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A molecular method for identification of the morphologically plastic invasive algal genera *Eucheuma* and *Kappaphycus* (Rhodophyta, Gigartinales) in Hawaii

Kimberly Y. Conklin · Akira Kurihara · Alison R. Sherwood

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Abstract A paucity of diagnostic morphological characters for identification and high morphological plasticity within the genera *Eucheuma* and *Kappaphycus* has led to confusion about the distributions and spread of three introduced eucheumoid species in Hawaii. Entities previously identified as *E. denticulatum*, *K. alvarezii*, and *K. striatum* have had profound negative effects on Oahu's coral reef ecosystems. The use of molecular tools to aid identification of algal species has been promising in other morphologically challenging taxa. We used three molecular markers (partial nuclear 28S rRNA, partial plastid 23S rRNA, and mitochondrial 5′ COI) and followed a DNA barcoding-like approach to identify *Eucheuma* and *Kappaphycus* samples from Hawaii. Neighbor-joining analyses were congruent in their separation of *Eucheuma* and *Kappaphycus*, and the resulting clusters were consistent with those revealed for global comparisons with the mitochondrial *cox*2-3 spacer and GenBank data. Based on these results, new insights were revealed into the distribution of these groups in Hawaii.

Keywords 28S rRNA · 23S rRNA · COI · LSU · UPA

Introduction

Carrageenans, or hydrocolloids derived from red algal cell walls, are used in numerous products (both edible and inedible) as thickening and stabilizing agents (Doty 1973; Abbott 1996). The value of the industry is estimated to be US$240 million annually (McHugh 2003), and the market for carrageenan has grown 5% per year since the 1970s (Bixler 1996; McHugh 2003). The Philippines is the leading producer of cultured *Eucheuma denticulatum* (N. L. Burman) F.S. Collins & Hervey and *Kappaphycus* spp. (Villanueva et al. 2008) and supplies approximately 70% of the world's semi-refined carrageenan (Llana 1991). Farming outside the Philippines has been profitable in only a few countries (Hurtado and Agbayani 2002; McHugh 2003). During the 1970s, increased demand for carrageenans resulted in several species of carrageenan-producing algae being brought to Hawaii (Smith et al. 2002). *Eucheuma denticulatum* and two species of *Kappaphycus* [*K. alvarezii* (Doty) Doty ex P.C. Silva and *K. striatum* (F. Schmitz) Doty ex P.C. Silva] were legally introduced to a northwestern reef bordering Moku O Loe in Kaneohe Bay, Oahu, for growth studies (Glenn and Doty 1981). In 1983, researchers dismissed concerns about the spread and potential impacts of *Kappaphycus* spp. in the bay (Russell 1983). By 1996, however, *Kappaphycus* spp. was estimated to be spreading at a rate of 250 m per year and had spread 6 km from the initial sites of introduction (Rodgers and Cox 1999). By 2002, reports indicated that *Kappaphycus* was heavily dominating some patch reefs in Kaneohe Bay, competing with corals by overgrowing colonies (Smith et al. 2002). Currently, *Kappaphycus* spp. continue to spread and have moved outside the bay and along the east coast of Oahu up to Hauula, which is approximately 14.0 km from Waikane town in north Kaneohe Bay (B. Hauk, personal communication).

Understanding the distributional spread of the three eucheumoid species in Hawaii is complicated by their morphological plasticity and paucity of diagnostic morphological characters for identification (Lluisma and Ragan 1995; Conklin and Smith 2005; Zuccarello et al. 2006).

K. Y. Conklin · A. Kurihara · A. R. Sherwood (✉)
Botany Department, University of Hawaii,
3190 Maile Way,
Honolulu, HI 96822, USA
e-mail: asherwoo@hawaii.edu

Misidentification has led to confusion over the distribution and spread of *E. denticulatum*, *K. alvarezii*, and *K. striatum* in Kaneohe Bay, Oahu. Increasingly, the use of molecular approaches has clarified identification of algal species in which morphological characters are poor or contradictory (e.g., Byrne et al. 2002; Kooistra and Verbruggen 2005; Garguilo et al. 2006; Guillemin et al. 2008). While previous studies have employed a variety of molecular markers (the mitochondrial *cox*2-3 spacer, the plastidal RuBisCo spacer, and *rbc*L gene, and the nuclear small-subunit ribosomal gene) to elucidate the systematics and taxonomy of *Kappaphycus* and *Eucheuma* (Lluisma and Ragan 1995; Fredericq et al. 1999; Zuccarello et al. 2006), here we assess the usefulness of the mitochondrial *cox*2-3 spacer plus a system of three additional short molecular markers to aid identification and clarify the distribution of these species on Oahu. These three markers are successfully being used to characterize red algal biodiversity in the Hawaiian Islands through the establishment of DNA sequence frameworks for as many red algal species as possible (see the Hawaiian Algal Database for details; http://algae.manoa.hawaii.edu/). Here, we present the results for the genera *Kappaphycus* and *Eucheuma*—poorly understood yet important taxa that are having profound negative effects on Oahu's coral reef ecosystems.

Materials and methods

A total of 15 collections of *Eucheuma denticulatum* and *Kappaphycus* spp. were included in the molecular analyses from various sites around the island of Oahu, Hawaii (Table 1). Five herbarium specimens representing *Eucheuma* and *Kappaphycus* from the Bernice Pauahi Bishop Museum (BISH) were sampled for DNA analysis, and ten fresh samples were collected by snorkeling. Fresh collections were cleaned of epiphytes under a dissecting scope and either frozen at −20°C or dried using silica gel as a desiccant. Formalin and herbarium vouchers were made with all remaining material. All morphological vouchers are presently located in the Sherwood Laboratory at the University of Hawaii and will ultimately be deposited at BISH.

