



A new clade of *Thalassia hemprichii* (Ehrenberg) Ascherson from the waters of North Sulawesi and West Papua, Indonesia revealed using chloroplast (rbcL) DNA

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Abstract. This study describes the genetic characteristics of *Thalassia hemprichii* in waters of North Sulawesi and West Papua, Indonesia using specific rbcL spacer primers. Samples of *T. hemprichii* were taken from Arakan (South Minahasa Regency), Tongkaina and Tanjung Merah (both in North Minahasa Regency), and Waisai (Raja Ampat Regency) in West Papua. The chloroplast DNA extraction was done using innuPrep DNA Micro Kit, edited with Geneious V6.0 software and compared with existing sequences in GenBank to infer molecular phylogenetic relationships between samples of this species and closely-related taxa. Neighbour-joining phylogenetic trees revealed two distinct clades: clade 1 (Tanjung Merah, Tongkaina, and Waisai), and clade 2 (Arakan), the latter tend to support an earlier study which showed distinct morphological structures such as longer nodes and underground structures (e.g. rhizome). Future studies should investigate in details genetic connectivity of *T. hemprichii*, especially in relation to oceanographic currents in the Indo-Pacific.

Key Words: *Thalassia hemprichii*, chloroplast DNA, neighbour-joining, seagrass, marine, North Sulawesi.

Introduction. In Southeast Asia, Indonesia has the largest seagrass area of 30,000 km², followed by the Philippines with only 978 km². The waters of North Sulawesi have a total seagrass area of around 200-300 km² (Kurniadewa et al 2003). The condition of seagrass beds in Indonesia based on the status by the Indonesian Ministry of Environment is categorized as being in an unhealthy condition. The Government of Indonesia through the Ministry of Environment issued in 2004 divided the seagrass beds into three groups that are categorized as healthy if the area of seagrasses covers > 60%, the category is less healthy if it covers 30-59.9%, and unhealthy if it covers between 0 and 29.9%. According to Alelo et al (2018), seagrasses *Thalassia hemprichii* in North Sulawesi is directly influenced by the type of sediment because the bulk of its biomass is buried in the substrate. It also has high carbon sequestration potential as determined by Tilaar et al (2019). Sedimentation occurs in the waters of North Sulawesi, mainly caused by anthropogenic activities such as traditional gold mining, conversion of land surface for agriculture and settlement; also naturally caused by erosion of surface land, aberration in coastal areas but it is also related to the situation of the region naturally rich in minerals (Paulus et al 2015). There is also evidence of negative effects of heavy metals on benthic macroalgae (Kepel et al 2018).

A number of studies have been done on the geographic distribution and genetic diversity of seagrasses in Indonesia (Kiswara 1992; Kurniadewa et al 2003; Wainwright et al 2018). The distribution of various types of seagrasses in Indonesia is more influenced by the geographical position of the island in Indonesia which is influenced by the Indonesian through Flow (Hernawan et al 2017a, b). The main current that crosses the Indonesian archipelago from the Pacific Ocean allows wide dispersal of seagrasses. North Sulawesi with the Sulawesi Sea is the first area to receive input from the main

water flows from the Pacific Ocean (Hasanudin 1998). Hernawan et al (2015) reported the *T. hemprichii* seagrass in Western Australia can be distributed through ocean currents up to 100 km. According to Waycott et al (2006), seagrasses in the Mediterranean are widely distributed and show the ability to adapt, and even evolve according to the environmental conditions in which they live. As a plant that has colonized waters for 100 million years ago, the Cretaceous Age, seagrass shows the ability of effective distribution (Den Hartog 1970; Orth et al 2006). The influence of current originating from the Pacific Ocean through the eastern part of the Philippines can disperse this seagrass species to Ryukyu Island in Japan, along with the Kuroshio Current (Arriessgado et al 2015).

