Systematics and genetic variation in commercial *Kappaphycus* and *Eucheuma* (Solieriaceae, Rhodophyta)

Giuseppe C. Zuccarello[1,*], Alan T. Critchley[2], Jennifer Smith[3], Volker Sieber[4], Genevieve Bleicher Lhonneur[2] & John A. West[5]

[1]*School of Biological Sciences, Victoria University of Wellington, P.O. Box 600, Wellington 6001, New Zealand;*
[2]*Degussa Texturant Systems France SAS, Baupte, France;* [3]*Department of Botany, University of Hawaii Manoa, Honolulu, HI 96822, USA;* [4]*Degussa Food Ingredients GmbH, Freising, Germany;* [5]*School of Botany, University of Melbourne, Victoria 3010, Australia*

*Author for correspondence: e-mail: joe.zuccarello@vuw.ac.nz

Abstract

The systematics and taxonomy of *Kappaphycus* and *Eucheuma* (Solieriaceae) is confused and difficult due to morphological plasticity, lack of adequate characters to identify species and commercial names of convenience. These taxa are geographically widely dispersed through cultivation. Commercial, wild and herbarium sources were analysed; molecular markers provided insights into taxonomy and genetic variation, and where sources of genetic variation may be located. The mitochondrial *cox2-3* and plastidal RuBisCo spacers were sequenced. There is a clear genetic distinction between *K. alvarezii* ("cottonii") and *K. striatum* ("sacol") samples. *Kappaphycus alvarezii* from Hawaii and some samples from Africa are also genetically distinct. Our data also show that all currently cultivated *K. alvarezii* from all over the world have a similar mitochondrial haplotype. Within *Eucheuma denticulatum* ("spinosum") most African samples are again genetically distinct. Our data also suggest that currently cultivated *E. denticulatum* may have been "domesticated" several times, whereas this is not evident for the cultivated *K. alvarezii*. The present markers used do not distinguish all the morpho-types known in cultivation (e.g. var. *tambalang*, "giant" type) but do suggest that these markers may be useful to assess introductions and species identification in samples.

Introduction

The seaweeds most commonly cultivated for the carrageenan industry belong to the genera *Kappaphycus* Doty and *Eucheuma* J. Agardh. These crops are almost entirely farmed and are usually referred to by the commercial names "cottonii", "sacol" and "spinosum". The formal taxonomy of these taxa has for a long time been in confusion due to misapplication of commercial and scientific names, the known general paucity of adequate morphological characters and the morphological plasticity of seaweeds. Much of the taxonomic confusion was addressed by the pioneering work of Maxwell Doty (Doty, 1985, 1988; Doty & Norris, 1985). Even in the detailed work of Doty, variability in the presence or absence of diagnostic morphological characters within taxa was noted, especially in non-ideal speci-

mens (i.e. non-reproductive specimens and specimens lacking typical attachment structures) and this was addressed by the caveat that descriptive paragraphs must carry the preamble "there is a tendency…" (Doty, 1988, p. 166).

Doty (1988), however, formally recognized certain species of *Eucheuma* as *Kappaphycus*, mostly based on their production of κ-carrageenan, and this generic circumscription has been supported, for the most part, by molecular studies (Fredericq et al., 1999; Aguilan et al., 2003). Nevertheless, questions remain as to the taxonomic identity of commercially produced strains. *Kappaphycus alvarezii* (Doty) Doty *ex* P. Silva is the most-grown commercial κ-carrageenan producer and many varieties and local strains are known (www.surialink.com). One of the commercially used 'strains' of *Kappaphycus* is the so-called "sacol" vari-

ety, but its scientific name is still unresolved. While it was originally considered to be *K. striatum* (Schmitz) Doty *ex* P. Silva (Trono, 1997), recent molecular investigations suggested that it could be a form of *K. cottonii* (Weber-van Bosse) Doty *ex* P. Silva (Aguilan et al., 2003). *Kappaphycus cottonii* is morphologically quite distinct from either *K. alvarezii* or *K. striatum* as it mostly forms prostrate branches. Culture studies have shown that many of the characters used to separate *Kappaphycus* species (e.g. habit, decumbent versus dichotomous) are found to segregate in tetraspore progeny (de Paula et al., 1999) from single plants, and it is likely that the identification of individual specimens based on morphology is unreliable.

Molecular markers have proven useful in not only elucidating red algal systematics but also in discovering genetic variation within red algal species. Commonly used intraspecific markers are the nuclear-encoded internal transcribed spacers of the ribosomal cistrons (ITS 1 and 2, e.g. Marston & Villalard-Bohnsack, 2002), the plastid-encoded RuBisCo spacer (e.g. Zuccarello et al., 2002) and the mitochondrial-encoded *cox2-3* spacer (Zuccarello & West, 2003), although these markers have their limitations, such as uniparental inheritance and limited variation (i.e. they do not reflect all the genetic variation found within groups). Studies using the RuBisCo spacer have shown that even single base pair changes could indicate reproductively isolated cryptic species (Zuccarello & West, 2003), while there is more variation within species at the *cox2-3* spacer region, due to its higher mutation rate (Zuccarello & West, 2002).

