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Low genetic diversity of *Enhalus acoroides* (L.f.) Royle from southeast coastal waters of Bali, Indonesia

Made Pharmawati[1], Luh Putu Wrasiati[2] and Sagung Chandra Yowani[3]

[1]Biology Department, Faculty of Mathematics and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali, **Indonesia**
[2]Department of Agro-industrial Technology, Faculty of Agricultural Technology, Udayana University, Kampus Bukit Jimbaran, Bali, **Indonesia**
[3]Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Udayana University, Kampus Bukit Jimbaran, Bali, **Indonesia**.
***Correspondence: made_pharmawati@unud.ac.id_** **Accepted: Received: 21-09-2019, Revised: 23-11-2019, Accepted: 29-11-2019 e-Published: 25-12-2019**

Enhalus acoroides (L.f.) Royle is one of the seagrass species widely distributed in Indonesia. In southeast coastal waters of Bali, Indonesia, *E. acoroides* can be found along Sanur Beach to Nusa Dua Beach. *Enhalus acoroides* acts as spawning and nursery ground for marine biota. The genetic diversity of *E. acoroides* needs to be evaluated to ensure the sustainability of *E. acoroides* populations. This research aimed to determine the genetic diversity of *E. acoroides* from southeast coastal waters of Bali, Indonesia using PCR-RAPD. Leaf samples were collected from Segara Ayu Beach, Semawang Beach and Samuh Beach, Bali. DNA isolation was done using CTAB buffer followed by extraction with chloroform and isoamylalcohol. PCR-RAPD were performed in 20 µl reaction and 11 RAPD primers were tested. PCR products were visualised using 1.8 % agarose gel electrophoresis stained with ethidium bromide. Results showed that only three primers produced polymorphic DNA bands. Furthermore, the three populations had low genetic diversity as measured by heterozygosity (He) where the values for the population from Segara Ayu Beach, Semawang Beach and Samuh Beach were 0.293, 0.142 and 0,244 respectively. Analysis of fixation index (F_{ST}) showed that population of Segara Ayu Beach differed with the population of Semawang Beach and Samuh Beach, while there was no differentiation between the population of Semawang Beach and Samuh Beach, Bali, Indonesia

Keywords: *E. acoroides,* genetic diversity, ISSR, RAPD

INTRODUCTION

Seagrass meadow is an important marine ecosystem due to its function as a nursery ground and spawning area for marine biotas. The dominant vegetation of seagrass meadow is seagrass of single species or two to 12 seagrass species (Kiswara and Winardi, 1999). It was reported that there are 60 seagrass species identified in the world, and 13 of them are found in Indonesia (Kuriandewa, 2009). In Bali, a recent study found that there were nine species of seagrass in coastal waters of southeast coastal line of Bali, Indonesia (Pharmawati et al., 2016).

One of the seagrass species is *Enhalus acoroides* (L.f.) Royle which has the largest and longest leaf blade and can grow up to 100 cm. At the southeast of Bali beaches, seagrass bed spreads from Sanur Beach to Mertasari Beach (Arthana, 2004). Seagrass populations are also found in beaches of Nusa Dua. One of the

beaches in Nusa Dua that dominated by *E. acoroides* is Samuh Beach.

Information on genetic diversity of *E. acoroides* is important for the sustainability of seagrass ecosystem. By analysing genetic diversity, conservation strategies for *E. acoroides* can be proposed. Priority in conserving the species can also be determined based on genetic diversity information.

Molecular marker is one tool used in analysing genetic diversity. There are several molecular markers available, one of them is PCR-RAPD (Polymerase Chain Reaction-Randomly Amplified Polymorphic DNA). PCR-RAPD is a dominant marker, simple genetic marker that does not require information on DNA sequences of target organism (Williams et al., 1990). PCR-RAPD employs single and short arbitrary primer that anneals to the unspecific site of DNA template and can produce polymorphic marker (Williams et al., 1990, Welsh et al., 1991). This technique is commonly used for population analysis for examples population of ginseng (Wang et al., 2016), chrysanthemum (Kumar et al., 2017) and wild apple (Kumar et al, 2018).