DNA extraction, PCR amplification, and sequencing

Genomic DNA was extracted from herbarium material using a modified Dellaporta et al. (1983) protocol described by Hughey et al. (2001) and from silica-dried and frozen material using a DNeasy Plant Mini Kit (Qiagen, USA). Polymerase chain reaction (PCR) was performed using an Eppendorf Mastercycler ep gradient S thermal cycler (Eppendorf, Germany). Four molecular regions were amplified: a portion of the nuclear 28S rRNA gene (large subunit, or LSU), a portion of the plastid 23S rRNA gene (Universal Plastid Amplicon, or UPA), the 5′ end of the mitochondrial cytochrome oxidase subunit I gene region (COI), and the mitochondrial *cox*2-3 spacer region. PCR was attempted for all samples for the first three regions, and several samples were selected to represent the different genetic clusters for amplification and sequencing of the *cox*2-3 spacer region, to allow comparison of our collections to those from other locations. PCR reactions (26.5 μL) consisted of 2.5 μL of 10X MangoTaq reaction buffer (Bioline, MA, USA), 1.5 μL of 50 mM $MgCl_2$, 1.5 μL of 1.0% bovine serum albumin solution, 1.0 μL of each primer (0.4 mM), 1.0 μL (20 mM) of each dNTP, 1.0 μL of MangoTaq DNA polymerase, 13.0 μL of nanopure water, and 1.0 μL of total genomic DNA. Herbarium extractions were diluted to 1:10, 1:50, or 1:100, while Qiagen extract DNA templates were used at full strength. PCR cycling conditions and primers followed those described in Sherwood and Presting (2007) for UPA, Saunders (2005) for COI, and Zuccarello et al. (2006) for the *cox*2-3 spacer. For the LSU region, two primers were used for amplification: nu28SF, 5′-GGAATCCGCYAAGGAGTGTG-3′ and nu28SR, 5′-GCAGGTAAGGGAAGTCGGCA-3′ (Sauvage et al., in review). These primers flank a region of approximately 668 nt (nucleotides) and are used extensively in the Sherwood Lab Rhodophyta Biodiversity Project on both florideophycean and bangiophycean red algae. Amplification conditions for the LSU region were as follows: initial denaturation at 94°C for 2 min, 40 cycles of 94°C (20 s)/55°C (30 s)/72°C (50 s), and a final cycle of 72°C for 5 min. Successful PCR products were purified using a Qiagen PCR purification kit (Qiagen). Purified PCR products were sequenced in both directions on an ABI 377XL DNA Sequencer (Applied Biosystems, USA). Sequences were assembled using Sequencher™ (Gene Codes, Ann Arbor, USA), and 21 additional *cox*2-3 spacer sequences of *Eucheuma* and *Kappaphycus* spp. for collections from Hawaii and other locations were retrieved from National Center of Biotechnology Information GenBank database for comparison to our newly generated *cox*2-3 spacer sequences (Table 1).

Analyses

Alignments for each of the four regions were generated using Clustal W (Thompson et al. 1994). All gaps and missing data were deleted from the datasets prior to analyses. We employed DNA barcode-like analyses to examine clusters of sequences as potential taxonomic units, a method that has been used in several red algal studies to estimate species boundaries (Saunders 2005, Robba et al. 2006, Sherwood et al. 2008). Neighbor-joining (NJ) analyses (Saitou and Nei 1987) were performed using

