Nat.Hist.Res.,Special Issue No.8 : 23-41. March 2005

Molecular phylogeny of the order Gelidiales (Rhodophyta)

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Abstract Phylogenetic relationships of the order Gelidiales were inferred on the basis of trees using nuclear-encoded SSU rDNA (small subunit of ribosomal DNA) and plastid-encoded *rbc*L (large subunit of the ribulose-1,5-bisphosphate carboxylase/oxgenase) sequences. These phylogenetic trees show three major clades, the *Gelidiella* clade that was the earliest diverging group within the order, the *Pterocladia/Pterocladiella* clade and the large *Gelidium*-complex clade. Each of the three types of secondary rhizoidal attachments (unicellular independent, peg, brush) is completely consistent with in the respective three major clades of the Gelidiales, which suggests that this morphological character reflects the phylogeny of this order. Three monophyletic groups were recognized in the *rbc*L tree of 21 local populations of *Pterocladiella* in Japan. They can be clearly recognized by discontinuity of morphological characters which remain distinct when grow under the same culture conditions. These results indicate that three groups represent distinct species, *Pterocladiella nana, P. tenuis* and *P. capillacea*, respectively. Two new species, *Gelidium tenuifolium* and *G. koshikianum* were described from Japan. Four species, *Gelidiella ligulata, G. pannosa, Pterocladiella caerulescens* and *P. caloglossoides*, were reported from Japan for the first time, and their diagnostic features were described.

Key words: Gelidiales, *Gelidiella*, *Gelidium*, molecular phylogeny, morphology, *rbcL*, *Pterocladiella*, Rhodophyta, SSU rDNA

Introduction

The red algal order Gelidiales currently includes 10 genera (*Acanthopeltis* Okamura, *Capreolia* Guiry et Womersley, *Gelidiella* Feldmann et Hamel, *Gelidium* Lamouroux, *Onikusa* Akatsuka, *Porphyroglossum* Kützing, *Pterocladia* J. Agardh, *Pterocladiella* Santelices et Hommersand, *Ptilophora* Kützing and *Suhria* J. Agardh) and approximately 140 species that are distributed worldwide (Santelices, 1990; Bailey and Freshwater, 1997). In Japan, six genera, i.e. *Acanthopeltis*, *Gelidiella*, *Gelidium*, *Onikusa*, *Pterocladiella* and *Ptilophora* are known, although separation of the genus *Onikusa* from the genus *Gelidium* (Akatsuka, 1986) is uncertain (Santelices, 1990; Freshwater *et al.*, 1995; Yoshida, 1998), and 26 species of Gelidiales are recognized in Japanese waters (Yoshida *et al.*, 2000).

Distinguishinf *Gelidium* and *Pterocladiella* (as *Pterocladia*) has always been notoriously difficult (Dixon and Irvine, 1977; Santelices, 1990). Several taxonomists have sought characters to separate *Gelidium* from *Pterocladiella* (as *Pterocladia*), which include cystocarpic structure (Fan, 1961; Santelices ,1991a, 1991b), hyphal distribution and shape of medullary cells (Okamura, 1934), basal bending at the point of branching of indeterminate laterals (Stewart, 1968), disposition of surface cortical cells (Akatsuka, 1970, 1981) and apical architecture (Rodríguez and Santelices, 1987). However, Rodríguez and Santelices (1988) claimed that these characters have only limited taxonomic value, and only reliable feature for distinguishing these genera is in the female reproductive structure. Female plants of *Gelidium* and *Pterocladia*, however, are only occasionally found, so that reliance on other vegetative features becomes a necessity (Santelices and Stewart, 1985).

Pterocladiella was recently separated from the genus *Pterocladia* by Santelices and Hommersand (1997) based on the development of carpogonia and cystocarps. The following three species that are assignable to either of these genera occur in Japanese waters: *Pterocladia*

nana Okamura (1932), *Pterocladia tenuis* Okamura (1934) and *Pterocladia densa* Okamura (1934). *Pterocladia tenuis* and *P. densa* were reduced to the synonymy of *Pterocladiella capillacea* (S. Gmelin) Santelices et Hommersand (Stewart, 1968, as *Pterocladia capillacea* [S. Gmelin Bornet et Thuret), and *P. nana* was also considered to be the dwarf form of the widespread *P. capillacea* (Santelices, 1991b, as *Pterocladia capillacea*). However, Akatsuka (1981) objected to this treatment on the grounds that Stewart (1968) used only a small amount of Japanese material and no quotation of Okamura's (1934) original description was made, and he proposed to treat *P. densa* and *P. tenuis* as distinct species. Although *P. nana* was included in *P. capillacea* by Santelices (1991b), morphological variability of *P. nana* has not been adequately analyzed in his work. Thus, the species of *Pterocladia* described by Okamura (1932, 1934) need to be reexamined.

Recent molecular phylogenetic studies of the Gelidiales using nuclear-encoded SSU rDNA (small subunit of ribosomal DNA) and plastid-encoded *rbc*L (large subunit of the ribulose-1,5-bisphosphate carboxylase/oxgenase) sequences resolved phylogenetic positions of several gelidialean species (Freshwater and Rueness, 1994; Freshwater *et al.*, 1995; Bailey and Freshwater, 1997; Patwary *et al.*, 1998). However, these studies included only a few Japanese species. In this study, I determined the nucleotide sequences of the nuclear-encoded SSU rDNA gene and plastid encoded *rbc*L gene of 18 species of Japanese gelidialean species, and discuss the phylogenetic relationships between gelidialean species.

The present paper is review of my studies of Gelidiales, and contains already published data (Shimada and Masuda, 1999, Shimada *et al.*, 1999, Shimada *et al.*, 2000a, 2000b, Shimada and Masuda, 2000).

Materials and Methods

DNA extraction

Total DNAs were extracted from 5 genera of 18 species (37 unialgally cultured strains) for phylogenetic analyses of Gelidiales (Table 1). Voucher specimens are deposited in the Herbarium of Graduate School of Science, Hokkaido University, Sapporo (SAP). Unialgal cultures were established from the tips of branchlets of field-collected plants and grown in PES medium (Provasoli, 1968) or Tris-buffered medium (van der Meer and Patwary, 1991) at 15℃ or 20℃ and 16:8 h LD cycle with the photon flux of 15-25 μ $Em^{-2}s^{-1}$

Blotted algal tissue was ground in liquid nitrogen. The frozen powder was rinsed with washing buffer (Kawahara *et al.*, 1995) 3-5 times until the supernatant became colorless to remove polysaccharides, and then UNSET buffer (Garriga *et al.*, 1984) was added to the rinsed pellet and this mixture was incubated on ice for 40 min. Then, an equal volume of phenol, chloroform and isoamyl alcohol mixture (25:24:1) was added and mixed gently for 10 min. The solution was centrifuged at 10000 rpm (7000 G) for 5 min. The upper aqueous phase was transferred to a new tube, and the extraction was repeated three times. CIA (chloroform:isoamyl alcohol = 24:1) mixture was added and mixed gently for 10 min. The solution was then centrifuged at 10000 rpm (7000 G) for 5 min. Total genomic DNA was precipitated with 0.2 M NaCl and 2.5 vol. 99.5% ethanol on ice for 10 min. This was followed by centrifugation at 12000 rpm (10000 G) for 15 min, the pellet was washed with cold 70% ethanol and air-dried. The pellet was redissolved in 50-100 μ l autoclaved distilled water.

PCR amplification and sequencing

The total DNA was used as the template for the polymerase chain reaction (PCR)(Saiki *et al.*, 1988). In this study, I used 6 pairs of primers: SR1-SR5, SR4-SR9 and SR8-SR12 for SSU rDNA (Nakayama *et al.*, 1996); F8-R643, F605-R1150 and F993-RrbcSstart for *rbc*L (Shimada *et al.*, 1999). The temperature-cycling protocol consisted of an initial denaturation step of 93℃, 1 min, followed by 35 cycles of 30 sec denaturation period at 94℃, 30 sec primer annealing at 55℃, and 45 sec extension at 72℃, then hold at 4℃. These PCR products were directly sequenced using a DNA autosequencer (ABI PRISM, 310 Genetic Analyzer) with dye-terminator method (Nakayama *et al.*, 1996).

Sequence analysis

SSU rDNA sequences were first aligned with the CLUSTAL W computer program (Thompson *et al.*, 1994) and then refined by eye. The *rbc*L sequences were aligned manually because

Table 1. List of species used in DNA extraction.

