

Biodiversity, Biogeography and Molecular Genetics of the Commercially Important Genera *Kappaphycus* and *Eucheuma*

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Abstract

Through the years, several strains and color morphotypes of *Kappaphycus* and *Eucheuma* have emerged in cultivation areas as a result of environmental and anthropogenic factors. This led to confusion in nomenclature of these seaweeds. However, the advent of molecular technology has brought notable changes to seaweed research over the past two decades. New molecular techniques have proved useful, particularly in the molecular identification and cataloging of economically important carrageenophytes, i.e. *Kappaphycus* and *Eucheuma* which were commercially introduced into many countries worldwide. The following chapter discusses the application and findings of molecular studies within the context of these red seaweeds, along with potential areas for future research.

2.1 Introduction

Kappaphycus and *Eucheuma* are two genera of red seaweeds which are economically important in the global market because they are valuable sources of natural phycocolloids, in the form of *kappa* and *iota* carrageenan, respectively. Originally, *Chondrus crispus* was gathered from the wild for carrageenan processing, however the natural population was not able to sustain the ever increasing demands of the indus-

try. Following years of research and development in field cultivation, carrageenan characterization and product development, *Kappaphycus* and *Eucheuma* were selected as the primary sources of raw materials for *iota* and *kappa* carrageenan extraction.

In addition to its use as a raw material for hydrocolloid, *Kappaphycus* has also been used experimentally in aquaculture feed for abalone (Capinpin and Corre 1996). The seaweed was also used with promising results as a sink or sponge for the extraction of organic and inorganic products from fish and invertebrate aquaculture in land-based, Integrated Multi-trophic Aquaculture (IMTA) systems (Rodrigueza and Montano 2007; Hayashi et al. 2008; Namukose et al. 2016). Positive results provided the impetus for further research on other “alternative” uses of eucheumatoid biomass. These commercial trials are crucial to the further development and sustainability of the cultivation industry. Likewise, initial results on the use of *Kappaphycus* as a source of bioethanol (Khambhaty et al. 2011; Meinita et al. 2012; Fakhruddin et al. 2014) were encouraging.

Kappaphycus and *Eucheuma* were successfully farmed in the Philippines in 1971 after almost five years of field cultivation trials (Doty 1973; Doty and Alvarez 1981; Hurtado et al. 2014, 2015). Presently, *Kappaphycus* is commercially farmed over a greater area than *Eucheuma*, in several

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countries above and below the 10° latitude (Kronen 2013; Krishnan and Narayanakumar 2013; Neish 2013; Hayashi et al. 2014; Msuya et al. 2014).

2.2 Biodiversity

The late Dr. MS Doty together with Drs. Abbott, Cheney and Norris worked diligently and contributed to the present nomenclature of *Kappaphycus* and *Euclidean*. A series of workshops and conferences were conducted under the California Sea Grant Program, University of California, La Jolla, California which resulted in notable publications in 1985 and 1988. These publications involved thorough examinations of external and internal morphologies of tetrasporophyte, carposporophyte, male and female gametophytes of *Euclidean* which contributed to the eventual separation of the genus *Kappaphycus* from *Euclidean*. Prior to this taxonomic separation, Doty and Norris (1985) classified *Euclidean* into four (4) sections (Table 2.1).

However, Doty (1988) classified *E. alvarezii* and *E. striatum* as *Kappaphycus alvarezii* and *K. striatum* (now *K. striatum*), respectively based on the following characteristics:

- (a) fronds are of many forms but commonly cylindrical, bearing simple blunt or spiny protuberances
- (b) protuberances are irregularly arranged or, in some cases, appearing in rows so segments become angular
- (c) branching is irregular but sometimes irregularly pinnate or, by chance opposite of falsely dichotomous
- (d) hyphal axial core usually present and vaguely cylindrical
- (e) carrageenan extracted is kappa
- (f) cystocarps are on main axes
- (g) no laterals are associated with cystocarp

Kappaphycus alvarezii, *K. striatus* and *Euclidean denticulatum* are endemic to the tropics, especially within the Coral Triangle, where the biodiversity of flora and fauna is very rich. Tanzania is also known to have natural populations of *E. denticulatum* (Sen 1991). *Kappaphycus* has been introduced to several countries, ranging from sub-tropical to tropical waters (Ask et al. 2003; Hurtado et al. 2015). At present,

there are several morphological plasticities and color morphotypes (Fig. 2.1a–m) of *K. alvarezii*, *K. malesianus*, *K. striatus* and *E. denticulatum*. Some of these are possibly morphological responses to crop selection and environmental conditions in the commercial farms (Hurtado 2013; Hurtado et al. 2016). Details of the biodiversity and distribution of *Kappaphycus* and *Euclidean* in Southeast Asia, where the holotypes of the two seaweeds were collected, were reported by Hurtado et al. (2016). Similarities and differences in their molecular systematics will be discussed in detail in the next section.

The first stock of *Kappaphycus* and *Euclidean* to be used for farming purposes was collected from the wild, which was later domesticated and formed the original stock (Doty 1973; Doty and Alvarez 1975, 1981). Vegetative cuttings are the only means to propagate the raw materials for carrageenan extraction (deemed to be called euclideanoids or carrageenophytes); the methods have remained the same to the present day. Cultivars of *Kappaphycus* and *Euclidean* have been transplanted to different areas of the Philippines where the favorable environment encouraged robust growth. Likewise, these seaweeds were introduced to other parts of the world. Due to several transplantations, in and out of the original area of growth in the Philippines, *Kappaphycus* and *Euclidean* cultivars adopted several phenotypic or morphological plasticities – probably in response to each new cultivation environment. The following shows some photos of various morphotypes of *K. alvarezii* (Fig. 2.1a–m), *K. striatus* (Fig. 2.2a–r), *K. malesianus* (Fig. 2.3a and b) and *E. denticulatum* (Fig. 2.4a–d).

2.3 Development of Genetic Markers for *Kappaphycus* and *Euclidean*

A genetic or molecular marker is a gene, or region of DNA, that can be used to identify and differentiate species. Although genetic markers serve various purposes, their application towards *Kappaphycus* and *Euclidean* stemmed mainly from the need for reliable identification of a very confusing array of color and morphological variants. *Kappaphycus* and *Euclidean* are morphologically plastic, in fact seaweeds of the same species may vary substantially in appearance. The accuracy of a molecular marker also allows for the identification of species in immature or damaged samples, e.g., broken thalli, carpospores etc. Thus, application of genetic marker technology allows an alternative, independent and reliable method for identification and therefore discrimination of these red seaweeds.

Genetic markers displaying notable levels of genetic variation were those most commonly used in the molecular studies of *Kappaphycus* and *Euclidean* (Conklin et al. 2009; Fredericq et al. 1999; Freshwater et al. 1994; Tan et al. 2012,

Table 2.1 Classification of *Euclidean* (Doty and Norris 1985)

Section	Section	Section	Section
<i>Euclidean</i> (iota)	Anaxiferae (iota)	Gelatiformia (kappa)	Cottoniformia (kappa)
	(Kraft 1972)		
<i>E. denticulatum</i>	<i>E. arnoldii</i>	<i>E. gelatinae</i>	<i>E. alvarezii</i>
<i>E. isiforme</i>			<i>E. striatum</i>
<i>E. uncinatum</i>			

