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***Kappaphycus alvarezii*'s Performance from Selection Based on Molecular Markers Cultivated in Tarakan**

(Maximum of 12 words)

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Abstract

The seaweed *Kappaphycus alvarezii* is a leading aquaculture commodity possessing high economic value that has been used as a raw material for various natural products, food, and pharmaceutical industries. The main challenge regarding its production, especially in Tarakan is the supply of superior seeds. Therefore, this study aims to obtain phylogenetic information and the cultivation performance of the selected seeds by identifying genetic sources based on molecular markers. DNA sequencing analysis was carried out using the molecular marker *cox2-3* spacer on a collection comprising 16 cultivars from all over the country (Indonesia). The four haplotypes representing *K. alvarezii* to be cultivated in the city were produced by phylogenetic clustering and further molecular analysis using *cox2-3* intergenic spacer mitochondrial DNA marker. These include the Kupang varieties and Natuna, also referred to as Lampung seedlings, which is a tissue culture plantlet used for development at SEAMEO-BIOTROP Bogor and propagated by the Lampung Marine Aquaculture Center (BBPBL), Tarakan. These varieties were genetically distances (pairwise comparisons based on mean Kimura 2-parameter model) ranging from 0.00-0.23 unit. The cultivation was carried out for 30 days in the waters of Tanjung Batu (Tj. Batu) Mamburungan, Tarakan, North Kalimantan, for 30 days. The result showed that the Natuna* as Lampung cultivar has superior performance compared to local cultivars (Takalar as Mamburungan Tanjung Batu, Tarakan, and Kupang). Furthermore, the genetic analysis with *cox2-3* spacer markers had the potential as an effective seed selection tool for tracing genetic information in the development of superior seedlings in aquaculture activities.

Keywords: algae, carrageenan, DNA sequencing, haplotype, phylogeny

1. Introduction

The seaweed *Kappaphycus alvarezii* is a blue carbon agent that possesses high economic value as the main ingredient for various natural products (Porse & Rudolph 2017; Rimmer *et al.* 2021). This species is also the main source of carrageenan, which is widely used in the food and pharmaceutical industries (Hayashi & Reis 2012). Recent studies reported that carrageenan has the potential for the treatment of COVID-19 (Frediansyah 2021). Its cultivation technology has been successfully developed by the Philippines and Indonesia for decades (Ask & Azanza, 2002).

Indonesia is the world's largest producer of *K. alvarezii* thanks to its tropical climate, geographical conditions, and rapid cultivation activities (Duarte *et al.* 2017). Traditionally, this seaweed is cultivated by tie-tie method using a long line that are then placed along the coastal shores that has a salinity range of 28-36 ppt (Simatupang *et al.* 2021). The process is simple, easy to duplicate, and does not require certain expertise or advanced technology (Cahyani *et al.* 2019).

Tarakan City in North Kalimantan is one of the regions in Indonesia where dried *K. alvarezii* is produced and supplied to major cities like Makassar, Bali, Jakarta, and Surabaya (Riatiga *et al.* 2017). The main challenge of producing biomass from this seaweed is to ensure that continuous supply of superior seeds for productive and sustainable cultivation can be achieved. Such seeds are expected to have higher growth rate that is in line with its production performance (Budiyanto *et al.* 2019) and are resistant to diseases as well as environmental changes (Azizi *et al.* 2018).

Numerous investigations have been conducted to find the best methods for providing superior seeds of this seaweed, including tissue culture seed technology and clonal selection (Kasim *et al.* 2021), mass

selection based on phenotypes of growth advantage and disease resistance (Dumilag *et al.* 2016; Pongmasak *et al.* 2009), and genetic identification based on molecular markers using the COI gene (Ratnawati *et al.* 2020). However, vegetative propagation of genetic sources of seaweed possessing high phenotype diversity has not been genetically confirmed, thus posing an obstacle in the efforts to improve seed quality. On the other hand, genetic information is useful for finding superior seeds candidate and improving the quality of cultivars (Robinson *et al.* 2013).

The taxonomic history of carrageenan-producing seaweeds from the genus *Kappaphycus* is complex, specifically concerning its species identification (Hurtado *et al.* 2017; Dumilag *et al.* 2018). The difficulties encountered during the early domestication of cultured *Kappaphycus* species may partly explain their low genetic base composition (Zuccarello *et al.* 2006; Halling *et al.* 2013), while the wide range of morphological variations is partly due to the influence of extrinsic or environmental factors. For example, the observed phenotypic variations among these species in the Philippines did not correspond to the differentiation at the genetic level, indicating that these morphological differences are influenced by strong extrinsic factors (Dumilag *et al.* 2018). The depth, water movement, and the exposure rate to drought have previously been attributed to explain the shape variations of the phenotypes in *K. cottonii* (Kraft 1969; Doty 1988; Pham 2002).

The use of mtDNA genes (*i.e.*, *cox-5P* and *cox2-3* spacer markers) for exploring genetic variations of *K. cottonii* in the Philippines has solved some taxonomic challenges in *Kappaphycus* (Dumilag *et al.* 2018). When compared to other markers (*e.g.* *cox2*, *rbcl*, and 18S rRNA), the *cox2-3* has been successfully used as a marker for the new *Kappaphycus* cultivar in the Philippines, mainly due to the availability of numerous comparable sequences deposited in the GenBank database (Roleda *et al.* 2021). Furthermore, a combination of *cox2-3* and *cox1* markers targeting the mitochondrial gene has provided essential information on the genetic diversity of *Kappaphycus* and *Eucheuma* in the Southeast Asian regions (Lim *et al.* 2014). Unfortunately, such mitochondrial markers could not be used for establishing a connection between genetic diversity and morphological variations in *Kappaphycus*, particularly during the process of discriminating phenotypes for cultivation improvement (Ratnawati *et al.* 2020).

Obtaining and comparing genetic information of *K. alvarezii* through DNA sequencing technology is crucial for selecting the desired quality of seaweed seeds for cultivation in Tarakan City. However, to the best of our knowledge, such effort has never been performed in Indonesia. Therefore, this study aims to obtain phylogenetic information and cultivation performance of the selected seaweed seeds that were cultivated in the waters of Tarakan City. Furthermore, we hope that the methodology applied in this study (*i.e.*, seed selection based on DNA sequence information) can potentially be used as an effective selection criterion of superior seeds for seaweed cultivation in Tarakan City, North Kalimantan.

2. Material and Methods

2.1 Collection of *K. alvarezii* Seed Varieties

Seventeen samples of *K. alvarezii* genome with different varieties were divided into four populations based on location, namely the Jawa population (Jepara, Situbondo); the commercial plantlet group from tissue culture SEAMEO Biotrop Bogor population (Tambalang*, Maumere brown/LC*, Maumere green/LH*, Natuna*, Kendari*); the Kalimantan population from central Indonesia (Sebatik, Nunukan, Tarakan, Bontang, Takalar); and the Indo-Timur population (Sumba, Kupang, Tual, Ambon, and Papua). The samples were rinsed with distilled water until clean from salt and debris, followed by crushing or grinding using mortar and pestle set with the addition of liquid nitrogen. Processed samples were, then transferred into Eppendorf tubes, and stored in a freezer at -4°C.

