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Relationships among *Eucheuma denticulatum*, *Eucheuma isiforme* and *Kappaphycus alvarezii* (Gigartinales, Rhodophyta) based on nuclear ssu-rRNA gene sequences

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Abstract

The nuclear genes encoding small-subunit ribosomal RNAs (ssu-rDNAs) of the carrageenophytes *Eucheuma denticulatum*, *E. isiforme* and *Kappaphycus alvarezii* were amplified by the polymerase chain reaction, cloned and sequenced. The sequences range from 1767 (*K. alvarezii*) to 1781 (*E. isiforme*) nucleotides in length, and have guanine + cytosine contents between 51.2% (*E. isiforme*) and 51.5% (*E. denticulatum*). Pairwise sequence identities among these sequences ranged from 97.6% to 98.5%, levels comparable to some intergeneric identities within Gracilariales. In phylogenetic analyses, the two *Eucheuma* ssu-rDNAs group stably together vis-a-vis the ssu-rDNA of *K. alvarezii*, and these three ssu-rDNAs form a monophyletic group within a larger grouping of other carrageenophytes. The results demonstrate quantitatively that analysis of nuclear-encoded ssu-rDNA sequences is likely to be useful in resolving taxonomic, phylogenetic and biogeographic questions among tribe Eucheumatoideae Doty.

Introduction

Most of the world's supply of carrageenan and carrageenan-like products is produced from tropical red seaweeds of the genera *Eucheuma* and *Kappaphycus* (Doty, 1987; Trono, 1992; Bixler, 1996). In 1993, approximately 28 000 tonnes of processed carrageenan, and a further 8700 tonnes of crude (chips, flour) or semirefined ('natural grade') product, entered the international market (Bixler, 1996). A large (70–80%) and growing proportion of the production is based on aquacultured *Eucheuma* and *Kappaphycus* (Ricohermoso, 1995).

Despite the economic importance of these algae and the importance of taxonomic identification to international trade and commercial utilization of carrageenan and carrageenan-like products, the systematics of *Eucheuma* and *Kappaphycus* is far from satisfactory. Doty (1988) suggested that the commercial harvest may involve more than 18 species. In the commercial trade these are lumped into three informal categories, 'cottonii', 'gelatinae' and 'spinosum'. However, this categorization is 'rife with taxonomic inconsistencies to such an extent that the names used should largely be ignored' (Doty, 1988).

Development of a satisfactory systematics has been hampered by the large size, robust morphologies, indeterminate growth, phenotypic variability and apparently infrequent sexual reproduction of these organisms. Because individuals can reach 5–10 kg in wet weight, with bulky fronds extending a meter or more in length, typically only small portions are preserved for taxonomic investigation, and many important features of reproductive morphology and post-fertilization devel-

opment are only poorly known. The taxonomic situation may have been further confused by the extensive transplantation of *Eucheuma* and *Kappaphycus* for mariculture, both within regions of endemism and from southeast Asia into east Africa. Few if any details of this transplantation, which is still continuing, are available in the open literature.

Initial steps toward an operational taxonomy were made by Doty and Norris (1985), who recognized four sections within *Eucheuma* based on carrageenan type and on seven morphological characters mostly associated with vegetative thalli. Later Doty (1988) revised this categorization and transferred *Eucheuma* section *Cottoniformia* to genus *Kappaphycus*. One of the distinguishing features of *Kappaphycus* is the production of kappa carrageenan, in contrast to iota and(or) other carrageenans in *Eucheuma*. However, Santos (1989) demonstrated that some members of Doty and Norris's section *Gelatiformia* yield mostly kappa carrageenan, while others yield only iota. The systematics of Eucheumatoideae endemic to regions other than Southeast Asia has also been reinvestigated: Cheney (1988) reviewed *Eucheuma* of the Atlantic Ocean and Caribbean regions and concluded that only one species (*E. isiforme*) with two varieties is present in the Florida-Caribbean basin. Nonetheless the systematics of Eucheumatoideae remains problematic, and Doty (1988) concluded that '(p)erhaps no basis for phylogeny exists at all now'.