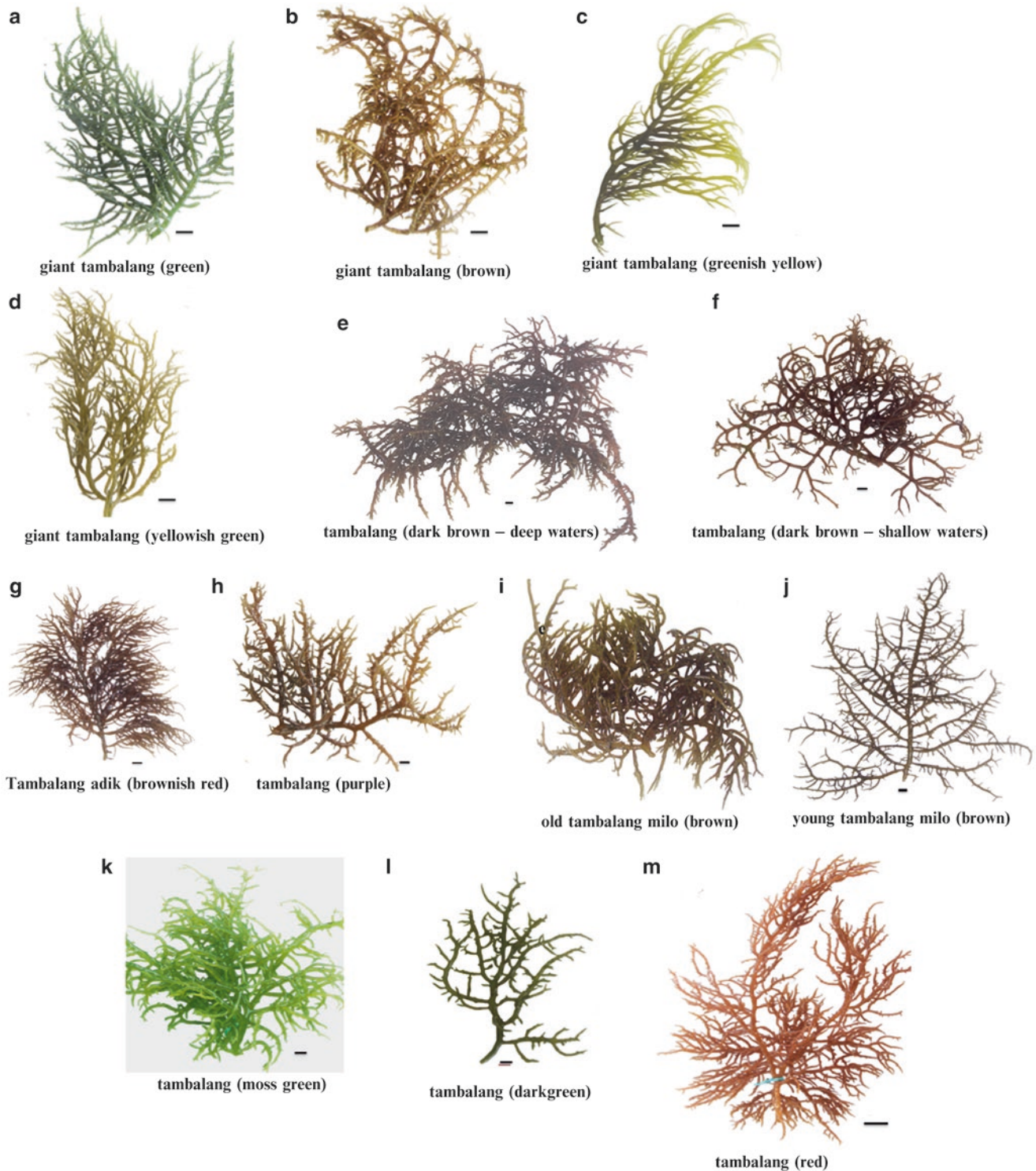


Fig. 2.1 (a–m) Several morphological plasticities and different color morphotypes of *K. alvarezii* (All photos courtesy of AQ Hurtado, bar = 1 cm)

2013, 2014; Zuccarello et al. 2006; Lim et al. 2014a; Zhao and He 2011). Other markers, i.e., random amplification of polymorphic DNA (RAPD) (Dang et al. 2008) and single locus markers (Maili et al. 2016) were also employed. Apart from molecular identification, genetic markers are also

invaluable in: (i). elucidating the phylogenetic relationship between *Kappaphycus* and *Euclima*; (ii). identifying seaweed strains with favorable traits for cultivation and commercialization; (iii). mapping the biodiversity and genetic

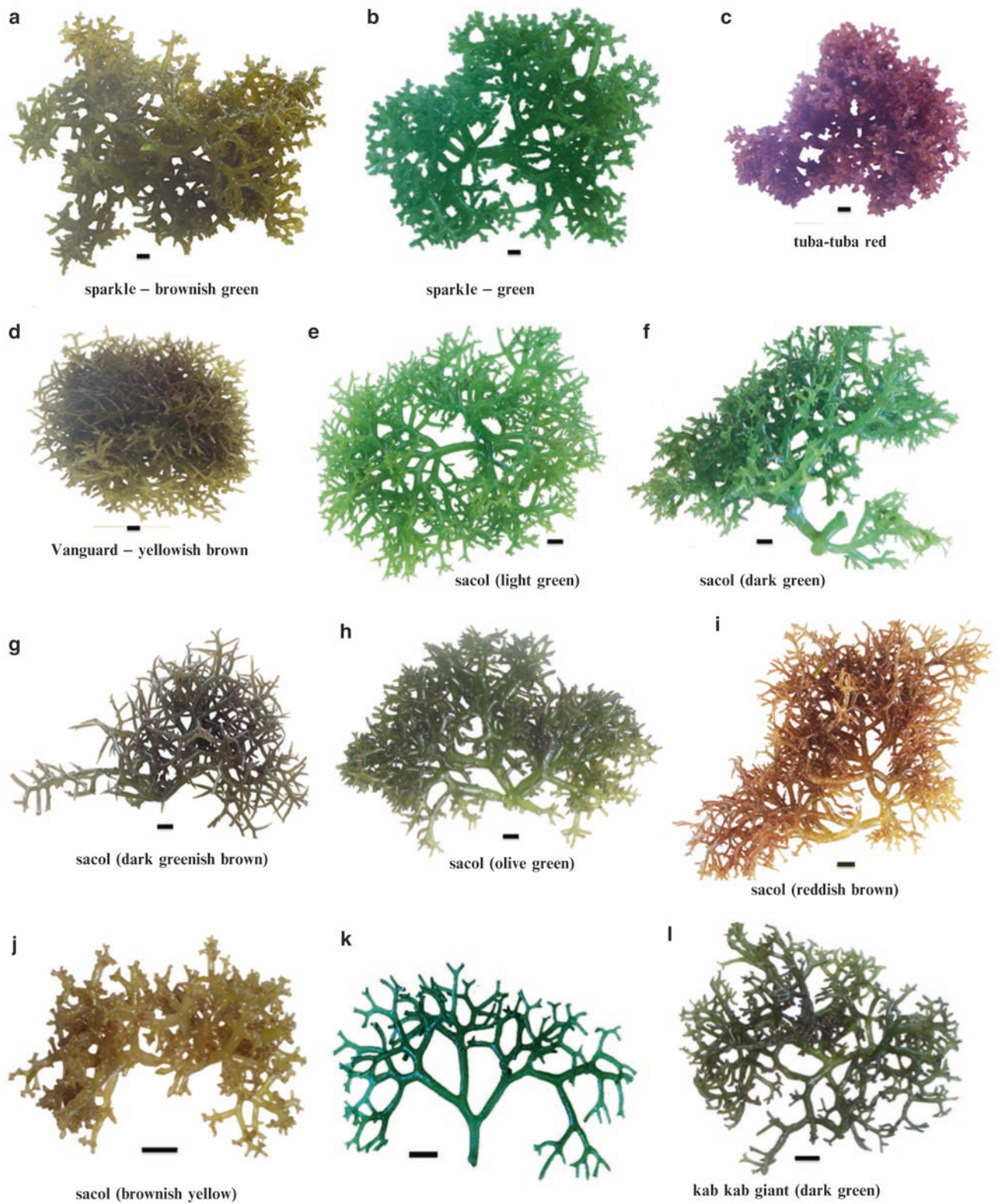


Fig. 2.2 (a–r) Several morphological plasticities and different color morphotypes of *K. striatus* (All photos courtesy of AQ Hurtado, bar = 1 cm)