2.2 DNA Extraction, PCR Amplification, Genetic Analysis and Seed Selection

The CTAB method of Doyle *et al.* (1990) was used for DNA extraction with a slight modification (that is, adding PVP 3%). PCR amplification was performed using a pair of *cox2-3* spacer primers (*i.e.*, Forward: 5'-GTACCWCTCTTTDRGRRKDAAATGTGATGC-3' and Reverse: 5'-GGAATACWAGATGRAAWGGATGTC-3') (Zuccarello *et al.* 1999). Each PCR mixture (50 µL) consisted of a 2 µL DNA template, 1.5 µL of each primer (10 mM), 25 µL MyTaq HS Red (15x) (Bioline), and 20 µL Nuclease Free Water (NSFW). Furthermore, the gradient PCR program was set at 94°C for 4 minutes; 5 cycles at 94°C, 45°C, and 72°C with a time of 1 minute each; 30 cycles at 94°C, 50°C, and 72°C with a time of 1 minute each; and the final elongation at 72°C for 10 minutes (Zuccarello *et al.* 1999). The quality of the PCR products was determined using electrophoresis via agarose gel (v/w 0.8%) added with 1 µL green fluorescent and observed using a UV transilluminator. When a good positive band is confirmed, the PCR products were sent to the 1st BASE Laboratory (<https://base-asia.com>) in Malaysia for Sanger Sequencing. The DNA sequences were compared against the NCBI GenBank database using the BLAST-N (Basic Local Alignment Search Tool for Nucleotide) program (<https://blast.ncbi.nlm.nih.gov>). The genetic

diversity was determined based on molecular phylogenetic analysis and haplotype network. Multiple sequence alignment was created and analyzed¹³ using MEGA version 11.0.10 (<https://www.megasoftware.net>), BLASTN/P, and BioEdit 7.2.5. (www.mbio.ncsu.edu/BioEdit/BioEdit.html). The Phylogenetic tree was constructed based on the maximum likelihood (ML) method (Tamura *et al.* 1993) with bootstrap 1000x from the nucleotide sequence of *cox2-3* spacer. Four varieties of candidate cultivar seeds were then selected from the genetic search results to observe their cultivation performance. The haplotype diversity in 3 population¹⁶ of Kalimantan, Indo-eastern varieties of seaweed, and tissue culture (SEAMEO-Biotrop) were analyzed using the DnaSP v6.12.03 software (Rozas *et al.* 2017).

2.3 Cultivation of Selected *K. alvarezii* Seeds

Randomized block design with three varieties of seedlings was selected based on the sequencing and control using 500 replicates of cultivated thallus clumps each. The initial weight of each clump (W_0) was set at 10 grams (Rama *et al.*, 2018). The maintenance period lasted 30 days at the Tanjung Batu, Mamburungan, Tarakan City, North Kalimantan (117°39'50.1624"E-3°15'54.9972"N) (Figure 1. Legend 3). Each of the three selected varieties represented a cluster formed from the phylogenetic analysis of molecular technology. The cultured seeds kept at the Tanjung Batu Mamburungan in the cultivation performance have been adapted previously for 1 month in the indoor tub of the Balai Benih Udang Pantai Amal (117°39'14.9994"E-3°15'53.03399"N Figure 1. Legend 1). The process continued with 3 months of cultivation using the treatment of the 2013 modified Gorontalo LP2BRL mass selection protocol (Pong Masak, *et al.* 2014) at Amal Beach, East Tarakan (117°39'50.1624"E-3°15'53.03399"N Figure 1. Legend 2).

Water quality parameters at Tanjung Batu Mamburungan were measured at the beginning of planting and every week until the end of maintenance. These include temperature, pH, salinity and DO in situ using a DO-meter 6+ Thermo Scientific Eutech. In situ brightness and current velocity were determined with Secchi disk and Flowatch FL-03, respectively²³. The chemical parameters of ex-situ in the form of ammonia (SNI 06-6989.30-2005), nitrate (Brucine), nitrite (SNI 06-6989.9-2004), phosphate (SNI 06-6989.31-2005), and turbidity (Turbidity meter WGZ-2000 ratio) were measured at the Water Quality Laboratory of FPIK-UBT. The yield level of carrageenan (AOAC International, 1990) and phytohormones levels (HPLC) were then analyzed at the Nutrition Laboratory of FPIK UBT and Bogor Agrochemical Residues Laboratory.

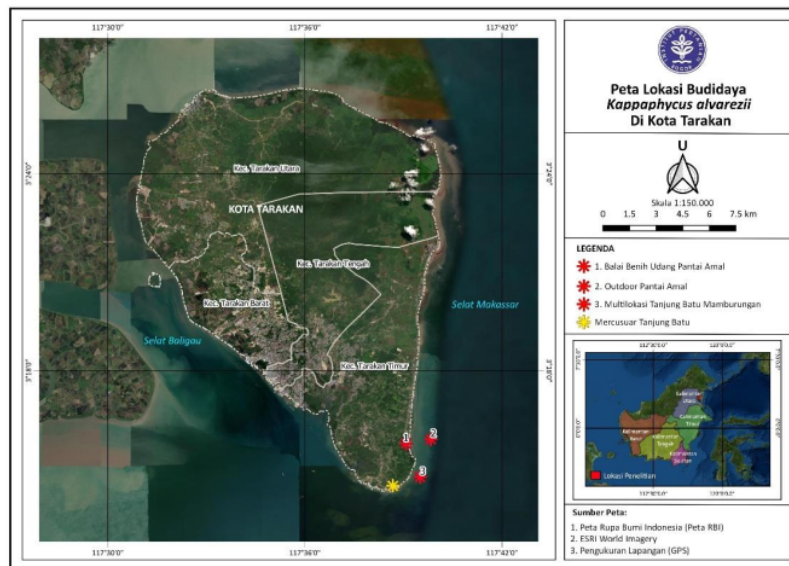


Figure 1. Map of Locations for *K. alvarezii* Cultivation in Tarakan City, North Kalimantan

2.4 Data Analysis

The performance of selected *K. alvarezii* seaweed varieties planted in the Tarakan cultivation site was analyzed to determine whether there are any differences regarding the genetic responses between the varieties, such as final weight, daily growth rate (DGR), percentage of carrageenan yields, and

phytohormones levels in relation to the environmental condition of the cultivation area for 30 days of experiment. A 95% confidence interval followed by the Tukey test was selected to statistically analyze the data using the Minitab 19 program (<https://www.minitab.com/en-us/>). Furthermore, water quality parameters of the aquaculture environment and climatological information acquired from the BMKG (Meteorology, Climatology, and Geophysical Agency) of Tarakan City were descriptively analysed.

