

Molecular Assessment of *Kappaphycus alvarezii* Cultivated in Tarakan based on *cox2-3* Spacer

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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 was analyzed using the molecular marker cyclooxygenase 2-3 intergenic spacer (*cox2-3* spacer) on the 16 cultivars collection from around Indonesia. Four haplotypes representing the *K. alvarezii* variant to be cultivated in Tarakan City were produced based on the phylogenetic clustering and further molecular analysis using a *cox2-3* spacer mitochondrial DNA marker. These include the Kupang and Natuna varieties, also referred to as Lampung seedlings, which is a tissue culture plantlets used for development at SEAMEO-BIOTROP Bogor and propagated by the Lampung Marine Aquaculture Center (BBPBL), Tarakan. These varieties have genetic distances (pairwise comparisons based on the mean Kimura 2-parameter model) ranging from 0.000-0.243 units. The cultivation was carried out for 30 days in the waters of Tanjung Batu (Tj. Batu) Mamburungan, Tarakan, North Kalimantan. The result showed that the Lampung cultivar (Natuna) has a superior daily growth rate (DGR) and carrageenan yield compared to the local cultivars (Mamburungan Tanjung Batu (Takalar), Tarakan, and Kupang). Furthermore, the genetic analysis with *cox2-3* spacer markers could be an effective seed selection tool for tracing genetic information in developing superior seedlings in aquaculture activities.

Keywords: Algae, carrageenan, DNA sequencing, phylogeny, tissue culture

Introduction

The seaweed *Kappaphycus alvarezii* is a blue carbon agent with 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 could potentially treat 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* due 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 placed along the coastal

shores with 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 ensuring a continuous supply of superior seeds for productive and sustainable cultivation. Such seeds are expected to have a higher growth rate that aligns with their production performance (Budiyanto et al., 2019) and are resistant to diseases and 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 (Fadilah et al., 2016), and genetic identification based on molecular markers using the Cytochrome Oxidase Subunit I (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 improving seed quality. On the other hand, genetic information helps find superior seed candidates and enhances cultivars' quality (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). At the same time, 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 solid extrinsic factors influence these morphological differences (Dumilag et al., 2018). The depth, water movement, and 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 mitochondrial DNA (mtDNA) genes (*i.e.*, *coi*-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). Compared to other markers (*e.g.*, *cox2*, *rbcL*, and 18S rRNA), *cox2*-3 has been successfully used for the new *Kappaphycus* cultivar in the Philippines. This is 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 to establish a connection between genetic diversity and morphological variations in *Kappaphycus*, particularly during 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. However, to our knowledge, such an effort has never been performed in Indonesia. Therefore, this study aims to obtain phylogenetic information and the cultivation performance of the selected seaweed seeds cultivated in Tarakan City waters. 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 for superior seeds for seaweed cultivation in Tarakan City, North Kalimantan.

Material and Methods

Collection of *K. alvarezii* Seed Varieties

Sixteen samples of the *K. alvarezii* genome with different varieties were divided into four populations based on location, *i.e.*, the Jawa population (Jepara), 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, then crushed or grinding using a mortar and pestle set with liquid nitrogen. Processed samples were transferred into Eppendorf tubes and stored in a freezer at -4°C.

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

The Cetyltrimethyl ammonium bromide (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'-GTACCWCTTTDRGRRKDAATGTGATGC-3' and Reverse: 5'-GGATCTACWAGATGRAAWGGATGTC-3') (Zuccarello et al., 1999). The *cox2*-3 spacer gene was used due to the tough seaweed taxonomy plastic and the lack of distinctive morphological features. 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 Mix (Bioline), and 20 µL Nuclease Free Water (NSFW). Furthermore, the 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 1.5% agarose-electrophoresis gel added with 1

μL of green fluorescent (SolutionRed Safe Nucleic Acid Staining Lot. Number: 21141) and observed using a UV transilluminator. The PCR products were sent to the 1st BASE Laboratory (<https://base-asia.com>) in Malaysia for Sanger Sequencing when a good positive band was confirmed. 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 analyzed 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 1000 \times from the nucleotide sequence of the *cox2-3* spacer. Four candidate cultivar seeds were selected from the genetic search results to observe their cultivation performance. The haplotype diversity in 3 populations of Kalimantan, Indo-eastern varieties of seaweed, and tissue culture (SEAMEO-Biotrop) was analyzed using the DnaSP v6.12.03 software (Rozas et al., 2017).