Table 1 List of samples analyzed in this study

Accession number	Species details	LSU[a] Genbank	UPA[b] Genbank	COI[c] Genbank	*cox*2-3 Genbank
00888	*E. denticulatum*; BISH 525087; Kaneohe Bay, Oahu, USA; 01 July 1998; coll. unknown.	FJ554841	FJ554863		FJ554859
00889	*E. denticulatum*; BISH 637523; Kaneohe Bay, Oahu, USA; 22 May 1994; coll. B. Cook.		FJ554865		
02865	*E. denticulatum*; Kaneohe Bay, Oahu, USA; Reef 41; 05 August 2007; coll. K. Conklin.	FJ554852			
03953	*E. denticulatum*; Kaneohe Bay, Oahu, USA; 13 August 2008; coll. B. Hauk (DAR).	FJ554847	FJ554872	FJ554855	FJ561733
00917	*K. alvarezii*; BISH 571059; Moku o Loe, Oahu, USA; 22 September 1987; coll. J. Fisher.	FJ554843	FJ554867		
02614	*K. alvarezii*; Moku o Loe, Oahu, USA; 24 March 2007; coll. J. Ozaki.		FJ554869	FJ554853	FJ554862
00916	*K. cottonii*, BISH 717106; Kaaawa, Oahu, USA; 16 June 2005; coll. J. Huisman.	FJ554842	FJ554866		
02780	*Kappaphycus* sp.; Kaaawa, Oahu, USA; 02 July 2007; coll. K. Conklin.		FJ554870		
02861	*Kappaphycus* sp.; Kaaawa, Oahu, USA; 28 July 2007; coll. K. Conklin.	FJ554845			
03773	*Kappaphycus* sp.; Maunalua Bay, Oahu, USA; 13 May 2008; coll. R. Richmond.	FJ554851	FJ554864		
03954	*Kappaphycus* sp.; Kaneohe Bay, Oahu, USA; 13 August 2008; coll. B. Hauk (DAR).	FJ554848	FJ554873	FJ554856	
03955	*Kappaphycus* sp., Kaneohe Bay, Oahu, USA; 13 August 2008; coll. B. Hauk (DAR).	FJ554849	FJ554874	FJ554857	FJ554861
03956	*Kappaphycus* sp.; Kaaawa, Oahu, USA; 13 February 2008; coll. K. McCoy (DAR).	FJ554850	FJ554875	FJ554858	
03957	*Kappaphycus* sp.; Kaaawa, Oahu, USA; 13 February 2008; coll. K. McCoy (DAR).	FJ554846	FJ554871	FJ554854	
00919	*K. striatum*; BISH 637533; Moku o Loe, Oahu, USA; 02 June 1994; coll. B. Cook.	FJ554844	FJ554868		FJ554860
E130	*K. alvarezii*; Zanzibar, Tanzania. (Zuccarello et al. 2006)				AY687436
E16	*K. alvarezii*; Madagascar. (Zuccarello et al. 2006)				AY687430
E3	*K. alvarezii*; commercial Venezuela. (Zuccarello et al. 2006)				AY687427
E71	*K. alvarezii*; store bought, Hawaii, USA. (Zuccarello et al. 2006)				AY687433
E57	*K. alvarezii*; Reef 44, Kaneohe Bay, Oahu, USA. (Zuccarello et al. 2006)				AY687432
E89	*K. striatum*; Jolo, Philippines. (Zuccarello et al. 2006)				AY687434
E117	*K. striatum*; Maratua Is., Indonesia. (Zuccarello et al. 2006)				AY687435
E48	*K. striatum*; Kudingareng Keke Is., S. W. Sulawesi, Indonesia. (Zuccarello et al. 2006)				AY687431
E108	*Kappaphycus cottonii* (Weber-van Bosse) Doty ex P.C. Silva; Panglao, Bhol. Philippines. (Zuccarello et al. 2006)				AY687426
E59	*Eucheuma* sp.; Kaneohe Bay, Hawaii, USA. (Zuccarello et al. 2006)				AY687425
E110	*Eucheuma* sp.; Dar es Salaam, Tanzania. (Zuccarello et al. 2006)				AY687424
E65	*Eucheuma platycladum* F. Schmitz; Mbudya Is., Tanzania. (Zuccarello et al. 2006)				AY687423
E111	*E. platycladum*; Chale Is., Kenya. (Zuccarello et al. 2006)				AY687422
E60	*E. denticulatum*; Rodrigues, Mauritius. (Zuccarello et al. 2006)				AY687439
E8	*E. denticulatum*; Madagascar. (Zuccarello et al. 2006)				AY687428
E13	*E. denticulatum*; Indonesia. (Zuccarello et al. 2006)				AY687429
E32	*E. denticulatum*; NW Sumba, Indonesia. (Zuccarello et al. 2006)				AY687437
E66	*Eucheuma* sp.; Mombasa, Kenya. (Zuccarello et al. 2006)				AY687418
E37	*Eucheuma isiforme* (C. Agardh) J. Agardh; Summerland Key, Florida. (Zuccarello et al. 2006)				AY687419
E2	*E. isiforme*. (Zuccarello et al. 2006)				AY687421
E35	*E. isiforme*; Bahia Honda State Park, Florida. (Zuccarello et al. 2006)				AY687420

Sherwood Lab accession numbers and GenBank accession codes are listed for all newly generated sequences as well as those obtained from GenBank for comparison. Preliminary species identifications based on morphological analysis are listed under species details. Full collection information, including latitude and longitude coordinates, can be obtained for our collections via the Hawaiian Algal Database (http://algae.manoa.hawaii.edu).

[a] *LSU* Partial nuclear 28S rRNA gene (large subunit)

[b] *UPA* Universal Plastid Amplicon (partial plastid 23S rRNA gene)

[c] *COI* 5' mitochondrial cytochrome oxidase subunit I gene

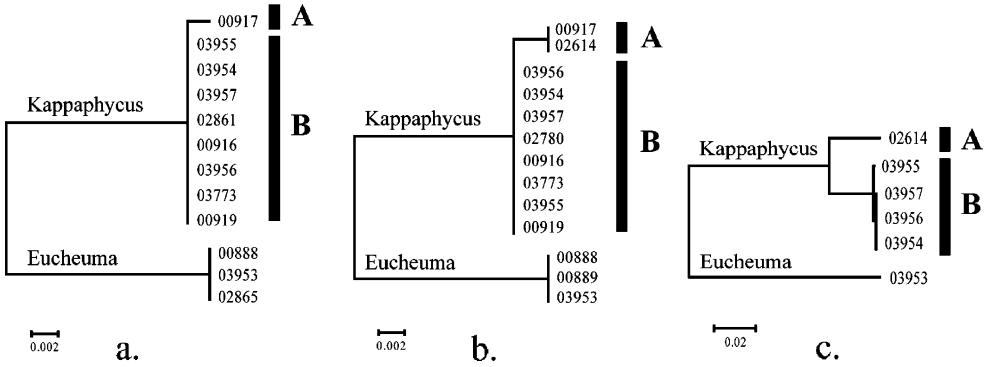

Fig. 1 Neighbor-joining analyses of the nuclear 28S rRNA region, or LSU (**a**), the plastid 23S rRNA region, or UPA (**b**), and the mitochondrial COI region (**c**) based on Kimura-2-parameter distances for *Eucheuma* and *Kappaphycus* samples from Oahu, Hawaii. *Kappaphycus* clusters (*A* and *B*) correlate to Zuccarello et al.'s (2006) clades based on *cox*2-3 spacer sequences. *Scale bars* indicate substitutions per site

MEGA version 4 (Tamura et al. 2007), based on Kimura 2-parameter distances (Kimura 1980).

