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Nucleotide Composition of the rbcL Gene Sequence on Cultivated *Kappaphycus alvarezii* in East Java – Indonesia

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Abstract: *Kappaphycus alvarezii* seaweed is an important fishery commodity in Indonesia because it contains carrageenan, an important main ingredient in various industries. Research on the genetic characterization of seaweed in Indonesia is important as a source of information and genetic data needed to develop aquaculture practices such as breeding programs. Therefore, this study was conducted to determine the nucleotide composition of the rbcL gene sequence in *Kappaphycus alvarezii* as part of a genetic characterization study of *Kappaphycus alvarezii* seaweed in East Java. This study used five samples taken in Banyuwangi, Situbondo and Sumenep and compared them with the *Kappaphycus alvarezii* seaweed database at GenBank. The method used was the PCR method, and the primer pairs used were F-577 5'-GTATATGAAGGTCTAAAAGGTGG-3' and R-753 5'-GCTCTTTCATACATATCTTCC-3'. Based on research, primers F-577 and R-753 succeeded in amplifying the rbcL gene in Kappaphycus alvarezii samples in a partial manner with a length of 295 – 299 bp. The DNA sequence results of the sample have a similarity of 100% to the database in GeneBank. Nucleotide composition analysis shows the existence of genetic variations in the DNA sequences studied.

Keywords: genetic characterization; *Kappaphycus alverezii*; nucleotide composition; rbcL gen

Introduction

Seaweed has become a source of food and several bioproducts used to meet human needs (Kim et al., 2017). Traditionally the fulfillment of this need was obtained from harvesting wild stock (Mantri, et al., 2016). However, with the increasing need for seaweed, seaweed cultivation is expected to meet this demand (Zuccarello and Paul, 2019). Therefore, there are many areas for seaweed cultivation spread across several regions in the world and one of the largest is in Indonesia (Grevo, 2004; Simatupang et al., 2021). *Kappaphycus alvarezii,* commercially known as *Eucheuma cottonii* (Bono, 2014) is a species of macroalga from the class Rhodophyceae (red algae). Over the last few decades, *Kappaphycus alvarezii* has become an important seaweed commodity due to its increasing demand as a major source of κ -carrageenan (Munoz et al. 2004). East Java is one of the seaweed-producing areas in Indonesia (Hidayah et al., 2020a; Hidayatulbaroroh, 2020). Seaweed production in East Java in 2015 was 608,132.5 tons, while in 2016 seaweed production was 645,274.1 tons (DKP East Java Province, 2016). Generally, the type of seaweed cultivated in East Java is *Kappaphycus alvarezii* (Andriyani et al., 2019).

Kappaphycus alvarezii is the leading industrial source of κ -carrageenan (Gereniu et al., 2017). κ-carrageenan is a polysaccharide commonly used in the food and cosmetic industry because of its gelling, stabilizing, thickening, and emulsifying properties (Necas and Bartosikova, 2013; Naseri et al., 2019). In addition, it is also used in the pharmaceutical and cosmetic industries, such as making lotions, toothpaste, and shaving foam (Ahsan, 2019).

Kappaphycus alvarezii are generally fleshy, cartilaginous and with smooth to tuberculous thalli despite the morphological plasticity commonly observed (Fadilah et al., 2016) Thalli are mostly

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cylindrical and have many, irregularly produced branches (Rudke, 2020). This type of algae is found in reddish, yellowish, brown, and green colors depending on the concentration of the phycoerythrin pigment (Tan et al., 2017). This alga is easy to cultivate and grows fast, increasing almost 4.5% wet weight daily (Jaelani et al., 2015).

Seaweed cultivation, especially of the carrageenophyte group, was first introduced in Bali using seedstocks from Bohol, the Philippines (Parenrengi et al., 2010; Porse and Rudolph, 2017). However, at this time, *Kappaphycus alvarezii* cultivation locations have spread in several coastal waters in East Java - Indonesia. namely Pamekasan Regency, Sumenep Regency, Sidoarjo Regency, Pasuruan Regency, Pasuruan City, Probolinggo Regency, Situbondo Regency, Banyuwangi Regency and Pacitan Regency (Fatmawati & Wahyudi, 2015; Andriyani et al., 2019; Hidayah et al., 2020b).