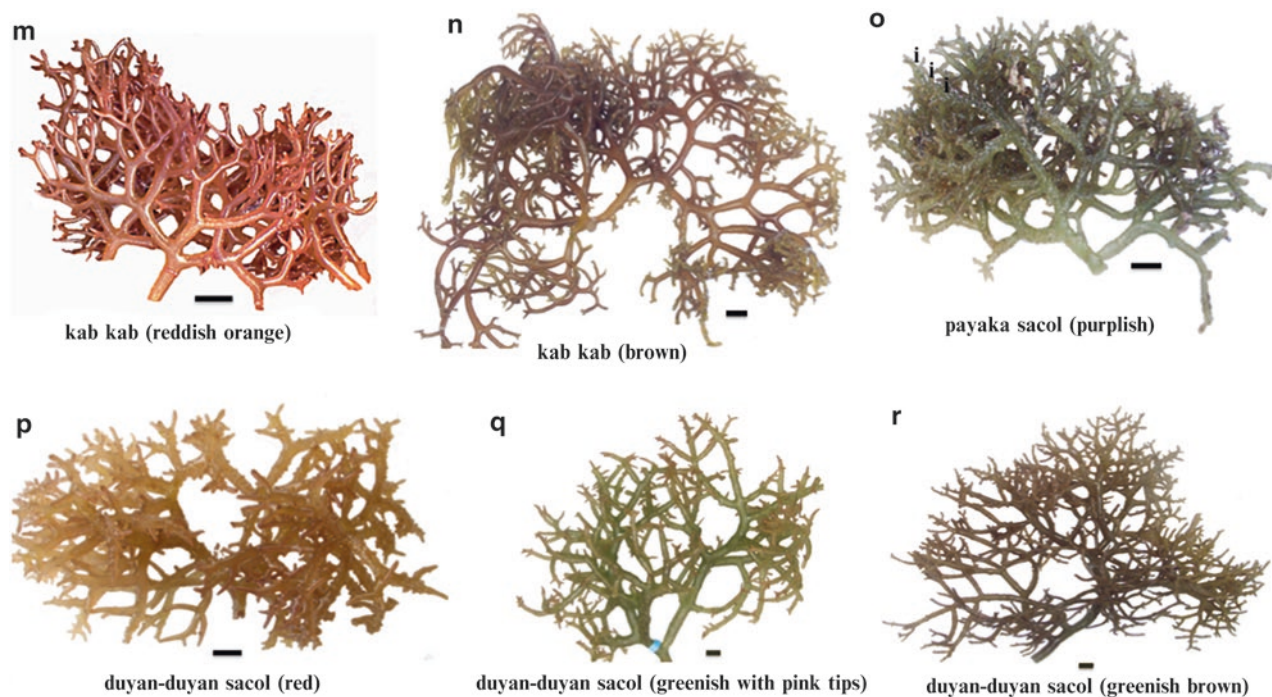
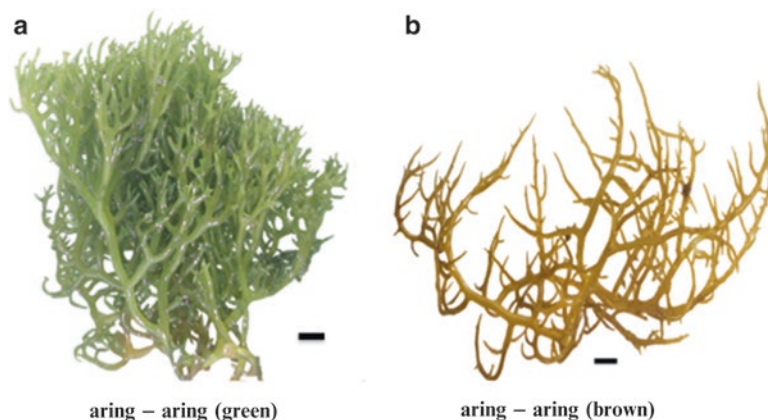


Fig. 2.2 (continued)

Fig. 2.3 (a and b) *K. malesianus* (Photo courtesy of AQ Hurtado and Tan J, bar = 1 cm)



diversity of these seaweeds on a global basis; (iv). detecting bio-invasive species, etc.

The first extensive use of genetic markers for *Kappaphycus* and *Euclidean* was conducted by Zuccarello and co-workers (2006), in which a mitochondrial-encoded *cox2–3* spacer and a plastid-encoded RuBisCO spacer were used for phylogenetic reconstruction of these seaweeds. The study demonstrated the usefulness of molecular markers in addition to revealing valuable insights into the phylogeny of *Kappaphycus*, *Euclidean* and *Betaphycus* seaweeds which had been collected from multiple sites around the world. The *cox2–3* spacer has since become a frequently used genetic marker due to its formidable resolving capabilities (Conklin et al. 2009; Dumilag and Lluisma 2014; Tan et al. 2012,

2013, 2014; Lim et al. 2014a). Over the last decade, several new DNA markers, i.e., mitochondrial *cox1* and *cox2*; nuclear internal transcribed spacer (ITS), as well as plastid *rbcL*, were designed or utilized for various purposes, including the detection of invasive *K. alvarezii* in Hawaii (Conklin et al. 2009), development of DNA barcodes (Tan et al. 2012), molecular identification and phylogenetics of species or varieties of *Kappaphycus* and *Euclidean* (Araújo et al. 2013; Tan et al. 2013; Zhao and He 2011; Dumilag and Lluisma 2014), species descriptions (Ganzon-Fortes et al. 2012; Tan et al. 2014), as well as genetic studies (Lim et al. 2014a). The details of these primers are summarized in Table 2.2

The resolution of genetic markers depends on multiple factors, including the number of phylogenetically informa-

Fig. 2.4 (a–d) Different color morphotypes of *E. denticulatum* (All photos courtesy of AQ Hurtado, bar = 1 cm)

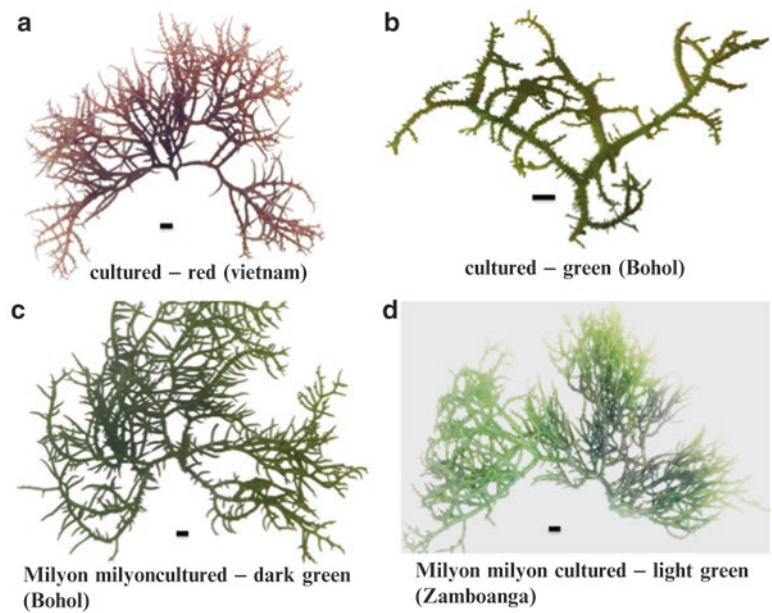


Table 2.2 Common genetic markers used in molecular studies of *Kappaphycus* and *Euचेuma*

