A molecular approach to investigate the phylogenetic basis of three widely used species groups in the red algal genus Ceramium (Ceramiales, Rhodophyta)

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M. SKAGE, T.M. GABRIELSEN AND J. RUENESS. 2005. A molecular approach to investigate the phylogenetic basis of three widely used species groups in the red algal genus Ceramium (Ceramiales, Rhodophyta). Phycologia 44: 353±360.

The taxonomy of Ceramium (Ceramiales, Rhodophyta) is difficult because of large and complex morphological variation. Three species groups are often recognised; one group that can develop fully corticated internodes, one group with ectocortic internodes, and one group with cortical spines. Two different molecular markers (the plastid rubisco spacer and one part of the large subunit [LSU] of nuclear ribosomal DNA) were used to investigate whether the three species groups have a phylogenetic basis. Sequence analyses of 15–17 North Atlantic species of Ceramium revealed highly conserved partial LSU sequences, whereas the rubisco spacer region was variable among, as well as within several species. Analyses of the rubisco spacer sequences, and in particular the combined data set, produced fairly resolved phylogenies with several well-supported relationships that were highly congruent with the informal species groups. These results suggest that cortical spines and internode cortication have evolved several times in Ceramium.

INTRODUCTION

Ceramium (Roth) is a cosmopolitan red algal genus with severe taxonomic as well as nomenclature problems (Dixon 1960; Maggs & Hommersand 1993; Boo & Lee 1994). The taxonomic problems are caused by a high degree of variation in the morphological characters that are used to delimit species. Whether this variation results from environmentally determined growth, genetic differences, or a combination of both these factors is uncertain (Maggs et al. 2002). Consequently, referring specimens to collective species names such as the ‘C. rubrum species complex’ and the ‘C. strictum species complex’ is common (see Garbary et al. 1978; Rueness 1978; Maggs & Hommersand 1993; Maggs et al. 2002; Gabrielsen et al. 2003). Species within the former group are characterised by their ability to form cortication that completely covers the internodes. In the British Isles, four fully corticated non-spiny species of Ceramium are currently recognised, as recently clarified by Maggs et al. (2002). These are C. pallidum (Nägeli ex Kützing) Maggs & Hommersand, C. secundatum Lyngbye, C. botryocarpum Griffiths ex Harvey, and C. virgatum Roth.

Species of Ceramium within the second group are characterised by ectocortic internodes. The illegitimate name ‘C. strictum’ (Silva et al. 1996) has been used both as a species name and representing a species group of North Atlantic Ceramium (e.g. Rueness 1977; South & Tittley 1986; Sears 1998). Most reports of C. strictum have referred to Harvey’s description in Phycologia Britannica (Harvey 1851, pl. 334), which currently is referred to as ‘C. strictum sensu Harvey’ in the British Isles (Maggs & Hommersand 1993). Delimitation of C. strictum sensu Harvey from other populations traditionally referred to as C. strictum in the North Atlantic, in particular Scandinavian entities, has been dealt with more recently. This has involved crossing experiments (Rueness 1978; Rueness & Kornfeldt 1992) and phylogenetic analyses of nuclear internal transcribed spacer (ITS2) and plastid rubisco spacer sequences among populations of C. strictum from five regions in the North Atlantic (Gabrielsen 2002; Gabrielsen et al. 2003).

In addition, a third group bearing characteristic cortical spines is identified in Ceramium (Dixon 1960; Maggs & Hommersand 1993; Millar 2002). Both fully and partially corticated species belong to this group, members of which have been extensively studied in the British Isles. These species are C. shuttleworthianum (Kützing) Silva, C. ciliatum (Ellis) Dudgeon, C. gaditanum (Clemente) Cremades & Pérez-Cirera, and C. echinotum J. Agardh (Dixon 1960; Maggs & Hommersand 1993).

In this study, sequences from the plastid-encoded rubisco spacer region and part of the nuclear ribosomal large subunit (LSU) gene from mainly North Atlantic specimens were analysed to investigate the possible phylogenetic basis of the three species groups in Ceramium. Furthermore, we evaluated the usefulness of these molecular markers in phylogenetic and taxonomic studies of Ceramium.

