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Morphological and Molecular Studies of Undescribed *Kappaphycus* Species

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Abstract Morphological and molecular studies were carried on an undescribed species of *Kappaphycus* (AS 12) from Sabah, Malaysia. The species displays unique and different physical characters from *Kappaphycus alvarezii*, *K. striatus*, *K. malesianus* and *Eucheuma denticulatum* in terms of branching patterns, thalli texture and colors. The phylogenetic position of this plant was inferred by *cox*2-3 spacer, intergenic transcribed spacer (ITS) region and RuBisCo spacer. Based on the results from this study, it is clear that the specimen AS 12 pointed to significant differences from *K. alvarezii*, *K. striatus*, *K. malesianus* and *E. denticulatum* has supported by both morphological and molecular analyses.

Keywords *cox*2-3 spacer; *Eucheuma*; ITS region; *Kappaphycus*; RuBisCo spacer

1 Introduction

This study is a morphological report of an undescribed species of *Kappaphycus* encountered during our sampling trips to Semporna, Sabah, Malaysia. The morphological characters exhibited in this plant are unique and different as compared to the related *Kappaphycus* and *Eucheuma* species. Its phylogenetic position is proposed to be examined using three molecular markers including mitochondrial-encoded *cox*2-3 spacer, nuclear-encoded ribosomal internal transcribed spacer (ITS) and plastid-encoded RuBisCo spacer, to clarify the taxonomical position of this entity at the species level.

The physical characteristic of *Kappaphycus* and *Eucheuma* tend to be variable where it is manipulated by both genetic make-up and environmental influences and apparently from spontaneous mutations (Neish, 2008). They are different by coloration, branch structure and other morphologies based on environments where they grow. They tend to be highly variable, enabling them to colonize better in variety of habitats and thrive in different environment regimes. The differences in their morphology maybe due to the interaction between light, water currents, water depth and nutrient availability (Santelices, 1999; Munoz et al., 2004; Thirumaran and Anantharaman, 2009; Góes and Reis, 2011).

2 Materials and Methods

Specimens for this study (AS 12) were obtained from Sebangkat Island with latitude of 4° 33' 18.8994" and longitude of 118° 39' 18.7806" in Semporna, Sabah on 16 October 2012. Gross external morphology was examined and described. Genomic DNA was extracted from approximately 100 mg of thalli ground in liquid nitrogen by using CTAB DNA extraction procedure outlined by Zuccarello et al. (2006). PCR amplifications of the *cox*2-3 spacer (Zuccarello et al., 2006), ITS region (White et al., 1990) and RuBisCo spacer (Tan et al., 2013) were carried out using primers shown in Table 1. PCR amplification was conducted using TopTaq DNA Polymerase (QIAGEN, Inc, USA) according to manufacturer's instruction. Cycling condition of the amplification was carried out as follow: initial denaturation at 94 ℃ for 3 min, followed by 30 cycles of each consisting denaturation at 94 ℃ for 30 sec, annealing at 50 - 56 ℃ according to primers used for 30 sec and extension at 72 ℃ for 1 min, and lastly final extension at 72 ℃ for 10 min. Annealing temperature was manipulated to obtain optimal PCR products. PCR products were purified by using the QIAquick Gel Extraction Kit (QIAGEN, Inc,

USA) in accordance with the manufacturer's protocol before direct sequencing. The sequences of the forward and reverse strands were determined by using an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, USA).

The similarity of the sequence was then verified using the Basic Local Alignment Search Tool (BLASTn). Maximum parsimony (MP) analyses were conducted using MEGA 5 software (Tamura et al., 2011). The MP trees were obtained using the Close-Neighbor-Interchange algorithm (Nei and Kumar, 2000) with search level 3 in which the initial trees were obtained with the random addition of sequence (10 replicates). All positions containing gaps and missing data were eliminated from the dataset (Complete Deletion option). The sequence divergences were calculated using Kimura's two-parameter distance bootstrapped using MEGA 5 with 1000 replications to calculate the standard deviation.

Table 1 Nucleotide sequences of the PCR primers for amplification of target regions.