Cultivation of Selected *K. alvarezii* Seeds

A 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), and 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 one month in the indoor tub (Satriani et al., 2022) 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 three 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.

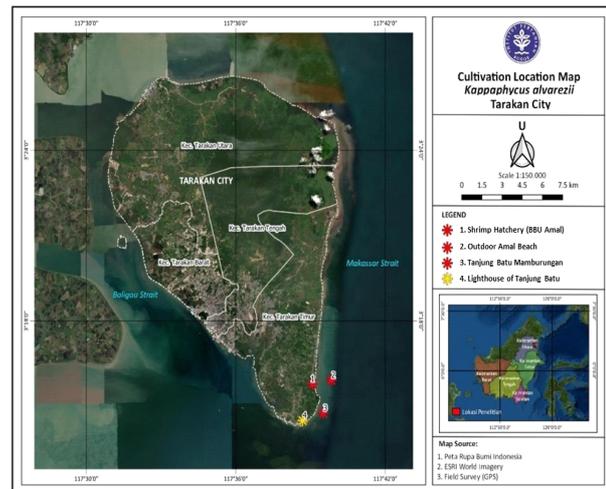


Figure 1. Locations for *K. alvarezii* Cultivation in Tarakan City, North Kalimantan.

These include temperature, pH, salinity, and DO *in situ* using a DO-meter 6+ Thermo Scientific Eutech. The Secchi disk and Flowatch FL-03 *in situ* determined brightness and current velocity, respectively. 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 Faculty of Fisheries and Marine Science University of Borneo Tarakan (FPIK-UBT). The yield level of carrageenan (AOAC International, 1990) and phytohormones levels (*high-performance thin layer chromatography*) were then analyzed at the Nutrition Laboratory of FPIK UBT and Bogor Agrochemical Residues Laboratory.

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 concerning the environmental condition of the cultivation area for 30 days of the 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 analyzed.

Results and Discussion

An Assessment *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), Mamburungan Tanjung Batu (Takalar) (No. 15), Tual (No. 16), and Maumere (No. 17 and 18) showed prominent amplification bands in contrast to other samples (Figure 2).

A study of the genetic diversity of *K. alvarezii* is essential 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.

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). Based on this phylogenetic analysis, the research's bootstrap value is 96%-97% (Figure 3), indicates 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 Lampung (Natuna) cultivar, Papua, and Sumba are grouped in one cluster. In contrast, seeds from other areas are grouped in another 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 selected as genetic sources for cultivating seeds in Tarakan. Each cluster chose one cultivar as a cultivation seed candidate (green circles) in Tarakan. According to the phylogenetic tree, Tarakan, Lampung (Natuna), and Kupang seeds were selected as representatives of each cluster. The Lampung (Natuna) cultivar is obtained from BBPBL Lampung nursery, originally from the first generation

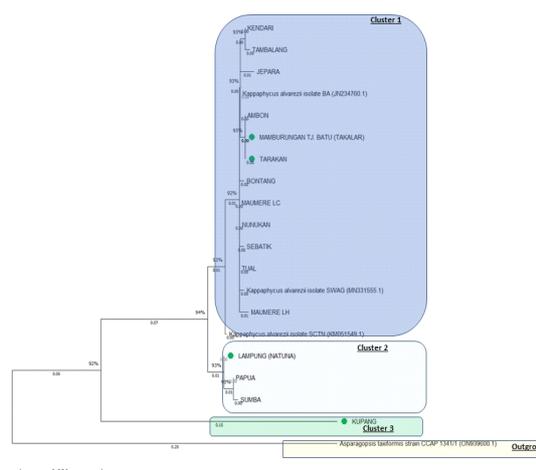


Figure 3. Phylogenetic reconstruction of *K. alvarezii* varieties based on the *cox2-3* spacer analyzed using the maximum likelihood (ML) method (1000× bootstrap replications). Green circles indicated the selected seed for cultivation experiment in Tanjung Batu Mamburungan Tarakan's seawater area. The 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 0.00-0.15, which indicates that from 100 nucleotide sequences, there are 0-15 different bases in each branch.