The previous study on genetic diversity of *E. acoroides* from Sanur Beach, Sindhu Beach and Semawang Beach, Sanur, Denpasar, Bali, Indonesia was conducted using PCR-RFLP of *trn*Q/*rp*S, ITS4/ITS5 and *trn*S/*trn*fM regions as well as sequencing of *trn*S/*trn*fM. The study failed to detect polymorphism (Pharmawati and Imaniar, 2016). This may indicate the homogenous populations of *E. acoroides* in Sanur coastal water, Bali and the low resolution of genetic markers used (Pharmawati and Imaniar, 2016). The ability of molecular markers to detect polymorphism at a population level is one parameter in choosing molecular markers. This study aimed to screen RAPD primers, determine the genetic diversity and population structure of *E. acoroides* from Segara Beach, Semawang Beach, Sanur, Denpasar, Bali and Samuh Beach, Nusa Dua, Badung, Bali.

MATERIALS AND METHODS

DNA Extraction

Leaf samples were collected from Segara Ayu Beach, Semawang Beach, both located in Sanur, Denpasar, Bali, and from Samuh Beach, Nusa Dua, Badung, Bali, Indonesia (Figure 1). DNA was isolated from 0.1 g frozen leaves using CTAB lysis buffer following Doyle and Doyle (1990) with modification (Pharmawati et al., 2004). Further extraction was done using chloroform: isoamyl alcohol (24:1). DNA was visualized using 1% agarose gel electrophoresis in TAE buffer (Tris Acetate EDTA) and stained with ethidium bromide. The electrophoresis was run at 100 V for 45 min (Sambrook et al., 1989). To estimate the DNA concentration, lambda DNA of known concentrations were loaded to the gel.

PCR-RAPD

PCR-RAPD was done in a total volume of 20 µl. The reaction consisted of 50 ng DNA template, 1 x PCR buffer, 0.2 mM dNTP, 3 mM $MgCl_2$, 1.5 µM primer and H_2O to make 20 µl reaction. The thermocycler was set for 1x initial denaturation for 5 minutes at 95ºC, followed by 30x of denaturation at 95ºC for 1 min, annealing at 37ºC for 50 sec, extension at 72ºC for 50 sec and 1 x final extension at 72ºC for 10 minutes. Eleven RAPD primer were used, nine from Operon Technology (OPA2, OPB8, OPB12, OPD11, OPD20, OPH3) and two from the University of British Columbia (UBC106 and UBC127). The primers were synthesised by 1stBase, Singapore and the sequences were shown in Table 1.

The PCR-RAPD products were separated using 1.8% agarose gel electrophoresis in TAE buffer, stained with ethidium bromide. As much as 10 µl of PCR product was loaded to the gel. The electrophoresis was run for 1 hour at 100V. The DNA fragments were visualized using UV transilluminator.

Data Analysis

Products of PCR-RAPD were scored 1 for presence fragment and 0 for an absent fragment. The binary data were subjected to iMEC (Online Marker Efficiency Calculator) an online program to calculate heterozygosity index (H), effective multiplex ratio (E), marker index (MI), discriminating power (D) and resolving power (R) (Amiryousefi et al., 2018).

The genetic distance between populations, Shannon's diversity index and the expected heterozigosity in each population were determined using GenAlEx 6.5 (Peakall and Smouse, 2012). AMOVA was used to calculate the diversity within and between populations.

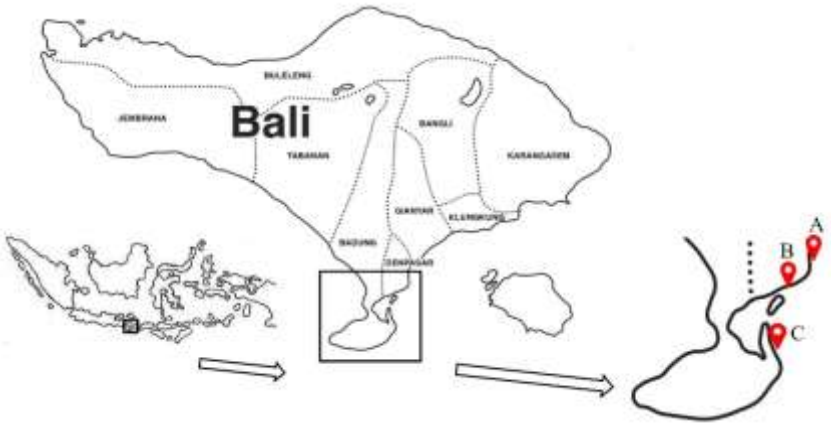

Figure 1; Sample Collection Sites at Segara Ayu Beach (A), Semawang Beach (B) and Samuh Beach, Bali, Indonesia. Map of Bali was modified from Tyler Yamin, https://archive.asia.si.edu/research/performing-indonesia/article-yamin.php

