Phylogeny and molecular identification of green macroalgae, *Ulva prolifera* (O. F. Müller, 1778) in Totok Bay, Maluku Sea, and Blongko waters, Sulawesi Sea, North Sulawesi, Indonesia

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Abstract. In this research, we provided a molecular identification of *Ulva prolifera* in Totok Bay (Maluku Sea) and Blongko waters (Sulawesi Sea), North Sulawesi, Indonesia, based on the genetic profile using plant/fungal DNA extraction kit. DNA was preserved at -20ºC for further analysis. The target DNA regions, namely rbcL, were amplified with universal DNA barcoding primers. The green macroalgae, *U. prolifera* was not confirmed as 100% similar with other *Ulva* species. The identification of this species is important, as it could be added into the vast taxonomic status of *U. prolifera*. Moreover, it serves as a basis of gene stock identification for coastal outbreak management of Indonesian macroalgae.
Key Words: seaweed, genetic, monophyletic, South Minahasa, Southeast Minahasa.

Introduction. *Ulva prolifera* (O. F. Müller, 1778) has synonym names such as *Enteromorpha clathrata, E. compressa, E. procera, E. prolifera, E. salina, E. simplex, Ulva compressa, U. enteromorpha, U. procera* and *U. simplex.* This species has light green thalli and dense mats of soft, delicate and hair-like hollow filaments. It is widely distributed in tropical regions, including the Philippines and Vietnam (Trono 1997), it is common along the coast of China and Japan (Trono 1998; Ohno & Takahashi 1988; Ohno et al 1999), but also in France (Ribier & Godineau 1984; Gayral & Cosson 1986; Caboic'h et al 1992). It can be found in the British Isles (Burrows 1991), Britain and Ireland (Hardy & Guiry 2006; Brodie et al 2007), east Asian countries (Ohno 1993; Park & Hwang 2011; Liu et al 2013), European Atlantic coasts (Pereira & Neto 2015), Blue Lagoon, Strait of Malacca, Malaysia (Asmida et al 2017), and Yellow Sea, China (Zang et al 2019). The green macroalgae, *U. prolifera* can generate green tides, creating serious environmental problems in the Yellow Sea, China (Liu et al 2016; Zang et al 2019).

Recently, *Ulva* sp. has been reported in Totok Bay and Blongko waters, Indonesia (Kepel et al 2018). The new record in North Sulawesi Province waters, Totok Bay (Maluku Sea) and Blongko waters (Sulawesi Sea), Indonesia, was based on molecular identification. Due to small-scale mining activities in the upstream that remove waste such as suspended matters, high turbidity and heavy metals enter the river and reach the estuary.of the Totok Bay. The sediment of Totok Bay contains higher arsenic concentrations than that of Blongko waters, 24 ppm and 2 ppm, respectively. Similar conditions occurred in *Ulva* sp. collected from Totok Bay waters, with 4.8 ppm of arsenic, and less than 0.5 ppm of arsenic in Blongko waters. In Totok Bay, other macroalgae were identified: green macroalgae *Halimeda opuntia* (Mantiri et al 2018), brown macroalgae *Padina australis* (Mantiri et al 2019a), and *Halimeda taenicola*, and *U. prolifera* (Mantiri et al 2019b). There are not many macroalgae that can live in Totok Bay. Only 5 species exist throughout the year. *Ulva* sp. is an algae that is able to live in Totok Bay compared to Blongko waters, where many species of macroalgae species live.

In recent publications there is no information about *U. prolifera* in Mantehage Island (Kepel et al 2019a) and Minahasa Peninsula, North Sulawesi Province (Kepel et al 2019b; Kepel et al 2020). This macroalgae is not found in Mantehage Island, although it is only a few nautical miles from the Minahasa Peninsula and this island is a part of Bunaken National Park. This present study aimed to prove the presence of *U. prolifera* by using phylogenetic analysis and molecular identification in Totok Bay and Blongko waters.

