

Diversity of Tropical Marine Macroalgae from Coastal Area of Sayang Heulang, West Java Indonesia

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Genetic diversity of nine marine macroalgae from coastal area of Sayang Heulang were assessed based on phenotypic and ribotyping analysis. Morphology of the macroalgae were classified based on the color of the organism such as red (4 samples), brown (3 samples) and green (2 samples). Genomic DNA from the organisms were isolated using CTAB method with slight modification. The 18S rRNA gene amplified by PCR method using standar primers for V4 region of 18S rRNA gene. The amplicons were sequenced and analysed. The results showed that all the sequences are closed to V4 region of 18S rRNA gene. Comparison among the nine sequences showed a variation of homology. The best homology was shown by the sequence of SB and TC (99,1%), both are brown color macroalgae. The lowest homology was shown by GU (red color) and TC (brown color) with the homology at around 64,6%. Further analysis by comparing among the same color and the cross color showed that the same color does not show genetically close each other. Phylogenic analysis showed that the three brown colors are closed each other, however the rest are seen different and far away each other.


Keywords: tropical macroalgae, ribotyping analysis, V4 region of 18S rRNA gene.

Macroalgae is a low marine plant classified as algae or more well-known as plant with thallus. Basically, macroalgae contains just root, stem and leaf which similar to higher plant. Macroalgae implified in group of Thallophyta adhered to substrate using holdfast. The organism contains chlorophyll for photosynthesis process and few pigments. According to Jiang, *et al* (2006), macroalgae, synonym of seaweed is collective term that refers to series of non-phylogenetic, multi cellular, macroscopic and eukaryotic organism.

A first classification of seaweeds are given by their ability to absorp light for the purpose of photosynthesis. Therefore, based on the parameter, the organisms are grouped as microalgae or marine microalgae (kelp or seaweed) in red algae (or rhodophytae, rodophycae), brown algae (or pheophytae, pheophycae) and green algae (or chlorophytes, chlorophycae) (Sulaiman, *et al*, 2015)

Littoral benthic organism may serve as useful tools to detect environmental changes

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since they integrate the environmental factors over time (Fredriksen, *et al.*, 2015). Littoral species are directly exposed to change in air and sea surface temperatures, as well as changes in ice cover and freshwater input and the littoral may thereby be considered as an “early warning habitat” of a shifting climate (Høglund, *et al.*, 2014). A good baseline understanding of species diversity is therefore important from a management perspective (Fredriksen, *et al.*, 2015). One of the main applications for analysis of genetic variation from marine species is genetic conversation. Marine ecosystems are potentially in a high-risk category, with estimates of approximately 10,000 species being transported daily, yet marine research lagged behind investigations of terrestrial and freshwater ecosystem (Frankham, *et al.*, 2002). The genetic pattern of given species or stock may be disturbed or totally lost even by small and localized disturbance of the environment due to man induced activities like over exploitation, environmental degradation etc. Individual with high level of genetic variation have greater prospect in aquaculture in terms of higher growth rate, developmental stability, viability and resistance to environmental stress and disease (Prasad, *et al.*, 2007)

Molecular systematic in seaweeds and other organisms had rapidly progressed with the use of PCR coupled by sequencing methods (Akhmaloka, *et al.*, 2006; Safika *et al.* 2013; Sun *et al.*, 2016). This molecular approach has been effective in addressing many phylogenetic questions that had not been solved using phenotypic characters (Sun, *et al.*, 2016). The nuclear 18S rRNA gene has been used extensively at various taxonomic levels for type of macroalgae such as *Hildenbrandia* from genus of Rhodophyta in North America (Sherwood, *et al.*, 2000) and *Prasiolales* from genus of Chlorophyta in Canada (Sherwood *et al.*, 1999). Recently, few reports on taxonomy and phylogeny of macro algae are available in Indonesia, however all of the studies based on phenotypic characters (Anggraeni *et al.*, 2008). Here we report the identification and classification of Indonesian macroalgae based on ribotyping analysis. The study was focused on isolation of genomic DNA from fresh marine macroalgae, amplification of specific region of 18S rRNA

gene, followed by analysis the 18S rRNA gene sequences.