Species	Locality (Date, voucher number in SAP)	Accession number SSU	Accession number rbcL
Acanthopeltis japonica Okamura	Shimoda, Shizuoka Pref. (25.ix.1996, 064829)	AB017664	AB017673
Acanthopeltis hirsuta (Okamura) Shimada *et al.*	Oryuzako, Miyazaki Pref. (11.vii.1996, 064845)	AB017666	AB017675
Gelidiella ligulata Dawson	Miyake Is., Shizuoka Pref. (14.vii.1998, 063883)	AB017669	AB017678
Gelidiella pannosa (Feldmann) Feldmann et Hamel	Ishigaki Is., Okinawa Pref. (6.iii.1999, 071773)	AB031300	
Gelidium divaricatum Martens	Nishiizu, Shizuoka Pref. (26.ix.1996, 064833)	AB017662	
Gelidium elegans Kuetzing	Awaji Is., Hyogo Pref. (16.v.1996, 064834)	AB017670	AB030623
Gelidium koshikianum Shimada *et al.*	Shimo-Koshiki Is. Kagoshima Pref. (31.vii.1997、070874)		AB030626
Gelidium linoides Kuetzing	Shimoda, Shizuoka Pref. (25.ix.1996, 064835)		AB030622
Gelidium pacificum Okamura	Enoshima, Kanagawa Prefecture (29.iii.1998, 064836)		AB030627
Gelidium pusillum (Stackhouse) Le Jolis	Awaji Is., Hyogo Pref. (1.x.1996, 064837)	AB017663	AB017679
Gelidium tenuifolium Shimada *et al.*	Shimoda, Shizuoka Pref. (25.ix.1996, 070868)		AB030628
Gelidium vagum Okamura	Jodogahama, Iwate Pref. (11.vi.1997, 064839)	AB017671	AB017680
Onikusa japonica (Harvey) Akatsuka	Shimoda, Shizuoka Pref. (25.ix.1996, 064840)	AB017667	AB017676
Pterocladiella caerulescens (Kuetzing) Santelices et Hommersand	Yonaguni Is., Okinawa Pref. (1.iii.1999, 071774)	AB031301	
Pterocladiella caloglossoides (Howe) Santelices	Ishigaki Is., Okinawa Pref. (4.iii.1999, 071778)	AB031302	
Pterocladiella capillacea (Gmelin) Santelices et Hommersand	Tomioka, Kumamoto Pref. (30.vii.1997, 065453)		AB023845
	Takedatsu, Oita Pref. (4.viii.1997, 065454)		AB023846
	Kiwado, Yamaguchi Pref. (19.xi.1998, 065463)		AB023855

Table 1. Continued.

Species	Locality (Date, voucher number in SAP)	Accession number SSU	Accession number rbcL
	Hinomisaki, Shimane Pref. (20.xi.1998, 065464)		AB023856
	Uradomi, Tottori Pref. (21.xi.1998, 065465)		AB023858
	Echizen, Hukui Pref. (22.xi.1998, 065460)		AB023852
	Unoura, Ishikawa Pref. (7.ix.1998, 065458)		AB023850
	Kasashima, Niigata Pref. (17.vi.1997, 065450)		AB023843
	Oga, Akita Pref. (19.vi.1997, 065451)		AB023844
	Taisei, Hokkaido (22.viii.1997, 065455)		AB023847
	Oshoro, Hokkaido (15.ix.1996, 065447)	AB017672	AB023841
	Shiriya, Aomori Pref. (7.iv.1998, 065456)		AB023848
	Onahama, Hukushima Pref. (13.vi.1997, 065449)		AB023842
	Hachijo Is., Shizuoka Pref. (9.vii.1998, 065457)		AB023849
	Hamashima, Mie Pref. (17.xi.1998, 065462)		AB023854
	Kushimoto, Wakayama Pref. (18.xi.1998, 065461)		AB023853
Pterocladiella nana (Okamura) Shimada *et al.*	Shimo-Koshiki Is. Kagoshima Pref. (31.vii.1997、065452)	AB031303	AB023840
Pterocladiella tenuis (Okamura) Shimada *et al.*	Shimoda, Shizuoka Pref. (25.ix.1996, 064842)	AB017672	AB017681
	Tsuyazaki, Hukuoka Pref. (4.xi.1998, 065459)		AB023851
	Hinomisaki, Shimane Pref. (20.xi.1998, 065483)		AB023857
	Enoshima, Kanagawa Pref. (5.i.1999, 065466)		AB023859
	Shimoda, Shizuoka Pref. (25.ix.1996, 064842)		AB017681

Table 2. List of GenBank species used in molecular phylogenetic analyses.

Species	Accession number	
	SSU	rbcL
Capleoria implexa Guiry et Womersley	U60344	L22456
Gelidiella acerosa (Forsskal) Feldmann *et* Hamel	AB017669	AB017678
Gelidium abbottiorum Norris		U16829
Gelidium allanii Chapman		L22458
Gelidium americanum (Taylor) Santelices	U60347	L22459
Gelidium attenuatum (Turner) Thuret		U00110
Gelidium canariense (Grunow) Seoane-Camba		L22460
Gelidium caulacantheum J. Agardh	U60343	U00103
Gelidium coulteri Harvey		U00105
Gelidium divaricatum Martens		U16828
Gelidium latifolium (Greville) Bornet *et* Thuret	U60350	U00112
Gelidium micropterum Kuetzing		U00446
Gelidium purpurascens Gardner		U00979
Gelidium pusillum (Stackhouse) Le Jolis CA U.S.A. (Ca)		U00984
Gelidium pusillum (Stackhouse) Le Jolis Canary Is. (CI)		U01003
Gelidium pusillum (Stackhouse) Le Jolis Norway (No)		U00999
Gelidium pusillum (Stackhouse) Le Jolis Puerto Rico (PR)		U00983
Gelidium sesquipedale (Clemente) Thuret		L22071
Onikusa pristoides (Turner) Akatsuka		U01044
Pterocladia lucida (R. Bornet ex Turner) J. Agardh	U60349	U01048
Pterocladiella melanoidea (Schousboe) Santelices *et* Hommersand	U60341	U01046
Ptilophora pinnatifida (J. Agardh) Norris	U60345	U16834
Ptilophora subcostata (Okamura) Norris	U60348	U16835
Suhria vittata (Lin.) J. Agardh		U00112
Gracilaria tikvahiae McLachlan	AF468911	U21357
Chondrus crispas Stackhouse	Z14140	U02984

no insertion/deletion mutations were detected. Sequences of 37 additional species were downloaded from GenBank and included in these alignments (Table 2).*Gracilaria tikvahiae* McLachlan and *Chondrus crispus* Stackhouse were used as outgroups for SSU rDNA and total *rbc*L analyses. In the selected *rbc*L analysis of the genus *Pterocladiella*, *Pterocladia lucida* (R. Brown ex Turner) J. Agardh was used as outgroup.

The maximum parsimony (MP) method was used to construct phylogenetic trees. Gaps in the SSU alignment were treated as missing data. MP analysis was performed PAUP 4.0b10 (Swofford, 2002). All sites were treated as unordered and equally weighted. Heuristic search option with random addition of sequences (10 replicates) and tree-bisection-reconnection branch swapping algorithm (TBR) were used for tree searching. Bootstrap analysis based on 1000 re-samplings (simple addition) of the data set (Felsenstein, 1985) was calculated to evaluate statistical reliability.

Secondary rhizoidal attachments and correlation of molecular and morphological data

Secondary rhizoidal attachments of 17 species of 9 genera were examined using cultured strains and field-collected plants. The correlation between morphological data, secondary rhizoidal attachments and phylogenetic tree of the SSU rDNA was analyzed.

Morphological observations of the *Pterocladiella* species.

I selected as many mature individuals as possible from 21 populations in Japan, and 104 total individuals were measured for the following vegetative characters: (1) length of axes; (2) maximum length of first-order branches; (3) maximum width of second-order branches; (4) branching intervals of axes at the middle third portion; and (5) branching intervals of first-order branches at the middle third portion. The following type materials from the Okamura herbarium housed in SAP were also examined: 1) the lectotype specimen of *Pterocladia nana*, collected at Yura-jima, Shimo-Koshiki Island, *Koshiki* Islands, Kagoshima Prefecture (19. vii.1919); 2) the lectotype specimen of *Pterocladia tenuis* collected at Enoshima, Kanagawa Prefecture (iii.1897); and 3) the lectotype specimen of *Pterocladia densa* collected at Uradomi, Tottori Prefecture (viii.1923).

Results and Discussion

Phylogeny of the Gelidiales

The strict consensus tree of SSU sequences is presented in Fig. 1 (28 equally parsimonious trees, 296 steps, CI = 0.818, RI = 0.820, RC = 0.670, HI = 0.182). The monophyletic clade of the genus *Gelidiella* was supported by 98% bootstrap value and was recognized as the earliest diverging lineage within the Gelidiales. *Pterocladiella* clade was supported by 100% bootstrap value. *Pterocladia* and *Pterocladiella* were also shown to be monophyletic (56% bootstrap value). The monophyletic large clade that includes rest of taxa (large *Gelidium*-complex clade) was supported by 52% bootstrap value.