Results

The final nuclear marker data set included 607–628 nt of the LSU (the number of nucleotides varied due to alignment gaps) and consisted of 12 samples. The final plastid marker data set included 370 nt of the UPA marker and consisted of 13 samples. The final COI gene data set included 617 nt (6 samples) while the *cox*2-3 spacer data set included 342–347 nt and consisted of 26 samples (a subset of our *Eucheuma* and *Kappaphycus* samples plus GenBank sequences).

Neighbor-joining (NJ) analyses of portions of the LSU, UPA, and COI revealed similar topologies and demonstrated that the *Eucheuma* samples are distinct from the *Kappaphycus* samples (Fig. 1). Within *Kappaphycus*, the nuclear and plastid markers showed a single nt difference while the mitochondrial marker showed 28 nt differences. The LSU and UPA markers showed no sequence divergence between samples in cluster B, while the COI marker yielded one nt difference between sample 03955 and samples 03954, 03956, and 03957 of cluster B.

The NJ analysis of the *cox*2-3 spacer region resulted in a tight clustering of our samples with previously sequenced collections from around the world (including sites from Oahu) (Fig. 2). Samples 00888 and 03953 exhibited no sequence divergence from a wild *E. denticulatum* sample from Indonesia, although two distinct *E. denticulatum* clusters are present in the analysis. Sample 02614 showed no sequence divergence from a commercial *K. alvarezii* sample from Venezuela, but again, several distinct clusters of this taxon are present in the analysis. Samples 00919 and 03955 showed no sequence divergence from *K. alvarezii* samples previously collected from Kaneohe Bay, Oahu. The taxonomic identities of members of these clusters are not clear and will require further investigation.

The three newer markers employed in this study (LSU, UPA, and COI) are comparable in their separation of the genera *Eucheuma* and *Kappaphycus* using NJ analyses (Fig. 1), and clusters of these analyses are consistent with those revealed for global comparisons with the *cox*2-3 spacer and GenBank data (Fig. 2). This lends support for the use of the LSU, UPA, and COI markers for the broader assessment of Hawaiian red algal biodiversity.

Discussion

DNA barcoding is based on short, single-marker sequence similarity comparisons to identify species (Hebert et al. 2003). Unfortunately, single-marker identifications may be confounded by varying levels of sequence divergence among taxa (i.e., divergence "cutoffs" may not be universally applicable across even closely related taxa) and, in any case, represent signals from only one of the possible sources of genetic information. We followed a DNA barcoding-like approach to identify *Eucheuma* and *Kappaphycus* samples from Hawaii, but used three molecular markers that represent the genetic sources in an algal cell (nucleus, plastid, and mitochondrion). Much confusion surrounds the morphological identification of this eucheumoid complex, and our results showed that these three short regions support congruent and clear separation of a *Eucheuma* sp. cluster and two *Kappaphycus* spp. clusters for samples collected from Oahu and revealed new insights into their distribution in Hawaii.

Nuclear, plastidial, and mitochondrial marker congruence to detect species of *Eucheuma* and *Kappaphycus*

This effort is the first of several studies currently being conducted to employ these three markers to examine