Material and Method

Sample collection. This study was conducted from June to October 2018. Samples of *U. prolifera* (Figure 1) were collected directly by hand. The research sites are Totok Bay (Maluku Sea), Southeast Minahasa Regency, and Blongko waters (Sulawesi Sea), South Minahasa Regency, North Sulawesi Province, Indonesia (Figure 2). Identified macroalgae were stored in proportional containers and labeled with classification information, location of discovery and collector. The determination of samples is based on the molecular identification.

Figure 1. Sample of *Ulva prolifera* from Totok Bay (A) and Blongko waters (B) in Minahasa Peninsula.

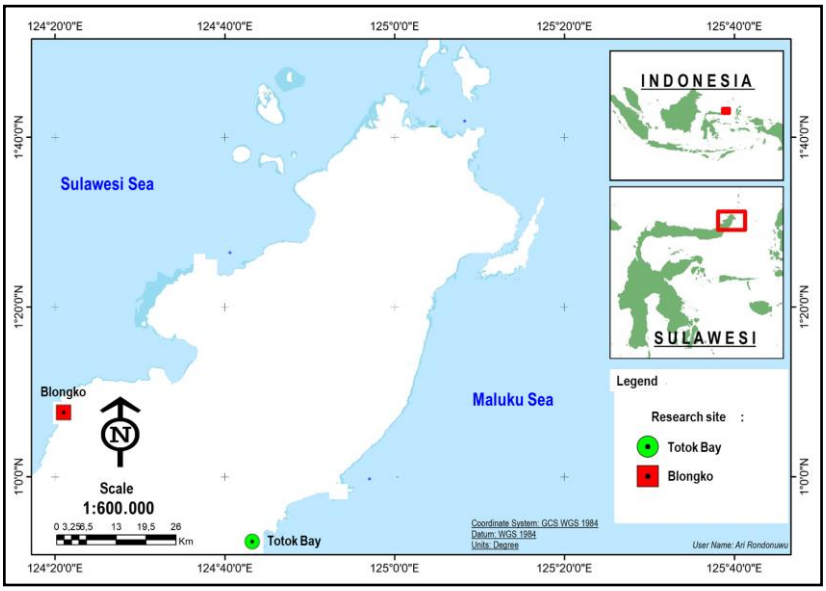

Figure 2. Sampling sites of *Ulva prolifera* in Totok Bay and Blongko waters.

DNA extraction, isolation, and amplification. Total genomic DNA of plant samples was extracted using Plant/Fungal DNA extraction kit (Norgen Bioteck, Canada) and DNA was preserved at -20ºC for further analysis. The targeted DNA regions, namely rbcL, were amplified with respective universal DNA barcoding primers (CBOL 2009). For the

rbcL gene, the following were used: rbcLa-F (5'-ATGTCACCACAAACAGAGACTAAAGC-3') and rbcLa-R (5-GTAAAATCAAGTCCACCRCG-3'). PCR was performed using a reaction mixture of a total volume of MyTaq™ HS Red Mix (Bioline) (PCR Kit), 20 µL 2x MyTaq™ HS Red Mix, 1.5 µL Primer Forward (10 µM), 1.5 µL Primer Reverse (10 µM), 15 µL MilliQ Water, and 2 µL DNA template. The PCR conditions were as follows: 1 cycle - 95ºC for 3 min, 35 cycles - 95ºC for 30 sec, 50ºC for 30 sec, and 72ºC for 30 sec, and 1 cycle - 72ºC for 1 min. Amplified PCR products of rbcL were checked on a 1.5% agarose gel electrophoresis for the respective bands (600 and 700 bps), which were sent to Malaysia's First Base Laboratory (Kuala Lumpur, Malaysia) for DNA sequencing.

Data analysis. The sequencing process was undertaken in Malaysia's First Base Laboratory through PT Genetika Science Indonesia. Editing and proofreading of the sequences were carried out using BioEdit software. The phylogenetic tree was created with the Maximum Likelihood Method (Lemey et al 2009) with 1000 bootstraps using the MEGA 6.0 software (Tamura et al 2011).

