A systematic study of the order Gelidiales (Rhodophyta) from Japan

Satoshi Shimada

2000

CONTENTS

ABSTRACT

Species of the red algal order Gelidiales that grow in Japanese waters were systematically analyzed using morphological, molecular phylogenetical and physiological methods on the basis of field and cultured materials.

Phylogenetic relationships of the species were inferred on the basis of trees using nuclear-encoded SSU rDNA (small subunit of ribosomal DNA), ITS1 (internal transcribed spacer 1) 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 type of secondary rhizoidal attachments, the unicellular independent type, peg type or brush type, is completely consistent with the respective three major clades of the Gelidiales, which suggests that this morphological character reflects the phylogeny of this order.

Acanthopeltis and *Yatabella* had been suggested to be derived from separate lineages by previous researchers who emphasized the difference of growth pattern. However, close relationships were recognized in the SSU rDNA, ITS1 and *rbc*L analyses. Cultured and field-collected materials of these algae show that *Acanthopeltis* and *Yatabella* possess a fundamentally similar growth pattern. The molecular data and morphological similarities indicate that *Acanthopeltis* and *Yatabella* are congeneric. The new combination, *Acanthopeltis*

hirsuta (Okamura) Shimada, Horiguchi et Masuda, comb. nov., was proposed.

Three monophyletic groups were recognized in the *rbc*L tree of 21 local populations of *Pterocladia/Pterocladiella*-complex in Japan. These three groups can be clearly recognized by the discontinuity of morphological measures including the length of the axes and first-order branches, width of the second-order branches and branching intervals of axes and first-order branches. The morphological distinctiveness of these three groups was also maintained under the same culture conditions. The molecular and morphological data indicate that these three groups represent distinct species, *Pterocladia nana* Okamura, *Pterocladia tenuis* Okamura and *Pterocladia densa* Okamura, respectively. *Pterocladia densa* is a synonym of *Pterocladiella capillacea* (Gmelin) Santelices et Hommersand. *Pterocladia nana* and *P. tenuis* are transferred to *Pterocladiella* on account of the production of *Pterocladiella*-type cystocarps. The following new combinations were therefore proposed: *Pterocladiella nana* (Okamura) Shimada, Horiguchi et Masuda and *Pterocladiella tenuis* (Okamura) Shimada, Horiguchi et Masuda.

Although *Gelidium subfastigiatum* Okamura and *Gelidium elegans* Kützing were reported to be conspecific, analysis of seasonal variations of their subterminally swollen branches resolved that characteristics of subterminally swollen branches can be used for distinction between these two species at least from winter to spring. The two species also had distinctions of ITS1 sequences and tolerance to lower

temperatures. These morphological, molecular and physiological data suggest that *G. subfastigiatum* and *G. elegans* are different species.

Two new species, *Gelidium tenuifolium* Shimada, Horiguchi et Masuda sp. nov. and *Gelidium koshikianum* Shimada, Horiguchi et Masuda sp. nov. were described from Japan. *Gelidium tenuifolium* with large-sized thalli is distinguished from species with such thalli by the production of wide, flattened and thin branches and the presence of an apical depression. *Gelidium koshikianum* with middle-sized thalli is distinguished from species with such thalli by having wide axes and short, unbranched, second- and third-order branches issuing at short intervals. In the molecular phylogenetic study using *rbc*L sequences, *Gelidium linoides* Kützing came to the position of the sister group to *G. tenuifolium* with 99 % bootstrap value, and *Gelidium koshikianum* and *G. allanii* Chapman were clustered together with 100 % bootstrap value.

Four species, *Gelidiella ligulata* Dawson, *Gelidiella pannosa* (Feldmann) Feldmann et Hamel, *Pterocladiella caerulescens* (Kützing) Santelices et Hommersand and *Pterocladiella caloglossoides* (Howe) Santelices, were reported from Japan for the first time, and their diagnostic features were described. Monoecious plants of *P. caerulescens* produce spermatangial sori on: i) fertile cystocarpic branchlets; ii) special spermatangial branchlets on a cystocarpic axis; and iii) branchlets of a special spermatangial axis. The latter two were newly found in this species.

ACKNOWLEDGMENTS

I wish to express my sincere thanks to Professor M. Masuda, Hokkaido University, who interested me in the order Gelidiales, directed the research, gave me many invaluable suggestions and carefully criticized the manuscript. I wish to thank sincerely Dr. T. Horiguchi, Hokkaido University, who kindly helped me with molecular analyses, gave me suggestions and carefully criticized the manuscript. I am grateful to Professor T. Ichimura and Professor H. Katakura, Hokkaido University for their critical reading of the manuscript. I am also grateful to Professor Emeritus T. Yoshida, Hokkaido University, Professor Emeritus S. Enomoto, Kobe University and Dr. S. Kawaguchi, Kyushu University, for their helpful advice and providing materials; Dr. K. Kogame, Hokkaido University and Dr. T. Abe, Hokkaido University, for their technical assistance and helpful discussion on analysis problems; Dr. J. Huisman of Murdoch University and M. Iwataki of Tokyo University, Y. Yamagishi, Hokkaido University, M. Tani, Hokkaido University, S. Uwai, Hokkaido University, who generously collected some of materials; Professor H. Ohba, University of Tokyo for the loan of the holotype specimen of *Acanthopeltis japonica*.

GENERAL INTRODUCTION

The red algal order Gelidiales currently includes 11 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, *Suhria* J. Agardh and *Yatabella* Okamura) and approximately 140 species that are distributed worldwide (Santelices 1990; Bailey & Freshwater 1997). In Japan, seven genera, i.e. *Acanthopeltis*, *Gelidiella*, *Gelidium*, *Onikusa*, *Pterocladiella*, *Ptilophora* and *Yatabella* 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 21 species of Gelidiales are recognized in Japanese waters (Yoshida 1998).

Distinction between *Gelidium* and *Pterocladia* has always been notoriously difficult (Dixon and Irvine 1977; Santelices 1990). Several taxonomists have sought characters to separate *Gelidium* from *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 1988). However, Rodríguez and Santelices (1987, 1988) claimed that these characters have only limited taxonomic value, and only reliable feature for distinguishing these genera is in the

female reproductive structure (Santelices & Stewart 1985; Rodríguiz & Santelices 1988). Female plants of *Gelidium* and *Pterocladia* are only occasionally found, so that reliance on other vegetative features becomes a necessity (Santelices & Stewart 1985). Recently, Santelices & Hommersand (1997) separated a new genus *Pterocladiella* from the genus *Pterocladia* based on the development of carpogonia and cystocarps. Then, Santelices (1997a, 1998) transferred four species from *Pterocladia* to *Pterocladiella*. In recent taxonomic reviews of a *Pterocladia/Pterocladiella*-complex (Santelices 1998, 1999), two species are remained in *Pterocladia* (only Australian species) and eight species (or ten species including two uncertain species) are included in *Pterocladiella*. Thus, we now need to seek vegetative characters to separate *Gelidium* from *Pterocladia/Pterocladiella*-complex, instead of *Gelidium* from *Pterocladia*.

Monotypic genera *Acanthopeltis* Okamura (sympodial growth) and *Yatabella* Okamura (monopodial growth) had been suggested to be derived from separate lineages by previous researchers who emphasized the difference of growth pattern. However, Okamura who described these two genera commented that *Yatabella* is similar to *Acanthopeltis* in its vegetative and reproductive features. The phylogenetic position of these genera, therefore, needs to be confirmed on the basis of molecular phylogenetic data.

A number of taxonomic studies of Japanese gelidialean species have been reported (Yatabe 1892; Okamura 1900, 1901, 1913, 1932, 1934, 1936; Segi 1955, 1957; Tanaka 1965;

Akatsuka 1982, 1986; Akatsuka & Masaki 1983). Although Yoshida (1998) recently reported 21 species of Japanese Gelidiales, he included uncertain species, such as *Gelidium decumbensum* Okamura and *Gelidium subfastigiatum* Okamura. Furthermore, small species belonging to *Gelidiella*, *Gelidium* and *Pterocladiella* in southern Japan have not been studied. Further taxonomic researches are needed to recognize Japanese gelidialean species.

Recent molecular phylogenetic studies of the Gelidiales using nuclear-encoded SSU rDNA (small subunit of ribosomal DNA), ITS1 (internal transcribed spacer 1) and plastid-encoded *rbc*L (large subunit of the ribulose-1,5-bisphosphate carboxylase/oxgenase) sequences resolved phylogenetic positions of several gelidialean species (Freshwater & Rueness 1994; Freshwater *et al.* 1995; Bailey & Freshwater 1997; Patwary *et al.* 1998). However, these studies included only a few Japanese species.

The present paper contains several analyses to resolve the above-mentioned problems. It is composed of 6 chapters. In Chapter 1, phylogenetic relationships of Japanese and other gelidialean species will be inferred on the basis of trees using the SSU rDNA, *rbc*L and ITS1 sequences. Secondary rhizoidal attachments will be evaluated whether this vegetative character reflects phylogeny of the Gelidiales or not.

In Chapter 2, the divergence of the SSU rDNA, *rbc*L and ITS1 sequences will be compared between monotypic genera *Acanthopeltis* and *Yatabella*, and growth patterns of these

genera will be reexamined on the basis of type materials, laboratory-cultured plants and several herbarium specimens.

The purpose of Chapter 3 is to elucidate the taxonomic status of three *Pterocladia* species described by K. Okamura. The sequences of *rbc*L gene for 21 local populations of a *Pterocladia/Pterocladiella*-complex will be determined, and morphological variation of these populations will be examined in order to assess the congruence of molecular and morphological data. Furthermore, the type specimens of the three species described by Okamura will be reexamined.

In Chapter 4, an uncertain species, *Gelidium subfastigiatum* Okamura, will be treated. Although this species was described by Okamura (1934), Akatsuka (1982) reduced it to the synonymy with *Gelidium elegans* Kützing. However, Yoshida (1998) did not adopt this Akatsuka's opinion in his recent review. The taxonomic status of this uncertain species will be confirmed by molecular, morphological and physiological methods.

In Chapter 5, two new species of *Gelidium*, *G. tenuifolium* sp. nov. and *G. koshikianum* sp. nov. will be described. These species have been collected along the warm coasts of central to western Japan. Then, phylogenetic positions of these two new species are discussed using *rbc*L sequences.

In Chapter 6, four small species, *Gelidiella ligulata* Dawson, *Gelidiella pannosa* (Feldmann) Feldmann et Hamel, *Pterocladiella caerulescens* (Kützing) Santelices et Hommersand and *Pterocladiella caloglossoides* (Howe) Santelices will be reported in Japan for the first time.

They have been collected at several localities of Okinawa Islands and Hachijo Island. Their phylogenetic positions are discussed using SSU rDNA sequences and secondary rhizoidal attachments.

Chapter 1

Chapter 1. Molecular phylogeny of Gelidiales

INTRODUCTION

Phylogenetic relationships among a number of gelidialean species and populations have been determinated based on plastid-encoded *rbc*L (large subunit of the ribulose-1,5-bisphosphate carboxylase/oxgenase) sequence analyses (Freshwater & Rueness 1994; Freshwater *et al.* 1995) and nuclear-encoded SSU rDNA (small subunit of ribosomal DNA) and *rbc*L sequence analyses (Bailey and Freshwater 1997). Recently, Patwary *et al.* (1998) presented phylogenetic relationships from nuclear-encoded SSU rDNA gene and ITS1 (internal transcribed spacer 1) sequences. However, only a few Japanese species have been included in these analyses. In this study, nucleotide sequences of the nuclear-encoded SSU rDNA gene, ITS1 sequence and plastid encoded *rbc*L gene of 11 species of Japanese gelidialean species which cover all the genera of the Gelidiales distributed in Japan will be reported, and phylogenetic relationships between gelidialean species in the world will be discussed.

Recently, new characters have been proposed as distinguishing ones between *Gelidium* and *Pterocladia/Pterocladiella*-complex, i.e. type of secondary rhizoidal attachments (De Gregorio & Perrone 1994; Perrone 1994) and medullary structures (Rodríguez & Santelices 1996). In this study, I will pay attention to the secondary rhizoidal attachments. Secondary rhizoidal attachments of 17

species belonging to 9 genera will be reported and their taxonomic value will be evaluated using molecular data.

MATERIALS AND METHODS

Sampling and DNA extraction

Total DNAs were extracted from 11 unialgally cultured strains (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 & Patwary 1991) at 15°C or 20°C and 16:8 h LD cycle with the photon flux of 15-25 $\mu Em^{-2}s^{-1}$.

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 μl 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 7 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 (Freshwater *et al.* 1994; Uwai unpublished, F8: 5'-GGTGTAATTCCATACGCTAAAATG-3', F605: 5'-CCATTTCATGCGTTGGAAAGAAAGAT-3', R643: 5'-AATTGAACGATTTACAGCTTCCAT-3'); TW81-RED5.8R for ITS1 (Goff *et al.* 1994). The temperature-cycling protocol consisted of an initial denaturation step of 93°C, 1 min, followed by 35 cycles of 30 sec denaturation period at 94°C, 30 sec primer annealing at 55°C, and 45 sec extension at 72°C, then hold at 4°C. 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 and ITS1 sequences were first aligned with the CLUSTAL W computer program (Thompson *et al.* 1994; Higgins *et*

al. 1996) and then refined by eye. The *rbc*L sequences were aligned manually because no insertion/deletion mutations were detected. Sequences of 50 additional gelidialean species were downloaded from GenBank and included in these alignments (Table 2). *Hildenbrandia rubra* (Sommerfelt) Meneghini and *Chondrus crispus* Stackhouse were used as outgroups for SSU rDNA (Ragan *et al.* 1994) and *rbc*L (Freshwater *et al.* 1994) analyses (Table 2).

The distance matrix method was used to construct phylogenetic trees. I used Kimura's two-parameter method (Kimura 1980) to calculate the distance matrix and neighbor-joining (NJ) method (Saitou & Nei 1987) to construct the trees. These procedures were performed using the CLUSTAL W computer program (Thompson *et al.* 1994; Higgins *et al.* 1996). Bootstrap analyses based on 100 resamplings of the data set (Felsenstein 1985) were 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 (Table 1). The correlation between morphological data, secondary rhizoidal attachments and phylogenetic trees of the SSU rDNA and *rbc*L was analyzed.

RESULTS

SSU rDNA analysis

Twenty two samples (21 species) were used for the SSU rDNA gene (1707 bp, including gaps) analyses. The phylogenetic tree obtained from NJ analysis is shown in Figure 1. The monophyletic clade of *Gelidiella acerosa* (Forsskål) Feldmann et Hamel and *Gelidiella ligulata* Dawson (the *Gelidiella* clade) was supported by 100% bootstrap value and was recognized as the earliest diverging lineage within the Gelidiales. *Pterocladiella* clade was also supported by 100% bootstrap value. *Pterocladia lucida* (Brown et Turner) J. Agardh, *Pterocladiella capillacea* (Gmelin) Santelices et Hommersand and *Pterocladiella melanoidea* (Schouboe ex Bornet) Santelices et Hommersand were also shown to be monophyletic (the *Pterocladia/Pterocladiella* clade), although the bootstrap support was slightly less than that for the other major clades.

The large clade that includes rest of taxa was referred to the large *Gelidium*-complex clade for the sake of convenience. In the large *Gelidium*-complex clade, three monophyletic clades, the *Ptilophora* clade (95% bootstrap value), the *Capreolia* clade comprised of *Capreolia*, *Gelidium divaricatum* Martens and *Gelidium caulacantheum* J. Agardh (90% bootstrap value), and the *Gelidium*-complex clade that includes *Gelidium* (excluding *G. divaricatum* and *G. caulacantheum*), *Onikusa*, *Acanthopeltis* and *Yatabella* (71% bootstrap value), were identified. The *Acanthopeltis* and *Yatabella* SSU rDNA sequences were identical. The

monophyletic clade of *G. vagum*, *G, elegans*, *G. latifolium* and *G. americanum* was supported by 85% bootstrap value.