Region	Primer sequence (5' – 3')		T_a (℃)	References
*cox*2-3	COX2for	GTACCWTCTTTDRGRRKDAAATGTGATGC	50	Zuccarello et al. 2006
spacer	COX3rev	GGATCTACWAGATGRAAWGGATGTC		
ITS region	NS7 forward	GAGGCAATAACAGGTCTGTGATGC	56	White et al. 1990
	ITS4	TCCTCCGCTTATTGATATGC		
RuBisCo	pRBCf	TGTGGACCTCTACAAACAGC	52	Tan et al. 2013
spacer	pRBCr	CCCCATAGTTCCCAAT		

Fig.1. Undescribed *Kappaphycus* sp. showing morphological differences from *K. alvarezii*, *K. striatus*, *K. malesianus* and *E. denticulatum*

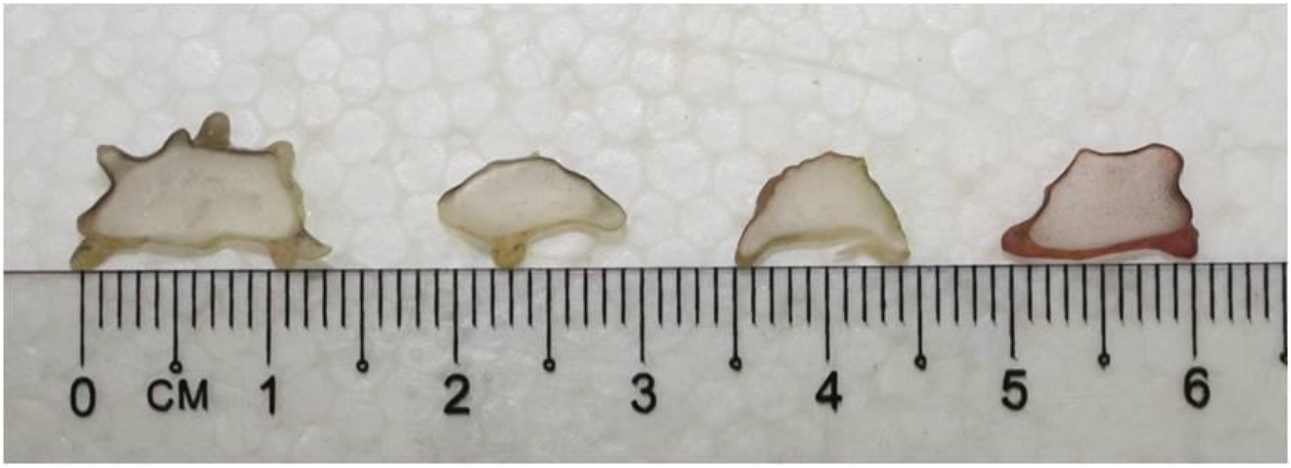

Fig.2. The diameter of thalli from different branches

Fig.3. Unevenly protrusions from the surface throughout the thalli

3 Results

3.1 Morphological observations

The specimen was found in sandy substrata attached on the dead corals by means of discoid holdfast. The plant is brownish-red to green in color with less than 15 cm in size (Fig. 1). Branching pattern is irregular to sympodial, indeterminate and thick. The basal stem expands into multiple primary branches. The subsequent branching are observed to be irregular dichotomous. Terminal branches are blunt-ended. The thallus is roughly in trapezoid shape with diameter less than 1.5 cm (Fig. 2). The thalli texture is characteristically hard and thick. The surface is unevenly rough with blunt protrusions (length < 0.5 cm) throughout the thalli (Fig. 3). The blunt protrusions are dense and determinate. The frequency of the protrusions is increased with each branching.

Table 2 Statistic of *cox*2-3 spacer, ITS region and RuBisCo spacer MP analyses.

Characters	*cox*2-3 spacer	ITS region	RuBisCo spacer
No. of taxa	12	10	11
Length (bp)	345	1024	259
No. of variable sites (%)	73 (20.98%)	251 (21%)	22 (8.03%)
No. of informative sites (%)	72 (20.69%)	212 (20.70%)	19 (6.93%)
MP tree length	87	218	20
Rescaled consistency index (RC)	0.93	0.98	0.91

3.2 Molecular analyses

In *cox*2-3 spacer, a total of 345 bp were aligned for 11 taxa from GenBank and specimen AS 12. Total of 73 sites were variable (20.98%) and 72 sites (20.69%) were parsimoniously informative (Table 2). The MP analysis tree length was 87 and the rescaled consistency index (RC) was 0.93. The sequences differed by up to 68 bp (19.54%) pairwise distance between *K. striatus* and AS 12 (data not shown).