3. Results and Discussion

3.1 An assesment *K.alvarezii* based on molecular marker

The PCR products of the *K. alvarezii* samples amplified using the *cox2-3* spacer primers were visualized using UVis-agarose electrophoresis with a 100 bp DNA ladder (Figure 2). Bands positioned within the 400 bp range indicated positive amplifications. In this study, we successfully amplified the *cox* gene of *K. alvarezii* from almost all observed samples. The *cox2-3* spacer amplification of the samples from Sumba (No.2), Takalar as Mamburungan Tanjung Batu (No. 15), Tual (No. 16), and Maumere (No. 17 and 18) showed prominent amplifications bands in contrast to other samples (Figure 2).

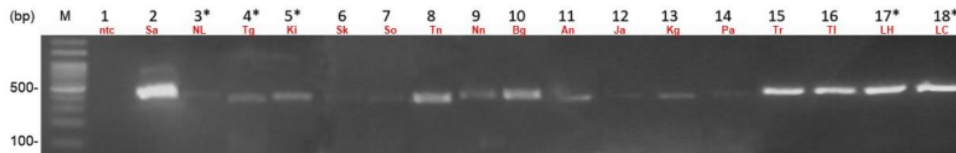


Figure 2. Amplified *cox2-3* spacer of *K. alvarezii* samples visualized on the agarose-electrophoresized gel (1.5% w/v). Sample column number: No. 1= no template control (ntc); No 2= Sumba (Sa); No. 3= Natuna* as Lampung (NL); No. 4= Tambalang (Tg); No. 5= Kendari (Ki); No. 6= Sebatik (Sk); No. 7= Situbondo (So); No. 8= Tarakan (Tn); No. 9= Nunukan (Nn); No. 10= Bontang (Bg); No. 11= Ambon (An); No. 12= Jepara (Ja); No. 13= Kupang (Kg); No. 14= Papua (Pa); No. 15= Takalar as Mamburungan Tanjung Batu (Tr); No. 16= Tual (Ti); No. 17= Maumere* LH (LH); and No. 18= Maumere* LC (LC). The asterisks (*) indicate tissue culture plantlets of *K. alvarezii* from SEAMEO-BIOTROP Bogor. Abbreviations: bp=base pair, M=Marker (100bp DNA Ladder; Geneaid).

A study of the genetic diversity of *K. alvarezii* is important to provide insight into management, conservation, and strain selection for aquaculture (Yow *et al.*, 2012). The amplicon bands displayed on the agarose gel during the electrophoresis stage were satisfactory and showed an amplicon of about 400 bp size (Figure 2). The *K. alvarezii*, 16 sequences of a *cox2-3* spacer of the research's sample, was successfully amplified and had a size range of 348-393 bp and all sample of the *K. alvarezii*'s *cox2-cox3* spacer nucleotide has high similarity with the *K. alvarezii* database GenBank (KM051549.1, MN331555.1, JN234760.1). Our 16 sequences of *K.alvarezii* of this sample research by fasta files open to the public are available to the corresponding author upon request.

The bootstrap values equaling up to 95% indicate that branching is accurate, consistent, and will not change if other phylogenetic trees are used (Madduppa *et al.* 2020), and based on this phylogenetic analysis research's bootstrap value is 96%-97% (Figure 3), the 16 seaweed seeds (excluding the sample from Situbondo that were not successfully sequenced) have a close genetic distance, except for seeds from Kupang (Figure 3). Seaweed seeds from Natuna* as Lampung cultivar, Papua, and Sumba are grouped in one cluster, while seed from other areas is grouped in other cluster with *K. alvarezii* from a database NCBI (*K. alvarezii* accession number of reference KM051549.1, MN331555.1, JN234760.1). The three main clusters described the grouping of 16 seaweed cultivars that were selected as genetic sources for cultivating seeds in Tarakan. Each cluster selected one cultivar as a cultivation seed candidate (green circles) in Tarakan. Tarakan, Natuna* as Lampung, and Kupang seeds were selected as representatives of each cluster according to the phylogenetic tree. The Natuna*as Lampung cultivar is obtained from BBPBL Lampung nursery, originally from the first generation of tissue culture plantlets of *K. alvarezii* from SEAMEO-Biotrop Bogor. Seed from Takalar as Mamburungan Tanjung Batu Tarakan was still selected as local control in this research location Mamburungan Tanjung Batu's seawater area cultivation.

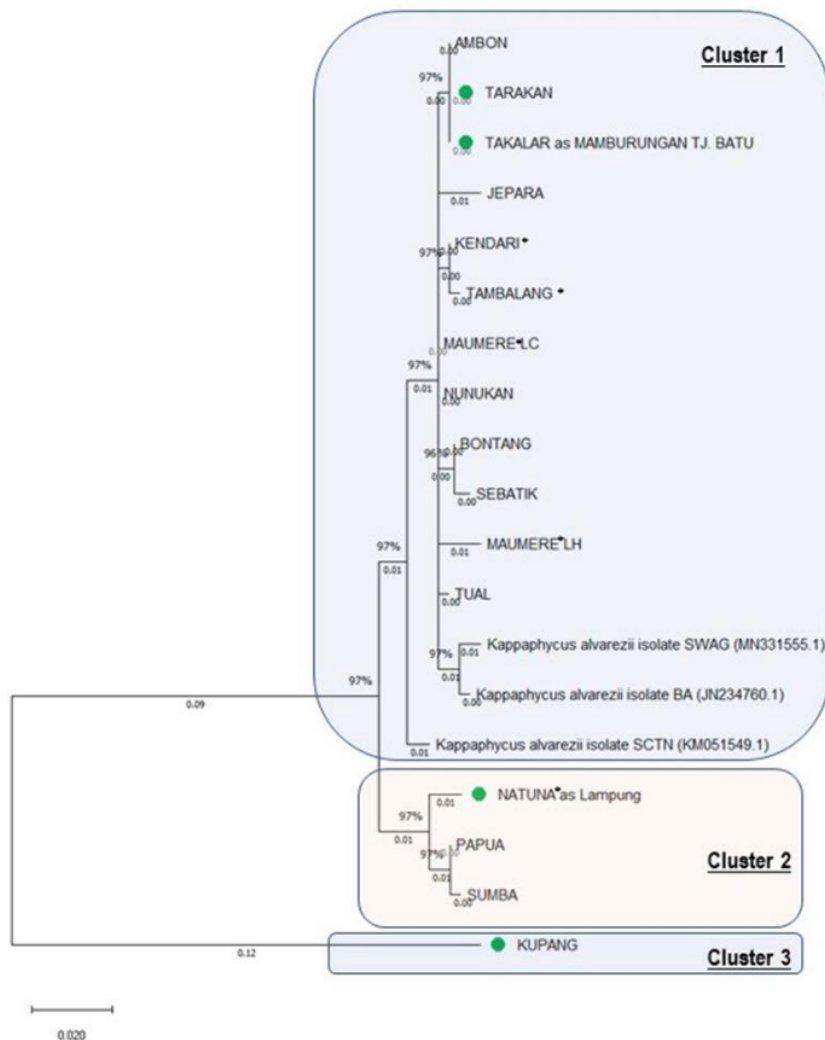


Figure 3. Phylogenetic reconstruction of *K. alvarezii* varieties based the *cox2-3* spacer analysed using the maximum likelihood (ML) method (1000× bootstrap replications). Green circles indicated the selected seed for cultivation experiment in Mamburungan Tanjung Batu Tarakan's seawater area. The percentage values at the nodes indicate the bootstrap support of the group or between sequences. In the phylogenetic scale formed, there is a scale of 0.00-0.12, which indicates that from 100 nucleotides sequences, there are 0-12 different bases in each branch.

