



## Genetic Diversity Analysis of Cultivated *Kappaphycus* in Indonesian Seaweed Farms using COI Gene

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### Abstract

Indonesia is a major player in the aquaculture of red algae, especially carrageenan producing 'euचेumatoids' such as *Kappaphycus* and *Euचेuma*. However, many current trade names do not reflect the evolutionary species and updated taxonomy, this is especially the case for euचेumatoid seaweeds that are highly variable in morphology and pigmentation. Genetic variation is also not known for the cultivated euचेumatoids in Indonesia. Therefore, this study aimed to determine the species and the level of genetic variation within species of cultivated euचेumatoids from various farms across Indonesia, spanning 150-1500 km, using the DNA barcoding method. Samples of seaweed were randomly collected at 14 farmed locations between April 2017 and May 2018. For this study the 5-prime end (~ 600 bp) of the mitochondrial-encoded cytochrome oxidase subunit one (COI) was amplified and sequenced. Morphological examination showed that the samples were quite variable in branching pattern and color. All samples collected from farms with floating line cultivation were identified based on COI sequences as *Kappaphycus alvarezii* and showed no variation in the COI gene. One farm sample with bottom-line cultivation was identified as *K. striatus*. The low genetic variation is in contrast to the phenotypic variation of samples, indicating that variation and phenotypic responses to environments is still found in samples with implications for growth rates and carrageenan yield and quality. Information about the genetic variation in stocks is important base knowledge for maintaining, expanding and continuing seaweed aquaculture.

**Keywords:** COI, Indonesia, *Kappaphycus*, phylogeny, species identification

### 1. Introduction

Seaweed aquaculture is a relatively new but already a large and growing enterprise around the world (FAO, 2016). As with all new primary industries, many basic biological questions still need to be determined, including the nature of potential pathogens, the resilience of the cultivated species to perturbations, and methods to continually improve these crops. Equally important is the identification and potential genetic diversity of the species under cultivation. Indonesia is a major player in seaweed aquaculture, especially in the growth and production of carrageenan-producing red seaweeds, the

euचेumatoids, including the genus *Euचेuma* and especially *Kappaphycus*.

In 1969, *Kappaphycus* was introduced in Indonesia and became known commercially as 'cottonii' in farms that were established in 1985 (Hurtado, Gerung, Yasir, & Critchley, 2014; Hurtado, Reis, Loureiro, & Critchley, 2014). Indonesia has more than 20 developed cultivation centers, with the main areas being in Sulawesi, Kalimantan, and Nusa Tenggara. Seaweed production in 2017 was 10.5 million tons and in 2018 was 10.35 million tons (wet weight) (Kementerian Kelautan dan Perikanan, 2019). Most of the seaweeds from cultivation are exported as raw material.

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Indonesian dried seaweed exports for 2017 was 173,624 tons and for 2018 was 192,276 tons (Badan Pusat Statistik, 2019).

The local production of seaweeds is accompanied by various trade names that do not reflect the evolutionary species, this is especially the case of the commercial red seaweeds that are highly variable in morphology. In Indonesia, the major seaweed crop is called 'cottonii', a name reflecting the previous taxonomic name *Euclidean cottonii* Weber Bosse which has been synonymized to *Kappaphycus alvarezii* (Doty) Doty ex P.C. Silva (Doty, 1988; Silva, Basson, & Moe, 1996). Another name used is 'sacol' for *Kappaphycus striatus* (F. Schmitz) Doty ex P.C. Silva (previously named *Euclidean striatum* F. Schmitz). The difference between *K. striatus* and *K. striatum* is an artefact of some practitioners using the masculine or neutral species name. *K. striatus* is the accepted taxonomic name, with *striatus* meaning "striped" in Latin (Guiry & Guiry, 2020).

A related genus is also cultivated in lesser amounts, i.e. *Euclidean denticulatum* (N. L. Buman) Collins & Hervey (previously named *Euclidean spinosum* J. Agardh, trade name 'spinosum'). There were also variety of names that have been formally described relating to the name of collection sites or morphologies (e.g., tambalang, ajak-assi, maumere; Doty, 1988). The genetic distinctness, based on DNA mitochondrial and plastid gene or spacer sequences, of these varieties has been questioned (Zuccarello et al., 2006), and this variation may just reflect plasticity in particular environments or local selection.