A total of 1024 bp in ITS region for 9 taxa from GenBank and specimen AS 12 were aligned. About 251 sites (21%) were variable and 212 sites (20.70%) were parsimoniously informative (Table 2). The MP analysis tree length was 218 and the RC was 0.98. The sequences differed by up to 161 bp (15.72%) pairwise distance between *E. denticulatum* and AS 12 (data not shown).

In RuBisCo spacer, a total of 259 bp for 10 taxa from GenBank and specimen AS 12 were aligned. A total of 22 sites were variable (8.03%) and 19 sites (6.93%) were parsimoniously informative (Table 2). The MP analysis tree length was 20 and the RC was 0.91. The sequences differed by up to 13 bp (4.74%) pairwise distance between *E. denticulatum* and AS 12 (data not shown).

Based on resulting phylogenetic trees inferred from *cox*2-3 spacer (Fig. 4), ITS region (Fig. 5) and RuBisCo spacer (Fig. 6), the specimen AS 12 was positioned within the *Kappaphycus* clade forming a sister group to the *K. alvarezii*, *K. striatus* and *K. malesianus*. All the sequences of this specimen based on *cox*2-3 spacer, ITS region and RuBisCo spacer were deposited in the GenBank nucleotide sequences database with the accession numbers: JN897022, KC571238 and JX997823.

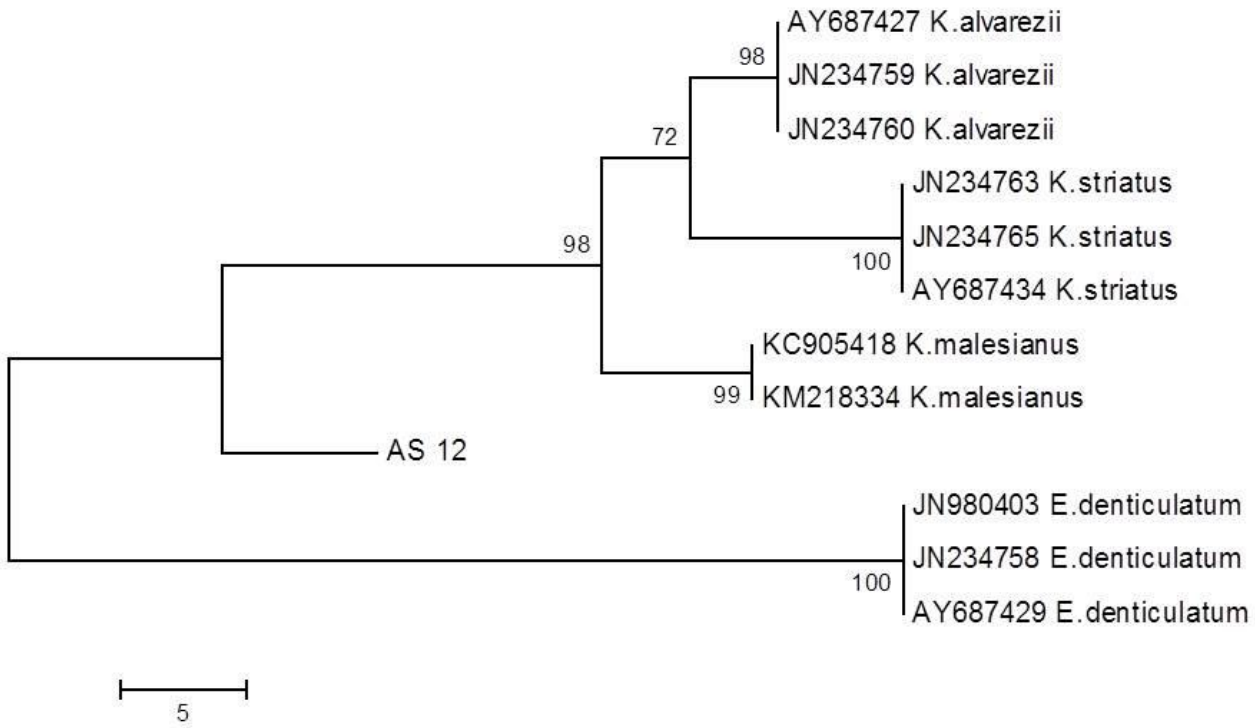

Fig.4. Phylogenetic tree based on the *cox*2-3 spacer sequences produced by the maximum parsimony method; support from 1000 bootstrap replicates is shown along the branches. Scale bar underneath the tree indicates the number of substitutions per site