MATERIAL AND METHODS

Material

A total of 43 samples representing the three species groups of Ceramium were analysed (Table 1). The North Atlantic samples were identified to species following the most recent treatments (Maggs & Hommersand 1993; Maggs et al. 2002; Ga-
Table 1. Collection data for specimens of *Ceramium* used in the phylogenetic analyses.

| Species                        | Sample code | Locality                          | GenBank accession number | Rubisco spacer | Collectors† | Voucher or isolate ref. |
|--------------------------------|-------------|-----------------------------------|--------------------------|----------------|--------------|------------------------|
| *C. botryocarpum* Griffiths ex Harvey | Chb         | N. Ireland, Down, Annalong        | AY253935                 | AY254306       | CAM         | #275/1                 |
| *C. ciliatum* (Ellis) Ducluzeau  | Ccil        | France, Banyuls-sur-mer           | AY253938                 | AY254307       | JR          | cil Banyuls            |
| *C. cimbricum* H. Petersen in Rosenvinge | Ccim.1      | Norway, Akershus, Snarøya         | AY253942                 | AY254373       | JR          | forn                   |
|                                | Ccim.2      | Norway, Vest-Agger, Jøssingfjorden | x²                      | PAÅ            | Jøss         |                        |
|                                | Ccim.3      | Denmark, Hirschholmene            | x                        | PMP            | C. Damn      |                        |
| *C. deslongchampsii* Chauvin ex Duby | Cde.1      | France, Finistère, Roscoff        | AY255474                 | FM             | deslRosc     |                        |
|                                | Cde.2      | Denmark, Fredrikshavn             | AY253941                 | AF543816¹      | PMP         | deslDann               |
|                                | Cde.3      | Ireland, Cork, Helen’s Bay        | x                        | CAM            | deslIRL      |                        |
| *C. diaphanum* (Lightfoot) Roth | Cdi.1       | Norway, Aust-Agger, Høvag         | AY253939                 | x              | JR          | Cer Hav                |
|                                | Cdi.2       | Norway, Hordaland, Espegrend      | AY255472                 | x              | JR          | E2-3                   |
| *C. echinotum* J. Agardh        | Cech        | England, Devon, Torquay           | AY255475                 | herb. C⁴       | 20792        |                        |
| *Ceramium* sp. 6. (C. elegans Ducluzeau?) | Csp6       | Adriatic Sea, Pirano              | AY255430                 | herb. O⁸       | Cer eleg     |                        |
| *C. pallidum* (Nägeli ex Kützing) Maggs & Hommersand | Cpa.2 | Norway, Hordaland, Kviturdvikpollen |                    |                |             |                        |
|                                | Cpa.4       | Norway, Aust-Ager, Lillesand      | AY253929                 | AY2543809⁴     | JR          | 98-9-1                 |
|                                | Cpa.7       | Norway, Vest-Ager, Farsund        | x                        | TG              | 25/10       |                        |
|                                | Cpa.8       | Norway, Hordaland, Eggholmen      | AY254303                 | MS              |             | 0305pall               |
|                                | Cpa.9       | Faeroe Islands, Torshavn          | x                        | ACS             |             | Cpa1lFæ #5             |
| *C. secundatum* Lyngbye         | Cse.1       | Faeroe Islands, Torshavn          | AY253936                 | AF543815³      | herb. O⁹     | lokSec                 |
|                                | Cse.2       | Norway, Hordaland, Store Kalsøy   | x                        | MS              |             | 0305Rusk               |
|                                | Cse.3       | Norway, Vestfold, Verdens Ende    | x                        | JR              |             | sec.v.end              |
|                                | Cse.4       | Norway, Sogn og Fjordane, Vågane  | x                        | herb. B⁷       |             | 97007806               |
|                                | Cse.17      | Germany, Sleesig-Holstein,        | AY253940                 | TG              | SWS         | 99-48-3                |