Red algae are difficult to identify to species due to their extreme phenotypic plasticity and lack of, or difficulty in finding, diagnostic morphological characters (Verbruggen, 2014). Molecular methods have been proven to be a reliable method of identifying species and determining the level of genetic variation within species. The so-called DNA barcoding of species for molecular identification has been used extensively to identify species in red algae, to discover hidden species diversity (i.e. cryptic species) as well as to discover the levels and distribution of genetic variation in habitats (Muangmai, Fraser, & Zuccarello, 2015).

The genetic diversity, relatedness and phylogeny of euclideanoids have been studied extensively (Zuccarello et al., 2006; Tan, Lim, & Phang, 2013; Tan et al., 2012; Dumilag, Orosco, & Lluisma, 2016), using both mitochondrial and plastid DNA markers. The earliest publication showed that both mitochondrial and plastid markers clearly separated the genera *Euclidean* and *Kappaphycus* from each other and separated species within these two genera

(Zuccarello et al., 2006). This study used the Rubisco spacer, an approximately 300 bp fragment that contains the short segment (3 prime end) of the *rbcl* gene and the spacer between *rbcl* and *rbcs* plus a portion (5 prime end) of the neighboring *rbcs* gene. The mitochondrial marker used was the spacer between the cytochrome oxidase subunit 2 (COX2) and subunit 3 (COX3), this marker is also about 350 bp in length. This study also showed that in cultivation, for the majority of samples, only one mitochondrial genotype was found throughout the world.

Partial mitochondrial cytochrome oxidase subunit I gene (COI-5P) is commonly used for identifying species (Hebert, Cywinska, Ball, & deWaard, 2003), and is longer than the previously mentioned markers (~ 600 bp). The COI marker is widely used for *Kappaphycus* identification for taxonomic purposes and phylogenetic relationships (Tan, Lim, & Phang, 2013). The COI marker of red algae for this gene is very robust and it has greater variation than the COX2-COX3 spacer in euclideanoids (Dumilag et al., 2018; Dumilag & Aguinaldo, 2017). Identification of *Kappaphycus* using COI has been performed in several countries: Hawaii (Conklin, Kurihara, & Sherwood, 2009), Malaysia (Tan, Lim, & Phang, 2013), and the Philippines (Dumilag, Orosco, & Lluisma, 2016).

Follow up studies in several countries also showed that cultivated genotypes have been colonized, and even have been replacing wild genotypes in non-cultivated habitats (Halling et al., 2013; Tano, Halling, Lind, Buriyo, & Wikström, 2015; Dumilag, Orosco, & Lluisma, 2016). It is clear that molecular methods can aid in determining species of red algae, however, these methods have not been extensively applied to cultivated euclideanoids or 'cottonii' in Indonesia. Therefore, an unequivocal identification of the species in production is still not known, nor is the genetic variation in cultivation farms. This study, therefore, aims to determine the species used in cultivation from various farms across Indonesia.

## 2. Material and Methods

### 2.1. Sample Collections

Samples were randomly collected in 14 farmed locations from western Indonesia (Sumatra) to eastern Indonesia (Maluku) between April 2017 and May 2018 (Table 1). Determination of the location as a representative of the cultivation centers was based on recommendations from the local government. Approximately 2 cm of the algal apical axes were placed in ample silica gel in ziplock bags for DNA preservation.

## 2.2. DNA extraction

DNA was extracted from 0.5 g of apical tips using a modified CTAB method (Zuccarello & Lokhorst, 2005; Zuccarello & Paul, 2019), including proteinase K (4 µg) in the lysis buffer. Precipitated DNA was dissolved in 50 µL of 0.1X TE (10 mM Tris, 0.1 mM EDTA). The 5-prime end, ~ 600 bp, of the mitochondrial-encoded COI was amplified using the following PCR mix. A 20 µL of PCR volume containing: 1X buffer, 2.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.5% bovine serum albumin (BSA), 1 U *Taq* polymerase (Vivantis, Selangor Darul Ehsan, Malaysia) and 7.5 pmol of the COI primers using GazF1 (5' TCAACAAATCATAAAGATATTGG 3') and GazR1 (5' ACTTCTGGATGTCCAAAAAYCA 3') (Saunders, 2005).

The PCR cycling conditions were: an initial denaturing at 94°C for 5 min; followed by 35 cycles of 95°C for 30 s, 45°C for 1 min and 72°C for 1 min, and a final elongation step of 72°C for 5 min. PCR products were checked for quality and quantity using 1% agarose gel electrophoresis. PCR products were commercially purified and Sanger sequenced at 1<sup>st</sup> BASE (Singapore).

