ISSR and RAPD primers selection for assessing genetic diversity of *Enhalusacoroides* (L.f.) Royle

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Abstract. Inter Simple Sequence Repeat (ISSR) and Random Amplified Polymorphic DNA (RAPD) are common PCR-based molecular markers used to study genetic diversity both between and within species. One of important steps in ISSR and RAPD analyses is primer selection to obtain bright PCR products. The aim of this study was to select ISSR and RAPD primers that produced scorable fragments of PCR product for genetic diversity evaluation of seagrass *Enhalusacoroides* (L.f.) Royle. The study also aimed to compare ISSR and RAPD technique in producing informative fragments. Enhalusacoroides is one seagrass species widely distributed in Indonesia. Seagrass samples were collected from Sanur Coastal Water and from Nusa Dua Coastal Water. DNA was extracted using CTAB method. Ten ISSR primers and eight RAPD primers were tested. Results showed that RAPD was more informative than ISSR. Among ten ISSR primers, only four primers resulted in scorable fragments in all samples, and only one among those primers gave polymorphic pattern. As many as 15 fragments have resulted from three ISSR primers and only 1 fragment was polymorphic. All RAPD primers produced scorable products, and three primers resulted in polymorphic fragments. A total of 42 RAPD fragments were produced and 4 from them were polymorphic fragments.

1. Introduction

Climate change is one of important environmental factor that affected genetic diversity of organism. Several changes in the climate include the increase of temperature of land and ocean, and the increase of CO₂ concentration in the atmosphere. These conditions can affect the ability and timing of plant and animal reproduction as well as distributions of species and sizes of population which in turn influence the genetic diversity and genetic structure [1, 2].

Changes in climate can be driven by human activities or due to natural disaster. Climate change affected both terrestrial and marine species including seagrass. Seagrass has high importance to human life. Seagrass supports fishery productivity by providing food, habitat, nursery areas [3]. *Enhalusacoroides* (L.f.) Royle is one of seagrass species which is easily distinguish from other species. It is the biggest seagrass species in Indonesia [4]. It has long and wide leaf and often form high density seagrass bed which become a shelter place for marine biota [5]. Environmental changes can have negative impact on seagrass habitat which become threat to seagrass. It was reported that in Shark Bay, Western Australia, the damage of seagrass meadow due to marine heatwave in 2010/2011...
reached 36% [6]. Adaptation to the changes in environmental is a main factor of populations to survive in their habitats. Adaptation of populations occur through selection of new mutation and selection of existing genetic variation [7]. Therefore, studies on genetic diversity is needed.

There are several techniques available to study genetic diversity including molecular techniques. ISSR (Inter Simple Sequence Repeat) and RAPD (random amplified polymorphic DNA) are two among many molecular techniques for assessing genetic diversity in population levels. Both ISSR and RAPD are PCR-based molecular techniques that easy to perform and only require single short oligonucleotide primer [8, 9]. ISSR amplify sequence DNA between repeat DNA sequence, while RAPD amplify random DNA fragments [8, 9, 10, 11].

In the ISSR and RAPD techniques, selection of primers is important step for optimization of ISSR and RAPD markers. Primers used should produce strong and reproducible amplified fragments. In addition to that, primers selected are primers that able to generate polymorphic pattern. This study aimed to screen ISSR and RAPD primers and select those that resulted in strong, reproducible and polymorphic DNA pattern for analysis of genetic diversity of *E. acoroides* from Semawang Beach, Sanur, Denpasar, Bali, Indonesia and Samuh Beach, Nusa Dua, Badung, Bali, Indonesia.

2. Materials and Method

2.1. Samples Collection

Leaf samples of *E. acoroides* were collected from Semawang Beach, Sanur, Denpasar, Bali, Indonesia and Samuh Beach, Nusa Dua, Badung, Bali, Indonesia (Figure 1). Seven samples were collected from each location. Individuals were collected randomly but they were separated at least 5 m apart to avoid clonal individuals. Leaf samples were wash thoroughly in running water and blotted dry in paper towel. Leaf samples were store at -80°C until further analysis.