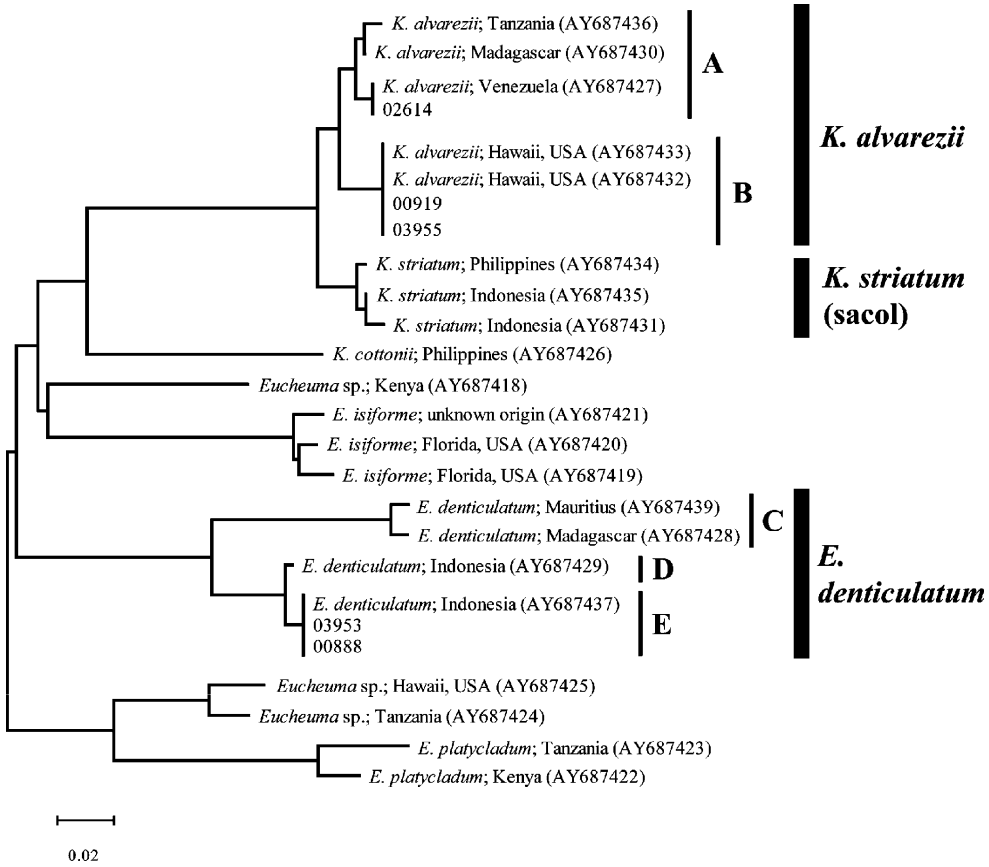

Fig. 2 Neighbor-joining analysis of *cox*2-3 spacer sequence data based on Kimura-2-parameter distances for *Eucheuma* and *Kappaphycus* samples from Hawaii and GenBank accessions. GenBank accession code or Sherwood Lab accession numbers are indicated in parentheses following the taxon labels. Species (*K. alvarezii*, *K. striatum* (sacol), and *E. denticulatum*) and cluster (*A*, *B*, *C*, *D*, and *E*) labels to the right of the tree refer to those from Zuccarello et al. (2006). *Scale bar* indicates substitutions per site

patterns of genetic variation in cryptic red algae in Hawaii. Here, we successfully distinguished morphologically confusing collections of *Eucheuma* and *Kappaphycus* that co-occur in Kaneohe Bay. The sequence comparisons based on the LSU, UPA, and COI markers were largely congruent and supported previous findings that separated the genera *Eucheuma* and *Kappaphycus* (Llana 1991; Fredericq et al. 1999; Zuccarello et al. 2006). Prior studies employed longer gene regions, such as 892–1443 nt of the *rbc*L gene (Fredericq et al. 1999), 1767–1781 nt of the SSU rRNA gene (Lluisma and Ragan 1995), or combined short, fast-evolving regions, such as the mitochondrial *cox*2-3 and plastidial RuBisCO spacers (Zuccarello et al. 2006), to elucidate the systematics and taxonomy of *Kappaphycus* and *Eucheuma*. In contrast, our markers are short regions (ranging from 320 to 628 nt) that easily amplify and sequence. These data allow us to separate our Hawaiian samples into a single *Eucheuma* cluster and two *Kappaphycus* clusters (Fig. 1), although some differences in ease of application were noted for the three markers. Amplification and sequencing of the COI marker were not successful with herbarium material from BISH when compared with fresh material. A combination of DNA degradation of herbarium specimens and a lower copy number for mitochondrial genes may account for the lower rate of amplification and sequencing success for this marker (A. Kurihara, personal communication). There were fewer problems with amplification and sequencing of the other two markers. Within *Kappaphycus*, the nuclear and plastidial marker alignments revealed one nt difference while the mitochondrial COI marker alignment showed 28 nt differences between two genetic clusters containing *Kappaphycus* samples. These data suggest that the markers have different levels of genetic resolution; a finding previously noted for members of the Batrachospermales for the UPA and COI regions (Sherwood et al. 2008). The LSU and UPA markers can be used to separate the genera *Eucheuma* and *Kappaphycus*, but may not be adequate to distinguish closely related species.

Confirmation of *Eucheuma* and *Kappaphycus* identifications using the *cox*2-3 spacer

The NJ analysis of the *cox*2-3 spacer sequences for a subset of our samples and worldwide sequences from GenBank complicate our species-level identification of *Eucheuma* and *Kappaphycus* samples (Fig. 2). Our *Eucheuma* samples (00888 and 03953) exhibited no sequence divergence from a *E. denticulatum* sample from Indonesia. This relationship was previously noted by the clustering of other Hawaiian *E. denticulatum* samples with Indonesian *E. denticulatum* samples in Zuccarello et al.'s (2006) *Eucheuma* and

Kappaphycus study employing the *cox*2-3 and RuBisCO spacers. This clade is only one of three clades of *E. denticulatum* presented by Zuccarello et al. (2006) and recovered in our analyses (Fig. 2: C, D, and E). Most of the earlier study's samples of *Eucheuma* could only be identified to genus. For these reasons, it is difficult to identify the Hawaiian *Eucheuma* species as *E. denticulatum* with confidence. Further analyses examining global molecular diversity of *Eucheuma*, coupled with DNA sequencing of type specimens or material from type localities, will be necessary to solve this taxonomic conundrum.