*rbc*L analysis

Forty one samples (36 species) were used for the *rbc*L gene (1467 bp) analyses. The phylogenetic tree obtained from NJ analysis is shown in Figure 2. Four monophyletic clades, the *Pterocladiella* clade, *Pterocladia* clade, *Gelidiella* clade and large *Gelidium*-complex clade, were evident with high bootstrap values (93-100%), although bootstrap values of their topological positions were less than 50%.

Monophyly of the large *Gelidium*-complex clade was supported by 97% bootstrap value and three clades that were shown in the SSU rDNA tree were also identified in this clade as the *Ptilophora* clade (100% bootstrap value), *Capreolia* clade (98% bootstrap value) and *Gelidium*-complex clade (100% bootstrap value). The *Gelidium*-complex clade was composed of three subclades, although the topological positions were not resolved. The *Gelidium coulteri* clade (*Gelidium coulteri* complex, Freshwater *et al.* 1995) contained *G. pusillum* (Stackhouse) Le Jolis (strains of Japan, California in U.S.A. and Puerto Rico), *G. capense* (Gmelin) Silva and *G. coulteri* Harvey. The *Acanthopeltis* clade contained *Onikusa japonica* (Harvey) Akatsuka, *Gelidium vagum* Okamura, *Acanthopeltis japonica* and *Yatabella hirsuta*. The monophyly of this subclade was supported by 100% bootstrap value and *Gelidium vagum* came to the position of the sister group to an *Acanthopeltis/Yatabella* group,

although the bootstrap value was low (53%). *Onikusa japonica* did not form a monophyletic clade with *Onikusa pristoides* (Turner) Akatsuka that was included in the *'Gelidium'* clade.

ITS1 analysis

Fifteen samples (14 species) in the *Gelidium*-complex clade and *Gelidium divaricatum* as outgroup were used for the ITS1 sequence (252 bp, including gaps) analyses. The phylogenetic tree was obtained from NJ analysis (Fig. 3). The three subclades that were described above were also supported by high bootstrap values: 97% for the *Gelidium coulteri* clade; 100% for the *Acanthopeltis* clade; 97% for the *'Gelidium'* clade. However, no bootstrap support (less than 52%) was obtained for the topological positions of these subclades.

Acanthopeltis and *Yatabella* formed a monophyletic group, and *G. vagum* that includes strains of Japan and the Pacific coast of Canada was recognized as the sister group of the *Acanthopeltis/Yatabella* group with 94% bootstrap value. *Onikusa japonica* was again not monophyletic with *Onikusa pristoides*. *Gelidium elegans* Kützing, *G. linoides* Kützing and *G. subfastigiatum* Okamura were recognized as a monophyletic group and came to the position of the sister group to the European species of *Gelidium* such as *G. sesquipedale* (Clemente) Turner, *G. arbuscula* (Montabne) Børgesen and *G. latifolium* (Greville) Bornet et Thuret.

Secondary rhizoidal attachments and correlation of molecular and morphological data

As Perrone (1994) has demonstrated, three types of secondary rhizoidal attachments were recognized in the present study (Figs 4-6); (1) the unicellular independent type was observed in *Gelidiella acerosa* (Fig. 4) and *Gelidiella ligulata*; (2) the peg type was found in *Pterocladia lucida*, *Pterocladiella capillacea* (Fig. 5) and *Pterocladiella* sp.; (3) the brush type was observed in *Ptilophora subcostata*, *Capreolia imprexa*, *Acanthopeltis japonica*, *Yatabella hirsuta*, *Onikusa japonica*, *Gelidium divaricatum*, *G. pusillum*, *G. elegans* (Fig. 6), *G. linoides*, *G. pacificum*, *G. vagum* and *G. subfastigiatum*. These three types of secondary rhizoidal attachments were correlated with the types of cystocarps (Fan 1961; Dawson 1953; Bailey & Freshwater 1997; Yoshida 1998) and correspond to the *Gelidiella* clade and *Pterocladia/Pterocladiella* clade in the NJ tree of the SSU rDNA gene (Fig 7), and the large *Gelidium*-complex clade in the *rbc*L gene tree, respectively.

DISCUSSION

Phylogeny of the Gelidiales

Results of the molecular analyses in this study were almost congruent with those of previous reports (Freshwater *et al.* 1995; Bailey & 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. These can be

summarized as follows: (1) 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 (*rbc*L analysis); (2) the genus *Gelidiella* was recognized as the earliest diverging lineage within this order with high bootstrap value in the SSU rDNA analysis; (3) the large *Gelidium*-complex clade contained three clades, the *Ptilophora* clade, the *Capreolia* clade and the *Gelidium*-complex clade (SSU rDNA and *rbc*L analyses); (4) the *Gelidium*-complex clade includes three subclades, the *Gelidium coulteri* clade (the *Gelidium coulteri* complex, Freshwater *et al.* 1995), the *Acanthopeltis* clade that is recognized for the first time and contains *Onikusa japonica*, *Gelidium vagum*, *Acanthopeltis japonica* and *Yatabella hirsuta*, all of which are distributed in the western and eastern Pacific, and the '*Gelidium*' clade that is divisible into four groups, the Indo-Pacific/Carribbean *Gelidium* complex, the European *Gelidium* complex, the *Suhria* clade and the European *Gelidium pusillum* clade as reported previously (Freshwater *et al.* 1995)(*rbc*L and ITS1 analyses); (5) *Onikusa japonica* was not monophyletic with the type species of the genus, *O. pristoides*, which was included in the *Suhria* clade (Freshwater *et al.* 1995)(*rbc*L and ITS1 analyses); (6) a Japanese strain of *Gelidium pusillum* was included in the *Gelidium coulteri* clade (*rbc*L and ITS1 analyses).

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 in each gene, i.e. the SSU rDNA is more conservative than the *rbc*L (Bailey & Freshwater 1997) and therefore the SSU rDNA is not suitable for the analysis of recently diverged taxa.

Freshwater *et al.* (1995) have suggested that certain genera, species and populations of Gelidiales can be assembled in groups based on geographic distribution. This statement is also confirmed to be valid in this study, as shown in the cases from (4) to (6) described above. *Gelidium pusillum* has been separated into three clades in the molecular analyses (Freshwater *et al.* 1995). In this study, a Japanese population of *Gelidium pusillum* was included in the *Gelidium 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 *Gelidium 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 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*. Rodríguez 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), *Onikusa pristoides* was included in the *Suhria* clade and the revival name *Suhria pristoides* (Turner) J. Agardh was suggested. I have demonstrated that *Onikusa japonica* is not closely related to *O. pristoides*. *Onikusa pristoides* is closely related to *Suhria* as suggested by Freshwater *et al.* (1995), while *Onikusa japonica* has close affinity with the *Acanthopeltis* clade. It seems appropriate to treat *Onikusa 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.

Acanthopeltis clade

Acanthopeltis japonica, *Yatabella hirsuta*, *G. vagum* and *O. japonica* were shown to be a monophyletic clade (*Acanthopeltis* clade) in both *rbc*L and ITS1 analyses with 100% bootstrap values. Although *Gelidium vagum* came to the position of the sister group of *Acanthopeltis/Yatabella*, SSU rDNA analysis showed *G. vagum* and *Acanthopeltis/Yatabella* to be in different lineages. In the SSU rDNA analysis, the low sequence divergence was detected among the member of the *Gelidium*-complex clade (only three bases change between *G.*

vagum and *Acanthopeltis/Yatabella*) and the tree might not reflect the true phylogeny within this clade. In this point, taking the results of *rbc*L and ITS1 analyses (100 % bootstrap value, respectively) into consideration, I conclude that *G. vagum* is included in the *Acanthopeltis* clade.

Spermatangial sori, which are exclusively formed on cystocarpic branchlets, have been reported for the following six gelidialean species: *Pterocladiella caerulescens* (Kützing) Santelices et Hommersand, *Gelidium howeii* Acleto, *G. mcnabbiana* (Dawson) Santelices, *G. pluma* Loomis, *Gelidium vagum* Okamura, *Onikusa japonica* (Okamura) Akatsuka (Santelices & Flores 1995). *Acanthopeltis japonica* and *Yatabella hirsuta* are also monoecious, but they have special spermatangial branchlets on the cystocarpic erect axes (Kaneko 1968; this study, figs 8, 9). Thus, all four species in the *Acanthopeltis* clade, *A. japonica*, *Y. hirsuta*, *O. japonica* and *G. vagum*, are monoecious. Although the *Acanthopeltis* clade is characterized by the production of monoecious gametophytes, other three monoecious species of *Gelidium*, *Gelidium howeii*, *G. mcnabbiana* and *G. pluma*, have not been included in molecular phylogenetic analyses. Taxonomic revision of species in the *Acanthopeltis* clade is needed to determine the phylogenetic positions of other three monoecious species of *Gelidium* and whether monoecism is limited in the *Acanthopeltis* clade or not.

Secondary rhizoidal attachments

Based on Figure 7, it can be concluded that: (1) *Gelidiella* is characterized by having unicellular independent attachments and the absence of sexual reproduction; (2) *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 a cystocarp (Bailey & Freshwater 1997); and (3) *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 & Hommersand 1997). Furthermore, in the *rbc*L tree I can conclude that: (4) all the members of the large *Gelidium*-complex clade (*Ptilophora*, *Capreolia*, *Acanthopeltis*, *Yatabella*, *Onikusa* and *Gelidium*) possess brush-type secondary rhizoidal attachments and carposporangia developing on both sides of the central plane of a cystocarp (Okamura 1900, 1901; Akatsuka 1986; Bailey & Freshwater 1997).

Three types of the secondary rhizoidal attachments were found to correspond to the three major clades resolved in the molecular study (the *Gelidiella* clade, *Pterocladia*/*Pterocladiella* clade and large *Gelidium*-complex clade). This means that the types reflect phylogenetic relationships of gelidialean algae.

In the case of *rbc*L analysis, affinities of *Pterocladia* and *Pterocladiella* have not been resolved clearly because of low bootstrap values. However, congruence of morphological characteristics and the SSU rDNA tree suggest monophyly of *Pterocladia* and *Pterocladiella* and robustness of the SSU rDNA tree, at least in this position. Although the genera *Pterocladia* and *Pterocladiella* have been shown to share the same type of secondary rhizoidal attachments, these two genera possess different patterns of the female reproductive system and developmental carposporophytes (Bailey & 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 & 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.

Chapter 2

Chapter 2. Phylogenetic affinities of two genera *Acanthopeltis* and *Yatabella* inferred from molecular and morphological analyses

INTRODUCTION

Acanthopeltis and *Yatabella* are monotypic and possess unique properties in the Gelidiales with regard to geographical distribution and morphology. The genus *Acanthopeltis* with *A. japonica* Okamura (in Yatabe 1892) is restricted to Japan (Yoshida 1998), Korea (Lee & Kim 1995) and the Philippines (Hurtado-Ponce *et al.* 1998). It has been said to have sympodial growth, whereas all other members of the Gelidiales show monopodial growth (Okamura 1900, 1901; Fan 1961; Santelices 1990). The erect axes are subcylindrical and numerous spinelike proliferations are arranged on leaflike structures. The genus *Yatabella* with *Y. hirsuta* Okamura (1900) is endemic to southern Japan and has subcylindrical erect axes beseted with numerous multifid-echinate ramuli.

On the basis of growth patterns, sympodial vs. monopodial, *Acanthopeltis* and *Yatabella* have been regarded to be derived from separate lineages in the Gelidiales (Fan 1961; Santelices 1990; Norris 1992). However, these two genera are similar to each other in other vegetative and reproductive features (Okamura 1900, 1901). Furthermore, *Acanthopeltis* and *Yatabella* were included in the same clade, *Acanthopeltis* clade, in the molecular analyses of Chapter 1.

In this study, I compared the sequence divergence of nuclear-encoded SSU rDNA, ITS1 and plastid-encoded *rbc*L between four samples of *Acanthopeltis japonica* and *Yatabella hirsuta*. I also reexamined growth patterns of *Acanthopeltis japonica* and *Yatabella hirsuta* on the basis of type materials, laboratory-cultured plants and herbarium specimens.

MATERIALS AND METHODS

DNA analysis

Methods for total DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of the *rbc*L gene were as described in Chapter 1. In Chapter 1, I determined the SSU rDNA, *rbc*L and ITS1 sequences of *Acanthopeltis japonica* (Shimoda, Shizuoka Prefecture, 25.ix.1996) and *Yatabella hirsuta* (Oryuzako, Miyazaki Prefecture, 11.vii.1996). In this study, additional SSU rDNA, *rbc*L and ITS1 sequences of *Acanthopeltis japonica* (Oryuzako, Miyazaki Prefecture, 3.viii.1997) and *Yatabella hirsuta* (Oryuzako, Miyazaki Prefecture, 3.viii.1997) were determined, and aligned these sequences by eye. The sequence divergence was, thus, compared between individuals from different localities of *Acanthopeltis japonica* and two individuals of *Yatabella hirsuta* from the same locality but collected at different time (Table 3).

Observations of growth patterns of *Acanthopeltis japonica* and *Yatabella hirsuta*

Growth patterns of *Acanthopeltis japonica* and *Yatabella hirsuta* were reexamined on the basis of the following specimens: (1) the holotype specimen of *A. japonica* (Fig. 10) collected at Misaki, Kanagawa Prefecture (iv.1885) and deposited in the University Museum, University of Tokyo (TI); (2) the holotype specimen of *Y. hirsuta* (Fig. 11) collected at Oryuzako, Miyazaki Prefecture (13.vii.1899) and deposited in herb. Okamura housed in SAP; (3) cultured plants shown in Table 1; (4) three herbarium specimens of *A. japonica* collected at Shimoda, Shizuoka Prefecture (8.vii.1983, SAP 062000), at Akiya, Kanagawa Prefecture (4.iii.1988, SAP 060389) and at Enoshima, Kanagawa Prefecture (15.iii.1930, SAP 060892); and (5) five herbarium specimens of *Y. hirsuta* collected at the type locality (23.iii.1948, SAP 062325; 3.iv.1948, SAP 062326; 3.viii.1958, SAP 062324; 1.vi.1962, SAP 061052; 11.vii.1996, SAP 064847).

RESULTS

Pairwise comparisons of DNA sequences between individual plants of *Acanthopeltis* and *Yatabella*

In pairwise comparisons, there is no sequence difference in the SSU rDNA among the four individuals, no sequence difference between *A. japonica* from Shimoda and two samples

of *Y. hirsuta* and only one substitution betweeen *A. japonica* from Oryuzako and two samples of *Y. hirsuta* in the *rbc*L, and one substitution and one gap between *A. japonica* and *Y. hirsuta* in the ITS1 (Table 4). In order to assess the effect of technical error, I have extracted DNA from the same individual twice and independently sequenced these genes. No sequence difference was found in these repeated experiments and thus the sequences reported here are free from the technical error.

Observations of growth pattern of *Acanthopeltis* and *Yatabella*

Growth patterns of erect axes of *Acanthopeltis* and *Yatabella* are shown in Figures 12-17. Isolated apical tips of branchlets of both species grew into creeping axes in laboratory culture, and these creeping axes formed erect axes 1-2 months after inoculation at 20°C. The erect axis of *A. japonica* became broad and grew into a leaflike structure, producing proliferations on both surfaces (Fig. 17, stage A1). Then one of the proliferations became broad and grew into a second leaflike structure (Fig. 12) that overtopped the parental leaflike structure (Fig. 17, stage A2). The second leaflike structure also produced proliferations on the surfaces, one of which grew into a third leaflike structure (Fig. 13 and Fig. 17, stage A3). This process was repeated many times and leaflike structures were piled up in three-dimensions (Fig. 14 and Fig. 17, stage A4, A5).

Yatabella hirsuta produced a number of multifid-echinate ramuli (Fig 15) to every direction at the surface, and several of them grew into lateral branches (Figs 15, 17). After the production of several branches (Fig. 17, stage Y1 and Y2), the axis (Y3, branch 1) was overtopped by one of the lateral branches, which was not leaflike and elongated like the parental axis (Fig. 17, stage Y3, branch 5). This branch (functioning as new axis) also produced a number of multifid-echinate ramuli to every direction at the surface, and several of them grew into lateral branches (Fig. 17, stage Y4, branches 6-9). The new axis (Y3, branch 5) was also overtopped by one of the branches (Fig. 17, stage Y5, branch 9), and the branch grew into a next axis. Field-collected specimens including the holotype specimen of *Y. hirsuta* have several overtopped branches (Fig. 16).

