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Kappaphycus malesianus sp. nov.: a new species of *Kappaphycus* (Gigartinales, Rhodophyta) from Southeast Asia

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Abstract A new species, *Kappaphycus malesianus*, is established as a new member of the genus *Kappaphycus*. Locally known as the "*Aring-aring*" variety by farmers in Malaysia and the Philippines, this variety has been commercially cultivated, often together with *Kappaphycus alvarezii* due to the similarities in morphology. Despite also producing kappa-carrageenan, the lower biomass of the *K. malesianus* when mixed with *K. alvarezii* ultimately affects the carrageenan yield. Morphological observations, on both wild and cultivated plants, coupled with molecular data have shown *K. malesianus* to be genetically distinct from its *Kappaphycus* congeners. The present study describes the morphology and anatomy of this new species as supported by DNA data, with additional morphological features for distinguishing between commercial *Kappaphycus* cultivars.

Keywords *Kappaphycus* · Carrageenophyte · Phylogenetics

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Introduction

According to Doty (1988), *Kappaphycus* consists of five species, currently named *Kappaphycus alvarezii* (Doty) Doty ex P. C. Silva, *Kappaphycus cottonii* (Weber-van Bosse) Doty ex P. C. Silva, *Kappaphycus inermis* (F. Schmitz) Doty ex H. D. Nguyen & Q. N. Huynh, *Kappaphycus procrusteanus* (Kraft) Doty and *Kappaphycus striatus* (F. Schmitz) Doty ex P. C. Silva, as well as one variety *K. alvarezii* var. *tambalang* (Doty). The latter variety was recorded as not being validly described (Guiry and Guiry 2013). Among the recognized species, at least two, i.e. *K. alvarezii* and *K. striatus*, are currently commercially cultivated worldwide for the kappa-carrageenan they produce.

The first attempt to domesticate *Kappaphycus* was initiated during the 1970s by Maxwell Doty (Doty and Alvarez 1975; Doty 1985; Trono 1992) to reduce reliance on harvests from dwindling wild populations and to increase productivity. *K. alvarezii* (as *Eucheuma alvarezii* then) was successfully cultivated and since then has been introduced into numerous countries, e.g. Africa, Brazil, China, Columbia, Fiji, Hawaii, India, Indonesia, Malaysia, Mexico, Singapore, Vietnam and among others for farming (Ask et al. 2003; Bindu 2010; Bixler and Porse 2010; Munoz et al. 2004; Neish 2003; Phang et al. 2010; Pickering 2006). The domestication of other *Kappaphycus* or *Eucheuma* plants gradually followed, most of which were undocumented, leading to an extensive (and confusing) gamut of farmed carrageenophytes, each with different local or commercial names. Tan et al. (2013) compared most locally derived varieties including the *K. alvarezii* var. *tambalang* (Doty), where many were not recognized as distinct species. However, there were still genetically different cultivars that remain unaddressed, one of which was the variety called "*Aring-aring*".

Locally called as "*Aring-aring*" variety in Malaysia, the name probably originated from the southern Philippines ("*Aring*" is not a valid vocabulary of the Malay language), and most likely refers to the same *Kappaphycus* species

reported by Villanueva et al. (2011). Capable of growing up to approximately 50 cm, cultivated "*Aring-aring*" has been reported to exhibit shades of brown and green, with irregular furcation of branches which are generally slimmer than those of *K. alvarezii* and *K. striatus*. The thalli have been observed to be smooth, fleshy, cartilaginous and flexible (Tan et al. 2013). The paucity of apparent and distinctive phenotypic characters has rendered the differentiation of "*Aring-aring*" varieties from the common *K. alvarezii* challenging, particularly to the untrained eye. This has often led to the mixed cultivation of both "*Aring-aring*" and *K. alvarezii* by local farmers, thus decreasing the effective carrageenan yield per farm. The potential reduction in carrageenan yield can be ascribed to the relatively slow growth rate and smaller biomass of the "*Aring-aring*" as compared to *K. alvarezii*, even though both species produce kappa-carrageenan. Wild specimens of "*Aring-aring*" earlier reported by Tan et al. (2013) appeared to be different from the morphologies observed in domesticated strains.

This study investigates the morphological and molecular aspects of the "*Aring-aring*" variety, along with comparisons to other *Kappaphycus* counterparts in order to verify its taxonomic status as a new species of *Kappaphycus*.

Materials and methods

Wild "*Aring-aring*" specimens were collected via snorkeling and scuba diving near the Karindingan and Sabangkat Islands, Sabah, Malaysia as well as Indonesia. Specimens, be they fertile or not, were sampled with the holdfast intact.

Morphological observations

Upon collection, the fresh specimens were photographed, and the external morphology subsequently observed and described. Free hand sections at several portions of the (fresh) plant were made and studied under a stereomicroscope (Olympus BX41). Certain specimens were stained with aniline blue for better contrast. The remaining specimen was then air-dried as a herbarium voucher and deposited in the University of Malaya Seaweed and Seagrasses Herbarium (KLU), Malaysia.

Molecular work

Small portions of the fresh thalli were excised and kept dry with silica gel for molecular studies.

Similar molecular protocols were used as that described by Tan et al. (2013), with the use of four genetic markers *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L for the phylogenetic inference of *Kappaphycus*. Primers and PCR parameters for all four DNA markers followed the original protocols by respective authors: *cox*1 (Geraldino et al. 2006; Yang et al. 2007), *cox*2 (Tan et al. 2012), *cox*2-3 spacer (Zuccarello et al. 1999; Zuccarello et al. 2006) and *rbc*L (Freshwater and Rueness 1994; Gavio and Fredericq 2002), of which the details have been summarized and discussed in Tan et al. (2012). Purified PCR products were sent to Lucigen (Taiwan) for sequencing.

Sequencing results were processed, and contigs generated using ChromasPro v1.5 (Technelysium Pty Ltd). Sequences obtained for the *Kappaphycus malesianus* within this study were compiled and aligned with associated *Kappaphycus* GenBank entries for each genetic marker. Ambiguous GenBank sequences were omitted from analyses. Multiple sequence alignment was carried out using ClustalX v2.0 (Larkin et al. 2007), and the resulting NEXUS file was input into (1) PAUP 4.0b10 (Swofford 2003) for maximum parsimony (MP) analyses and (2) Kakusan v4 (Tanabe 2011) to generate best fit nucleotide substitution models for subsequent maximum likelihood (ML) and Bayesian inference (BI) analyses.