This work aimed to: (1) determine the levels of genetic variation in commercially grown species of *Kappaphycus* and *Eucheuma*; (2) clarify some of the taxonomic confusion in commercial strains and wild strains of *Kappaphycus* and *Eucheuma*; (3) determine which geographic regions contain samples with ecologically superior genotypes or with genetic variation that is potentially useful to the industry.

Materials and methods

Samples were for the most part obtained from commercial supplies. Thalli were selected from the corners of representative bales delivered from suppliers for industrial extraction of carrageenan. Samples were placed in silica gel until DNA extraction. Although drying and storage methods may have differed, nearly all samples were adequate for DNA extraction and am-

plification. Often exact provenance (specific region, farm) of the samples was unknown. Other samples were collected (Hawaii and Indonesia) and dried immediately in silica gel. Hawaiian samples collected at Kane'ohe Bay spanned the range of morphologies at this site where material is introduced and invasive, and contained many cystocarpic or tetrasporic specimens. Other samples were removed from herbarium sheets and processed as below. All samples used are listed in Table 1.

DNA extractions were performed using a modified CTAB extraction procedure. Dried samples (approx. 1 cm tip) were pulverized using a shaking mill (Retsch, type MM200) and then placed in a microfuge tube containing 500 μL of CTAB extraction buffer (2% CTAB, 0.1 M Tris-HCl (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 1% PEG 8000) plus 50 μg RNAse A and 80 g Proteinase K (Promega, Madison, USA). Samples were then placed at 60°C for 30 min, mixing occasionally. Two extractions using an equal volume of chloroform:isoamyl alcohol (24:1), mixing, and spinning for 10 min at 12,000 g were preformed. DNA was precipitated with an equal volume of 100% isopropanol, the tube inverted and placed at room temperature for 30 min. The sample was spun for 30 min at 12,000 g and decanted and then washed in 70% ethanol, air-dried and 50 μL of 0.1 X TE buffer was added.

Amplification of the plastid-encoded RuBisCo spacer followed procedures outlined in Zuccarello et al. (1999b). Amplification of the mitochondrial-encoded *cox2-3* spacer and sequencing followed procedures outlined in Zuccarello et al. (1999a). Sequences were aligned by eye. Phylogenetic relationships were inferred with PAUP*4.0b10 (Swofford, 2002). Data sets from different genomic regions were tested for incongruence using the partition homogeneity test (PHT) (Farris et al., 1994) as implemented in PAUP* (1000 replicates, 5 random additions, 100 trees per addition saved). A combined dataset was subjected to maximum-parsimony (MP) analysis, using the heuristic search option, 500 random sequence additions, 100 trees per addition saved, TBR branch swapping, unordered and unweighted characters, and gaps treated as missing data. The program Modeltest version 3.06 (Posada & Crandall, 1998) was used to find the model of sequence evolution that best fits each data set by a hierarchical likelihood ratio test ($\alpha = 0.01$). When the best sequence evolution model had been determined, maximum-likelihood searches were performed in PAUP* using the estimated parameters (substitution model, gamma distribution, proportion of invariable

Table 1. Samples used in molecular analysis

Code	Sample information
1	1 PIL (Dublin) Zamboanga del Norte, Philippines
2	*E. isiforme*
3	'cottonii', commercial Venezuela
4	'cottonii', 3096, Philippines
5	'cottonii', Panama
6	'cottonii', 3005, Indonesia
7	*K. striatum*, Madagascar, wild collected
8	'denticulatum', 2189, Madagascar
13	'spinosum', 3000, Indonesia
14	'spinosum', 3127, Philippines
15	'spinosum', 3036, Tanzania
16	Madagascar, '*striatum*', cultivated
17	Bongae, Tani Tauf. (Dublin)
18	Calaguas, Philippines
19	Zamboanga, Philippines (Sacol)
20	Jolo, Philippines (Socal) E. Dublin
21	Mindoro, Philippines (Lopez)
22	'cottonii', 3054, Tanzania, (*K. alvarezii*)
23	'cottonii', Jolo, Philippines (Dublin)
24	Zamboanga, Philippines (Marcel)
32	*E. spinosum*, L992029702, Snellius II, 15/9/84, NE Sumba, Indonesia
33	*E. spinosum*, L991332595, Snellius II, 14/9/84, NE Sumba, Indonesia
34	*E. johnstonii* S&G, L94034716, California (?) W.van Bosse
35	*E. isiforme*, L01791 Bahia Honda State Park, Florida, USA
37	*E. isiforme*, L987276949, Summerland Key, Florida, USA
44	*E. denticulatum*, L992 274 234, Bone Tambung Is., S.W. Sulawesi, Indonesia
45	*E. denticulatum*, L992274274, Langkai Is., S.W. Sulawesi, Indonesia
46	*E. denticulatum*, L370570, Dar es Salaam, Tanzania,
48	*K. striatum*, L993114217, Kudingareng Keke Is. S.W. Sulawesi, Indonesia
49	*K. striatum*, L992274420, Langkai Is., S.W. Sulawesi, Indonesia
50	"cottonii" 03 240, Oct 2003, Vietnam
51	*K. alvarezii*, R: Oct 2003, Colombia
53	'cottonii' (best)-Solomon Islands (1), Nov 2003
54	*K. alvarezii*, 4264–2, (John West)
55	'cottonii' 03 241, Oct 2003, Vietnam, large
56	*E. denticulatum*, Apr 2003, AH-RU3–3, Philippines.
57	SS1, Reef 44, Kane'ohe Bay, Hawaii
58	GS1, Reef 44, Kane'ohe Bay, Hawaii
59	C2A, Kane'ohe Bay, Hawaii
60	*E. odontophorum* var. *mauritianum*, HEC 14606, Cotton Bay, Rodrigues, Mauritius