|                                |             | Geltinger fjord                    | x                        | herb. O⁸       |             |                        |
|                                | Cse.32      | Finland, Turku-Pori, Rauma        | AY253928                 | AY254299       | TG, SWS     | 98-17-5                |
|                                |             |                                  |                          |                 |             |                          |
| *C. siliculosus* (Kützing) Maggs & Hommersand | Csi.1     | Ireland, Clare, Fanore            | AY253930                 | AY2543808⁴     | CAM         | #172                   |
|                                | Csi.2       | Spain, Asturas                    | x                        | SF              |             | CerAstur               |
|                                | Cte.3       | Norway, Froga, Hallangspollen     | x                        | x               |             | paraOsl                |
|                                | Cte.5       | Norway, Nesodden, Flaskebakk     | x                        | TG, SWS         |             | 98-42-5                |
|                                | Cte.17      | Germany, Sleesig-Holstein,        | AY253940                 | Herb.           |             |                        |
|                                |             | Geltinger fjord                    | x                        | Kützing⁹          |             | 940.112.256            |
|                                | Cte.32      | Finland, Turku-Pori, Rauma        | AY253928                 | AY254299       | TG, SWS     | 98-17-5                |
| *C. virgatum* Roth             | Cvi.1       | Germany, Kieler Förde             | AY253937                 | AY254304       | TG, JR      | 98-4-1                 |
|                                | Cvi.2       | Norway, Akershus, Snarøya         | x                        | JR, MS          |             | snar                   |
|                                | Cvi.3       | Norway, Aust-Ager, Lillesand      | x                        | JR              |             |                        |
|                                | Cvi.4       | Norway, Vest-Ager, Farsund        | x                        | TG              | no2 2510    |                        |
|                                | Cvi.5       | Norway, Hordaland, Busepollen     | x                        | JR              |             | rubbuse                |
|                                | Cvi.6       | Faroe Islands, Torshavn           | x                        | ACS             |             | local                  |
| *Ceramium* sp. 1               | Csp1.2      | USA, New Jersey, Tuckeron         | AY253931                 | AY543813³      | PE          | CerPE                  |
|                                | Csp1.3      | Canada, Nova Scotia, Antagonish   | AY254300                 | DG              | CerDG#22    |                        |
| *Ceramium* sp. 2               | Csp2        | Spain, Gran Canaria, Las Palmas   | AY253933                 | AY543812       | JR          | las palm               |
| *Ceramium* sp. 3               | Csp3        | Norway, Hordaland, Espegrend      | AY253934                 | AY543814       | TG, JR      | 98-39-1                |
| *Ceramium* sp. 4⁰              | Csp4.1      | N. Ireland, Donegal, Marble Hill  | AY253932                 | AY254302       | CAM         | CerDoneg               |
|                                | Csp4.3      | Wales, Anglesey                   | x                        | DG              | CerUK       |                        |
|                                | Csp4.4      | N. Ireland, Down, Annalang        | x                        | CAM             | CerDown     |                        |
| *Spyridia filamentosa* (Wulfen) Harvey | Sfi | Spain, Mallorca                    | AY253943                 | AY255476       | JR          | spyridia f              |
brielsen et al. 2003). Unialgal cultures were used for the analyses in most cases; these were established from field-collected material and grown as described by Gabrielsen et al. (2003). Two samples were obtained as isolated DNA from Dr C. A. Maggs, Queen’s University of Belfast (Csi.1 of C. siliculosum and Cbo. of C. botryocarpum; Table 1).

