

PHYLOGENETICS ANALYSIS OF MARINE AND COASTAL SPECIES USING 18S rRNA SEQUENCE

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ABSTRACT

Phylogenetics analysis using 18S rRNA sequence was done on some marine and coastal organisms that have been used as traditional medicine in Indonesia. Proper and accurate identification of coastal and marine organisms producing bioactive compound is important to support drug discovery. Ribosomal RNA has been described as one of most useful and most used for molecular chronometer. In this experiment a part of 18S rRNA gen was amplified using PCR employing 300F and 1400R primers. PCR product then was cloned using pGEM-T vector (Promega) and transformed into *Escherichia coli*. The recombinant *Escherichia coli* were sequenced by applying universal primer and internal primer. The alignment of sequenced product and phylogenetic analysis indicated that sample 1 closely related to *Chaetomorpha crassa*, sample 2 closely related to *Ptilophora pinnatifida*, sample 3 closely related to *Flagellaria indica*, and sample 4 closely related to *Wallemia ichthyophaga*.

Keywords: phylogenetic analysis, 18S rRNA sequence.

ANALISIS FILOGENETIK SPESIES LAUT DAN PESISIR PANTAI MENGGUNAKAN URUTAN 18S rRNA

ABSTRAK

Analisis filogenetik dengan menggunakan urutan nukleotida 18S rRNA telah dilakukan terhadap beberapa organisme laut dan pesisir pantai yang biasa digunakan sebagai obat tradisional di Indonesia. Identifikasi yang tepat dan akurat perlu dilakukan terhadap organisme laut dan pesisir pantai yang memproduksi senyawa bioaktif yang penting untuk penemuan obat baru. RNA ribosom merupakan kronometer molekul yang paling bermanfaat dan paling sering digunakan. Pada penelitian ini bagian dari gen 18S rRNA di amplifikasi menggunakan sepasang primer 300F dan 1400R. Produk PCR kemudian dikloning menggunakan vektor pGEM-T (Promega) dan ditransformasi ke *Escherichia coli*. DNA plasmid rekombinan disekuensing menggunakan primer universal dan primer internal. Hasil penjajaran urutan nukleotida dan analisis filogenetik menunjukkan

bahwa sampel 1 dekat hubungannya dengan *Chaetomorpha crassa*, sampel 2 dekat hubungannya dengan *Ptilophora pinnatifida*, sampel 3 dekat hubungannya dengan *Flagellaria indica*, dan sampel 4 dekat hubungannya dengan *Wallemia ichthyophaga*.

Kata kunci: analisis filogenetik, 18S rRNA.

INTRODUCTION

Nature has continuously provided mankind with a broad and structurally diverse arsenal of pharmacologically and nutraceutically active compounds that continue to be utilised as health food and highly effective drugs to prevent and combat a multitude of deadly diseases or as lead structures for the development of novel synthetically derived drugs that mirror their models from nature (Kingston & Newman, 2002). Ethnically, marine organisms have been used to prevent and cure various diseases especially in Indonesia and other Asian countries.

Indonesian ethnic groups have been using various marine organisms as traditional medicine for decades. Coastal and small island communities consume various coastal and marine organisms to maintain strength and healthy. Modern technology has approved that marine organisms contained various useful pharmaceutical compounds. Those organisms are possible to be used as sources of pharmaceutical or nutraceutical (Balzarini *et al.* 2001; Hilbe *et al.* 2001; Sotomayor *et al.* 2002).

Proper and accurate identification of a coastal and marine organisms producing bioactive compound is certainly important to support drug discovery. To date, more than 15,000 marine natural products has been reported in Marinlit data base. By knowing the species name, possible bioactive compound can be assessed via this data base. This opportunity surely shortens the discovery time and increase the chance to find a novel compound. In addition, identification of producer organisms is also essential for developing coastal and marine culture to secure the long term supply of biomass to sustain marine pharmaceuticals and nutraceuticals production.