2.2. DNA Extraction

DNA was extracted from 0.2 g frozen leaf following Doyle and Doyle [12] with modification [13]. The lysis buffer contained CTAB buffer (2% CTAB, 1.4 M NaCl, 50 mM EDTA, 100mM Tris pH 8.0). Frozen leaf was grounded with mortar and pestle and 1 ml lysis buffer was added. The mixture was incubated at 65°C for 30 min. Same volume of chloroform : isoamylalcohol (24:1) was added and vortexed. After that, the mixture was centrifuged at 14.000 for 10 min and supernatant was transferred to a new tube. Cold isopropanol was added and incubated at -20°C for 2 hours. Centrifugation was done at 14.000 for 5 min. Supernatant was discarded and pellet was wash with 70% ethanol. After centrifugation at 14.000 rpm for 5 min, the pellet was air dried. The pellet was dissolved in 100 µl sterile H₂O. Electrophoresis was done in 1% agarose in TAE buffer. As much as 3 µl DNA solution was loaded to gel. As a comparison, lambda DNA at different concentrations were loaded next to the samples. Electrophoresis was run at 100V for 45 min. Gel was stained with ethidium bromide and DNA was observed under uv transilluminator [14].

2.3. ISSR and RAPD

ISSR and RAPD were conducted each in 20 µl reaction that consisted of 1x PCR buffer (Promega), 200 µM dNTP, 3 mM MgCl₂, 1.5µM primer, 1 U Taq polymerase (Promega) and 50 ng DNA template. Ten ISSR primers and eight RAPD primers were screened (Table 1). The PCR cycle for ISSR was 1 cycle initial denaturation at 95°C for 5 min, followed by 30 cycles consisted of denaturation at 95°C for 50 sec, annealing at 48°C and 50°C (depending on the primer used) for 50 sec and elongation at 72°C for 50 sec. Final elongation was done for 10 min at 72°C. The PCR thermal cycles for RAPD was 1 cycle initial denaturation at 95°C for 5 min, followed by 30 cycles consisted of denaturation at 95°C for 50 sec, annealing at 37°C for 50 sec and elongation at 72°C for 50 sec. Final elongation was done for 10 min at 72°C.

2.4. Data Analysis

DNA fragments of ISSR and RAPD products were scored 1 if present and 0 if absent. Percentage of polymorphism was calculated and compared between ISSR and RAPD. Marker index (MI),
discriminating power (D) and resolving power (R) were calculated using iMEC: Online Marker Efficiency Calculator [15]. The dendrogram showing relationships between samples was analysed using NTSYS [16].

**Fig 1.** Locations of *E. acoroides* sample collection; 1=Semawang Beach, 2=Samuh Beach.

Electrophoresis of PCR products was done in 1.8% agarose in TAE buffer. As many as 10 µl PCR product was loaded to the gel. Electrophoresis was done for 1 hr at 100V. Gel was stained with ethidium bromide and observed using uv transilluminator [14].

**Table 1.** List of primers used, GC content and their annealing temperature.

| Type of Marker | Primer Name | Sequence | GC Content (%) | Annealing Temperature |
|----------------|-------------|----------|----------------|-----------------------|
| ISSR           | UBC805      | (TA)8C   | 5.88          | 36                    |
|                | UBC810      | (GA)8T   | 47.05         | 48                    |
|                | UBC812      | (GA)8A   | 47.05         | 48                    |
|                | UBC820      | (GT)8C   | 52.9          | 50                    |
|                | UBC825      | (AC)8T   | 47.05         | 48                    |
|                | UBC830      | (TG)8G   | 52.9          | 50                    |
|                | UBC855      | (AC)8YT  | 44.4-50      | 52                    |
|                | UBC864      | (ATG)6   | 33.3          | 48                    |
|                | UBC868      | (GAA)6   | 33.3          | 48                    |
|                | UBC890      | *VHV(GT)7| 41.2-58.8    | 42                    |
| RAPD           | OPA2        | TGCCGAGCTG| 70            | 36                    |
|                | OPA3        | AGTCAGCCAC| 60            | 36                    |
|                | OPB4        | GGACTGGAGT| 60            | 36                    |
|                | OPB8        | GTCCACACGG| 70            | 36                    |
|                | OPB12       | CCTTGACGCA| 60            | 36                    |
|                | OPD11       | AGCGCCATTG| 60            | 36                    |
|                | OPD20       | ACCCGGTCA| 70            | 36                    |
|                | OPH3        | AGACGTCCCG| 70            | 36                    |