Results and Discussion

Genetic characteristics. Genetic identification carried out to confirm genetics of green macroalgae from Totok Bay (DMLBT) and Blongko waters (DMLBL). Electrophoresis of mitochondrial DNA Cytochrome Oxidase 1 (CO1) genes of green macroalgae with amplicon lengths is approximately 600-700 bp. Genetic identification results show that green macroalgae samples from Totok Bay (DMLBT) were identified as *Ulva prolifera*. The alignment of the nucleotide sequences of mitochondrial DNA CO1 gene from samples was compared with the other data available in the NCBI database.

The results indicate that green macroalgae sampled are genetically the same species. The proofreading results from the forward and reserve sequences combined with the sequence of the two samples (DMLBT and DMLBL) are presented in Table 1.

Table 1

Proofreading results of forward and reserve sequence of samples DMLBT (Buyat Bay) and DMLBL (Blongko waters)

Sample code	*Proof reading results*
DMLBT	AGGTACTGGCTTTAAAGCTGGTGTAAAAGATTACCGTTTAACTTATTACACGCCTGAT TATCAGGTAAAAGATACTGATATTTTAGCAGCGTTCCGTATGACTCCTCAACCAGGAG TACCGGCAGAAGAAGCTGGTGCGGCTGTTGCTGCTGAATCATCAACAGGTACTTGG ACAACTGTATGGACTGATGGTTTAACATCTTTAGATCGTTATAAAGGTCGTTGTTACG ACATTGAACCATTAGGAGAAGACGATCAATATATTGCTTATATTGCTTATCCTTTAGAT TTATTTGAAGAAGGGTCAGTTACAAACTTATTTACTTCAATTGTAGGTAACGTTTTTGG TTTTAAAGCTTTACGTGCTTTACGTTTAGAAGATCTACGTATTCCGCCAGCTTACGTTA AAACATTCCAAGGTCCGCCTCACGGTATTCAGGTTGAACGTGATAAATTAAACAAATA TGGTCGTGGTTTATTAGGTTGTACAATTAAACCAAAATTAGGTCTTTCAGCTAAAAAC TATGGACGTGCCGTTTATGAATGTTTA
DMLBL	AGGTACTGGCTTTAAAGCTGGTGTAAAAGATTACCGTTTAACTTATTACACGCCTGAT TATCAAGTAAAAGATACTGATATTTTAGCAGCATTCCGTATGACTCCTCAACCAGGAG TACCGGCAGAAGAAGCAGGTGCGGCTGTTGCTGCTGAATCATCAACAGGTACTTGG ACAACTGTATGGACTGATGGTTTAACATCTTTAGATCGTTATAAAGGTCGTTGTTATG ACATTGAACCATTAGGAGAAGATGATCAATATATTGCTTATATTGCTTATCCTTTAGAT TTATTTGAAGAAGGTTCAGTTACAAACTTATTTACTTCAATCGTAGGTAATGTTTTTGG TTTTAAAGCTTTACGTGCTTTACGTTTAGAAGATTTACGTATTCCACCAGCTTATGTTA AAACATTCCAAGGTCCACCTCACGGTATTCAGGTTGAACGTGATAAATTAAACAAATA TGGTCGTGGTTTATTAGGTTGTACAATTAAACCAAAATTAGGTCTTTCAGCTAAAAAC TATGGACGTGCTGTTTATGAGTGTTTA

The green macroalgae sampled from Totok Bay and Blongko waters were identified in the GenBank, using the BLAST method. The samples were identified as *U. prolifera* with a query cover value of 100%, E-value of 0.0 and identity value of 99% (Table 2). Based on the results of BLAST analysis, it can be concluded that these DNA sequences have a high degree of similarity to the DNA sequences available in the Genbank. According to Claverie & Notredame (2003), if the value of E-value is less than 0.4, then the DNA sequence has a similarity or high homology.