The strict consensus tree of *rbc*L sequences is presented in Fig. 2 (10 equally parsimonious trees, 1855 steps, CI = 0.44, RI = 0.614, RC = 0.271, HI = 0.56). Four monophyletic clades, the *Gelidiella* clade, *Pterocladia* clade, *Pterocladiella* clade and large *Gelidium*-complex clade, were evident with high bootstrap values (98-100%), although bootstrap values of their topological positions were less than 50%.

Results of the molecular analyses in this study were almost congruent with those of previous reports (Freshwater *et al.*, 1995; Bailey and Freshwater, 1997; Patwary *et al.*, 1998). Several points were improved and additional information was obtained by including additional Japanese species and non-gelidialean outgroups in the analyses: three major clades were recognized; the *Gelidiella* clade (SSU rDNA and *rbc*L analyses), *Pterocladia/Pterocladiella* clade (SSU rDNA analysis) and large *Gelidium*-complex clade (SSU rDNA and *rbc*L analyses); and the genus *Gelidiella* was recognized as the earliest diverging lineage within this order with high bootstrap value in the SSU rDNA analysis. There is a tendency that a better bootstrap support is obtained for the analysis of early branches in the SSU rDNA, while better bootstrap values can be obtained in the analysis of the *rbc*L among recently diverged groups. This is due to the different level of conservativeness of each gene, i.e. the SSU rDNA is more conservative than the *rbc*L (Bailey and Freshwater, 1997) and therefore the SSU rDNA is not suitable for the analysis of recently diverged taxa.

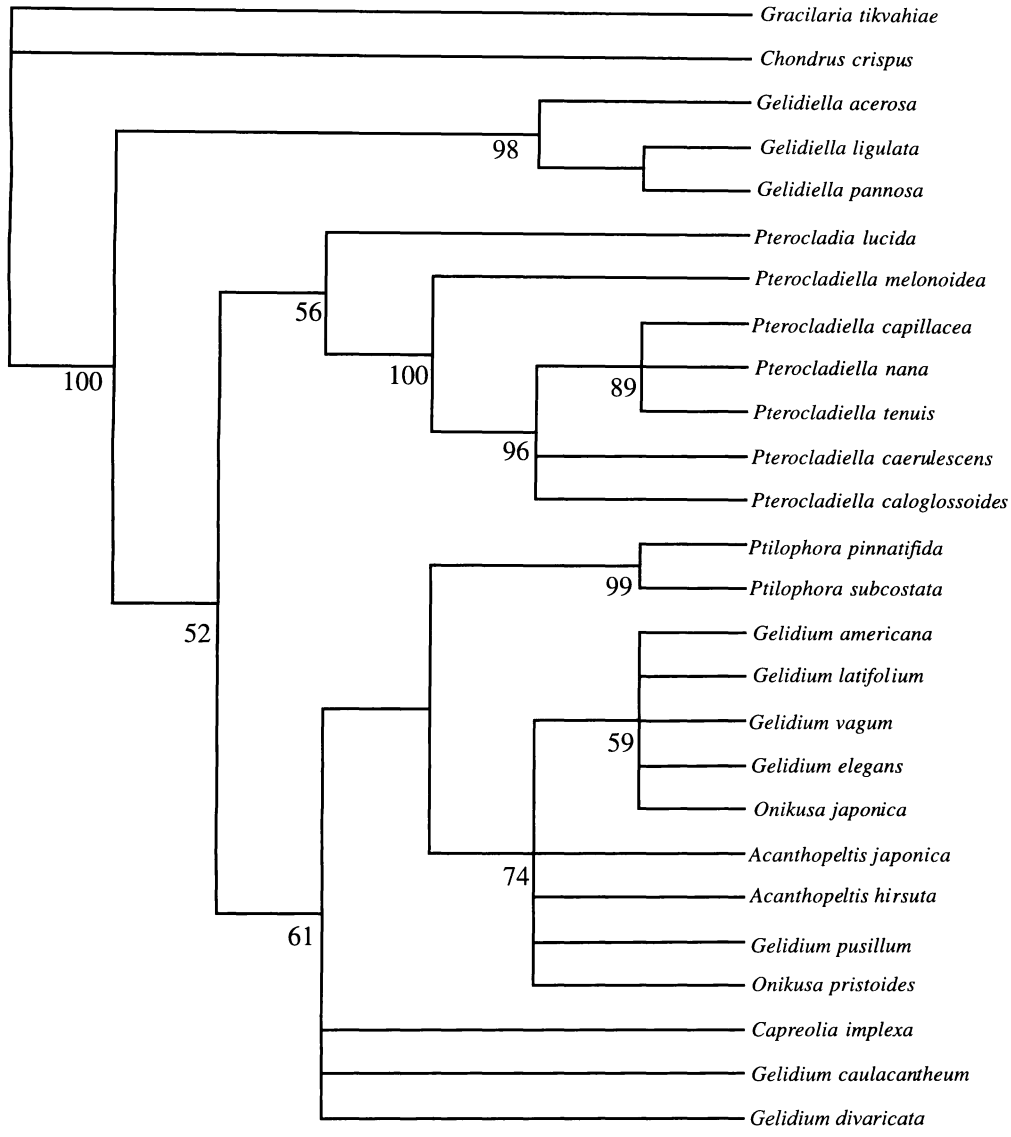


Fig. 1. Strict consensus tree of 28 most parsimonious trees constructed from an analysis of SSU rDNA sequences from Gelidiales. *Gracilaria tikvahiae* and *Chondrus crispus* were used as outgroups. The numbers under the branches represent full heuristic bootstrap values (1000 replicates) greater than 50%.

As Perrone (1994) has demonstrated, three types of secondary rhizoidal attachments were recognized in the present study; (1) the unicellular independent type was observed in *Gelidiella*; (2) the peg type was found in *Pterocladia* and *Pterocladiella*; (3) the brush type was observed in *Ptilophora*, *Capreolia*, *Acanthopeltis*, *Onikusa* and *Gelidium*. These three types of secondary rhizoidal attachments were correlated with the types of cystocarps (Fan, 1961; Dawson, 1959; Bailey and Freshwater, 1997; Yoshida, 1998) and correspond to the three large clades of the SSU rDNA gene (Fig 3). Based on Figure 3, it can be concluded that: (1) *Gelidiella* is characterized by having unicellular independent attachments and the absence of sexual reproduction; (2a) *Pterocladia* is characterized by having peg-type secondary rhizoidal attachments, nutritive filaments only arising from the third-order filament basal cells on the carpogonial side of the central axis, and carposporangia developing only on one side of the central plane of