Genetic marker	Primer	Primer sequence*	Annealing temperature (Tm)	References
<i>Cox1</i>	COXI43F	5'-TCAACAAATCATAAAGATATTGGWACT-3'	52 °C	Geraldino et al. (2006) and Yang et al. (2007)
	C622F	5'-CCTGTNTTAGCAGGWGCTATTACAATGC-3'		
	C880R	5'-ACAGTATACATATGATGNGCTCAAAC-3'		
	COXI1549R	5'-AGGCATTTCTCAAANGTATGATA-3'		
<i>Cox2</i>	Cox2_for	5'-GTACCWTCCTTDRGRRKDAAATGTGATGC-3'	50 °C	Tan et al. (2012)
	Cox3_rev	5'-GGATCTACWAGATGRAAWGGATGTC-3'		
<i>Cox2-3 spacer</i>	Kcox2_F71	5'-TTCAAGATCCTGCAACTCC-3'	51 °C	Zuccarello et al. (1999a)
	Kcox2_R671	5'-ATTTCACTGCATTGGCCAT-3'		
<i>rbcL</i>	F-7	5'-AACTCTGTAGTAGAACGNACAAG-3'	50 °C	Freshwater and Rueness (1994) and Gavio and Fredericq (2002)
	F-577	5'-GTATATGAAGGTCTAAAAGGTGG-3'		
	R-753	5'-GCTCTTTCATACATATCTTCC-3'		
	R-rbcS start	5'-GTTCTTTGTGTTAATCTCAC-3'		
RuBisCO spacer	RuBisCO_for	5'-TGTGGACCTCTACAAACAGC-3'	55 °C	Zuccarello et al. (1999b)
	RuBisCO_rev	5'-CCCCATAGTTCCCAAT-3'		

* Ambiguous nucleotide codes are in accordance to IUPAC: K = G/T; R = A/G; W = A/T; N = A/T/C/G

tion characters, interspecific divergences and intraspecific variations. According to Tan and co-workers (2012), the mitochondrial-encoded *cox2-3* spacer was the most suitable marker for molecular identification, basic molecular phylogeny and DNA-barcoding (discussed below) of *Kappaphycus* and *Euचेuma*, considering the large database of DNA data already available in GenBank. However, the *cox2-3* spacer, along with *cox2*, were more suitable at delineating intergeneric and inter-specific relationships. Phylogenetic analysis of higher taxa requires a combination of both *cox1* and *rbcL* which are genetically more conservative (Tan et al.

2012). In terms of phylogeny, a multi-gene approach, involving DNA from different organelles, is most likely to best represent the evolutionary pathways of these commercially important rhodophytes, regardless of the taxonomic rank being analyzed.

DNA-barcoding is the use of short, universal genetic markers in DNA in order to determine its identity, based on online databases. The technique was first introduced by Herbert and co-workers (2003a, b, 2004). Although conceptually similar to the use of genetic marker(s) for the molecular identification of organisms, DNA-barcoding emphasizes

the preferable use of just one DNA barcode for rapid and reliable identification, particularly by non-experts. Thus, the assessment and selection of DNA-barcodes are generally more thorough and comprehensive, also taking into account the universality of the DNA-barcode, popularity, ease of amplification, length of the DNA barcode (short barcodes may contain less information, whereas longer ones may take too long to analyze), in addition to resolving power, as well as inter-specific divergences and intra-specific variations (Jinbo et al. 2011; Meier et al. 2008; Meyer and Paulay 2005; Tan et al. 2012). The mitochondrial *cox2* and *cox2-3* spacers were identified as suitable DNA-barcodes for *Kappaphycus* and *Eucheuma* (Tan et al. 2012), however the extent of their effectiveness will become more evident as an increasing number of species are sampled.

2.4 Molecular Identification of *Kappaphycus* and *Eucheuma*

The morphologically plastic nature of eucheumatoid seaweeds (e.g. *Betaphycus*, *Eucheuma* and *Kappaphycus*) was well known in the early 1980s (Doty 1985, 1988; Doty and Norris 1985), whereby different species of seaweeds may resemble one another, or conspecifics appearing different between individuals. The issue led to confusion in the identification of these seaweeds, which not only impeded taxonomic studies, but affected farmers as well as the entire carrageenan industry. The confusion was exacerbated by the use of colloquial and commercial names which differ amongst countries (Doty 1985; Doty and Norris 1985; Zuccarello et al. 2006). For example, the common trade name for *Kappaphycus alvarezii* (formerly known as *Eucheuma alvarezii*) is “cottoni” which is not to be confused with *Kappaphycus cottonii* (Weber-van Bosse) Doty ex P.C.Silva, a different species within the genus. Another example involves the use of the local term “*Aring-aring*” which refers to *K. malesianus* in Malaysia but *K. alvarezii* in the Philippines (Dumilag and Lluisma 2014; Tan et al. 2013, 2014).

Comprehensive work, mostly by MS Doty led to the eventual classification of eucheumatoid seaweeds into the genera used at present, viz. *Betaphycus*, *Eucheuma* and *Kappaphycus* (Doty 1985, 1988; Doty and Alvarez 1975; Doty and Norris 1985). The delineation of these genera was based on morphological and biochemical characteristics of these red seaweeds. Some distinctive morphological characters included: branching patterns, the presence of spines, cystocarp structure, as well as cell arrangement (microscopy required). The most notable biochemical differences between *Betaphycus*, *Eucheuma* and *Kappaphycus* is the type of carrageenan produced, namely *Beta* (β), *Iota* (ι) and *Kappa* (κ) carrageenan, respectively.

Although the morphological and biochemical features allow for the inter-generic distinctions to be made between *Betaphycus*, *Eucheuma* and *Kappaphycus*, these features were less reliable in differentiation at the species level (Lim et al. 2014b; Tan et al. 2013; Zuccarello et al. 1999). Species identification has proven to be exceptionally challenging in Southeast Asia which harbors a rich diversity of eucheumatoids. For example, Tan and co-workers reported at least eight varieties of *Kappaphycus* that were recognized by the locals in Sabah, Malaysia (Lim et al. 2014b; Tan et al. 2013; Phang et al. 2010). Likewise, numerous varieties of *Kappaphycus* and *Eucheuma* are also cultivated in Indonesia and the Philippines (Lim et al. 2014a). Without a reliable means of identifying the farmed seaweeds, most farmers end up planting mixed populations of *Kappaphycus* and *Eucheumaseaweeds*. This translates to profit losses in the carrageenan industry in Malaysia as the harvested *Kappaphycus* and *Eucheumaseaweeds* produce different types of carrageenan which have to be separated prior to processing (Lim et al. 2014b; Tan et al. 2013; Phang et al. 2010). In other countries, concerns were mainly due to the uncontrolled spread of invasive seaweed strains arising from the commercially introduced *Kappaphycus alvarezii* (Araújo et al. 2013; Conklin et al. 2009; Dumilag and Lluisma 2014; Zhao and He 2011). These issues promoted the need for the application of molecular techniques in identification of *Betaphycus*, *Eucheuma* and *Kappaphycuseaweeds* (Araújo et al. 2013; Conklin et al. 2009; Dumilag and Lluisma 2014; Lim et al. 2014a; Tan et al. 2012, 2013, 2014; Zhao and He 2011; Zuccarello et al. 2006; Maili et al. 2016). The identity of a seaweed is usually determined via genetic comparison against DNA data within an online database such as GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>). Despite the ease and convenience of this method, care has to be taken in verifying the authenticity and reliability of DNA sequences available within the repository. In addition, the confirmation of a seaweed species should always be supported with morphological examination.