Table 2

Nucleotide sequence identifying through BLAST analysis

Sample code	*Species outcome*	*BLAST*			
		Access code of NCBI	*Query cover (%)*	*E-value*	*Identity value (%)*
DMLBT	*U. prolifera*	AB598812.1	100	0.0	99
DMLBL	*U. prolifera*	JQ963229.1	100	0.0	99

Phylogeny and relatedness. Genetic identification results show that green algae samples from Totok Bay (DMLBT) and samples from Blongko waters (DMLBL) were identified as *U. prolifera*. The alignment of the nucleotide sequences of mitochondrial DNA CO1 gene from samples with the other data available in the NCBI database is presented in Figure 3. Figure 3 showed that the green macroalgae samples from Totok Bay and from Blongko waters were in a different clade and identified as *U. prolifera*. The phylogenetic tree confirmed that the identified species were *U. prolifera* (JQ963229.1) in Blongko waters and *U. prolifera* (AB598812.1) in Totok Bay. Our research shows that *U. prolifera* of Totok Bay is monophyletic with some individuals from China (JQ963247.1, JQ963232.1, JQ963230.1), and *U. prolifera* of Blongko waters is monophyletic with other individuals from China (JQ963229.1, JQ963236.1, JQ963231.1). They are all mutually related to each other, this being supported by paired distance analysis and phylogenetic tree reconstruction using maximum likelihood. The results show that some individuals of *U. prolifera* from Totok Bay are closely related to some individuals of *U. prolifera* from Blongko waters.