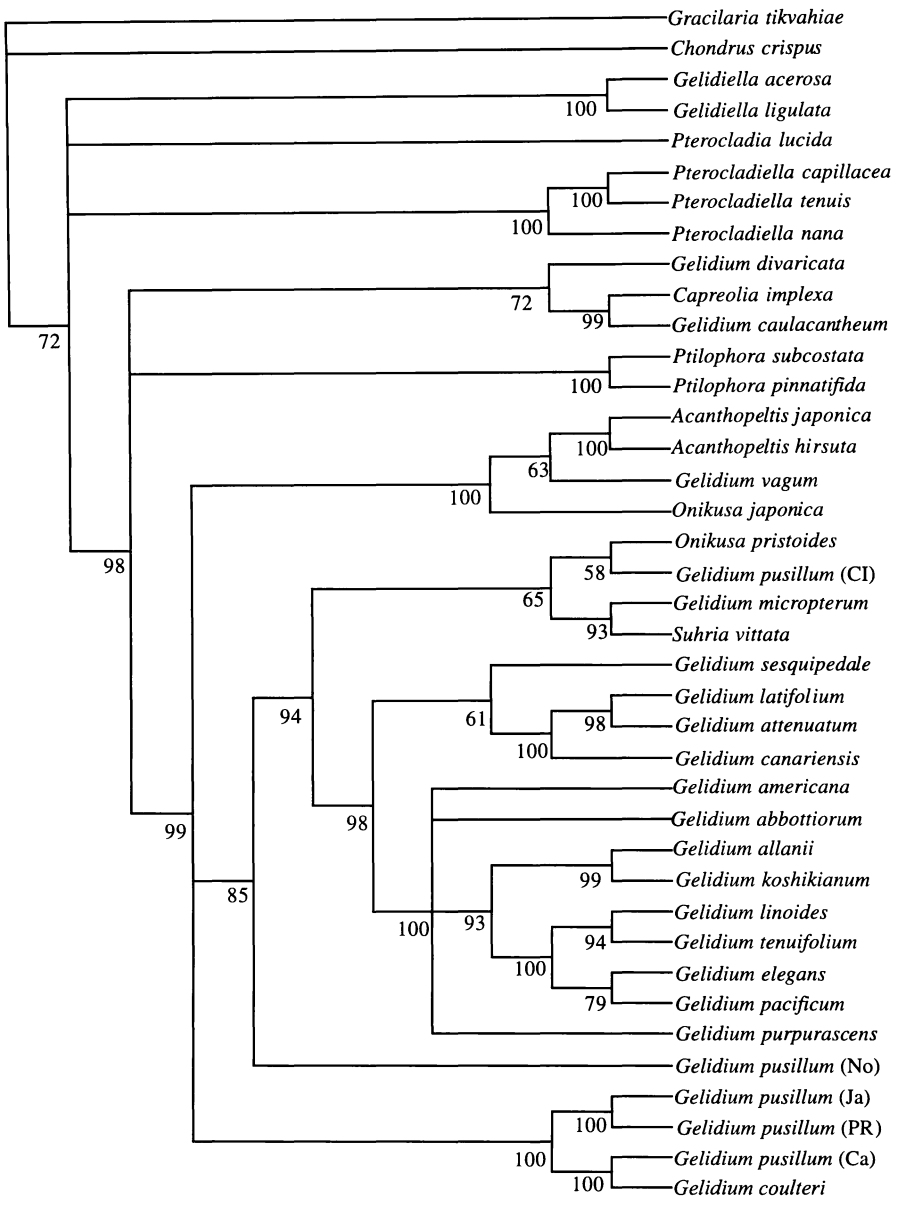


Fig. 2. Strict consensus tree of 10 most parsimonious trees constructed from an analysis of rbcL sequences from Gelidiales. *Gracilaria tikvahiae* and *Chondrus crispus* were used as outgroups. The numbers under the branches represent full heuristic bootstrap values (1000 replicates) greater than 50%.

a cystocarp (Bailey and Freshwater, 1997); (2b) *Pterocladiella* is characterized by the possession of peg-type secondary rhizoidal attachments, nutritive filaments arising from third-order filament basal cells adjacent to the central axis, and carposporangia developing on all sides of the central axis except where gonimoblasts attach to the floor of a cystocarp cavity (Santelices and Hommersand, 1997); and (3) all the members of the large Gelidium-complex clade (*Ptilophora, Capreolia, Acanthopeltis, Onikusa* and *Gelidium*) possess brush-type secondary rhizoidal attachments and carposporangia developing on both sides of the central plane of a cystocarp (Okamura, 1900; Akatsuka, 1986; Bailey and Freshwater, 1997).

The three types of secondary rhizoidal attachments were found to correspond to the

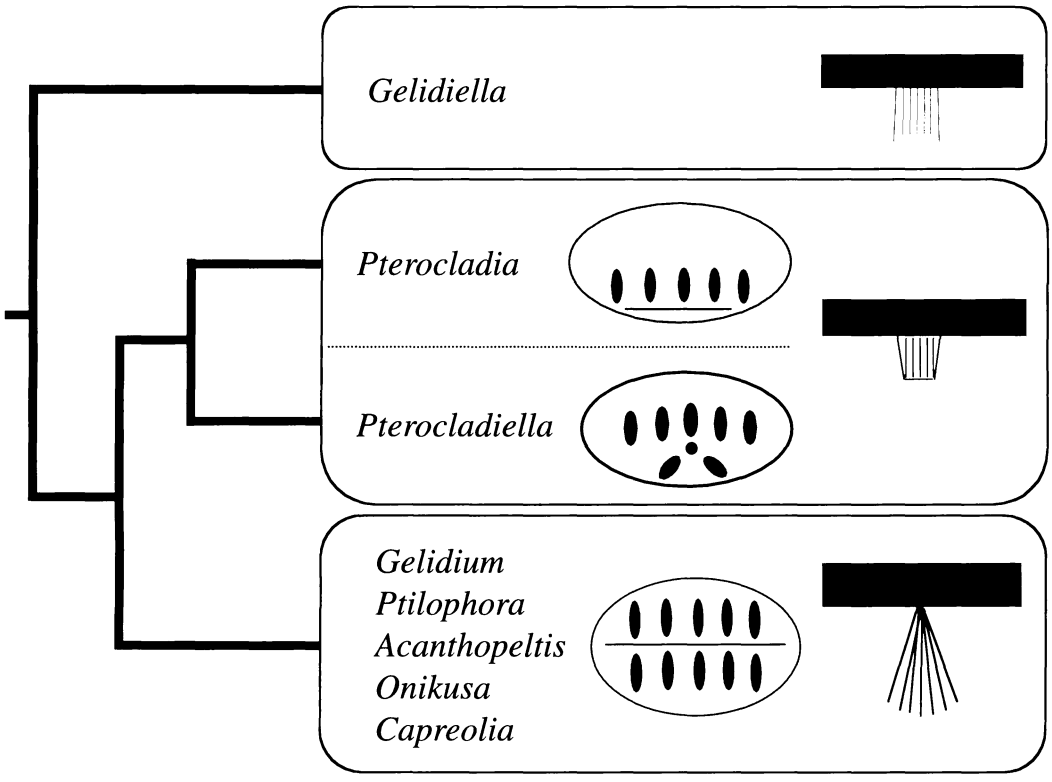


Fig. 3. Correlation of morphological data and SSU tree topology. The types of secondary rhizoidal attachments and developing carposporophyte were used as morphological data.

three major clades resolved in the molecular study. This means that the types reflect phylogenetic relationships of gelidialean algae. Although the genera *Pterocladia*(2a) and *Pterocladiella* (2b) have been shown to share the same type of secondary rhizoidal attachments, these two genera possess different development of the female reproductive system and carposporophytes (Bailey and Freshwater, 1997). This indicates that the reproductive system has evolved faster than the morphology of secondary rhizoidal attachments.

Once the usefulness of secondary rhizoidal attachments as a taxonomic criterion is established, it can be used as an aid to sort out taxonomic problems that are seen in several genera such as *Gelidiella*, *Pterocladia/Pterocladiella* and *Gelidium*, even when only small amounts of material or sterile individuals are available. For example, *Gelidiella calcicola* Maggs et Guiry is known to possess peg-type attachments (Maggs and Guiry, 1987), and this suggests that the species belongs to either *Pterocladia* or *Pterocladiella* rather than to *Gelidiella* or *Gelidium* (Norris, 1992). Such suggestion should prompt further taxonomic researches as well as molecular studies on the species.

Some taxonomic problems remains in the phylogenetic analysis of the Gelidiales. *Gelidium pusillum* has been separated into three clades in the molecular analyses (Freshwater *et al.*, 1995). In this study, a Japanese population of *G. pusillum* was included in the *G. coulteri* clade that contains the Pacific/Caribbean populations of *G. pusillum*, and this clade was separated from European or Eastern Atlantic populations of *G. pusillum*. It is obvious that sequencing of specimens from the type locality (Sidmouth, Deveon, England) of *G. pusillum*, and taxonomic revision of the species is needed. The genus *Onikusa* offers another problem. The genus was erected on the basis of *G. pristoides* from South Africa as the type species (Akatsuka, 1986) and includes *O. japonica* (Akatsuka, 1986) from Japan and Taiwan and *O. foliacea* (Okamura) R. E. Norris (1992) from