Molecular techniques have proven useful for resolution of phylogenetic relationships in hundreds of instances where morphological features are infrequent, obscure or contradictory. Comparative analysis of sequences of homologous genes or proteins is especially powerful because relationships can be inferred quantitatively and confidence intervals or other statistics calculated. Among red algae, most analysis has focused on nuclear genes encoding small-subunit ribosomal RNAs (Bird *et al.*, 1992a, b, 1994; Ragan *et al.*, 1994; Saunders & Kraft, 1994; Oliveira *et al.*, 1995; Saunders *et al.*, 1995), although 5S rRNAs (Lim *et al.*, 1986), *rbcL* (Freshwater *et al.*, 1994) and to a lesser extent other genes (reviewed by Ragan & Gutell, 1995) have been examined as well.

As a first step toward an eventual molecular-sequence survey of tribe Eucheumatoideae, herein we report ssu-rDNA sequences of *Eucheuma denticulatum* and *Kappaphycus alvarezii* from the Philippines, and of the 'typical form' of *E. isiforme* from the Caribbean.

Materials and methods

Eucheuma denticulatum (N. L. Burman) Collins et Hervey, brown form, and *Kappaphycus alvarezii* (Doty) Doty, brown 'Giant Tambalang' form, were collected at an experimental mariculture site in Bolinao, Pangasinan, Philippines on 21 December 1992. The parent stocks had respectively been transplanted from Bohol, Philippines in 1991, and from Batangas, Philippines in 1990; both had originated in the Sulu Sea region (G. C. Trono, pers. commun.). The 'typical form' of *E. isiforme* (C. Ag.) J. Ag. (Dawes *et al.*, 1974) was collected on the Caribbean side of Spanish Harbor Key, Florida on 16 March 1994. Samples were cleaned of epiphytes and packed in silica gel for shipment. Voucher specimens have been deposited in algal herbarium of the National Research Council (NRCC) as NRCC 10941, 10940 and 11003 in the order cited.

DNA was extracted from 5–g samples of the silica-dried material by the method of Rice and Bird (1990), and nuclear ssu-rDNAs were amplified by the method of Sogin (1990) as modified (Bird *et al.*, 1990) and cloned into pAMP 1 vector (Gibco BRL, Gaithersburg, MD). Inserts from cloned *E. denticulatum* and *K. alvarezii* ssu-rDNAs were sequenced manually, while those from *E. isiforme* were subjected to automated fluorescence sequencing on an ABI 373A equipped with Stretch^{TM} upgrade (Applied Biosystems, Foster City, CA). Sequencing primers and other procedures were as described by Bird *et al.* (1992a) and Ragan *et al.* (1994).

Sequences were added visually (Raff *et al.*, 1994) into a matrix of red algal ssu-rDNA sequences (Ragan & Gutell, 1995) aligned on the basis of secondary and higher-order structures as revealed by covariation of nucleotides (Gutell, 1993; Gutell *et al.*, 1994). Columns of the initial matrix corresponding to sequences of PCR primers or to ambiguously alignable regions, and those that were largely empty, were deleted to yield a 39 species × 1600 nucleotide-position matrix. The ssu-rDNA of the cryptomonad *Guillardia theta* Hill & Wetherbee (as *Cryptomonas* sp. Φ) was defined as the outgroup. Alternatively, a subset of 12 ssu-rDNAs (*E. denticulatum*, *E. isiforme*, *K. alvarezii*, *Furcellaria lumbricalis* (Huds.) Lamour., *Chondrus crispus* Stackh., *Mastocarpus stellatus* (Stack. in With.) Guiry, *Lomentaria baileyana* (Harv.) Farl., *Grateloupia filicina* (Lamour.) C. Ag. var. *luxurians* A. & E. Gepp, *Bonnemaisonia hamifera* Hariot, *Griffithsia globulifera* Harv. and *Gelidium vagum*

Table 1. Lengths (in nucleotides), nucleotide and G+C compositions of ssu-rDNAs. (Numbers may not sum to 100.0% due to rounding.)