Field-collected specimens of *Acanthopeltis japonica* (Fig. 10) and *Yatabella hirsuta* (Figs 11, 16) are profusely branched. In *A. japonica* profuse branching may be due to the formation of a further leaflike structure apically from some leaflike structures that formed a single leaflike structure proximally, the apical leaflike structure growing in a manner similar to that of the proximal one. On the other hand, such branching in *Y. hirsuta* is due to the production of branches with sympodial growth.

DISCUSSION

Taxonomic treatment of *Acanthopeltis* and *Yatabella*

When Okamura (1900) described a new genus *Yatabella* with a new species *Y. hirsuta*, he noticed that this species is similar to *A. japonica* in its vegetative and reproductive features. However, the difference in growth patterns, sympodial vs. monopodial, let Okamura to separate these two species at the generic level.

In the present study I have demonstrated that *Acanthopeltis* and *Yatabella* possess a fundamentally similar growth pattern. Sympodial growth (the production of overtopped leaflike structures) occurs repeatedly and regularly with short intervals in *Acanthopeltis*, whereas sympodial growth (the formation of overtopped branches) does infrequently and irregularly in *Yatabella*. In the latter lateral branches are formed monopodially on a particular branch, and one of such branches formed near the apex of the parental branch shows sympodial growth. I think that Okamura (1900) might be misled by the frequent, monopodial emergence of lateral branches that obscure sympodial growth.

In the molecular analyses, *Acanthopeltis* and *Yatabella* were recognized as a monophyletic group in the *rbc*L and ITS1 analyses, and a similar result was obtained with the SSU rDNA analysis (Chapter 1). Then, there are no sequence differences in the SSU rDNA gene, or one substitution between these two species in the *rbc*L gene, and there are two substitutions in the ITS1 sequences (Table 4). These results indicate that *Acanthopeltis* and *Yatabella* are closely related. Taking morphological and molecular closeness into consideration, I think that there is no reason to separate these taxa at the generic rank any more.

Are *A. japonica* and *Y. hirsuta* conspecific?

In Freshwater and Rueness (1994) *rbc*L sequences were generated for multiple samples of *G. pulchellum* (6 samples), *G. pusillum* (4 samples), and *G. latifolium* (8 samples): sequence divergence values within these species were 0.5-1.8%, 0.2-0.5%, and 0.3-0.8%, respectively. Two samples of *G. elegans* showed a sequence divergence of 0.4%, and 9 samples of *Pterocladiella capillacea* showed a range of 0.2-1.4% (Freshwater *et al.* 1995). Compared to those differences, the divergence between *Acanthopeltis* and *Yatabella* (0.0-0.1%) is very low. The low sequence divergence between *A. japonica* and *Y. hirsuta* even suggests conspecificity of these two taxa.

There are only two base substitutions in the ITS1 sequence. Such a close range of sequence divergence in the ITS sequences have been observed within same species in fungi (Gardes *et al.* 1991), in diatoms (Zechman *et al.* 1994), in the red alga *Chondrus crispus* Stackhouse (Chopin *et al.* 1996) and between different species in the brown algal genus *Fucus* (Leclerc *et al.* 1998). As Bird *et al.* (1992) pointed out, the taxonomic significance of molecular sequence divergence must be evaluated on a case-by-case basis. In this case, the sequence divergence found between *A. japonica* and *Y. hirsuta* falls into either inter- or intraspecific variations of other taxa as shown above. However, on the morphological and ecological grounds I believe that these species are not the same species.

Acanthopeltis japonica and *Yatabella hirsuta* occur sympatrically in Oryuzako, Miyazaki Prefecture, Japan, growing on the subtidal rocks. Yet, the morphology of each species can be clearly distinguished in the field and no intermediate forms have been observed at Oryuzako. In the culture study, morphological distinctiveness of both species, such as branch morphology and branching patterns are also maintained under the same culture conditions. Although I have not been able to attempt crossing experiments yet, these facts strongly suggest that these two algae are not conspecific. In this case, therefore, the sequence divergence of two base pairs in the ITS1 sequence is probably indicative of the species difference. As Leclerc *et al.* (1998) reported, the low sequence divergence of *A. japonica* and *Y. hirsuta* can be explained either as the result of very recent separation between the taxa or by a slower substitution rate within the species.

The sequence divergence (one base change) found within *rbc*L sequences of *A. japonica* from different localities (Shimoda and Oryuzako) is interesting, because one of them possesses exactly the same sequence as that of *Y. hirsuta*. Since *A. japonica* and *Y. hirsuta* possess identical sequences in the SSU rDNA gene it is highly possible that these taxa originally possessed identical sequences in the *rbc*L gene as well. Unless supposed the convergence, it is hard to explain the situation that two species originally had one base difference and became identical in the Shimoda population. I believe that it is much more possible to think that these two species originally had the same sequences and a mutation

has occurred in *A. japonica* of the Oryuzako population and its base change has been maintained. This intraspecific variation can be seen in other gelidialean species as demonstrated by Freshwater and Rueness (1994).

Concluding remarks

Acanthopeltis and *Yatabella* are congeneric on the basis of morphological similarities, no sequence difference in the SSU rDNA, and a close range of sequence divergence in the *rbc*L genes and ITS1 sequences. The following new combination is therefore proposed.

***Acanthopeltis hirsuta* (Okamura) Shimada, Horiguchi et Masuda, comb. nov.**

Basionym: *Yatabella hirsuta* Okamura, Illustrations of the Marine Algae of Japan 1: 1, pl. 1, 1900.

Chapter 3

Chapter 3. The confirmation of the status of three *Pterocladia* species described by K. Okamura

INTRODUCTION

Pterocladiella was recently separated from the genus *Pterocladia* based on the development of carpogonia and cystocarps by Santelices and Hommersand (1997). 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 (1991), 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.

In this study, I determined nucleotide sequences of plastid-encoded *rbc*L gene for 21 populations of the species described by Okamura (1932, 1934). I also examined morphological variation in these samples in order to assess the congruence of molecular and morphological data. Furthermore, the type specimens of the three species described by Okamura were reexamined and compared to my materials. Based on these studies, I will confirm the taxonomic status of the three *Pterocladia* species described by Okamura (1932, 1934).

MATERIALS AND METHODS

Sampling

Twenty one unialgal cultures were established from excised tips of branchlets of plants collected from 21 local populations in Japan (Table 5). Voucher specimens are deposited in the Herbarium of Graduate School of Science, Hokkaido University, Sapporo (SAP 064842, 065448-065496). Unialgal cultures were grown in Tris-buffered medium (van der Meer & Patwary 1991) at 15°C and 16:8 h LD cycle with the photon flux of 15-25 $\mu Em^{-2}s^{-1}$.

Molecular analysis

Methods for total DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of the *rbc*L gene were as described in Chapter 1. For this study, sequences of three

additional species, i.e. *Pterocladia lucida* (Brown et Turner) J. Agardh (U01048), *Pterocladiella capillacea* (Gmelin) Santelices et Hommersand (type locality Mediterranean, Gmelin 1768) (U01888-Italy, U01889-Spain and U01898-Australia) and *Pterocladiella melanoidea* (Schouboe ex Bornet) Santelices et Hommersand (U01046) were downloaded from GenBank and incorporated into the analysis. *Pterocladia lucida* (Brown et Turner) J. Agardh was used as outgroup. The distance matrix method was used to construct phylogenetic trees. For the distance matrix method, I used Kimura's (1980) two-parameter method to calculate the distance matrix and neighbor-joining (NJ) method (Saitou & Nei 1987) to construct the tree using the CLUSTAL W computer program (Thompson *et al.* 1994; Higgins *et al.* 1996). Bootstrap analysis based on 100 resamplings of the data set (Felsenstein 1985) was calculated to evaluate statistical reliability.

Morphological observations

The following specimens were used for morphological observations: (1) voucher specimens for culture studies (Table 5) and specimens collected at these localities at different time, and (2) cultured plants listed in Table 1. I selected as many mature individuals as possible from each population (Table 5), 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 character number (2), (3) and (5) were not measure by Stewart (1968). I also measured the length and width of the tetrasporangia and carposporangia. The following type materials from the Okamura herbarium housed in SAP were examined: 1) the lectotype specimen of *Pterocladia nana*, which was designated in this study, 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). Furthermore, the type material of *Gelidium decumbensum* Okamura was examined to clarify its status, as Okamura (1934) comments that this species is similar to *Pterocladia tenuis*.

RESULTS

*rbc*L analysis

Nucleotide sequences of the *rbc*L gene were determined for 21 local populations of *Pterocladia/Pterocladiella* (Fig. 18). Eight different *rbc*L sequences (same length, 1467 bp) were found within the 21 samples sequenced: (1) Oshoro, Taisei, Oga, Kasashima, Unoura, Echizen, Uradomi, Hinomisaki #304, Kiwado (Sea of Japan), Takedatsu (Seto Inland sea) and

Shiriya (Pacific); (2) Enoshima and Shimoda (Pacific); (3) Onahama and Hachijo Island (Pacific); (4) Hamashima and Kushimoto (Pacific); (5) Shimo-Koshiki Island (Pacific); (6) Tomioka (Pacific); (7) Tsuyazaki (Sea of Japan); and (8) Hinomisaki #314 (Sea of Japan).

The phylogenetic tree obtained from NJ analysis is shown in Figure 19. Three monophyletic clades were identified: group 1 comprised of only the Shimo-Koshiki Island strain, group 2 consisting of the Tsuyazaki, Hinomisaki #314, Enoshima and Shimoda strains (98% bootstrap value), and group 3 including the rest of Japanese strains and *P. capillacea* from Australia, Spain and Italy (82% bootstrap value). The sequence divergence values between/within three groups are as follows: 4.5-4.8% (group 1 and 2); 4.6-5.0% (group 1 and 3); 0.5-1.1% (group 2 and 3); 0.0-0.4% (within group 2); and 0.0-0.5% (within group 3). The Hinomisaki #304 and #314 strains were resolved in different clades emphasizing the overlapping geographical distribution of the two taxa represented by the group 2 and group 3 clades (Fig. 18).

Observations of morphological variations

Group 1 plants (Fig. 20) are reddish-brown in 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.

Tetrasporangia are irregularly disposed at the apices of axes and branches. Cruciately divided sporangia are 44-48 μm long by 20-40 μm wide. Cystocarps are formed near the apices of branches. A cystocarp is attached to one side of the cystocarp floor and produces chains of carpogonia from the remaining three sides. Nutritive filaments grow centripetally and form a virtually solid cylinder around the central axis. Carposporangia are 30-40 μm long by 16-20 μm wide. Spermatangia were not found in our specimens.

Group 2 plants (Figs 21, 22) are 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. Tetrasporangia are irregularly disposed at the apices of branches. Cruciately divided sporangia are 48-52 μm long by 20-36 μm wide. Cystocarps are formed near the apices of branches. The cystocarpic structure is similar to that of group 1 plants, and carposporangia are 28-36 μm long by 16-22 μm wide. Spermatangia were not found in our specimens.

Group 3 plants (Figs 23, 24) are 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. Tetrasporangia are irregularly disposed at the apices of

branches. Cruciately divided sporangia are 40-48 μm long by 16-28 μm wide. Cystocarps are formed near the apices of branches. The cystocarpic structure is similar to that of group 1 plants, and carposporangia are 28-40 μm long by 20-24 μm wide. Spermatangial sori are formed at the apices of branches of male gametophytes. Spermatangial mother cells are elongated and 8-10 μm long by 1.5-2.0 μm wide.

No significant differences of positions and dimensions of reproductive structures were found between the three groups. I then, analyzed vegetative morphology. 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. 25): 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.

Populations of the three groups recognized by the molecular analysis were given respective group specific symbols in Figure 25. Group 1 is distinguished from group 2 by all five dimentions measured (Figs 25-a, 25-b, 25-c, 25-d), and from group 3 by the lengths of axes and first-order branches (Fig. 25-a) and branching intervals of axes (Fig. 25-c). Group 2 differs from group 3 in the width of second-order branches (Fig. 25-b) and branching intervals of first-order branches (Fig. 25-d).

In my culture study, morphological distinctiveness of these three groups, such as short axes in group 1 (Fig. 26), wide branches and long branching intervals in group 2 (Fig. 27) and slender branches and short interval branchlets in group 3 (Fig. 28) were also maintained under the same culture conditions, i.e. 15°C and 16:8 h LD cycle.

DISCUSSION

Taxonomic treatment of three groups

When Okamura (1932, 1934) described three species of *Pterocladia* (*P. nana*, *P. tenuis* and *P. densa*) from Japan, he commented that *P. nana* is distinguished from the other two species by dwarf thalli. Furthermore, he stated that *P. tenuis* and *P. densa* are similar to each other, but the latter can be separated from the former by having a slender branchlets with shorter intervals. He also mentioned that *P. tenuis* possesses broader branches than those of the cosmopolitan species *Pterocladia capillacea*. Unfortunately, Stewart (1968) and Santelices (1991) did not seem to pay much attention to these diagnostic features, which might lead these authors to recognize the three species as conspecific.

Analysis of *rbc*L sequences resolved three separate monophyletic clades (group 1-3) in the Japanese *Pterocladia/Pterocladiella*. These three groups can also be distinguished from each other on morphological grounds. Group 1 is characterized by small thalli; group 2 is

distinguished from the other two groups by having wide branches of the second-order with longer branching intervals; and group 3 is separated from group 1 by larger thalli and from group 2 by possessing more slender second-order branches with shorter branching intervals. In culture experiments, the morphological characteristics of each group are maintained under the same conditions. Although I have not been able to conduct crossing experiments yet, the congruence of molecular and morphological data strongly suggests that these three groups represent different species.

In order to elucidate precise relationships between these three groups and the three species of *Pterocladia* described by Okamura (1932, 1934), I reexamined the type materials of *Pterocladia nana*, *P. tenuis* and *P. densa*.

In describing *Pterocladia nana*, Okamura (1932) illustrated four specimens (pl. 278, figs 1-3), which were collected at Yura-jima, Shimo-Koshiki Island, Koshiki Islands (vii.1919), according to the label of the specimens that are deposited in the Okamura Herbarium (SAP). However, none of the specimens were designated as the holotype. The Okamura Herb. has two specimens of *Pterocladia nana* designated in Okamura's writing as 'Type' on the cover sheet that were illustrated in his later publication (Okamura 1934, pl. 33, fig. 9). Although he wrote that these specimens were collected from Koshiki Islands (as Koshiki-dima), his label shows that they were collected at Seto, Kii Prov. (Seto, Shirahama, Wakayama Prefecture) in July 1930. There are numerous specimens in the Okamura Herb. which were

designated by Okamura as 'Type' even for species that were not established by himself. They are not actually nomenclatural types but specimens used for published illustrations by Okamura himself. The specimens illustrated in his later publication (Okamura 1934, pl. 33, fig. 9) reach 3 cm in height and did not fit his original description stating 'plant attaining 2 cm in height', whereas those illustrated in his original publication (Okamura 1932, pl. 278, figs 1-3) are 1.5-2.5 cm in height. Okamura divided the latter specimens into two groups in his herbarium, 'regular type' and 'large type'. I select one of the regular type, which is more compatible with the type description, as the lectotype specimen (Fig. 29).

The axis of the lectotype specimen of *Pterocladia nana* is 1.9 cm long and produces first-order branches up to 0.9 cm long at intervals of 0.2-1.4 mm that form second-order branches up to 0.6 mm wide at intervals of 0.2-1.1 mm. Based on these measures, I identify the group 1 taxon as *P. nana*.