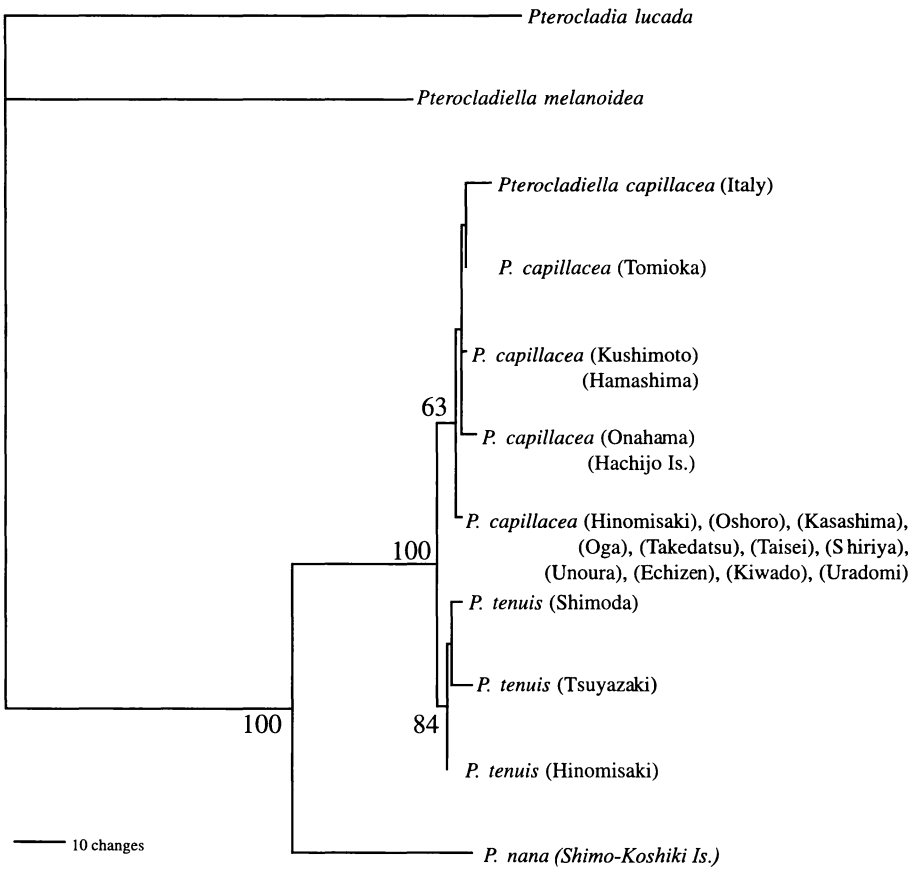


Fig. 4. Phylogenetic tree inferred from rbcL sequences with the maximum parsimony (MP) method. The numbers at each node indicate bootstrap values (1000 replications) greater than 60%.

Japan. According to Akatsuka (1986), the aggregation of surface cells in tetrads and the presence of abundant proliferations are the main features that distinguish *Onikusa* from *Gelidium*. Rodrĭguez and Santelices (1988) and Santelices (1990), however, pointed out that such features are not robust to separate these two genera. In the previous molecular analysis (Freshwater *et al.*, 1995), *O. pristoides* was included in the *Suhria* clade and the revival name *S. pristoides* (Turner) J. Agardh was suggested as the correct placement for this species. I have demonstrated that *O. japonica* is not closely related to *O. pristoides*. *Onikusa pristoides* is closely related to *Suhria* as suggested by Freshwater *et al.* (1995), while *O. japonica* has close affinity with the *Acanthopeltis* clade. It seems appropriate to treat O. japonica as another genus. However, more information on morphology of *O. japonica* is needed prior to the formal taxonomic action. As Freshwater *et al.* (1995) reported, the *Capreolia* clade and *Suhria* clade contain other *Gelidium* species. This result requires more morphological data for suitable taxonomic treatment of these species.

Species of *Pterocladiella* in Japan

The phylogenetic tree of the species of *Pterocladiella* in Japan is shown in Figure 4. Three species, *P. nana*, *P. tenuis* (84% bootstrap value) and *P. capillacea* (63% bootstrap value), were shown to be monophyletic clades, respectively. The sequence divergence values between/within three species are as follows: 4.5-4.8% (*P. nana* and *P. tenuis*); 4.6-5.0% (*P. nana* and *P. capillacea*); 0.5-1.1% (*P. tenuis* and *P. capillacea*); 0.0-0.4% (within *P. tenuis*); and 0.0-0.5% (within *P. capillacea*).

Morphological data was shown in Fig.5. *Pterocladiella nana* (Fig. 6,7) is reddish-brown in

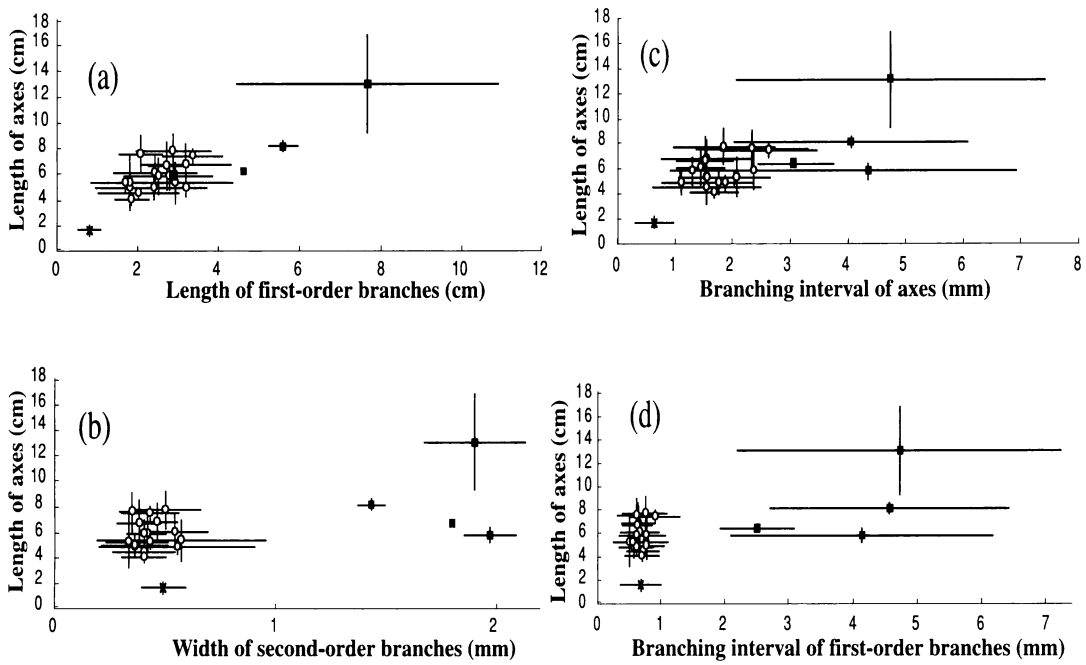


Fig. 5. The means and standard deviations of dimensions in each population are plotted in the following combinations: (a) length of axes vs. maximum length of first-order branches; (b) length of axes vs. maximum width of second-order branches ; (c) length of axes vs. branching intervals of axes; (d) length of axes vs. branching intervals of first-order branches. *: *Pterocladiella nana* ; ■ : *Pterocladiella tenuis* ; ○ : *Pterocladiella capillacea*

color and 1.0-2.4 cm high. Up to three orders of branches are produced. First-order branches are borne regularly pinnately at intervals of 0.1-1.6 mm and reach 0.4-1.5 cm long. These branches bear irregularly pinnately arranged second-order branches 0.2-0.6 mm wide at intervals of 0.1-1.4 mm.

Pterocladiella tenuis (Fig. 8,9) is dark red in color and 5.2-18.0 cm high. Up to four orders of branches are formed. First-order branches are borne regularly pinnately at intervals of 1.0-16.0 mm and reach 2.6-14.2 cm long. These branches bear regularly (sometimes irregularly) pinnately arranged second-order branches 1.4-2.3 mm wide at intervals of 1.0-11.0 mm.

Pterocladiella capillacea (Fig. 10,11) is dark red in color and 3.7-9.8 cm high. Up to five orders of branches are produced. First-order branches are borne regularly pinnately at intervals of 0.5-4.4 mm and reach 0.9-4.6 cm long. These branches bear regularly pinnately arranged second-order branches 0.2-1.0 mm wide at intervals of 0.1-1.6 mm.

For the analysis of morphological variations within and between the populations, the mean and standard deviations of each population are plotted in the following combinations in order to clearly highlight morphological distinctiveness between groups (Fig. 5): a) length of axes vs. maximum length of first-order branches; b) length of axes vs. maximum width of second-order branches; c) length of axes vs. branching intervals of axes; d) length of axes vs. branching intervals of first-order branches. *Pterocladiella nana* is distinguished from *P. tenuis* by all five dimensions measured (Fig. 5-a, 5-b, 5-c, 5-d), and from *P. capillacea* by the lengths of axes and first-order branches (Fig. 5-a) and branching intervals of axes (Fig. 5-c). *Pterocladiella tenuis* differs from *P. capillacea* in the width of second-order branches (Fig. 5-b) and branching intervals of first-order branches (Fig. 5-d).