## 2.3. Phylogeny and DNA Diversity Measurement

Samples were sequenced in both directions using the amplification primers. Sanger sequence traces were assembled, edited and extracted to an alignment in Geneious Prime (<https://www.geneious.com>). COI sequences of various haplotypes (sequence variants) and species of *Kappaphycus* were also downloaded from National Center of Bioinformatics Institute (NCBI) (<https://www.ncbi.nlm.nih.gov/>) and added to the alignment. Alignment was done using ClustalW, no indel were observed in the alignment.

A maximum-likelihood analysis was implemented using IQ-tree (Trifinopoulos, Nguyen, Haeseler, & Minh, 2016). IQ-tree was used to select the molecular evolution model for each codon position (Modelfinder) and construct a maximum-likelihood (ML) trees with 500 bootstrap approximations. A divergent sequence of *Kappaphycus* (*Kappaphycus* sp. VYT 2013a, GenBank Accession Number KC192782) was used as an outgroup. Base pair differences between samples were determined using MEGA6 ([www.megasoftware.net](http://www.megasoftware.net)) (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

## 3. Results and Discussion

### 3.1. Morphology of Seaweed

Samples were collected from floating lines throughout Indonesia (Table 1), plus a single sample

from a bottom-line farm in Poto Tano, West Nusa Tenggara. Floating line samples were quite variable in morphology (branching pattern and color). Generally, observation of morphology showed three types of color variation: brown (Pangkep, Bontang, Bantaeng, Lombok, Mamuju, Sumbawa, Maumere), red-brown (Manado, Kupang, Banten, Gorontalo, Saumlaki, Tual) and green-brown (Lampung) with many branches (Figure 1). The samples from Sumatra, West Nusa Tenggara and Java have brown color with many branches and small thalli, different from those collected in Sulawesi and Maluku, which have brown and red-brown color with a large thallus and many branches.

Strains from Lampung are a known tissue culture variant that has been widely developed by the farmers. Previous studies on *K. alvarezii* and color variation from several areas in Malaysia, have also found a range of colors from brown, green, to yellow and pink (Tan, Lim, & Phang, 2013). Sample from Sumbawa, West Nusa Tenggara was identified as *K. striatus* with green color and compact thalli (Figure 2; Table 1). *K. striatus* or sacol (Dumilag, Orosco, & Lluisma, 2016; Ask & Azanza, 2002) is a seaweed that is generally cultivated in this area. *K. striatus* generally has green and yellow-green color with dense and compact thalli, giving a cauliflower-like appearance; the surface was smooth and cartilaginous, and had occasional blunt protrusions (Tan, Lim, & Phang, 2013).

### 3.2. Analysis of DNA Sequences

PCR amplification was successful after various DNA dilutions (up to 100-fold). The edited sequences were aligned to a COI data set consisting of multiple species and isolates of *Kappaphycus* (*K. alvarezii*, *K. malensiaus*, *K. striatus*) downloaded from NCBI GenBank (Figure 2). The aligned dataset was 627 bases long. The molecular evolution models used for the maximum-likelihood tree (1<sup>st</sup> codon position= K2P+I; 2<sup>nd</sup> codon= F81+F; 3<sup>rd</sup> codon= HKY+F) produced a phylogeny with a log likelihood score of -1342.6655 (Figure 2). All samples from floating lines (1-13) were identified based on COI sequences as *K. alvarezii* and grouped with other *K. alvarezii* samples (Figure 2; Table 1).

*K. alvarezii* from all samples also had identical haplotypes (no sequence variation), which was identical to the major cultivated haplotype (GenBank accession number KT316556) Dumilag et al., 2016) (Table 1). The bottom-line sample (14) was identified using COI sequences as *K. striatus* (Figure 2; Table 1). There were 27 basepair differences (4%) between the COI sequence of samples of *K. alvarezii* and that of *K. striatus* from Indonesia.