Pterocladiella caerulescens (Kützing) Santelices et Hommersand is similar in gross morphology to *P. nana*. This species was first described by Kützing (1868) under the name of *Gelidium caerulescens* from material collected at New Caledonia, later transferred to *Pterocladia* by Santelices (1976), and recently transferred to *Pterocladiella* by Santelices and Hommersand (1997). This species is distributed in the Caribbean Sea and the tropical Pacific: Venezuela (Rodríguez de Ríos 1992), Line Islands (Dawson 1959), Australia (Price and Scott 1992), Hawaii (Santelices 1976, 1977, 1978) and Indonesia (Hatta and Prud'Homme van

Reine 1991) as well as its type locality New Caledonia (Kützing 1868). There are two features which distinguish *P. caerulescens* from *P. nana*: (1) *P. caerulescens* is monoecious (Santelices & Flores 1995), whereas our material and Okamura's specimens of *P. nana* do not produce spermatangial patches on the carpogonium-producing branchlets; and (2) *P. caerulescens* is light green, dark green, or almost blackish in color (Santelices 1977), while *P. nana* is reddish-brown in color. On the basis of these differences, I consider *P. nana* a distinct species from *P. caerulescens*. The cystocarpic structure of *P. nana* is the *Pterocladiella*-type (Okamura 1932; present study, Fig. 32). *Pterocladia nana* should therefore be transferred to the genus *Pterocladiella*.

***Pterocladiella nana* (Okamura) Shimada, Horiguchi et Masuda, comb. nov.**

Basionym: *Pterocladia nana* Okamura, Icon. Japanese Alg. 6(6), p. 53, pl. 278, figs 1-14, 1932.

The axis of the lectotype specimen of *Pterocladia tenuis* (Fig. 30) is 12.4 cm long and bears first-order branches up to 6.1 cm long at intervals of 2.0-5.5 mm that issue second-order branches up to 1.7 mm wide at intervals of 2.0-4.5 mm. These dimentions agree with those found in this study for the group 2 specimens, and I identify group 2 as *P. tenuis*. The cystocarpic structure of *P. tenuis* is the *Pterocladiella*-type (Okamura 1913, as *Pterocladia capillacea*; present study, Fig. 33). *Pterocladia tenuis* should therefore be transferred to the genus *Pterocladiella*.

***Pterocladiella tenuis* (Okamura) Shimada, Horiguchi et Masuda, comb. nov.**

Basionym: *Pterocladia tenuis* Okamura, J. Imp. Fisher. Inst. 29, p. 62, pl. 29, pl. 30, fig. 3, pl. 33, figs 1-3, 1934.

The axis of the lectotype specimen of *Pterocladia densa* (Fig. 31) 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 dimentions agree with those determined in this study for the group 3 specimens and I identify group 3 as *P. densa*. The type illustration of *Pterocladiella 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), Spain and Australia were included in the group 3 clade. The morphological similarity and molecular closeness suggest that *P. densa* should be considered to be a synonym of *Pterocladiella capillacea*.

Status of *Gelidium decumbensum* Okamura

Okamura (1934) described *Gelidium decumbensum* (Fig. 34) based on only two specimens collected at Enoshima, Kanagawa Prefecture. He commented that this species is similar to *Pterocladia tenuis* (Okamura 1934), and there is a

possibility that it might be an abnormal form of another alga (Okamura 1936). I reexamined the lectotype specimen of *G. decumbensum*. The specimen has the peg-type secondary rhizoidal attachments that are exclusively found in *Pterocladia* and *Pterocladiella* (Perrone 1994; Chapter 1), a broad axis up to 2 mm wide and broad second-order branches up to 2.5 mm wide, and long branching intervals of axis (1.0-5.5 mm) and first-order branches (1.5-7.0 mm). These features indicate that *G. decumbensum* is a synonym of *P. tenuis*.

Chapter 4

Chapter 4. A reassessment of the taxonomic status of *Gelidium subfastigiatum* Okamura

INTRODUCTION

Gelidium subfastigiatum Okamura was described from materials collected from the northern part of Japan (Okamura 1934). In the original description of this species (Okamura 1934), he commented that this species is most similar to *Gelidium elegans* Kützing (as *G. amansii* Lamouroux), but this species can be distinguished from *G. elegans* by more robust thalli that have subterminally swollen branches and tooth-like sterile branchlets (Okamura 1934). Akatsuka (1982) argued that robustness is not an adequate criterion for distinguishing *G. subfastigiatum* from *G. elegans* (as *G. amansii*), due to its variability, and he reduced *G. subfastigiatum* to the synonymy of *G. elegans* (as *G. amansii*). However, he did not compare morphology of widened branches between these species, and Yoshida (1998) suspended Akatsuka's taxonomic treatment of *G. subfastigiatum*.

In this study, seasonal variation of subterminally swollen branches of *G. elegans/G. subfastigiatum* complex were examined on the basis of periodic samplings at two localities including the type locality of *G. subfastigiatum*. Nuclear-encoded ITS1 sequences for 26 samples of 20 populations of this complex were determined. Furthermore, morphology of subterminally swollen branches and tolerance to lower temperature in these samples were examined in order to assess the congruence of molecular, morphological and

physiological data. Based on these studies, I will confirm the taxonomic status of the *G. subfastigiatum*.

MATERIALS AND METHODS

Sampling and morphological observations

In examination of seasonal variations of subterminally swollen branches, the following specimens were used: Oshoro (Fig. 41, number 11), Hokkaido, (9.vi.1995, 21.vii.1995, 12.viii.1995, 13.x.1995, 11.xii.1995, 15.ii.1996, 6.iii.1997, 16.iv.1997, 22.v.1997, 2.xi.1997, 2.ix.1999); and Yura (Fig. 41, number 2), Awaji Island, Hyogo Prefecture (17.iv.1995, 29.vi.1995, 19.x.1995, 30.i.1996, 16.v.1996). I selected three plants from each collection and measured the maximum width and thickness of ten second-order branches (1-2 mm below the apex).

Unialgal cultures were established from excised tips of branchlets of plants collected from 20 local populations in Japan (Table 6). They were grown in PES medium (Provasoli 1968) at 15°C, 16:8 h L:D (light:dark) with the photon flux of 15-25 $\mu Em^{-2}s^{-1}$. Voucher specimens are deposited in the Herbarium of Graduate School of Science, Hokkaido University, Sapporo (SAP 071898-071935). Parental plants of these unialgal cultures were also measured for the maximum width and thickness of ten second-order branches (1-2 mm below the apex).

Molecular analysis

Methods for total DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of the ITS1 sequences were as described in Chapter 1. ITS1 sequences of 26 cultures from 20 local populations in Japan were determined (Table 6).

Tolerance to lower temperature

Small tips (2-3 mm long) of branches of 21 unialgal strains, which were grown at 15°C and 16:8 h LD cycle with the photon flux of 15-25 $\mu Em^{-2}s^{-1}$ for 8-40 months, were transferred to 10°C and 16:8 h LD for one day and then to 5°C and 16:8 h LD for one day. These were finally transferred to 3°C and maintained under 8:16 h LD with the photon flux of 30-40 $\mu Em^{-2}s^{-1}$ for 3 months. Color (dark red or whitish) and length of branches were observed for every week: when the branches become whitish (died), the cultures were terminated.

RESULTS

Seasonal variation of subterminally swollen branches

For analysis of seasonal variations of subterminally swollen branches of materials from Oshoro and Yura, means and standard deviations of widths and thicknesses are plotted, respectively (Fig. 35). Subterminally swollen branches of Oshoro population showed a clear seasonal change in width:

they are widened (493-544 μm) from February to April (Fig. 36), becoming slender (379 μm) from May onward, reaching 187-211 μm in width from June to November (Fig. 37), but becoming wider (367 μm) from December onward. These subterminally swollen branches of the Oshoro populations showed a similar seasonal change in thickness: they are thickened (820-955 μm) from February to May, becoming thinner (500 μm) from June onward, reaching 390 μm in thickness in September, and then becoming thicker (558 μm) from November onward. Corresponding branches of the Yura population showed no significant seasonal variation in width and thickness: they are 199-221 μm in width (Figs 38, 39) and 398-493 μm in thickness throughout the year.

Morphology of subterminally swollen branches of parental plants of unialgal strains

Parental plants of unialgal strains, which were collected at several localities (Table 7) from December to May, were selected to examine morphology of subterminally swollen branches, because two types of branches between the above-mentioned Oshoro and Yura populations are clearly shown in these season. The means and standard deviations of widths and thicknesses are shown in Table 7. There are two groups that are clearly distinguishable by width and thickness: four samples, #13, #16, #152, and #157, are narrow (174-216 μm in width) and thin (362-458 μm in thickness) than other seven samples, #43, #45, #47, #48, #143, #161, and #177 (426-508 μm in width and 850-986 μm in thickness). Two

samples collected at the same site and on the same date in Oga, Akita Prefecture (6.iv.1998) have a clear morphological gap of subterminally swollen branches: (#157) 216 μm (±30.98) in width and 396 μm (±39.78) in thickness; and (#161) 508 μm (±91.51) in width and 986 μm (±165.47) in thickness.

ITS1 sequences

Two different ITS1 sequences, type 1 (203 bp) and type 2 (204 bp), were found within the 26 sequences. Only one difference (gap) has been found in these two types (Fig. 40). The geographical distribution of these types is shown in Figure 41: (type 1) Izura, Kitayamazaki, Hachinohe, Shiriya, Oshoro, Tomari, Tappi, one sample at Oga, Atsumi, Shitsumi, Hinomisaki, and Tsuyazaki; and (type 2) Cape of Sata, Yura, Kushimoto, Hamashima, Shimoda, Tateyama, one sample at Oga, Sasakawanagare, and Kiwado. All the six samples collected at Oshoro (6.iii.1997, 2.ix.1999) had type 1 ITS1 sequence. Two samples collected at the same site and same date in Oga, Akita Prefecture (6.iv.1998) had different sequences: #157 had type 1; and #161 had type 2.

Tolerance to lower temperature

Nine samples (#10, #13, #16, #20, #24, #89, #152, #157, and #309) that have type 1 ITS1 sequence became white in five to eight weeks (Table 8). Twelve samples (#43, #45, #57, #60, #67, #73, #92, #143, #161, #177, #253, and #306) that have

type 2 ITS1 sequence did not change color and they were slightly elongated.

DISCUSSION

Morphology of subterminally swollen branches

Okamura (1934) described *Gelidium subfastigiatum* with his comment that this species is distinguished from *G. elegans* by subterminally swollen branches. However, Akatsuka (1982) reduced it to the synonym with *G. elegans* (as *G. amansii*), although Yoshida (1998) did not adopt this Akatsuka's opinion in his recent review.

Analysis of seasonal variations of subterminally swollen branches between materials from Oshoro (type locality of *G. subfastigiatum*) and Yura resolved clearly that subterminally widened and thickened branches were observed only from December to May in the Oshoro population. These branches, however, became slender and thinner from June to November and cannot be distinguished from materials of Yura whose branches showed no seasonal variation (always slender and thin). As Okamura described (1934), there are two types of branches; however, clearly subterminally swollen branches can be formed during only limited season. The lectotype specimen of *G. subfastigiatum* (Fig. 42), which was illustrated in the original description (Okamura 1934), was collected at Oshoro in March 1920, so that it possesses subterminally widened and thickened branches. However, widened and thickened branches of *G. subfastigiatum* change

to slender and thinner ones during a half of year, and I think this is a reason why *G. subfastigiatum* is not easily distinguishable from *G. elegans*.

The occurrence period of subterminally widened and thickened branches correspond to the coldest season in Japan, and temperatures in this period are 3-10 °C at Oshoro (Fig. 43). The branches may become subterminally widened and thickened under such lower temperatures.

Is *G. subfastigiatum* an independent species?

It is important to interpret certain morphological differences with the combination of other features such as geographical distribution, molecular and physiological aspects and when one decides whether the morphological differences indicate species differences or merely represent intraspecific variations. In this case, the production of widened and thickened branches, which is a diagnostic character of *G. subfastigiatum*, was observed in the Oshoro population from December to May and in the parental plants of other localities (#43, #143, #161, and #177) of unialgal cultures that were collected from December to May at several localities of Japan. One sample at Oga (#161) showed the widened and thickened branches, but another sample at Oga (#157) had slender and thinner branches just like other three parental plants (#13, #16, and #152) of unialgal cultures. Two types (widened and thickened or slender and thinner) of branches, therefore, can be clearly distinguished in the field even if materials occur

sympatrically (in Oga, Akita Prefecture), and no intermediate forms have been observed from December to May. Furthermore, types of ITS1 sequence and tolerance to lower temperatures were found to correspond to these two types of the branches: 1) slender and thin type has type 1 ITS1 sequence and cannot tolerate lower temperatures; 2) whereas widened and thicken type has type 2 ITS1 sequence and can tolerate lower temperatures. These congruence of morphological, molecular and physiological data strongly suggests that these two groups are different species. As Okamura (1934) described, I identify the slender and thin type as *G. elegans*, and the widened and thickened type as *G. subfastigiatum*, respectively.

Speciation of *G. subfastigiatum*

In this study, I was able to demonstrate that *G. subfastigiatum* has tolerance to lower temperature and is distributed along the coast of the Sea of Japan and the Pacific coast of northern Honshu. These results suggest that *G. subfastigiatum* might be evolved from *G. elegans* in the Sea of Japan or the northern Pacific coast of Japan, as predicted by Okamura (1934). *Gelidium subfastigiatum* might have obtained the tolerance to lower temperatures and extended its geographical range to the northern part of Japan. Further investigation is needed to understand the nature of the tolerance to lower temperatures in these species, for example, what gene of *G. subfastigiatum*

functions which genes of *G. subfastigiatum* expressed exclusively in lower temperatures.

Chapter 5

Chapter 5. Two new species of *Gelidium*, *G. tenuifolium* and *G. koshikianum*, from Japan

INTRODUCTION

Gelidium is the largest genus in the Gelidiales and includes approximately 100 species in the world (Nelson *et al.* 1994). Although in Japanese waters 14 species of *Gelidium* have been known (Yoshida 1998), there are still several species for which further investigations are necessary: for example, *Gelidium amamiense* Tanaka et Nozawa and *Gelidium isabelae* Taylor have not been reported since Tanaka (1965). Furthermore, some unrecorded or undescribed species may be present in Japanese waters (Shimada, unpubl. obs.) so that taxonomic studies on this genus growing around Japanese coasts are apparently needed.

By the recent molecular analyses, it has been shown that species of the genus *Gelidium* are clustered into several monophyletic clades, each representing specific geographic area (Freshwater *et al.* 1995; Chapter 1). Many Japanese *Gelidium* species are included in Indo-Pacific/Carribbean *Gelidium* complex; however, a few species such as *G. pusillum* (Stackhouse) Le Jolis, *G. divaricatum* Martens and *G. vagum* Okamura have been found to be positioned outside the Indo-Pacific/Carribbean *Gelidium*-complex clade and formed a clade together with other Pacific small species (*G. pusillum* in the *Gelidium coulteri* complex) or that with other genera (*G. divarivcatum* in the *Capreolia* clade, and *G. vagum* in the *Acanthopeltis* clade) (Chapter 1).

It is, therefore, important to make clear its phylogenetic position by molecular means when a new species is described.

In the present chapter, *Gelidium tenuifolium* and *G. koshikianum* are described as new species from the Pacific coast of Japan. Their phylogenetic positions within the genus *Gelidium* are discussed with the aid of a molecular phylogenetic study using *rbc*L sequences.

MATERIALS AND METHODS

Specimens of *Gelidium tenuifolium* were collected at Touji (25.ix.1996) and Shirahama (25.ix.1996, 28.iii.1998), Shimoda, Shizuoka Prefecture and Naminoura (29.ix.1997), Kushimoto, Wakayama Prefecture (Fig. 44). Specimens of *G. koshikianum* were collected at Nagahama (31.vii.1997), Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture and Yamatategami (2.viii.1997), Makurazaki, Kagoshima Prefecture (Fig. 44). The majority of materials were fixed and preserved in 10% formalin-seawater. Some were dried as voucher herbarium specimens and were deposited in the Herbarium of the Graduate School of Science, Hokkaido University, Sapporo (SAP 070862-070877). Some plants were transported live to Hokkaido University for culture studies. Unialgal cultures were established from excised apical tips of branchlets of plants and grown in PES medium (Provasoli 1968) at 15°C, 16:8 h L:D (light:dark) with the photon flux density of 15-25 $\mu Em^{-2}s^{-1}$.

