Genetic diversity information of seagrass *Enhalus acoroides* and *Cymodocea rotundata* for the local genetic conservation at North Maluku

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Abstract. This study is aimed at analyzing the molecular identification and the level of the genetic diversity of seagrass species as a basis information for the local genetic conservation. Sampling of both species, *Enhalus acoroides* and *Cymodocea rotundata*, was carried out at four small islands at North Maluku, namely Hiri, Ternate, Maitara, and Tidore Island. Locus used in genetic analysis is the chloroplast genome (*rbcL* and *matK*). The results of this work indicated that the *rbcL* genetic marker were able to identify species well but were low in discrimination and divergence of species, thus is not give the good results for genetic diversity level. Conversely, *matK* genetic marker, although a little weak in identifying species, have good results in the discrimination and divergence of species. *matK* genetic marker were able to provide a description of genetic diversity level. The results of this study found that the level of genetic diversity of *E. acoroides* (*H₂*: 13; *Hₑ*: 0.862; π: 0.273) and *C. rotundata* (*H₂*: 19; *Hₑ*: 0.975; π: 0.119) were higher at Tidore Island compared to the three others. This indicated that the high genetic diversity at Tidore Island can be used as a basis for local genetic conservation and maintenance of biodiversity.

1. Introduction

The Indo-Pacific region is the distribution of tropical seagrass bioregion with the highest number of seagrass species compared to other bioregions namely around 24 species of seagrass [1]. The territorial waters of Indonesia, where is part of the Indo-Pacific region, are known to have a wide distribution of seagrasses, where to date there have been discovered around 13 species of seagrasses including the new seagrass species, *Halophila sulawesii*, which was investigated by [2]. *Enhalus acoroides* and *Cymodocea rotundata* are two important types of seagrass invented in Indonesian waters [3], [4]. Ecologically these two types of seagrass have an important role where *Enhalus acoroides* can act as a microhabitat and shelter for various marine biota such as fish and shrimp, while *Cymodocea rotundata* acts as a source of nutrition for dugongs [5]. However, the global population of seagrass *E. acoroides* which is a climax species with a large body morphology, long-lived, and dioecious is in a decreased status in a number of places. While the global population of *C. rotundata*, although still in stable condition, this species which is a pioneer species and forms fringing beds,
often affected by the development of coastal areas and other anthropogenic activities which are local threats to their existence [6].

Seagrasses live in a very dynamic shallow water environment because they are influenced by the dynamics of the sea itself and human activities on the terrestrial areas. Local threats that can affect seagrass habitats and populations include development of coastal areas, dredging, shipping activities, runoff and eutrophication [6]. Through changes in natural disturbance and an increase in the frequency of anthropogenic disturbances, it is important to understand how these changes will affect biodiversity at the most fundamental level, namely genetic diversity [7]. The decline in seagrass habitat and population makes an important understanding of seagrass genetic diversity [8]. Genetic diversity in seagrass ecosystems provides an insight into the mode of reproduction and potential adaptation and is therefore an integral part of conservation strategies for coastal ecosystems [9]. Additionally, genetic diversity and genetic structure of populations are the key components of seagrass resilience and contribute to understanding management and conservation [10].

Seagrass *E. acoroides* and *C. rotundata* can be found in the North Maluku Islands including in Hiri Island, Ternate Island, Maitara Island, and Tidore Island [11], [12]. Coastal development activities such as construction of coastal areas, coastal reclamation and other anthropogenic activities have become one of the factors causing the degradation of seagrass habitat at these locations. The decline on seagrass population due to habitat degradation through those activities can cause limited light which stimulate a decrease in genetic diversity because certain genotypes become incompatible with their environmental conditions [13]. For this reason, genetic methods are needed through application of genetic markers, namely rbcL and matK which are the locus of the chloroplast gene that has been used for identification, and detection of the diversity and phylogenetic analysis of seagrass [8]. Molecular identification of seagrass and phylogenetic analysis with molecular markers is still not widely done in Indonesia [14]. To date, there is still very little molecular data to explain the genetic diversity of seagrasses and their genetic relationship in the water of North Maluku, particularly the seagrass populations of *E. acoroides* and *C. rotundata*. According to [14] genetic diversity information is truly indispensable for ecosystem conservation, population management, and maintenance of biodiversity in an aquatic environment. The objectives of this work are to identify molecular and determine the level of genetic diversity of *E. acoroides* and *C. rotundata* in North Maluku waters based on rbcL and matK molecular markers as a basis for local seagrass genetic conservation efforts in the study area.