Organism	A	G	C	T	G+C%	Length
E. denticulatum	23.9	30.1	21.5	24.6	51.5	1777
E. isiforme	23.9	30.0	21.2	25.0	51.2	1781
K. alvarezii	24.1	30.1	21.3	24.6	51.4	1767

Okam., with *Curdiea flabellata* Chapm. as outgroup) was selected, and a 1742-position matrix was constructed from full-length sequences (excluding only the regions corresponding to PCR primers).

Unless otherwise stated, phylogenetic trees were inferred using PHYLIP version 3.53c (Felsenstein, 1989) implemented under UNIX on a Sun 10/61 workstation, with randomized order of sequence input and global rearrangements where relevant. Distance matrices were calculated using the PHYLIP program DNADIST under a generalized Kimura two-parameter ('maximum-likelihood') model, neighbor-joining trees were inferred using NEIGHBOR, and parsimony trees were inferred using DNAPARS (39-species matrix) and DNAPENNY (12-species matrix). Analyses were bootstrapped ($n = 100$ replicates) using SEQBOOT and the inference program(s) mentioned above, and results were displayed as majority-rule consensus trees (Margush & McMorris 1981) using CONSENSE. Nonparametric tests of alternative (user-defined) topologies were conducted as described by Felsenstein (1985) following Templeton (1983) under parsimony, and by Kishino and Hasegawa (1989) under maximum likelihood, using programs DNAPARS and DNAML respectively. Maximum-likelihood trees were inferred using fastDNAml (Olsen *et al.*, 1994).

Results

Single sequences corresponding to intronless nuclear ssu-rRNA genes were successfully amplified from each of these three algae. Data on length (including amplification primers) and nucleotide composition are summarized in Table 1. The sequences, with annotation, have been deposited in GenBank under accession numbers U25437 (*K. alvarezii*), U25438 (*E. isiforme*), and U25439 (*E. denticulatum*).

Pairwise identities among the complete aligned sequences (including amplification primers), expressed in percent, are: *E. denticulatum* - *E. isiforme*, 98.5%; *E. denticulatum* - *K. alvarezii*, 98.0%; *E. isiforme* - *K. alvarezii*, 97.6%.

Maximum-likelihood analysis of the 39×1600 rDNA sequence matrix reveals that the two *Eucheuma* ssu-rDNAs are more closely related to each other than either is to the ssu-rDNA of *K. alvarezii* (Fig. 1). Parsimony and neigbor-joining analyses gave essentially identical results (not shown) which differed topologically from the maximum-likelihood tree, if at all, only in the relative positions of the ssu-rDNAs of *Dasya baillouviana* (Gmel.) Mont. and *Rhodomela confervoides* (Huds.) Silva; the relationship among the *Eucheuma* and *Kappaphycus* ssu-rDNAs was in every case identical to that shown in Fig. 1. The guanosine-plus-cytosine (G + C) contents of these sequences range from 43.1–52.1% (44.9–52.1% within Rhodophyta, 47.5–52.1% within Florideophycidae, 50.4–51.2% within Gigartinales), making it unlikely that differences in G + C content (Lockhart & Penny, 1993) bias the topology in the region of the tree near *Eucheuma* and *Kappaphycus* ssu-rDNAs.

Bootstrap support for the *E. denticulatum–E. isiforme* clade ranged from 98–100% with the more-conservative (1600-position) matrix, but dropped to 77–82% with the essentially complete (i.e. noisier) 1742-position matrix. Bootstrap support for the *Eucheuma–Kappaphycus* clade was 100% in all analyses. A clade of *Eucheuma*, *Kappaphycus*, *Chondrus* and *Mastocarpus* ssu-rDNAs was resolved in all analyses, with 90–97% and 99–100% bootstrap support from the more-conservative and essentially complete matrices respectively. *Furcellaria lumbricalis* ssu-rDNA was usually resolved as the sister group to the *Eucheuma–Kappaphycus* clade, although with much weaker bootstrap support.