(*Continued on next page*)

Table 1. (*Continued*)

Code	Sample information
61	*Eucheuma spinosum*, HEC 11474, Pemba Is., Tondooni, Tanzania
62	'cottonii' Parara, Philippines, Feb 2003
63	'cottonii', Calaguas, Philippines, May 2003
64	*Betaphycus philippinensis*, Type locality, HG-PH267, DapDap, Bulusan, Sorsogon, Luzon, Philippines
65	*E. platycladum*, HEC 11311, Mbudya Is. (near Dar es Salaam), Tanzania
66	*Eucheuma* sp. HG 6933, Tudor Creek, Mombasa, Kenya,
68	C2B, Kane'ohe Bay, Hawaii
69	C3A, Kane'ohe Bay, Hawaii
70	C3B, Kane'ohe Bay, Hawaii
71	O1, Hawaii, store bought
72	GS2, Reef 44, Kane'ohe Bay, Hawaii
73	SS2, Reef 44, Kane'ohe Bay, Hawaii
74	GS3, Reef 43, Kane'ohe Bay, Hawaii
75	GS4, Reef 43, Kane'ohe Bay, Hawaii
76	GS5, Coconut Is., Kane'ohe Bay, Hawaii
77	SS3, Reef 44, Kane'ohe Bay, Hawaii
78	SS4, Reef 29, Kane'ohe Bay, Hawaii
79	SS5, Reef 29, Kane'ohe Bay, Hawaii
80	C2C, Kane'ohe Bay, Hawaii
81	C2D, Kane'ohe Bay, Hawaii
82	C3C, Kane'ohe Bay, Hawaii
83	C3D, Kane'ohe Bay, Hawaii
84	GS6, Coconut Is., Kane'ohe Bay, Hawaii
85	SS6, Reef 29, Kane'ohe Bay, Hawaii
86	'*Kappaphycus*', Colombia, new cultivation trial-Raul, Jan 2004
87	Panama, recent commercial supply-fine form
88	Panama, recent commercial supply-large form
89	'cottonii' Flower-type (Sacol) (Marcel sample)-13/2/04, Jolo, Philippines
90	'cottonii' Giant, (Marcel sample)-15/2/04, Jolo, Philippines
91	'cottonii' 'tambalang', (Marcel sample)-13/2/04, Jolo, Philippines
92	Kane'ohe Bay, Hawaii 'cottonii-like', 6/2/04, Prostrate large branch
93	'*E. denticulatum*', Kane'ohe Bay, Hawaii, 6/2/04
94	'*E. denticulatum*-like', Kane'ohe Bay, Hawaii, 6/2/04, Prostrate lateral branch B/
95	Philippines, sample A2, Feb 04
96	Philippines, sample A1, Feb 04
97	Philippines, "C", Feb 04
99	Kane'ohe Bay, Hawaii 6/2/04, B/, lateral branch
100	Philippines, Mr Dublin material. 2 local names-Adic-Adic; Maka-Purdoy
101	Philippines, Mr Dublin material. 3 local names-Pataka; Flower, sacol
102	'Eucheuma tip', Panama (commercial supply)

(*Continued on next page*)