Ribosomal RNA (rRNA) has been described as one of the most useful and most used of the molecular chronometer. It shows a high degree of functional consistency that evolves in clock like manner. The bases evolve at different rates in each domain of the molecule, so a wide range of phylogenetic relationship can be inferred from their sequence divergence. The small subunit rRNA is the most used of the ribosomal RNA. Its length of 1600-2000 bases provides enough information to reliably infer evolutionary relationship from sequence comparison, whilst its slow rate of evolution allows it to be used in universal phylogenetic trees (Van der Peer *et al.*, 1993; Baker *et al.*, 2001).

Molecular technique based on 18s rRNA gene sequencing is an identification method of organism that is relatively new and developed since 1996. In Indonesia,

however, this method is hardly used as a tool to identify organisms. Development of this technique especially in Indonesia will contribute significantly to the science and technology development regarding the biodiversity of marine resources. Applying this molecular technique for biodiversity identification will enhance the chance of Indonesian scientist to contribute in International scientific journal as molecular techniques is the most accurate and acceptable method. Previous investigation showed that molecular identification lead to the finding of new species of tropical marine biodiversity.

This study described the genetic diversity of some Indonesian marine organisms using 18S rRNA sequences. The samples consist of marine organisms used as traditional medicine by various local communities in Indonesia including Buton, Mentawai, Ternate and Seribu Island. The long term objective of this research is to develop a culture of coastal and marine organisms used as traditional medicine to conserve marine biodiversity and support the development of marine nutraceutical industry in Indonesia.

MATERIALS AND METHODS

Samples

Coastal and marine organisms were provided by the Department of Aquatic product technology, Faculty of Fisheries and Marine Science, Bogor Agricultural University (IPB). The samples consisted of marine organisms used as traditional medicine in Mentawai, Ternate and Seribu Island that have been proven containing bioactive compound. The bioactivity test was done by the Department of Aquatic Product Technology, IPB. The samples included two seaweed and two coastal plants. The list of samples and their biological activity are presented in table 1.

Table 1. List of coastal and marine samples.

No.	Sample code	Local name	Common name	Biological activity
1.	Sample-1	Rumput rambut	Seaweed	Antioxidant and antimicrobes
2.	Sample-2	Rumput merah	Seaweed	Antioxidant and cytotoxic
3.	Sample-3	Seksekoat	Coastal plant	Antioxidant and cytotoxic
4.	Sample-4	Kangkung laut	Coastal plant	Antioxidant and cytotoxic

Samples were collected in bottles containing ethanol (p.a). Samples were washed by using aquadest and then air dried. Furthermore, they were crushed by applying liquid nitrogen. In some cases, glass bead was added to break the tissue more efficiently.

DNA Extraction

The DNA genomes were extracted using six different methods; (1) Standard method by adding SDS 20% (Jones *et.al*, 1997); (2) Standard method by adding CTAB (Wilkie *et.al*, 1997), (3) DNA isolation protocol for red seaweed (Wittier *et*.

al., 2000); (4) DNeasy Plant Mini kit (Qiagen); (5) Wizard DNA genomic purification kit (Promega); (6) Nucleon Phytopure kit (Amersham). Isolated DNA was run using 1% agarose gel electrophoresis.

18S rRNA Gene Amplification and Cloning

Approximately 100 ng genomic DNA was amplified by PCR in 50 µl solution consisted of 2.5 U Taq DNA polymerase (MDBio), 3 mM MgCl₂, and eukaryote 18S rRNA gene-specific primers 300F and 1400R (Hinkle, 1997). The PCR cycle includes initial denaturing 5 min at 95°C; 30 cycles; 1 min at 95°C; 1 min at 35°C; 2 min at 70°C followed by a single period at 70°C for 10 min. 1.4 kb PCR products were run on 1% agarose gel electrophoreses and purified using GFX™ Gel Band Purification kit (Amersham). The purified PCR products were ligated into a p-GEM-T (Promega) vector and transformed into competent cell JM 109 *Escherichia coli*. White colonies were selected for plasmid recombinant characterization.