Although phylogenetic analyses are typically conducted to infer the best (i.e. shortest, most-parsimonious or maximally likely) trees, alternative topologies are sometimes only slightly worse solutions. Using the nonparametric Templeton-Felsenstein under parsimony or the Kishino-Hasegawa test under maximum likelihood, one may determine whether specified alternative topologies can be rejected statistically. More specifically, an alternative topology is rejected if it is more than 1.96 standard deviations worse than the best (i.e. shortest or most-likely) known tree, measured in number of steps (in parsimony analysis) or likelihood units (in likelihood analysis). Two alterna-

Table 2. Non-parametric tests of alternative topologies (see Fig. 2) under the Templeton-Felsenstein (T-F) and Kishino-Hasegawa (K-H) tests, based on more-conservative (39 species x 1600 sites) and essentially full (12 species x 1742 sites) sequence matrices.

	T-F test:			K-H test:		
	Steps	s.d.[1]	Worse?	Likelihood	s.d.	Worse?
More-conservative matrix:						
Topology A	441	—	—	−4687.76820	—	—
Topology B	447	2.8293	yes	−4719.46337	14.5640	yes
Topology C	448	2.6466	yes	−4721.25248	14.1171	yes
Essentially complete matrix:						
Topology A	655	—	—	−5515.44057	—	—
Topology B	657	2.4502	no	−5525.26835	8.2824	no
Topology C	659	2.0006	yes	−5527.22669	7.6843	no

[1] Standard deviation in steps (T-F test) or likelihood units (K-H test). A topology is rejected if it costs more than 1.96 standard deviations (s.d.) additional steps (T-F test), or is more than 1.96 s.d. less likely (K-H test). Thus with the essentially complete matrix, Topology B cannot be rejected under the T-F test because it costs only 657-655= 2 additional steps, while the rejection level is 1.96(2.4502)= 4.80 steps; but with the same matrix, Topology C is rejected under the same test, because it costs 659-655= 4 additional steps while the rejection level is now 1.96(2.0006)= 3.92 steps.

tive topologies were tested (Table 2, Fig. 2): *E. denticulatum* as a sister group to an *E. isiforme* - *K. alvarezii* clade (topology B), and *E. isiforme* as a sister group to an *E. denticulatum* - *K. alvarezii* clade (topology C). The results are presented in Table 2.

Discussion

The maximum-likelihood (Fig. 1), parsimony and neighbor-joining (not shown) analyses of the ssu-rDNA sequence data all indicate that the geographically isolated but morphologically very similar species *E. denticulatum* (from the Philippines) and *E. isiforme* (from the Florida Keys) are more closely related to each other than either is to *K. alvarezii* from the Philippines. Nonparametric tests based on the more-conservative matrix (i.e. the better-aligned sequence regions) indicate that alternative interpretations, in which *Kappaphycus* is included within *Eucheuma*, can be rejected statistically (Table 2). That these alternative topologies are not (or are only just) rejectable when the essentially complete matrix is analysed, is a consequence of the decrease in signal-to-noise ratio brought about by inclusion of sequence regions that are ambiguously alignable (hence perhaps non-homologous) or that require many gaps for alignment, or both.

It is not possible to conclude from these results that genus *Eucheuma* (as currently circumscribed) is necessarily holophyletic, i.e. that all *Eucheuma* species descend from a common ancestor unique to themselves and exclusive of *Kappaphycus*. Although by ssu-rDNA sequence analysis, *E. denticulatum* and *E. isiforme* (both members of section *Eucheuma*: Doty & Norris, 1985) are more closely related to each other than either is to *K. alvarezii*, other species of *Eucheuma* (e.g. belonging to sections *Gelatiformia* or *Anaxiferae*) might diverge with, or more basally in the rDNA tree than, *K. alvarezii*. Santos (1989) has already suggested, based on comparison of carrageenan characteristics, that two members of section *Gelatiformia*, *E. platycladum* Schmitz and *E. odontophorum* Birg, might better be placed in genus *Kappaphycus*. This can be resolved by substantial additional sequencing.