The phylogenetic tree among *K. alvarezii* from several places in Indonesia is presented in Figure 3. Tree topology shows the 19 observed seaweed seeds have a close genetic distance, except seeds from Kupang. Seaweed seeds from Natuna* as Lampung, Papua, and Sumba are grouped in one cluster, while seeds from other areas are grouped in other clusters. These results indicated Kupang variant has more mutations in the *cox2-3* spacer sequence. These mutations could occur due to different environmental conditions, such as salinity, sediment, waves, and currents. Geographically, the Wallace line divided the Indonesian waters into three regions, including West, Central, and East. This division indicates differences in the characteristics of reef exposures, which is an ecosystem that is overgrown with algae (Kadi 2005). The coastal waters in several islands of Central Indonesia have short and steep reef exposures (drop-offs), large waves, and strong currents. Simatupang *et al.* (2021) also found that salinity, temperature, DO, pH,

and nitrate in the Indonesian sea are highly varied and can affect the production and product quality of *K. alvarezii*.

The mitochondrial encoded *cox2-3* spacer was the most suitable marker for molecular identification, basic molecular phylogeny, and DNA barcoding of *Kappaphycus*, considering the large database of DNA data already available in GenBank (Tan *et al.* 2012). The NCBI nucleotide BLAST of *K. alvarezii* *cox2-3* spacer sequences from Indonesia indicated high sequence similarity with other *cox* sequences available in the NCBI GenBank database (Table 1). The highest similarity to Genbank NCBI can be characterized by the query cover value (95%-100%) and percent identity (82.70%-99.47% based on the data in this study), whose values are close to 100% (Triandiza and Madduppa, 2018).

Table 1. Results of NCBI nucleotide BLAST for *K. alvarezii* cultivars using *cox2-3* spacer

No.	Origin	Species	Length (bp)	Query Cover	Percent Ident.	Accession Reference
1.	Sumba	<i>K. alvarezii</i>	349	100%	96.14%	KM051549.1
2.	Natuna* as Lampung	<i>K. alvarezii</i>	389	100%	96.93%	KM051549.1
3.	Tambalang*	<i>K. alvarezii</i>	389	99%	98.71%	MN331555.1
4.	Kendari*	<i>K. alvarezii</i>	385	100%	98.44%	MN331555.1
5.	Maumere (LC)*	<i>K. alvarezii</i>	374	98%	99.47%	JN234760.1
6.	Sebatik	<i>K. alvarezii</i>	391	95%	99.20%	JN234760.1
7.	Tarakan (Amal)	<i>K. alvarezii</i>	393	100%	97.73%	MN331555.1
8.	Nunukan	<i>K. alvarezii</i>	371	99%	97.96%	MN331555.1
9.	Bontang	<i>K. alvarezii</i>	348	100%	98.21%	JN234760.1
10.	Ambon	<i>K. alvarezii</i>	370	99%	98.44%	JN234760.1
11.	Jepara	<i>K. alvarezii</i>	370	100%	97.72%	MN331555.1
12.	Kupang	<i>K. alvarezii</i>	350	99%	82.70%	KM051549.1
13.	Papua	<i>K. alvarezii</i>	393	100%	95.18%	KM051549.1
14.	Takalar as Mamburungan Tj. Batu	<i>K. alvarezii</i>	352	100%	97.73%	JN234760.1
15.	Maumere (LH)*	<i>K. alvarezii</i>	381	97%	99.20%	JN234760.1
16.	Tual	<i>K. alvarezii</i>	378	99%	98.67%	JN234760.1

Note:

The asterisks (*) indicate tissue culture plantlets of *K. alvarezii* from SEAMEO BIOTROP

Green highlights: Selected cultivar as a representative from each cluster in a phylogenetic tree cultivated in Mamburungan Tj. Batu Tarakan.

Query coverage is the percentage of nucleotide length of the query that matches the existing sequence in the GenBank database. At the same time, per cent identity matches the query sequence and the aligned database sequence (Claverie and Notredame, 2007). The query value of this research ranges from 95% to 100%, with a percent identity of 82.70-99.47%. The results of the nucleotide alignment on the NCBI GenBank upon the DNA samples of this study identified *K. alvarezii*.

The kinship between populations was determined based on genetic distance parameters (Nei, 1987), and then statistical analysis of the genetic tree reconstruction also required species identification. The kinship relationship between *K. alvarezii* Kupang and Jepara (Table 1) is the furthest apart (0.23), explaining that there is a genetic divergence between *alvarezii* Kupang and Jepara. This difference in genetic distance is thought to be due to the different distribution patterns of the two species, thus providing a small chance of meeting and causing no gene flow between the studied populations. In general, the mitochondrial DNA marker *cox2-3* spacer is used as an important coding region in the identification of *K. alvarezii* because mitochondrial DNA has a higher mutation rate than nuclear DNA, has a higher number of copies, is a gene that is inherited directly from the parent, and considering the large database of DNA data already available in GenBank (Amorim *et al.*, 2009; Tan *et al.*, 2012).

After the BLAST (www.blast.ncbi.nlm.nih.gov) (Table 1) results had been obtained, a phylogeny tree was reconstructed using the Maximum Likelihood (ML) tree (Figure 3) method using a bootstrap number of 1000 with the Kimura-2 Parameter model (Table 2). In the phylogenetic tree branches (Figure 3), several bootstrap values are present, and they function to unveil the accuracy of the phylogenetic tree branches (Horiike *et al.*, 2009). This tree was created using 16 sequences of *K. alvarezii* of this current study with an additional 3 sequences from GenBank. Bootstrap values equal 96%-97%; if the bootstrap value is higher than 70%, this means a permanent or significant branching (Coenye *et al.* 2003).

The genetic distance describes the variation level between varieties, with a higher and lower value indicating high diversity and uniformity. Reconstruction of phylogenetic trees needs to be supported by the results of genetic distance analysis on species (Akbar *et al.*, 2014). The genetic distances between these sequences are shown in Table 2. Analyses were conducted using the Kimura 2-parameter (K2P) model (Kimura 1980; Tamura *et al.* 2013). The analysis involved 16 sequences of our samples and 3 sequences of interspecies access from GenBank. The K2P distance is the most effective model when genetic distances

are low (Nijman and Aliabadian, 2010). The range of genetic distance within species *K. alvarezii* 0.00-0.23 (Table 2), where is the maximum value 0.23 between Kupang and Jepara.