Some of the samples were taken from herbarium material. We included the lectotype of C. echinotum from 1837 (lectotypified by C. A Maggs 20 September 1990). A specimen from western Norway (Flora), identified as C. nodosum in Lein et al. 1999 (= C. virgatum acc. to Maggs et al. 2002), was probably misidentified and belongs to C. secundatum (Cse.4; Table 1). Herbarium material of a specimen described as C. elegans (Roth) Ducluzeau from the Adriatic Sea was also included (Ceramium sp. 6, Table 1).

Some of the samples listed in Table 1 (e.g. Ceramium spp. 1–4) were sequenced for the rubisco spacer in a previous study (Gabrielsen et al. 2003). These data were included in the present analyses.

A cultured isolate of Spyridia filamentosa (Wulfen) Harvey (Ceramiaceae) was used as outgroup, in addition to the rubisco spacer sequence of Antithamnion sp., from Kostrewa et al. (1990) (GenBank accession number X54532). According to Maggs & McIvor (2002), this taxon is Antithamnionella spirographidis (Schiffner) Wollaston.

DNA isolation, PCR, and sequencing

The isolated cultures were washed in distilled water and blotted dry. The DNA was extracted from 2–15 mg blotted material using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. The DNA from the herbarium specimens was extracted as described by Gabrielsen et al. (2003).

Amplification of the rubisco spacer region was performed with primers taken from Goff et al. (1994), with polymerase chain reaction (PCR) conditions as described by Gabrielsen et al. (2003). The forward primer Z (Freshwater et al. 1999) and the reverse primer 28F (Hamby et al. 1988) were used to amplify one of the most variable regions of LSU (Freshwater et al. 1999). Amplifications were performed in reaction volumes of 50 μl containing 1 × PCR buffer (Qiagen), 200 μM of each deoxyribonucleotide (dNTP), 2 mM MgCl₂, 0.3 μM primer (DNA Technology, Aarhus, Denmark), 0.5 U AB Tag DNA Polymerase (AB gene, Epsom, England), and 5 μl of 100 × diluted DNA. The thermocycler was programmed for 4 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 52°C, and 75 s at 72°C. The cycles were followed by 7 min at 72°C for extra elongation.

Amplified PCR products were either sequenced manually using the Thermo Sequenase Radiolabeled Terminator Cyclic Sequencing Kit (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer’s specifications, or run on an ABI Prism 377 DNA sequencer (Perkin Elmer, Boston, USA) using the Big-dye Terminator Cycle Sequencing Kit (Perkin Elmer). The sequences were run in both directions. The sequencing profiles obtained by automated sequencing were checked and edited using Chromas version 1.6 (Technelysium Pty Ltd, Tewantin, Australia).

Phylogenetic analyses

The sequences were aligned using ClustalW in BioEdit version 5.0.9 (Hall 1999) and corrected manually. Protein coding sequences flanking the rubisco spacer were identified by the presence of start and stop codons (e.g. Brodie et al. 1998). Phylogenetic analyses using maximum parsimony and maximum likelihood (ML) were performed on each region separately and on a combined data set (see below). Possible saturation of the sequences was examined graphically by plotting the Ti/Tv ratio against the uncorrected P distance. Because some mutational saturation was indicated between the two outgroup sequences and the Ceramium sequences in both regions (not shown), phylogenetic analyses were repeated excluding the outgroup species.

All parsimony analyses were performed using heuristic searches with 10 random addition replicates and TBR branch swapping, either excluding gaps or including gaps as single evolutionary events. Parsimony analyses with the two gap treatments produced similar results, and only the analyses including gaps are presented. The model of sequence evolution that best fit each data set for ML analysis was found by a hierarchical likelihood ratio test using Modeltest version 3.06 (Posada & Crandall 1998). The same models were used to estimate sequence divergences of the rubisco spacer region and partial LSU for species of Ceramium.

The combined data set was tested for character incongruence using the incongruence length difference test (ILD; Farris et al. 1994) implemented as the partition homogeneity test in PAUP* (Swofford 2001). If the ILD is the difference between the number of steps required by individual and combined analyses, and the distribution of the ILD statistics can be estimated by randomisation as described by Cunningham (1997). P > 0.05 indicates lack of incongruence, thus allowing the data sets in question to be combined. The species for which only rubisco spacer sequences were obtained were excluded from the combined alignment. Randomisations for the partition homogeneity test were performed 10,000 times.

Support for individual branches was evaluated using bootstrap analysis (Felsenstein 1985) as implemented in PAUP* (Swofford 2001) with 1000 bootstrap data sets generated for
RESULTS

Rubisco spacer region

A total of 30 rubisco spacer region sequences were obtained (Table 1). The alignment generated from these sequences consisted of 341 positions, of which the rubisco spacer ranged from 93 to 105 bp in species of Ceramium (100 bp in Antithamnion sp. and 107 bp in S. filamentosus), and the flanking regions were 85 bp (rbcL) and 139 bp (rbcS) in all taxa. The difference in sequence lengths between Ceramium species was mainly due to an 11 bp indel found in C. shuttleworthiam, C. diaphanum, C. deslongchampsii, C. cimbricum, and C. echinotum. A total of 127 characters were variable, and 81 (64 excluding the outgroups) were parsimony-informative.