MP was carried out using PAUP 4.0b10 with the following parameters: heuristic searches with 1,000 bootstrapping replications, 100 stepwise random sequence addition and tree bisection reconnection branch swapping. *Eucheuma denticulatum* specimens—*E. denticulatum* (Burman) Collins & Hervey and *E. denticulatum* (Burman) Collins & Hervey var. *endong* Trono & Ganzon-Fortes (Ganzon-Fortes et al. 2011)—were preset as outgroups prior to MP runs. ML trees were generated using raxmGUI (Silvestro and Michalak 2011) using the ML+ thorough bootstrap module with the GTR+GAMMA model. Twenty independent searches were conducted, each with 1,000 non-parametric bootstrap replicates. For the BI analysis, two sets of four Monte Carlo Markov Chain runs extended for two million generations using Mr. Bayes v3.2.1 (Huelsenbeck et al. 2001; Ronquist and Huelsenbeck 2003), with trees sampled every 500th generation. Convergence of log likelihood values were assessed using Tracer v1.5 (http://196tree.bio.ed.ac.uk/software/tracer), and trees saved prior to convergence were discarded as burn-ins. Remaining trees were used to construct a 50 % majority-rule consensus tree. All resulting phylogenetic trees were viewed and processed using Figtree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

***Kappaphycus malesianus* J. Tan, P. E. Lim et S. M. Phang sp. nov.**

Description: Kappa-carrageenophyte. Plants are mostly multi-axis, up to 40 cm, and holdfast discoid. Thalli are thin cylindrical; diameter of non-fertile plants is 0.8 cm or less, and up to 1.4 cm in cystocarpic plants. Non-cystocarpic thalli are cartilaginous, smooth and flexible. Branching is opened and irregular. Branch apices are flexuous, slender and tapering towards light. Internally, it is comprised of cortical and medullary cells. Pit connections and tyloses are present. Cystocarps throughout thalli, hemispheric to umbonate. Fusion cell and

gominoblast are at the core. Carposporangia clavate or subclavate and in interconnected rows. Carpospores are brown, globose to isodiametric, 20–30 μm.

Holotype: KLU PSM12732 (sample 216) collected by Mr. Adibi Rahiman on 18 January 2013, deposited in the University of Malaya Seaweeds and Seagrasses Herbarium (KLU), Malaysia.

DNA sequences from holotype: KC905309 (*cox*1), KC905207 (*cox*2), KC905419 (*cox*2-3 spacer) and JC905218 (*rbc*L).

Type locality: Seagrass beds in the Celebes Sea, west of the Karindingan Island (N04°23.280′, E118°37.680′) Sabah, Malaysia. Sample occurring at the littoral zone, attached to hardened corals or rocky substrata, coexisting with members of the genera *Acanthophora*, *Gracilaria*, *Hypnea* and other seagrasses.

Etymology: The name is given based on the word *Malesia*, referring to the geographical region where the species is found.

Isotypes: KLU PSM12733–12735 (samples 217–219, respectively), Karindingan Island (N04°23.280′, E118°37.680′), Sabah, Malaysia.

Paratypes: Karindingan Island (N04°20.169′, E118°40.448′), Sabah, Malaysia (KLU PSM12448, KLU PSM12450, KLU PSM12460, KLU PSM12466); Sabangkat Island (N04°35.701′, E118°39.422′), Sabah, Malaysia (KLU PSM12062, KLU PSM12063, KLU PSM12064); Pulau Panjang (S2°59.298, E132°15.132), Papua, Indonesia (SMAR/0053).

All *K. malesianus* specimens in this study were collected from the wild. Photographs of the holotype and isotypes are shown in Fig. 1, whereas those of the paratypes are provided as Online Resource 1. GenBank accession numbers and details of the isotypes and paratypes of *K. malesianus* are summarized in Table 1.

Wild *K. malesianus* plants are generally multi-axis, although individuals with single axis are also observed. Plants are capable of growing up to 40 cm in size, with shades of green, and producing kappa-carrageenan. The main axis of the plant arises from a discoid holdfast, where new main axes may arise immediately or from the main axis itself. Undamaged axes are cylindrical, basically less than 0.8 cm in size; cystocarpic plants have the tendency to display thicker thalli diameter of up to 1.4 cm. The stipe, if applicable, is smooth, plain and thickening upwards, with sufficient tensile strength and flexibility to keep the plant intact during strong currents.

Thalli consist predominantly of secondary branches and occasionally up to tertiary. The branching pattern is irregularly dichotomous, with axes mostly sympodial. Branch apices indeterminate, flexuous, slender, attenuated and pointed; however, terminal branches of cystocarpic plants tend to exhibit larger diameters with relatively rounded apices. Plant thalli, when grazed off or damaged, will first regenerate cortical cells over the affected areas, where new outgrowths may subsequently arise. This will produce a ring-like callus on the damaged part, especially apparent and common in wild specimens.

Fig. 1 *Kappaphycus malesianus*. **a** Sample 216 (holotype). **b** Sample 219 (isotype). **a**, **b** Cystocarpic plants. **c** Sample 217 (isotype). **d** Sample 218 (isotype). **c**, **d** Non-cystocarpic plants [*scale bar* = 5 cm]

Anatomically, the cortical and medullary cells are apparent and recognizable. A mucilage layer 10–20 μm thick can be seen enclosing the entire thallus surface. The cortex is composed of elongated outer cortical cells (3–7 μm width × 10–13 μm height) which are arranged radially in pairs (Fig. 2d) along the circumference and inner cortical cells that are predominantly elongated (8–20 μm width × 10–20 μm height). Granular material, presumably starch, is particularly dense within the inner cortical cells, thus restricting vacuole size although a sporadic few do have larger, centralized vacuoles. Cells within the cortex are pigmented and adjacently linked by pit connections. Sizes of outer cortical cells are somewhat uniform throughout the plant, whereas inner cortical cells tend to become larger and rounder further away from the apex.