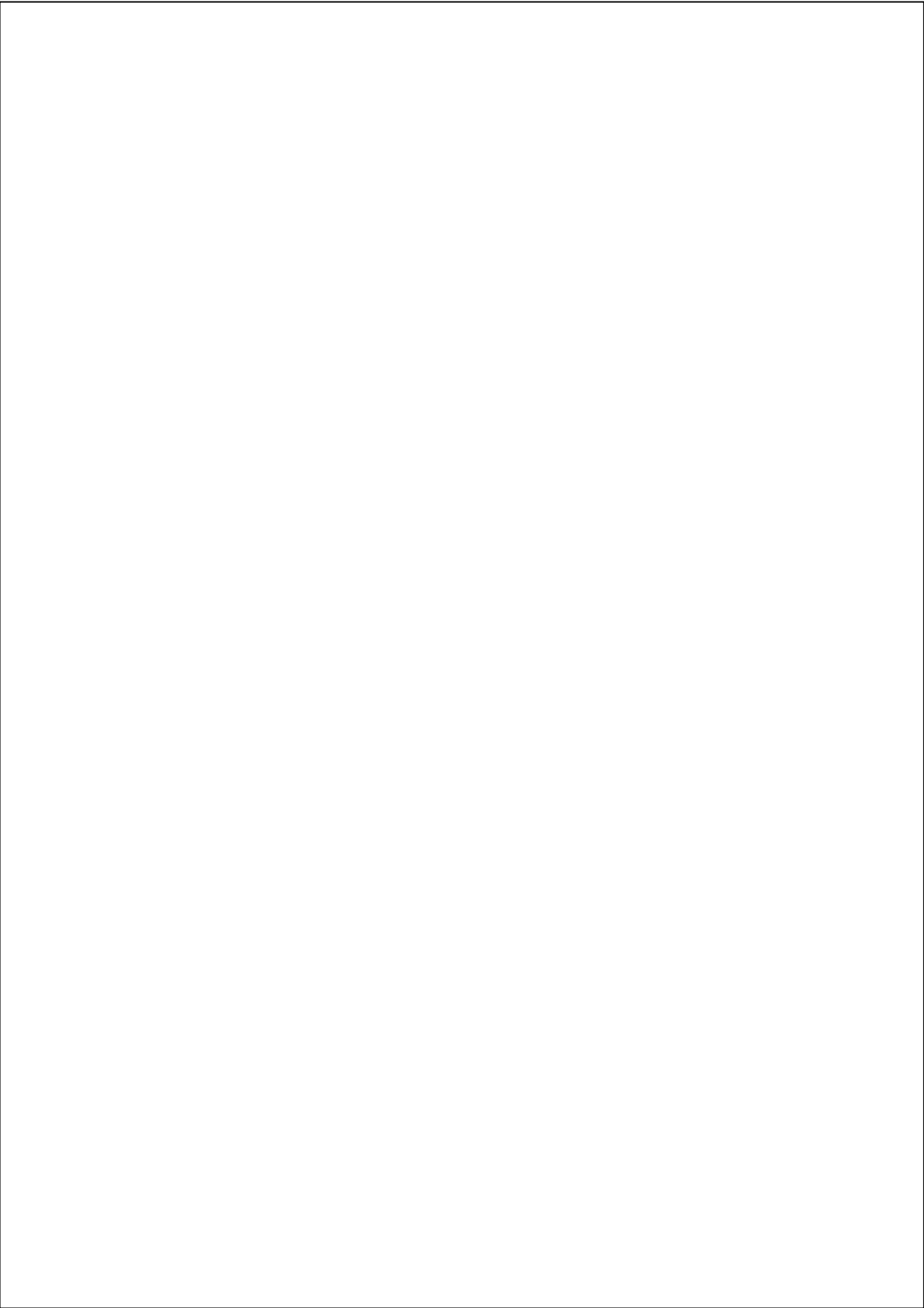


Table 2. Pairwise comparisons based on K2P model distance within *K. alvarezii* species

No	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	AMBON																			
2	BONTANG	0.01																		
3	JEPARA	0.01	0.01																	
4	KENDARI*	0.01	0.01	0.01																
5	KUPANG	0.21	0.21	0.23	0.22															
6	MAUMERE* LC	0.00	0.00	0.01	0.00	0.21														
7	MAUMERE* LH	0.01	0.01	0.02	0.01	0.21	0.01													
8	NATUNA* as Lampung	0.04	0.04	0.04	0.04	0.22	0.04	0.04												
9	NUNUKAN	0.00	0.00	0.01	0.00	0.21	0.00	0.01	0.04											
10	PAPUA	0.04	0.04	0.04	0.04	0.22	0.03	0.04	0.01	0.03										
11	SEBATIK	0.01	0.00	0.02	0.01	0.22	0.01	0.02	0.04	0.01	0.04									
12	SUMBA	0.04	0.04	0.05	0.04	0.22	0.04	0.04	0.02	0.04	0.00	0.04								
13	TAKALAR as MAMBURUNGAN TJ.BATU	0.00	0.00	0.01	0.01	0.21	0.00	0.01	0.04	0.00	0.04	0.01	0.04							
14	TAMBALANG*	0.01	0.01	0.01	0.00	0.22	0.01	0.02	0.04	0.01	0.04	0.01	0.04	0.01						
15	TARAKAN	0.00	0.01	0.01	0.01	0.21	0.00	0.01	0.04	0.00	0.04	0.01	0.04	0.00	0.01					
16	TUAL	0.01	0.00	0.01	0.01	0.21	0.00	0.01	0.03	0.00	0.04	0.01	0.04	0.01	0.01	0.01				
	<i>Kappaphycus alvarezii</i> isolate_SWAG_(MN331555.1)																			
17	<i>Kappaphycus alvarezii</i> isolate_SWAG_(MN331555.1)	0.01	0.01	0.02	0.01	0.22	0.01	0.02	0.04	0.01	0.04	0.01	0.05	0.01	0.02	0.01	0.01			
18	<i>Kappaphycus alvarezii</i> isolate_BA_(JN234760.1)	0.01	0.01	0.01	0.01	0.22	0.01	0.01	0.04	0.01	0.04	0.01	0.04	0.01	0.01	0.01	0.01	0.01		
	<i>Kappaphycus alvarezii</i> isolate_SCTN_(KM051549.1)																			
19	<i>Kappaphycus alvarezii</i> isolate_SCTN_(KM051549.1)	0.02	0.02	0.02	0.02	0.22	0.01	0.02	0.03	0.01	0.03	0.02	0.03	0.02	0.02	0.02	0.02	0.02	0.02	0.02

The genetic diversity analysis showed that Indonesia's samples of *K. alvarezii* have several haplotypes. Seaweed populations from Biotrop* Bogor, Kalimantan, and Indo-Eastern have three, five, and five haplotypes, respectively. A total of 3 populations with 15 sequences were analyzed for haplotype and nucleotide diversities (Table 3). In *cox2-3 spacer* primers, the variation of sequences (*polymorphic site*) causes many haplotypes, so it also impacts the high value of haplotype diversity (Hd) is 0.7–1. In contrast, nucleotide diversity (π) varies from low 0.004 to high 0.088. Unfortunately, one of the two varieties representing the Jawa population (*i.e.*, Situbondo) could not be analyzed for their genetic diversity (*i.e.*, haplotypes and nucleotide diversities) because the Situbondo sample was not successfully sequenced. Such genetic diversity analyses require a minimum of 2 individuals or samples as representative of a population (*i.e.* the Jawa population) (Rozas *et al.* 2017), which could not be fulfilled in the present study.