Tabel 1; Primer Name and Sequence, DNA Band Size, Number of DNA Band and Percentage of Polymorphic DNA

Primer Name	Sequence	DNA Band Size	Number of DNA Band	Number of Polymorphic Band	% Polymorphic Band
OPA2	TGCCGAGCTG	325-750	7	0	0
OPA3	AGTCAGCCAC	448-600	3	0	0
OPB4	GGACTGGAGT	435-820	6	0	0
OPB8	GTCCACACGG	418-1380	10	4	40
OPB12	CCTTGACGCA	540-900	3	2	66.7
OPD11	AGCGCCATTG	382-1288	7	0	0
OPD20	ACCCGGTCAC	288-1460	8	4	50
OPH3	AGACGTCCCG	395-650	3	0	0
OPH9	TGTAGCTGGG	286-824	4	0	0
UBC106	CGTCTGCCCG	smear	-	-	-
UBC127	ATCTGGCAGC	450-1100	6	0	0
Total			57	10	
Average			5.18	0.91	14.24

RESULTS

DNA was successfully extracted using a modified CTAB lysis buffer. The increase of EDTA concentration in the buffer from 20 mM (Doyle and Doyle, 1990) to 50 mM (Pharmawati et al., 2004) reduced the degradation of DNA. The concentration of DNA was determined by comparing the brightness and thickness of DNA band to a series of lambda DNA with known concentrations. The concentrations of DNA from 0.1 g leaf sample ranged from 50 ng μl^{-1} to 180 ng μl^{-1}.

Eleven RAPD primers were tested in PCR-RAPD analysis. Ten primers resulted in clear, bright and scorable DNA bands. The total number of DNA bands amplified was 57. Among the ten RAPD primers produced clear bands, only three primers amplified polymorphic DNA bands. Table 1 shows primer names and sequences, DNA band size, number of DNA band amplified and percentage of polymorphic bands. A representative of PCR-RAPD profile of *E. acoroides* from Segara Ayu Beach, Semawang Beach and Samuh Beach was shown in Figure 2.

Primers that produced polymorphic bands were analysed further for heterozygocity index (H), effective multiplex ratio (E), marker index (MI), discriminating power (D) and resolving power (R) (Table 2).

The genetic distances between populations were calculated based on Nei genetic distance and Nei genetic identity using GenAlEx (Table 3). The highest Nei genetic distance value (0.153) was between the population of *E. acoroides* in Segara Ayu Beach and Semawang Beach, while the smallest distance was between the population in Semawang Beach and Samuh Beach (0.015).

Similarly, using Nei genetic identity, *E. acoroides* population in Segara Ayu Beach and Semawang Beach had the lowest value, indicating the differences among the two populations, while *E. acoroides* from Semawang Beach and Samuh Beach had the highest value.

The genetic differentiation between populations was analysed using AMOVA and the result show that molecular variation between populations was only 13%, while the variation within population was 87%. Based on fixation index (F_{ST}) analysis using an F_{ST} analog (PhiPT), the population of *E. acoroides* at Segara Ayu Beach showed differences with the population at Semawang Beach (P=0.002). The population at Segara Ayu Beach and population at Samuh Beach were also different (P=0.045), while the population at Semawang Beach did not differ with the population at Samuh Beach (P=0.145) (Table 4).

Enhalus acoroides population of Segara Ayu Beach had the highest number of alleles (1.9), number of effective alleles (1.468) and the average value of expected heterozigosity (0.293). The average expected heterozigosity in Semawang Beach was 0.142 and in Samuh Beach was 0.244. Similar patterns were observed for Shannon's diversity index where the population of Segara Ayu Beach had the highest index (0.449) and the population at Semawang Beach demonstrated the lowest index (0.230) (Table 5).

The water conditions in each population covering temperature, pH and salinity was shown in Table 6. The conditions at three locations was almost similar. The human activities were high at the three locations including fishing and boating activities.