The medulla is comprised of primary cells that occupy a large part of the diameter, and secondary cells concentrated at the core (Fig. 2a, c). In transections branches 1–1.5 cm from the apex, the primary cells are mostly elongated and irregularly hexagonal (100–200 μm width × 200–300 μm height) close to the cortical cells, becoming isodiametric and larger towards the core. In longitudinal views, primary cells are ovoid to somewhat hexagonal (60–130 μm width × 100–200 μm height) as well. The medullary cells nearer to the cortex radiate outwards and gradually becoming parallel to the

Table 1 Details of samples used in this study

No.	Sample name	Nature/reference	Sampling information	Collection code (KLU)	GenBank accession no.			
					*cox*1	*cox*2	*cox*2-3 spacer	*rbc*L
Samples collected in this study								
1	*Kappaphycus malesianus* 216 [H]	Cystocarpic	West of Karindingan Island, Sabah, Malaysia (18 January 2013)	PSM12732- UMSS0671	KC905309	KC905207	KC905419	KC905218
2	*Kappaphycus malesianus* 217 [I]	Uncertain		PSM12733-UMSS0672	KC905310	KC905208	KC905420	KC905219
3	*Kappaphycus malesianus* 218 [I]	Uncertain		PSM12734-UMSS0673	KC905311	KC905209	KC905421	KC905220
4	*Kappaphycus malesianus* 219 [I]	Cystocarpic		PSM12735-UMSS0674	KC905312	KC905210	KC905422	KC905221
5	*Kappaphycus malesianus* 188 [P]	Cystocarpic	West of Karindingan Island, Sabah, Malaysia (03 July 2012)	PSM12448-UMSS0593	KC905313	KC905211	KC905423	KC905222
6	*Kappaphycus malesianus* 190 [P]	Uncertain		PSM12450-UMSS0595	KC905314	KC905212	KC905424	KC905223
7	*Kappaphycus malesianus* 200 [P]	Uncertain		PSM12460-UMSS0605	KC905315	KC905213	KC905425	KC905224
8	*Kappaphycus malesianus* 206 [P]	Cystocarpic		PSM12466-UMSS0611	KC905316	KC905214	KC905426	KC905225
9	*Kappaphycus malesianus* 92 [P]	Cystocarpic	Sabangkat, Sabah, Malaysia (15 November 2010)	PSM12062-UMSS0233	KC905317	KC905215	KC905427	KC905226
10	*Kappaphycus malesianus* 93 [P]	Cystocarpic		PSM12063-UMSS0234	JX624033	JX624062	JN663786	JX624004
11	*Kappaphycus malesianus* 94 [P]	Cystocarpic		PSM12064-UMSS0235	KC905318	KC905216	KC905428	KC905227
12	*Kappaphycus malesianus* P1 [P]	Uncertain, damaged	Pulau Panjang, Papua, Indonesia (23 September 2012)	SMAR/0053/230912/UNRAM/KI	KC905319	KC905217	KC905429	KC905228
GenBank samples								
13	*Kappaphycus* sp. "*Bola-bola*"	Mon	Philippines	–	EU334416	–	–	–
14	*Kappaphycus* sp. 2614	Con	Hawaii	–	FJ554853	–	–	–
15	*Kappaphycus* sp. 3957	Con	Hawaii	–	FJ554854	–	–	–
16	*Kappaphycus* sp. 3954	Con	Hawaii	–	FJ554856	–	–	–
17	*Kappaphycus* sp. 3955	Con	Hawaii	–	FJ554857	–	–	–
18	*Kappaphycus* sp. 3956	Con	Hawaii	–	FJ554858	–	–	–
19	*Kappaphycus* sp. "*Tambalang*"	Mon	Philippines	–	EU334415	–	–	–
20	*K. cottonii* D-12	Thi	Malaysia	–	–	–	JN897022	–
21	*K. cottonii*	Mon	Philippines	–	EU334417	–	–	–
22	*K. alvarezii* 1999N	Llu	Philippines	–	–	–	–	AF489872
23	*K. alvarezii* 1999C	Llu	Philippines	–	–	–	–	AF489870
24	*K. alvarezii*	Agu	Philippines	–	–	–	–	AF481498
25	*K. alvarezii*	Fre	Philippines	–	–	–	–	AF099694
26	*K.* "*cottonii*"	Agu	Philippines	–	–	–	–	AF481499
27	*K.* "*cottonii*"	Fre	Philippines	–	–	–	–	AF099695
28	*Kappaphycus* sp. "*Sacol*"	Agu	Philippines	–	–	–	–	AF481500
29	*K.* "*alvarezii*" 1999H	Llu	Philippines	–	–	–	–	AF489871

Specimens in addition to those utilized in Tan et al. (2012, 2013). Dashes (−) indicate non-available data. Square brackets "[ ]" indicate sample typification for *K. malesianus*: [H] holotype, [I] isotype, [P] paratype. Specimens possibly misidentified were labeled with quotation marks

Agu Aguilan et al. (2005), *Con* Conklin et al. (2009), *Fre* Fredericq et al. (1999), *Llu* Lluisma A. O. (unpublished), *Mon* Montes et al. (2008), *Thi* Thien et al. (unpublished)

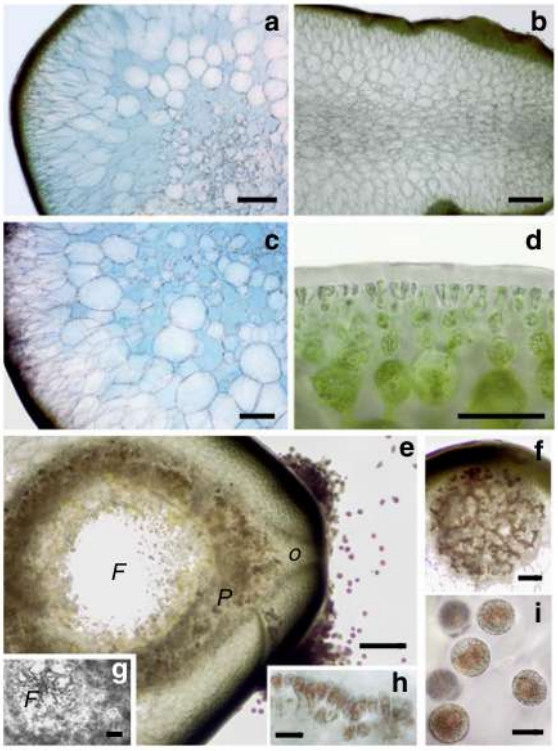

Fig. 2 Microscopic observations of *K. malesianus*. **a** Transection of segment 1–1.5 cm from the tip. **b** Longitudinal section of the tip. **c** Transection of segment approx. 10 cm from the tip. **d** Cortical cells. **e** Anatomy of cystocarp when cut directly from the center (*F*=location of fusion cell; *P*=placental envelope; *O*=ostiole). **f** Anatomy of cystocarp when cut slightly off center, showing the interconnected carposporangial networks. **g** Fusion cell radiating gominoblast filaments. **h** Carposporangia within the placental envelope. **i** Carpospores [*scale bars*: **a**–**c**, **e**, **f**=250 μm; **g**=100 μm; **d**, **h**, **i**=25 μm]

axis when approaching the center. In cross sections, the secondary medullary cells occur as somewhat uniformly sized, isodiametric or spherical cells (50–120 μm in diameter) scattered throughout the core, with some primary cells occasionally embedded within. Tyloses present especially around the core. Longitudinal sections revealed the filamentous and pseudoparenchymatous nature of the medullary cells (Fig. 2b). The axis is composed of a mixture of large and elongated primary medullary cells (60–80 μm width×130–210 μm length), with smaller isodiametric (50–60 μm in diameter) or irregularly elongated secondary cells (40–50 μm width×5–110 μm length) filling in the intercellular spaces. These cells are all orientated in parallel to the axis. Towards the base of the plant, an overall increase in medullary cell sizes is observed. Cell boundaries appear to be more undulated as well. Primary cells (130–300 μm width×250–430 μm length) displayed increased irregularity of cell shapes and relatively coarser cell boundaries, whereas the distribution of secondary cells (100–130 μm in diameter) becomes more erratic around the core, often interspersed with large primary medullary cells. Pit connections are observed within the medulla.