*V=A, C or T; H=A, C or G; Y=C or T

**3. Results and Discussion**

DNA was successfully extracted from frozen leaf of *E. acoroides* using modified Doyle and Doyle [12] method. The concentration of DNA ranged from 66.7 ng/µl to 266.7 ng/µl. Among 10 ISSR
primers screened, only 5 ISSR primers (UBC805, UBC810, UBC812, UBC825 and UBC890) amplified scorable fragments for all 14 samples. Two ISSR primers, UBC864 and UBC868 only amplified DNA fragment in 2 and 8 samples respectively, while three ISSR primers (UBC820, UBC815, and UBC830) resulted in smeared product for all samples (Table 2). In contrast to ISSR primers, the eight RAPD primers used produced scorable PCR products for all samples (Table 2). From five ISSR primers that produced scorable fragments, only one primer (20%) showed polymorphic fragment. Among eight RAPD primers, three primers (37.5%) produced polymorphic fragments. Representatives of ISSR and RAPD gel electrophoresis were shown in Figure 2 and 3.

The total number of DNA fragment amplified was 22 in ISSR and 42 in RAPD (Table 2). However, in ISSR the total number of fragments amplified in all samples was 15. Four fragments out of 42 fragments were polymorphic in RAPD marker, while one fragment from 15 ISSR fragments was polymorphic. ISSR and RAPD primers that produced polymorphism were further analysed for several characters including marker index (MI), discriminating power (D) and resolving power (R).

| Marker Type | Primer Name | Number of Fragment | Number of Sample Amplified | Fragment Size (bp) | Number of Polymorphic Fragment | % Polymorphism |
|-------------|-------------|--------------------|----------------------------|---------------------|-----------------------------|---------------|
| ISSR        | UBC805      | 2                  | 14                         | 200-295             | 0                          | 0             |
|             | UBC810      | 2                  | 14                         | 710-908             | 0                          | 0             |
|             | UBC812      | 7                  | 14                         | 315-887             | 1                          | 14.3          |
|             | UBC820      | 0                  | 0                          | -                   | -                          | -             |
|             | UBC825      | 4                  | 14                         | 400-802             | 0                          | 0             |
|             | UBC830      | 0                  | 0                          | -                   | -                          | -             |
|             | UBC855      | 0                  | 0                          | -                   | -                          | -             |
|             | UBC864      | 1                  | 2                          | 600                 | 0                          | 0             |
|             | UBC868      | 2                  | 8                          | 210-340             | 0                          | 0             |
|             | UBC890      | 4                  | 14                         | 496-1340            | 0                          | 0             |
| Total       |             | 22                 |                            | 1                   |                            |               |
| Average     |             | 2.2                |                            |                     |                            |               |
| RAPD        | OPA2        | 6                  | 14                         | 325-750             | 0                          | 0             |
|             | OPA3        | 3                  | 14                         | 448-600             | 0                          | 0             |
|             | OPB4        | 6                  | 14                         | 435-820             | 0                          | 0             |
|             | OPB8        | 7                  | 14                         | 418-1380            | 2                          | 28.6          |
|             | OPB12       | 3                  | 14                         | 542-900             | 1                          | 33.3          |
|             | OPD11       | 7                  | 14                         | 382-1288            | 0                          | 0             |
|             | OPD20       | 7                  | 14                         | 420-1460            | 0                          | 0             |
|             | OPH3        | 3                  | 14                         | 375-718             | 1                          | 33.3          |
| Total       |             | 42                 |                            | 4                   |                            |               |
| Average     |             | 5.25               |                            |                     |                            |               |

