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Short Communication

# DNA Barcoding of Red Algae (Rhodophyta) in Ternate Island Sea, North Maluku, Indonesia

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## Abstract

Ternate Island is located on the North Maluku Sea. The North Maluku Sea region includes the Wallacea area and the world's coral triangle. One of the organisms commonly found in this sea is red algae. This study aimed to determine the red algae species by phylogenetic tree analysis based on the rbcL gene as a DNA marker. The preserved red algae tissue samples were extracted with the Geneaid GP100 DNA Extraction Kit Plant. The DNA sample was amplified and then visualized by 1% agarose gel electrophoresis. The amplicon products were sequenced and then aligned with the rbcL gene database that was available at the NCBI gene bank. The phylogenetic tree was constructed using the UPGMA method. The results showed that red algae were identified into four species: namely *Gibsmithia hawaiiensis* (98.65%), C_rbcL sample was identical to *Amansieae* sp. (91.50%), D_rbcL sample was identical to Peyssonnelia sp. (95.54%), and G_rbcL sample was similar to *Portieria hornemanniI* (96.15%). Based on phylogenetic tree analysis, *Gibsmithia hawaiiensis* from North Maluku is closely related to species from Raja Ampat, West Papua, Indonesia, followed by the Philippines species. Special findings were found to carry out phylogenetic reconstructions that can answer inter-species kinship. The general finding is that the markers used can be used for phylogenetic construction. Phylogenetic construction of *Peyssonnelia* sp. in North Maluku is related to species from South Africa. North Maluku's *Portieria hornemannii* is closely related to a species from Korea.

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## 1. Introduction

Ternate Island is located on the North Maluku Sea. The waters in the North Maluku region include the Wallacea area and the world's coral triangle (Achmad *et al*., 2023). This geographical condition has consequences for the high marine biodiversity in the waters of North Maluku (Akbar *et al*., 2014). This geographical condition makes this area rich in marine biodiversity, including the island of Ternate. One of the marine organisms which are commonly found in this sea is red algae. On Ternate Island, these organisms grow and develop in subtidal and intertidal areas with biological mechanisms attached to mangrove roots, coral reefs, and hard substrates (Achmad *et al*., 2023). The distribution of red algae in the intertidal region makes it easier for this species to be exploited (Subagio and Kasim, 2019). In Indonesian waters, 452 species of red algae were identified (Oryza *et al*., 2017; Annisaqois *et al*., 2018).

Red algae have an important role in aquatic bioecology, such as the balance of ecosystems and food chains. In addition to having an important function in the environment, red algae have a high economic value, because they can be used as raw materials in the food, cosmetic, and pharmaceutical industries. Utilization of red algae in the food industry is based on the content of gelatin, carrageenan, porphyrin, furcular, and phycobilin pigments (consisting of phycoerythrin and phycocyanin) which are food reserves that contain lots of carbohydrates. While the use of red algae in the cosmetic and pharmaceutical industries is because this type of algae contains triterpenes, glycosides, steroids, flavonoids, and alkaloids. These compounds have biological activities as anticancer, antibacterial, antioxidant, antidiabetic, and immunomodulator (Chojnacka *et al*., 2012; Amaranggana and Wathoni, 2017; Amelia and Tanod, 2016; Ghazali *et al*., 2018; Istifada and Saptarini, 2018; Julyasih *et al*., 2020; Singkoh *et al*., 2019; Winowoda *et al*., 2020; Aris *et al*., 2021).

The high use of red algae is a sign that this organism needs to be protected and preserved to avoid extinction (Samman and Achmad, 2023). For this reason, research is needed on the molecular biology identification of red algae in the Ternate Island Sea. The purpose of this identification is to obtain the genetic quality, characteristics, or character of a species, and to support taxonomic identification, so that it can be used for breeding and conservation purposes (Ragan *et al*., 1994; Rohani-Ghadikolaei *et al*., 2011; Djakatara *et al*., 2018; Annisaqois *et al*., 2018). Genetic information can be used as a conservation database to determine policy strategies and the existence of a biota (Akbar *et al*., 2014; Achmad *et al*., 2019; 2023).

Research on red algae in Ternate Island seas has been reported but only related to diversity and distribution (Samman and Achmad, 2023). Research on molecular red algae in Indonesia has been carried out, but there are still many types of red algae that have not been identified until now, especially in the waters of North Maluku. The lack of molecular information related to red algae indicates that research using molecular markers is very limited. The use of molecular markers is important to clarify the study of the morphology and genetic quality of a species. The use of rbcL gene sequences has been recorded in various studies, showing that these marker genes are quite potential and useful for studying genetic variation in natural populations (Lim *et al*., 2013; Djakatara *et al*., 2018; Annisaqois *et al*., 2018; Ratnawati *et al*., 2020; Meinita *et al*., 2021; Wirawan *et al*., 2021; Hamdan *et al*., 2013). Previous studies were still limited so we started to do DNA info and barcodes red algae in the Ternate Island Sea. The identification of red algae based on the rbcL gene is expected to provide scientific information as a basis for developing sustainable resource potential.