MATERIALS AND METHODS

Sampling and Morphology Identification

Sampling of macro algae has been carried out by purposive sampling method in certain coordinate point in Coastal Area of Sayang Heulang, West Java, Indonesia. The Selected macroalgae were weight and identify its phynotypic based on standard handbook of algae identification (Mckenzie *et al.*, 2003). The samples were stored on temperature -80 °C until DNA isolation.

DNA Isolation

DNA Isolation was carried out by using CTAB method (Philips, *et al.*, 2006). 100 mg of wet sample was cleaned and polished with liquid nitrogen in mortar by using pestle. This step was repeated three times. The sample was then mixed with 500 μ L extraction buffer and placed in sterile microtube 1.5 mL. The sample was incubated in water bath at 60 °C for 30 min. The microtube was centrifuged for 10 min in 8.000 g. The supernatant was moved to another sterile microtube and mixed with chloroform: isoamyl alcohol (24:1), then centrifuged again in same condition. The upper phase was collected into sterile microtube. The mixture was added by pure ice cold isopropyl alcohol in the same volume of aqueous phase, then mixed gently and incubated in freezer at -20 °C for 30 min. After incubation, the mixture was centrifuged and the pellet was collected. Furthermore the pellet was washed by 70% alcohol, re-centrifuged, removed aqueous phase and let the pellet dried (air dried) for overnight. Finally the pellet was added by 50 μ L TE buffer and tapped gently to dissolved.

DNA Amplification

Amplification of 18S rRNA gene fragment was conducted with RAPD-PCR method. The primer used was standard sequence for the VU region (Tables). Generally all RAPD-PCR process was carried out using composition of reaction mixture with total volume of 25 μ L. The PCR mix consists of 1 μ L DNA template, 10 μ M for each primer with total volume 5 μ L, 2.5 μ L of 2 mM dNTPs mixture, 2.5 μ L of PCR buffer 10X, 2 μ L of 25 mM MgCl₂, 1 unit of Tag DNA

polymerase and ddH₂O until total volume up to 25 μ L. RAPD-PCR process was run with Thermal Cycler Bio-Rad. Total cycles of RAPD-PCR was carried out for 36 times with pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec and extension in 72 °C for 1 min. Final extension was run for 10 min.

DNA Sequencing Analysis

DNA sequencing was done at Macrogen Inc., South Korea. The process involved Dye Terminator method (3'-dye labeled

dideoxynucleotide triphosphate) which consists of template preparation, sequencing reaction, PCR product purification and electrophoresis with scanning fluorescence.

Phylogenetic Analysis

Phylogenetic tree was constructed by DNA Baser v4.0.7 (licence: Demo) to view its contig, Notepad++ to observe, choose and join the contig analysis. BioEdit was used to edit sequence alignment and MEGA 6.06 to construct phylogeny tree (Tamura, *et al.*, 2011).

Table 1. Primers used for amplification of U4 region of 18S rRNA gene

Forward	5' – CAG CAG CCG CGG TAA TTC C-3'
Reverse	5' – CCC GTG TTG AGT CAA ATT AGG C-3'

Table 2. Percent similarity of the U4 region sequences among the samples. Brown samples (SP, TC and SB); Green samples (GM1 and UF); Red samples (GA, HSp, GV and GSp)

	SP	TC	SB	GM1	UF	GA	HSp	GV	GSp
SP	100								
TC	98.58								
SB	98.40	99.12							
GM1	97.86	98.04	98.58						
UF	74.25	74.48	75.51	75.58					
GA	67.03	67.28	68.43	68.93	67.11				
HSp	65.38	65.63	66.82	67.61	67.55	91.46			
GV	64.61	64.86	66.07	65.92	66.10	84.17	85.90		
GSp	67.84	68.08	69.23	69.56	68.86	86.55	88.88	95.82	100

Table 3. Gen bank accession number of nine samples. SC (Sample Code); AN (Accession Number)

No	SC	AN
1	GA	MG680736
2	GSp	MG680737
3	HSp	MG680738
4	SB	MG680739
5	SP	MG680740
6	TC	MG680741
7	UF	MG680742
8	GM1	MG680743
9	GV	MG462720

RESULTS AND DISCUSSION

Sampling and Classification

Nine macroalgae were successfully isolated from coastal area of Sayang Heulang. Morphological identification of the organisms were based on algae identification sheet (McKenzie, *et al.*, 2003). The macroalgae were classified as brown, green and red macroalgae. Brown macroalgae consisted of three samples coded as SB, SP, and TC. While green consisted two samples coded as UF and GM and last group was red macroalgae consisted four samples coded as GA, GV, GS and HS (data not shown).