103	'Eucheuma tip'-2, Panama (commercial supply)
104	Kane'ohe Bay, Hawaii 6/2/04, lateral branch a/
105	Philippines, sample B2, Feb 04
106	'cottonii', Vietnam, Mar 2004
107	'cottonii', Vietnam, Mar 2004, -2
108	*K. cottonii*, HG-PH125, Dumaluan Beach, Panglao, Bohol. Philippines
109	*E. spinosum*, Tanzania, HEC11401, Pemba Is., Tanzania
110	*Eucheuma* sp., HEC11501, Kunduchi, Dar es Salaam, Tanzania
111	*E. platycladum*, HEC9452, Chale Is., Kenya
113	*E. denticulatum* Bali A, Nusa Lembongan-Ceningan Channel, Indonesia
114	*E. denticulatum* Bali B, wild, Tanjung Aan, Lombok, Indonesia
115	*E. denticulatum* Bali C, Tanjung Aan, Lombok, Indonesia
116	*E. denticulatum* Bali D, Tanjungan, Nusa Lembongan, Indonesia
117	*Eucheuma* sp. L BER03-464, F30, Maratua Is., Indonesia, wild sample
118	*Betaphycus philippinensis* 4, Dancalan, Bulusan, Sorsogun, Luzon, Philippines
119	*K. striatum* 6, western side Hilutangal Is. Cebu, Philippines
120	*K. alvarezii* Bali E, Nusa Lembongan-Cennigan Channel, Indonesia
121	*K. alvarezii* Bali F, Brown, Gunung Payon, Nusa Dua, Bali, Indonesia
122	*K. alvarezii* Bali G, Gerupak Lombok, Indonesia
123	*K. alvarezii* Bali H, Tanjungan, Nusa Lembongan, Indonesia
124	*K. alvarezii* Bali I, Tanjung Aan, Lombok, Indonesia
125	*K. alvarezii* Bali J, Red, Gunung Payon, Nusa Dua, Bali, Indonesia
126	*K. alvarezii*, BZ1 (brown strain) sporophyte (Edison de Paula)
127	*K. alvarezii*, BZ2 (brown strain) sporophyte (Edison de Paula)
128	*K. alvarezii*, BZ3 (brown strain) female (Edison de Paula)
129	*K. alvarezii* BZ5, sporophyte (Edison de Paula)
130	*E. striatum* 5, Zanzibar, Tanzania
131	*Kappaphycus striatum* 2, Little Santa Cruz Is. Mindanao, Philippines
132	*K. cottonii* 3, western side Hilutangal Is. Cebu, Philippines
133	*Kappaphycus alvarezii*, Bali K, sumba-type, Gunung Payon, Nusa Dua, Bali
134	*E. denticulatum* 8, Zanzibar, Tanzania
135	*Betaphycus speciosum* 1, Ravin Reef, Rottenest Is. WA, Australia
137	*K. striatum* BZ4, green strain (Edison de Paula)

Samples given names derived from suppliers if available. (L = National Herbarium Netherlands Leiden; HEC = Herbarium Eric Coppejans, HG = Herbarium Gent).

sites, transition–transversion ratio). Maximum likelihood (ML) was used to construct the most likely tree from the data set. Support for individual internal branches was determined by bootstrap analysis (Felsenstein, 1985), as implemented in PAUP*. For bootstrap analysis, 1000 bootstrap data sets were generated from resampled data (5 random sequence additions, 1,000,000 rearrangements per replicate). Haplotype networks (gene genealogies) were calculated using TCS 1.13, (Clement et al., 2000) that produces an estimation of gene genealogies for DNA sequences.

Sequences are deposited in Genbank (*cox2-3* spacer: AY687417–39; RuBisCo spacer: AY687400–16).

Results

Molecular phylogeny

The results from the PHT test indicated that the mitochondrial and plastid data sets are not significantly different from each other and could be combined ($p = 0.689$). This combined data set contained only samples from which both sequences were available and had 83 taxa and 668 characters, 193 of which were potentially informative. Maximum-parsimony produced 461 trees of 461 steps ($CI = 0.6573$) (Figure 1). Maximum likelihood (estimated evolution model = HKY85; Ti/Tv ratio = 6.0729, proportion of invariable sites = 0.515, gamma parameter = 2.9212; $-ln$ L = 3007.31052) produced a topology identical to the MP tree.

The tree topology shows that the *Kappaphycus* samples are distinct from the *Eucheuma* samples. One sample from the Philippines identified as *K. cottonii* (108) collected in the wild is a sister sample to the mainly cultivated species *K. alvarezii* and *K. striatum*. Within *Kappaphycus* there is a clear distinction between the two species *K. alvarezii* and *K. striatum* (the "sacol" strain). Within *K. striatum* the two wild-collected samples (48, 117) are distinct from the cultivated samples. Within the *K. alvarezii* clade three main groups are found: (1) A sample from Africa (plus other African samples as seen in the haplotype network, see below); (2) samples from Hawaii; and (3) cultivated samples from around the world.