## 2. Material and Methods

### *2.1 Place and Time*

The research was conducted from October to December 2021 at Ternate Island Sea (Figure 1). Red algae tissue (9 ind.) was preserved with 96% Ethanol solution, then stored (15°C) in a 15 ml Falcon tube during DNA work. This reservation aimed to maintain the quality of the DNA sample to be extracted. A total of nine samples of red algae that had been preserved then proceeded to the DNA extraction stage. The secondary data on red algae for the purpose of making phylogenetic data was downloaded from the NCBI website (https://www.ncbi.nlm.nih.gov/). Research equipment used were GPS Garmin, tube, cutter, underwater camera cannon, and scuba diving. The research materials were using 90% ethanol and fresh water.

### *2.2 DNA Extraction*

The DNA extraction was carried out at the Fisheries Hydrobiology Laboratory, Gadjah Mada University. This extraction was conducted using the Geneaid GP100 DNA Extraction Kit Plant. The results of DNA extraction were then used as DNA templates in the amplification of the Rubisco gene through the PCR (Polymerase Chains Reaction) process.

#### *2.2.1 DNA amplification*

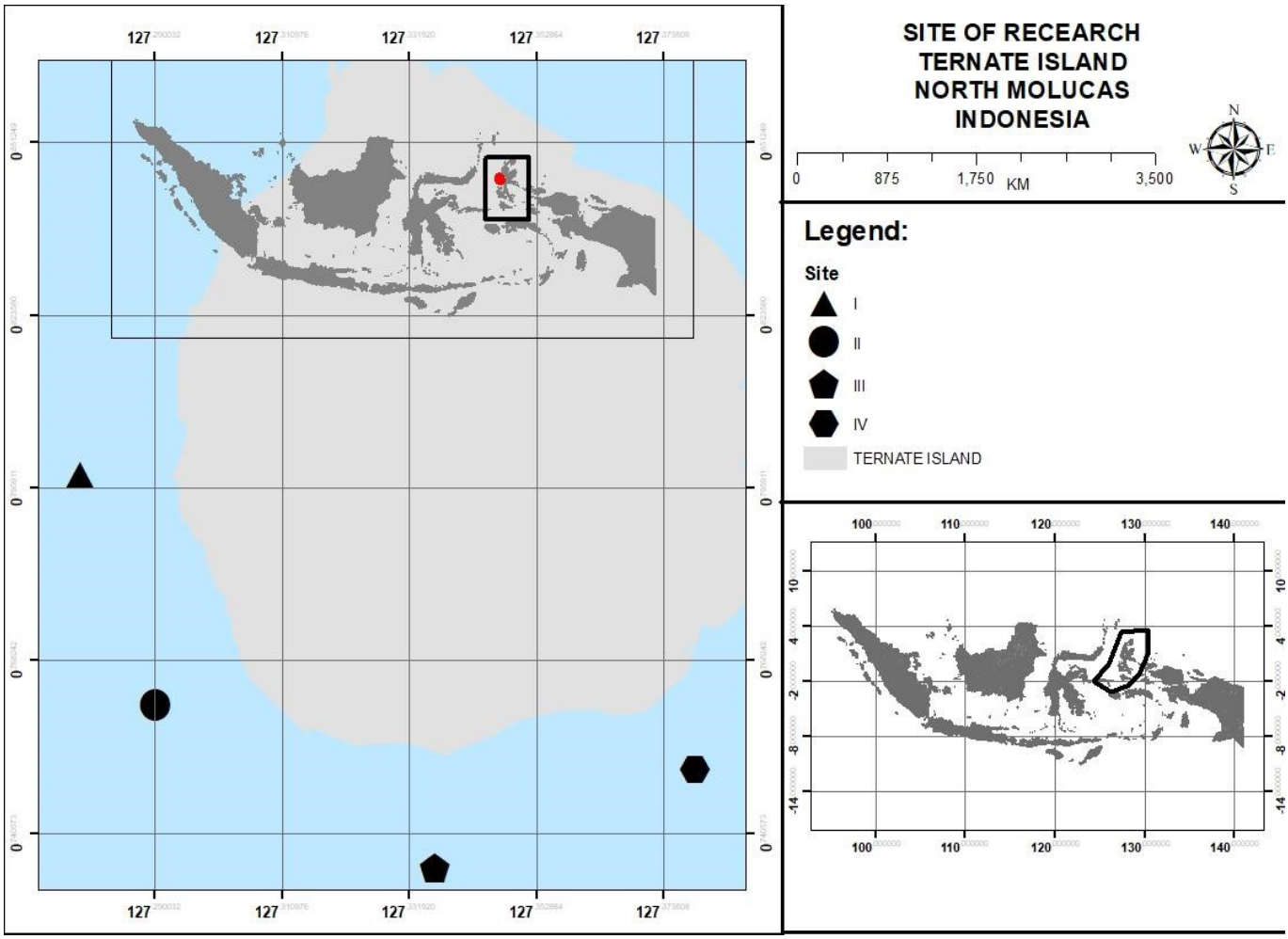


**Figure 1.** Research site of red algae tissue in Ternate Island.

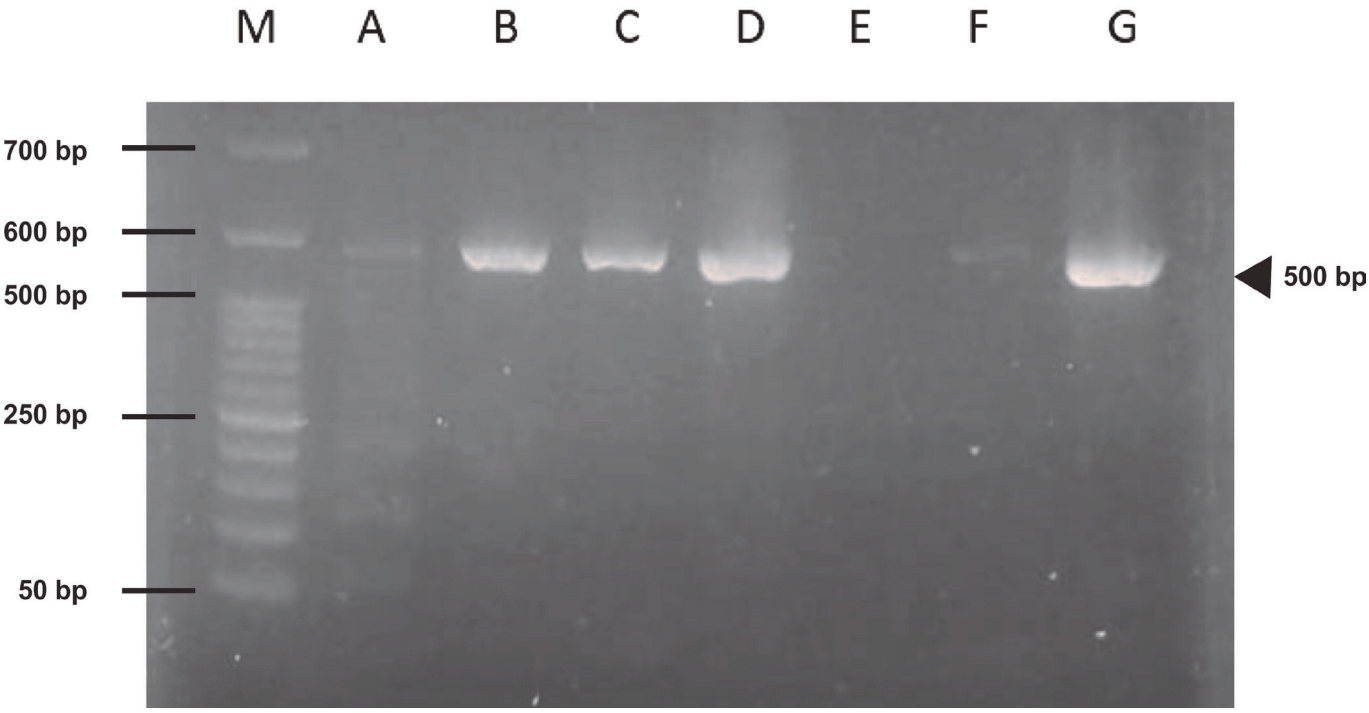


**Figure 2.** Visualization of 1% agarose gel amplification of the rbcL Gene. Note: M: 100 bp marker, A-G: red algae sample

**Table 1**. The similarity of the red algae rubisco gene on Ternate Island, North Maluku

| No | Sample | Base Length | Percent identity | Species | Acc. number |
|---|---|---|---|---|---|
| 1 | B_*rbcL* | 472 bp | 98.65% | *Gibsmithia hawaiiensis* | KM392339.1 |
| 2 | C_*rbcL* | 524 bp | 91.50% | *Amansieae* sp. | MF094038.1 |
| 3 | D_*rbcL* | 467 bp | 95.54% | *Peyssonnelia* sp. | EU349141.1 |
| 4 | G_*rbcL* | 522 bp | 96.15% | *Portieria hornemannii* | AF212185.1 |