Apart from the stipe, cystocarpic plants have rough surfaces throughout the axes and branches, in which swollen cystocarps may arise. These plants may exhibit significantly larger thalli diameter (<1.4 cm) on certain axes, whereas the remaining axes retain normal size (as earlier described). Cystocarps are especially preponderant (approximately 0.2 cm apart in the densest parts) but irregularly dispersed on the larger, more fertile thalli. These matured and highly fertile cystocarps are hemispherical, umbonate to bell-shaped (about 45 mm wide and high), with dark round patches (pericarp and its contents) at the peak. A slight protrusion may be present on the ostiole. On the other hand, less matured cystocarps on the smaller axes tend to be small, less umbonate and slightly pointed. These cystocarps are irregularly distributed.

Internally, the female reproductive structure consists of a pericarp (<1.5 mm in diameter) enclosing a loosely connected fusion cell at the core, which is often removed during dissection (Fig. 2e). The cortical region surrounding the pericarp is relatively thicker and pigmented, where increased amounts of cortical cells are observed. The outer cortical cells become smaller and packed (2–5 μm width×8–10 μm height), whereas the inner cortical cells become particularly elongated and more layered (3–7 μm width×13–20 μm). The cortex extends until the ostiole of the cystocarp. Medullary cells (200–300 μm thick when measured from the hemispheric apex) surrounding the pericarp are abundant and isodiametric to ovoid (20–30 μm width×30–40 μm length), becoming more elongated and larger when near the pericarp. The fusion cell (~120 μm width and length) radiates gominoblast filaments within what seems to be a gel-like medium (Fig. 2g). These filaments appear to be connected to carposporangia which occur on the outermost regions of the placental envelope (~150–200 μm thick in matured cystocarp bodies), defined as the space in between the pericarp and gominoblast (Doty 1988). Carposporangia are linked and occur in multiple rows of 35–40 μm thickness (Fig. 2h), ultimately constituting an interconnected structure surrounding the internals (Fig. 2f), which is often observable when the cystocarp is longitudinally excised slightly off the centre. The carposporangia are mostly clavate or subclavate (<12 μm in width, <25 μm in length), with dense, granule-filled cytoplasm and small vacuoles. Carpospores are brown in colour (especially at the core) with dense, granulated cystoplasm (Fig. 2i). Ranging from globose to isodiametric (20–30 μm when released), spores often accumulate in massive numbers within the carpospores chambers (in the placental envelope) prior to ejection through the ostiole.

Tetraspores or male reproductive organs were not observed in non-cystocarpic plants (217 and 218).

Molecular phylogeny

*cox*1, *cox*2, *cox*2-3 spacer and *rbc*L sequences were successfully amplified for all 12 samples of *K. malesianus* in this study. Associated GenBank accession numbers were summarized in Table 1. *Kappaphycus cox*2-3 spacer sequences obtained from the GenBank are similar to those used in Tan et al. (2012, 2013) and will not be elaborated. Additional *cox*1 and *rbc*L sequences were included for the respective datasets, also included in Table 1. DNA sequences for the *cox*1, *cox*2, *cox*2-3 spacer and *rbc*L genetic markers were easily amplified, sequenced and aligned, generating multiple sequence alignment lengths of 1,413, 575, 341 and 1,464 bp, respectively. The *cox*1 sequences were truncated to a final length of 582 bp for better comparisons with GenBank entries. Nucleotide compositions for each marker data set coincided with those described earlier by Tan et al. (2012, 2013).

For all four data sets, clear delineation of *Kappaphycus* was observed from the *E. denticulatum* outgroups. All phylogenetic trees displayed similar topology for the phylogenetic reconstruction of *Kappaphycus*, with minor variations caused by few GenBank sequences. For simplicity, only the *cox*2-3 spacer (Fig. 3) and *cox*1 (Fig. 4) phylogenetic trees were depicted in this context, whereas the *cox*2 and *rbc*L trees were provided as supplementary data (Online Resource 2 and 3, respectively). All genetic markers have unanimously shown that *K. malesianus* is genetically distinct from other members of the genus *Kappaphycus*. Although the *cox*1 marker, at its short length, was unable to clearly define phylogenetic relatedness between most *Kappaphycus* congeners, the *cox*2, *cox*2-3 spacer and