Table 1. Identification of samples using COI gene

No.	Sample name	Location	GPS coordinates	Date of collection	Sample identification	COI haplotype (GenBank accession number)
1	Pangkep	Boddie, Pangkep, South Sulawesi	S 4°35'45.42", E 119°24'55"	May, 2017	<i>K. alvarezii</i>	KT316556
2	Bontang	Bontang, East Kalimantan	N 0°07'37.28", E 117°31'35.35"	May, 2017	<i>K. alvarezii</i>	KT316556
3	Bantaeng	Bantaeng, South Sulawesi	S 5°29'32.82", E 119°54'12.36"	May, 2017	<i>K. alvarezii</i>	KT316556
4	Manado	Kulu, North Minahasa, Manado, North Sulawesi	N 1°41'43.02", E 124°57'19.98"	May, 2017	<i>K. alvarezii</i>	KT316556
5	Lombok	Serewe, East Lombok, West Nusa Tenggara	S 8°54'1.75", E 116°30'41.04"	May, 2017	<i>K. alvarezii</i>	KT316556
6	Banten	Tirtayasa, Serang, Banten	S 5°57'49.09", E 106°17'48.678"	April, 2017	<i>K. alvarezii</i>	KT316556
7	Kupang	Kupang, East Nusa Tenggara	S 10°9'1.81", E 123°32',31"	May, 2017	<i>K. alvarezii</i>	KT316556
8	Gorontalo	Langge, North Gorontalo, Gorontalo	N 0° 48'57", E 122° 50' 52"	May, 2017	<i>K. alvarezii</i>	KT316556
9	Mamuju	Mamuju, West Sulawesi	S 2°37'55.43", E 118°57'38.86"	April, 2017	<i>K. alvarezii</i>	KT316556
10	Lampung	South Lampung, Lampung, Sumatra	S 5°43'84", E 105°47'898"	May, 2017	<i>K. alvarezii</i>	KT316556
11	Saumlaki	Saumlaki, Southeast west Maluku, Maluku	S 7°58'51", E 131°17'46"	May, 2018	<i>K. alvarezii</i>	KT316556
12	Maumere	Kojadoi, Sikka Maumere, East Nusa Tenggara	S 8°29'39.7", E 122°24'4.4"	May, 2018	<i>K. alvarezii</i>	KT316556
13	Tual	Tual, Southeast Maluku, Maluku	S 5°40'12", E 132°44'56"	May, 2018	<i>K. alvarezii</i>	KT316556
14	Sumbawa	Poto Tano, Sumbawa, West Nusa Tenggara	S 8°42'27,7", E 116°46'32,9"	May, 2018	<i>K. striatus</i>	KC192776

While the carrageenan industry in Indonesia has expanded immensely, an accurate identification of the carrageenan-producing algae in cultivation is often missing. This study sampled eucheumatoids across the expanse of Indonesia and cultivation areas and shows that crops of 'cottonii' (Hurtado et al., 2014; Ask & Azanza, 2002) were all belong to the species *K. alvarezii*. This study also shows that genetic variation, as measured in mitochondrial COI

haplotypes, is limited in the cultivated *K. alvarezii* in Indonesia.

This low genetic variation in cultivation has already been reported (Zuccarello et al., 2006; Dumilag, Salvador & Halling, 2016) and is believed to be due to the clonal (fragmentation) method of cultivation and transportation around the country and the world. The world-wide cultivated haplotype was determined using the mitochondrial COX2-COX3 spacer (Zuccarello et