In Malaysia, the application of the several mitochondrial and the plastid-based genetic markers has led to the clear identification of several varieties of seaweed. For example, the “*Aring-aring*” variety of *Kappaphycus*, which is morphological similar to *K. alvarezii* was shown to be a different species, now described as *K. malesianus* J. Tan, P. E. Lim et S.M. Phang (Lim et al. 2014b; Tan et al. 2013, 2014). Likewise, the commonly cultivated “*Cacing*” variety was shown to be *Eucheuma* instead of *Kappaphycus*. These findings had immediate practical implications in that they minimized the cultivation of mixed populations of *Kappaphycus* and *Eucheumaseaweeds* within the country, thereby decreasing processing issues and in so doing increasing the value of the biomass. Similar approaches were applied to farmed and wild (growing on the seabed far away from any seaweed

farms) specimens elsewhere in a first attempt to identify the bio- and genetic diversity of these seaweeds.

2.5 Molecular Phylogenetics of *Kappaphycus* and *Eucheuma*

Molecular phylogeny is often performed alongside molecular identification as both studies involve the same DNA sequences or dataset. Unlike molecular identification which employs the use of genetic data for species identification, molecular phylogeny infers relationships between various species (or taxa) of organisms. As genotype is inherently linked to phenotype, so molecular-based phylogenetic results are often used to supplement morphologically-based phylogeny, or taxonomy. Often this analysis reveals evolutionary lineages as well as the genetic distribution of organisms from various regions of the world, which is particularly important in strain selection and the detection of bio-invasions (Conklin et al. 2009; Tan et al. 2012; Zuccarello et al. 2006).

Molecular phylogeny of seaweeds, at the family and genus level, was reported during the 1990s, mostly relying on the plastid-encoded *rbcL* gene (Fredericq et al. 1999; Freshwater et al. 1994). Freshwater and co-workers (1994) reported the paraphyly of *Eucheuma*, which was supported by subsequent studies employing different sets of molecular markers (Tan et al. 2013; Zuccarello et al. 2006). The phylogeny of *Betaphycus*, *Eucheuma* and *Kappaphycus* (Fig. 2.5) is based mainly on the mitochondrial *cox2-3* spacer marker due to the large number of sequences available in GenBank.

The *cox2-3* spacer phylogenetic tree (Fig. 2.5) has shown that *Kappaphycus* and *Eucheuma* are genetically different. However, the phylogenetic resolution of the genetic marker was not sufficient to infer the relationships between *Betaphycus*, *Eucheuma* and *Kappaphycus*. The paraphyly of *Eucheuma* was observed with the inclusion of *Eucheuma isiforme* specimens from America (Doty 1988; Doty and Norris 1985; Zuccarello et al. 2006).

The genus *Kappaphycus* was inferred to be monophyletic although the taxonomic position of the poorly studied *K. cot-*

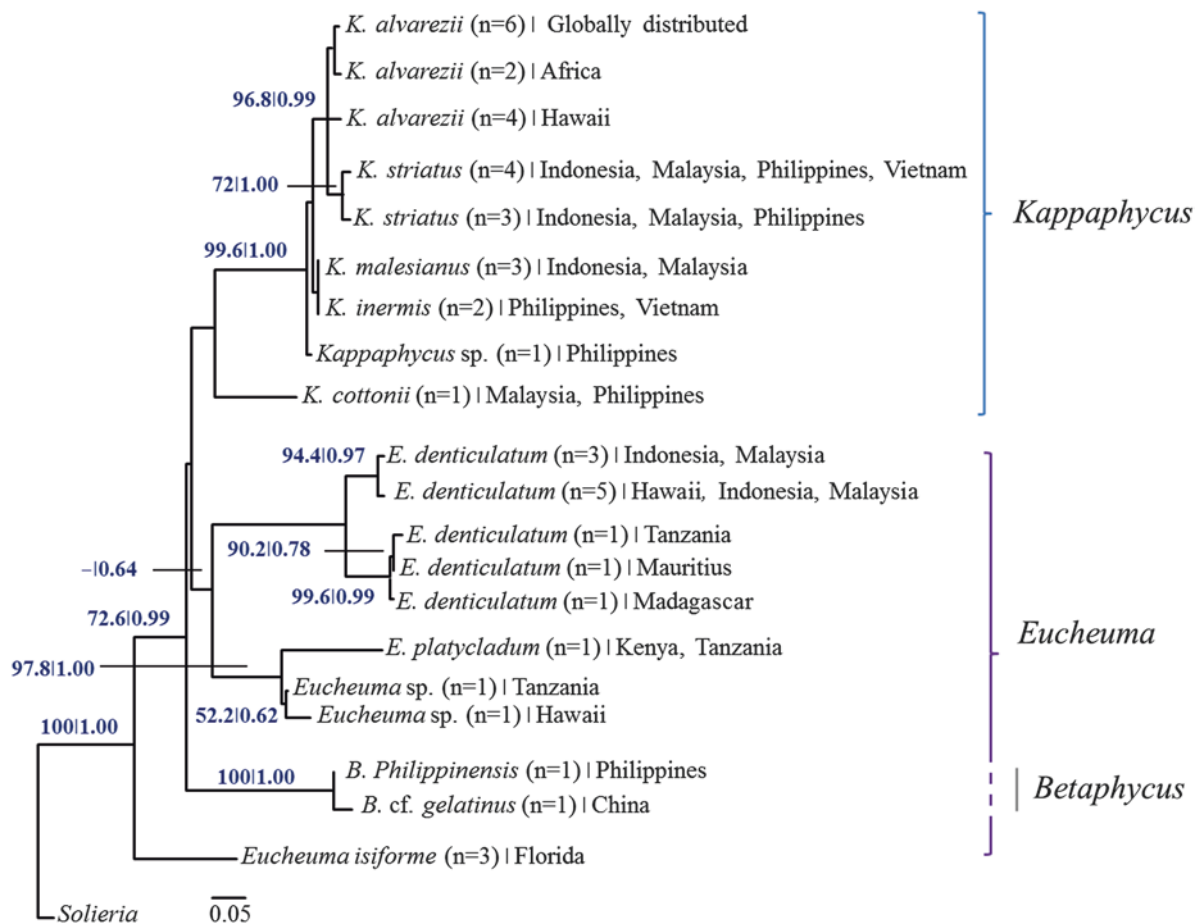


Fig. 2.5 Simplified phylogenetic tree showing the current relationships between *Betaphycus*, *Eucheuma* and *Kappaphycus* based on the mitochondrial-encoded *cox2-3* spacer. Nodal values denote Maximum

Likelihood bootstrap support and Bayesian posterior probabilities (Phylogenetic tree constructed based on DNA data adopted from Dumilag and Lluisma 2014; Tan et al. 2013; Zuccarello et al. 2006)

tonii is unresolved (Doty 1988; Doty and Norris 1985; Zuccarello et al. 2006). It is also worth noting that DNA records of *K. procrusteanus* (Kraft) Doty 1988, first reported from the Philippines, were unavailable (Tan et al. 2014). Based on genetic results, three different genotypes of *K. alvarezii* were reported (Tan et al. 2013; Zuccarello et al. 2006; Conklin et al. 2009): (i). A *K. alvarezii* strain that is globally distributed, and is likely the commercial strain initiated in the Philippines; (ii). A genotype unique to Africa; and (iii). a strain exclusive to the Hawaiian islands. These genotypes were not known to be morphologically different from one another. Based on the *cox2–3* spacer, the globally distributed *K. alvarezii* specimens were genetically closer to those collected from Africa (Genetic Distance, GD, of <0.9%) than those from Hawaii (GD of <2.1%). Comparative growth and carrageenan studies between these genotypes may lead to the identification of potentially better cultivars.

Likewise, two different genotypes of *Kappaphycus striatus* were observed within Southeast Asia (GD < 1.2%; *cox2–3* spacer), in which one of the genotypes is constituted of commercially cultivated strains, whereas the other involves specimens from the wild. On the other hand, *K. malesianus*, a newly described species (Tan et al. 2014) was inferred to form a sister clade to *K. inermis* from the Philippines, based on both mitochondrial and plastid-encoded genetic markers (Dumilag and Lluisma 2014). Sampling efforts by Lim and co-workers (2014a) revealed several new genotypes of *Kappaphycus* which may be potential new species worth further research.