2. Materials and method
2.1. Study site
Seagrass samples of *E. acoroides* and *C. rotundata* were collected from May to August 2017 and May 2018 at four small islands in North Maluku, namely Ternate Island, Maitara Island, Tidore Island, and Hiri Island (Figure 1).
Samples of seagrass *E. acoroides* and *C. rotundata* were taken using the linear quadratic transect method where the transect line was drawn along 100 m perpendicular to the coastline and the distance between the sampling points was 20 m. At each sampling point, the square of the observation (1m x 1m) was then taken as samples of young seagrass leaves from each species. Samples of seagrass leaves of *E. acoroides* and *C. rotundata* were washed with running water to clean the dirt and epiphytes that were attached, dried with a tissue and then cut to a size of ± 3cm, then put in plastic ziplock where was added with silica gel.

2.2 DNA extraction, amplification, and sequencing

The DNA extraction process was carried out using the Dneasy Plant Mini Kit from Qiagen which already has an extraction protocol. The target genome used in this study is chloroplasts. The target gene segment is amplified using a PCR (Polymerase Chain Reaction) machine.

The primers used were primers of the chloroplast locus (two primers), namely *matk* (P6465'TAATTTACGATCAATTCC3’ and P6475’GTTCTAGCACAAAGAAGTCCG-3); and *rbcL* (P609; 5’-GAAAATCAATGTCCTACCCTG-3, and P610; 5’-ATGTCACCAACAGACTAAAGC-3) [8]. The amplification process uses a master mix with a total volume of 27 µl consisting of 3 µl DNA templates, 12.5 µl MyTaq HS Red Mix, 1.25 µl of primers (*rbcL* and *matk*), and 9 µl ddH2O. The denaturation process at 94°C, 30 seconds, annealing at 52°C, 35 seconds, and extension at 70°C for 1 minute, with 35 cycles at *matk*; while for *rbcL* the denaturation process was carried out at 94°C for 1 minute, annealing at 57°C for 2 minutes and the extension process at 72°C for 2 minutes, with 35 cycles.

The PCR product was assessed by using electrophoresis gel made from 0.5 gr of agarose 1%, TBE buffer 50 ml and EtBr (ethidium bromide) dye 3 µl and then visualized using MINI Alphamager ultraviolet machine. Positive PCR products through DNA electrophoresis visualization are sent to the 1st Base sequencing facility in Malaysia for sequencing.

2.3 Data analysis

DNA sequences obtained from sequencing results were aligned and edited using *clustal W* from MEGA 6.0 (*Molecular Evolutionary Genetic Analysis*) software. The DNA sequences obtained were compared with the DNA sequences in the database (*GenBank*) to confirm the species were *E. acoroides* and *C. rotundata*. Analysis of the level of genetic diversity, namely haplotype diversity (*Hd*) and nucleotide diversity (*π*) using DnaSP software [15].

3. Results and discussion

3.1 Molecular identification and nucleotide base of *E. acoroides* and *C. rotundata* based on *rbcL* and *matk* molecular markers

Multilocus molecular markers, *rbcL* and *matk*, have been recommended as the main barcodes of plants [16]. DNA barcodes involve sequences of standard DNA regions and have proven to be powerful tools for species identification [17]. Molecular identification results of the two seagrass species, *E. acoroides* and *C. rotundata*, from the four small islands (Hiri, Ternate, Maitara, and Tidore) using the BLAST (*Basic Local Alignment Search Tool*) based on the molecular markers *matk* and *rbcL* are shown in Table 1. These results indicated that the molecular markers *rbcL* provide better results in identifying species molecularly than *matk*. *MatK* molecular markers are only able to identify about 65% or 54 sequence data of *E. acoroides* which show species similarity with sequential data from GenBank with 95.61-100% similarity level, while *rbcL* molecular markers show 100% results similar to GenBank data.