**Table 2.** The genetic distance of the North Maluku B rbcL sample with the NCBI database

| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | B_rbcl_ North Maluku | | | | | | | | | |
| 2 | *G hawaiiensis* (KM392339.1) | 0.009 | | | | | | | | |
| 3 | *G dotyi* (KT310688.1) | 0.087 | 0.076 | | | | | | | |
| 4 | *G hawaiiensis* (KM392322.1) | 0.036 | 0.026 | 0.063 | | | | | | |
| 5 | *G hawaiiensis* (KM392323.1) | 0.036 | 0.026 | 0.063 | 0.000 | | | | | |
| 6 | *G hawaiiensis* (KM392326.1) | 0.036 | 0.026 | 0.063 | 0.000 | 0.000 | | | | |
| 7 | *G hawaiiensis* (KY851884.1) | 0.012 | 0.002 | 0.074 | 0.024 | 0.024 | 0.024 | | | |
| 8 | *G hawaiiensis* (KY851885.1) | 0.012 | 0.002 | 0.074 | 0.024 | 0.024 | 0.024 | 0.000 | | |
| 9 | *Gibsmithia* sp. (KT310706.1) | 0.056 | 0.046 | 0.082 | 0.019 | 0.019 | 0.019 | 0.043 | 0.043 | |

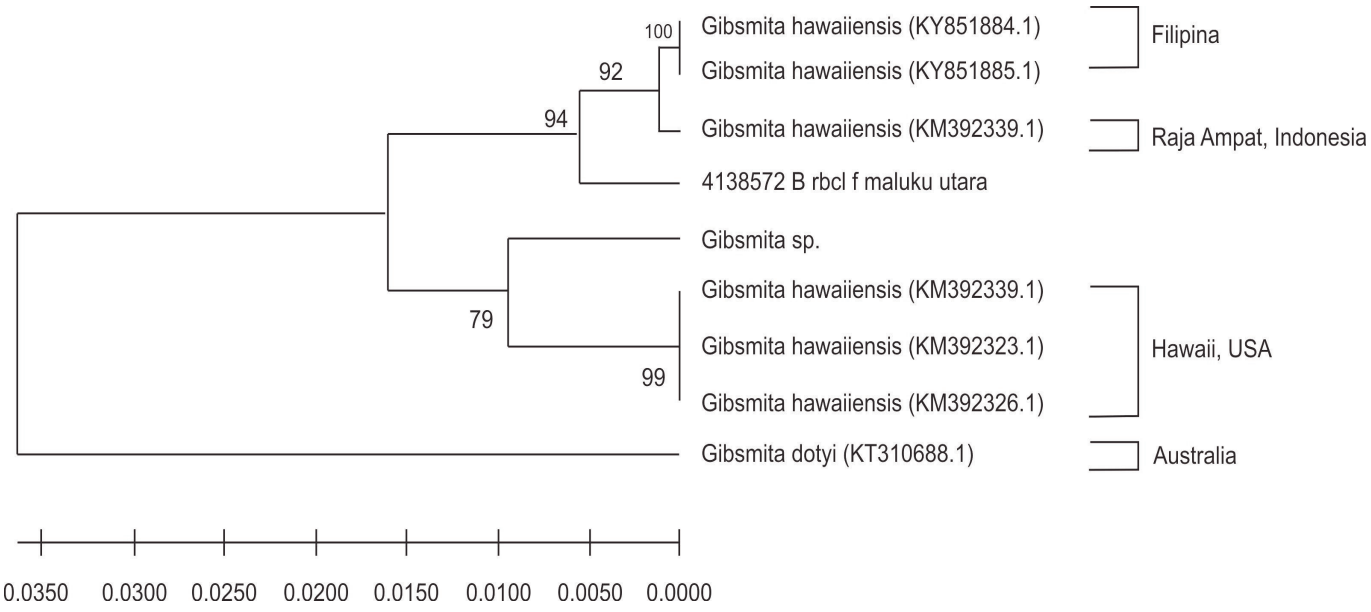


**Figure 3.** Phylogenetic tree of rbcL gene of group B using UPGMA method.

The rbcl is a standard DNA marker used for DNA barcoding of algae (Hengkengbala *et al*., 2018; Alshehri *et al*., 2019). Protocol standard in laboratory the rbcL gene fragment was amplified using the PCR (*Polymerase Chains Reaction*) method with primers rbcL F (GTAATTCCATATGCTAAAATGGGG) and rbcL R (ACATTTGCTGTTGGAGTYTC). A total of 50 L mixed solution consisting of 5 L of sample DNA, 25 L of MyTaq HS Red Mix (Bioline), 1 L primers, namely F and R, and distilled water($dH_2O$) was reacted with a PCR temperature profile. The PCR steps included initial denaturation at 95°C for three minutes, denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, extension at 72°C for one minute, and final extension at 72°C for five minutes. The number of cycles used was 30 cycles. Furthermore, the PCR product was visualized using 1% agarose gel electrophoresis.