Samples of *Eucheuma denticulatum* are also divided into three groups, though the composition of the three groups is not as clear as for *K. alvarezii*. The first group contains samples from Africa, including a sample identified as *E. odontophorum* var. *mauritianum* (60). A
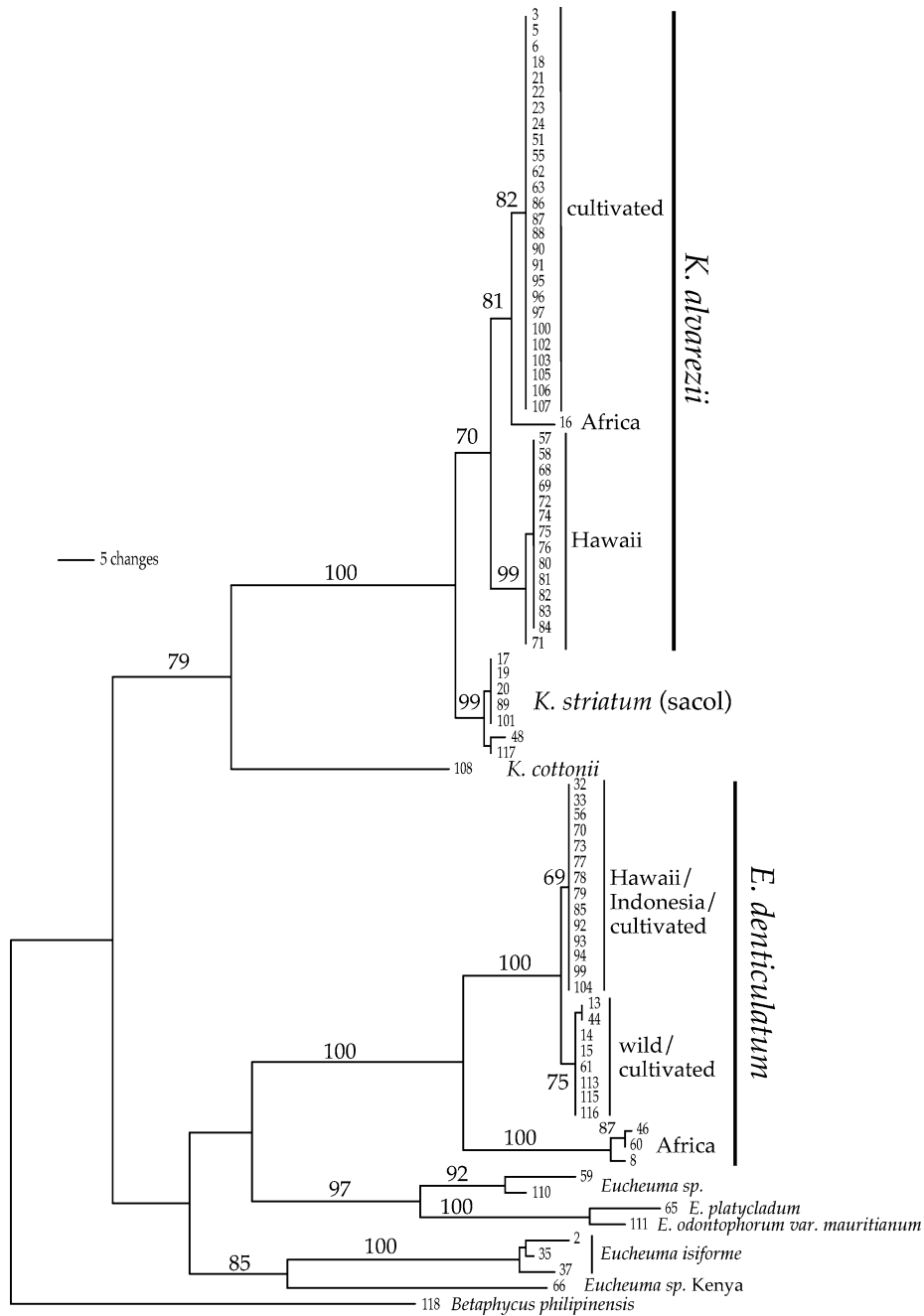
Figure 1. One of the 461 most-parsimonious trees from a combined *cox2-3* and RuBisCo spacer data set (668 characters, 461 steps, CI = 0.6573), Numbers on branched = MP bootstrap support >69% (1000 replicates).

second group contains the samples from Hawaii, plus some wild samples from Indonesia (32, 33) and a cultivated sample from the Philippines (56); the final group contains wild samples from Indonesia (44) and Tanzania (61) plus cultivated samples from the Philippines, Indonesia and Tanzania (15).

Other samples of *Eucheuma* were sequenced, with many of these only identified to genus. Three samples of *E. isiforme* (C. Agardh) J. Agardh grouped together. There is also a supported grouping of a sample from Tanzania (110) and a sample collected from Hawaii (59).
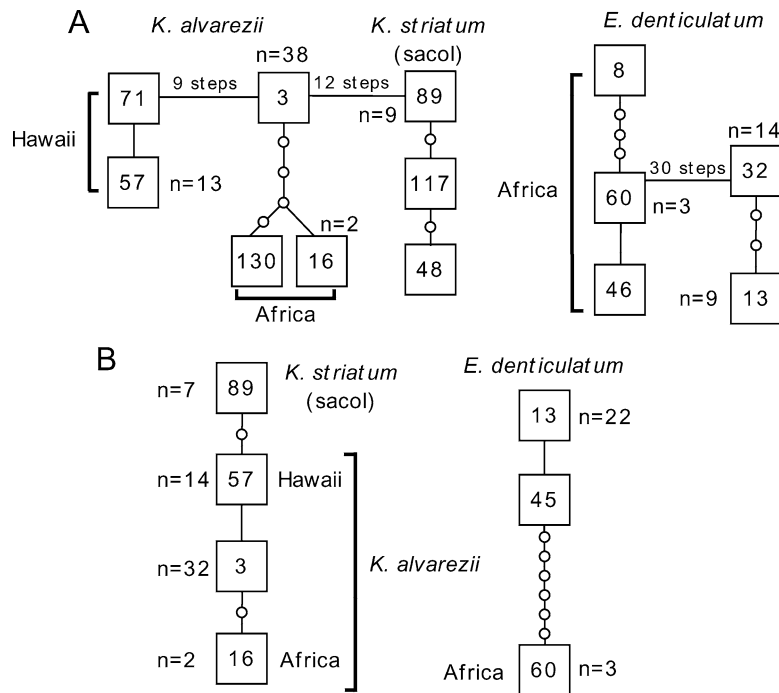
Figure 2. Haplotype networks of samples of *Kappaphycus alvarezii* and *K. striatum* and *Eucheuma denticulatum*. n = number of samples. Line indicates a point mutation, empty circle = intermediate hypothetical haplotype. (A) *cox2-3* spacer haplotypes: 71 = 71; 57 (*n* = 13; 57, 58, 68, 69, 72, 74, 75, 76, 80, 81, 82, 83, 84); 89 (*n* = 9; 17, 19, 20, 89, 101, 131, 132, 133, 137); 117 = 117; 48 = 48; 3 (*n* = 38; 3, 5, 6, 18, 21, 22, 23, 24, 51, 54, 55, 62, 63, 86, 87, 88, 90, 91, 95, 96, 97, 100, 102, 103, 105, 106, 107, 119, 120, 121, 122, 123, 124, 125, 126, 127); 16 (*n* = 2; 7, 16); 130 = 130; 8 = 8; 60 (*n* = 3; 60, 109, 134); 46 = 46; 32 (*n* = 14; 32, 33, 56, 70, 73, 77, 78, 79, 85, 92, 93, 94, 99, 104); 13 (*n* = 9; 13, 14, 15, 44, 61, 113, 114, 115, 116). (B) RuBisCo spacer haplotypes: 89 (*n* = 7; 17, 19, 20, 48, 89, 101, 117); 57 (*n* = 14; 57, 58, 68, 71, 72, 74, 75, 76, 80, 81, 82, 83, 84); 3 (*n* = 32; 1, 3, 4, 5, 6, 18, 21, 22, 23, 24, 49, 50, 51, 53, 54, 55, 62, 63, 86, 87, 88, 90, 91, 95, 96, 97, 100, 102, 103, 105, 106, 107); 16 (*n* = 2; 7, 16); 13 (*n* = 22; 13, 14, 15, 32, 33, 44, 56, 61, 70, 73, 77, 78, 79, 85, 92, 93, 94, 99, 104, 113 115, 116); 45 = 45; 60 (*n* = 3; 8, 46, 60).