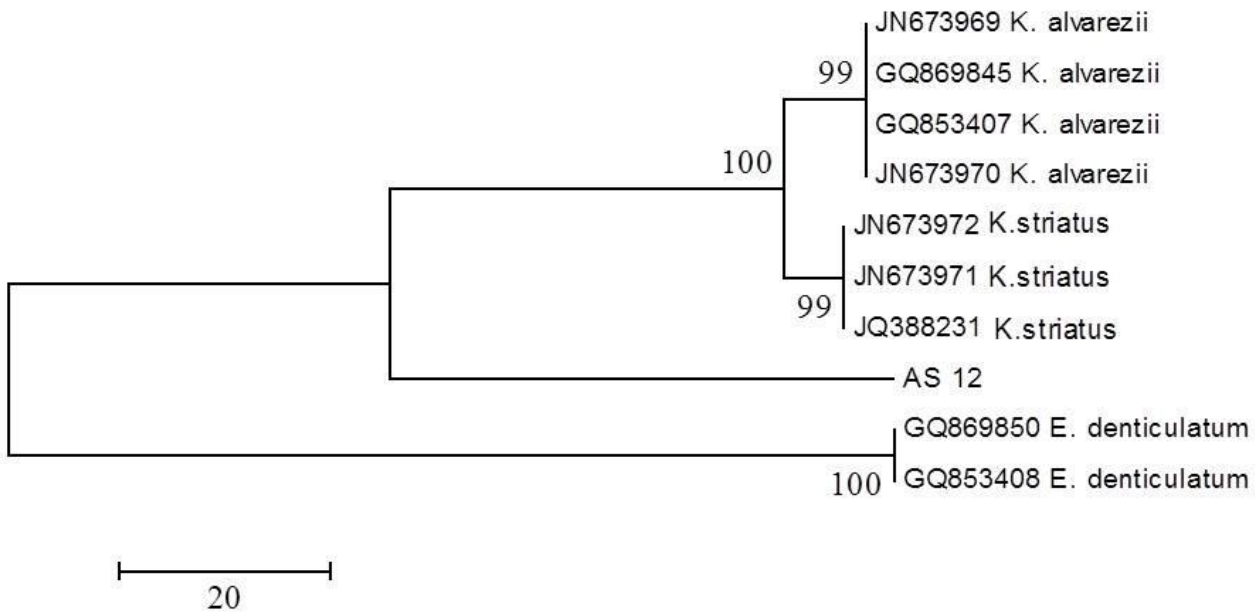

Fig.5. Phylogenetic tree based on the ITS region sequences produced by the maximum parsimony method; support from 1000 bootstrap replicates is shown along the branches. Scale bar underneath the tree indicates the number of substitutions per site. ITS region sequence for *K. malesianus* is not available in GenBank