In my culture study, morphologically distincti characters of these three groups, such as short axes in *P. nana* (Fig. 12), wide branches and long branching intervals in *P. tenuis* (Fig. 13) and slender branches and short interval branchlets in *P. capillacea* (Fig. 14) were also main-

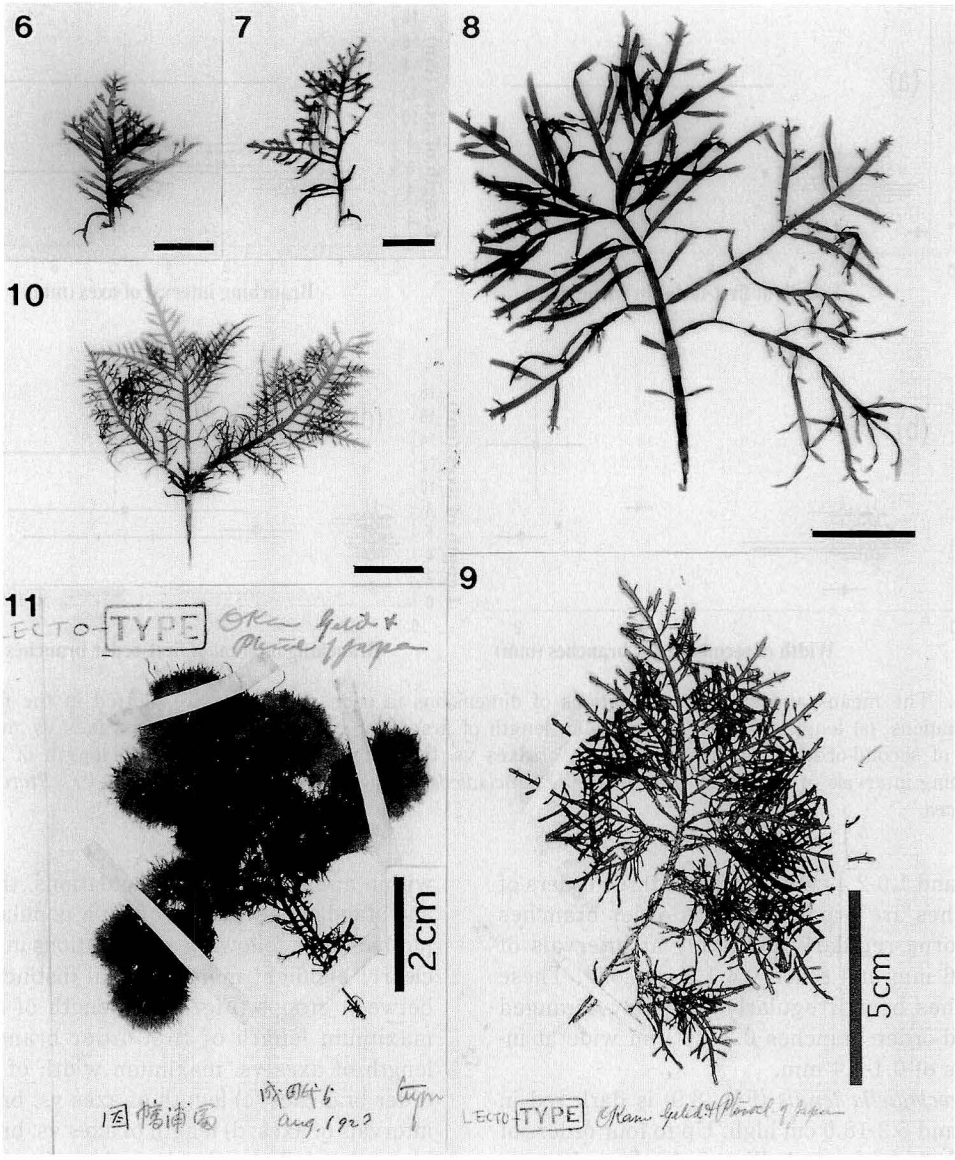


Fig. 6—11. Formalin/seawater-preserved specimens and type material of the *Pterocladia* species described by Okamura.

Fig. 6. *Pterocladiella nana*, collected at the southernmost locality Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture. Scale bar = 5mm.

Fig. 7. Lectotype specimen of *Pterocladia nana* collected at Yura-jima, Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture (19.vii.1919, Okamura Herb. in SAP). Scale bar = 5mm.

Fig. 8. *Pterocladiella tenuis*, collected at the Pacific coast of central Japan (Shimoda, Shizuoka Prefecture). Scale bar = 2cm.

Fig. 9. Lectotype specimen of *Pterocladia tenuis* collected at Enoshima, Kanagawa Prefecture (iii.1897, Okamura Herb. in SAP).

Fig. 10. *Pterocladiella capillacea*, collected at the northernmost locality, Oshoro, Hokkaido. Scale bar = 1cm.

Fig. 11. Lectotype specimen of *Pterocladia densa* collected at Uradomi, Tottori Prefecture (viii.1923, Okamura Herb. in SAP).

tained under the same culture conditions, i.e. 15 ℃ and 16:8 h LD cycle.

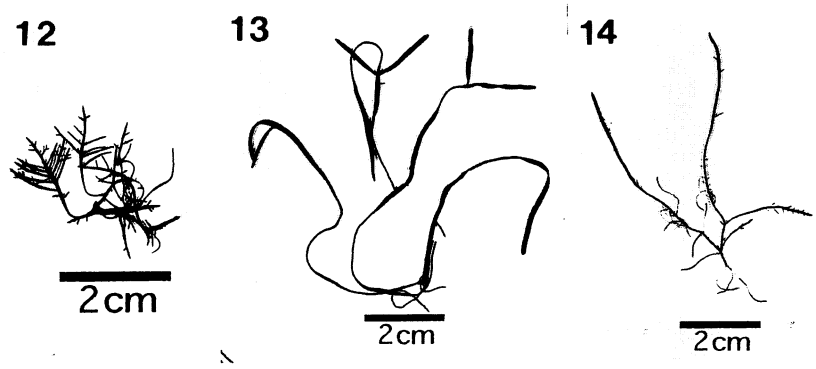


Fig. 12—14. Five-month-old cultured plants grown at 15_C and 16:8 h LD cycle.
Fig. 12. *Pterocladia nana* (Koshiki Island, Kagoshima Prefecture) showing short axes and branches with short intervals.
Fig. 13. *Pterocladia tenuis* (Shimoda, Shizuoka Prefecture) showing broad branches with long branching intervals.
Fig. 14. *Pterocladiella capillacea* (Tomioka, Kumamoto Prefecture) showing slender branches that issue ultimate branchlets with short intervals.

Analysis of *rbc*L sequences resolved that three species belong to separate monophyletic clades. These three species can also be distinguished from each other on morphological grounds. *Pterocladiella nana* is characterized by small thalli; *P. tenuis* is distinguished from the other two groups by having wide branches of the second-order with longer branching intervals; and *P. capillacea* is separated from *P. nana* by larger thalli and from *P. tenuis* by possessing more slender second-order branches with shorter branching intervals. Although I have not been able to conduct crossing experiments yet, the congruence of molecular and morphological data strongly suggests that these three species represent different species.

In order to elucidate precise relationships between these three species and the three species of *Pterocladia* described by Okamura (1932, 1934), I reexamined the type materials of *Pterocladia nana*, *P. tenuis* and *P. densa*. Based on morphological observations, type materials of *P. nana* (Fig. 7) and *P. tenuis* (Fig. 9) were congruent with our material of *P. nana* (Fig. 6) and *P. tenuis* (Fig. 8), respectively.

The axis of the lectotype specimen of *P. densa* (Fig. 11) is 6.2 cm long and produces first-order branches up to 3.7 cm long at intervals of 0.8-2.6 mm that form second-order branches up to 0.4 mm wide at intervals of 0.1-0.8 mm. These dimensions agree with those determined in this study for the *P. capillacea*. The type illustration of *P. capillacea* (Gmelin, 1768, pl. 15, fig. 1, as *Fucus capillaceus* S. Gmelin) shows similar characteristics to *P. densa*, i.e. gradually decreasing widths from axes to second-order branches, and having slender branchlets that are formed at short intervals. In our molecular analysis, the sequence of the population of *P. capillacea* from Italy (type locality, Mediterranean) was included in the *P. capillacea* clade. The morphological similarity and molecular closeness suggest that *P. densa* should be considered to be a synonym of *Pterocladiella capillacea*

New species of *Gelidium*

Shimada *et al.*, (2000b) described two new species of *Gelidium*, *G. tenuifolium* and *G. koshikianum* from Japan. *Gelidium tenuifolium* (Fig. 14) with large-sized thalli (up to 30 cm tall) is distinguished from species with such thalli by the production of wide, flattened and thin branches (up to 2 mm wide and 60-80 μm thick), the presence of an apical depression, and simple determinate branches. *Gelidium koshikianum* (Fig. 15) with middle-sized thalli (5-8 cm tall) is distinguished from species with such thalli by having wide axes (up to 2.5 mm wide) and short (2.0-3.2 mm), unbranched, second- and third-order branches issuing at short intervals (0.8-1.4 mm). In phylogenetic analyses of *rbc*L sequences (Fig. 2), four *Gelidium* species that