The emergence of seagrass species is phenotypically similar but can be found in different geographical locations and it is "sibling species" or "twin species", which was first proposed by Den Hartog (1970). For example, *Halodule wrightii* is only found in the tropical Atlantic Ocean and the widespread *Halodule* (*H. uninervis* and *H. pinifolia*) has a widespread distribution in the Indo-West Pacific. The discovery of several new taxa of seagrasses is highly possible as they occupy different substrates (Den Hartog 1970; Phillips & Menez 1988; Short & Coles 2001; Waycott et al 2004; Wagey 2017).

Identifying specimens using DNA barcodes has a high degree of accuracy by compiling with public DNA libraries such as the Barcode of Life Database and GenBank (Hebert et al 2004; Clark et al 2016). Identification and analysis of the relationship between genus-level and up to molecular levels at the DNA will be able to answer the limitations of morphological identification (Paulus et al 2015). The role of the ribulose-bisphosphate carboxylase/oxygenase (rbcL) enzyme, located in the chloroplast genome, is the right choice for determining phylogenetic relationships at higher taxonomic levels (Tanaka et al 1997; Stoeckle et al 2011). Research on genetic diversity about seagrasses to date has been lacking and is more focused on ecological and evolutionary studies (Uchiyama 1996; Waycott et al 1997; Procaccini & Mazzella 1996; Raniello & Procaccini 2002; Alberto et al 2003; Waycott et al 2006; Wagey & Calumpong 2013). Since the last decade, systematic molecules in plants have developed rapidly with *in-vitro* fertilization of DNA amplification and DNA sequencing and in systematic angiosperms, this molecular approach has been effective in dealing with many phylogenetic questions that cannot be solved using character phenotypes (Tanaka et al 1997; Stoeckle et al 2011).

Wagey (2018) emphasized the need for more detailed studies on the biology of seagrasses in Indonesia, particularly in North Sulawesi, a highly diverse marine ecosystem but subjected to anthropogenic threats such as pollution and other activities. Furthermore, Katuuk et al (2018) found out that morphologically, the samples of this species from Arakan showed distinct features (e.g. longer node lengths) as compared to the samples from other localities. Such morphological differences cannot be explained by the type of substrate alone. Similar morphometric study done by Wagey (2017) on two congeneric seagrass (*Cymodocea serrulata* and *C. rotundata*) showed that morphological variations are still consistent with each species regardless of locations. Wagey & Calumpong (2013) underscores that despite significant morphological variations of two species of *Halodule* (*H. uninervis* and *H. pinifolia*), genetic structures (rbcL plastid markers) were maintained. This study was conducted to provide baseline information on the genetic diversity of seagrasses in North Sulawesi. In this study, the researchers provide some preliminary insights as to the molecular phylogeny of the turtle seagrass (*Thalassia hemprichii*). Specifically, this study compared the chloroplast DNA (rbcL) sequences from *T. hemprichii* samples from four localities in Indonesia: Arakan (South Minahasa Regency), Tongkaina and Tanjung Merah (both in North Minahasa Regency), and Waisai (Raja Ampat Regency) in West Papua.

Material and Method

Sampling areas. Seagrass samples were taken from four Indonesian locations, three sites in North Sulawesi and one site in West Papua (Figure 1A-C). Arakan (VKA) [1°22'42.99"N, 124°33'34.07"E] is located in South Minahasa Regency. Two sites are located in North Minahasa Regency: Tongkaina (VKT) [1°35'24.93"N, 124°48'32.46"E] and Tanjung Merah (VKTM) [1°21'52.61"N, 125° 4'50.29"E]. The fourth site is in

Bonkawir (0°24'33.79"S, 130°52'59.62"E), Waisai town in Raja Ampat Regency, West Papua (VKP). The samples were taken from January 10, 2018 to July 25, 2019.