### *2.3 Sequencing*

The DNA amplification results were sent to the first BASE Lab for sequencing purposes. The results of the forward and reverse sequences were reviewed using a chromatogram. The DNA sequences were then aligned with Clustal-W using the MEGA 10 program (https://www.megasoftware.net/). The matched amplicon sequences were further analyzed to determine their identity.

### *2.4 Data Analysis*

The amplicon sequences were aligned with the rbcL gene sequence database in the BLAST program of the National Center for Biotechnology Information (NCBI) on the website https://blast.ncbi.nlm.nih.gov for similarity testing.

The phylogenetic tree was constructed based on the sample rbcL gene using the UPGMA method and MEGA 5 (Tamura *et al*., 2013). The bootstrap method with 1000 replications was used to support the accuracy and strength of phylogenetic branching (Nei and Kumar, 2000). Genetic distance was calculated using the pairwise distance (p-distance) method (Nei and Kumar, 2000).

## 3. Result and Discussion

### *3.1 Molecular Characteristics and Identification*

The results of DNA amplification were obtained in 467-524 base pairs (bp) (Figure 2). The length of DNA bases was found in other studies, namely Djakatara *et al*. (2018), which is 1000-1200 bp using primers F-577 (for) and R-753 (rev), and Annisaqois *et al*. (2018), using primers rbcL F-7 (for) and R-753 (rev) obtained 1400-1600 bp and immersed rbcL F-577 (for) and R-753 (rev) with DNA base lengths of 900-1400 bp in *Kappaphycus* sp. Hengkengbala *et al*. (2018) found thick DNA bands at positions around 300–400 bp (1kb marker, Solis Biodyne). Result research Alshehri *et al*. (2019) DNA sequence length 5263 bp, where it ranges from 610-753 (mean length 658 bp).

The differences in DNA base length (bp) may be due to differences in the use of local, primers, the number, and the quality of samples. In general, the results of amplification with the rbcl gene have high DNA quality and normal base length. The results of the amplification of the rbcL gene showed high concentrations (thick bands in samples B, C, D, and G) (Figure 2).

The results of the similarity test found four species of red algae, namely B_rbcL was identical to *Gibsmithia hawaiiensis* (98.65%), C_rbcL sample was similar to *Amansieae* sp. (91.50%), D_rbcL sample was identical to *Peyssonnelia* sp. (95.54%), and G_rbcL sample was identified *Portieria hornemanni* (96.15%) (Table 1). In general, the species found are scattered in the North Maluku Sea but have not been identified or published. Molecular identification is very fast and can clarify morphological information. Classification information using molecular information is very informative so that it becomes a change in scientific knowledge.

### *3.2 Phylogenetic Tree Analysis and Genetic Distance*

The reconstruction of the phylogenetic tree found differences (Figure 3, Figure 4, Figure 5, and Figure 6). The rbcL gene phylogenetic tree sample of group B showed that all species of the genus Gibsmithia used in this tree were in one large clade or were monophyletic. Monophyletic groups are species with varying numbers that form clades because they come from a common ancestor.

The B_rbcL sample on Ternate Island has the closest relation to *Gibsmithia hawaiiensis* (KM392339.1) from Raja Ampat, West Papua, Indonesia, and is closely related to a species from the Philippines. The closeness of this kinship is made possible by the influence of geographical location and almost the same climatic characteristics in the Pacific Ocean region (Figures 3, Figure 4, Figure 5, and Figure 6). North Maluku, the Philippines, and West Papua are included in the area around the Wallace line, where the imaginary line groups flora and fauna, which is a transition between Asia and Australia (Woelkerling, 1990; Waryono, 2001).