Research on *Eucheumaseaweeds* has been generally less extensive than that of *Kappaphycus*, which may be attributed to: (i). sampling difficulty; (ii). relatively lower economic value and (iii). the vast number of species available, many of which may be synonymous. Three main genotypes of *E. denticulatum*, the most widely cultivated *Eucheuma* species, were reported (Lim et al. 2014a; Tan et al. 2013; Zuccarello et al. 2006): two genotypes which are cultivated in Southeast Asia (and countries where they have been introduced) and one genotype exclusive to Africa. A genetic distance of <9.1% was observed between the Southeast Asian and African genotypes of *Eucheuma*. The taxonomic positions of other *Eucheuma* species were poorly resolved and can only be inferred when more samples become available.

The genus *Betaphycus* is also poorly studied, although its taxonomic differentiation from *Eucheuma* and *Kappaphycus* was reported from earlier studies (Doty 1988; Doty and Norris 1985; Dumilag et al. 2014; Tan et al. 2013; Zuccarello et al. 2006). Dumilag and co-workers (2014) suggested that the type of cell wall polysaccharide may not be a reliable character in the taxonomy of *Betaphycus* due to the presence of hybrid carrageenan structures (Chopin et al. 1999). The authors also suggested the possible conspecificity of *B. philippinensis* and *B. speciosus* based on morphological and molecular analyses.

Phylogenetic studies of *Betaphycus*, *Eucheuma* and *Kappaphycus* within the past decade (Conklin et al. 2009; Dumilag et al. 2014; Dumilag and Lluisma 2014; Tan et al. 2012, 2013, 2014; Zuccarello et al. 2006) have revealed valuable insights into the taxonomic positioning of these species. Although the commonly used genetic markers, viz., *cox1*, *cox2*, *cox2–3* spacer, *rbcL* were quite capable of species delineation, resolving inter-generic relationships would most likely require slower-evolving markers, or the use of concatenated datasets involving nuclear, plastid and mitochondrial genetic regions.

2.6 Genetic Diversity of *Kappaphycus* and *Eucheuma*

Documenting the genetic diversity of *Kappaphycus* and *Eucheuma* provides promising benefits to the seaweed industry, which include: marker-assisted selection of good seaweed strains for cultivation, DNA-barcoding, monitoring of invasive strains of *Kappaphycus* or *Eucheuma*, as well as studies on biodiversity and phylogeography. Lim et al. (2014a) reported for the first time, a collaborative effort between major carrageenan producers within Southeast Asia to document the genetic diversity of *Kappaphycus* and *Eucheuma* within the region. The study involved a large-scale sampling and study program for *Kappaphycus* and *Eucheuma* involving Malaysia, the Philippines, Indonesia and Vietnam. The study employed the use of the mitochondrial-encoded *cox1* and *cox2–3* spacers to generate haplotype (DNA polymorphism) networks of these seaweeds. Haplotype networks based on the concatenated *cox1* and *cox2–3* spacer markers of *Kappaphycus* and *Eucheuma* are summarized in Figs. 2.6 and 2.7, respectively.

In brief, the study revealed many different haplotypes within Southeast Asia for most species of *Kappaphycus*, as well as *Eucheuma denticulatum*. A total of seven known haplotypes was revealed for *K. alvarezii*, seven for *K. striatus*, four for *K. malesianus*, as well as two unknown *Kappaphycus* spp. from Vietnam and the Philippines. The main haplotype of *K. alvarezii* “KA” is cultivated throughout Southeast Asia and is likely to be distributed globally. Interestingly, three different major genotypes of *K. striatus* were observed in Southeast Asia, namely “KSA” which was present in Malaysia, Indonesia, Philippines and Vietnam; “KSB” which was observed in Malaysia and Indonesia, and “GUI2” which was exclusive to the Philippines. These genotypes are morphologically similar and may potentially be cryptic species. The major haplotype of *K. malesianus* “MY216” (including cultivars), showed 75 nucleotide differences from *K. alvarezii* “KA”. The two unidentified *Kappaphycus* species recorded a nucleotide difference of four and 13 steps from

Fig. 2.6 Haplotype networks of *Kappaphycus* based on the concatenated *cox1-cox2-3* spacer markers. Each line represents a point mutation (a step) whereas circles indicate hypothetical haplotypes. Common haplotypes are represented by larger circles (not drawn to scale) (Haplotype network adopted from Lim et al. 2014a, b)

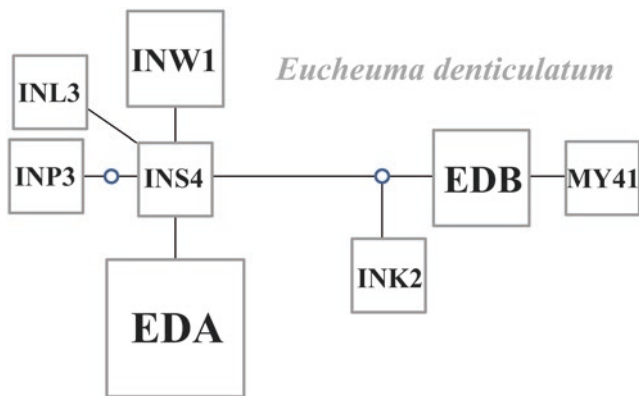
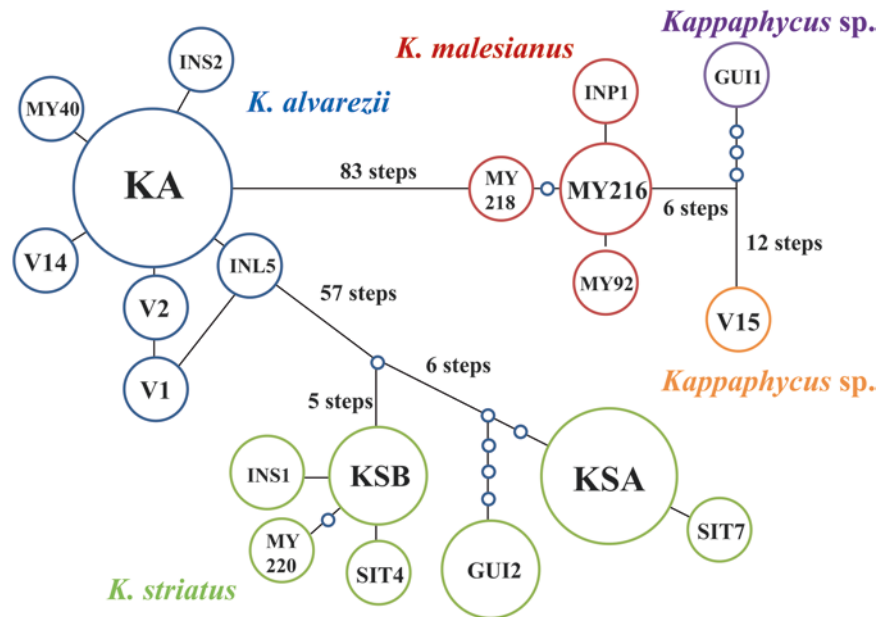


Fig. 2.7 Haplotype network of *Eucheuma* based on the concatenated *cox1-cox2-3* spacer markers. Each line represents a point mutation (a step) whereas circles indicate hypothetical haplotypes. Common haplotypes are represented by larger circles (not drawn to scale) (Haplotype network adopted from Lim et al. 2014a)

“MY216”, which inferred genetic relatedness to *K. malesianus*, although there was no geographical pattern associated with these seaweeds.