Accurately naming species (identification) and also the diversity of seaweed organisms is rather difficult to determine (Zuccarello et al., 2006; Conklin et al., 2009; Dumillag et al., 2016). This is due to the similarity of morphological characteristics and plasticity in most of the seaweed organisms (Lim et al., 2014; Verbruggen, 2014; Dumilag et al., 2023). Accurate identification information on cultivated seaweed species is necessary to develop cultivation practices such as breeding programs.

In many studies, *rbc*L sequences have been reported, and it is clear that these primers have great potential and benefit for studying the genetic variation of natural populations (Freshwater et al., 1994; Reddy et al., 2018; Yanuhar et al., 2019). In Rhodophyta, this gene is usually applied as a species-level marker for phylogenetic studies having a high mutation rate (Yang et al., 2013; Li et al., 2016). The ribulose bisphosphate carboxylase (*rbc*L) sequence method has been also widely used as genetic characterization because it can be easily repeated for related species (Lim et al., 2017; Tan et al., 2012; Alshehri et al, 2019). Based on this, the researchers wanted to study the rbcL gene by observing the nucleotide composition, especially of *Kappaphycus alvarezii* seaweed cultivated in East Java. This research is expected to be able to complete the genetic characterization data of *Kapappaphycus alvarezii* which is useful for breeding programs.

Method

Sample Collection

Sample were randomly collected in 3 farmed locations in Banyuwangi (2 samples), Sumenep (1 samples) and Situbondo (2 samples). Determination of the cultivation centers was based on recommendation from the local government. Each sample was documented using a camera for morphological observation. Approximately 50 mg of *Kappaphycus alvarezii* were place in ziplock bags for DNA preservation. Each sample is given a sample code BBr, BGr, MGr, SBr, SGr.

DNA Extraction and Amplification

DNA was extracted from 50 mg of alga using the KIT Genomic DNA Mini Kit Plant (Geneaid), following the manufacturer's instruction. The gene amplification process was carried out using the GO Taq Green PCR Mix method with several combinations of *rbc*L primers as the target gene for amplification. The master mix (Go Taq Green) was made by adding 18 µl of ddH2O, 2.5 µl of primer 1 and 2 of primer, 25 µl of Go Taq Green and 2 µl of DNA extraction. Amplification was carried out at a final volume of 50 µl. rbcL primer using F-577 5'-GTATATGAAGGTCTAAAAGGTGG-3' and R-753 5'-GCTCTTTCATACATATCTTCC-3' (Tan et al., 2012).

Cycle program used with pre-denaturation temperature settings of 94°C for 4 min, denaturation of 94°C for 1 min (35 cycles), annealing of 51°C for 30 s, elongation of 72°C for 1,5 min, and post-elongation 72°C for 10 min (Tan et al., 2012). PCR product were checked for quality and quantity using 0,8% agarose gel electrophoresis. PCR product were commercially purified and Sanger sequenced at Genetic Indonesia.

Data Analysis

Chromatogram reading, editing, and DNA alignment were performed using the MEGA 11 software (Tamura et al., 2021). Sequence alignment was performed using the ClustalW algorithm then the sequence data for each sample was calculated for its nucleotide composition using MEGA 11 software. All sequences were then aligned with the GeneBank database with BLAST (Basic Local Search Tool) (https://blast.ncbi. nih.gov/) for species confirmation.

Figure 1. Sampling of *Kappaphycus alvarezii*

Result and Discussion

The results showed that the amplification of the *Kappaphycus alvarezii* rbcL gene resulted in a fragment length of 295 – 299 bp. Based on the analysis using the nucleotide BLAST method, the seaweed samples studied had 100% similarity with *Kappaphycus alvarezii* in the GeneBank database. Therefore, the samples of seaweed studied included the species *Kappaphycus alvarezii*. The results of the rbcL gene amplification can be seen in Figure 2.