Haplotype network

Haplotype networks were produced for all samples of *K. alvarezii* and *K. striatum*, and *E. denticulatum* with both the *cox2-3* spacer sequences (*n* = 93) (Figure 2A) and the RuBisCo spacer sequences (*n* = 82) (Figure 2B). The grouping seen for these species in the overall phylogeny (Figure 1) is again evident, but with increased sample sizes.

More haplotypes and more variation (point mutations) are seen in the *cox2–3* spacer network. Within *K. alvarezii* three groups are seen. The Hawaiian samples which contain two haplotypes: the single store-bought sample (71) (bought fresh in Honolulu and presumably collected from a location on Oahu); and the samples from Kane'ohe Bay (haplotype 57, *n* = 13), a location known to contain an invasive population of *K. alvarezii* (Conklin & Smith, 2004; Smith et al., 2002). This Hawaiian group is separated by 9 muta-

tions from the cultivated samples of *K. alvarezii* (haplotype 3, *n* = 38), comprising samples from the Philippines, Indonesia, Vietnam, Panama, Colombia, and a sample from Tanzania (22), plus samples identified as var. *tambalang* (91) and 'giant' (90). This cultivated *K. alvarezii* group is 4–5 mutation steps different from the three other African samples, all identified as *K. striatum* of which two are wild-collected (130, 7) and one is cultivated (16). The main cultivated *K. alvarezii* haplotype (3) is 12 steps different from the samples of the "sacol" variety. The main "sacol" haplotype (haplotype 89, *n* = 9) contains samples not identified as variety "sacol" (17, 133), plus wild samples identified as *K. cottonii* (132) and *K. striatum* (131). The two divergent haplotypes of *K. striatum* (117, 48) are both wild-collected plants from Indonesia. There are five *cox2-3* spacer haplotypes of our sampled *E. denticulatum*. Again three groups are seen, corresponding to: (1) samples from

Hawaii ($n = 14$); (2) cultivated and wild samples (haplotype 13, $n = 9$) from Indonesia, Tanzania and the Philippines; and (3) three haplotypes of samples from Madagascar, Mauritius and Tanzania (8, 46, 60).

The RuBisCo spacer haplotypes (Figure 2B) form groups congruent with the *cox2-3* spacer haplotype, though less variation is found in this genetic region plus the sample size is slightly smaller. For example, only one RuBisCo spacer haplotype is found for all the "sacol" samples versus three for the *cox2-3* spacer, and only one haplotype for the *K. alvarezii* samples from Hawaii versus two for the *cox2–3* spacer. For *E. denticulatum* the same RuBisCo spacer haplotype occurs in all Hawaiian and non-African or cultivated samples (haplotype 13, $n = 22$). African samples are quite distinct from the other *E. denticulatum* samples.

Discussion

Our data show that the genetic regions used are useful for resolving inter- and intra-specific relationships. The utility of these short genetic markers (approximately 300–350 base pairs) in resolving intra- and inter-specific relationships that are supported by longer regions has been documented (Zuccarello et al., 2002) and means that sample sizes can be drastically increased for equal time and money. Shorter segments are also more easily amplified in less well-preserved material (i.e. herbarium specimens, commercially dried samples, etc.).