The extent of pairwise sequence difference among ssu-rDNAs of the few species of *Eucheuma* and *Kappaphycus* so far investigated indicates that analysis of ssu-rDNA sequences holds considerable promise as the basis for a future taxonomy of these organisms. Pairwise differences among *E. denticulatum*, *E. isiforme* and *K. alvarezii* (1.5% to 2.4%) are comparable in magnitude to those observed between the gracilarialean genera *Hydropuntia* and *Curdiea* (1.5%: Bird *et al.*, 1992b), between *Hydropuntia* and *Gracilaria* species (0.8% to 1.4%: Bird *et al.*, 1992b), and among

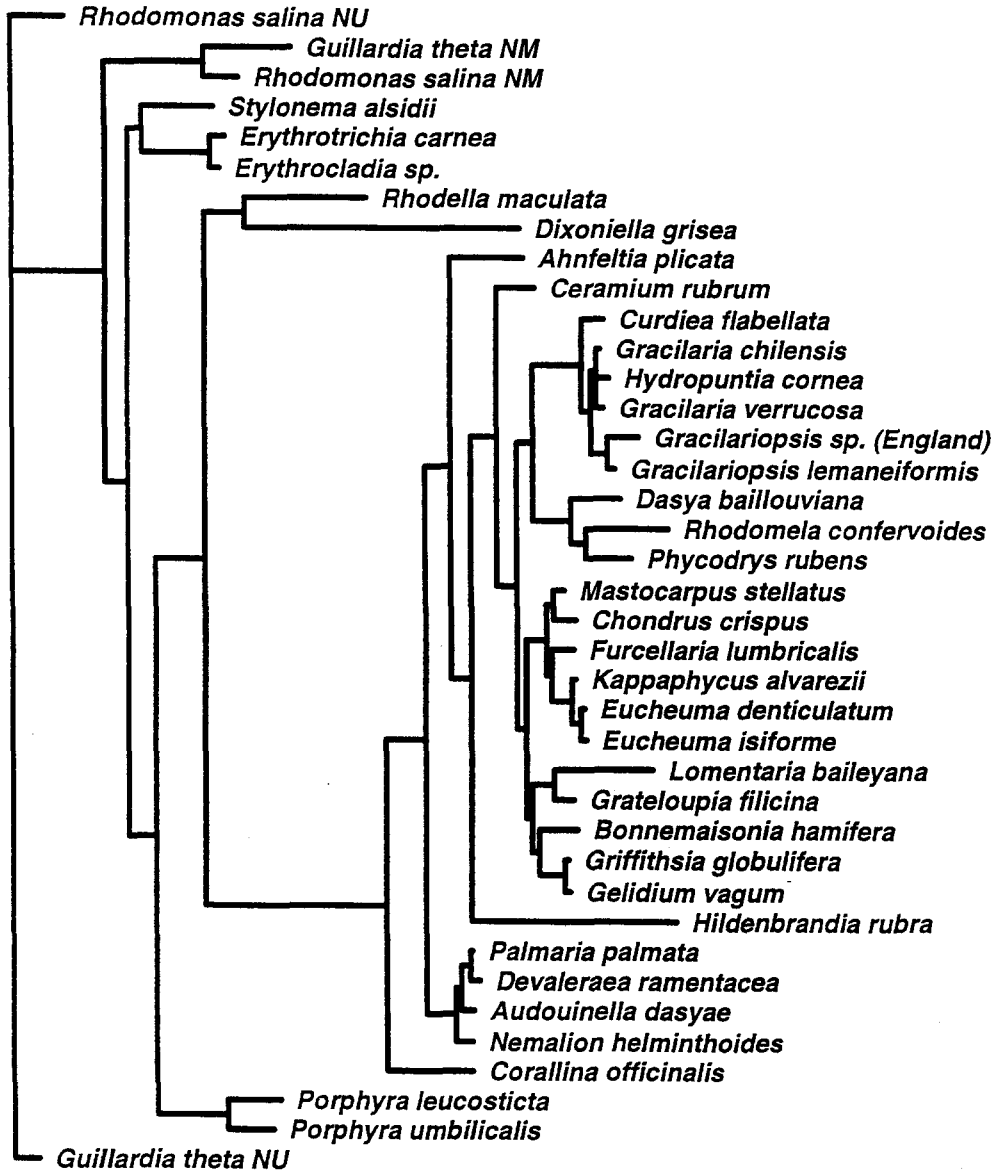

Fig. 1. Maximum-likelihood (fastDNAml) tree inferred from 39 species × 1600 nucleotide position ssu-rDNA matrix. For cryptomonads: NU, nuclear ssu-rDNA; NM, nucleomorph ssu-rDNA.

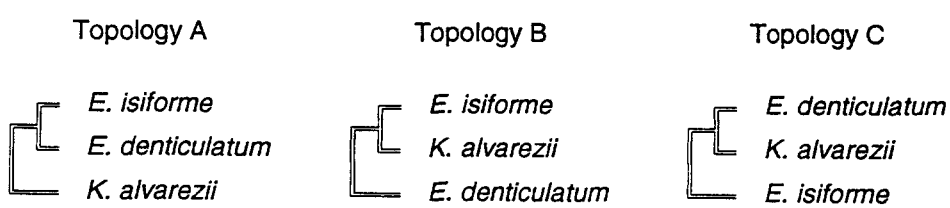

Fig. 2. Topology A (optimal solution), and alternative topologies B and C (see text and Table 2).

geographically dispersed populations of *Gracilariopsis* (2.3% to 3.4%: Bird *et al.*, 1994). They are also comparable to differences among eight acrochaetialean and palmarialean algae (0.3% to 2.6%: Saunders *et al.*, 1995), but are smaller than differences among eight species of *Porphyra* (5.3% to 12.6%: Oliveira *et al.*, 1995). In most cases nuclear ssu-rDNA sequences will probably be insufficiently variable to be useful in finer-scale differentiation of species, strains or isolates, or in studies of biogeography. In such cases analysis of more-variable sequences such as *rbcL* (Freshwater *et al.*, 1994), nuclear or plastid rDNA internal transcribed spacers (Goff *et al.*, 1994), or the *rbcL-rbcS* spacer (Destombe & Douglas, 1991; Goff *et al.*, 1994; Bird, 1995) may provide the necessary resolution.

