. Representatives of digested DNA patterns were presented in Figure 2.

Table 2. Summary of digestion using restriction enzyme

| Primer | Size of PCR product (bp) | Restriction enzyme | Size of DNA after digestion (bp) |
|---|---|---|---|
| P672 / P673 | 1000 | *Pst*1 | 1000 |
| | 1000 | *Hind*III | 1000 |
| | 1000 | *Mva* | 1000 |
| | 1000 | *Bam*HI | 1000 |
| | 1000 | *Rsa* | 600+400=1000 |
| | 1000 | *Hin*fI | 400+380+220=1000 |
| | 1000 | *Eco*RI | 400+380+220=1000 |
| ITS4 / ITS5 | 550 | *Mva* | 550 |
| | 550 | *Rsa* | 550 |
| | 550 | *Hind III* | 550 |
| | 550 | *Hin*fI | 330+220=550 |
| P674 / P675 | 800 | *Rsa* | 800 |
| | 800 | *Hind*III | 800 |
| | 800 | *Mva* | 800 |

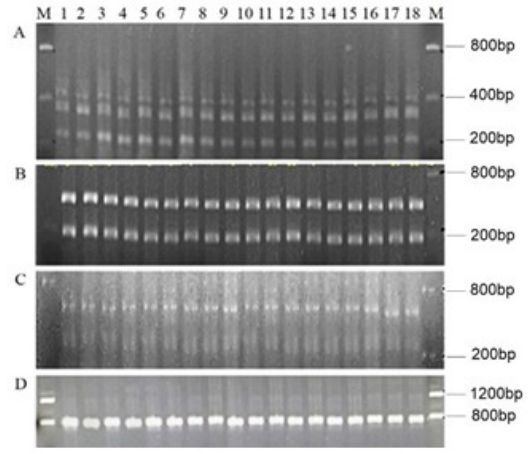


Figure 2. Patterns of PCR products digested using restriction enzymes. PCR product using primer P672/P673 digested with *Hin*fI (A) and *Rsa* (B). PCR product using primer ITS4/ITS5 digested with *Hin*fI (C). PCR product using primer P674/P675 digested using *Mva* (D)

CTCGGCCATCTCTCCGAGAGACAATCTCCATTTTATTCCTATCCATAGTACATAGCCATAGGAATCCATATACTA
ACGATTTGTAGATGCAGCAACCCACCCTAGAGGGGAATCTAAATTTCGATGTATCCATCTGTATACGGATTACAG
TATGTATATCCGTACATTTAGGAAGTCCATACGAAATTCCGCTAAACGCATTTCATGTCTAAACCGAAATCCAAG
AAAGAGTGAATGGTAATAAGTTCTAATAAGTTCTTAAGAATCCATGGGGGGATTCCTGATGAAATCCCTCCACGA
TGCATTTCATCCCAATTTGGACTCAGCGAGGGATCCAATGGTATAGTTCATTTGTTAGTAGTTTGGAGGATTACA
AGCATGATTCTTGCTTTCCAATTGGCTGTTTTTGCATTAATTGCAACCTCATCCATCCTATTGATTAGTGTACCC
GTTGTATTTGCTTCTTCGGATGGTTGGTCAAGTAACAAAAAGATTGTCTTTTCTGGTACATCATATGGATTGGAT
TAGTCTTTCTGGTAGCTATCCTGAATTCTCTTATCTCTTGAATCTCTTTGGTTTGGTACCGGATCCAAATCTATA
TACCTTTCCTTCTCAAAAATGCGGATTCTGAGACAATGGAAATGAAATCGGACTGAATTTCCTATTTAGGAGAAT
CATCAATCCTAATG

Figure 3. The sequence of *trn*S/*trn*fM fragment of *E. acoroides*

Restriction enzyme *Pst*I, *Hind*III, *Mva*, *Bam*HI did not cut PCR product of P672/P673. PCR products of primer P674/P675 and ITS4/ITS5 were not digested by *Rsa*, *Hind*III and *Mva*. Table 2 shows that using 14 primer-enzyme combinations there was no polymorphism at 18 *E. acoroides* samples from Sanur coastal region. This result indicates that the population of *E acoroides* in Sanur Coastal region is quite homogenous.Based on size estimation using agarose electrophoresis, the length of PCR products using primer *trn*S/*trn*fM was 1500 bp, while from sequencing analysis only 689 bp of DNA was clearly sequenced (Figure 3). Multiple sequence alignment of 18 individual of *E. acoroides* showed identical base sequences.

Identification of the sequence of *trn*S/*trn*fM was done using Basic Local Alignment Search Tool (BLAST). The sequence had 97% level of similarity to *Phragmipedium schlimii* and 96% level of similarity to *Ravenala madagascariensis* and *Thaumatococcus danielii*. This may because there is no DNA sequence of *trn*S/*trn*fM fragment of *E. acoroides* in the National Center for Biotechnology Information (NCBI) database.

Both PCR-RFLP and sequencing technique failed to detect variation in *E. acoroides* from Sanur Beach, Sindhu Beach and Semawang Beach, Sanur, Bali. Population of *E. acoroides* at Sanur Beach, Sindhu Beach and Semawang Beach are open population and not isolated by any barrier. Based on the geographic condition, it is predicted that there is gene flow between populations. The presence of gene flow leads to genetic connectivity [26].

A study on development of microsatellite markers for *E. acoroides* found that only 4 pairs of primer out of 36 primer pairs detected polymorphism in *E. acoroides* from Li'Angang and Xincungang, Hainan Province, China [27]. Low genetic diversity was also found in seagrass species *Posidonia oceanica*. In this species, very limited sexual recombination was detected due to clonal propagation [28].

Rhizome extension is a major factor in development of seagrass meadow. In general, the ability of seagrass to form wide seagrass bed is because of its vegetative growth through the growth and dominancy of rizhome [29]. It has been stated that propagation by sexual reproduction and establishment of seedling have little contribution to shoot recruitment in development of seagrass bed [30].

Genetic monomorphism in aquatic plants was detected using allozymes and random amplified polymorphic DNA (RAPD) [31]. This indicated the dominancy of clonal propagation than sexual propagation [31, 32. 33] as well as the low resolution of the methods used [31]. It was suggested that *E. acoroides* grown at Sanur Beach, Sindhu Beach and Semawang Beach, Bali is probably grown through vegetative and well developed clonal growth. However, the used of PCR-RFLP and sequencing of limited genome regions is not sufficient to draw robust conclusion. By using 18 samples, this preliminary study indicated that PCR-RFLP of chloroplast and nuclear fragments, as well as sequencing of *trn*S/*trn*fM showed low resolution.

## CONCLUSION

PCR-RFLP of chloroplast and nuclear DNA fragments failed to detect polymorphism of *E. acoroides* from Sanur coastal water, Bali, Indonesia. The sequences of *trn*S-*trn*fM fragment from 18 individual were identical in all samples.

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