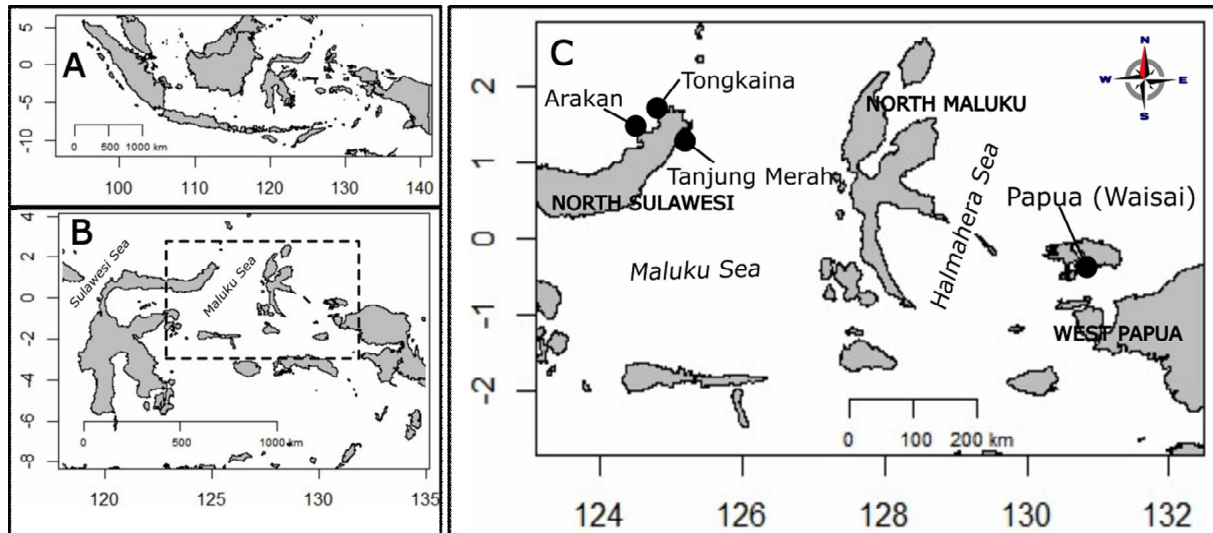


Figure 1. Map showing the Indonesian archipelago (A) and sampling sites (B & C): Arakan (VKA) in South Minahasa Regency, Tongkaina (VKT), Tanjung Merah (VKTM) in North Minahasa Regency, and Waisai in West Papua (VKP).

Research procedure. In each site, samples were collected by hand and cleaned from sediments attached to seawater then seagrasses samples were stored in cool boxes during transportation from several sampling locations. Seagrass samples were prepared in the UNSRAT MIPA laboratory for DNA analysis, using PCR, Electrophoresis, and Spectrophotometer. The chloroplast DNA was extracted using innuPrep DNA Micro Kit (Analytik Jena, Germany). DNA was then amplified using PCR (polymerase chain reaction) using Kapa Taq Readymix PCR kit following the Kapa Biosystems protocol. PCR products with the two primers were then sent to First Base CO in Malaysia for sequencing.

Data analysis. Nucleotide sequences were then edited using Geneious software V6.0 and MEGA version 6. Data in the form of DNA barcodes were further confirmed by NCBI (National Center for Biotechnology Information) GenBank (Clark et al 2016), and summarized in the form of a table of divergences of *rbcL* pairs of several types of *T. hemprichii*. Nucleotide sequences were compared with those available online in the database of NCBI using BLAST (Basic Local Alignment Search Tool) for comparison with existing *rbcL* sequences.

Results. PCR results from seagrass specimens VKA, VKT, VKTM, VKP are base pairs separated using electrophoresis as shown in Figure 2. DNA from PCR is visualized using UV-Transilluminator, and the success of PCR was determined by the presence of a single DNA band with a length of around 724 bp.

All seagrass samples went through the PCR process and based on identification with electrophoresis all base pair spots were around 724 bp, which is a typical base pair count of seagrass DNA. The divergence of the *rbcL* pair of several types of seagrasses is shown in Table 1. Divergences from seagrass samples studied and compared with NCBI BOLD bank genes found several types of closest relatives of *T. hemprichii*, such as *Ottelia alismoides*, *Enhalus acoroides*, *Halophila decipiens*, and *Thalassia testudinum*.