The scarcity of *Eucheuma* spp. (and *Betaphycus*) samples impeded the genetic documentation of these carrageenophytes. Only *E. denticulatum* was sampled in Lim et al. (2014a) which hinted at the rarity of other *Eucheuma* spp., at least within the vicinity of shores and farms in Southeast Asia. Two main haplotypes of *E. denticulatum* (which differed by ten nucleotides), namely “EDA” and “EDB” were sampled within the region. Based on the preliminary study, Lim and collaborators (2014a) believed that numerous

additional haplotypes and new species of *Kappaphycus* and *Eucheuma* are present within the seas of Southeast Asia, particularly within the Coral Triangle. However, sampling the thousands of islands within this region will require concerted efforts, particularly in terms of funding and logistics between Southeast Asian countries in order to comprehensively document the genetic diversity of these commercially important rhodophytes. Similarly, genetic diversity studies could be performed for countries or continents with native populations of *Kappaphycus* or *Eucheuma*, e.g. Africa and Hawaii.

2.7 Introduction of *K. alvarezii* to Non-indigenous Areas

Non-indigenous means a species occurs outside its native range generally arising by human influences. It is synonymous to “non- native,” “alien” and “exotic” (Lodge et al. 2006). Species that spread widely beyond the location of initial establishment, become locally abundant, or spread into natural areas, are referred to as invasive and this depends on time and spatial scale. Within the Areschougiaceae, 8 from the total of 96 species have been successful invaders (Williams and Smith 2007), including members of the genera *Kappaphycus*, *Eucheuma* and *Sarconema*, many of these were intentionally introduced for commercial carrageenan production (Zemke-White and Smith 2006). Amongst those red species which has been introduced to many places outside their original location of the Philippines are *Kappaphycus alvarezii*, *K. striatus* and *Eucheuma denticulatum* (Ask et al. 2003; Hurtado et al. 2016; Fig. 2.8).

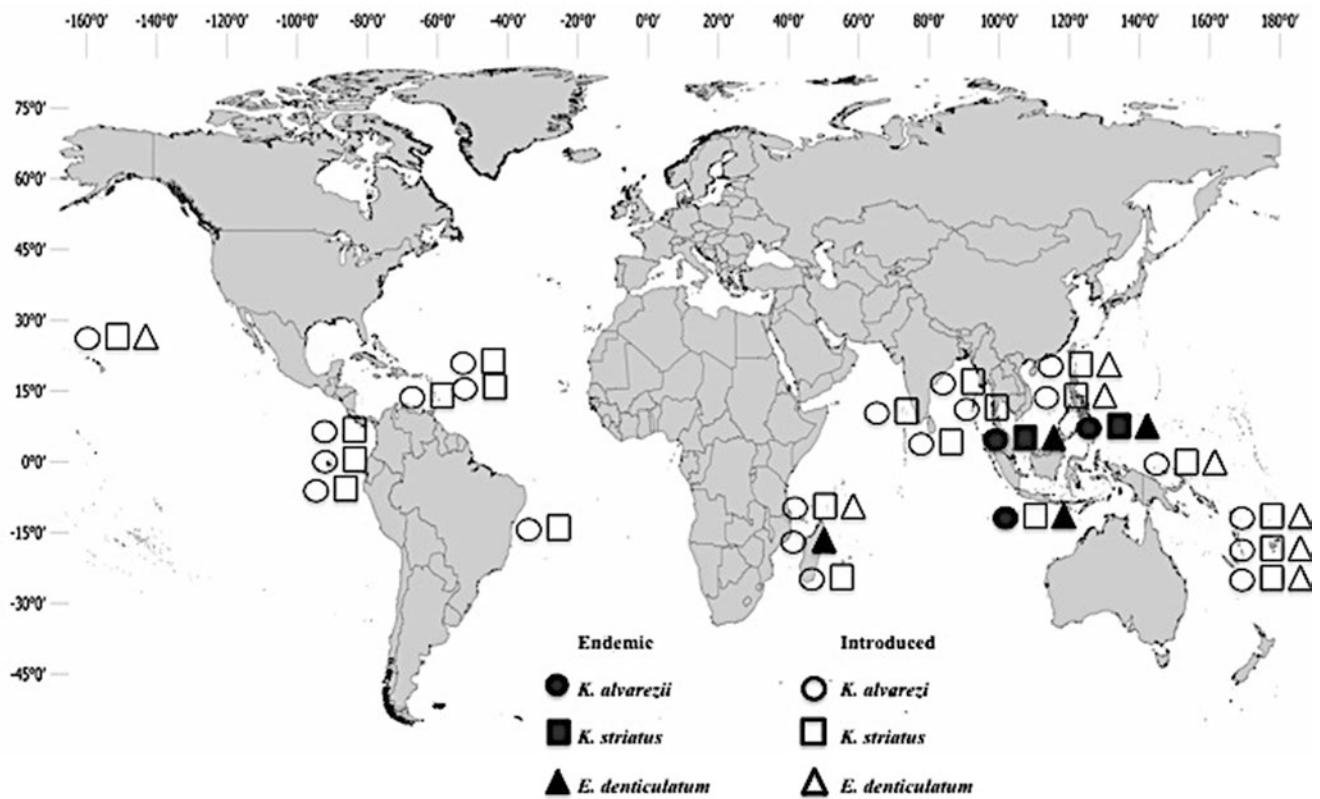


Fig. 2.8 Endemic and introduced *K. alvarezii*, *K. striatus* and *E. denticulatum*

Of the almost 30 countries where these seaweeds were introduced, only Hawaii and India reported invasion on corals. The introduction of *K. alvarezii*, *K. striatus* and *E. denticulatum* in areas which were not indigenous was always made with good intentions, i.e. for research purposes and ultimately for commercial cultivation, since these species bring economic gains to seaweed farmers as well as to the whole seaweed carrageenan industry (Porse and Rudolph 2017).

The only reason for the introduction of *Kappaphycus alvarezii*, *K. striatus* and *E. denticulatum* outside the Philippines, Indonesia and Malaysia was for their use as raw materials for aquaculture purposes. These seaweeds were intentionally introduced in 1974 to late 1976 into the fringing reef surrounding the Hawai'i Institute of Marine Biology (HIMB) at Coconut Island (Moku o Lo'e), Kane'ohe Bay, O'ahu, Hawaiian Islands for experimental research and commercial aquaculture projects (Doty 1978; Russell 1983). Though the pilot-scale evaluations were positive, even after several years, no commercial cultivation was initiated in the area. Sadly further to Prof Doty's death and subsequent lack of sustained attention, the experimental pilot farms were abandoned, the cultivars which had been grown there then very surprisingly gave rise to "alien species", which began to expand their range beyond the cultivation sites, as claimed by Woo (1999), Rodgers and Cox 1999; Smith et al. (2002), Conklin and

Smith (2005). The remarkable observations were that once "benign strains", were able to reproduce via sporulation and further more attach (via rhizoids), something unknown (or unobserved) to that point in cultivation sites. These were definitely important lessons learned. The success of the non-indigenous eucheumatoid algae may have been the result of a variety of factors including adaptation to low and high-wave energy environments, extraordinary phenotypic plasticity, chemical or physical defense from herbivory and diverse physiological characteristics that lead to rapid growth rates from vegetative propagation (Borowitzka 1981; Duffy and Hay 1990; Holmlund et al. 1990; Hay et al. 1994; Bolser and Hay 1996; Hay 1997; Paul 1997). Despite the absence of reproductive plants in Ka'ne'ohe Bay, O'ahu, vegetative propagation from small fragments allowed these seaweeds to spread within the vicinity to a limited extent, as no growth of *Kappaphycus* was found outside the Bay, probably due to its heavy weight which limits long dispersal (Woo 1999).