Sequencing and Sequence Analysis

Transformed cells were purified using Qiagen mini-prep kit and sequenced initially with PCR primers 300F and 1400R. Sequencing was carried out using dideoxy Sanger Dye-terminator method. Sequence was aligned using Seqman in Lasergene and novel operational taxonomic unit (OTUs) were sequenced with the internal sequencing primer and primers specific to the pGEM-T (Promega) vector, SP6 and T7. The sequence fragments were assembled and edited in Lasergene Seqman and the consensus sequences were compared with other 18S rRNA genes in the GenBank using NCBI BLAST. Each OTU sequence was aligned with closely related sequences identified from BLAST search and representative in-group and out-group taxa using Clustal in Lasergene MegAlign. Phylogenetic tree was inferred using Clustal method with weighted residue weight table in Lasergene MegAlign.

RESULTS AND DISCUSSION

DNA Genome Extraction

Genomic DNA was isolated using six different protocols. The results indicated that the third protocol (Wittier *et. al.*, 2000) was the most reproducible one. Among four samples of coastal and marine organisms, all samples were successfully isolated using method 1-4. During DNA extraction, it was found that coastal plant has very hard cell wall. This conditions required hard physical treatment that might cause chemical damage influencing extraction process (Millan, 1992). The metabolites of coastal and marine organisms such as phenolic compounds, tannin, polyphenol, and polysaccharide could also affect PCR process. In addition, co-isolation of hydrocolloid might influence the DNA polymerase activity. This found during DNA isolation using the first protocol, the isolated DNA was viscous so it was not able to be amplified. However, genomic DNA extraction methods still need to be optimized for other samples.

The most reproducible protocol which is the second one is the modification of terrestrial plant DNA isolation technique. Modification were made to minimize polysaccharide co-isolation (Wattier *et.al.*, 2000). Unfortunately, for sample 3 (seksekoat) this technique was not successful. It produced green color that influenced PCR process. The genomic DNA of this sample then isolated using DNeasy Plant Mini kit (Qiagen).

PCR and Cloning

1.4 kb fragment PCR product is presented in Figure 1. This result showed that the 300F and 1400R primers that was used to amplified 18S rRNA gene of terrestrial plant was suitable to amplified marine plant.

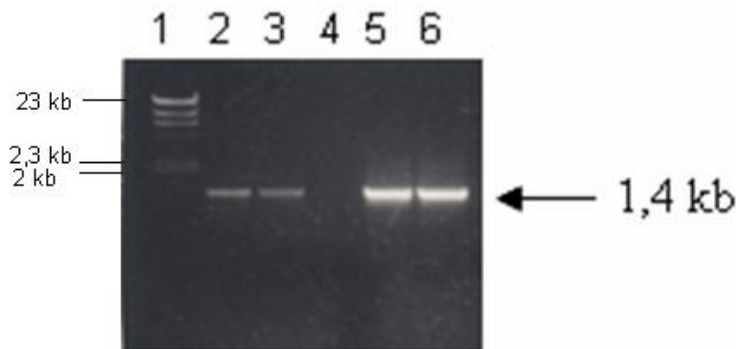


Figure 1. PCR products of 1,4 kb 18S rRNA gene; line (1) DNA λ /HindIII; (2) Sample 3; (3) Sample 4; (4) negative control; (5) Sample 1; (6) Sample 2.

All genomic DNA was give positive PCR result using specific primer for 18S rRNA. All positive PCR products then was purified and inserted to pGem-T vector and cloned in *E. coli*. All samples was success cloned in *E. coli*. Table 2 presented result of PCR and cloning of all samples.

Tabel 2. Genomic DNA extraction, PCR and cloning result.