Our molecular phylogeny clearly indicates that the two genera *Eucheuma* and *Kappaphycus* are distinct, but that *Eucheuma* could be paraphyletic (no bootstrap support for its distinction from *Kappaphycus*). Our choice of *Betaphycus* as an outgroup was based on a mid-point rooted tree that indicated that the *Betaphycus* sequences were the most divergent. Analyses with *E. isiforme* as an outgroup, a suggested sister taxon to the remaining *Eucheuma/Kappaphycus/Betaphycus* species (Fredericq et al., 1999), placed *Betaphycus philippinensis* Doty (4) as a sister taxon to *Kappaphycus* and therefore made *Eucheuma* paraphyletic, although it did not change the relationships within *Kappaphycus* or the remaining *Eucheuma* samples (data not shown). More research into the higher level relationships of these genera is needed, that should include wide-spread geographic and taxonomic sampling and use of molecular data from types (Hughey et al., 2001).

Kappaphycus

A lone sample identified morphologically as *K. cottonii* (103) is a highly divergent sister taxon to the remaining *Kappaphycus* samples. The true identity of this sample will require clarification. Our data clearly indicate that *K. alvarezii* can be divided into several supported evolutionary lineages. Firstly, there is a lineage that contains mostly, but not exclusively, samples grown as the "sacol" variety. Of note is that two wild collections of this variety from Indonesia are distinct from the cultivated samples, based on the *cox2–3* spacer. The taxonomic status of the "sacol" variety of *Kappaphycus* is problematic. It is clear that the morphological variability is such that species can only be "approximately" identified on the sole basis of morphology. Previous molecular studies suggested that the "sacol" variety was *K. cottonii*. This was based on only one sample identified as *K. cottonii* and one of the "sacol" variety using the *rbc*L gene (Aguilan et al., 2003). Incorporation of the terminal end of the *rbc*L gene of the Aguilan et al. (2003) samples into our RuBisCo spacer data set shows that these samples (AF481500 and AF481499) are part of our clade of the "sacol" variety. Our data indicate that the "sacol" variety is probably a distinct *Kappaphycus* species, although its proper name will depend on more sampling of wild collections and continued morphological examination. We suggest the use of the epithet *K. striatum* for this variety for now. The only other samples that could be considered as *K. striatum* are the cultured and wild samples from Africa (Tanzania and Madagascar) that have unique haplotypes (Figure 2) but are nested within the *K. alvarezii* samples (Figure 1).

Another lineage that forms a distinct evolutionary group is the set of samples from Hawaii. This was surprising as these samples were introduced to Hawaii, presumably from the Philippines, but are distinct from all other *Kappaphycus* samples. The unique genotypes, as expressed in unique haplotypes, of these samples may explain their invasive nature in Hawaii (Conklin & Smith, 2005). The final lineage contains cultured samples of *K. alvarezii* from around the world. Obviously these organellar markers do not reflect all the genetic variation existing in these samples as they can be morphologically quite variable and even contain genetically stable variants (var. *tambalang*). It appears that "cultivated" *K. alvarezii* is already found throughout the world in cultivation.

The RuBisCo spacer haplotypes again reveal the geographic/taxonomic patterns seen in the *cox2-3*

haplotype data and this is reflected in the combined phylogeny, although the level of variation is lower compared to the mitochondrial marker. The RuBisCo spacer is known to be more conserved and in other red algae more closely indicates reproductively isolated or partially isolated groups (Zuccarello et al., 2002; Zuccarello & West, 2003) or morphologically identifiable species (Brodie et al., 1996) with only 1–2 bp changes. It is therefore likely that all four groups of *Kappaphycus* studied are reproductively isolated from each other (African, cultivated, Hawaiian, "sacol" samples). Due to the rarity of reproduction in "cultivated" *Kappaphycus* this may be difficult to test but important if crop improvement by hybrid formation is pursued.

Eucheuma

The phylogeny, including single samples from herbaria, indicates that many 'species' of *Eucheuma* exist. In some cases we thought it prudent not to try a preliminary identification and left the samples as unidentified. It is clear that a complete monograph, including a molecular data set, of *Eucheuma* is desperately needed. Our sampling of *E. denticulatum* is much lower than for *Kappaphycus*, partially reflecting its lower commercial production (www.surialink.com). Again three groups are seen although they do not clearly reflect geographic/commercial patterns as seen in *K. alvarezii*. An African group consists of wild and cultivated samples. A mainly Hawaiian group also contains wild samples from Indonesia (32, 33) and a cultivated sample from the Philippines (56), plus a group containing both wild and cultivated samples from Indonesia, the Philippines and Tanzania. It is likely that the cultivation history of *E. denticulatum* is different from that for *K. alvarezii* with the species brought into cultivation several times in various locations. *E. denticulatum* is known to have been cultivated in larger volumes than at present, due to greater market demands for κ-carrageenan from *Kappaphycus* species. Our haplotype data again show that the *cox2–3* spacer is more variable in this species than the RuBisCo spacer. The RuBisCo spacer is not able to distinguish the predominantly Hawaiian *cox2-3* haplotype from the predominantly cultivated haplotype.