Amplification of V4 Region of 18S rRNA gene

Isolation of total DNA from macroalgae was carried out using CTAB method. The DNA was seen on agarose gel before used for amplification of 18S rRNA gene fragment (data not shown). V4

region of 18S rRNA genes were amplified using a pair of primer (Table 1). The primer will amplify at around 650 bp long fragment. In V4 region of 18S rRNA gene (Hadziavdic, *et al.*, 2014). The result showed that single amplicons appear on agarose gel with the size at around 650 bp (Figure 1). This suggested that the primers amplified only the correct fragment of VU region of 18S rRNA gene.

Sequence and Ribotyping Analysis of The Amplicons and Analysis

All of (nine) amplicons were sequenced and analysed. Homological analysis showed that the amplicons appeared high homology to 18S rRNA gene. Comparison among the sequences of the amplicons showed variation of some homology (Table 2). The best homology was shown between SB and TC samples with the homology of 99%. Both TC and SB samples belong to brown macroalgae. In contrast, the less homology was

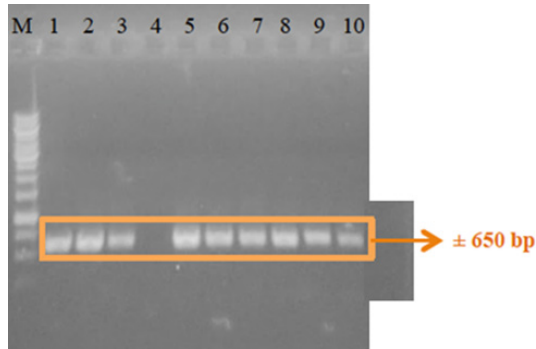


Fig. 1. Electropherogram of amplicons on agarose gel electrophoresis. M (Marker); 1-10 (member of sample)

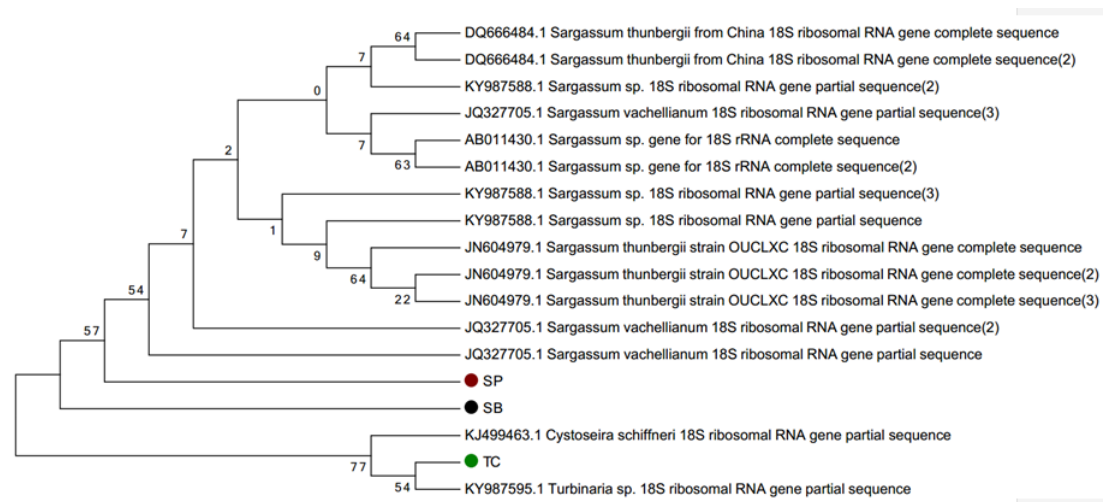


Fig. 2. Phylogenetic tree of brown samples SP, SB, and TC (samples of brown macroalgae)