No.	Sample code	Local name	Genomic DNA isolation (different methods)	PCR	Cloning
1.	Sample-1	Rumput rambut	Positive (method 1, 2 3)	Positive	Positive
2.	Sample-2	Rumput merah	Positive (method 3)	Positive	Positive
3.	Sample-3	Seksekoat	Positive (method 4)	Positive	Positive
4.	Sample-4	Kangkung laut	Positive (method 2,3)	Positive	Positive

Sequence Analysis

Sample-1

Morphologically, the sample-1 (Figure 2) was identified as *Chaetomorpha sp.* It is marine algae intake by marine mollusk *Discodoris sp* living in coastal area of Buton. The mollusk was used traditionally as a prodisiax agent. The alga was found to contain antimicrobial and antioxidant compounds. The identification of these bioactive compounds is in progress. In addition Lee *et al.*, (2004) reported that sulfate polysaccharide from green algae including *Chaetomorpha crassa* showed potent anti-HSV-1 activities with IC_{50} of 0.38 - 8.5 $\mu\text{g}/\text{mL}$. These results revealed that some sulfated polysaccharides from green algae could be potential candidates of antiviral agents which might act on different stages in the virus replication cycle.

Phylogenetic tree analysis of sample 1, by using Clustal method with weighted residue weight table, showed that it is closely related to *Chaetomorpha crassa* (Figure 3). It is green algae with taxonomic group of *Eukaryota-Viriplantae-Chlorophyta-Ulvophyceae-Cladophorales-Cladophoraceae-Chaetomorpha*. The molecular identification showed in agreement to morphological identification. It indicated that the genomic DNA isolation method and PCR process are suitable for this specific sample.



Figure 2. Sample-1 and reference photo of the *Chaetomorpha crassa*.

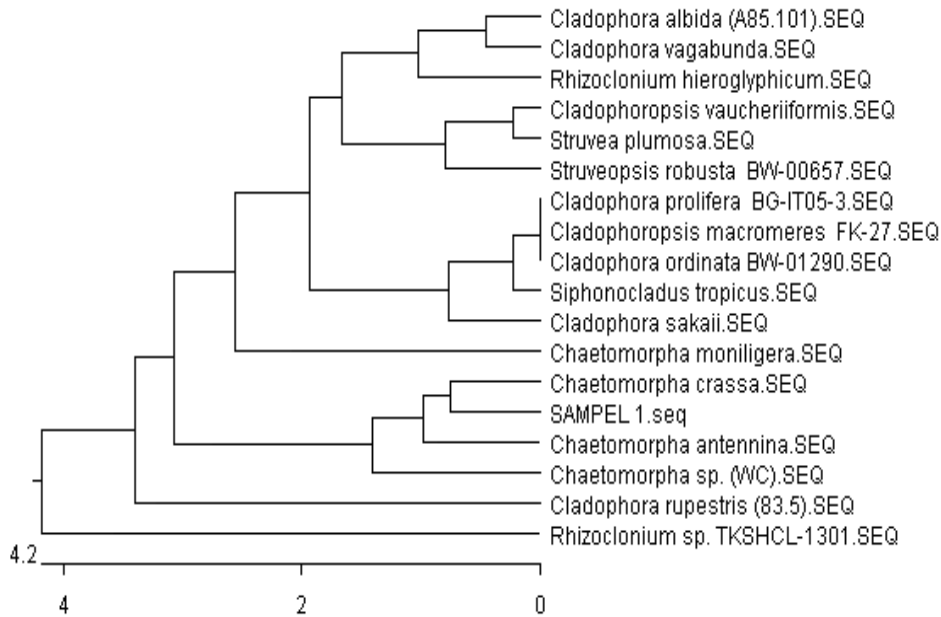


Figure 3. Phylogenetic tree using Clustal method, based on alignment of 1200-bp 18S rRNA genes sequence fragment of sample-1.

Sample-2

Sample-2 is also marine algae having local name as rumput merah. The alga was found to contain antioxidant and cytotoxic compound against A2780 cancer cell line. And the identification of these bioactive compounds is in progress. Morphological identification was unable to name its genus. Phylogenetic tree analysis of sample-2, using Clustal method with weighted residue weight table, showed that it is closely related to *Ptilophora pinnatifida* (Figure 4). *Ptilophora pinnatifida* is red algae with taxonomical group: *Eukaryota-Rhodophyta-Florideophyceae-Gelidiales-Gelidiaceae-Ptilophora*. The sample-2 and reference photo are presented in Figure 5.