Likewise, the molecular identification of *C. rotundata* where *matk* molecular markers were able to identify approximately 80% or 76 sequence data showing the similarity of species with sequence data from GenBank from a total of 95 sequence data analyzed, while *rbcL* markers are around 88.42% or 83 sequence data with GenBank data. According to [18], species discrimination with plant barcodes is usually lower than CO1 in animals. Furthermore [19] stated that the limitations of the chloroplast
gene for species identification are acceptable for plant DNA barcodes. Although \( \text{matK} \) is one of the most developed plastid genomes, it usually results in a success rate of PCR and sequencing around 70% in angiosperms [18]. Conversely, the \( \text{rbcL} \) barcode area is easily amplified, sequenced and aligned and is a useful backbone for barcode data sets, even though it has only modest discrimination [16].

Table 1. Molecular verification of seagrass \( E. \) acoroides and \( C. \) rotundata using BLAST in North Maluku waters based on molecular markers \( \text{matK} \) and \( \text{rbcL} \)

| Species        | Primer | The number of fragment sequences analyzed | Length of base pair (bp) | Molecular verification (with sequence data of GenBank) | Similarity (%) | Accession Code |
|----------------|--------|------------------------------------------|-------------------------|------------------------------------------------------|----------------|----------------|
|                |        | Corresponding                             | Not Corresponding        |                                                      |                |                |
| \( E. \) acoroides | \text{matK} | 83                                       | 525                     | 65% (54 sample)                                      | 35% (29 sample) | 95.61-100      | AB002569.1     |
|                | \text{rbcL} | 85                                       | 515                     | 100%                                                 | 0              | 100            | JN225336.1     |
| \( C. \) rotundata | \text{matK} | 95                                       | 891                     | 80% (76 sample)                                      | 20% (19 sample) | 99.74-100      | KX526628.1     |
|                | \text{rbcL} | 94                                       | 487                     | 88.42% (83 sample)                                   | 11.58% (11 sample) | 100            | MH547225.1     |

The nucleotide base component consisting of Purine bases (Adenine and Guanine) and Pyrimidine bases (Thymine/Uracil and Cytosine) have the possibility to undergo both transitional and transversal substitution. The content of Thymine and Uracil bases in \( \text{matK} \) and \( \text{rbcL} \) \( E. \) acoroides sequence fragments was found to be the highest at 39.01% and 29.12% respectively, while the lowest was found in the Guanine (G) base of 20.97% for \( \text{rbcL} \) and Adenine (A) bases 16.99% for \( \text{matK} \) with the total A + T is higher than C + G. The matrix of the nucleotide substitution of \( \text{matK} \) and \( \text{rbcL} \) locus of species, \( E. \) acoroides shown in Table 2.

Table 2. Nucleotide substitution probability matrix based on the molecular markers of \( \text{matK} \) and \( \text{rbcL} \) of \( E. \) acoroides

|        | \( \text{matK} \) | \( \text{rbcL} \) |     |     |     |     |
|--------|------------------|------------------|-----|-----|-----|-----|
|        | \( \text{A} \)  | \( \text{T} \)  | \( \text{C} \)  | \(\text{G}\) | \( \text{A} \)  | \( \text{T} \)  | \( \text{C} \)  | \( \text{G} \)  |
| \( \text{A} \) | 8.88*            | 4.16*            | 7.66*          | 6.38*           | 21.96*         | 4.16*            | 17.89*          |
| \( \text{T} \) |                |                  |                | 10.27*          | 23.93*         |                |                |
| \( \text{C} \) |                |                  |                |                | 5.02*          |                |                |
| \( \text{G} \) |                |                  |                |                |                |                |                |

Note: *Transition ; ^Transversal

While the \( C. \) rotundata fragment is somewhat different from \( E. \) acoroides where the highest is found in the Adenine bases for both \( \text{rbcL} \) and \( \text{matK} \) respectively 39.98% and 34.35%, while the lowest is found in the Guanine base respectively 14.94% and 14.67% with a total A + T higher than C + G.
The nucleotide composition of other seagrass species such as *Zostera marina* also shows the higher content of Thymine (32.5%) and the lowest Guanine (17.4%), but has a high total G + C (35.46%) [20]. The matrix of the nucleotide substitution of *matK* and *rbcL* locus of species *C. rotundata* shown in Table 3.