Sample 02614 showed no sequence divergence from a *K. alvarezii* sample from Venezuela. No Hawaiian samples included in Zuccarello et al.'s (2006) study clustered previously with this "cultivated" *K. alvarezii* clade (Fig. 2: A), although collections had been made from this same locality (Moku o Loe, Kaneohe Bay). This result is not surprising, however, given that Moku o Loe was the site of an experimental seaweed farm in the 1970s for at least three eucheumoid species in Kaneohe Bay (Russell 1983; Glenn and Doty 1990). In contrast, sample 00919 (identified morphologically as *K. striatum*) showed no sequence divergence from *K. alvarezii* samples, which were previously collected from Kaneohe Bay, Oahu. This second "*K. alvarezii*" lineage (Fig. 2: B) was described by Zuccarello et al. (2006) and consisted of haplotypes unique to Hawaii. Inconsistencies in species identifications based on morphology are probably very common for these eucheumoid taxa. Zuccarello et al. (2006) included samples in their analysis identified initially as *K. striatum* from Tanzania and Madagascar which clustered in their *K. alvarezii* clade A. In contrast, their "sacol" variety samples were initially identified as *K. cottonii* based on a study by Aguilan et al. (2003), but did not cluster with another sample morphologically identified as *K. cottonii*. Zuccarello et al. (2006) concluded that the "sacol" variety was probably a distinct *Kappaphycus* species, but more sampling and morphological examination was needed. The clade was re-named *K. striatum*, which still appears to be the commercially accepted species for the "sacol" variety (Hurtado et al. 2008) until a proper name is determined. Thus, it is not surprising to find that our 00919 sample, which was originally identified as *K. striatum*, has the same sequence as samples identified as *K. alvarezii* given the lack of distinct and consistent morphological characters for this genus. As mentioned above, given the morphological plasticity of *Eucheuma* and *Kappaphycus* species, confirmed application of taxonomic names to genetic clusters will require DNA sequencing of type specimens. To minimize confusion surrounding the *Eucheuma* and *Kappaphycus* species in Hawaii, we will use Zuccarello et al.'s (2006) clades as terminology for the Hawaiian entities (*Eucheuma* clade E, *Kappaphycus* clade A, and *Kappaphycus* clade B) and await the results of future studies for species identifications.

Revised distributions of *Eucheuma* and *Kappaphycus* on Oahu

Based on these results, new insights have been gained into the distribution of these species around Oahu. Degradation of the Kaneohe Bay patch reefs appears to be proceeding by overgrowth of a species that has been generally identified as *Kappaphycus* sp. (Rodgers and Cox 1999; Woo 2000; Conklin and Smith 2005). Here, we tentatively revise its identification to *Eucheuma* clade E (Fig. 2) based on analyzed sequences of samples from Kaneohe Bay. Further use of these molecular markers should be considered when sampling the dominant algal species overgrowing other patch reefs in the bay.

Movement of eucheumoid species outside Kaneohe Bay has sparked well-founded concern. The identification of the entity that has spread as far as Kaaawa (and potentially Hauula) on the east shore of Oahu and been found in drift in Maunalua Bay on the southeast shore of Oahu as *Kappaphycus* clade B (and not *E. denticulatum*) was surprising (Fig. 3). This is an important finding for understanding the interplay of invasive character display and spread of a morphologically plastic species complex. Contrary to previous assumptions, two species need to be controlled: *Eucheuma* clade E and its overgrowth and movement throughout Kaneohe Bay, as well as *Kappaphycus* clade B and its expansion outside Kaneohe Bay. Their individual reproductive strategies may explain how both species are successful in their respective ways. According

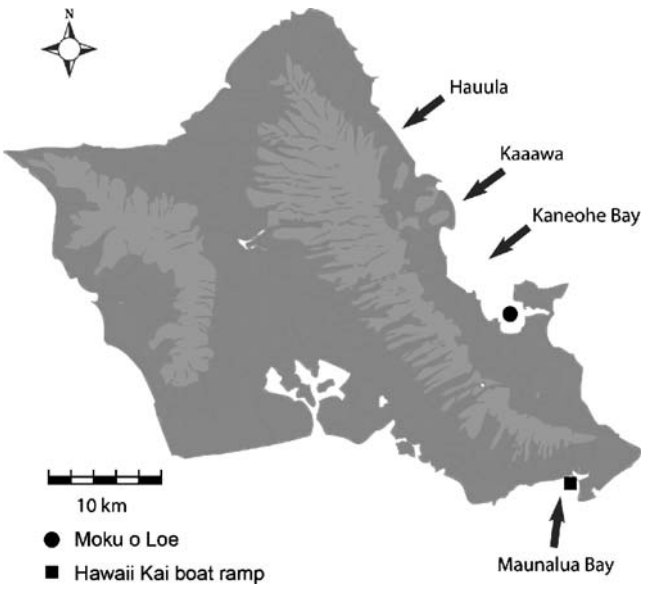

Fig. 3 Map of Oahu and *Eucheuma* and *Kappaphycus* collection sites

to Abbott (1999), tetrasporangial and spermatangial plants of *E. denticulatum* have not been seen in Hawaii and cystocarpic plants are rare. However, tetrasporangial and cystocarpic materials have been found among herbarium specimens at BISH for both *Kappaphycus* species (Table 2). Our current understanding suggests that *Eucheuma* clade E is principally spreading via vegetative propagation, and this strategy has not only allowed the alga to successfully invade the patch reefs it does reach, but, to date, has also confined the alga within the bay. *Kappaphycus* clade B, on the other hand, has been found both north and south of Kaneohe Bay. Numerous collections of small *Kappaphycus* clade B plants attached to rocks in Kaaawa suggest that this species is spreading north outside the bay along the east shore of Oahu via dispersal of tetraspores or carpospores from populations inside the bay or populations outside the bay. Ocean circulation patterns along the east side of Oahu support this northern movement from Kaneohe Bay (Qiu et al. 1997). Following the single collection of a large *Kappaphycus* clade B plant found in the drift in Maunalua Bay on the south shore, algal surveys were done in the area of the Hawaii Kai boat ramp. No other plants were found (B. Hauk, personal communication). It is speculated that this specimen was discarded from a vessel using the boat ramp after a fishing trip to Kaneohe Bay.