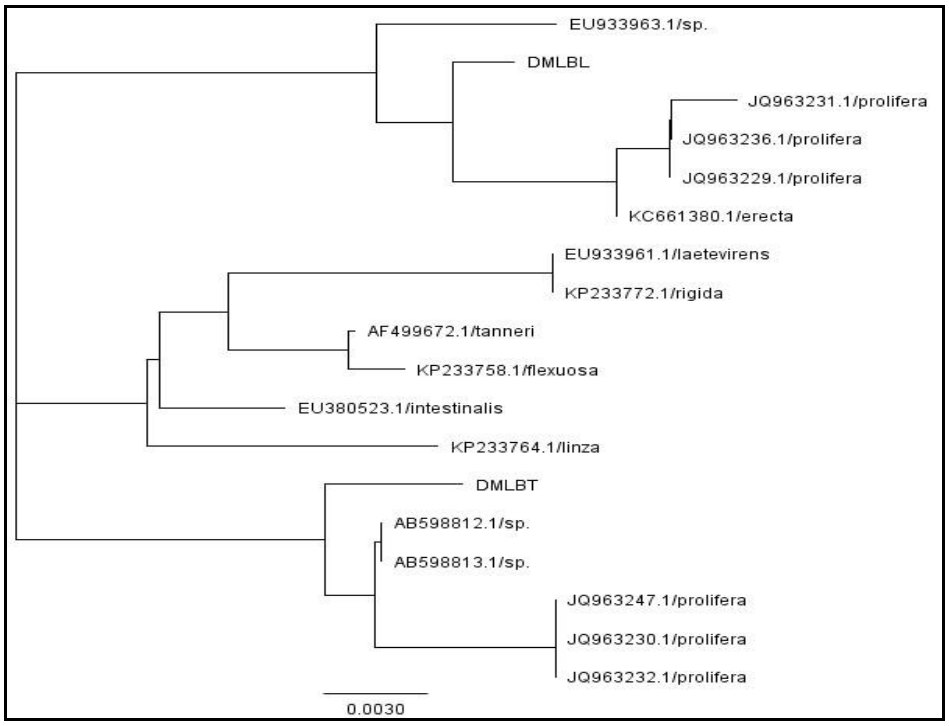

Figure 3. Phylogenetic tree of *Ulva prolifera.*

Table 3

Pairwise distance analysis of *Ulva prolifera*

No	*Specimen*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*
1	EU933963.1/sp.	100																	
2	DMLBL	99.1	100																
3	JQ963231.1/*prolifera*	98.4	98.9	100															
4	JQ963236.1/*prolifera*	98.5	99.1	99.8	100														
5	KC661380.1/*erecta*	99.0	99.6	99.8	100	100													
6	JQ963229.1/*prolifera*	98.5	99.1	99.8	100	100	100												
7	DMLBT	97.5	97.5	96.5	96.7	97.1	96.7	100											
8	AB598812.1/sp.	97.5	97.5	96.5	96.7	97.1	96.7	99.5	100										
9	AB598813.1/sp.	97.5	97.5	96.5	96.7	97.1	96.7	99.5	100	100									
10	JQ963247.1/*prolifera*	96.9	96.9	97.1	97.3	97.1	97.3	98.9	99.5	99.5	100								
11	JQ963230.1/*prolifera*	96.9	96.9	97.1	97.3	97.1	97.3	98.9	99.5	99.5	100	100							
12	JQ963232.1/*prolifera*	96.9	96.9	97.1	97.3	97.1	97.3	98.9	99.5	99.5	100	100	100						
13	EU933961.1/*laetevirens*	97.6	97.8	97.1	97.3	97.7	97.3	97.1	97.5	97.5	97	96.9	96.9	100					
14	KP233772.1/*rigida*	97.6	97.8	97.1	97.3	97.7	97.3	97.1	97.5	97.5	97	96.9	96.9	100	100				
15	KP233764.1/*linza*	97.3	97.6	96.5	96.7	97.1	96.7	97.5	97.8	97.8	97	97.3	97.3	97.8	97.8	100			
16	EU380523.1/*intestinalis*	97.4	97.6	96.5	96.7	97.1	96.7	97.8	98.4	98.4	98	97.8	97.8	98.4	98.4	98.7	100		
17	AF499672.1/*tanneri*	97.5	97.6	96.9	97.1	97.5	97.1	97.6	98.0	98.0	98	97.5	97.5	98.7	98.7	98.7	99.0	100	
18	KP233758.1/*flexuosa*	97.3	97.6	96.9	97.1	97.5	97.1	97.5	97.8	97.8	97	97.3	97.3	98.5	98.5	98.0	99.0	99.8	100

Note: DMLBL from Blongko waters, DMLBT from Totok Bay.

The current pattern in Blongko, western part of the Minahasa Peninsula (Sulawesi Sea) is different from the one in Totok Bay, eastern part of the Minahasa Peninsula (Maluku Sea). Nevertheless, it allows the widespread spores of this species. According to an academic manuscript of the Government of North Sulawesi Province (2017), the water conditions in North Sulawesi are heavily influenced by the dynamics occurring in the western Pacific Ocean. Its very open nature to the Pacific Ocean allows the path of a thermohaline current flow from the Pacific Ocean to the Indian Ocean, which is later known as the cross-Indonesian current (ARLINDO) as part of the world's Great Conveyor Belt current trajectory. This current circulation system only occurs for mid-water masses and remains valid throughout the year. The mass of water from Mindanao (Mindanao Current) that travels along the east coast of Mindanao to the Sulawesi Sea, partly flowing into the Makassar Strait, is partly turning eastwards down the north coast of North Sulawesi Peninsula and then going east to the Pacific Ocean. In the northwestern region of this coastal current, Halmahera, an island in North Maluku Province, east of North Sulawesi Province, reunites with the current of the branches of Mindanao that came from the north between Sangihe and Talaud Islands and meets with water that went out off the Maluku Sea, then merged with the North Equatorial (the equator current counter).

Conclusions. The green macroalgae species found in Totok Bay and Blongko waters is identified as *Ulva prolifera*, and after phylogenetic analysis, the results obtained showed 97-98% similarity with other *Ulva* species found in the genbank. Several individuals of *U. prolifera* from Totok Bay and Blongko waters Bay are closely related to individuals from China. This is supported by the P-distance value of 96.5 to 100 and the phylogenetic tree topology, which indicates a monophyletic clade.

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