Our results indicate many interesting aspects of the evolutionary history of the genera *Kappaphycus* and *Eucheuma*. These data also highlight the limitations in our taxonomic knowledge and the need for more variable markers that can lead to a better analysis of genotypes that correlate with economically and ecologically important variables (colloid quality and content, growth rate, warm water susceptibility, disease resistance, invasive potential) in these commercially important seaweeds.

References

Aguilan JT, Broom JE, Hemmingson JA, Dayrit FM, Montano MNE, Dancel MCA, Ninonuevo MR, Furneaux RH (2003). Structural analysis of carrageenan from farmed varieties of Philippine seaweed. Bot. Mar. 46: 179–92.

Brodie J, Hayes PK, Barker GL, Irvine LM (1996) Molecular and morphological characters distinguishing two *Porphyra* species (Rhodophyta, Bangiophycidae). Europ. J. Phycol. 31: 303–08.

Clement M, Posada D, Crandall KA (2000) TCS: A computer program to estimate gene genealogies. Mol. Ecol. 9: 1657–59.

Conklin EJ, Smith JE (2005) Abondance and spread of the invasive red algae *Kappaphycus* spp., in Kane'ohe Bay, Hawai'i and an experimental assessment of management options. Biological Invasions, 1029–39.

Doty MS (1985) *Eucheuma alvarezii sp. nov.* (Gigartinales, Rhodophyta) from Malaysia. In Abbott, I. A. (ed.), Taxonomy of Economic Seaweeds. California Sea Grant College Program, La Jolla, California, 37–45.

Doty MS (1988) Prodomus Ad systematica eucheumatioderum: A tribe of commercial seaweeds related to *Eucheuma* (Solieriaceae, Gigartinales). In Abbott, I. A. (ed.), Taxonomy of Economic Seaweeds. California Sea Grant Program, La Jolla, California, 159–207.

Doty MS, Norris JN (1985) *Eucheuma* species (Solieriaceae, Rhodophyta) that are major sources of carrageenan. In Abbott, I. A. (ed.), Taxonomy of Economic Seaweeds. California Sea Grant College Program, La Jolla, California, 47–61.

de Paula EJ, Lima Pereira RT, Ohno M (1999) Strain selection in *Kappaphycus alvarezii* (Solieriaceae, Rhodophyta) using tetraspore progeny. J. Appl. Phycol. 11: 111–21.

Farris JS, Källersjö M, Kluge AG, Bult C (1994) Testing for significance of incongruence. Cladistics 10: 315–19.

Felsenstein J (1985) Confidence intervals on phylogenies: An approach using the bootstrap. Evolution 39: 783–91.

Fredericq S, Freshwater DW, Hommersand MH (1999) Observations on the phylogenetic systematics and biogeography of the Solieriaceae (Gigartinales, Rhodophyta) inferred from *rbc*L

sequences and morphological evidence. Hydrobiologia 399: 25–38.

Marston M, Villalard-Bohnsack M (2002) Genetic variability and potential sources of *Grateloupia doryphora* (Halymeniaceae, Rhodophyta), an invasive species in Rhode Island waters (USA). J. Phycol. 38: 649–58.

Hughey JR, Silva PC, Hommersand MH (2001) Solving taxonomic and nomenclatural problems in Pacific Gigartinaceae (Rhodophyta) using DNA from type material. J. Phycol. 37: 1091–109.

Posada D, Crandall KA (1998) Modeltest: Testing the model of DNA substitution. Bioinformatics 14: 817–18.

Smith JE, Hunter CL, Smith CM (2002) Distribution and reproductive characteristics of nonindigenous and invasive marine algae in the Hawaiian Islands. Pac. Sci. 56: 299–315.

Swofford DL (2002) PAUP*. Phylogenetic analysis using parsimony (*and Other Methods). Version 4. Sinauer Associates, Sunderland, Massachusetts.

Trono GC (1997) Field guide: Atlas of the seaweed resources of the philippines. National Bookstore, Inc., Manila, Philippines, 306.

Zuccarello GC, Burger G, West JA, King RJ (1999a) A mitochondrial marker for red algal intraspecific relationships: variable within populations and maternally inherited. Mol. Ecol. 8: 1443–1448.

Zuccarello GC, West JA, Kamiya M, King RJ (1999b) A rapid method to score plastid haplotypes in red seaweeds and its use in determining parental inheritance of plastids in the red alga *Bostrychia* (Ceramiales). Hydrobiologia 401: 207–214.

Zuccarello GC, West JA (2002) Phylogeography of the *Bostrychia calliptera/B. pinnata* complex (Rhodomelaceae, Rhodophyta) and divergence rates based on nuclear, mitochondrial and plastid DNA markers. Phycologia 41: 49–60.

Zuccarello GC, Sandercock B, West JA (2002) Diversity within red algal species: Variation in world-wide samples of *Spyridia filamentosa* (Ceramiaceae) and *Murrayella periclados* (Rhodomelaceae) using DNA markers and breeding studies. Europ. J. Phycol. 37: 403–17.

Zuccarello GC, West JA (2003) Multiple cryptic species: Molecular diversity and reproductive isolation in the *Bostrychia radicans/B. moritziana* complex (Rhodomelaceae, Rhodophyta) with focus on North American isolates. J. Phycol. 39: 948–59.