Table 3. Nucleotide substitution probability matrix based on molecular markers of *matK* and *rbcL* of *C. rotundata*

|       | A   | T     | C      | G      |
|-------|-----|-------|--------|--------|
| *matK*| A   | 2.42^* | 1      | 22.66^*|
| T     | 1.91^* | -     | 4.74^* | 0.89^* |
| C     | 1.91^* | 11.49^* | -     | 0.89^* |
| G     | 48.67^* | 2.42^* | 1^     | -      |

|       | A   | T     | C      | G      |
|-------|-----|-------|--------|--------|
| *rbcL*| A   | 5.39^* | 3.86^* | 8.88^* |
| T     | 4.94^* | -     | 18.27^* | 4.04^* |
| C     | 4.94^* | 25.5^* | -     |        |
| G     | 10.87^* | 5.39^* | 3.86^* | -      |

Note: ^Transition; *Transversal

Generally, this study found that the transition value of *E. acoroides* was greater than the transversal value based on *matK* molecular markers, while the purine transition value was higher than pyrimidine bases based on *rbcL* molecular markers. While *C. rotundata* shows pyrimidine transition values are higher than purine bases based on *rbcL* markers, while purine transition values are greater than pyrimidine bases based on *matK*. This could have an influence on the base order of each individual seagrass *E. acoroides* and *C. rotundata* which contribute to the formation of a level of genetic diversity. According to [21] that one estimation of genetic diversity can be seen in the diversity of nucleotides formed where the differences in nucleotides may stimulate the process of genetic changes. Furthermore [22] reported that the genetic variation that was produced can be the basis for seagrass species to adapt to environmental changes.

3.2. Genetic diversity of seagrass *E. acoroides* and *C. rotundata* based on *rbcL* and *matK* molecular markers

The results of the analysis of the level of genetic diversity of the both species, *E. acoroides* and *C. rotundata* at all four small islands (Hiri, Ternate, Maitara, and Tidore) indicated that the molecular markers of *matK* provided the better results than *rbcL* (Table 4 and Table 5).

This can be seen from the 54 *E. acoroides* sequence data analyzed, found a total of 17 haplotype were formed in the study area, while from 85 *rbcL* sequence data, only two haplotypes were formed (Table 4). The results of this study indicated that Tidore Island has a higher genetic diversity where the number of haplotype found in the population of *E. acoroides* is 13 haplotypes and is supported by the high values of haplotype diversity (Hd: 0.862) and the nucleotide diversity (π: 0.273) at the island, compared with the other two island namely Ternate Island (3 haplotypes) and Maitara Island (4 haplotypes) which have the lower values (Table 4).
Table 4. Genetic diversity of *E. acoroides* in the North Maluku Islands based on *matK* and *rbcL* markers

| Population | N  | Genetic diversity |           |           |
|------------|----|-------------------|-----------|-----------|
|            |    |                   | \(H_n\)   | \(H_d\)   | \(\pi\)   |
| **matK**   |    |                   |           |           |
| Tidore     | 21 | 13                | 0.862     | 0.273     |
| Maitara    | 20 | 4                 | 0.284     | 0.057     |
| Ternate    | 13 | 3                 | 0.295     | 0.092     |
| **rbcL**   |    |                   |           |           |
| Tidore     | 32 | 1                 | 0         | 0         |
| Maitara    | 29 | 1                 | 0         | 0         |
| Ternate    | 22 | 2                 | 0.091     | 0.001     |
| Hiri       | 2  | 1                 | 0         | 0         |

Note: haplotype number \((H_n)\); haplotype diversity \((H_d)\); nucleotide diversity \((\pi)\)