Table 1

List of divergences of RCB Pair of several types of *Thalassia hemprichii*

No	Sample	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	<i>Cymodocea rotundata</i>	100														
2	<i>Stratiotes aloides</i>	95.1	100													
3	<i>Elodea nuttallii</i>	94.6	97.5	100												
4	<i>Blyxa japonica</i>	94.3	98.1	98.1	100											
5	<i>Ottelia ovalifolia</i>	94.6	98.5	98.5	99.4	100										
6	<i>Ottelia alismoides</i>	94.6	98.5	98.5	99.4	99.7	100									
7	VKTM	79.7	83.3	82.1	82.8	82.7	82.8	100								
8	VKT	79.7	83.3	82.1	82.8	82.7	82.8	100	100							
9	VKP	79.7	83.3	82.1	82.8	82.7	82.8	100	100	100						
10	<i>Enhalus acoroides</i>	90.1	93.8	92.9	93.8	93.5	93.7	87	87	87	100					
11	<i>Thalassia testudinum</i>	90.3	94	92.7	93.7	83.7	93.8	87	86.9	86.9	97.8	100				
12	<i>Thalassia hemprichii</i>	90.6	94.6	93.2	94.1	94.1	94.3	88	87.7	87.7	98.5	98.8	100			
13	VKA	90.6	94.6	93.2	94.1	94.1	94.3	88	87.7	87.7	98.5	98.9	100	100		
14	<i>Halophila decipiens</i>	90.3	93.7	93.2	93.5	93.8	94	85	85.2	85.2	96.8	96.5	97.1	97.1	100	
15	<i>Halophila engelmannii</i>	90.1	94.1	93.4	93.7	94	94.1	86	85.7	85.7	97.4	96.8	97.4	97.4	95.5	100

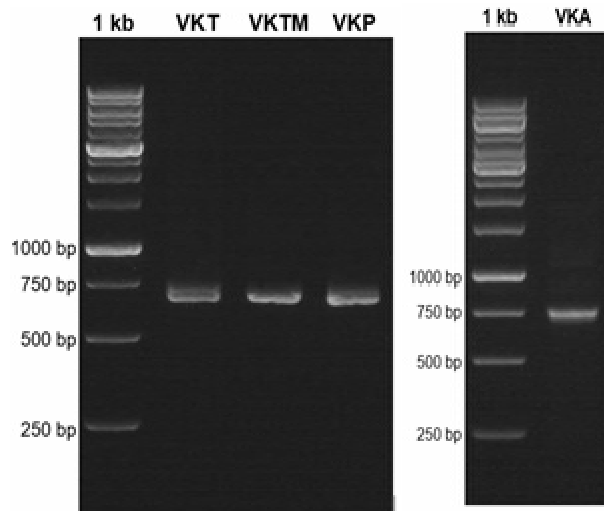


Figure 2. VKT, VKTM, VKP, and VKA seagrass electrophoresis.

Seagrass originating from Tanjung Merah (VKTM), Tongkaina (VKT), and from outside the island of Sulawesi namely from Papua Island (VKP), are in one of the closest group, with a value of 79.7, and this group (VKTM, VKT, and VKP) appears closely related to *O. alismoides* (94.6) rather than with *E. acoroides* (90.1). The genus *Thalassia* is distributed throughout the islands in Indonesia (Den Hartog 1970; Wagey 2015; Hernawan et al 2017a, b). *T. hemprichii* from Arakan (VKA) appeared to be distinct compared to that from other sampling sites as shown graphically in greater details based on the neighbor-joining phylogenetic tree in Figure 3.