Sections were made by hand using a razor blade. Tissues of fixed materials were stained with 0.5% (w/v)

cotton blue in a lactic acid/phenol/glycerol/water (1:1:1:1) solution and mounted in 50% glycerol-seawater on microscope slides.

Methods for total DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of the *rbc*L gene were as described in Chapter 1. For this study, I determined six *rbc*L sequences: *Gelidium linoides* Kützing collected at Shirahama, Shimoda, Shizuoka Prefecture (25.ix.1996)(AB030622); *Gelidium elegans* Kützing collected at Touji, Shimoda, Shizuoka Prefecture (25.ix.1996)(AB030623); *Gelidium koshikianum* collected at Nagahama, Shimo-Koshiki Island, Koshiki Islands (31.vii.1997)(AB030626); *Gelidium pacificum* Okamura collected at Enoshima, Kanagawa Prefecture (29.iii.1997)(AB030627); and *Gelidium tenuifolium* collected at Touji, Shimoda, Shizuoka Prefecture (25.ix.1996, #37) and at Shirahama, Shimoda, Shizuoka Prefecture (28.iii.1997, #139)(AB030628). Twenty-one additional species, i.e. *Gelidium pusillum* (Japanese material)(AB017679), *Gelidium capense* (Gmelin) Silva (L22461), *Gelidium coulteri* Harvey (U00105), *Gelidium micropterum* Kützing (U00446), *Suhria vittata* (Linnaeus) J. Agardh (U00112), *Gelidium sesquipedale* (Clemente) Thuret (L22071), *Gelidium pulchellum* (Turner) Kützing (U01822), *Gelidium canariense* (Grunow) Seoane-Camba (L22460), *Gelidium latifolium* (Greville) Bornet et Thuret (U00112), *Gelidium attenuatum* (Turner) Thuret (U00110), *Gelidium floridanum* Taylor (U00106), *Gelidium purpurascens* Gardner (U00979), *Gelidium serrulatum* J. Agardh (U01042), *Gelidium americanum* (Taylor) Santelices (L22459), *Gelidium*

abbottiorum Norris (U16829), *Gelidium pteridifolium* Norris, Hommersand et Fredericq (U16833), *Gelidium robustum* (Gardner) Hollenberg et Abbott (U01041), *Gelidium allanii* Chapman (L22458), *Gelidium elegans* from Chiba Prefecture (U16830), *Gelidium pacificum* from Chiba Prefecture (U16832) and *Ptilophora pinnatifida* (J. Agardh) Norris (U16834) were downloaded from GenBank and included in the alignment. *Ptilophora pinnatifida* was used as an outgroup.

The distance matrix, maximum parsimony, and maximum likelihood methods were used to construct phylogenetic trees. For the distance matrix method, I used Kimura's (1980) two-parameter method to calculate the distance matrix and neighbor-joining (NJ) method (Saitou & Nei 1987) to construct the tree using the CLUSTAL W computer program (Thompson *et al.* 1994; Higgins *et al.* 1996). Bootstrap analysis based on 100 resamplings of the data set (Felsenstein 1985) was calculated to evaluate statistical reliability. Maximum parsimony (MP) analysis was implemented with the PAUP program (version 3.1.1.; Swofford 1993) using heuristic search. Bootstrap analysis (100 replications) was made using PAUP. The maximum likelihood (ML) method was implemented with the fastDNAml program (version 1.0.6c; Olsen *et al.* 1994) using the global search option.

RESULTS

Gelidium tenuifolium sp. nov.

Axes erecti, 15-30 cm alti, cartilaginei, purpureorubri ad brunneoli-rubri, teres ad subteres, 480-720 μm diametro in parte basali, sursum compressescentes, attingentes 1.4-2.0 mm in latitudine et 280-340 μm in crassitudine in parte medio, oppositim vel alternatim-distichim ramosi 4-plo vel 5-plo; rami laterales numerosi breves, simplices, determinati sed aliqui indeterminati; rami indeterminati ordinis primae ad tertiae teres ad subteres, 400-560 μm diametro in parte proximali, sursum compressescentes ad complanatescentes, attingentes usque ad 2 mm in latitudine et 60-80 μm in crassitudine in parte distali; axes et rami indeterminati apicibus obtusis; cullulae apicales axium et ramorum indeterminatorum vulgo immersae in depressione apicali. Tetrasporangia irregulariter disposita ad apices ramorum determinatorum brevium, cruciatim vel decussatim divisa, 44-60 μm longa et 24-40 μm lata; cystocarpia facta in parte medio ramorum determinatorum brevium; spermatangia ignota.

Erect axes 15-30 cm tall, cartilaginous, purplish to brownish-red, terete to subterete, 480-720 μm in diameter in the basal region, becoming compressed upward, reaching 1.4-2.0 mm in width and 280-340 μm in thickness in the mid-region, oppositely or alternate-distichously branched 4 or 5 times; many lateral branches short, undivided, determinate but a few indeterminate; indeterminate first- to third-order branches terete to subterete, 400-560 μm in diameter in the proximal region, becoming compressed to flattened upward, reaching up to 2 mm in width and 60-80 μm in thickness in

the distal portion; axes and indeterminate branches having obtuse apices; apical cells of axes and indeterminate branches usually immersed in the apical depression. Tetrasporangia irregularly disposed at the apices of short determinate branches, cruciately or decussately divided, 44-60 μm long and 24-40 μm wide; cystocarps formed in the middle portion of short determinate branches; spermatangia unknown.

Holotype and type locality: A cystocarpic specimen (SAP 070868; Fig. 2), collected at Shirahama, Shizuoka Prefecture, on 28 March 1998 by S. Shimada.

Etymology: The specific epithet refers to the thin upper portions of branches.

Japanese name: Usuba-tengusa.

Plants forming tufts on bedrock in the lower intertidal to upper subtidal zones. They are 15-30 cm tall (Fig. 45, 46), cartilaginous, and purplish to brownish red in color. Erect axes arise from creeping axes, which attach to the substratum by brushlike secondary rhizoidal attachments (Fig. 47) that are up to 680 μm in length and 10-14 μm in diameter. The creeping axes are subterete, up to 1.7 cm in length and 300-400 μm in diameter and are branched irregularly. Erect axes are terete to subterete, 480-720 μm in diameter in the basal region, becoming compressed upward, reaching 1.4-2.0 mm wide and 280-340 μm thick in the mid-region and becoming flattened distally (1.8 mm wide and 80-100 μm thick). They are oppositely or alternate-distichouly branched 4 or 5 times. The vast majority of laterals become

short simple determinate branches and a few of them grow into indeterminate branches (Fig. 48). Indeterminate first- to third-order branches are terete to subterete, 400-560 μm in diameter in the proximal region, becoming compressed to flattened upward, reaching up to 2 mm wide and 60-80 μm thick in the distal portion. Axes and indeterminate laterals have obtuse apices.

A dome-shaped apical cell is evident at the apices of axes and branches, as is typical of the Gelidiales. The majority of apical cells of axes and indeterminate branches are immersed in the apical depression (Fig. 49). Basal portions of axes (Fig. 50) consist of a medulla composed of a few large cells up to 80 μm in diameter and numerous rhizines (slender, thick-walled, internal, hyphalike filaments), and a cortex composed of 3 or 4 layers of smaller cells 4-8 μm in diameter. Distal portions of branches consist of a medulla composed of 8-12 layers of cells up to 16 μm in diameter and a cortex composed of 1 or 2 layers of smaller cells 2-4 μm in diameter, and there is no rhizines (Fig. 51).

Tetrasporangia are irregularly disposed at the apices of short determinate branches (Fig. 52). Cruciately or decussately divided sporangia are 44-60 μm long by 24-40 μm wide (Fig. 53). Cystocarps are formed in the middle portion of short determinate branches (Fig. 54), and are bilocular, having a distinct ostiole in each side. Carposporangia are 40-72 μm long by 16-40 μm wide (Fig. 55). Spermatangia were not found in our specimens.

***Gelidium koshikianum* sp. nov.**

Axes erecti, 5-8 cm alti, cartilaginei, aurantiaci ad purpureorubri, teres ad subteres, 320-480 μm diametro in parte basali, sursum compressescentes, attingentes 2.5 mm in latitudine et 240-360 μm in crassitudine in parte medio, ferentes ramos laterales ordinum usque ad trium in modo opposito in intervallis 0.8-1.4 mm; rami indeterminati ordinis primae teres ad subteres, 400-640 μm diametro in parte proximali, sursum compressescentes, attingentes usque ad 1.6 mm in latitudine et 200-240 μm in crassitudine in parte medio. Tetrasporangia irregulariter disposita ad apices ramorum determinatorum brevium, cruciatim vel decussatim divisa, 48-64 μm longa et 20-44 μm lata; cystocarpia facta in parte medio ramorum determinatorum brevium; spermatangia ignota.

Erect axes 5-8 cm tall, cartilaginous, orange to purplish red, terete to subterete, 320-480 μm in diameter in the basal region, becoming compressed upward, reaching up to 2.5 mm in width and 240-360 μm in thickness in the mid-region, bearing lateral branches of up to three orders in an opposite manner at intervals of 0.8-1.4 mm; first-order indeterminate branches terete to subterete, 400-640 μm in diameter in the proximal region, becoming compressed upward, reaching up to 1.6 mm in width and 200-240 μm in thickness in the mid-region. Tetrasporangia irregularly disposed at the apices of short determinate branches, cruciately or decussately divided, 48-64 μm long and 20-44 μm wide;

cystocarps formed in the middle portion of short determinate branches; spermatangia unknown.

Holotype and type locality: A tetrasporangial specimen (SAP 070874; Fig. 12), collected at Nagahama, Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture, on 31 July 1997 by S. Shimada.

Etymology: The specific epithet refers to the name of Koshiki Islands including the type locality where this species was first collected by the first author.

Japanese name: Satsuma-tengusa.

Plants forming tufts on bedrock in the middle to lower intertidal zones. They are 5-8 cm tall (Fig. 56, 57), cartilaginous, and orange to purplish red in color. Erect axes arise from creeping axes, which attach to the substratum by brushlike secondary rhizoidal attachments (Fig. 58) that are up to 360 μm in length and 10-12 μm in diameter. The creeping axis is subterete, up to 400 μm in diameter and is branched irregularly. Erect axes are terete to subterete, 320-480 μm in diameter in the basal region, becoming compressed upward, reaching up to 2.5 mm wide and 240-360 μm thick in the mid-region. The erect axes produce lateral branches of up to three orders in an opposite manner at intervals of 0.8-1.4 mm. The vast majority of second- and third-order laterals remain short (2.0-3.2 mm) and unbranched. First-order indeterminate branches are terete to subterete, 400-640 μm in diameter in the proximal region, becoming compressed upward, reaching up to 1.6 mm wide and

200-240 μm thick in the mid-region. Apices of axes and branches are acute.

A dome-shaped apical cell is evident at the apices of creeping axes and branches (Fig. 59), as is typical of the Gelidiales. Basal portions of axes consist of a medulla composed of a few large cells 40-60 μm in diameter and numerous rhizines, and a cortex composed of 3 or 4 layers of smaller cells 2-4 μm in diameter. First-order branches consist of a medulla composed of 10 to 14 layers of cells up to 30 μm in diameter and a cortex composed of 1 or 2 layers of smaller cells 2-4 μm in diameter in the mid-region (Fig. 60). Rhizines of first-order branches are abundant in the inner cortex, but rare in the central medulla.

Tetrasporangia are irregularly disposed at the apices of short determinate branches (Fig. 61). Tetrasporangia are divided in a cruciate manner (Fig. 62) or a decussate manner, and some intermediate conditions between these two are found (Fig. 63). Mature tetrasporangia are 48-64 μm long by 20-44 μm wide (Figs 62, 63). Cystocarps are formed in the middle portion of short determinate branches (Fig. 64), and are bilocular, having a distinct ostiole in each side. Carposporangia are 36-64 μm long by 14-30 μm wide (Fig. 65). Spermatangia were not found.

*rbc*L analysis

Two sequences of *G. tenuifolium* (#37 and #139) were completely identical. The phylogenetic tree obtained from NJ analysis is shown in Figure 66. *Gelidium tenuifolium* was

included in the Japanese *Gelidium*-complex clade (99 % bootstrap value) that included three other Japanese *Gelidium* species. *Gelidium linoides* came to the position of the sister group to *G. tenuifolium* (99 % bootstrap value). Four substitutions (0.3% divergence) were found between *Gelidium linoides* and *G. tenuifolium*. *Gelidium koshikianum* was clustered with *G. allanii* (100 % bootstrap value), and this clade came to the position of the sister group to the Japanese *Gelidium*-complex clade (83 % bootstrap value). Six substitutions (0.4% divergence) were found between *Gelidium koshikianum* and *G. allanii*. Intraspecific divergence between Shimoda and Chiba populations of *Gelidium elegans* was 0.2% (three substitutions), while in *Gelidium pacificum* 0.4% divergence (six substitutions) was found. Other two phylogenetic methods (MP and ML) showed the same topology for the branching of Japanese species and *G. allanii*. (Figs 67, 68).

DISCUSSION

Gelidium tenuifolium

Gelidium tenuifolium is one of the largest species in this genus. Some species with large-sized thalli are known in the Pacific ocean: *Gelidium linoides*, *G. pacificum*, *G. elegans* (Japan), *G. latiusculum* Okamura (Taiwan), *G. nudifrons* Gardner, *G. purpurascens* Gardner, *G. robustum* (Gardner) Hollenberg et Abbott (California), *G. asperum* (C. Agardh)

Greville, and *G. australe* J. Agardh (Australia) (Okamura 1934, 1935; Abbott & Hollenberg 1976; Womersley 1994).

Primarily, *G. tenuifolium* is characterized by the nature of upper portion of branches that are wide, flattened and thin, and possess apical depressions that are formed at obtuse apices. Secondarily, it bears short, pinnate, simple determinate branches. Although most branches had apical depressions that are formed at obtuse apices, a few branches possess acute apex. Rodríguez and Collado-Vides (1996) have demonstrated that apex morphology is changeable depending on developmental stages of the thallus, and each species has its own specific apical morphology. The presence of both depressed and acute apices in *G. tenuifolium*, thus, representing presence of branches in different developmental stages. It is important to note that most branches of this species possess apical depressions. Therefore despite the fact that some part of a plant possess acute apices, I regard apical depressions is one of the diagnostic characters for *G. tenuifolium*, because closely related species (*G. linoides*, *G. elegans* and *G. pacificum*) are known to have acute apices only.

Gelidium tenuifolium can be distinguished from other large sized species as follows: *Gelidium linoides* differs from *G. tenuifolium* in having linear branches (200-500 μm in width, Table 9), acute apices of branches and apical cell at apex (Okamura 1934; Table 9); *Gelidium pacificum* and *G. latiusculum* have slightly bulged midribs (Okamura 1934, 1935; Table 9); *Gelidium elegans* differs from *G. tenuifolium* in having darker reddish color, subterete to slightly

compressed branches, acute apices and elongating and branching branchlets (Okamura 1934; Table 9); *Gelidium nudifrons* is compressed to flattened throughout (Abbott & Hollenberg 1976); *Gelidium robustum* possesses thicker (up to 1.7 mm) axes (Abbott & Hollenberg 1976); *Gelidium asperum* and *Gelidium australe* possess subterete to slightly compressed branches (Nelson *et al.* 1994; Womersley 1994).

Freshwater *et al.* (1995) have suggested that certain genera, species and populations of the order Gelidiales can be assembled in groups based on geographical distribution. In this study, too, four *Gelidium* species including *G. tenuifolium* chiefly distributed in Japan are clustered together with 99 % bootstrap value (Japanese *Gelidium*-complex clade).