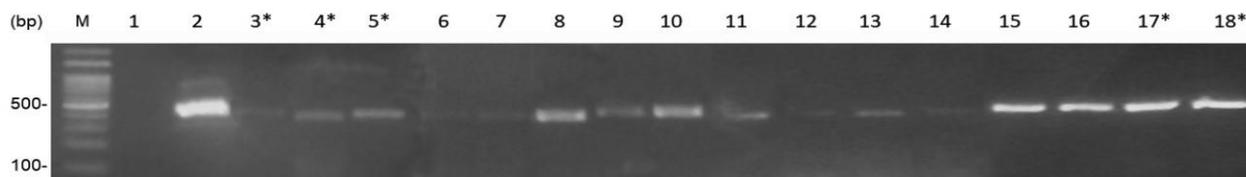


Figure 2. Amplified *cox2-3* spacer of *K. alvarezii* samples visualized on the agarose-electrophoresis gel (1.5% w/v). Sample column number: No. 1= no template control; 2= Sumba; 3= Lampung (Natuna); No. 4= Tambalangi; 5= Kendari; 6= Sebatik; 7= Situbondo; 8= Tarakan; 9= Nunukan; 10= Bontang; 11= Ambon; 12= Jepara; 13= Kupang; 14= Papua; 15= Mamburungan Tanjung Batu (Takalar); 16= Tual; 17= Maumere LH; and 18= Maumere LC. The asterisks (*) indicate tissue culture plantlets of *K. alvarezii* from SEAMEO-BIOTROP Bogor—abbreviations: bp=base pair, M=Marker (100bp DNA Ladder; Geneaid).

of tissue culture plantlets of *K. alvarezii* from SEAMEO-Biotrop Bogor. Seed from Mamburungan Tanjung Batu (Takalar) was still selected as local control in this research location Tanjung Batu Mamburungan's seawater area cultivation.

The phylogenetic tree among *K. alvarezii* from several places in Indonesia is presented in Figure 3. Tree topology shows that the 19 observed seaweed seeds have a close genetic distance, except those from Kupang. Seaweed seeds from Lampung (Natuna), 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. The Wallace line geographically divided the Indonesian waters into the West, Central, and East regions. This division indicates differences in the characteristics of reef exposures, an ecosystem 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 extensive database of DNA data already available in GenBank (Tan et al., 2012). The NCBI nucleotide BLAST of the *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).

Query coverage is the percentage of nucleotide length of the query that matches the existing sequence in the GenBank database. At the same time, percent identity matches the query and aligned database sequences (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). Besides, statistical analysis of gene tree reconstruction also required species identification. The kinship relationship between *K. alvarezii* Kupang and Jepara; also Kupang and Sumba (Table 1) are the furthest apart (0.243), explaining 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

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 Number of Reference	Accession Number of Origin (Genbank)
1.	Sumba	<i>K. alvarezii</i>	349	100%	96.14%	KM051549.1	OP136984
2.	Lampung (Natuna)	<i>K. alvarezii</i>	389	100%	96.93%	KM051549.1	OP136980
3.	Tambalang*	<i>K. alvarezii</i>	389	99%	98.71%	MN331555.1	OP136986
4.	Kendari*	<i>K. alvarezii</i>	385	100%	98.44%	MN331555.1	OP136976
5.	Maumere (LC)*	<i>K. alvarezii</i>	374	98%	99.47%	JN234760.1	OP136978
6.	Sebatik	<i>K. alvarezii</i>	391	95%	99.20%	JN234760.1	OP136983
7.	Tarakan	<i>K. alvarezii</i>	393	100%	97.73%	MN331555.1	OP136987
8.	Nunukan	<i>K. alvarezii</i>	371	99%	97.96%	MN331555.1	OP136981
9.	Bontang	<i>K. alvarezii</i>	348	100%	98.21%	JN234760.1	OP136974
10.	Ambon	<i>K. alvarezii</i>	370	99%	98.44%	JN234760.1	OP136973
11.	Jepara	<i>K. alvarezii</i>	370	100%	97.72%	MN331555.1	OP136975
12.	Kupang	<i>K. alvarezii</i>	350	99%	82.70%	KM051549.1	OP136977
13.	Papua	<i>K. alvarezii</i>	393	100%	95.18%	KM051549.1	OP136982
14.	Mamburungan Tj.Batu (Takalar)	<i>K. alvarezii</i>	352	100%	97.73%	JN234760.1	OP136985
15.	Maumere (LH)*	<i>K. alvarezii</i>	381	97%	99.20%	JN234760.1	OP136979
16.	Tual	<i>K. alvarezii</i>	378	99%	98.67%	JN234760.1	OP136988