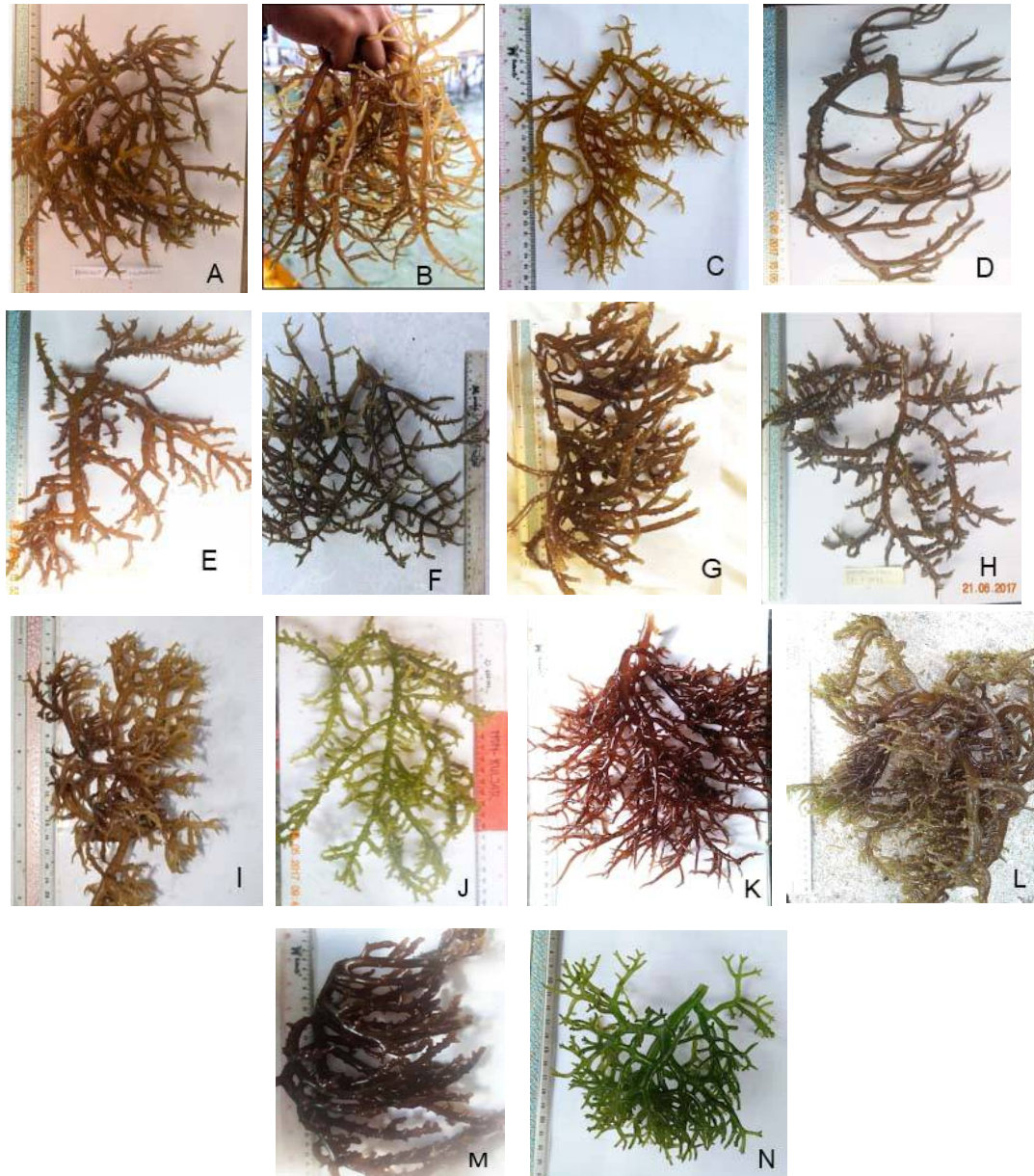


Figure 1. Morphological and color variation in samples collected around Indonesia. A= Pangkep (South Sulawesi), B= Bontang (East Kalimantan), C= Bantaeng (South Sulawesi), D= Manado (North Sulawesi), E= Lombok (West Nusa Tenggara), F= Banten, G= Kupang (East Nusa Tenggara), H= Gorontalo, I= Mamuju (West Sulawesi), J= Lampung, K= Saumlaki (Southeast West Maluku), L= Maumere (East Nusa Tenggara), M= Tual (Southeast Maluku), N= Sumbawa (West Nusa Tenggara).

al., 2006) and was designated as E3. This E3 haplotype is also found in plants with the KALV COI haplotype (GenBank accession number KT316556) (Dumilag et al., 2016), the haplotype found in all Indonesian *K. alvarezii* examined in this study.

Information about genetic variation is an important factor in maintaining, expanding and continuing seaweed aquaculture. Based on COI haplotypes, this study showed that variation in the Indonesian strains of *Kappaphycus* is very low in cultivation, including

samples acclimated and under cultivation at scale across a broad range of sites that have been propagated from fragmentation or tissue culture. The low genetic variation in *Kappaphycus* is in contrast to the phenotypic variation of samples, indicating that genetic variation, and phenotypic responses to environments is still found in samples but cannot be identified using the COI marker. We recommend that native genetic variants be looked for in Indonesia to diversify the genetic stock across the country. These



Figure 2. Maximum-likelihood phylogeny of partial COI DNA sequences of *Kappaphycus* samples from around Indonesia plus identified sequences from GenBank with their accession numbers. Indonesian sequences were mostly *K. alvarezii*, plus one *K. striatus*. Scale bar = substitution/site

variants should be tested for useful aquaculture and carrageenan gel traits.

#### 4. Conclusion

Our study revealed that *K. alvarezii* from floating lines on farms across Indonesia was identical to the major cultivated haplotype (GenBank accession number KT316556) based on COI sequences. Species from bottom-line sample was identified as *K. striatus* using COI sequences. There was no variation in the cultivated Indonesian strains of *K. alvarezii* used in this study. This finding was in contrast to the phenotypic variation of samples. These results indicated that phenotypic responses to environments and underlying genetic factors are still found in samples. This has implications for growth rates as well as carrageenan yield and quality in these samples in Indonesia.

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