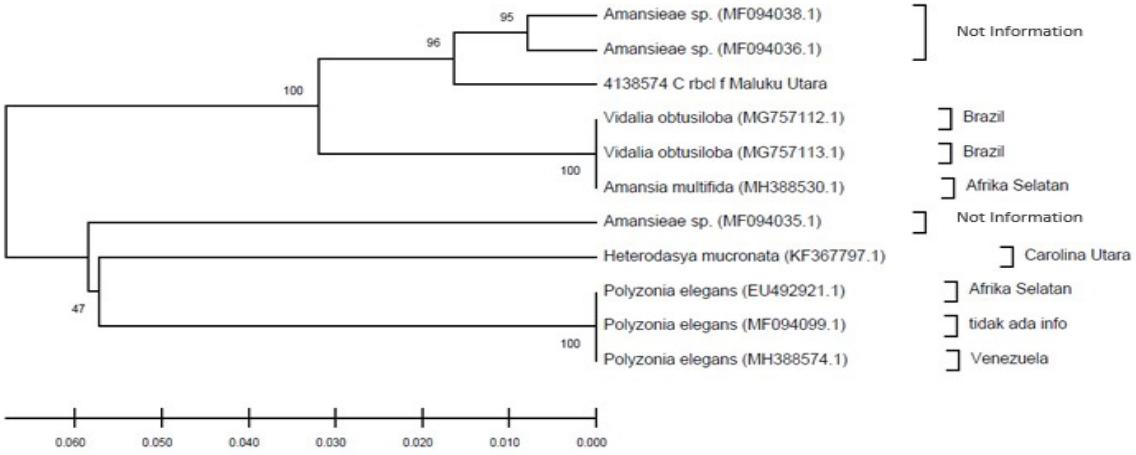


**Figure 4.** Phylogenetic tree of rbcL gene of group C using UPGMA method

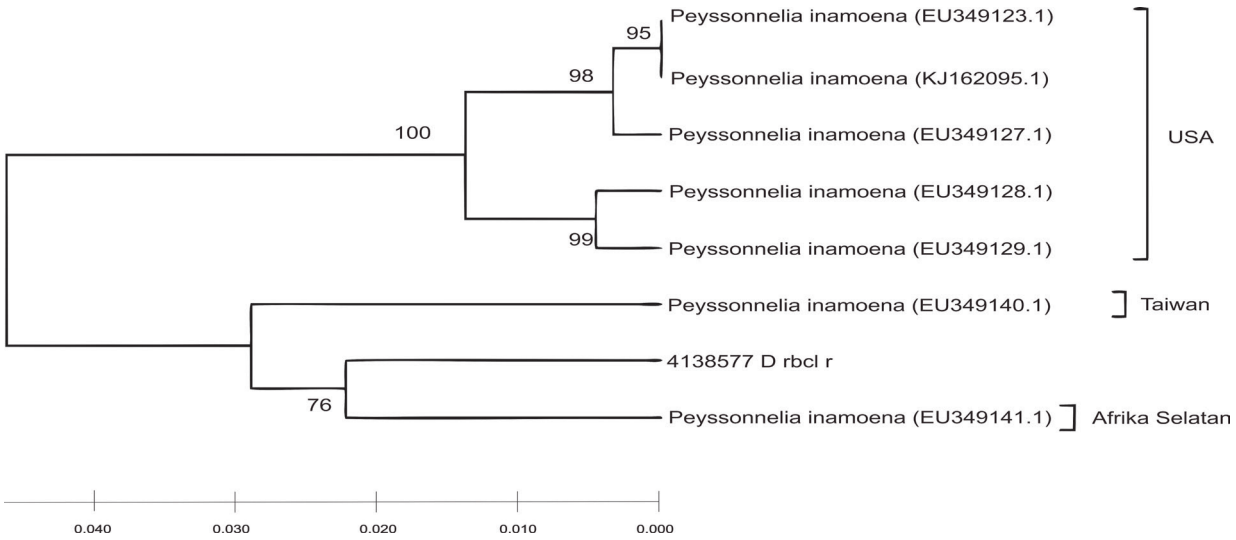


**Figure 5**. Phylogenetic tree of rbcL gene of group D using UPGMA method

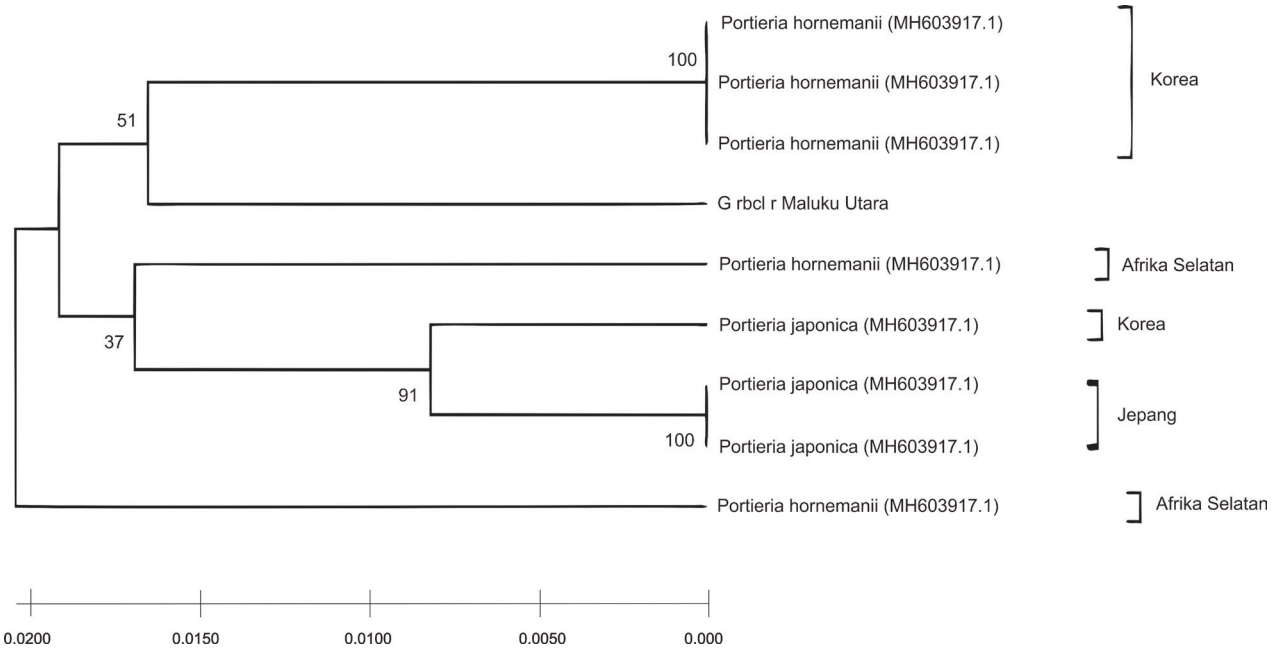


**Figure 6.** Phylogenetic tree of the sample group of rbcL gene using the UPGMA method

The phylogenetic reconstruction was supported by the results of genetic distance analysis (Table 2). The B_rbcL sample result from Ternate Island against sequences from Raja Ampat, Philippines, Australia, and Hawaii were 0.009, 0.012, 0.056-0.087, and 0.012-0.036, respectively. This value is considered low, so the genetic distance is very close. Genetic distance is declared close if the value ranges from 0.010 to 0.099, moderate if the value ranges from 0.100-0.990, and far if the value ranges from 1-2. Two or more individuals are said to have genetic closeness if the resulting genetic distance value is not more than 0.1 (Nei and Kumar, 2000). The closeness of the genetic distance shows the close genetic relationship between the sequences which is characterized by the least variation or difference in base pairs between the sequences used. Akbar and Aris (2018) genetic connectivity show the closeness between populations. The low genetic distance value indicates the closeness of the kinship relationship (Achmad *et al.*, 2019). It can be said that the sequence has a low level of diversity. A high bootstrap value of >70 indicates a reliability in data set of constructed phylogenetic (Pasaribu *et al.*, 2023).