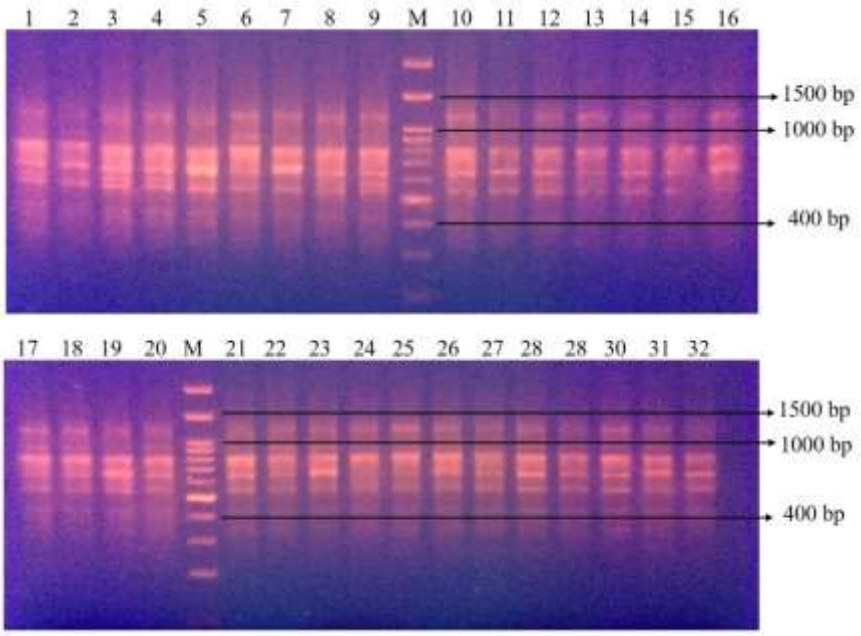

Figure 2; Products of PCR-RAPD Using Primer OPB8. Sample no 1-9 were from Segara Ayu Beach, sample no 10-20 were from Semawang Beach and sample no 21-32 were from Samuh Beach, Nusa Dua, Bali. M is 100bp DNA size marker

Tabel 2; Statistic of Polymorphism of RAPD Primers

Primer Name	H	E	MI	D	R
OPB8	0.378	7.468	0.0088	0.442	2.562
OPB12	0.413	2.125	0.0091	0.500	0.375
OPD20	0.458	5.156	0.0092	0.584	2.062

D = discriminating power; E = effective multiplex ratio; H = expected heterozygosity; MI = marker index; R = resolving power

Table 3; Pair-wise Population Matrix Using Nei Genetic Distance (Below Diagonal) and Nei Genetic Identity (Above Diagonal)

	Segara Ayu	Semawang	Samuh
Segara Ayu	0	0.858	0.892
Semawang	0.153	0	0.985
Samuh	0.115	0.015	0

Table 4; F_{ST} Analogue (PhiPT) of Three *E. acoroides* Populations (Below Diagonal), and The Probability (Above Diagonal)

	Segara Ayu	Semawang	Samuh
Segara Ayu	0	0.002	0.045
Semawang	0.257	0	0.145
Samuh	0.083	0.048	0

Table 5; Number of Different Alleles, Shannon Index and Expected Heterozygosity

Population	N	NA	NE	% Polymorphic Loci	Shannon Index	Expected Heterozygosity
Segara Ayu	9	1.9	1.468	90	0.449±0.065	0.293±0,048
Semawang	11	1.3	1.221	60	0.230±0.078	0.142±0.054
Samuh	12	1.7	1.394	80	0.378±0.078	0.244±0.057

N= number of samples, NA= number of alleles, NE= number of effective alleles

Table 6; Environmental Condition of *E. acoroides* Sites

Location Area	Coordinate	Temperature*	pH*	Salinity*	Substrate
Segara Ayu Beach	-8.682726 and 115.264401	27.33ºC	8.25	33.33‰	Mix of (1) Muddy sand, (2) Sand with rocky rubble mix
Semawang Beach	-8.707327 and 115.262459	27.53ºC	8.3	31.33‰	Mix of (1) Sand, (2) Sand with rocky rubble mix (3) Rocky sand
Samuh Beach	-8.795556 and 115.232699	26.67ºC	8.34	31‰	Mix of (1) Sand, (2) Sand with rocky rubble mix (3) Rocky sand

*The values were the average from 3 measurements at different points in the area

DISCUSSION

In the DNA extraction stage, liquid nitrogen was not used during the grinding process of leaf sample. Therefore, there was a risk of DNA degradation. The increase of EDTA concentration from 20 mM to 50 mM helped reducing DNA degradation. EDTA prevents DNA degradation by chelating Mg^{2+} which is a cofactor for nuclease activity (Tiwari et al., 2012).