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 identifying *K. alvarezii*. This is due to 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 were 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). Several bootstrap values are present in the phylogenetic tree branches (Figure 3), unveiling 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 three additional 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. The 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). This analysis involved sixteen sequences of our samples and three sequences of interspecies access from GenBank, and one sequence outgroup 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* is 0.00-0.243 (Table 2).

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

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	20
1	AMBON																				
2	BONTANG	0.006																			
3	JEPARA	0.012	0.012																		
4	KENDARI	0.006	0.006	0.006																	
5	KUPANG	0.234	0.226	0.243	0.237																
6	MAUMERE_LC	0.003	0.003	0.009	0.003	0.231															
7	MAUMERE_LH	0.009	0.006	0.015	0.009	0.223	0.003														
8	LAMPUNG_(NATUNA)	0.033	0.033	0.039	0.033	0.23	0.03	0.033													
9	NUNUKAN	0.003	0.003	0.009	0.003	0.23	0	0.006	0.03												
10	PAPUA	0.039	0.039	0.045	0.039	0.239	0.036	0.039	0.006	0.036											
11	SEBATIK	0.006	0	0.012	0.006	0.231	0.003	0.009	0.033	0.003	0.039										
12	SUMBA	0.042	0.042	0.048	0.042	0.243	0.039	0.042	0.009	0.039	0.003	0.042									
13	MAMBURUNGAN_TJ_BATU_(TAKALAR)	0	0.003	0.012	0.006	0.231	0.003	0.009	0.033	0.003	0.039	0.006	0.042								
14	TAMBALANG	0.009	0.006	0.009	0.003	0.241	0.006	0.012	0.036	0.006	0.042	0.003	0.045	0.009							
15	TARAKAN	0	0.006	0.012	0.006	0.234	0.003	0.009	0.033	0.003	0.039	0.006	0.042	0	0.009						
16	TUAL	0.003	0	0.006	0.003	0.228	0	0.006	0.03	0	0.036	0.003	0.039	0.003	0.006	0.003					
17	Kappaphycus_alvarezii_isolate_SWAG_(MN331555.1)	0.006	0.006	0.012	0.006	0.226	0.003	0.006	0.033	0.003	0.039	0.006	0.042	0.006	0.009	0.006	0.003				
18	Kappaphycus_alvarezii_isolate_BA_(JN234760.1)	0.003	0.003	0.009	0.003	0.231	0	0.003	0.03	0	0.036	0.003	0.039	0.003	0.006	0.003	0	0.003			
19	Kappaphycus_alvarezii_isolate_SCTN_(KM051549.1)	0.012	0.012	0.018	0.012	0.231	0.009	0.012	0.021	0.009	0.027	0.012	0.03	0.012	0.015	0.012	0.009	0.012	0.009		
20	Asparagopsis_taxiformis_strain_CCAP_1341/1_(ON939600.1)	0.326	0.318	0.331	0.324	0.352	0.321	0.322	0.303	0.321	0.302	0.323	0.307	0.323	0.329	0.326	0.319	0.317	0.321	0.312	

(*polymorphic site*) causes many haplotypes, so it also impacts the high value of haplotype diversity (Hd) is 0.7–1. In contrast, nucleotide diversity (δ) 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

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

sequenced. Such genetic diversity analyses require a minimum of 2 individuals or samples to represent a population (*i.e.*, the Jawa population) (Rozas et al., 2017), which could not be fulfilled in the present study.

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} describes 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

Table 4. AMOVA based on three populations

Analysis of variation	*d.f	Percentage of variation (%)	F_{ST}	Significance
Among population	3	1,5	0,014	$P>0.05$
Within population	12	98,5		
Total		100		

Note: *d.f. = degree of freedom

0-1; the closer to 1, the stronger it is so that the populations are increasingly isolated, *i.e.*, 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.