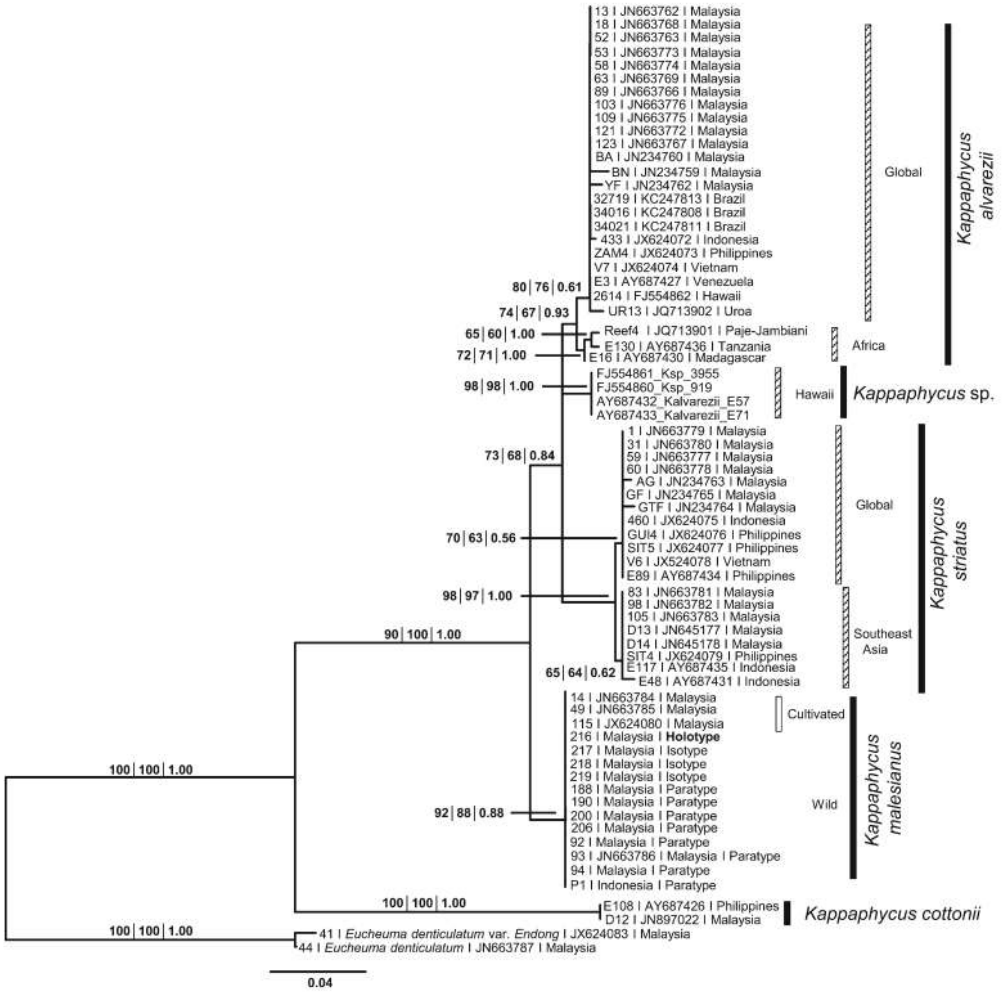

Fig. 3 Maximum likelihood 50 % majority-rule consensus tree based on the *cox*2-3 spacer. Nodal supports are arranged as ML bootstrap support | MP bootstrap support | Bayesian posterior probabilities. *Bars with oblique lines* indicate locality of samples. *White and grey bars* represent cultivated and wild *K. malesianus* samples, respectively. The holotype, isotypes and paratypes are also designated for *K. malesianus*

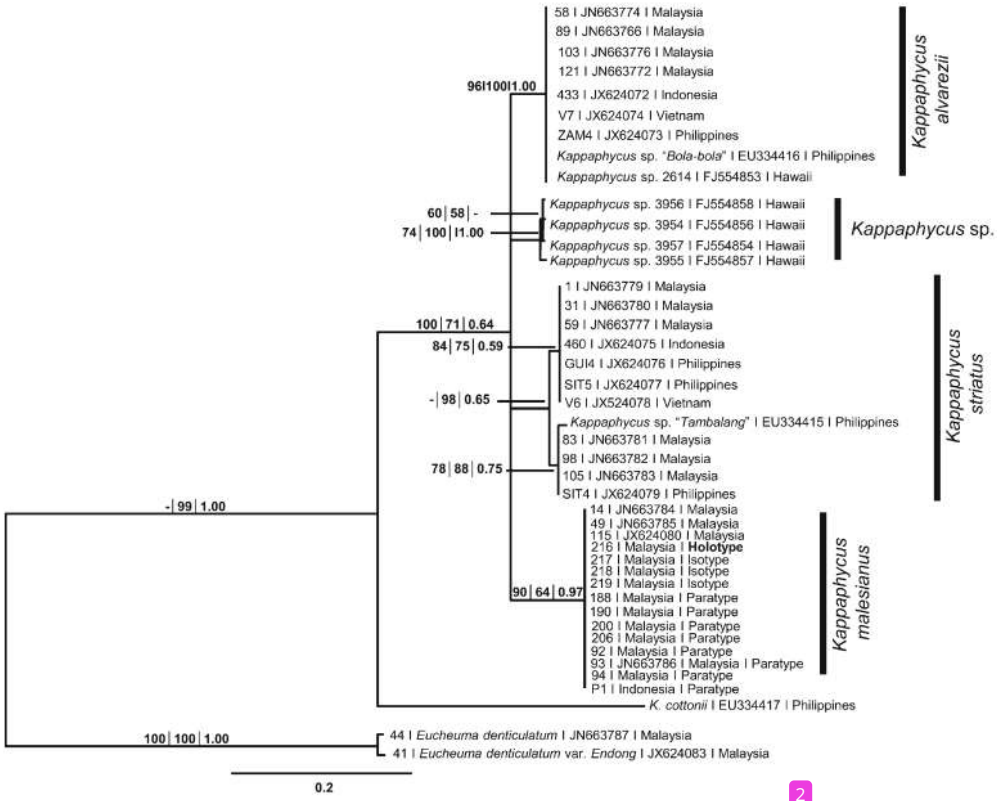

Fig. 4 Maximum likelihood 50 % majority-rule consensus tree based on the *cox*1. Nodal supports are arranged as ML bootstrap support | MP bootstrap support | Bayesian posterior probabilities. The holotype, isotypes and paratypes are also designated for *K. malesianus* samples

*rbc*L have inferred *K. malesianus* to be sister to both *K. alvarezii* and *K. striatus* with high support (>97 % for ML and MP; 1.00 for BI). The *cox*2 DNA marker was capable of detecting intraspecific genetic differences within *K. malesianus* specimens. To date, most *K. malesianus* samples are from Malaysia, and one from Pulau Panjang, Papua, Indonesia.

Phylogenetic interpretation will be based on the more common *cox*2-3 spacer, supplemented by the other DNA markers whenever applicable. *K. alvarezii* consisted of two subclades (ML=74 %, MP=67 %, BI=0.93), namely the most common *K. alvarezii* (Doty) Doty ex P. C. Silva cultivar that was introduced and farmed throughout the globe (ML=80 %, MP=76 %, BI=0.61), and *K. alvarezii* unique to Africa (ML=65 %, MP=60 %, BI=1.00). Certain *Kappaphycus* sp. sampled from the Hawaiian Islands collectively form a strongly supported monophyletic clade based on the *cox*2-3 spacer (ML=98 %, MP=98 %, BI=1.00). The taxonomic position was also supported by the *cox*1 marker (ML=74 %, MP=100 %, BI=0.99). However, the relationship between these Hawaiian samples and *K. alvarezii* or *K. striatus* is not resolved by merely using single DNA markers. *K. striatus* specimens were grouped into two subclades as well (ML=98 %, MP=97 %, BI=1.00), where one is *K. striatus* (F. Schmitz) Doty ex P. C. Silva, supposedly cultivated worldwide (ML=70 %, MP=63 %, BI=0.56), whereas the other is another *K. striatus* genotype which is until today, only reported from Southeast Asia (ML=65 %, MP=64 %, BI=0.62). *K. cottonii* (Weber-van Bosse) Doty ex P. C. Silva samples, albeit the small amount of sequences, were inferred to be sister to all the other aforementioned *Kappaphycus* members by both *cox*2-3 spacer (ML=100 %, MP=100 %, BI=1.00) and *cox*1 (ML=–, MP=99 %, BI=1.00). Even though older *rbc*L GenBank entries of *K.* "*cottonii*" were also available along with several *Kappaphycus* sp. with local Philippine names (Online Resource 3), several were shown to be wrongly identified based on DNA data (labeled with quotation marks in Table 1, Fig. 4 and Online Resource 3). No GenBank records of *K. inermis* (F. Schmitz) Doty ex H. D. Nguyen & Q. N. Huynh and *K. procrusteanus* (Kraft) Doty were available.