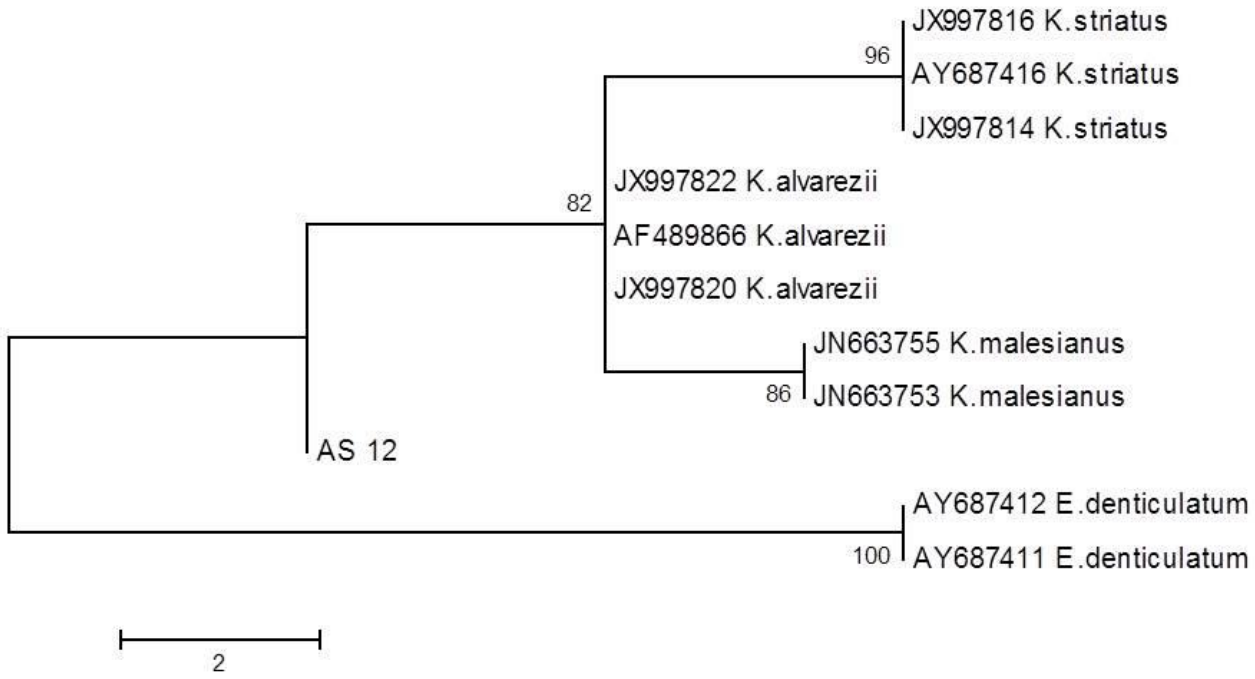

Fig.6. Phylogenetic tree based on the RuBisCo spacer sequences produced by the maximum parsimony method; support from 1000 bootstrap replicates is shown along the branches. Scale bar underneath the tree indicates the number of substitutions per site

4 Discussion

A morphological analysis has been done for *Kappaphycus* and *Eucheuma* samples based on plant size, color, branch diameter, branching patterns and thalli texture (Tan et al., 2013). Branch diameter, branching patterns and thalli texture are the main differentiating criteria for *K. alvarezii*, *K. striatus*, *K. malesianus* and *E. denticulatum*. Specimen AS 12 has the largest branch diameters, followed by *K. alvarezii*, *K. striatus*, *K. malesianus* and *E. denticulatum*. Specimen AS 12 has low branching frequency as compared to *K. striatus* (highest branching frequency), *K. malesianus* and *K. alvarezii*. The main morphological difference between *K. alvarezii*, *K. striatus*, *K. malesianus* and specimen AS 12 is that *K. alvarezii* is characterized by its long and cylindrical thalli and sparse branches with sharp pointed, *K. striatus* is characterized by stubby and thick cylindrical branches with blunt and forked tips, which resemble a cauliflower shape (Hurtado et al., 2008), *K. malesianus* is characterized by smoother and slender to flexuous terminal branches without blunt protuberances scattered throughout its thalli (Tan et al., 2014), while specimen AS 12 is characterized by its thick and trapezoid-like thalli covered with dense short protrusions. Morphologically the specimen AS 12 can be readily distinguished from *K. alvarezii*, *K. striatus*, *K. malesianus* and *E. denticulatum* by having entire margins and the short protrusions as compared to other *Kappaphycus* species and *E. denticulatum*. Unlike the fleshy and cartilaginous nature of *Kappaphycus* species, the thallus of the specimen AS 12 is harder and inflexible. The size of plant is obviously smaller than *K. alvarezii*, *K. striatus*, *K. malesianus* and *E. denticulatum*.

The mitochondrial-encoded *cox*2-3 spacer, nuclear-encoded ribosomal internal transcribed spacer (ITS) and plastid-encoded RuBisCo spacer were selected because they are adequate molecular markers for the determination of species boundaries according to number of studies carried out on different orders of red algae including *Kappaphycus* and *Eucheuma* (Zuccarello et al., 2006; Conklin et al., 2009; Zhao and He, 2011; Tan et al., 2013). Our molecular phylogenetic analyses revealed its uniqueness, with a sufficient amount of gene sequence divergence from its putative relatives. The high level of congruence and bootstrap values between trees of *cox*2-3 spacer, ITS region and RuBisCo spacer datasets showed the distinctness of the specimen AS 12. The specimen AS 12 was placed in *Kappaphycus* clade, as a sister taxon to *K. alvarezii*; *K. striatus* and *K. malesianus*. This specimen is believed to have shared the same common ancestor with that of *Kappaphycus* species. In addition, there is no affinity of the specimen AS 12 to the gene sequences available in GenBank and it might probably be a new species or variety. The exact taxonomic status of this entity remained unresolved and its true identity will require further clarification.

Author's contributions

V. Y. Thien collected the samples and conducted the experiments. W. T. L. Yong and G. J. W. L. Chin supervised the samples preparation, analysis and characterization. All authors read and approved the final manuscript.

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