The best model for ML reconstruction of the rubisco spacer region sequences was the substitution model of Hasegawa et al. (1985) with unbalanced base composition (A = 0.38, C = 0.19, G = 0.13, T = 0.30) and rate heterogeneity according to the gamma distribution (shape parameter 0.302). The well-supported branches of the ML tree (Fig. 1) were also found in the parsimony analysis, in which 12 equally most parsimonious trees of 271 steps were found (CI = 0.59, CIX = 0.50, RI = 0.61; not shown, bootstrap values in Fig. 1). Analyses excluding the two outgroup species did not alter the tree topology, but somewhat higher bootstrap values were obtained in the ML analyses (not shown). Within the highly supported Ceramium ingroup, five divergent (11.5–20.1%) species representing both the spiny group and the non-spiny group with ecorticate internodes were basal in the tree, but their relationships were not well resolved (Fig. 1). The remaining species formed a clade (with moderate support in the parsimony analysis) consisting of two large subclades. One of the subclades was moderately supported (54% and 84%) and included several species with ecorticate internodes (Ceramium spp. 1–4, C. tenuicorne, and C. siliquosum) and one of the species that can develop full cortication (C. pallidum). A close relationship between C. siliquosum and C. pallidum was well-supported (85–92%) in both analyses, whereas the remaining relationships were not well resolved (Fig. 1).

The other subclade was not supported and included representatives of all three species groups. Within this subclade, one highly supported group (94–99%) consisted of C. botryocarpum as sister to a weakly supported group (55–57%) containing C. secundatum (Fig. 1). The relationships among the remaining three species (C. ciliatum, C. virgatum, and Ceramium sp. 6) were not resolved.
Large subunit

A total of 25 partial LSU sequences were obtained (Table 1), ranging from 303 bp in *S. filamentos"a* to 318 bp in *C. cimbricum*. The alignment included a total of 9 coded gap positions and 320 nucleotides, of which 78 were variable and 19 were parsimony-informative (15 excluding the outgroup species).

The best model for ML reconstruction of the partial LSU sequences was a general time-reversible model with balanced nucleotide frequencies and rate heterogeneity according to the gamma distribution (shape parameter 0.16). The partial LSU sequences showed low levels of variation, and several species had identical sequences (not shown). Two equally most parsimonious trees of 59 steps were found in the parsimony analysis (CI = 0.81, CIx = 0.63, RI = 0.73). All phylogenetic trees from the LSU analyses were poorly resolved (not shown). The only moderately- to well-supported clade in the analyses of the LSU sequences consisted of all the species except the four most basal ones in the trees (*C. cimbricum, C. deslongchampsi"a, C. shuttleworthianum, and C. diaphanum*).

Combined analyses

The partition homogeneity test indicated that the rubisco spacer and partial LSU sequences were not significantly different from each other (*P* = 0.81) and thus could be combined. The analyses of the combined data set result in more robust phylogenies than when each region was analysed separately. Analyses without an outgroup produced higher bootstrap values than the analyses including it, particularly in the ML analyses, and only the analyses without the outgroup are shown. The best model for ML reconstruction of the combined data set was a general time reversible model with unequal base frequencies (*A* = 0.33, *C* = 0.19, *G* = 0.21, *T* = 0.27) and rate heterogeneity according to the gamma distribution (shape parameter 0.08).

The well-supported branches of the ML tree (not shown, bootstrap values in Fig. 2) were similar to those obtained in the parsimony analysis, in which 26 equally most parsimonious trees of 230 steps were found (CI = 0.68, CIx = 0.54, RI = 0.61; Fig. 2). The relationships among three of the basal species (*C. cimbricum, C. deslongchampsi"a, and C. shuttleworthianum*) were still not resolved, but there was moderate bootstrap support (74–79%) for *C. diaphanum* as sister to the remaining species (Fig. 2). Additionally, there was high bootstrap support (79–91%) for the monophyletic group including *C. tenuicorne* as sister to *C. siliqusum–C. pallidum* (Fig. 2).