The geographical distance between populations can significantly affect genetic diversity, even in haplotypes. This theory is supported by Bramandito et al. (2018), which affirm that the greater the distance between populations, the greater the difference in base order. According to Nei (1987), 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 a large population size. According to Yang *et al.* (2021), the heterogeneity is increased 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 a *cox2-3* spacer indicates that there is no geographic population structuring or subdivision, and the possibility of a *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 Mamburungan Tanjung Batu (Takalar)), 5 (Tual), and 6 (Sebatik). Meanwhile, haplotype 3 (Papua) has the highest similarity with haplotype 4 (Sumba).

Nine haplotypes were found from the haplotype network analysis results (Figure 4) of the *cox2-3* spacer. 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 Mamburungan Tanjung Batu (Takalar)). There are seven unique haplotypes (*unique/private haplotypes*), namely (haplotypes 3 to 9, Papua, Sumba, Tual, Sebatik, Jepara, Kupang, and Lampung (Natuna)). This 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

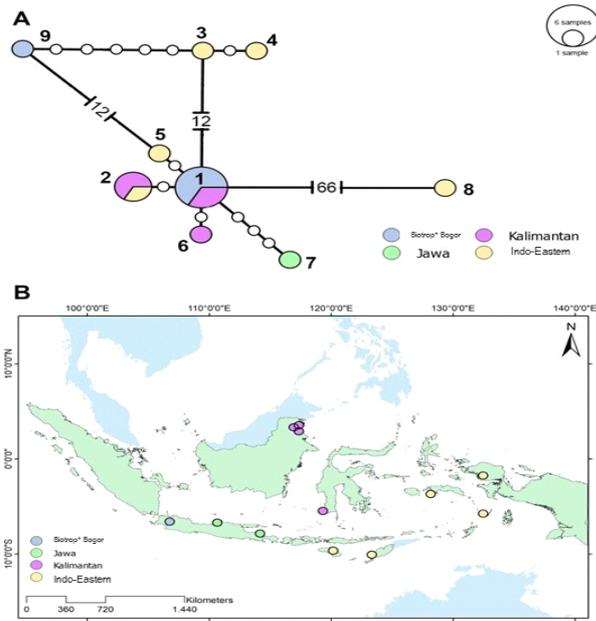


Figure 4. (A) Haplotype network diagram constructed using the Minimum Spanning Network method (Bandelt et al., 1999) on PopART software. (B) Map of Indonesia showing the locations of the four groups, namely Biotrop* Bogor, Jawa, Kalimantan, and Indo-Eastern. The map was processed from ArcMap 10.3. Description: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

inter-sample mixes with each other 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 developing 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 a risk of

plant susceptibility to disease because most *Kappaphycus* cultivated are clones (Roleda et al., 2021). Consequently, increasing plant genetic diversity, a strong defense against infection, is essential to the seaweed industry (Ekroth et al., 2019).

Performance of Cultivated Seaweed

The 30 days cultivation experiment showed significant differences among the four varieties (Table 5). Cultivars from Lampung (Natuna) 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 Mamburungan Tj. Batu (Takalar) was the lowest ($28.76 \pm 1.42\%$).

Based on the molecular analysis, Lampung (Natuna), Tarakan, and Kupang represent the cultivars of Western, Central, and Eastern Indonesia, respectively. Afterwards, these cultivars are to be cultivated in the seawater area of Tanjung Batu Mamburungan, Tarakan City (Figure 1). Since 2016, almost local farmers in this area have developed Mamburungan Tj. Batu of Takalar as a cultivar obtained by the sponsorship of the Dinas Perikanan Tarakan. In this study, Mamburungan Tanjung Batu (Takalar) was used to control the cultivation performance of the other cultivars.

Seaweed phenotypes must represent traits of high economic importance, for example, final weight, DGR, phytohormones levels, and carrageenan yield percentage. The Lampung (Natuna) has the highest value across all phenotypes (Table 5). Furthermore, the highest value of the final weight phenotype was obtained by the Lampung (Natuna), followed by Mamburungan Tanjung Batu (Takalar) and Tarakan, and the lowest was Kupang (Figure 6). When the average value of DGR is at least 3%, it is categorized

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

No.	Phenotype	P-value	Cultivars (Average±Standard Deviation)			
			Mamburungan Tj. Batu (Takalar) control	Lampung (Natuna)	Tarakan	Kupang
1	Final weight (gr)	0	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	0	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	28.76±1.42 ^c	42.6±1.94 ^a	34.89±1.07 ^b	31.07±1.94 ^{bc}