The phylogenetic tree of the rbcL gene in sample group C (Figure 3) showed that the B_rbcL sample from Ternate Island is in the same clade as *Amansieae* sp. (MF094038.1 and MF094036.1), but the origin of the database is unknown. Another clade shows that the species of *Amansia multifida* is in the same group as the species of *Vidalia obtusiloba*. Amnesia and Vidalia are genera that are in the same tribe namely Amansia (Schmitz and Hauptfleisch, 1897), which means that they are evolutionarily related, but in the phylogenetic tree analysis based on the rbcL gene, it is not good enough to separate the two genera. Research on the genetic identification of the Amansieae has not been done much, so the limited availability of the database in the Genbank has caused the analysis of the kinship of the Amansieae in Indonesia to be minimal. The genetic distance of the C_rbcL samples from Ternate Island to the sequences from NCBI was quite varied (Table 3). The genetic distance of the North Maluku C_rbcL sample is very low against the *Amansieae* sp. (MF094036.1 and MF094038.1) and Vidalia obtusiloba (MG757113.1), namely 0.032-0.034 and 0.080, respectively. Meanwhile, the genetic distance to Polyzonia Elegans *Heterodasya mucronata* is moderate, with a range of 0.150-0.160.

The phylogenetic tree of the rbcL gene in sample group D (Figure 5) showed that the D_rbcL sample from Ternate Island is in the same clade as *Peyssonnelia* sp. originating from South Africa. Besides having close kinship, *Peyssonnelia* sp. it also has a high similarity value to the sample sequence of D_rcbL in Ternate Island (95.54%). Even though they are geographically far apart, this kinship is possible because of the similarity of genetic and environmental characteristics. Similarities or environmental disturbances can maintain and disrupt genetic quality, although population genetic