**DISCUSSION**

Sequence divergences and utility of the genetic markers

Progress in analyses of molecular data has undoubtedly clarified parts of the intriguing history of *Ceramium* (e.g. Gabrielsen *et al.* 2002, 2003; Maggs *et al.* 2002; Cho *et al.* 2003a, b; Seo *et al.* 2003). As expected, molecular markers were also informative in the present study, although varying degrees of information were obtained from the analysed regions. Analyses of the combined data set resulted in the most robust tree topologies (Fig. 2), even if the sequenced segment of the LSU region was highly conserved and not useful for distinguishing among several of the species. On the other hand, exceptions
do exist, and *C. cimbricum* was quite divergent from the other species. The sequenced segment of the LSU, which includes one of the most variable regions observed in an analysis of Gelidiales (Freshwater et al. 1999), is thus more appropriately used at higher taxonomic levels.

The rubisco spacer region was considerably more variable with sequence divergences ranging from 0.3% to 25.1%. It seems, however, that the relatively short spacer region may be of limited value for separating closely related species (Goff et al. 1994; Brodie et al. 1998; Kamiya et al. 1998; Müller et al. 1998). Findings of both inter- and intraspecific variation in the rubisco spacer sequences in *Ceramium* is a result also known from other red algal genera (Kamiya et al. 1998; Rueness & Rueness 2000; Zuccarello et al. 2000). In a recent study, high sequence divergence rates were found among some Pacific species of *Ceramium*, but no variation was found within species (Cho et al. 2003b).

The divergence between *C. secundatum* and *C. botryocarpum* (0.3–0.7%) was of a similar magnitude as the divergences among different isolates of *C. pallidum* (0–0.7%). To further study the relationship between *C. secundatum* and *C. botryocarpum*, we also sequenced the ITS2 region of *C. botryocarpum* (T.M. Gabrielsen, unpublished observations) and compared it to that of *C. secundatum* by Gabrielsen et al. (2003). The ITS2 sequence divergence between these two species (3.8%) was higher than the *rbcL* sequence divergence (1.8–2.7%) found by Maggs et al. (2002). It is worth noticing that intraspecific ITS divergences in *Ceramium* were distinctly lower than the lowest interspecific ITS divergence (Gabrielsen et al. 2003, this study). However, this is not the case with *rbcL* sequences from *C. botryocarpum* and *C. secundatum* (Maggs et al. 2002).

**Molecular systematics**

The corticated *C. virgatum* appears to be most closely related to the incompletely corticated *Ceramium* sp. 6 (Fig. 1), with a sequence divergence of 5.3%. *Ceramium* sp. 6 was assigned to *C. elegans* (Roth) Ducluzeau by the collector. Currently, *C. elegans* has been treated as varieties under both *C. diaphanum* (Lightfoot) Roth (Silva et al. 1996) and *C. siliquosum* (Kützing) Maggs & Hommersand (Furnari et al. 1999; Garreta et al. 2001). The material that we analysed as *Ceramium* sp. 6 was not closely related to our sequences of any of these species, and the identification of this specimen must await further investigation.

The monophyletic relationship found between *C. virgatum* and *C. secundatum–C. botryocarpum* in the British Isles (Maggs et al. 2002), was not found in our analyses. The different results of these two studies are probably explained by different taxon sampling, combined with the fact that other molecular markers were used.

The non-spiny ecorticate species in this study were genetically distinct, but the present analyses did not resolve the relationship among *Ceramium* spp. 1–4 (Fig. 2). The only exception was some weak bootstrap support for the American *Ceramium* sp. 1 as the first-diverging lineage (Fig. 2). On the other hand, the relationship that included *C. tenuicorne* as sister to *C. siliquosum–C. pallidum* (Fig. 2), was well supported, as it was in previous analyses of ITS2 and rubisco spacer sequences by Gabrielsen et al. (2003). Unlike those

results, we find no support for the sister relationship of *Ceramium* spp. 2 and 4 (Fig. 2). It is clear, however, that divergent lineages of non-spiny ecorticate species can be identified with certainty, almost exactly corresponding to five geographic regions (see Gabrielsen et al. 2003). Several crossing experiments between these lineages have furthermore supported these findings (Rueness 1978; Rueness & Kornfeldt 1992; Gabrielsen et al. 2003).