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

as good (Fadilah et al., 2016). Almost all cultivars had DGR values exceeding 5%. Only Kupang varieties had a low DGR value of 2.94% due to osmosis factors caused 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 final weights (W_f) (Figure 5). However, when planting, the initial weight (W_0) for all seeds used was ± 10 grams, uniform for all cultivars. Fadilah et al. (2016) showed that the total thallus length of the selected seaweed was 66.94% longer ($p < 0.05$) than the external control

and 48.35% longer than the internal control. The total sugar content of selected seaweed was 15.52% higher than the internal control and 16.42% higher than the external control ($p > 0.05$). During mass selection, selected and internal control seaweeds were affected by environmental conditions in the south Gorontalo Waters. The result aligns with Mastuti (2017), who states plants generally have a high degree of developmental plasticity and display various tissue or organ types. Phytohormones can increase this regenerative capacity and are factors that greatly determine the success of the growth and differentiation of plant cells (Mastuti, 2017). These morphological characteristics are related to different physiological activities, including DGR and phytohormone content in each cultivar type (Fadilah et al., 2016).



Figure 5. Variation of thallus morphology at day 30 from various cultivars.

The environmental water quality parameters of cultivation showed a range that supported the growth of *K. alvarezii* (Table 6). Based on climatological data (Figure 6) 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, sunshine duration, 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, Mamburungan, Tarakan City, and 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

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	8.2	8.18	8.25	8.3	≥ 5 (SNI)
3	pH	-	7.78	7.88	7.96	7.83	7.8	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	0.37	0.11	0.07	≤ 0.300 (KepMen LH 51/2004)
8	Nitrate	mg/L	0.526	0.761	0.115	0.241	0.43	0.39	0.040-0.1 (Effendi, 2003)
9	Nitrite	mg/L	0.005	0.014	0.031	0.047	0.01	0.001	≤ 0.06 (CCRM, 1987)
10	Phosphate	mg/L	0.22	0.187	0.228	0.041	0.23	0.108	0.02-1.00 (Lakitan, 2000)
11	Turbidity	NTU	1	0.6	1	3.2	0.4	0.8	-

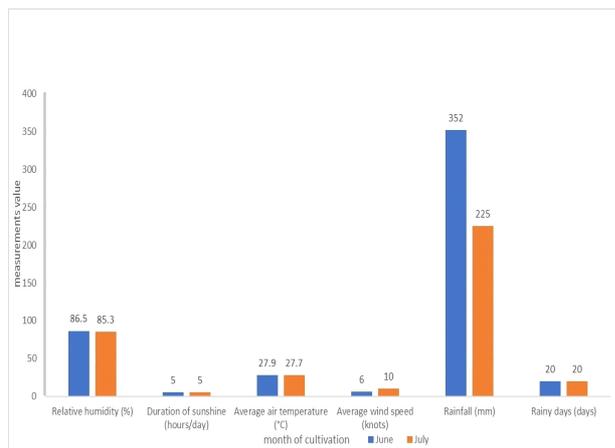


Figure 6. Climatological data during the cultivation period (Source: BMKG Tarakan City 2021).

study, the highest phytohormones in the form of kinetin-IAA ratio were obtained by Lampung (Natuna), followed by Tarakan, Mamburungan Tanjung Batu (Takalar), and the lowest was Kupang (Table 5). In carrageenan extraction, the highest yield was obtained from Lampung (Natuna), followed by Tarakan, Kupang, and Mamburungan Tanjung Batu (Takalar) 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 solar energy exposure are significantly influenced by the differences in the geographical areas of West, Central, and East Indonesia. Photosynthesis is the foremost important factor influencing the growth of algae tissue (Figure 6), where energy changes affect the average gain 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 Lampung (Natuna) cultivar need 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 consider global climate challenges, adaptive development of temperature tolerance, rapid

growth response, increased concentration of desired carrageenan molecules, resistance to polluting organisms (irritant 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 0.00-0.243 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 Tanjung Batu, Mamburungan Tarakan City, the Lampung (Natuna) 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 seed selection in Tarakan or other water areas.

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Supplementary Materials

Supplementary materials is not available for this article.

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