Table 3. Genetic diversity of *K. alvarezii* based on sample size (n), haplotype number (Hn), haplotype diversity (Hd), and nucleotide diversity (π) from three populations

Population	n	Genetic diversity		
		Hn	Hd	π
Biotrop* Bogor	5	3	0.7	0.014
Kalimantan	5	5	1	0.004
Indo-Eastern	5	5	1	0.088

Based on the hierarchical Analysis of Molecular Variance/AMOVA (Table 4), the genetic diversity within the population was higher than among the populations. The pairwise comparison of the genetic differentiation indexes (F_{ST}) showed that three populations have a low F_{ST} (0.014). F_{ST} can see whether there is gene flow between samples or not, even though the value of F_{ST} (0.014) is weak genetic structuring. The F_{ST} value is 0-1; the closer to 1 it means the stronger it is so that the populations are increasingly isolated from each other, *i.e.*, there is no gene flow (Madduppa *et al.*, 2021). Table 4 shows that the genetic variation is higher within the population than among the population, indicating that it is genetically more heterogeneous because each sample has a different haplotype.

Table 4. AMOVA based on 3 populations

Analysis of variation	*d.f	Percentage of variation (%)	F_{ST}	Significance
Among population	3	1.50	0.014	$P > 0.05$
Within population	12	98.50		
Total		100		

*d.f. = degree of freedom

The geographical distance between populations can significantly affect genetic diversity, even in haplotypes. This theory is supported by Bramandito *et al.* (2018), which affirms that the greater the distance between populations, the greater the difference in base order. According to Nei (1981), the haplotype diversity values in the Indo-Eastern population are to be included in the high category. The high value of haplotype diversity is inferred to be due to the large population size still available in the local seawaters. The migration factor will also significantly affect the high value of haplotype diversities for a population, and this is because a population migration will result in inter-population encounters that allow for interbreeding and gene mixing between population members (Akbar *et al.* 2014; Pertiwi *et al.* 2017; Akbar *et al.* 2020). According to Kliman *et al.* (2008), high genetic diversity reflects large population size. According to Yang *et al.* (2021), the heterogeneity is high if the variation is greater in the population (within a population) in table 4. A high value of F_{ST} (~0.9) indicated strong subdivision and low gene flow between populations (Madduppa *et al.* 2021). The low F_{ST} (0.014) result in *cox2-3* spacer indicates that there is no geographic population structuring or subdivision, and the possibility of *cox2-3* spacer is more conserved (Zuccarello *et al.* 2006; Tan *et al.* 2012).

The circle size shows the number of samples that have the haplotype (Figure 4). The result showed that the Kalimantan cultivars have a similar haplotype distribution with Biotrop* Bogor (haplotype 1) and Indo-Eastern (haplotype 2) varieties, except for the Sebatik haplotype (haplotype 6). Haplotype 1 (Maumere* LC, Maumere* LH, Kendari*, Tambalang*, Bontang, and Nunukan) has the highest similarity with haplotype 2 (Ambon, Tarakan, and Takalar as Mamburungan Tanjung Batu Tarakan), 5 (Tual), and 6 (Sebatik). Meanwhile, the haplotype 3 (Papua) has the highest similarity with haplotype 4 (Sumba).