Likewise, the genetic diversity of *C. rotundata* in which of 76 *mat K* fragments analyzed found 35 haplotypes in the study area, while of 83 *rbcL* fragments there were eight haplotypes formed (Table 5). Application of *rbcL* markers for *C. rotundata* genetic diversity level indicated that Maitara Island has a higher level of genetic diversity where six haplotypes were found with a haplotype diversity \((H_d)\) value of 0.743 and nucleotide diversity \((\pi)\) of 0.224 compared to the other three islands. However, *matK* markers provided a better description of the level genetic diversity in which of 35 haplotype formed. Tidore Island has the highest number of haplotypes of 19 haplotype and was supported by high haplotype diversity \((H_d: 0975)\) and nucleotide diversity \((\pi: 0.119)\) (Table 5).

Table 5. Genetic diversity of *C. rotundata* in the North Maluku Island based on *matK* and *rbcL* markers

| Population | N  | Genetic diversity |           |           |
|------------|----|-------------------|-----------|-----------|
|            |    |                   | \(H_n\)   | \(H_d\)   | \(\pi\)   |
| **matK**   |    |                   |           |           |
| Ternate    | 20 | 14                | 0.953     | 0.077     |
| Tidore     | 26 | 19                | 0.975     | 0.119     |
| Hiri       | 13 | 8                 | 0.897     | 0.039     |
| Maitara    | 17 | 9                 | 0.904     | 0.090     |
| **rbcL**   |    |                   |           |           |
| Ternate    | 30 | 1                 | 0         | 0         |
| Tidore     | 25 | 5                 | 0.357     | 0.107     |
| Hiri       | 11 | 3                 | 0.473     | 0.185     |
| Maitara    | 17 | 6                 | 0.743     | 0.224     |

Note: haplotype number \((H_n)\); haplotype diversity \((H_d)\); nucleotide diversity \((\pi)\)

Generally, the results of this study indicated that the application of the use of *matK* molecular markers provided better results in describing the level of genetic diversity of seagrass *E. acoroides* and *C. rotundata* in the study area compared to *rbcL*. Previous studies have shown that *rbcL* has a very low divergence rate [23], [17]. Data obtained in this study also showed that this was related to the low ability of *rbcL* divergence. This also affects the assessment of the level of genetic diversity of populations of *E. acoroides* and *C. rotundata* based on the two molecular markers used.

The high genetic diversity at Tidore Island may indicate that the environmental conditions or habitats are still in good condition in supporting genetic diversity in these locations. The low genetic diversity of *E. acoroides* at Ternate Island may be caused by high anthropogenic activity in the coastal
area. According to [13] that seagrass population which grew in the intertidal areas which were disturbed, having a low genetic diversity compared to populations that grew in pristine environments. This study found the level of genetic diversity of seagrass *E. acoroides* and *C. rotundata* was higher at Tidore Island compared to the other three islands, namely Ternate, Maitara, and Hiri. The high level of genetic diversity on Tidore Island is most likely related to the high diversity of seagrass species at the island [11]. Information on high genetic diversity and species diversity at Tidore Island can contribute as a basis for local genetic conservation and maintenance of biodiversity at the island. This information is important because it is related to the development of coastal areas on the islands such as construction of coastal buildings, coastal reclamation and tourist destinations where these activities causing the degradation of seagrass habitat and seagrass loss at these locations. Thus there needs to be high efforts to protect seagrass beds in the study area, where [24] Bjork et al. (2008) recommended that seagrass beds with high species diversity must be protected, and Duarte et al. (2018) recommended that population that are genetically diverse as source population for restoration for management and conservation on population with low genetic diversity.

4. Conclusion
This work reported that application of *matK* molecular markers was better in determining the level of genetic diversity of seagrass *E. acoroides* and *C. rotundata* compared with *rbcL* markers although it was slightly weak in amplifications and sequencing for molecular identification. This study also found levels of genetic diversity of both seagrass species, *E. acoroides* and *C. rotundata*, were higher at Tidore Island compared to the other three islands, namely Hiri Island, Ternate Island, and Maitara Island. This can be used as a basic consideration in seagrass genetic conservation efforts locally, considering seagrass ecosystems are the important coastal ecosystem for small islands in the North Maluku Islands.

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