Discussion

Distribution

Wild populations of *K. malesianus* appear to be largely abundant around the shallower, intertidal parts of the Sabangkat

Island (Tan et al. 2013) and Karindingan Island of Sabah, Malaysia. It is not unexpected should this species be discovered throughout the Sulu Sea, Celebes Sea or the Makassar Strait considering the similar ecological niches. The geographical coverage of this species is probably more extensive since it has been recorded as far as Papua, Indonesia. Although not observed in the present study, wild populations of *K. malesianus* are expected to be abundant in the Philippines as well, as reported by Villanueva et al. (2011). It is known that *K. malesianus* is commonly farmed and called "*Aring-aring*" in Malaysia and the southern Philippines.

Morphology

As mentioned by Doty (1988) and many other subsequent publications, morphological studies on *Kappaphycus* are taxonomically challenging considering the morphologically plasticity as well as the paucity of distinctive identification criteria. The massive sizes and the gradual secretion of moisture from the thalli have rendered transportation and proper preservation of these carrageenophytes taxing as well. The loss of structure upon dehydration, degradation of hydrocolloids (Doty and Norris 1985) and DNA in older, type exsiccata have also hindered taxonomic studies on *Kappaphycus*, even when molecular technologies are available.

Although cultivated *K. malesianus* are easily accessible, healthier, larger (up to 50 cm) and often in undamaged conditions, it is believed that the "true" morphology of these plants would be altered upon domestication as a result of deviations in environmental conditions from that of the original habitat. Thus, cultivars were not used in this study. However, DNA results have shown that cultivated "*Aring-aring*" plants (Tan et al. 2012, 2013) are genetically similar to the wild ones used in this study. Specimens collected from the wild are often exposed to environmental damages, often resulting in alterations to morphology, causing confusion even to seasoned taxonomists. Despite the lack of apparent characteristics, external morphology was capable of differentiating among *Kappaphycus* species to an extent (Doty 1985, 1988; Doty and Norris 1985), provided the specimens are whole, undamaged with sufficient morphological characters. Table 2 is provided as a summary of putative morphological characteristics of each *Kappaphycus* species which will hopefully aid scientists and farmers in differentiating especially commercial species. *K. malesianus* ("*Aring-aring*" then) was reported to produce slightly lower gel strength (>500 g cm^{-2}; 1.2 % gel) compared to *K. alvarezii* (>550 g cm^{-2}) (Phang et al. 2010).

Externally, *K. malesianus* plants (both wild and cultivars) morphologically resemble *K. alvarezii*, where both display irregular branching and slender to flexuous terminal branches, thus often misidentified by locals. The main features separating *K. malesianus* from *K. alvarezii* are the relatively smaller thallus diameter and plant size. Non-cystocarpic plants rarely exceed 0.8 cm in thallus diameter at any point of the plant, with overall sizes of less than 40 cm, whereas *K. alvarezii* plants can easily achieve diameters of approximately 1.5 cm and up to 2 m large (Doty 1985; Tan et al. 2013). Owning to the slimmer diameters, non-fertile *K. malesianus* plants are less robust and flexible throughout; a characteristic not seen in healthy *K. alvarezii*, where the thalli often snap when pressure is applied. The thalli of non-cystocarpic plants are also relatively smoother compared to those of *K. alvarezii*. Although unexplainable, blunt protuberances often scattered throughout the thalli of *K. alvarezii* (*Buaya* variety) and *K. striatus* (*Durian* variety) cultivars (Tan et al. 2013). These protrusions are not observed in *K. malesianus* cultivars. Although colour is not a good criterion for species identification, *K. malesianus* plants are, to date, only reported in shades of green and brown (cultivars often in lighter colours), unlike *K. alvarezii* which may range from green, brown, yellow to pink.

K. malesianus can be easily distinguished from *K. striatus* by the smaller thallus diameter, branching pattern and overall shape. The thalli of non-cystocarpic *K. striatus* plants are much like those of *K. alvarezii*, being thicker and more robust as compared to *K. malesianus*. Branches of *K. striatus* are dense, with generally less than 2 cm between branches. Branches are angularly dichotomous, with branching degrees of up to quaternary or even quinary in non-damaged and non-cystocarpic plants. Branch apices are mostly rounded or forcipate. Growing of branches towards light is not as conspicuous as seen in *K. alvarezii* and *K. malesianus* due to the heavy branching. The dense yet short branching of *K. striatus* gives rise to a compact, compressed and overall isodiametric shape which is also observed (albeit less obvious) in wild *K. striatus* samples.

Studies on *K. cottonii*, *K. inermis* and *K. procrusteanus* are relatively scarce, possibly due to rarity. Apart from a tiny but wild *K. cottonii* from Sabah, wild or cultivated plants resembling morphological descriptions of other *Kappaphycus* such as *K. inermis* and *K. procrusteanus* were not encountered in Malaysia. Despite efforts in studying the phenotypic characteristics of type or non-type herbaria of *K. cottonii*, *K. inermis* and *K. procrusteanus*, information on these red algae are still based on pre-existing literatures, considering the significant variations in morphology between fresh and dried materials (Doty and Norris 1985; Doty 1988).