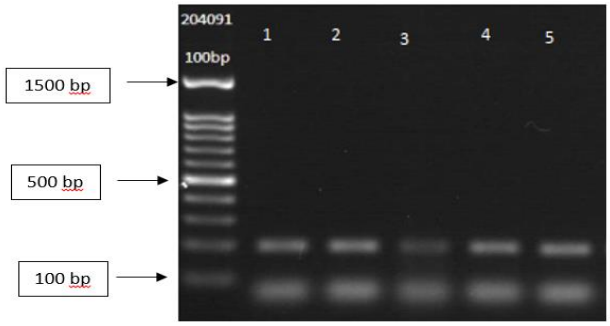

Figure 2. PCR Amplification seaweeds sampel 1) BBr, 2) BGr, 3) MGr, 4) SBr, 5) SGr

Electrophoresis results showed that *Kappaphycus alvarezii* DNA was successfully amplified using the primer pairs (Fig. 1). The results of the amplification of the rbcL *Kappaphycus alvarezii* gene showed a clear and single band pattern, as indicated by electrophoresis, which was visualized with a UV-Transluminator. According to Ardiana (2009), good electrophoresis results are indicated by thick and clear DNA bands and almost no smears. The results of DNA visualization have different band thicknesses, which can be caused by differences in the concentration of extracted DNA used for PCR (Tampanguma et al. 2020).

From the length of these fragments, the average nucleotide base composition of the five seaweed samples was T/U = 32.54%, C = 13.39%, A = 35.06%, G = 19.00%, while the average – the average nucleotide composition of A+T= 67.6% and the average nucleotide composition of C+G= 32.29. Complete data on the nucleotide base composition of each sample can be seen in the Table 1.

Table 1. Nucleotide Base composition *Kappaphycus alvarezii* sample from each location

Sample	Nucleotide Base (%)					
	T(U)	C	A	G	A+T	C+G
SGr	32.54	13.89	32.25	18.31	67.79	32.2
SBr	32.77	13.85	35.14	18.24	67.91	32.09
MGr	32.44	13.71	35.11	18.72	67.55	32.43
BBr	32.17	10.39	34.65	22.77	66.82	33.16
BGr	32.66	14.14	35.01	18.18	67.67	32.32
Avg	32.54	13.39	35.06	19.00	67.6	32.39

If the rbcL gene sequence data from the *Kappaphycus alvarezii* sample and the *Kappaphycus alvarezii* sequence data from the GeneBank database are aligned, a fragment length of 197 bp will be obtained. The nucleotide composition of each rbcL gene sequence can be seen in table 2.

Nucleotide is the smallest unit in the DNA sequence. DNA consists of nucleotide monomers consisting of 3 parts: pentose sugars, nitrogenous bases (A, T, G, and C), and phosphate groups (Toha, 2001). Table 2 shows that the difference in the composition of the T nucleotides in the sample rbcL gene sequence and the GeneBank database sequence is between 0 - 0.5%, in C nucleotides it is 0.51 - 0.47%, in A nucleotides it is 0.51 - 1, 61% and in G nucleotides there is no difference. The difference in composition A + T is 0.51 - 1.11% and the difference in composition C + G is 0.47 - 0.51%.

Based on the nucleotide base composition data above, the nucleotide bases in the *Kappaphycus alvarezii* sample were dominated by A (adenine) and T (thymine) base bonds so that the rbcL gene of this species was categorized as an AT-rich group (Kolondam et al., 2012). The percentage of AT content is also higher than the percentage of CG content. This is because the nucleotide composition in chloroplasts mainly consists of nucleotide bases Adenine (A) and Thymine (T) (Nei & Kumar, 2000). The AT hydrogen bond consists of 2 hydrogen bonds which are weaker than the GC hydrogen bond, which has three hydrogen bonds (Astarini et al., 2021).

The difference in the nucleotide composition of the rbcL gene indicates the presence of genetic variation in the seaweed (Hengkebala et al., 2018). In general, genetic variation is caused by random mating, very large population sizes, migration, mutation, recombination, and natural selection (Hartl & Jones, 1998). Variations in the nucleotide bases that make up DNA can be used to describe the level of genetic diversity (Irmawati, 2016). Genetic diversity is the diversity within species and refers to any variation either at the level of nucleotides, genes, chromosomes, or an individual's entire genome (Yow, 2013). Genetic diversity is the lowest level (hierarchy) in the level of biodiversity (Yusron, 2015). In addition, according to Claverie & Notradame (2006), analysis of nucleotide base composition can be used to determine the level of primitiveness of organisms through evolution and mutation rates compared to Adenosine (A) and Thymine (T) nucleotide base pairs in DNA strands. A lower percentage of CG content indicates that the species is more primitive (Hapsari, 2015).