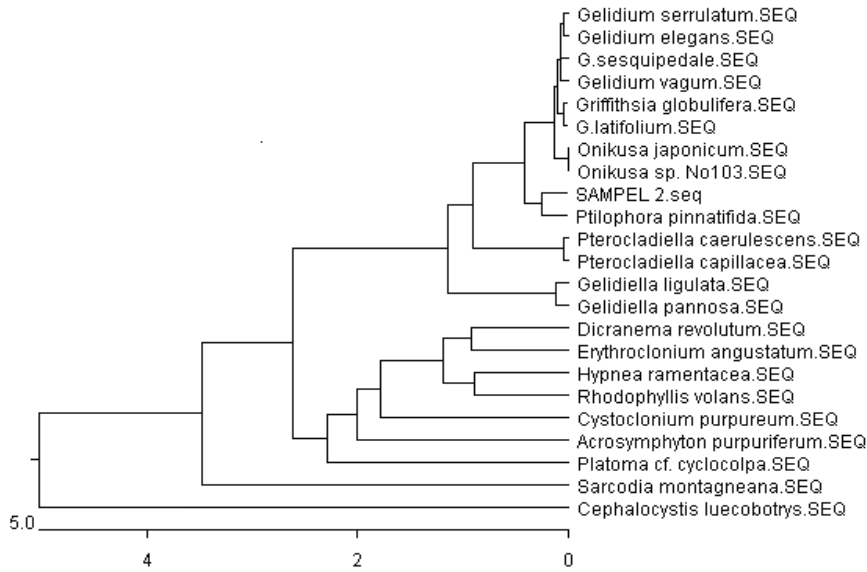


Figure 4. Phylogenetic tree using Clustal method, based on alignment of 1200-bp 18S rRNA genes sequence fragment of sample-2.



Figure 5. Sample-2 and reference photo of *Ptilophora pinnatifida*

Sample-3

Sample-3 is named locally as seksekoat. It is coastal plant used as traditional medicine in Mentawai. The bioassay showed that the plant contained antioxidant and cytotoxic compound against A2780 cancer cell line. The identification of bioactive compound is in progress.

Phylogenetic tree analysis of sample-3, using Clustal method with weighted residue weight table, showed that it is closely related to *Flagellaria indica* (Figure 6). The sample-3 and reference photo are presented in Figure 7. *Flagellaria indica* is a liane species which climbs on trees using crochets at the top of the leaf or crawls on the ground, with taxonomical group of *Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta; Spermatophyta; Magnoliophyta; Liliopsida; Poales; Flagellariaceae; Flagellaria*.



Figure 6. Phylogenetic tree using Clustal method, based on alignment of 1200-bp 18S rRNA genes sequence fragment of sample-3.

Flagellaria indica is green coastal plant that can also grow up to 1500 m above sea level. It is found in Asia including Indonesia, Africa, Australia and Pacific. Asians Regional Centre for Biodiversity Conservation reported that *Flagellaria indica* is used as hair tonic/alopecia. It is also used as antipyretic agent and appetizer in Indonesia and Thailand.

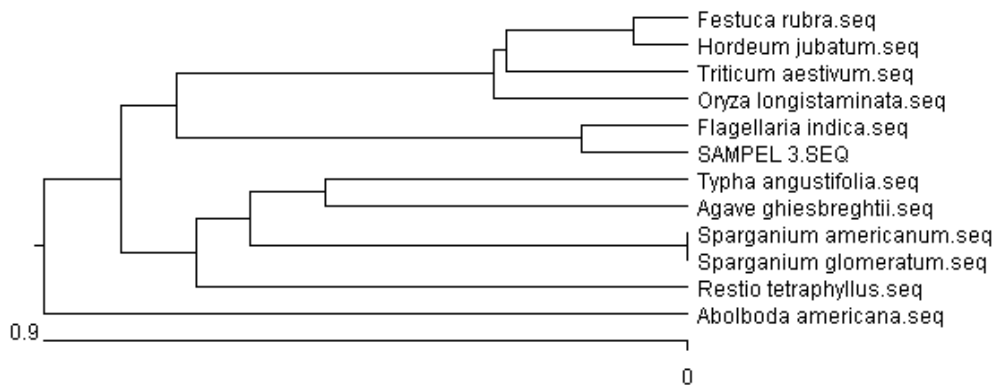


Figure 7. Sample-3 and reference photo of *Flagellaria indica*.