These results were unexpected. Previous studies suggested that *K. alvarezii* (formerly *E. striatum* "tambalang") is the dominant eucheumoid alga in Kaneohe Bay. Glenn and Doty (1990) revealed that *K. alvarezii* had a larger average relative growth rate (5.06% per day) than both *K. striatum* and *E. denticulatum* (3.50% per day). This has recently been supported in Brazil (Bulboa and de Paula 2005), where *K. alvarezii* has been shown to grow faster than *K. striatum* under all experimental conditions. Russell (1983) provided some insight into why *Eucheuma* clade E (as *E. denticulatum*) and *Kappaphycus* clade B (as *K. striatum*) could be more successful than *Kappaphycus* clade A (as *K. alvarezii*) in the Kaneohe Bay. Russell (1983) showed that *K. alvarezii* (as *E. striatum* "tambalang") was biologically limited because it was not found to produce viable spores, while it could reproduce by vegetative fragmentation. *Kappaphycus alvarezii* was speculated to have no life-history mechanism for dispersal over deep water or out of depressions and channels. Further use of these molecular markers should be considered in creating up-to-date detailed distributional maps of all three groups of *Eucheuma* and *Kappaphycus* around Oahu.

Molecular identifications play a crucial role in clarifying these algae, revealing the complex nature of introduced eucheumoid species to Oahu. There is a dire need for further *Eucheuma* and *Kappaphycus* taxonomic and ecological research. Much of the confusion surrounding the *Eucheuma* and *Kappaphycus* complex arises from the morphological plasticity of these species and the small number of diagnostic morphological characters. Molecular approaches will allow field biologists to corroborate eucheumoid species identifications and thus better associate specific invasive characteristics to a species on an ecological level.

DNA sequencing of type specimens is strongly recommended before taxonomic names are applied to genetic clusters. This approach is critical for this growing and fairly unregulated industry. Recently, Chandrasekaran et al. (2008) reported that *K. alvarezii* is invading and establishing on both dead and live coral in the Gulf of Mannar Marine Biosphere Reserve (GoM) in India since its introduction in 2000–2002 from the Philippines for commercial production of carrageenan. This runs counter to the assumption that the alga would restrict itself to sand-covered habitats and not compete with native corals in the GoM (Chandrasekaran et al. 2008). Molecular tools can assist in the identification of potentially invasive strains of

Table 2 List of tetrasporangial and cystocarpic material for *Kappaphycus* specimens deposited in the Bernice P. Bishop Museum

BISH accession number	Species	Collection details	Reproductive stage
553112	*K. alvarezii*	Kaneohe Bay, Oahu; 08 October 1987	Cystocarpic
553094	*K. alvarezii*	Kaneohe Bay, Oahu; 03 September 1986	Cystocarpic
571171	*K. alvarezii*	Kaneohe Bay, Oahu; 10 December 1987	Cystocarpic
553084	*K. alvarezii*	Kaneohe Bay, Oahu; 11 September 1986	Cystocarpic
637531	*K. alvarezii*	Kaneohe Bay, Oahu; 02 June 1994	Cystocarpic
552946	*K. alvarezii*	Kaneohe Bay, Oahu; 07 June 1976	Tetrasporangial
553082	*K. striatum*	Kaneohe Bay, Oahu; 04 June 1984	Cystocarpic
553091	*K. striatum*	Kaneohe Bay, Oahu; 25 June 1986	Cystocarpic
553092	*K. striatum*	Kaneohe Bay, Oahu; 25 June 1986	Cystocarpic
553083	*K. striatum*	Kaneohe Bay, Oahu; no collection date	Cystocarpic
552941	*K. striatum*	Kaneohe Bay, Oahu; 18 July 1974	Tetrasporangial

Eucheuma and *Kappaphycus* before they are introduced to new sites, and focus management and control efforts on these problematic algae.

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References

Abbott IA (1996) Ethnobotany of seaweeds: clues to uses of seaweeds. Hydrobiologia 326/327:15–20

Abbott IA (1999) Marine red algae of the Hawaiian Islands. Bishop Museum Press, Honolulu, Hawaii

Aguilan JT, Broom JE, Hemmingson JA, Dayrit FM, Montaño MNE, Dancel MCA, Niñonuevo MR, Furneaux RH (2003) Strutural analysis of carrageenan from farmed varieties of Philippine seaweed. Bot Mar 46:179–192

Bixler HJ (1996) Recent developments in manufacturing and marketing carrageenan. Hydrobiologia 326/327:35–57

Bulboa CR, de Paula EJ (2005) Introduction of non-native species of *Kappaphycus* (Rhodophyta, Gigartinales) in subtropical waters: comparative analysis of growth rates of *Kappaphycus alvarezii* and *Kappaphycus striatum in vitro* and in the sea in south-eastern Brazil. Phycol Res 53:183–188

Byrne K, Zuccarello GC, West J, Liao M-L, Kraft GT (2002) *Gracilaria* species (Gracilariaceae, Rhodophyta) from southeastern Australia, including a new species, *Gracliaria perplexa* sp. no.: morphology, molecular relationships and agar content. Phycol Res 50:295–311