Gelidium linoides was shown to be most closely related to *G. tenuifolium* morphologically, and four substitutions (0.3% divergence) were found between the two species in *rbc*L sequence. In Gelidiales, intraspecific sequence divergence has been known to be a range of 0.0-1.8%, while the value for interspecific divergence is 0.0-11.5% (Freshwater *et al.* 1994; Bailey & Freshwater 1997; Chapter 2, 3). Thus, the sequence divergence found between *G. tenuifolium* and *G. linoides* falls into either intra- or interspecific variations of other taxa as shown above. As Bird *et al.* (1992) pointed out, the taxonomic significance of molecular sequence divergence must be evaluated on a case-by-case basis, and it is important to interpret these values with the combination of other features such as morphology and geographical distribution when one decide whether these

values indicate species difference or merely an intraspecific difference. In this case, the geographical distributions of *G. tenuifolium* and *G. linoides* almost overlap and they often occur sympatrically: for example, in Shirahama, Shimoda, Shizuoka Prefecture, the two species co-occur. However, the two species are clearly distinguished morphologically in the field as mentioned above (Table 9), and no intermediate forms have been observed. These facts strongly indicate that these two entities are separate species rather than intraspecific taxa. Therefore, the sequence divergence of four base pairs in the *rbc*L sequence is indicative of the species difference.

Gelidium koshikianum

Gelidium koshikianum is one of middle-sized species in this genus. In the Indo-Pacific regions and Carribbean Sea, a number of middle-sized species have been reported. These include *G. tenue* Okamura, *G. vagum*, *G. yamadae* Fan (Japan), *G. planiusculum* Okamura, *G. kintaroi* (Okamura) Yamada (Taiwan), *G. americanum* (Taylor) Santelices, *G. serrulatum* J. Agardh, *G. johnstonii* Setchell et Gardner, *G. floridanum* Taylor (Pacific coast of America and Carribbean Sea), *G. abbottiorum* Norris (South Africa), and *G. allanii* Chapman (New Zealand) (Setchell & Gardner 1924; Gardner 1927; Okamura 1934, 1935; Taylor 1943, 1960; Segi 1955; Chapman 1969; Abbott & Hollenberg 1976; Stewart & Norris 1981; Norris 1990; Rodríguez 1991).

Gelidium koshikianum can be distinguished from other middle-sized species as follows: *G. tenue* possesses slightly broader lateral branches (up to 2 mm) and lacks short second- and third-order determinate branches (Okamura 1934); *G. vagum* has twisted axes and branches (Okamura 1934); *G. yamadae* has densely arranged branches (Okamura 1935, as *G. densum* Okamura; Fan 1951); *G. planiusculum* has numerous, linear branches (Okamura 1935); *G. kintaroi* has clavate branches (Okamura 1934, as *G. clavatum* Okamura; Segi 1955); *G. americanum* has obtuse apices of branches and lacks short second- and third-order determinate branches (Taylor 1943; Santelices 1976; Rodríguez 1991); *G. serrulatum* has serrate branchlets (Rodríguez 1991; Taylor 1960); *G. johnstonii* has glossy color and flattened branchlets (Setchell & Gardner 1924; Segi 1955; Stewart & Norris 1981); *G. floridanum* has very notable congestion of reproductive branchlets (Taylor 1943; Rodríguez 1991); *G. abbottiorum* has longer branching intervals (3-4 mm) (Norris 1990); and *G. allanii* has very prominent apical cells, narrower axes (up to 0.5 mm), longer second- and third-order branches and tetrasporangia formed in elongate patches with sterile margins (Chapman 1969; Adam 1994; Nelson *et al.* 1994; Table 9).

Gelidium allanii was shown to be most closely related to *G. koshikianum* in the analysis of *rbc*L sequences, and six substitutions were found. *Gelidium allanii* was described from New Zealand and found at only one locality at the time of establishment (Chapman 1969). To date, it has been found from six localities in New Zealand and thought to be endemic to northern New Zealand with a highly localized distribution

(Nelson *et al.* 1994). The geographical distributions of both species, therefore, are widely separated from each other. Although our molecular data indicate that these two entities are closely related, the vegetative and reproductive morphology of *G. koshikianum* and *G. allanii* can be clearly distinguished as mentioned above (Table 9). Taking these clear differences in morphology into consideration, I conclude that these entities should be treated as independent species.

Chapter 6

Chapter 6. First report of *Gelidiella ligulata*, *Gelidiella pannosa*, *Pterocladiella caerulescens* and *Pterocladiella caloglossoides* in Japan

INTRODUCTION

A number of tropical species of *Gelidiella* and *Pterocladiella* (*Pterocladia*) have been reported in the Pacific Ocean (Dawson 1954; Santelices 1977; Hatta & Prud'homme 1991; Price & Scott 1992). However, only one species, *Gelidiella acerosa* (Forsskål) Feldmann et Hamel, has been reported in Japan (Yamada & Tanaka 1938; Segawa & Kamura 1960; Akatsuka 1973; Ohba & Aruga 1982). In this study, four tropical species, *Gelidiella ligulata* Dawson, *Gelidiella pannosa* (Feldmann) Feldmann et Hamel, *Pterocladiella caerulescens* (Kützing) Santelices et Hommersand and *Pterocladiella caloglossoides* (Howe) Santelices are newly reported in Japanese waters from Miyake Island and Yaeyama Islands. Their phylogenetic positions within the order Gelidiales are discussed with the aid of molecular phylogenetic study using nuclear-encoded SSU rDNA sequences and secondary rhizoidal attachments.

MATERIALS AND METHODS

Specimens of *Gelidiella ligulata* were collected at Izu-misaki (13.vii.1998) and Benkene-misaki (14.vii.1998), Miyake Island (Fig. 69). Specimens of *Gelidiella pannosa* were collected at Agarizaki (24°27'30"N, 122°02'40"E;

3.iii.1999), Yonaguni Island, and Oohama (24°20'10"N, 124°12'00"E; 4.iii.1999) and Nosoko (24°30'40"N, 124°15'00"E; 6.iii.1999), Ishigaki Island, Okinawa Prefecture. Specimens of *Pterocladiella caerulescens* were collected at Sonai (24°27'55"N, 123°00'10"E; 1.iii.1999), Irizaki (24°26'40"N, 122°54'00"E; 2.iii.1999) and Agarizaki (3.iii.1999), Yonaguni Island, and Oohama (4.iii.1999), Ishigaki Island. Specimens of *Pterocladiella caloglossoides* were collected at Oohama (4.iii.1999), Ishigaki Island. The majority of specimens were fixed and preserved in 10% formalin-seawater and then, some were dried, or mounted in 30% Karo on microscope slides as voucher specimens that are deposited in the Herbarium of the Graduate School of Science, Hokkaido University, Sapporo (SAP 063883-063886, 071771-071778). Some plants were transported live to Hokkaido University for culture studies. Unialgal cultures were established from excised apical tips of branchlets of plants and were grown in Tris-buffered medium (van der Meer & Patwary 1991) at 20°C, 16:8 h L:D (light:dark) with the photon flux of 15-25 $\mu Em^{-2}s^{-1}$.

Methods for total DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing of the SSU rDNA gene were as described in Chapter 1. For this study, I determined four sequences: #326 *Gelidiella pannosa* collected at Nosoko, Ishigaki Island (AB031300); #321 *Pterocladiella caerulescens* collected at Sonai, Yonaguni Island (AB031301); #328 *Pterocladiella caloglossoides* collected at Oohama, Ishigaki Island (AB031302); #83 *Pterocladiella nana* (Okamura) Shimada, Horiguchi et Masuda collected at Teuchi,

Shimo-Koshiki Island, Koshiki Islands (31.vii.1997)(AB031303). Additional 13 species, i.e. *Gelidium latifolium* (Greville) Bornet et Thuret (U60350), *Gelidium americanum* (Taylor) Santelices (U60347), *Acanthopeltis japonica* Okamura (AB017664), *Capreolia implexa* Guiry et Womersley (U60344), *Ptilophora subcostata* (Okamura) Norris (U60348), *Ptilophora pinnatifida* (J. Agardh) Norris (U60345), *Pterocladiella capillacea* (Gmelin) Santelices et Hommersand (AB017672), *Gelidiella ligulata* Dawson (AB017669), *Gelidiella acerosa* (Forsskål) Feldmann et Hamel (U60342), *Gracilaria tikvahiae* MacLachlan (M33640), and *Chondrus crispus* Stackhouse (Z14140) were downloaded from GenBank and included in the alignment. *Gracilaria tikvahiae* and *Chondrus crispus* were used as outgroups. SSU rDNA sequences were first aligned with the CLUSTAL W computer program (Thompson *et al.* 1994; Higgins *et al.* 1996) and then refined by eye. The alignments are available from the first author upon request. The distance matrix method was used to construct phylogenetic tree. For the distance matrix method, I used Kimura's (1980) two-parameter method to calculate the distance matrix and neighbor-joining (NJ) method (Saitou & Nei 1987) to construct the tree using the CLUSTAL W computer program (Thompson *et al.* 1994; Higgins *et al.* 1996). Bootstrap analysis based on 100 resamplings of the data set (Felsenstein 1985) was calculated to evaluate statistical reliability.

RESULTS

***Gelidiella ligulata* Dawson 1953: 81, pl. 3, figs 3-5**

Type locality: Cabeza Ballena, Baja California.

Distribution: Tropical regions in the Pacific (Kraft & Abbott 1998).

Japanese name: Sasaba-shimatengusa (new name).

Plants tuft on bedrock in the middle intertidal zone of sheltered shores or in tidal pools. They are up to 4.5 cm tall (Fig. 70) and are dark red to purplish red in color. Individual plants consist of a creeping axis and erect blades. The creeping axis attaches to the substratum by unicellular independent attachments (Figs 71, 72) that are 50-240 μm in length and 10 μm in diameter. The creeping axis is subterete, 300-500 μm in diameter and is branched irregularly. 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 (Fig. 73), but become lanceolate with age (1-3 mm wide, 100-270 μm thick). They are usually simple, but are sometimes irregularly to dichotomously branched (Fig. 74). Blade margins are undulate and sometimes ruffled. Subterete to lanceolate proliferations issue from both sides of blades pinnately (Fig. 74), injured (perhaps grazed) ends of blades (Fig. 75) and blade surfaces. A dome-shaped apical cell is evident at the apices of creeping axes (Fig. 76) and erect blades, as is typical of the Gelidiales. Both creeping axes and erect blades (Fig. 77) 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 (slender, thick-walled, internal, hyphalike filaments) are absent throughout creeping axes and erect blades. Reproductive structures were not found in our specimens.

***Gelidiella pannosa* (Feldmann) Feldmann et Hamel 1934: 534, f. 1, 2**

Basionym: *Echinocaulon pannosum* Feldmann (1931).

Type locality: Biarritz, France.

Distribution: Tropical to temperate coasts in the world (Kraft & Abbott 1998).

Japanese name: Ito-shimatengusa (new name).

Plants grow gregariously on bedrock in the upper intertidal zone and are dark red. Individual plants consist of a creeping axis and numerous erect axes (Fig. 78), both of which are terete to subterete. Creeping axes attach to the substratum by unicellular independent attachments (Fig. 79) that are up to 200 μm long by 10-25 μm wide. They are 80-135 μm in diameter and are branched irregularly. 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.

A dome-shaped apical cell is evident at the apices of creeping axes and erect axes (Fig. 80), as is typical of the Gelidiales. 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

(Fig. 81). Rhizines (slender, thick-walled, internal, hypha-like filaments) are absent throughout the thalli.

Tetrasporangia and sexual reproductive structures were not found in our specimens.

***Pterocladiella caerulescens* (Kützing) Santelices et Hommersand 1997: 118**

Basionym: *Gelidium caerulescens* Kützing (1868).

Type locality: Vieillard, New Caledonia.

Distribution: Tropical regions in the Pacific (Santelices 1998).

Japanese name: Ao-obakusa (new name).

Plants tuft on bedrock in the upper intertidal zone, are up to 3.3 cm tall and greyish to blackish green in color. Individual plants consist of a creeping axis and numerous erect axes (Fig. 82). Erect axes arise from a creeping axis, which attach to the substratum by peglike secondary rhizoidal attachments (Fig. 83) that are up to 360-480 μm in length and 95-145 μm in diameter. The creeping axes are subterete, 200-350 μm in diameter and are sparingly branched. Erect axes 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-distichouly 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.

A dome-shaped apical cell is evident at the apices of axes and branches, as is typical of the Gelidiales. The majority of apical cells of axes and branches are immersed in the apical depression (Fig. 84). 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 (Fig. 85). Rhizines of first-order branches are abundant in the central medulla.

Tetrasporangia are irregularly disposed at the apices of axes and branches (Fig. 86). Cruciately or decussately divided sporangia are 24-36 μm long by 44-64 μm wide (Figs 87, 88).

Cystocarps are formed near the apices of branches (Fig. 89). Nutritive filaments grow centripetally and form a virtually solid cylinder around the central axis (Fig. 90). A cystocarp is attached to one side of the cystocarp floor and produces chains of carposporangia from the remaining three sides (Fig. 91). Carposporangia are 20-30 μm long by 14-22 μm wide.

The majority of cystocarpic plants bear spermatangial sori on the cystocarpic branchlets. Some of them produce spermatangial sori on spermatangial branchlets (Fig. 92) that are independently formed from cystocarpic branchlets on the erect axes. A few cystocarpic plants bear a special erect axis that produce only spermatangial branchlets (Fig. 93). These three types of spermatangial sori are formed on

different individuals and/or the same ones. Two spermatangia are cut off from an elongated spermatangial mother cell, and are 2-3 μm in diameter (Figs 94, 95).

***Pterocladiella caloglossoides* (Howe) Santelices 1998: 244**

Basionym: *Gelidium caloglossoides* Howe (1914).

Type locality: San Lorenzo Island, Peru.

Distribution: Tropical regions in the Pacific and Indian ocean (Santelices 1998).

Japanese name: Hime-obakusa (new name).

Plants grow gregariously on bedrock in the upper intertidal zone and are purplish red. Individual plants consist of a creeping axis and erect axes (Fig. 96). Creeping axes are subterete to compressed, 110-160 μm in diameter, attach to the substratum by peglike attachments (Fig. 97) 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.

A dome-shaped apical cell is evident at the apices of creeping axes and erect axes (Fig. 98), as is typical of the Gelidiales. 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. 99). Rhizines of first-order branches are rare in the central medulla.

Tetrasporangia are regularly arranged in transverse rows (Fig. 100), 8-16 per row, and are ovate in surface view, 16-32 μm in diameter. They are divided cruciately or decussately (Fig. 101). Sexual reproductive structures were not found in our specimens.

SSU rDNA analysis

The phylogenetic tree obtained from NJ analysis is shown in Figure 102. *Gelidiella ligulata* and *G. pannosa* was included in the *Gelidiella* clade (100 % bootstrap value) with *G. acerosa*. *Pterocladiella caerulescens* and *P. caloglossoides* were included in the *Pterocladiella* clade (99 % bootstrap value), and they were clustered with *Pterocladiella capillacea* and *P. nana* (91 % bootstrap value).

DISCUSSION

Gelidiella ligulata

The genus *Gelidiella* currently includes 22 species that are distinguished from members of other genera in the Gelidiales by the absence of rhizines. The species of *Gelidiella* have been characterized by thallus habit, thallus size (height and diameter/width), axis symmetry, tetrasporangial location and tetrasporangial arrangement (Kraft & Abbott 1998). A few species are known to have flattened thalli: *G. bornetii* (Weber-van Bosse) Feldmann et Hamel (1934), *G. feldmannii* Baardseth (1941), *G. indica* Sreenivasa Rao (1970) and *G.*

ligulata. Of these species, *G. bornetii* and *G. feldmannii* differ from the Japanese material by having very narrow blades up to 300 μm wide (Weber-van Bosse 1926, as *Gelidium bornetii* Weber-van Bosse) and 500 μm wide (Baardseth 1941), respectively. *Gelidiella indica* and *G. ligulata* are more similar to the Japanese material. These two species have lanceolate erect blades. Dawson (1953) did not describe tetrasporangia, but Womersley and Bailey (1969) reported tetrasporangial lateral branches of their material from the Solomon Islands. Similarly, *G. indica* has tetrasporangial lateral branches in the upper to middle part of the erect axes (Sreenivasa Rao 1970, fig. 4c). One marked difference between *G. indica* and *G. ligulata* is the presence/absence of deciduous branches. For *G. ligulata*, Dawson (1953, p. 81) described older blades becoming closely pinnately branched near the extremities, and readily deciduous and apparently serving as a vegetative means of reproduction, whereas Sreenivasa Rao (1970) reported that lateral branches of *G. indica* are not generally deciduous. Pinnate branches that are very similar to those of Dawson's alga (1953, pl. 6, fig. 5) have been found in the Japanese material (Fig. 74), but they are not deciduous. At present the deciduous nature of Dawson's (1953) material, whether those deciduous branches function as propagules or are artifacts during preservation, has not been confirmed. *Gelidiella indica* seems to represent matured stages of *G. ligulata* and the latter (Dawson 1953) has nomenclatural priority over the former (Sreenivasa Rao 1970). However, it is prudent to maintain *G. ligulata* and *G. indica* as separate species until

fully matured plants of the former alga from its type locality are collected and these two algae are thoroughly compared. I refer our material to *G. ligulata* that has nomenclatural priority over *G. indica*.