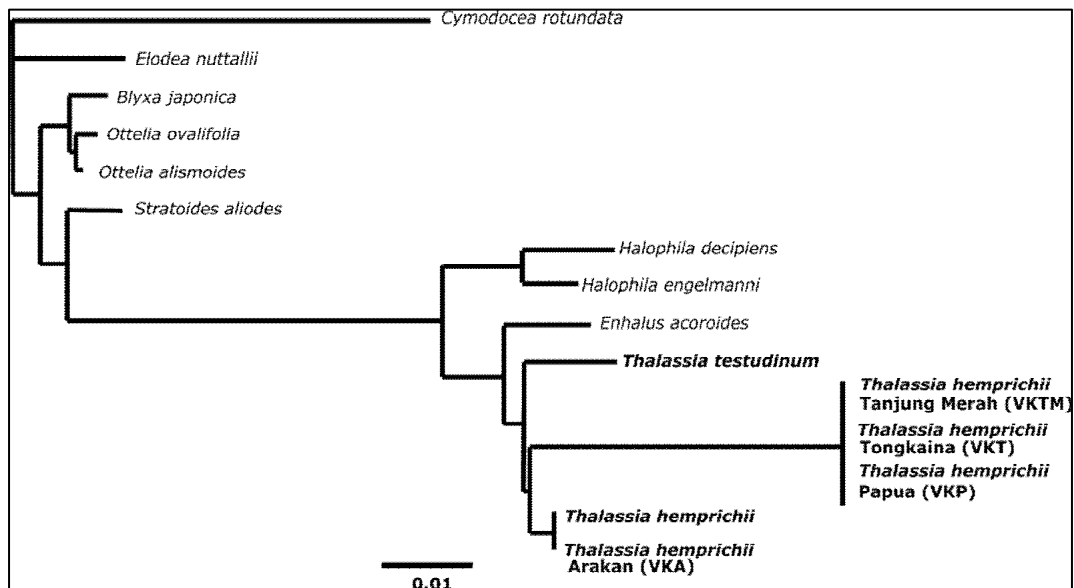


Figure 3. Phylogenetic (Neighbor-Joining) tree showing two clades of *T. hemprichii* (clade 1: VKTM, VKT, VKP, and clade 2: VKA).

Discussion. Using phylogenetic analysis of *rbcL* sequences, this study found genetic differentiation in the turtle grass (*T. hemprichii*) with two clades identified. This study found a distinct clade of *T. hemprichii* in Arakan, supporting an earlier study by Katuuk et al (2018), on the morphology of *T. hemprichii* in Northern Sulawesi, Indonesia. The specimens in Arakan reportedly had narrower leaves, longer nodes, and higher underground structures (e.g. rhizomes) compared to the rest of the specimens from two sites (Katuuk et al 2018). Such variations cannot be explained by the type of substrate. It should be pointed out that Arakan is located in the western section of the North

Sulawesi and has a different oceanographic conditions (e.g. influence of northeast current which connects to the Mindanao eddy) as shown by Masumoto et al (2001).

A more recent study (Kansil et al 2020) showed no morphological difference of this species in relation to environmental variables, probably due to the proximity of the sampling stations (i.e. environmental variables are least likely varied) that they sampled.

Other studies showed moderate genetic diversity of *T. hemprichii* from Awur Bay Jepara, Indonesia (Faozi et al 2019) barcoded using Internal Transcribed Spacer gene (ITS). This might be due to the fact that this species can be dispersed to several hundred kilometers by ocean currents (Waycott et al 2004, 2006; McMahon et al 2017) or other vectors such as birds and fish (Wu et al 2016). Wagey (2015), in his study on the genetics of two species of *Halodule* (*H. uninervis* and *H. pinifolia*) in Central Visayas, Philippines suggested that the morphological variations of these species may be influenced by environmental factors but may not be directly detectable using rbcL sequences (see also Wagey & Calumpang 2013).

The limited number of samples at hand and rbcL sequences available in existing databases prevented the authors from making generalizations. More work has to be done, including detailed morphological characteristics of these distinct clades reported by this present study. This species occurs in nearby countries such as the Philippines (Meñez et al 1983) and a comparison can also be made as to the genetic distance between Philippine and Indonesian *T. hemprichii* populations.

Conclusions. In this study, rbcL DNA sequences showed (based on neighbour-joining phylogenetic tree) two distinct clades: clade 1 (Tanjung Merah, Tongkaina, and Waisai), and clade 2 (Arakan), the latter clade tends to support an earlier study which showed distinct morphological structures such as longer nodes and underground structures (e.g. rhizome). Future studies should investigate in details genetic connectivity of *T. hemprichii*, especially in relation to oceanographic currents in the Indo-Pacific.

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