Chandrasekaran S, Nagendra NA, Pandiaraja D, Krishnankutty N, Kamalakannan B (2008) Bioinvasion of *Kappaphycus alvarezii* on corals in the Gulf of Mannar, India. Curr Sci (Bangalore) 94:1167–1172

Conklin EJ, Smith JE (2005) Abundance and spread of the invasive red algae, *Kappaphycus* spp., in Kaneohe Bay, Hawaii and an experimental assessment of management options. Biol Invasions 7:1029–1039

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparations: version II. Plant Mol Biol Rep 1:19–21

Doty MS (1973) Farming the red seaweed, *Eucheuma*, for carrageenans. Micronesica 9:59–73

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 398/399:25–38

Garguilo GM, Morabito M, Genovese G, De Masi F (2006) Molecular systematics and phylogenetics of Gracilariacean species from the Mediterranean Sea. J Appl Phycol 18:497–504

Glenn EP, Doty MS (1981) Photosynthesis and respiration of the tropical red seaweeds, *Eucheuma striatum* (Tambalang and Elkhorn varieties), and *E. denticulatum*. Aquat Bot 10:353–364

Glenn EP, Doty MS (1990) Growth of the seaweeds *Kappaphycus alvarezii*, *K. striatum*, and *Eucheuma denticulatum* as affected by environment in Hawaii. Aquaculture 84:245–255

Guillemin M-L, Ait Akki S, Givernaud T, Mouradi A, Valero M, Destombe C (2008) Molecular characterisation and development of rapid molecular methods to identify species of Gracilariaceae from the Atlantic coast of Morocco. Aquat Bot 89:324–330

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. Proc R Soc Lond B 270:313–321

Hughey JR, Silva PC, Hommersand M (2001) Solving taxonomic and nomenclatural problems in Pacific Gigartinaceae (Rhodophyta) using DNA from type material. J Phycol 37:1091–1109

Hurtado AQ, Agbayani RF (2002) Deep-sea farming of *Kappaphycus* using the multiple raft, long-line method. Bot Mar 45:438–444

Hurtado AQ, Critchley AT, Trespoey A, Bleicher-Lhonneur G (2008) Growth and carrageenan quality of *Kappaphycus striatum* var. *sacol* grown at different stocking densities, duration of culture and depth. J Appl Phycol 20:551–555

Kimura M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120

Kooistra WHCF, Verbruggen H (2005) Genetic patterns in the calcified tropical seaweeds *Halimeda opuntia*, *H. distorta*, *H. hederacea*, and *H. minima* (Bryopsidales, Chlorophyta) provide insights in species boundaries and interoceanic dispersal. J Phycol 41:177–187

Llana EG (1991) Production and utilisation of seaweeds in the Philippines. INFOFISH International 1/91:12–17

Lluisma AO, Ragan MA (1995) Relationships among *Eucheuma denticulatum*, *Eucheuma isiforme*, and *Kappaphycus alvarezii* (Gigartinales, Rhodophyta) based on nuclear ssu-rRNA gene sequences. J Appl Phycol 7:471–477

McHugh DJ (2003) A guide to the seaweed industry. Technical Report 441. Food and Agriculture Organization of the United Nations, Rome, Italy

Qiu B, Koh DA, Lumpkin C, Flament P (1997) Existence and formation mechanism of the North Hawaiian ridge current. J Phys Oceanog 27:431–444

Robba L, Russell SJ, Barker GL, Brodie J (2006) Assessing the use of the mitochondrial *cox1* marker for use in DNA barcoding of red algae (Rhodophyta). Am J Bot 93:1101–1108

Rodgers SK, Cox EF (1999) Rate of spread of introduced rhodophytes *Kappaphycus alvarezii*, *Kappaphycus striatum*, and *Gracilaria salicornia* and their current distributions in Kaneohe Bay, Oahu, Hawaii. Pac Sci 53:232–241

Russell DJ (1983) Ecology of the imported red seaweed *Eucheuma striatum* Schmitz on Coconut Island, Oahu, Hawaii. Pac Sci 37:87–107

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Saunders GW (2005) Applying DNA barcoding to red macroalgae: a preliminary appraisal holds promise for future applications. Philos Trans R Soc Lond B 360:1879–1888

Sherwood AR, Presting GG (2007) Universal primers amplify a 23S rDNA plastid marker in eukaryotic algae and cyanobacteria. J Phycol 43:605–608

Sherwood AR, Vis ML, Entwisle TJ, Necchi O Jr., Presting GG (2008) Contrasting intra versus interspecies DNA sequence variation for representatives of the Batrachospermales (Rhodophyta): insights from a DNA barcoding approach. Phycol Res 56:269–279

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

Tamura K, Dudley J, Nei M, Kumar S (2007) *MEGA4*: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680

Villanueva RD, Montaño MNE, Romero JB (2008) Iota-carrageenan from a newly farmed, rare variety of eucheumoid seaweed–"endong". J Appl Phycol doi:10.1007/s10811-008-9356-y

Woo MML (2000) Ecological impacts and interactions of the introduced red alga, *Kappaphycus striatum*, in Kaneohe Bay, Oahu. MSc Thesis. Honolulu: University of Hawaii

Zuccarello GC, Critchley AT, Smith JE, Sieber V, Bleicher-Lhonneur G (2006) Systematics and genetic variation in commercial *Kappaphycus* and *Eucheuma* (Solieriaceae, Rhodophyta). J Appl Phycol 18:643–651