When the outgroups were excluded from the phylogenetic analyses, similar tree topologies were found, suggesting that the basal *Ceramium* sequences actually represent early branching lineages. Traditionally, some of these lineages (e.g. *C. cimbricum* and *C. diaphanum*) have been confused with members of the *C. strictum* group. However, the sequence divergence and phylogenies found in the present study support more recent studies that considered these species to be distinct (Rueness 1992; Maggs & Hommersand 1993; Rueness & Boo 1994). *Ceramium cimbricum*, for instance, distinguished itself rather markedly. This species is very divergent from all the others in both rubisco spacer (13.3–25.1%) and LSU (9.2–13.3%) sequences. Furthermore, when three recently published rubisco spacer sequences from other *Ceramium* species were included in the analyses (GenBank accession numbers AY178518, AY178523, AY178524), it showed that *C. cimbricum* belongs to a moderate to well-supported monophyletic group of Pacific species (not shown). This group was placed as a sister-group to the remaining species, and included the ecorticate *C. tenerrimum* (Martens) Okamura, the fully corticated *C. kondoi* Yendo and *C. boydenii* Gepp. This is an interesting result, particularly when a recent study has shown that Pacific species of *Ceramium* are paraphyletic with respect to *Campylaephora J. Agardh and Reinboldiella De Toni* (Seo et al. 2003). Because of this, phylogenetic analyses of the genus can be seriously affected by taxon sampling. All things considered, a Pacific origin of *Ceramium cimbricum* might explain the pattern described above, as well as the long branches seen in the other phylogenetic trees (Figs 1, 2). The inclusion of new taxa, did not, however, clarify the relationships among *C. diaphanum*, *C. echinotum*, *C. deslongchampii*, and *C. shuttleworthinum*.

**Evolution of spines and cortication**

Spine morphology and the development and arrangement of the cortical bands are assumed to be diagnostic characters appropriate to delimit species of *Ceramium* (e.g. Dixon 1960; Womersley 1978; Maggs & Hommersand 1993). However, the three widely used species groups of *Ceramium* did not correspond to groups found in the phylogenetic analyses (Figs 1, 2). Representatives with spines were found among the early-diverging lineages as well as within the major clade that obtained high bootstrap support in the combined analysis. Similar results have been found when the phylogeny of spiny and ecorticate non-spiny species from Korea were analysed based on *rbcL* sequences (Cho et al. 2003b). In addition, it has been demonstrated that spine morphology differs considerably among different species (Dixon 1960), as well as within species (Millar 2002), which further supports the idea that spines evolved independently in different lineages.

Non-spiny, fully corticated species occurred in several different, partly highly supported clades that also contained non-
spiny species with eccentric internodes. The non-spiny, corticated species *C. pallidum* is most closely related to the two incompletely corticated species *C. siliquosum* and *C. tenuicorne* (see also Gabrielsen et al. 2003). Recently, Cho et al. (2003a, b) showed similar contradicting patterns among Pacific species. The degree of cortication has been shown to vary conspicuously even within a single species. It is known that specimens of both *C. pallidum* and *C. virgatum* have been observed with eccentric internodes, in addition to fully corticated ones (Maggs & Hømmersand 1993; Maggs et al. 2002). In fact, various studies have shown that cortication patterns are highly influenced by varying growth conditions (Garbary et al. 1978; Suh & Lee 1984; Cormaci & Motta 1987). These results suggest that the presence of cortical spines and the cortication patterns of internodes are homoplastic characters that cannot be used to infer species relationships in *Ceramium*. Additionally, because the genus is not monophyletic based on rubisco spacer and *psbA* gene sequences (Seo et al. 2003), further study is needed to unravel the phylogeny of *Ceramium* and related genera.

**ACKNOWLEDGEMENTS**

We thank Lise Broch for maintaining the cultures and Sissel A. Brubak for technical assistance. Thanks are due to the collectors of material (Peter Edwards, Stein Fredriksen, David Garbary, Tone Jacobsen, Francis Magne, Poul Møller Pedersen, Snore W. Steen, Anne-Cathrine Sørlie, and Per Arvid Åsen), and in particular Christine A. Maggs for providing DNA of *C. siliquosum* and *C. botryocarpum* as well as several cultures. We also thank two anonymous reviewers for valuable comments on this manuscript. This project was funded by EU under the program Biobase (MAS3-CT98-0160).

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Received 23 March 2004; accepted 27 December 2004

Communicating editor: R. Waaland