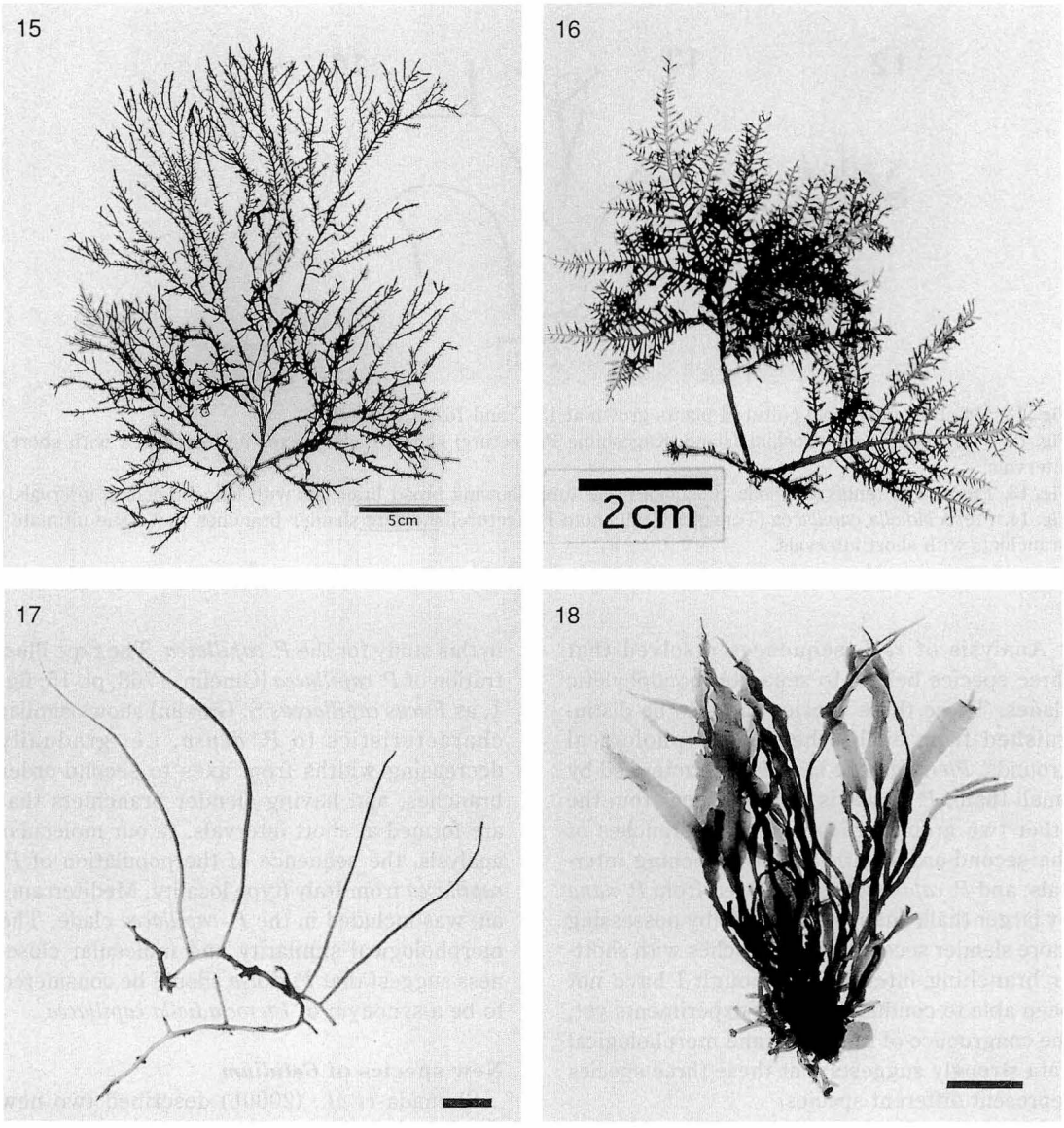


Fig. 15. Type specimen of *Gelidium tenuifolium*. Holotype specimen collected at Shirahama, Shimoda, Shizuoka Prefecture (28.iii.1998; SAP #070868).
Fig. 16. Type specimen of *Gelidium koshikianum*. Holotype collected at Nagahama, Shimo-Koshiki Island, Koshiki Islands, Kagishima Prefecture (2.viii.1997; SAP #070874).
Fig. 17. *Gelidiella pannosa*. Formalin/seawater-preserved specimen collected at Nosoko, Ishigaki Island, Okinawa Prefecture (3.iii.1999; SAP #071771). Scale bar = 1mm.
Fig. 18. *Gelidiella ligulata*. Formalin/seawater-preserved specimen of collected at Izu-misaki, Miyake Island, Tokyo. (13.vii.1998; SAP #063883). Scale bar = 1cm.

are chiefly distributed in Japan (*G. linoides*, *G. tenuifolium*, *G. elegans* and *G. pacificum*) were clustered together with 100% bootstrap value (Japanese *Gelidium*-complex clade). *Gelidium linoides* Kützing was in the position of a sister group to *G. tenuifolium* with 94% bootstrap value. There were four substitutions (0.3% divergence) between *G. linoides* and *G. tenuifolium* sequences. *Gelidium koshikianum* and *G. allanii* Chapman were clustered together with 99% bootstrap value and they formed a sister group to the Japanese *Gelidium*-complex clade

with 93% bootstrap value. There were six substitutions (0.4% divergence) between *G. koshikianum* and *G. allanii* sequences.

New records of Gelidiales from Japan

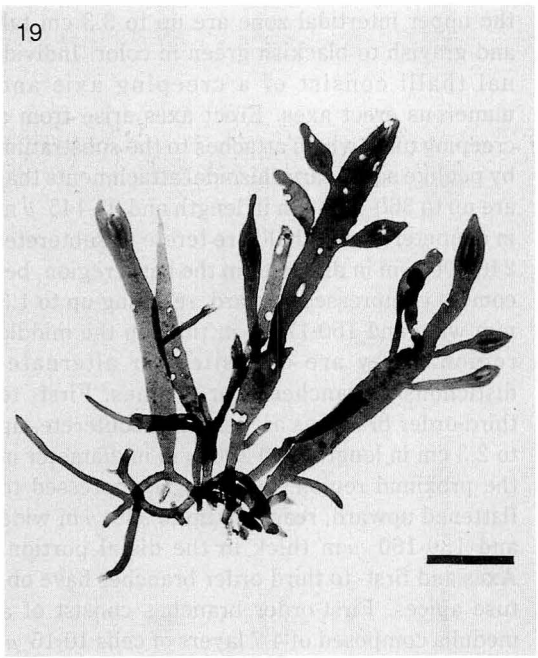
Shimada and Masuda (1999, 2000) newly reported four species of Gelidiales from Japan. *Gelidiella pannosa* grows gregariously on bedrock in the upper intertidal zone and are dark red. Individual thalliconsist of a creeping axis and numerous erect axes (Fig. 16), all of which are terete to subterete. Creeping axes attach to the substratum by unicellular independent attachments that are up to 200 μm long by 10-25 μm wide. The majority of branches grow into erect axes and some become creeping branches that grow like the parental axis. Erect axes are up to 8 mm high and 65-115 μm wide. They are usually simple, but are sometimes irregularly branched. Thalli consist of a medulla composed of 3-5 layers of cells 6-12 μm in diameter and a cortex composed of 1 or 2 layers of slightly smaller cells 4-10 μm in diameter. Rhizines (slender, thick-walled, internal, hypha-like filaments) are absent throughout the thalli.

Gelidiella ligulata tufts on bedrock in the middle intertidal zone of sheltered shores or in tidal pools are up to 4.5 cm tall (Fig. 17) and are dark red to purplish red in color. Individual thalli consist of a creeping axis and erect blades. The creeping axis attaches to the substratum by unicellular independent attachments that are 50-240 μm in length and 10 μm in diameter. Erect blades arise from the creeping axis. They are terete (250-350 μm in diameter) at the proximal portion, gradually expanding and become flattened. The blades are fan-shaped when young, but become lanceolate with age (1-3 mm wide, 100-270 μm thick). They are usually simple, but are sometimes irregularly to dichotomously branched. Blade margins are undulate and sometimes ruffled. Subterete to lanceolate proliferations issue from both sides of blades pinnately, injured (perhaps grazed) ends of blades and blade surfaces. Both creeping axes and erect blades consist of a medulla composed of 10-18 layers of cells 6-40 μm in diameter and a cortex composed of 2-3 layers of smaller cells 3-5 μm in diameter. Rhizines are absent throughout creeping axes and erect blades.