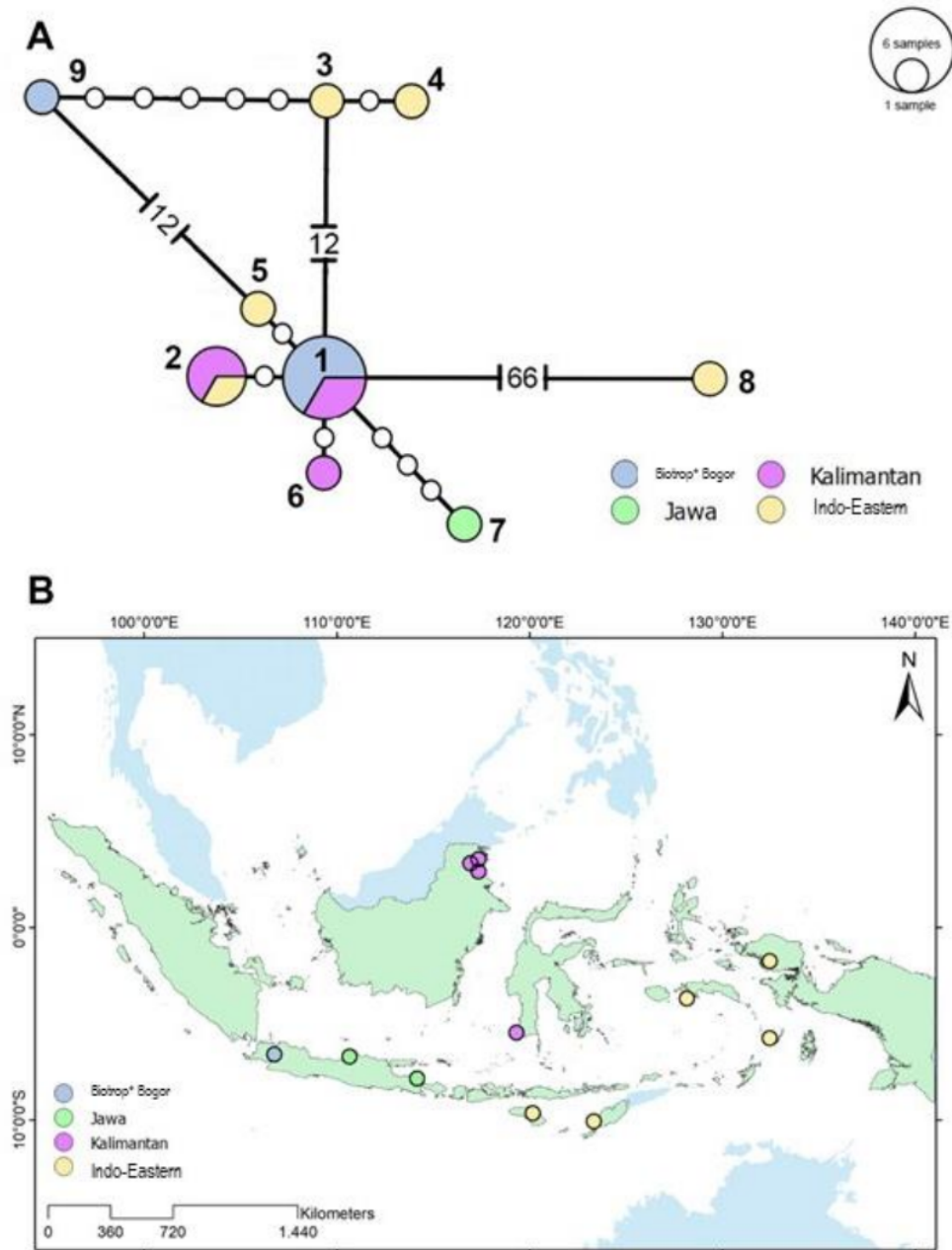


Figure 4. (A) Haplotype network diagram constructed using the Minimum Spanning Network method (Bandelt *et al.* 1999) on PopART software. (B) Map of Indonesia showing the locations of the 4 groups, namely Biotrop* Bogor, Jawa, Kalimantan, and Indo-Eastern. The map was processed from ArcMap 10.3. Description: Haplotype 1 (Maumere* LC, Maumere* LH, Kendari*, Tambalang*, Bontang, Nunukan); Haplotype 2 (Ambon, Tarakan, Takalar as Mamburungan Tj. Batu); Haplotype 3 (Papua); Haplotype 4 (Sumba); Haplotype 5 (Tual); Haplotype 6 (Sebatik); Haplotype 7 (Jepara); Haplotype 8 (Kupang); Haplotype 9 (Natuna* as Lampung). The white circles show mutations that are less than 5, numbers in brackets show the number of mutations of more than 5, while the bold number indicates the *n*-th haplotype.

From the haplotype network analysis results (Figure 4) of the *cox2-3* spacer, a total of 9 haplotypes were found. Haplotype one is the most common because it is owned by six samples (Maumere* LC, Maumere* LH, Kendari*, Tambalang*, Bontang, and Nunukan). Furthermore, five samples owned haplotype two (Ambon, Tarakan, and Takalar as Mamburungan Tanjung Batu). There are seven unique haplotypes (*unique/private haplotypes*), namely (haplotypes 3 to 9, Papua, Sumba, Tual, Sebatik, Jepara, Kupang, and Natuna* as Lampung), which means that each unique haplotype is only owned by one sample. However, the haplotype analysis results did not show whether the haplotype groupings or linkages describe the distribution pattern based on geographic area, which may be due to the inter-sample mixes with each other and being spread by ocean currents, especially in wild specimens. For example, samples from Indo-Eastern were not clustered, but haplotype 5 (Tual) was more closely connected to Biotrop* Bogor and Kalimantan than Papua and Sumba. It is suspected that the population of Eastern Indonesia is an area with high genetic diversity, so it is suitable as a candidate stock of seeds for the development of sustainable cultivation of *K. alvarezii* seaweed.

The plasticity in response to abiotic and biotic factors resulted in various morphologies within the same species (Leliaert *et al.* 2014). This is consistent with the monoculture practice of *Kappaphycus* seaweed cultivation, where farmers generally rely on a fragmentation system with vegetative propagation, through thallus cuttings for each harvest cycle. This results in low cultivar genetic diversity and risk of plant susceptibility to disease because most *Kappaphycus* cultivated are clones (Roleda *et al.*, 2021). Consequently, increasing plant genetic diversity, which is a strong defense against infection is essential to the seaweed industry (Ekroth *et al.* 2019).

3.2 Performance of Cultivated Seaweed

The 30 days cultivation experiment showed significant differences among the four varieties (Table 5). Cultivars from Natuna* as Lampung exhibited the highest carrageenan yield ($42.6 \pm 1.94\%$), followed by those from Tarakan ($34.89 \pm 1.07\%$) and Kupang ($31.07 \pm 1.94\%$), while Takalar as Mamburungan Tj. Batu was the lowest ($28.76 \pm 1.42\%$).

Table 5. The performance of selected *K. alvarezii* cultivated for 30 days in Tanjung Batu, Mamburungan, Tarakan City

No.	Phenotype	P-value	Cultivars			
			Takalar as Mamburungan Tj. Batu	Natuna* as Lampung	Tarakan (Amal)	Kupang
1.	Final weight (gr)	0.000	54.27 ± 29.68^{ab}	56.28 ± 29.81^a	50.66 ± 30.19^b	24.34 ± 13.86^c
2.	Daily Growth Rate/DGR (%/day)	0.000	5.63 ± 0.20^a	5.74 ± 0.36^a	5.40 ± 0.21^a	2.94 ± 0.34^b
3.	Phytohormones content (mg/L)	0.022	2.29 ± 0.11^b	3.22 ± 0.05^a	2.91 ± 0.69^{ab}	2.24 ± 0.03^b
4.	Carrageenan yield (%)	0.000	28.76 ± 1.42^c	42.6 ± 1.94^a	34.89 ± 1.07^b	31.07 ± 1.94^{bc}

Description: The numbers in the same row followed by different superscript letters show significant differences at the 5% test level (Tukey).

Based on the molecular analysis, Natuna* as Lampung, Tarakan (Amal), and Kupang represent the cultivars of Western, Central, and Eastern Indonesia, respectively, to be cultivated in the seawater area of Mamburungan Tanjung Batu, Tarakan City (Figure 1). Since 2016, all local farmers in this area have only developed Takalar, known as Mamburungan Tj. Batu seed as a cultivar obtained by the community with the assistance of the Dinas Perikanan Tarakan. In this study, Takalar as Mamburungan Tanjung Batu was used to control the cultivation performance of the other three cultivars.

Phenotypes need to support genotype characteristics such as cultivation performance including final weight, DGR, phytohormones levels, and carrageenan yield percentage. The Natuna* as Lampung has the highest value across all phenotypes (Table 5). Furthermore, the highest value of the final weight phenotype was obtained by the Natuna* as Lampung, followed by Takalar as Mamburungan Tanjung Batu, Tarakan (Amal), and the lowest was Kupang (Figure 6). When the average value of DGR is at least 3%, then it is categorized as good (Fadilah *et al.* 2016). Almost all cultivars had DGR values exceeding 5% and only Kupang varieties had a low DGR value of 2.94% due to osmosis factors by the difference in salinity between the waters of Central Indonesia (Tarakan 30-31 ppt) and the East Nusa Tenggara (NTT) in the Eastern part, which generally has higher salinity values (Kupang 34-36 ppt) (Simatupang *et al.* 2021).