**Table 3.** The genetic distance of the Ternate Island C rbcL sample with the NCBI database

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| C_rbcl_f_North_Maluku | | | | | | | | | | | |
| *Polyzonia elegans* (EU492921.1) | 0.151 | | | | | | | | | | |
| *Polyzonia elegans* (MF094099.1) | 0.151 | 0.000 | | | | | | | | | |
| *Polyzonia elegans* (MH388574.1) | 0.151 | 0.000 | 0.000 | | | | | | | | |
| *Heterodasya mucronata* (KF367797.1) | 0.160 | 0.114 | 0.114 | 0.114 | | | | | | | |
| *Vidalia obtusiloba* (MG757112.1) | 0.080 | 0.136 | 0.136 | 0.136 | 0.144 | | | | | | |
| *Vidalia obtusiloba* (MG757113.1) | 0.080 | 0.136 | 0.136 | 0.136 | 0.144 | 0.000 | | | | | |
| *Amansia multifida* (MH388530.1) | 0.080 | 0.136 | 0.136 | 0.136 | 0.144 | 0.000 | 0.000 | | | | |
| *Amansieae* sp. (MF094035.1) | 0.150 | 0.115 | 0.115 | 0.115 | 0.123 | 0.124 | 0.124 | 0.124 | | | |
| *Amansieae* sp. (MF094038.1) | 0.034 | 0.135 | 0.135 | 0.135 | 0.130 | 0.056 | 0.056 | 0.056 | 0.124 | | |
| *Amansieae* sp. (MF094036.1) | 0.032 | 0.125 | 0.125 | 0.125 | 0.128 | 0.056 | 0.056 | 0.056 | 0.126 | 0.016 | |

flows continue to flow at great geographic distances (Yusron, 2005; Kristanto and Kusrini, 2007; Leatemia *et al*., 2021). Factors that affect the growth of marine macroalgae include substrate salinity, nutrients both from the substrate and water mass, waves, currents, depth, and light intensity (Waryono, 2001). There are 115 species names from the genus *Peyssonnelia* on Algaebase.org. Morphologically, the genus *Peyssonnelia* has also been identified in various water areas in Indonesia (Thenu, 1997; Sukiman *et al*., 2014; Aziz and Chasani, 2020).

The genetic distance shown by the D_rbcL sample in Ternate Island to the reference sequence was relatively small, i.e., 0.045-0.098. This shows that there is a low difference in the value of variation for the species that have been recorded in the NCBI database. *Peyssonnelia* is a genus of the family *Peyssonneliaceae* (Decaisne, 1841) that has the most species members, namely 88 species (algaebase.org), compared to other genera. The wide distribution area, influenced by supportive environmental factors, causes the genus to be found in various water areas.

The rbcL gene phylogenetic tree sample of group G (Figure 6) showed that the G_rbcL sample from Ternate Island was in the same clade as the Korean species *Portieria hornemannii*. This shows that evolutionarily, Ternate Island species have a close relationship with Korean species but based on the low bootstrap value (51) of the data used in the reconstruction of this phylogenetic tree, it still needs to be reconsidered.

The genetic distance of the G_rbcL sample to the reference was quite low, ranging from 0.031-0.046, indicating the low level of gene diversity contained in the Portieria data used. This is slightly different from other studies that have been conducted (Ji *et al*., 2008; Leliaert *et al*., 2018). Portieria is used as a model for marine algae to understand the evolutionary processes that result in the biogeographical patterns of seaweeds. As a result, the highest Portieria species diversity is found in the Indo-Malaysian Archipelago (IMA). With various supporting data, it is concluded that the long geological history played an important role in shaping the Porteria diversity in the region. The foregoing findings are very informative and coupled with our phylogenetic reconstruction.

Identification of red algae based on rbcL as DNA marker in this study found four species, namely B_rbcL sample was identical to *Gibsmithia hawaiiensis* (98.65%), C_rbcL sample was identical to *Amansieae* sp. (91.50%), D_rbcL sample was identical to *Peyssonnelia* sp. (95.54%), and G_rbcL sample was identical to *Portieria hornemanniI* (96.15%). The phylogenetic tree analysis using UPGMA method can also group species that have evolutionary closeness with a relatively high bootstrap value. Thus, it can be concluded that rbcL gene is quite effective in identifying red algae on Ternate Island, North Maluku.

## 4. Conclusion

Red algae in the waters of Ternate Island identified similarity of >90%. The rbcL gene can be used for molecular identification of seaweed. This can be seen from a high degree of accuracy, indicating that rbcl gene can also be used to see evolutionary processes, genetic diversity, and kinship. Phylogenetics explains that red algae species from Ternate Island have an affinity with other Asian and African regions. Indications of kinship occur through the phenomenon of global flows, but the accuracy of this hypothesis can only be determined if a rapid population study is carried out.

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## Authors' Contributors

The planning, research, analysis, and writing of the manuscript were all done by the writers. M Janib Achmad and Nebuchadnezzar Akbar all contributed thoughts to the study, analysis, drafting, and completion of the manuscripts. Data on alga was gathered by Firdaut Ismail and Rustam E Paembonan. Ardan Samman made maps, tabulated data and added discussion. Dondy Arafat and Beginer Subhan added literature, correcting words, and writing errors in numbers and letters.

## Conflict of Interest

The authors declare that they have no conflict of interest.

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