Management strategies were implemented to reduce the spread of the invasive *Kappaphycus* in Hawaii. Manual removal of the biomass of *Kappaphycus* was time consuming for large-scale removals, though a modified dredging is under test. Populating the area with *Tripneustes gratilla* as bio-control in reducing the biomass of *Kappaphycus* was promising (Conklin and Smith 2005), but the low palatability

of the algae to native herbivores seemed not to show positive effect as claimed further by the authors.

A similar example leading to the notoriety of cultivated eucheumatoids was later provided by an example from another side of the world. *Kappaphycus alvarezii* (a Philippine-derived macroalga) was introduced by PepsiCo into the Gulf of Mannar (GoM) Marine Biosphere Reserve, South India for mariculture in 2000. However, at about the same time as matters in Hawaii were becoming evident, the ecological threat from this invasive alga to coral species in GoM was first reported by Pereira and Verlecar (2005) followed by Tewari et al. in 2006 and later picked-up by the Daily Spreadsheet (Anon. 2006; Vijayalakshmi 2007). These reports were affirmed by the report of Chandrasekaran et al. (2008) who made qualitative observations and quantitative analysis on the bio-invasion of *K. alvarezii* on corals in the Kurusadai Island of the GoM. The authors observed that *K. alvarezii* had successfully “invaded” and established by attachment on both dead and live corals, specifically *Acropora nobilis* and *A. formosa* in Kurusadai Island; death of the corals followed due to shadowing and smothering effects. The invasive populations occurred as either mono-specific beds or mixed with other marine communities on live and dead corals, coral rubble and pavement. Furthermore, the authors observed an extraordinary phenotypic plasticity in terms of color and shape of the thallus, i.e. thickness of its major axis, morphological features and frequency of primary and secondary branching. In terms of the cover of coral by *K. alvarezii* (sq. cm/0.5 sq. m) between two sites studied, there were significant differences ($P < 0.05$), i.e. a higher cover of *K. alvarezii* was measured towards the open sea; but there was no significant difference between colonization of areas of corals or sand. The first report was further bolstered by Kamalakannan et al. (2010) reporting the unusual appearance of *K. alvarezii* on the brown seaweed *Turbinaria* sp. was observed. The space between the lateral “plates” (or ‘cups’) of *Turbinaria* sp. provided an ideal settlement surface for *K. alvarezii*, providing protection from wave action and favoring the profuse growth of secondary branches of *K. alvarezii* between the plates, this was directly in contrast to the smothering effect on the top of coral plates.

However, the earliest reports on the invasion of *K. alvarezii* at Kurusadai Is. was disputed by Mandal et al. (2010) which presented findings based on a one-year study (2008–2009) in the same area. Their rigorous sampling revealed that *K. alvarezii* was confined to just two separate patches of 105 m × 55 m and 8 m × 9 m, located at the southeastern part of Kurusadai Island with an actual extent of canopy coverage of 76.7 m² – accounting for less than 0.0035% of the total coral reef area. The daily growth rate of the *K. alvarezii* at Kurusadai Is. was very small at 0.7% day⁻¹. *K. alvarezii* was not observed in the coral reef area of the adjoining Pullivalal and Poomarichan Islands or the Palk Bay cultivation sites.

The authors claimed that the lack of functional reproductive material, low spore viability and the absence of microscopic phases in the life cycle of this alga coupled with the abundant presence of herbivores may have restricted the spread of this alga; so, on reflection, after much controversy and negative publicity the continued invasion of *K. alvarezii* at Kurusadai Island is considered remote. Today, commercial *Kappaphycus* farming has gained wide acceptance by the coastal fisher-folks as a source of livelihood in India (Krishnan and Narayanakumar 2013; Periyasamy et al. 2014a, b, 2015).

2.8 Future Studies

Although recent molecular studies have emphasized solving fundamental issues pertaining to the identification of commercial *Kappaphycus* and *Eucheuma* (and *Betaphycus*) seaweeds, the authors believed that enhanced applications of molecular tools will provide necessary information for management decisions and options for future development. Taken together, these will be able to bring about further, economically important changes to the seaweed industry. In addition, the advent of efficient molecular techniques and enhanced computing power is expected to expedite molecular research of these seaweeds. Of the potential research that remains to be done, the authors recommend three key areas for improvement:

2.8.1 Wider and Deeper Approach to Sampling

More specimens are undoubtedly required to better understand (any aspects of) *Kappaphycus* and *Eucheuma*. Recent sampling approaches focused on specimen collection from wet markets, seaweed farms or seashores. As many cultivated seaweeds are vegetatively propagated from one particular species (Ask and Azanza 2002; Ask et al. 2003; Hurtado et al. 2001), they share similar, if not identical genotypes. The same applies to specimens collected near seaweed farms or those washed ashore, which often originated from broken thalli of farmed cultivars (and are thus genetically identical). This situation leads to a general under-estimation of genetic diversity of these seaweeds. Therefore, the search for new species or potential cultivars of *Kappaphycus* and *Eucheuma* should involve the collection of specimens from sea beds via scuba-diving, far away from seaweed farms to minimize the chances of collecting broken thalli from farmed seaweeds. Apart from genetic difference, seaweeds collected from the wild are often present in cystocarpic form (rarely seen in cultivars), which is useful in identification or taxonomic description. The various species of *Kappaphycus* and *Eucheuma* originally collected and described from the Coral

Triangle (many of which were not reported again) also imply that the natural diversity has not been adequately described, and there may yet be many species and varieties of carrageenophytes to be collected and evaluated for cultivation. This also applies to the Hawaiian Islands, Africa, etc., where native populations of *Kappaphycus* and *Euclidean* have been reported in their taxonomic records. In addition, reinvigorated sampling efforts should also be focused at type localities of rare and poorly studied seaweeds, e.g. *K. procrusteanus*, *K. cottonii*, *E. arnoldii* etc.

2.8.2 Taxonomic Revision and Comprehensive Genetic Studies

The taxonomy of *Kappaphycus* and especially *Euclidean* require revision, especially when more specimens are collected for analysis. Although the application of molecular methods has greatly aided in the identification and phylogenetic understanding of these seaweeds, further progress has been impeded by the general under-sampling of several important species. This again stresses the importance of more comprehensive sampling. The application of a multi-genetic approach, preferably with genes from different organelles, will not only aid in the identification of (particularly poorly studied) seaweeds, but will better document the evolutionary pathway of *Kappaphycus* and *Euclidean* seaweeds, which are some of the most important seaweeds in marine agronomy on a global basis. These studies will serve as important supplementary data in taxonomic revisions. The increasing prevalence of genome sequencing methods will also contribute to the field of phylogenomics, genetic selection, breeding and genetic manipulation.

2.8.3 Putting Genetic Information to Use

It is important that the genetic results generated from the molecular studies of *Kappaphycus* and *Euclidean* be applied on a commercial scale, so as to benefit the carrageenan industry as a whole. Molecular results can be applied under various circumstances: (i) the use of genetic markers in the identification of new strains or varieties of seaweeds, particularly those from the wild (which are often deformed or damaged); (ii) the use of molecular markers in marker-assisted identification and selection of good genotypes for cultivation; (iii) genetic diversity studies to catalog the biodiversity of seaweeds within a region, as well as to monitor the occurrence of invasive seaweed species; (iv) genomic studies to identify genes that govern “quality traits” of seaweeds (e.g. rapid growth, disease resistance, good carrageenan yields and quality, etc.) and to further select them either via

breeding and/or genetic engineering. Naturally, the application of scientific knowledge out in the field necessitates a proper platform linking academia to entrepreneurs as well as to the farmers. The improvement of the euclidean seaweed industry will also benefit from research grants, incentives as well as financial aids from local government and authorities. Finally, collaborative efforts will also strengthen research and business ties.

The achievement of these key points will lead to better farm practices and management as well as the cultivation of excellent and robust cultivars. These translate to better consistency and efficiency in the production of quality carrageenan per farm, which in turn generates revenue for the entire carrageenan industry, as well as the economy of the country.

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