According to Doty (1988), *K. cottonii* thalli are compressed to flattened above the basal segment; prostate, irregular in form, or with linear segments sometimes in heads, or decumbent, or decurrent from their place of origin; with irregular occurrences of protuberances or branches. Literature has shown that *K. inermis* plants are differentiated from other *Kappaphycus* based on inflated branch apices, central axial region with regular border of cells of nearly uniform medium-sized cells not seen in other *Kappaphycus*, and the presence of a supposedly "conical" holdfast. *K. procrusteanus*, being the only *Kappaphycus* reported to display foliose blades or fronds, can be distinguished from other

Table 2 Putative morphological comparisons among *Kappaphycus* species

No.	Name	Distinctive morphological characters	Colour	Known distribution
1	*K. alvarezii* (Doty) Doty ex P. C. Silva	Large size (<2 m); thick and robust (<1.5 cm diameter in non-cystocarpic plants); multi-axis, often with one primary axis; opened and irregular branching patterns; slender apices. Cultivated samples may display bluntprotuberances throughout the thalli	Brown, green, yellow, pink	Commercially distributed and cultivated worldwide. Wild specimens (some of which invasive) mostly in the tropics. Fertile specimens uncommon in Southeast Asia
2	*K. striatus* (F. Schmitz) Doty ex P. C. Silva	Plants isodiametric; branching angularly dichotomous, short and dense (<2 cm apart); branch apices mostly rounded. Cultivated samples may display blunt protuberances throughout the thalli. Cystocarp-bearing plants isodiametric, with relatively less branching (but frequencies still higher than that of cystocarpic *K. alvarezii* and *K. malesianus*).	Brown, green, yellow, pink	Distributed and cultivated worldwide, though not as much as *K. alvarezii*. Wild specimens mostly in the tropics
3	*K. cottonii* (Weber-van Bosse) Doty ex P. C. Silva	Thalli compressed or flattened; decumbent or decurrent; irregular protuberances and branching.	–	Not known to be cultivated. Wild specimens reported from China, Malaysia, Philippines, Tanzania and Vietnam
4	*K. inermis* (F. Schmitz) Doty ex H. D. Nguyen & Q. N. Huynh	Presence of inflated branch apices; "conical" holdfast where multiple axes arise; border of cells of nearly uniform medium sized cells in central axial region	–	Not known to be cultivated. Wild specimens reported from the east coast of Africa, southwestern Indian Ocean and Vietnam
5	*K. procrusteanus* (Kraft) Doty	Presence of coarse, whole blades or smooth fronds (2–3.5 mm thick). Tiny cystocarps (1–2 mm across, 1 mm height)	Reddish brown	Not known to be cultivated. Reported only from the Philippines
6	*K. malesianus* (J. Tan, P. E. Lim and S. M. Phang)	Overall smaller (<50 cm) than *K. alvarezii*; slimmer <0.8 cm; <1 cm in cultivars) and pliable thalli or branches (particularly in thalli not bearing cystocarps); branching opened and irregular; terminal branches slender	Brown, green (lighter colours in cultivars)	Known to be cultivated in Malaysia and the Philippines. Wild populations currently reported in Malaysia and Indonesia

Dashes (−) indicate non-available data

Kappaphycus congeners with ease (Doty 1988; Kraft 1969). The outlined morphological characters of *K. cottonii*, *K. inermis* and *K. procrusteanus* were not seen in *K. malesianus*. Nevertheless, the morphological descriptions available for *K. cottonii* and especially *K. inermis* are perhaps too general at this point and require revision when samples become available. Revisions should be supplemented with solid molecular data so as to avoid erroneous identifications.

K. malesianus samples 217 and 218 do not exhibit any tetraspores, male or female reproductive structures; it is thus difficult to define the life stage of these specimens due to similarities in gross morphology. However, resembling *K. alvarezii*, *K. striatus* and possibly all other *Kappaphycus*, *K. malesianus* demonstrates a triphasic alternation of generations. Although tetrasphorophytic reproductive structures were not observed in the present study, *K. malesianus* samples (and other valid *Kappaphycus* species) probably produce zonately divided tetrasporangia as those reported in *K. alvarezii* (Ask and Azanza 2002; Doty 1987; Okuda and Neushul 2008), *K. cottonii* (Nguyen and Huynh 1995) and *K. procrusteanus* (Kraft 1969).

Cystocarp-bearing *Kappaphycus* plants are morphologically different from non-fertile plants. Common in wild populations, these fertile female gametophytic plants are often subjected to environmental stress and damages, making on-site identifications a challenge. Wild, cystocarpic *K. alvarezii* is uncommon in Malaysia, or perhaps even the Southeast Asia, making direct interspecific comparisons difficult. However, based on original *K. alvarezii* descriptions (Doty 1985), the swollen, generally hemispherical nature of cystocarps appeared to be similar to that seen in *K. striatus* and *K. malesianus*. The dimensions of fresh cystocarps do not seem to vary between these species as well, although it is noted that cystocarps of *K. procrusteanus* are comparatively smaller, at 1–2 mm width× 1 mm height (Kraft 1969). Details on cystocarpic *K. cottonii* and *K. inermis* plants are, to the authors' knowledge, unrecorded as of now. Observations have shown that for wild, cystocarpic *K. striatus* and *K. malesianus* (and possibly *K. alvarezii*), immature cystocarps appear as smaller, cone-shaped protuberances which may be confusing during species identification. These structures may be throughout the entire plant or occasionally on smaller axes of an otherwise fully matured cystocarpic plant. This statement is made based on studies involving larger amounts of cystocarpic *Kappaphycus* samples and serves as an amendment to that reported in Tan et al. (2013) in which these spiny cystocarps were erroneously described as characteristics of *K. striatus*.

Cystocarpic plants tend to exhibit thicker thalli diameters in certain branches than non-fertile plants. Still, the aforementioned morphological characteristics of *K. alvarezii*, *K. striatus* and *K. malesianus* are still applicable to an extent in differentiating non-damaged, cystocarpic plants. Cystocarp-bearing *K. striatus* can be identified based on the relatively short and denser branching (though not as frequent as non-fertile plants) and compressed, overall isodiametric shape. Identification criteria between cystocarpic *K. alvarezii* and *K. malesianus* remained vague at this time due to the lack of cystocarpic *K. alvarezii* samples, but it may still be possible to differentiate them by plant size, thallus diameter and pliability. Fertile *K. alvarezii* plants were reported to have diameters up to 3 cm by Doty (1988), which can also be used as a rough characteristic for species differentiation.