The number of RAPD primers that produced polymorphic fragment was only 3 out of 11 primers tested. This indicated that the genetic diversity of *E. acoroides* at Segara Ayu Beach, Semawang Beach and Samuh Beach was quite low. This supported the previous study on *E. acoroides* from Sanur coastal water, Bali using PCR-RFLP of chloroplast and nuclear DNA as well as sequencing of chloroplast DNA *trn*S/*trn*fM fragment, where no polymorphism was detected (Pharmawati and Imaniar, 2016). A study of *E. acoroides* from Li'Angang and Xincungang, Hainan Province, China using microsatellite markers reported that among 36 primer pairs developed, only 4 pairs showed polymorphism (Gao et al., 2012).

Among 3 RAPD primers, primer OPB8 has the highest resolving power, followed by OPB20. Each of these two primers generated four polymorphic bands which led to higher resolving power. Resolving power of a primer shows the suitability of a marker system for identification. It indicates the number of specimens distinguished by the primer (Prevost and Wilkinson, 1999). Therefore, primer OPB8 and OPB20 can be a priority of choice in analysis of genetic diversity *of E. acoroides* using RAPD marker.

Low genetic distances were observed between *E. acoroides* populations. This means

that the three populations had close relationships. Analysis of population differentiation (F_{ST}), also showed similar results where the F_{ST} values in the matrix of pairwise F_{ST} for populations comparison were low. The highest FS_T value was between the population at Segara Ayu Beach and Semawang Beach and statistical analysis showed that these two populations were different from each other. The population of Segara Ayu also differed with the population at Samuh Beach. The population at Semawang Beach and Samuh Beach showed no differentiation. This means that there is genetic connectivity between the last two populations.

The low genetic variation detected in this study may due to vegetative distribution dominancy of *E. acoroides*. It was reported that the dominant characteristic of seagrass is its vegetative growth through rhizome extension (Waycott and Procaccini, 2006). In general, high rate of vegetative reproduction of marine angiosperms led to the statement that recruitment of seagrass from seed is very seldom and only had a little contribution (Lacap et al., 2002, Ruiz-Montoya et al., 2015). This will lead to low genetic variation of a population. Although vegetative distribution seemed to be dominant in this study, the sexual reproduction for propagation also occurred, detected as molecular variation within and between population.

The connectivity between populations was due to seed dispersal of *E. acoroides* (Yu et al., 2019). *Enhalus acoroides* has large fruit pod which contains approximately 11.8 ± 4.04 seeds. The fruit may float following the currents up to 41 km while floating seeds only travel less than 5 km (Lacap et al., 2002, Kendrick et al., 2012). The distance from Semawang Beach to Samuh Beach is approximately 10.37 km, thus gene flow may occur. In a study of *E. acoroides* populations at a long geographical distance, covering Indo-Malay Archipelago, it was found that there was significant population differentiation detected using microsatellite DNA (Putra et al., 2018). The formation of the Sunda Shelf was predicted to contribute to the isolation of *E. acoroides* by preventing dispersal (Putra et al., 2018).

Molecular markers can show spatial distribution of genetic diversity (Kendrick et al., 2017). The low genetic diversity of *E. acoroides* populations from Segara Ayu Beach, Semawang Beach and Samuh Beach, Bali, suggested that extension of rhizome may occur over a long distance. However, there were several RAPD bands that only slightly shared among populations indicated that seeds also as a mean of long-distance dispersal.

CONCLUSION

Genetic diversity of *E. acoroides* in southeast coastal water of Bali, Indonesia was quite low as reflected by a low percentage of polymorphic DNA band and low heterozygosity. Vegetative reproduction may be dominant in this area although gene flow through seed dispersal also occurred at low levels.

CONFLICT OF INTEREST

The authors declared that the present study was performed in the absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors conducted sample collection, DNA extraction and manuscript preparation. MP design the experiments and performed PCR dan data analyses. All authors have read the manuscript.

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