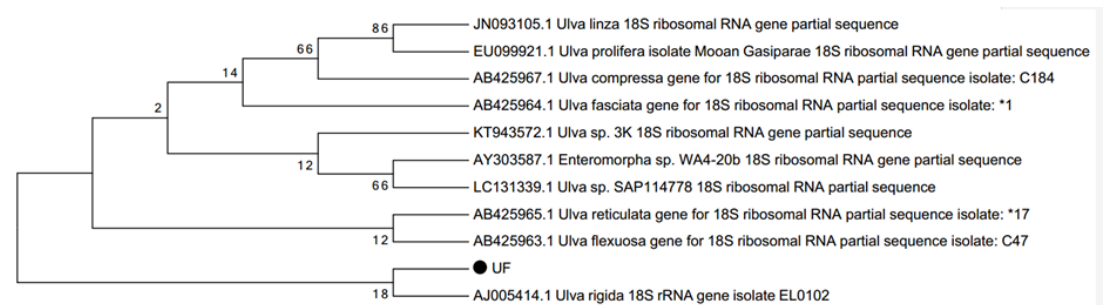


Fig. 3. Phylogenetic tree of green samples UF (sample of green macroalgae)

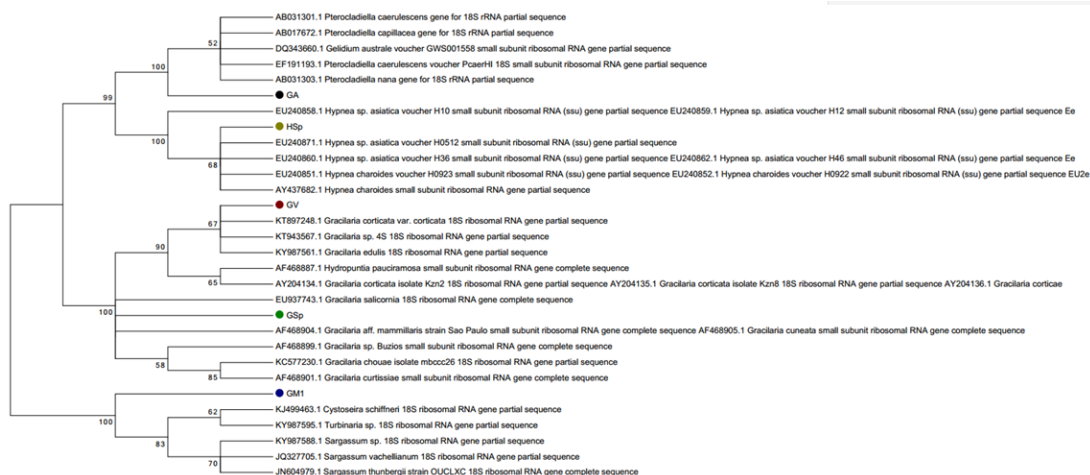


Fig. 4. Phylogenetic tree of red samples GA, HSp, GV, and GSp (red samples); GM1 (green sample)

shown by SP and GV samples with homology of 64,6%. SP sample is member of brown macroalgae meanwhile GV sample is member of the red color algae.

Further analysis by comparing the sequence among the same colors and cross color showed that the lowest homology for the same color was shown by GM1 and UT samples, both are green samples, with the similarity at around 75,5%. Moreover, the highest homology for different color was shown by SB and GM samples with the homology of 98,6%. These data suggest that the same color does not appear genetically close each other.

Phylogenetic Analysis of the Macroalgae

Phylogenetic analysis of each samples were constructed with other 18S rRNA gene fragment based on the best similarity of the sequences. The brown samples are clustered on one branch (Figure 2), where TC sample is close to *Turbinaria* sp (KY 987 595), meanwhile SP and SB samples are not seem branch to all others but close to *Sargassum*.

The phylogenetic of the green samples are a bit surprising. The UF sample clustered to the 18S rRNA gene of *Ulva* (Figure 3), meanwhile, GMI is close to *Cystoseira*, *Turbinaria* and *Sargassum* (Figure 4). The lost genes are belong to the brown color macroalgae.

Phylogenetic at the red samples showed that HSp, GV and GSp are very close to *Hypnea* and *Gracilaria* respectively (Figure 4). While GA is

seen out of the branch which is close to *Gelidium anstrack*, *Pterocladia*.. Based on the homology of the sequence GA is close to *Gelidium*. The 18S rRNA gene of the nine sequences were deposit in the genbank (Table 3).

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