In this study, ISSR marker was less informative than RAPD marker. Smear PCR products were observed when using primer UBC830 and UBC855. The smear PCR product of ISSR could be due to unspecific binding of the primer to DNA template. PCR enhancing agent such as formamide, DMSO or glycerol should to be added to improve PCR product by reducing smear background [17]. To obtain successful ISSR products, the SSRs of primer pairs must be located within a short distance on the same chromosome [18]. This allows amplification of DNA fragment located between SSRs. ISSR primer UBC820 did not produce any PCR product. It could be no annealing site of primer UBC820 to the template.
Three RAPD primers produced polymorphism and the primers were further analysed. Primers that resulted in monomorphic profile were not included in the analysis. Table 3 summarised the analyses of ISSR and RAPD primer used in E. acoroides from Semawang Beach, Sanur, Bali and Samuh Beach, Nusa Dua, Bali, Indonesia. Primer OPB8 had the highest resolving power (R) (1.143) and primer OPB12 had the lowest R value (0.286). However, OPB12 has the highest discriminating power. Resolving power indicates the ability to differentiate between analyzed samples, while differentiating power is the possibility of two randomly chosen individuals showed different patterns [15].

Levels of polymorphism depend on the species tested. In this study, the result was discordant with many previous studies of intraspecific variation in plant using ISSR and RAPD. In analysis of genetic variation of Vigna mungo ISSR showed more polymorphism than RAPD [19]. Higher polymorphism of ISSR compared to RAPD was also observed in Cicer arietinum [20], Malus domestica [21] and Alpinia galangal [22]. ISSR and RAPD were both effective in detection of variation in Olea europaea ssp. Oleaster where both ISSR and RAPD showed 100% polymorphism [23]. However, a study reported that in barley, RAPD detected 100% polymorphism, while ISSR had 66.67% polymorphism [24]. Higher polymorphism detected by RAPD was also found in a study on genetic diversity of Citrullus colocynthis [25].

The relationships of the 14 E. acoroides samples was analysed using NTSYS based on coefficient of similarity. In general, the samples were not grouped based on their growing locations (Figure 4). There were two grouped of samples, each group consisted of samples from Semawang Beach and Samuh Beach. Several samples had similarity coefficient of one indicating similarity.
More primers need to be screened and PCR condition needs to be optimized to obtained more scorable DNA fragments of ISSR and RAPD.

Table 3. Characteristic of ISSR and RAPD primers that produced polymorphic fragments in E. acoroides

| Marker Type | Primer Name | Marker Index | Discriminating Power | Resolving Power |
|-------------|-------------|--------------|----------------------|-----------------|
| ISSR        | UBC812      | 0.012        | 0.195                | 0.571           |
| RAPD        | OPB8        | 0.018        | 0.368                | 1.143           |
|             | OPB12       | 0.021        | 0.495                | 0.286           |
|             | OPH3        | 0.015        | 0.268                | 0.857           |
| Average of RAPD |          | 0.018        | 0.377                | 0.762           |

Fig 4. Genetic Relationships of 14 E. acoroides samples from Semawang Beach and Samuh Beach Based on ISSR and RAPD Markers.

4. Conclusion

The results showed that among ten ISSR primers, only four primers resulted in scorable fragments in all samples, and only one among those primers gave polymorphic patterns. As many as 15 fragments have resulted from three ISSR primers and only 1 fragment was polymorphic. All RAPD primers produced scorable products, and three primers resulted in polymorphic fragments. A total of 42 RAPD fragments were produced and 4 from them were polymorphic fragments.

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