Microscopically, *K. malesianus* plants bore close resemblance to *K. alvarezii* in terms of cellular components, although the range of cell sizes for *K. malesianus* are generally smaller, which might be the reason for its pliability. Apart from the presence of unique, uniform cells in the axial core reported only in *K. inermis* (Doty 1988), the lack of differentiating characters on other *Kappaphycus* has rendered species identifications based on anatomy unreliable, at least for now. Although tyloses or thylles were reported for *K. alvarezii* (Doty 1985) and *K. procrusteanus* (Kraft 1969), little is known of these outgrowths, which probably do not hold much value for species differentiation. Cystocarpic contents observed in *K. malesianus* appear to be mostly similar to those described in earlier studies (Agardh 1876, 1892; Doty 1985, 1988; Kraft 1969). The cystocarp is composed of a hollow cavity presumed to be formed by the breakdown of a large fusion cell (Kraft 1969). The fusion cell radiates thin projections that develop into gominoblast filaments or sterile paraphyses within this supposedly gel-filled chamber, which is often lost in thin, longitudinal hand sections (Doty 1988; Kraft 1969). Gominoblast filaments elongate centrifugally into specialized carpospores chambers within the placental envelope, where terminal carposporangia arise; meanwhile, sterile filaments span the placental envelope and merge with nutritive tissues there (Doty 1985; Kraft 1969). The clavate or subclavate caposporangia structures within the carpospore chambers occur in multiple rows which are interconnected (Fig. 2f), surrounding the fusion cell cavity. Easily seen when cystocarps are longitudinally cut slightly off the center of the hemisphere, these carposporangia networks do not entirely enclose the internal contents, leaving "open spaces" for matured carpospores to be conveyed towards the ostiole for release. Similar carposporangia patterns were observed in cystocarps of *K. striatus* (results not shown). Again, the anatomical structures of cystocarps do not present much value for species differentiation due to the many information gaps within this particular genus.

Molecular systematics

The improvements in molecular biology have brought about huge advancements to the taxonomic studies of red algae, particularly so for genera with apparent morphological variations, i.e. *Kappaphycus* and the closely related *Eucheuma*.

Following the pioneering work by Zuccarello et al. (2006), it became evident that molecular data are an important, if not essential, component in the taxonomic establishment or reformation of taxa associated with *Kappaphycus* or *Eucheuma*. The simplicity and versatility of DNA technology enabled accurate, supportive information for the identification or phylogenetic inferences of these carrageenophytes (Barros-Barreto et al. 2013; Halling et al. 2012; Tan et al. 2012, 2013; Zuccarello et al. 2006), even when the specimens of interest are tiny, deformed or damaged. The application of molecular taxonomy has also narrowed down the number of available *Kappaphycus* species, showing that many colloquial names or local varieties are not valid from a genotypic standpoint (Tan et al. 2012, 2013). These preliminary results have intensified sampling efforts over wider geographical areas which may harbour uncommon *Kappaphycus* species.

*cox*1, *cox*2, *cox*2-3 spacer and *rbc*L genetic markers were shown to be capable of delineating members of the genus *Kappaphycus*. However, *cox*2-3 spacer (Fig. 3) and *cox*1 (Fig. 4) were given more emphasis within this context due to the larger numbers of available GenBank references, and also their suitability as DNA barcodes for *Kappaphycus* (and *Eucheuma*) (Tan et al. 2012). The phylogenetic patterns of *Kappaphycus* and *Eucheuma* coincide with those obtained in earlier studies (Barros-Barreto et al. 2013; Conklin et al. 2009; Halling et al. 2012; Tan et al. 2012, 2013; Zuccarello et al. 2006). Although the *cox*1 DNA marker, with its short length of 582 bp, merely resolved *K. alvarezii*, *Kappaphycus* sp. (Hawaii), *K. striatus* and *K. malesianus* as polytomy; the usage of the full *cox*1 length of 1,411 bp managed to resolve the phylogenetic relatedness among these *Kappaphycus* (Tan et al. 2012), generating topologies similar to that of *cox*2, *cox*2-3 spacer and *rbc*L.

The monophyly and genetic distinctiveness of *K. malesianus* is evident based on all four molecular markers, serving as strong supporting data to the morphological observations for species establishment. The *cox*2 DNA marker was able to provide better resolution of *K. malesianus* specimens at an intraspecific level, clustering samples 49 with 115, and samples 200, 206, 216 with 219, with each pair occurring one node into the *K. malesianus* clade. This reveals the inherent genetic diversity within *K. malesianus* that are not observed as clearly by other genetic markers. However, no defined morphological differences were observed in spite of these genetic variations. Plants of different life stages show no noticeable genetic differences as well. Therefore, life stage identification of *K. malesianus* and other *Kappaphycus* is still largely dependent on anatomical studies on fertile plants or perhaps ploidy studies (Zitta et al. 2011).

*cox*1 and *rbc*L phylogenetic trees have shown that several GenBank entries were misidentified, including two Philippine *K.* "*cottonii*" *rbc*L sequences (AF481499 and AF099695) from earlier studies. It is postulated that these misidentifications were caused by the lack of distinct morphological characters for *K. cottonii* and the confusion between commercial names, i.e. *cottonii*, and basionyms, i.e. *Eucheuma cottonii*. Large-scale, collaborative samplings have also shown that the true *K. cottonii* is probably not commercially cultivated in Southeast Asia, despite the crops often being termed "*K. cottonii*" by farmers. Although few in comparison, there are five DNA sequences (*cox*1: EU334417, *cox*2-3 spacer: AY687426 and JN897022, RuBisCO spacer: AY687409 and AF489869) of *K. cottonii* within GenBank to date which are deemed correct for genotypic reference. No DNA sequences of *K. inermis* and *K. procrusteanus* were available at the time of writing. Attempts in sequencing DNA from herbaria collections were generally futile, probably due to poor preservation or simply due to the oldness of the samples. Taxonomists may have to rely on neotypes for DNA sequences, at least until better techniques or more effective genetic markers are developed for such type herbaria.

Currently, large databases of *Kappaphycus* DNA sequences are mostly derived from cultivars, predominantly from the Philippines, Malaysia, Hawaii, Brazil and Africa. Results have shown that most, if not all, of the most commercially cultivated *Kappaphycus* are of the same few genotypes, albeit some minor differences between haplotypes. This is not unexpected since these plants, most of which originate from the Philippines, were extensively distributed vegetatively to different parts of the world for cultivation over the last four decades. Nevertheless, molecular results have also shown the existence of different *Kappaphycus* genotypes throughout the world which would otherwise remained undiscovered if based on morphology alone. The authors believe that there are undoubtedly more *Kappaphycus* species out there and agree that subsequent taxonomic research should be more focused on *Kappaphycus* and *Eucheuma* from wild populations, which will reveal the true biodiversity and genetic richness of these carrageenophytes. This also increase the chances of finding, collecting and archiving *K. inermis* and *K. procrusteanus* plants which will fill in the missing information gaps associated with the genus.

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