In the ssu-rDNA sequence tree (Fig. 1) the two species of *Eucheuma* and *K. alvarezii* are positioned among other red algae in a way largely consistent with existing taxonomy, which is based largely on morphological characters of sexual reproduction and post-fertilization development. Thus, among the algae studied here, six gigartinalean species (*Mastocarpus stellatus*, *Chondrus crispus*, *Furcellaria lumbricalis*, *K. alvarezii*, *E. denticulatum* and *E. isiforme*) constitute a monophyletic group which is incongruent with Gigartinales only by absence of *Grateloupia filicina*, which instead groups with *Lomentaria baileyana* (Rhodymeniales). These six species are all carrageenophytes, while *Grateloupia* species (Craigie, 1990) and *Lomentaria catenata* Harv. (Takano *et al.* 1994) have been described as accumulating a carrageenan-agar hybrid. By contrast, the distribution of agarophytes (e.g. *Porphyra* species, *Ahnfeltia plicata* (Huds.) Fr., *Ceramium nodulosum* (Lightf.) Ducluz (as *C. rubrum*), *Gelidium vagum*, *Rhodomela confervoides*, and members of order Gracilariales) suggests a paraphyletic or even polyphyletic derivation of agaroid biosynthesis. Similar analyses based on a larger database of red algal ssu-rDNAs (G. W. Saunders, unpublished; M. A. Ragan, unpublished) reinforce these conclusions, and Freshwater *et al.* (1994) provide greater phyletic detail based on sequences of *rbcL*. We anticipate that further comparative analysis of ssu-rDNA and other gene sequences will help illuminate relationships among commercially important red algae, and thereby among components of the biosynthetic pathways for agars and carrageenan.

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