Gelidiella pannosa

Four small-sized *Gelidiella* have been reported from the western Pacific: *G. adnata* Dawson, *G. bornetii* (Weber-van Bosse) Feldmann et Hamel, *G. myrioclada* (Børgesen) Feldmann et Hamel and *G. pannosa* (Dawson 1954; Hatta & Prud'homme 1991; Kraft & Abbott 1998). *Gelidiella bornetii* can be distinguished from the other three species by the flattened creeping and erect axes (Kraft & Abbott 1998). *Gelidiella myrioclada* is characterized by alternately, oppositely or pectinately branched erect axes (Børgesen 1934). *Gelidiella adnata* is most similar to *G. pannosa*. However, there is marked difference between these two species in two points (Kraft & Abbott 1998). *Gelidiella adnata* has a dense rank of short unicellular independent rhizoids that develop along the entire undersurface and stichidia that are formed on special short erect axes, whereas *G. pannosa* has units of unicellular independent rhizoids in several portions and terminal stichidia on fertile branches. Although I cannot observe tetrasporangial stichidia of Japanese material, it has the *G. pannosa* like attachment (Feldmann and Hamel 1934), and I refer our material to this species.

Pterocladiella caerulescens

Pterocladiella caerulescens is one of monoecious species in the Gelidiales (Santelices & Flores 1995). Spermatangial sori, which are exclusively formed on cystocarpic branchlets, have been reported for this alga together with the following five gelidialean species: *Gelidium howeii* Acleto, *G. mcnabbiana* (Dawson) Santelices, *G. pluma* Loomis, *Gelidium vagum* Okamura, *Onikusa japonica* (Okamura) Akatsuka (Santelices & Flores 1995). *Acanthopeltis japonica* and *Acanthopeltis hirsuta* (Okamura) Shimada, Horiguchi et Masuda are also monoecious, but they have special spermatangial branchlets on the cystocarpic erect axes (Kaneko 1968; Chapter 1). In this study, three types of spermatangial sori were found in Japanese materials of *P. caerulescens* on: i) fertile cystocarpic branchlets; ii) special spermatangial branchlets of a cystocarpic axis; and iii) branchlets of a special spermatangial axis. The latter two types are reported from *P. caerulescens* for the first time.

Pterocladiella caloglossoides

In the original description by Howe (1914), *Pterocladiella caloglossoides* was characterized by the repent flattened thalli throughout, regularly arranged tetrasporangia, and a single row of medullary cells. However, this species shows a wide range of morphological variations: creeping axes being subterete to flattened and the length of erect axes ranging from 0.8 mm to 1.2 cm (Howe 1914; Santelices 1977; Norris 1987). Japanese material is more similar to South African

(Norris 1987) specimens that have more terete creeping axes and longer erect axes than those of the original description. However, all the materials identified as *P. caloglossoides* (Howe 1914; Santelices 1977; Norris 1987) show a regular arrangement of tetrasporangia that is a unique feature of this species among the genus *Pterocladiella*. The Japanese material also has regularly arranged tetrasporangia, so that I refer them to *P. caloglossoides*.

Secondary rhizoidal attachments

Three types of secondary rhizoidal attachments are known in the Gelidiales: i) unicellular independent type; ii) peg type; and iii) brush type (Chapter 1). This study revealed that *Gelidiella ligulata* and *G. pannosa* has the unicellular independent type, and *P. caerulescens* and *P. caloglossoides* have the peg type. Each type of secondary rhizoidal attachments is completely consistent with the corresponding genus clade in SSU rDNA tree with high bootstrap values, which suggests that this morphological character reflects the phylogeny of this order.

References

REFERENCES

ABBOTT I.A. & HOLLENBERG G.J. 1976. *Marine Algae of California.* Stanford University Press, Stanford, 827 pp.

ADAM N.M. 1994. *Seaweeds of New Zealand.* Canterbury University Press, Christchurch, 360 pp.

AKATSUKA I. 1970. Morphology of the cortical layer of some species of Gelidiales. *The bulletin of Japanese Journal of Phycology* **18**: 72-76.

AKATSUKA I. 1973. Marine algae of Ishigaki Island and its vicinity in Ryukyu archipelago. *The bulletin of Japanese Journal of Phycology* **21**: 39-42. (in Japanese)

AKATSUKA I. 1981. Use of names *Pterocladia tenuis* Okamura and *P. densa* Okamura. *Japanese Journal of Phycology* **29**: 272 (in Japanese).

AKATSUKA I. 1982. Preliminary observations and literature analysis of morphological variability in some Japanese species of *Gelidium* (Gelidiaceae, Rhodophyta) and an evaluation of criteria used in their discrimination. *Nova Hedwigia* **36**: 759-774.

AKATSUKA I. 1986. Surface cell morphology and its relationship to other generic characters in non-parasitic Gelidiaceae (Rhodophyta). *Botanica Marina* **29**: 59-68.

AKATSUKA I. & MASAKI T. 1986. *Beckerella irregularis* sp. nov. (Gelidiales, Gelidiaceae) from Japan. *Bulletin of Faculty of Fisheries, Hokkaido University.* **34**: 11-19.

BAARDSETH E. 1941. The marine algae of Tristan da Cunha. *Res. Norw. Sci. Exped. Tristan da Cunha* **9**: 1-173.

BAILEY J.C. & FRESHWATER D.W. 1997. Molecular systematics of the Gelidiales: inferences from separate and combined analyses of plastid *rbc*L and nuclear SSU rDNA gene sequences. *European Journal of Phycology* **32**: 343-352.

BIRD C.J., RICE E.L., MURPHY C.A. & RAGAN M.A. 1992. Phylogenetic relationships in the Gracilariales (Rhodophyta) as determined by 18S rDNA sequences. *Phycologia* **31**: 510-522.

BØRGESEN F. 1934. Some Indian Rhodophyceae especially from the shores of the Presidency of Bombay: IV. *Bull. Musc. Inf., Royal Botanic Gardens, Kew* **1934**: 1-30.

CHAPMAN V.J. 1969. *The marine algae of New Zealand. Part III: Rhodophyceae Issue 1: Bangiophycidae and Florideophycidae (Nemalionales, Bonnemaisoniales, Gelidiales)*. J. Cramer, Lehre, 113 pp, 38 pls.

CHOPIN T., BIRD C.J., MURPHY C.A., OSBORNE J.A., PATWARY M.U. & FLOC'H J.-Y. 1996. A molecular investigation of polymorphism in the North Atlantic red algae *Chondrus crispus* (Gigartinales). *Phycological Research* **44**: 69-80.

DAWSON E.Y. 1953. Marine red algae of Pacific Mexico. Part 1. Bangiales to Corallinaceae subf. Corallinoideae. *Allan Hancock Pacific Expeditions* **17**: 1-171.

DAWSON E.Y. 1954. Marine plants in the vicinity of the Institut Océanographique de Nha Trang, Viêt Nam. *Pac. Sci.* **8**: 373-469

DAWSON E.Y. 1959. Changes in Palmyra Atoll and its vegetation through the activities of man. *Pacific Naturalist* **1**: 1-51.

De Gregorio S. & Perrone C. 1994. Rhizoid ontogenesis in *Gelidiella* Feldmann et Hamel (Gelidiales, Rhodophyta). *Giorn. Bot. Ital.* **128**: 1085-1087.

Dixon P.S. & Irvine L.M. 1977. *Seaweeds of the British Isles*, Vol. 1, *Rhodophyta*, part 1, *Introduction, Nemaliales, Gigartinales*. British Museum (Natural History) London. xi + 252pp.

Fan K.-C. 1961. Morphological studies of the Gelidiales. *University of California Publications in Botany* **32**: 315-368.

Feldmann J. 1931. Notes sur quelques Algues marines de Tunisie. *Station Océanography Salammbô*, Notes **24**: 1-20.

Feldmann J. & Hamel G. 1934. Observations sur quelques Gelidiacées. *Revue Génnerale de Bottanique* **46**: 528-49.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using bootstrap. *Evolution* **39**: 783-791.

Freshwater D.W., Fredericq S., Butlar B.S., Hommersand M.H. & Chase M.W. 1994. A gene phylogeny of the red algae (Rhodophyta) based on plastid *rbc*L. *Proceedings of the National Academy of Sciences of the United States of America* **91**: 7281-7285.

Freshwater D.W., Fredericq S. & Hommersand M.H. 1995. A molecular phylogeny of the Gelidiales (Rhodophyta) based on the analysis of plastid *rbc*L nucleotide sequences. *Journal of Phycology* **31**: 616-632.

Freshwater D.W. & Rueness J. 1994. Phylogenetic relationships of some European *Gelidium* (Gelidiales, Rhodophyta) species, based on *rbc*L nucleotide sequence analysis. *Phycologia* **33**: 187-194.

GARDES M., WHITE T.J., FORTIN J.A., BRUNS T.D. & TAYLOR J.W. 1991. Identification of indigenous and introduced symbiotic fungi in ectomycorrhizae by amplification of nuclear and mitochondrial ribosomal DNA. *Canadian Journal of Botany* **69**: 180-190.

GARDNER N.L. 1927. New species of *Gelidium* in the Pacific coast of North America. *University of California Publcations in Botany* **13**: 273-318.

GARRIGA G., BERTRAND, H. & LAMBOWITZ, A.M. 1984. RNA splicing in *Neurospora* mitochondria: nuclear mutants defective in both splicing and 3′ end synthesis of the large rRNA. *Cell* **36**: 623-634.

GMELIN S.G. 1768. *Historia Fucorum*. 4 pls., 239 pp., 35 pls. St. Petersburg.

GOFF L.F., MOON D.A. & COLEMAN A.W. 1994. Molecular delineation of species and species relationships in the red algal agarophytes *Gracilariopsis* and *Gracilaria* (Gracilariales). *Journal of Phycology* **30**: 521-537.

GUIRY M.D. & WOMERSLEY H.B.S. 1993. *Capreolia implexa* gen. et sp. nov. (Gelidiales, Rhodophyta) in Australia and New Zealand; an intertidal mat-forming alga with an unusual life history. *Phycologia* **32**: 266-277.

HATTA A.M. & PRUD'HOMME VAN REINE W.F. 1991. A taxonomic revision of Indonesian Gelidiales (Rhodophyta). *Blumea* **35**: 347-380.

HIGGINS D.G., THOMPSON J.D. & GIBSON T.J. 1996. Using CLUSTAL for multiple sequence alignments. *Methods in Enzymology* **266**: 383-402.

HOWE M.A. 1914. The marine algae of Perú. *Memories of the Torrey Botanical Club* **15**: 1-185.

HURTADO-PONCE A.Q., CHAVOSO E.A.J. & PARAMI N.P. 1998. Assessment of the seaweed-seagrass resource of Mararison Island, Cilasi, Antique, Philippines. *Phycological Research* **46**: 175-181.

KANEKO T. 1968. Morphological and developmental studies of Gelidiales II. On *Acanthopeltis japonica* Okamura. *Bulletin of Faculty of Fisheires, Hokkaido University* **19**: 165-172.

KAWAHARA T., MURAKAMI N., SETOGUCHI H. & TSUMURA Y. 1995. Procedures of plant DNA extraction for phylogenetic analysis. *Proceedings of the Japan Society of Plant Taxonomists* **11**: 13-32. (in Japanese with English abstract)

KIMURA M. 1980. A simple method for estimating rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* **16**: 111-120.

KRAFT G.T. & ABBOTT I.A. 1998. *Gelidiella womersleyana* (Gelidiales, Rhodophyta), a diminutive new species from the Hawaiian Islands. *Botanica Marina* **41**: 51-61.

KÜTZING F.T. 1868. *Tabulae Phycologicae*. Vol. 18, 35 pp., 100 pls. Published by the author, Nordhausen.

LECLERC M.C., BARRIEL V., LECOINTRE G. & DE REVIERS B. 1998. Low diveregence in rDNA ITS sequences among five species of Fucus (Phaeophyceae) suggests a very recent radiation. *Journal of Molecular Evolution* **46**: 115-120.

LEE H.-B. & KIM J.-I. 1995. Notes on Gelidiales species from Korea. In: *Taxonomy of Economic Seaweeds*. Vol. V (Ed. by

I.A. Abbott), pp. 161-174. California Sea Grant College Program, La Jolla, CA.

MAGGS C.A. & GUIRY M.D. 1987. *Gelidiella calcicola* sp. nov. (Rhodophyta) from the British Isles and Northern France. *British Phycological Journal* **22**: 417-434.

NAKAYAMA T., WATANABE S., MITSUI K., UCHIDA H. & INOUYE I. 1996. The phylogenetic relationship between the Chlamydomonadales and Chlorococcales inferred from 18S rDNA sequence data. *Phycological Research* **44**: 47-55.

NELSON W.A., KNIGHT G.A., FALSHAW R., FURNEAUX R.H., FALSHAW A. & LYNDS S.M. 1994. Characterisation of the enigmatic, endemic red alga *Gelidium allanii* (Gelidiales) from northern New Zealand-morphology, distribution, agar chemistry. *Journal of Applied Phycology* **6**: 497-507.

NORRIS R.E. 1987. *Pterocladia* (Gelidiales, Rhodophyta), a genus previously unknown in South Africa, as it occurs in Natal. *South African Journal of Botaty* **53**: 39-43.

NORRIS R.E. 1990. A critique on the taxonomy of an important agarophyte *Gelidium amansii*. *Japanese Journal of Phycology* **38**: 35-42.

NORRIS R.E. 1992. A proposed phylogenetic scheme for the Gelidiales. In: *Taxonomy of Economic Seaweeds*. Vol. III (Ed. by I.A. Abbott), pp. 151-171. California Sea Grant College Program, La Jolla, CA.

OHBA H. & ARUGA Y. 1982. Seaweeds from Ishigaki Island and adjacent islets in Yaeyama Islands, southern Japan. *Japanese Journal of Phycology* **30**: 325-431.

OKAMURA K. 1900. *Illustrations of the Marine Algae of Japan*. Vol. I, No. 1, pp. 1-14, pls. 1-5. Tokyo.

OKAMURA K. 1901. *Illustrations of the Marine Algae of Japan.* Vol. I, No. 2, pp. 15-28, pls. 6-10. Keigyosha, Tokyo.

OKAMURA K. 1913. *Icones of Japanese Algae.* Vol. 3(3), pp. 39-54, pls 111-115. Published by the author, Tokyo.

OKAMURA K. 1932. *Icones of Japanese Algae.* Vol. 6(6), pp. 49-62, pls 276-280. Published by the author, Tokyo.

OKAMURA K. 1934. On *Gelidium* and *Pterocladia* of Japan. *Journal of the Imperial Fisheries Institute* **29**: 47-67, pls 16-33.

OKAMURA K. 1935. On *Gelidium* species of Taiwan. *Nippon Gakujutsu Kyokai Hokoku.* **10**: 441-443 (in Japanese).

OKAMURA K. 1936. *Nippon Kaiso-shi.* Uchida Rokakuho, Tokyo. 9 + 6 + 964 + 11 pp.

OLSEN G.J., MATSUDA H., HAGSTROM R. & OVERBEEK R. 1994. fastDNAml: a tool for construction of phylogenetic tree od DNA sequences using maximum likelifood. *Computer Applications in the Biosciences* **10**: 41-48.