The four cultivars studied at the time of evaluation of cultivation performance at the end of the harvest period (30 days) showed different morphological weights/W_t (Figure 5). However, at the time of planting, the weight size (W₀) for all seeds used was ±10 grams, uniform for all cultivars. These morphological characteristics are related to different physiological activities, including DGR, and phytohormone content in each cultivar type.



Figure 5. Variation of thallus morphology W₃₀ from various cultivars at harvest of cultivation

Seaweed growth is influenced by environmental factors, including the condition of the seawater where it is cultivated (Table 6) and the weather/season (Figure 6) at the planting. In general, environmental conditions in the sea water area in Mamburungan, Tanjung Batu, Tarakan is still suitable for supporting the growth and cultivation of *K. alvarezii*.

Table 6. Parameters of water quality for cultivation in Tanjung Batu Mamburungan, Tarakan City (30 days of *K.alvarezii* cultivated)

No	Variable	Unit	27 June 2021	4 July 2021	11 July 2021	18 July 2021	25 July 2021	27 July 2021	Optimum Level
1	Temperature	°C	30	30	30	30	29	30	26-32 (SNI)
2	DO	mg/L	8.29	8.14	8.20	8.18	8.25	8.30	≥5 (SNI)
3	pH	-	7.78	7.88	7.96	7.83	7.80	7.98	6.8-8.8 (SNI)
4	Salinity	ppt	31	31	31	31	31	31	28-34 (SNI)
5	Brightness	cm	75	75	75	70	75	75	≥70 (SNI)
6	Current Speed	km/hour	1.1	0.6	1	1.2	0.8	1.1	-
7	Ammonia	mg/L	0.102	0.115	0.289	0.370	0.110	0.070	≤0.300 (KepMen LH 51/2004)
8	Nitrate	mg/L	0.526	0.761	0.115	0.241	0.430	0.390	0.040-0.1 (Effendi, 2003)
9	Nitrite	mg/L	0.005	0.014	0.031	0.047	0.010	0.001	≤0.06 (CCRM, 1987)
10	Phosphate	mg/L	0.220	0.187	0.228	0.041	0.230	0.108	0.02-1.00 (Lakitan, 2000)
11	Turbidity	NTU	1	0.6	1	3.2	0.4	0.8	-

Climatology Secondary Data (BMKG Kota Tarakan, 2021)

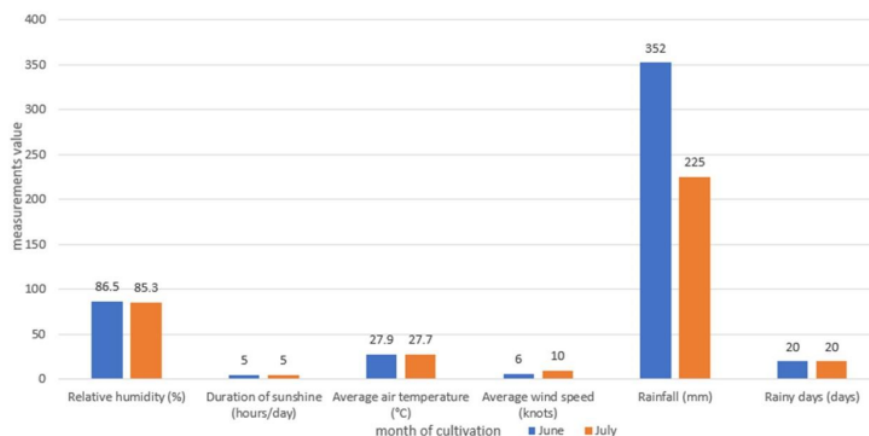


Figure 6. Climatological data during the cultivation period (Source: BMKG Tarakan City 2021)

The environmental water quality parameters of cultivation showed a range that supported the growth of *K. alvarezii* (Table 6). Based on climatological data from the BMKG of Tarakan City (2021), rainfall was relatively (352 mm) in the cultivation period in the early weeks of planting in June, and became moderate during the second week to the end of the study (225 mm). The values of other climatological data information such as relative humidity, duration of sunshine, average air temperature, average wind speed, and rainy days in June and July are not significantly different (Figure 6). They are still suitable for supporting cultivation conducted in the waters of Tanjung Batu, Tarakan City, North Kalimantan.

The growth and development of *K. alvarezii* seaweed are influenced by phytohormones, which act as growth regulators (Fadilah *et al.* 2016). In this study, the highest phytohormones in the form of kinetin-IAA ratio were obtained by Natuna* as Lampung, followed by Tarakan (Amal), Takalar as Mamburungan Tanjung Batu, and the lowest was Kupang (Table 5). In carrageenan extraction, the highest yield was obtained from Natuna* as Lampung, followed by Tarakan (Amal), Kupang, and Takalar as Mamburungan Tanjung Batu varieties. Meanwhile, carrageenan is a primary metabolite resulting from photosynthetic activity in the form of linear sulfate polysaccharides from D-galactose and 3,6 anhydrous-D-galactose extracted from Rhodophyceae class seaweed which is a cell wall filler (Campo *et al.* 2009). The carrageenan yield required by the industry is 20%, and the percentage in this study exceeded the prerequisites above and was generally higher than those reported by Simatupang *et al.* (2021).

The photosynthetic and adaptation responses of *K. alvarezii* to the exposure of solar energy are significantly influenced by the differences in the geographical areas of West, Central, and East Indonesia. Photosynthesis is the main important factor influencing the growth of algae tissue (Figure 6), where energy changes affect the average growth and division of algae cells (Mulyani *et al.* 2018). Furthermore, the growth of algae is strongly influenced by extrinsic and intrinsic factors such as type, strain, thallus used, and age. Extrinsic factors that play a significant role include the state of the physical environment, chemical nutrient waters, and management of aquaculture systems by humans (Hong *et al.* 2010; Hung *et al.* 2019; Budiyo *et al.* 2019).

New candidate strains of superior cultivar *K. alvarezii* in the form of Natuna* as Lampung cultivar needs to be developed in each geographically different cultivation area in Indonesia to expand the growing season and increase production. In addition, the superiority needs to take into account global climate challenges, adaptive development of temperature tolerance, rapid growth response, increased concentration of desired carrageenan molecules, resistance to polluting organisms (disturbing pests), and strain resistance to disease (Kim *et al.* 2017).

CONCLUSION

The *cox2-3* spacer DNA sequencing analysis on 16 samples of *K. alvarezii* seaweed seedlings cultivated in Tarakan (North Kalimantan) showed a genetic distance of 0.00-0.23 unit with seven unique haplotypes. Furthermore, the phylogenetic tree reconstruction resulted in 3 clusters representing the

selection of superior cultivar candidates. Compared to local and other cultivars cultivated in the seawater area of Mamburungan Tanjung Batu Tarakan, the Natuna* as Lampung cultivar produced the highest carrageenan yield, and it can also be developed as a candidate for superior seeds. Therefore, the *cox2-3* spacer marker has the potential to be applied as an effective and efficient tool for developing superior seeds selection in Tarakan or other water areas.

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