Pterocladiella caerulescens tufts on bedrock in the upper intertidal zone are up to 3.3 cm tall and grayish to blackish green in color. Individual thalli consist of a creeping axis and numerous erect axes. Erect axes arise from a creeping axis, which attaches to the substratum by peglike secondary rhizoidal attachments that are up to 360-480 μm in length and 95-145 μm in diameter. Erect thalli are terete to subterete, 240-300 μm in diameter in the basal region, becoming compressed upward, reaching up to 1.3 mm wide and 150-175 μm thick in the middle region. They are oppositely or alternate-distichously branched 2 or 3 times. First- to third-order branches are terete to subterete, up to 2.3 cm in length, 110-200 μm in diameter in the proximal region, becoming compressed to flattened upward, reaching up to 940 μm wide and 130-160 μm thick in the distal portion. Axes and first- to third-order branches have obtuse apices. First-order branches consist of a medulla composed of 4-7 layers of cells 10-15 μm in diameter and a cortex composed of 3 or 4 layers of slightly smaller cells 4-8 μm in diameter in the middle region. Rhizines of first-order branches are abundant in the central medulla.

Pterocladiella caloglossoides grows gregariously on bedrock in the upper intertidal zone and is purplish red. Individual thalli consist of a creeping axis and erect axes (Fig. 19). Creeping axes are subterete to compressed, 110-160 μm in diameter, attach to the substratum by peg-like attachments that are 135-455 μm long by 65-90 μm wide. Erect axes are up to 3 mm tall, terete to subterete, 60-240 μm in diameter in the basal region, becoming flattened upward, reaching up to 700 μm wide and 65-115 μm thick in the middle region. They are usually simple, but are sometimes irregularly branched. Erect axes consist of a medulla composed of a single layer of cells 8-16 μm in diameter and a cortex composed of 2-4 layers of slightly smaller cells 4-12 μm in diameter (Fig. 20). Rhizines of first-order branches are rare in the central medulla.

The SSU rDNA phylogenetic tree obtained from MP analysis is shown in Figure 1. *Gelidiella pannosa* and *Gelidiella ligulata* were included in the *Gelidiella* clade (98% bootstrap value) with *G. acerosa*. *Pterocladiella caerulescens* and *P. caloglossoides* were included in the *Pterocladiella* clade (100% bootstrap value), and



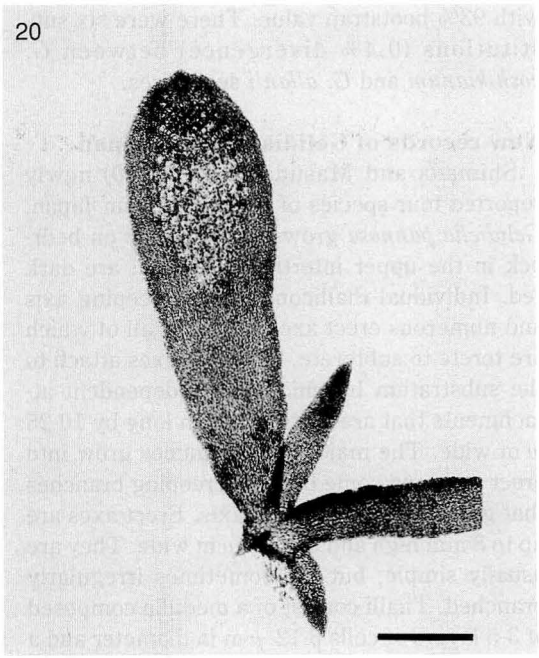


Fig. 19. *Pterocladiella caerulescens*. Formalin/seawater-preserved specimen of collected at Sonai, Yonaguni Island, Okinawa Prefecture (1.iii.1999; SAP #071774). Scale bar = 2mm.
Fig. 20. *Pterocladiella caloglossoides*. Formalin/seawater-preserved specimen of collected at Oohama, Ishigaki Island, Okinawa Prefecture (4.iii.1999; SAP #071778). Scale bar = 1mm.

they were clustered with Japanese three species of *Pterocladiella* with 96% bootstrap value.

Acknowledgments

I would like to thank Dr. M. Miyata and other staff of the Natural History Museum and Institute, Chiba, and Dr. M. Notoya of Tokyo University of Fisheries for their invitation to present this paper at the 14th Natural History Symposium on "Present and Future on Molecular Biology of Useful Seaweeds".

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(Manuscript received 15 December 2003 ; accepted 24 March 2003.)

紅藻テングサ目における分子系統学的解析

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紅藻テングサ目は世界中に分布し11属約150種が記載されている。しかし，このグループは外部形態の変異が激しく独立性の不明確な分類群を多く含み，属や種の分類形質の妥当性も統一された見解は得られていなかった。この状態を打破するため，日本産紅藻テングサ目の野外藻体及び培養藻体をもとに，形態学的・分子系統学的な手法を用いて系統分類学的研究を行い，テングサ類の種間・属間の系統関係や個体群レベルの遺伝的多様性を解明してきた。

本研究では，日本全国61箇所をまわりテングサ類を採集し，枝先を切り単藻培養化とした。DNA の抽出は単藻培養株から行った。テングサ目全体の系統推定を行うため，核コードの SSUrRNA 遺伝子及び葉緑体コードの rbcL 遺伝子の塩基配列を決定し分子系統学的解析を行った。これらの系統樹はテングサ目が３つの大きなクレード，テングサ目で最初に分岐する Gelidiella クレード，Pterocladia / Pterocladiella クレード及び large Gelidium comlex クレードからなることを示した。この３つのクレードは嚢果の特徴と一致した。しかし，実際問題として野外藻体で嚢果をつけている藻体は極わずかで，属の同定に使えない。テングサ属とオバクサ属は寒天原藻としての需要も多く，また種類数も多いことから分類学的にも重要な２属であるにも関わらず，これまで両属を生殖器官以外で区別するのは非常に困難であった。そこで，嚢果に頼らず他の形態的特徴で両属の区別が出来ないか探ることにした。通常，野外で採集した藻体は200ml のプラスチック容器に入れて持ち帰り研究室で単藻培養化するのだが，持ち帰る間に匍匐糸が伸び，二次的仮根が形成されているのを観察することができた。しかも，この二次的仮根には３つのタイプがあって容易に区別することが出来た。そこで，この二次的仮根のタイプを８属24種で調べ上げ，分子系統樹にのせてみた。すると Gelidiella クレードの種類は単独仮根，Pterocladia / Pterocladiella クレードの種類はくぎ状仮根，large Gelidium comlex クレードの種類はふさ状仮根を持ち，３つの大きなクレードと二次的仮根のタイプがぴったり一致していた。

邦産オバクサ属として，*Pterocladia nana*，*P. tenuis* および *P. densa* が岡村金太郎によって記載されたが，その後の外国の研究者によりこれら３種とも *Pterocladiella capillacea* の synonym であると報告されていた。しかし，この外国の研究者による報告は扱った標本，形質とも原記載を無視したもので，標本数も少なすぎることから再検討が必要であるといわれてきた。そこで本研究では，日本各地の藻体を元にこのオバクサ complex を分類学的見直すため，分子系統学的・形態学的な解析を進めた。日本各地のオバクサ21個体群から rbcL 遺伝子の塩基配列を決めて系統樹を構築したところ，オバクサ complex は３つのクレードに分かれた。次に，分子系統に用いた藻体と同一個体群の藻体から成熟個体をできるだけ選び合計109個体を元に形態の比較を行ったところ，藻体の長さ，第一枝の長さ，第二枝の太さ，軸の分枝間隔，第一枝の分枝間隔などの形態形質で３つのグループを認識することができた。さらに，分子や形態データを取った藻体の単藻培養株を同一条件で培養させると，上述の形態的特徴が固定されていることもわかった。つまり，日本産オバクサ complex は遺伝的に３つのグループにわかれ，そのグループは形態的にも特徴を持っており，しかも，その特徴は同一条件の培養実験で固定されていたのだ。このことからこれら３グループはそれぞれ独立した種であると結論付けた。

テングサ目の２つの新種と４つの日本新産を報告した。新種 *Gelidium tenuifolium*（ウスバテングサ）は大型の藻体で，枝は幅広く扁平で薄く頂端が窪み，分子系統解析ではキヌクサと近縁であることが推察された。もう１つの新種 *Gelidium koshikianum*（サツマテングサ）は中型の藻体で，軸幅が広く狭い間隔で短く分枝しない第二位枝及び第三位枝が発出し，分子系統解析ではニュージーランド特産の *Gelidium allanii* と近縁であることが推察された。さらに，沖縄や三宅島から日本新産 *Gelidiella pannosa*（イトシマテングサ），

Gelidiella ligulata（ササバノシマテングサ），*Pterocladiella caerulescens*（アオオバグサ），*Pterocladiella caloglossoides*（ヒメオバクサ）を報告した。