Table 2. Base Nucleotide Composition *Kappaphyucus alvarezii* sample and from GenBank

Sample	Nucleotide Base (%)					
	T(U)	C	A	G	A+T	C+G
SGr	31.47	10.69	34.52	23.35	65.99	34.04
SBr	31.97	10.65	34.01	23.35	65.98	34.00
MGr	31.47	10.69	35.11	23.35	66.58	34.03
BBr	31.97	10.69	34.65	23.35	66.62	34.04
BGr	31.47	10.65	35.01	23.35	66.48	34.00
MT946315.1*	31.97	11.16	33.5	23.35	65.47	34.51
JX623985.1*	31.97	11.16	33.5	23.35	65.47	34.51
JX069175.1*	31.47	11.16	33.5	23.35	65.47	34.51
JX623999.1*	31.97	11.16	33.5	23.35	65.47	34.51
JX624003.1*	31.47	11.16	33.5	23.35	65.47	34.51
KF687988.1*	31.97	11.16	33.5	23.35	65.47	34.51

Note: *sample *Kappaphycus alvarezii* fron GeneBank

The level of genetic diversity of a species is determined by processes that occur in nature such as natural selection, migration, mutation and genetic drift (Marston & Bohnsack, 2002). In general, mutations are changes in gene composition that occur spontaneously in an allele or chromosome, resulting in genetic diversity appearing in individuals/species (Valero, et al., 2001). Mutations that occur in nucleotide sequences can be caused by environmental influences and the activity of mutagenic compounds and can change the nucleotide arrangement in DNA and will cause translation errors in the nucleotide chain (Nur & Syahrudin, 2016; Kim, et al., 2017). The level of genetic diversity of each population varies and mainly depends on variations in population size, dispersal patterns and abilities between populations, and evolutionary relationships (Yow, 2011). Chemical, physical and biological factors cause continuous environmental changes over a short or long period of time (Jump et al., 2008).

Nucleotide base substitution probability values were also calculated in this study. In protein coding genes, there are two types of nucleotide base substitutions. Transition substitutions are changes between purine bases (A and G) or pyrimidine bases (C and T). While transversion is the change from a purine base to a pyrimidine base or vice versa. The probability value of nucleotide base substitution in this study has a high percentage of transition substitution, namely 14.5878%. The value of the probability of nucleotide base substitution was determined using the K80 Maximum Likelihood (ML) analysis model.

Table3. Nucleotide Substitution Probability *Kappaphycus alvarezii* with Maximum Likelihood (ML) Analysis

From/To	A	T	C	G
A	-	*5.2061*	*5.2061*	**14.5878**
T	*5.2061*	-	**14.5878**	*5,2061*
C	*5.2061*	**14.5878**	-	*5,2061*
G	**14.5878**	*5.2061*	*5,2061*	-

Note: each value indicates the probability of substituting one base (row) with another (column). Transition substitutions are indicated in bold and transversion substitutions are indicated in *italics*.

In general, transition substitution in nucleotide base sequences occurs more easily than transversion substitution. This is because each purine and pyrimidine base has the same molecular structure. Kocher et al. (1995) explained that most of the nucleotide substitutions at the species level are transitional because they have more or less the same structure and nitrogen-carbon rings. In the table above, the transition substitution percentage between purine and pyrimidine bases has the same value, namely 14.5878%. This is related to the frequency of each nucleotide in the base composition mentioned above.

Conclusion

Based on research, primers F-577 and R-753 succeeded in amplifying the rbcL gene in Kappaphycus alvarezii samples in a partial manner with a length of 295 – 299 bp. The DNA sequence results of the sample have a similarity of 100% to the database in GeneBank. Nucleotide composition analysis shows the existence of genetic variations in the DNA sequences studied.

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Author Contributions

The first author, Alin Asyabil contributed to designing the research, conducting the research, and writing the research articles. The second author, Yenny Risjani, played a role in guiding research to writing articles. A third author, Lawrence M. Liao contributed to guiding the writing of the article. All

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Conflicts of Interest
The authors declare no conflict of interest.

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