Sample-4

Sample-4 is named locally as kankung laut. It is coastal plant used as traditional medicine in Seribu Island and Ternate. The bioassay showed that the plant contained antioxidant and cytotoxic compound against A2780 cancer cell line. The identification of bioactive compound is in progress. Morphological identification showed that the plant belonged to the species of *Ipomea sp* (Figure 8).

Phylogenetic tree analysis of sample-4, using Clustal method with weighted residue weight table, showed that it is closely related to *Wallemia ichthyophaga* (Figure 9).



Figure 8. *Ipomea sp.*

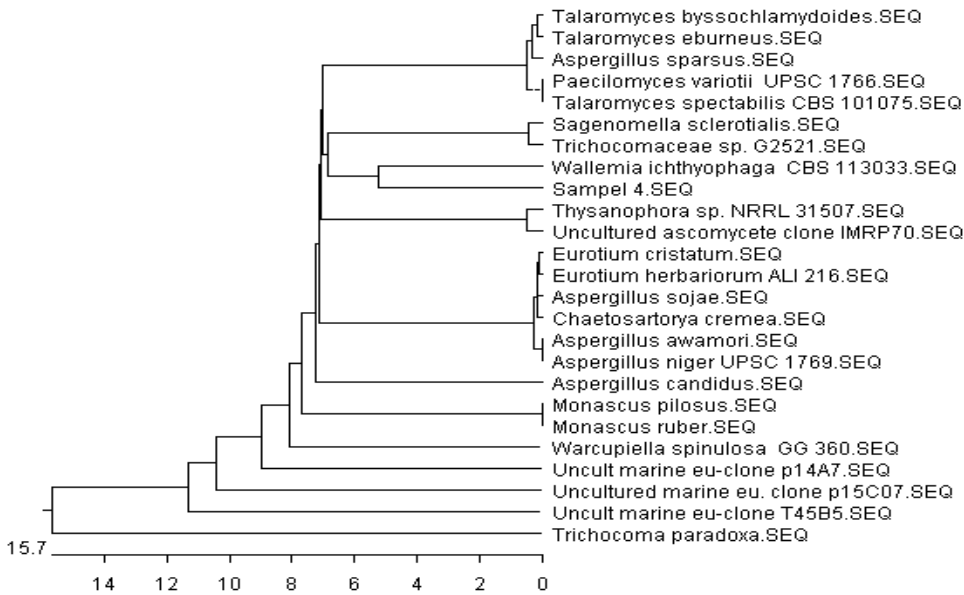


Figure 9. Phylogenetic tree using Clustal method, based on alignment of 1200-bp 18s rRNA genes sequence fragment.

Molecular identification indicated that the isolated DNA could be the symbiotic microorganisms of *Ipomoea sp. Wallemia ichthyophaga* is an eukaryota with the taxonomical group of fungi; *Ascomycota; mitosporic Ascomycota; Wallemia*. Genus *Wallemia* comprises xerophilic species. Based on parentheses ultra structure it has been linked to the *Filobasidiales* (basidiomycetes). Species show a unique type of conidiogenesis, including basauxic development of fertile hyphae, segregation of conidial units more or less basipetally, and disarticulation of conidial units into mostly four arthrospore-like conidia. *Wallemia* is found in air, soil, dried food (causing spoilage) and salty area. It can be isolated from hyper saline water (Zalar *et al.*, 2005)

CONCLUSION

Molecular technique based on 18s rRNA gene sequencing was successfully applied to identified coastal and marine organisms used as traditional medicine in Indonesia. This method is more sensitive than morphological identification, and therefore could be applied to identify organism that difficult to identification morphologically. In this research, all samples were successfully identified molecularly. Furthermore, due to different type of cell wall, genomic DNA extraction method for each samples need to be modified.

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