PATWARY M.U., SENSEN C.W., MACKAY R.M. & VAN DER MEER J.P. 1998. Nucleotide sequences of small-subunit and internal transcribed spacer regions of nuclear rRNA gene support the autonomy of some genera of the Gelidiales (Rhodophyta). *Journal of Phycology* **34**: 299-305.

PERRONE C. 1994. Diagnostic and taxonomic value of the rhizoids in the Gelidiales: some considerations. *Giornale Botanico Italiano* **128**: 1088-1091.

PRICE I.R. & SCOTT F.J. 1992. *The turf algal flora of the Great Barrier Reef. Part 1. Rhodophyta.* James Cook University of North Queensland, Townsville. xii + 266 pp.

PROVASOLI L. 1968. Media and prospects for the cultivation of marine algae. In: *Cultures and Collections of Algae.* (Ed. by Watanabe A. & Hattori A.), pp. 63-75. Proceedings of the United States Japan Conference, The Japanese Society of Plant Physiologists, Tokyo.

RAGAN M.A., BIRD C.J., RICE E.L., GUTELL R.R., MURPHY C.A. & SINGH R.K. 1994. A molecular phylogeny of the marine red algae (Rhodophyta) based on the nuclear small-subunit rRNA gene. *Proceedings of the National Academy of Sciences of the United States of America* **91:** 7276-7280.

RODRÍGUEZ D. & COLLADO-VIDES L. 1996. Architectural models for apical patterns in *Gelidium* (Gelidiales, Rhodophyta): hypothesis of growth. *Phycological Research* **44:** 95-100.

RODRÍGUEZ D. & SANTELICES B. 1987. Patterns of apical structure in the genera *Gelidium* and *Pterocladia* (Gelidiales, Rhodophyta). *Hydrobiologia* **151/152:** 199-203.

RODRÍGUEZ D. & SANTELICES B. 1988. Separation of *Gelidium* and *Pterocladia* on vegetative characters. In: *Taxonomy of Economic Seaweeds.* Vol. II (Ed. by I.A. Abbott), pp. 115-125. California Sea Grant College Program, La Jolla, CA.

RODRÍGUEZ DE RIOS N. 1992. Estudios taxonomicos en agarofitas de Venezuela II. Notas sobre el genero *Pterocladia* J. Agardh (Rhodophyta, Gelidiales). *Ernstia* 2: 77-93.

RODRÍGUEZ N. 1991. Estudios taxonomicos en agarofitas de Venezuela I. Notas sobre el genero *Gelidium* Lamouroux (Rhodophyta, Gelidiales). *Ernstia* **1:** 5-20.

SAIKI R.K., GELFAND D.H., STOFFEL S., SCHARF S.J., HIGUCHI R., HORN G.T., MULLIS K.B. & ERLICH H.A. 1988. Primer-directed

enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**: 487-491.

SAITOU N. & NEI M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and Evolution* **4**: 406-425.

SANTELICES B. 1976. Taxonomic and nomenclatural notes on some Gelidiales (Rhodophyta). *Phycologia* **15**: 165-173.

SANTELICES B. 1977. A taxonomic review of Hawaiian Gelidiales (Rhodophyta). *Pacific Science* **31**: 61-84.

SANTELICES B. 1978. The morphological variation of *Pterocladia caerulescens* (Gelidiales, Rhodophyta) in Hawaii. *Phycologia* **17**: 53-59.

SANTELICES B. 1990. New and old problems in the taxonomy of the Gelidiales (Rhodophyta). *Hydrobiologia* **204/205**: 125-135.

SANTELICES B. 1991a. Intrageneric differences in cystocarp structure in *Gelidium* and *Pterocladia*. *Hydrobiologia* **221**: 1-17.

SANTELICES B. 1991b. Variations in cystocarp structure in *Pterocladia* (Gelidiales, Rhodophyta). *Pacific Science* **45**: 1-11.

SANTELICES B. 1997a. The sexual reproductive development of *Pterocladieaa bulbosa* (Loomis) comb. nov. (Gelidiales, Rhodophyta). *Cryptogamia Algology* **18**: 297-307.

SANTELICES B. 1997b. The spermatangial sorus of *Gelidiella acerosa* (Gelidiellaceae, Gelidiales). In: *Taxonomy of Economic Seaweeds*. Vol. VI (Ed. by I.A. Abbott), pp. 77-87. California Sea Grant College Program, La Jolla, CA.

SANTELICES B. 1998. Taxonomic review of the species of *Pterocladia* (Gelidiales, Rhodophyta). *Journal of Applied Phycology* **10**: 237-252.

SANTELICES B. 1999. Taxonomic status of the species originally ascribed to the genus *Pterocladia* (Gelidiales, Rhodophyta). In: *Taxonomy of Economic Seaweeds.* Vol. VII (Ed. by I.A. Abbott), pp. 71-80. California Sea Grant College Program, La Jolla, CA.

SANTELICES B. & FLORES V. 1995. Spermatangial sori on cystocarpic branchlets of species of *Gelidium* and *Pterocladia* (Gelidiales, Rhodophyta). *Phycologia* **34**: 337-341.

SANTELICES B. & HOMMERSAND M. 1997. *Pterocladiella*, a new genus in the Gelidiaceae (Gelidiales, Rhodophyta). *Phycologia* **32**: 114-119.

SANTELICES B. & STEAWRT J.G. 1985. Pacific species of *Gelidium* Lamouroux and other Gelidiales (Rhodophyta), with keys and descriptions to the common or economically important species. In: *Taxonomy of Economic Seaweeds.* Vol. I (Ed. by I.A. Abbott & J.N. Norris), pp. 17-31. California Sea Grant College Program, La Jolla, CA.

SAUNDERS G.W. & KRAFT G.T. 1994. Small-subunit rRNA gene sequences from representatives of selected families of the Gigartinales and Rhodymeniales (Rhodophyta). 1. Evidence for the Plocamiales ord. nov. *Canadian Journal of Botany* **72**: 1250-1263.

SEGAWA S. & KAMURA S. 1960. *Marine Flora of Ryukyu Islands.* Extension Service, University of the Ryukyus, Naha, 72 pp. (in Japanese)

SEGI T. 1955. The species of the genus *Gelidium* from Japan and its vicinity (I). *Report of the Faculty of Fisheries, Prefectural University of Mie* **2**: 124-137.

SEGI T. 1957. The species of the genus *Gelidium* from Japan and its vicinity (II). *Report of the Faculty of Fisheries, Prefectural University of Mie* **2**: 456-462.

SETCHELL W.A. & GARDNER N.L. 1924. New marine algae from the Gulf of California. *Proceedings of the California Academy Science* **12**: 695-949.

SREENIVASA RAO P. 1970. Systematics of Indian Gelidiales. *Phycos* **9**: 63-78, pls 1, 2.

STEWART J.G. 1968. Morphological variation in *Pterocladia pyramidale*. *Journal of Phycology* **4**: 76-84.

STEWART J.G. & NORRIS J.N. 1981. Gelidiaceae (Rhodophyta) from the northern Gulf of California, Mexico. *Phycologia* **20**: 273-284.

SWOFFORD D.L. 1993. *PAUP: Phylogenetic Analysis Using Parsimony*, Version 3.1.1. *User Manual*, by D. L. Swofford and D. P. Begle. Laboratory of Molecular Systematics, Smithonian Institution, Washington, DC, 257 pp.

TANAKA T. 1965. Studies on some marine algae from Southern Japan-VI. *Memoirs of the Faculty of Fisheries, Kagoshima University* **14**: 52-71.

TANAKA T. & ITONO H. 1972. The marine algae from the Island of Yonaguni-II. *Memoirs of the Faculty of Fisheries, Kagoshima University* **21**: 1-14.

TAYLOR W.R. 1943. Marine algae of Haiti collected by H. H. Bartlett in 1941. *Papers of the Michigan Academy of Science, Arts, and Letters* **28**: 143-63, pls. 1-4.

THOMPSON J.D., HIGGINS D.G. & GIBSON T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22**: 4673-4680.

VAN DER MEER J.P. & PATWARY M.U. 1991. Genetic alleviation of the self-fertilization complication when hybridizing monoecious *Gelidium vagum*. *Hydrobiologia* **221**: 167-179.

YAMADA Y. & TANAKA T. 1938. The marine algae from the Island of Yonakuni. *Institute of Algological Research, Faculcy of Science, Hokkaido Imperial University* **2**: 53-86.

YATABE R. 1892. *Icongraphia Florae Japonicae*. Vol. I, No 2, pp. 157-158. Maruzen, Tokyo.

YOSHIDA T. 1998. *Marine Algae of Japan*. 1222 pp. Uchida Rokakuho Publishing, Tokyo. (in Japanese)

WEBER-VAN BOSSE A. 1926. Papers from Dr. Th. Mortensen's Pacific Expedition 1914-16. XXXIII. Algues de l'Expédition danoise aux îles Kei. Vidensk. Medd. Dan. Naturhist. Foren. Kobenhavn **81**: 57-155.

WOMERSLEY H.B.S. 1994. *The Marine Benthic Flora of Southern Australia. Part IIIA*. Australian Biological Resources Study, Canberra, 508 pp.

WOMERSLEY H.B.S. & BAILEY A. 1969. Marine algae of the Solomon Islands. *Philosophical Transactions of the Royal Society of London* B **259**: 257-352.

ZECHMAN F.W., ZIMMER E.A. & THERIOT E.C. 1994. Use of ribosomal DNA internal trascribed spacers for phylogenetic studies in diatoms. *Journal of Phycology* **30**: 507-512.

Figures & Tables

Table 1. List of species used in DNA extraction and rhizoid observation.

Species	Locality (Date, voucher number in SAP)	DNA	rhizoid
Acanthopeltis japonica Okamura	Shimoda, Shizuoka Prefecture (25.ix.1996, 064829)	*	*
Capreolia implexa Guiry et Womersley	Tamarama, Australia (23.iv.1998, 064831)		*
Gelidiella acerosa (Forsskål) Feldmann et Hamel	Ginowan, Okinawa Prefecture (11.vi.1998, 064832)		*
Gelidiella ligulata Dawson	Miyake Is., Shizuoka Prefecture (14.vii.1998, 063883)	*	*
Gelidium divaricatum Martens	Nishiizu, Shizuoka Prefecture (26.ix.1996, 064833)	*	*
Gelidium elegans Kützing	Awaji Is., Hyogo Prefecture (16.v.1996, 064834)	*	*
Gelidium linoides Kützing	Shimoda, Shizuoka Prefecture (25.ix.1996, 064835)	*	*
Gelidium pacificum Okamura	Enoshima, Kanagawa Prefecture (29.iii.1998, 064836)		*
Gelidium pusillum (Stackhouse) Le Jolis	Awaji Is., Hyogo Prefecture (1.x.1996, 064837)	*	*
Gelidium subfastigiatum Okamura	Oshoro, Hokkaido (6.iii.1997, 064838)	*	*

Table 1. Continued.

Species	Locality (Date, voucher number in SAP)	DNA	rhizoid
Gelidium vagum Okamura	Jodogahama, Iwate Prefecture (11.vi.1997, 064839)	*	*
Onikusa japonica (Harvey) Akatsuka	Shimoda, Shizuoka Prefecture (25.ix.1996, 064840)	*	*
Pterocladia lucida (Brown et Turner) J. Agardh	Scarborough, Perth (7.xii.1997, 064841)		*
Pterocladiella capillacea (Gmelin) Santelices et Hommersand	Shimoda, Shizuoka Prefecture (25.ix.1996, 064842)	*	*
Pterocladiella sp.[1]	Sandakan, Malaysia (16.v.1998, 064843)		*
Ptilophora subcostata (Okamura) Norris	Naminoura, Wakayama Prefecture (29.ix.1996, 064844)		*
Yatabella hirsuta Okamura	Oryuzako, Miyazaki Prefecture (11.vii.1996, 064845)	*	*

[1] This alga has *Pterocladiella*-type unilocular cystocarps and this is similar to *P. minima* (Guiry et Womersley) Santelices et Hommersand (1997), described from Australia. However, it has much larger erect axes (4-7 mm high) than the latter (0.5-1.5 mm high).

Table 2. List of species used in the molecular study and GenBank accession number.

Species	accession number		
	SSU	*rbc* L	ITS1
Acanthopeltis japonica Okamura	AB017664	AB017673	AB017682
Capreolia implexa Guiry et Womersley	U60344[1]	L22456[2]	
Gelidiella acerosa (Forsskål) Feldmann et Hamel	U60342[1]	L22457[2]	
Gelidiella ligulata Dawson	AB017669	AB017678	
Gelidium abbottiorum Norris		U16829[2]	
Gelidium allanii Chapman		L22458[2]	
Gelidium americanum (Taylor) Santelices	U60347[1]	L22459[2]	
Gelidium arbuscula (Montagne) Børgesen			Y11956[3]
Gelidium attenuatum (Turner) Thuret		U00110[2]	
Gelidium canariense (Grunow) Seoane-Camba		L22460[2]	Y11961[3]
Gelidium capense (Gmelin) Silva		L22461[2]	Y11962[3]
Gelidium caulacantheum J. Agardh	U60343[1]	U00103[2]	
Gelidium coulteri Harvey		U00105[2]	
Gelidium divaricatum Martens	AB017662	U16828[2]	AB017692
Gelidium elegans Kützing	AB017670	U16830[2]	AB017688
Gelidium floridanum Taylor		U00106[2]	
Gelidium latifolium (Greville) Bornet et Thuret	U60350[1]	U00112[2]	Y11965[3]

Table 2. Continued.

Species	accession number		
	SSU	*rbc* L	ITS1
Gelidium linoides Kützing			AB017689
Gelidium micropterum Kützing		U00446[2]	
Gelidium pteridifolium Norris, Hommersand et Fredericq		U16833[2]	
Gelidium pulchellum (Turner) Kützing		U01822[2]	
Gelidium purpurascens Gardner		U00979[2]	
Gelidium pusillum (Stackhouse) Le Jolis CA U.S.A.		U00984[2]	
Gelidium pusillum (Stackhouse) Le Jolis Canary Is. (CI)		U01003[2]	
Gelidium pusillum (Stackhouse) Le Jolis Japan (Ja)	AB017663	AB017679	AB017691
Gelidium pusillum (Stackhouse) Le Jolis Norway (No)		U00999[2]	
Gelidium pusillum (Stackhouse) Le Jolis Puerto Rico (PR)		U00983[2]	
Gelidium robustum (Gardner) Hollenberg et Abbott		U01041[2]	
Gelidium serrulatum J. Agardh		U01042[2]	
Gelidium sesquipedale (Clemente) Thuret		L22071[2]	Y11963[3]
Gelidium subfastigiatum Okamura			AB017690
Gelidium vagum Okamura Ja	AB017671	AB017680	AB017687
Gelidium vagum Okamura Ca			Y11952[3]
Onikusa japonica (Harvey) Akatsuka	AB017667	AB017676	AB017685

Table 2. Continued.

Species	accession number		
	SSU	*rbc* L	ITS1
Onikusa pristoides (Turner) Akatsuka	U60353[1]	U01044[2]	Y11964[3]
Pterocladia lucida (Brown et Turner) J. Agardh	U60349[1]	U01048[2]	
Pterocladiella capillacea (Gmelin) Santelices et Hommersand Japan (Ja)	AB017672	AB017681	
Pterocladiella capillacea (Gmelin) Santelices et Hommersand U.S.A.	U60346[1]	U01896[2]	
Pterocladiella melanoidea (Schousboe ex Bornet) Santelices et Hommersand	U60341[1]	U01046[2]	
Ptilophora pinnatifida (J. Agardh) Norris	U60345[1]	U16834[2]	
Ptilophora subcostata (Okamura) Norris	U60348[1]	U16835[2]	
Suhria vittata (Linnaeus) J. Agardh		U00112[2]	
Yatabella hirsuta Okamura	AB017666	AB017675	AB017684
Chondrus crispus Stackhouse	Z14140[4]	U02984[5]	
Hildenbrandia rubra (Sommerfelt) Meneghini	L19345[4]	U04174[5]	

[1] Bailey & Freshwater (1997), [2] Freshwater et al. (1995), [3] Patwary et al. (1998), [4] Ragan et al. (1994) and [5] Freshwater et al. (1994)

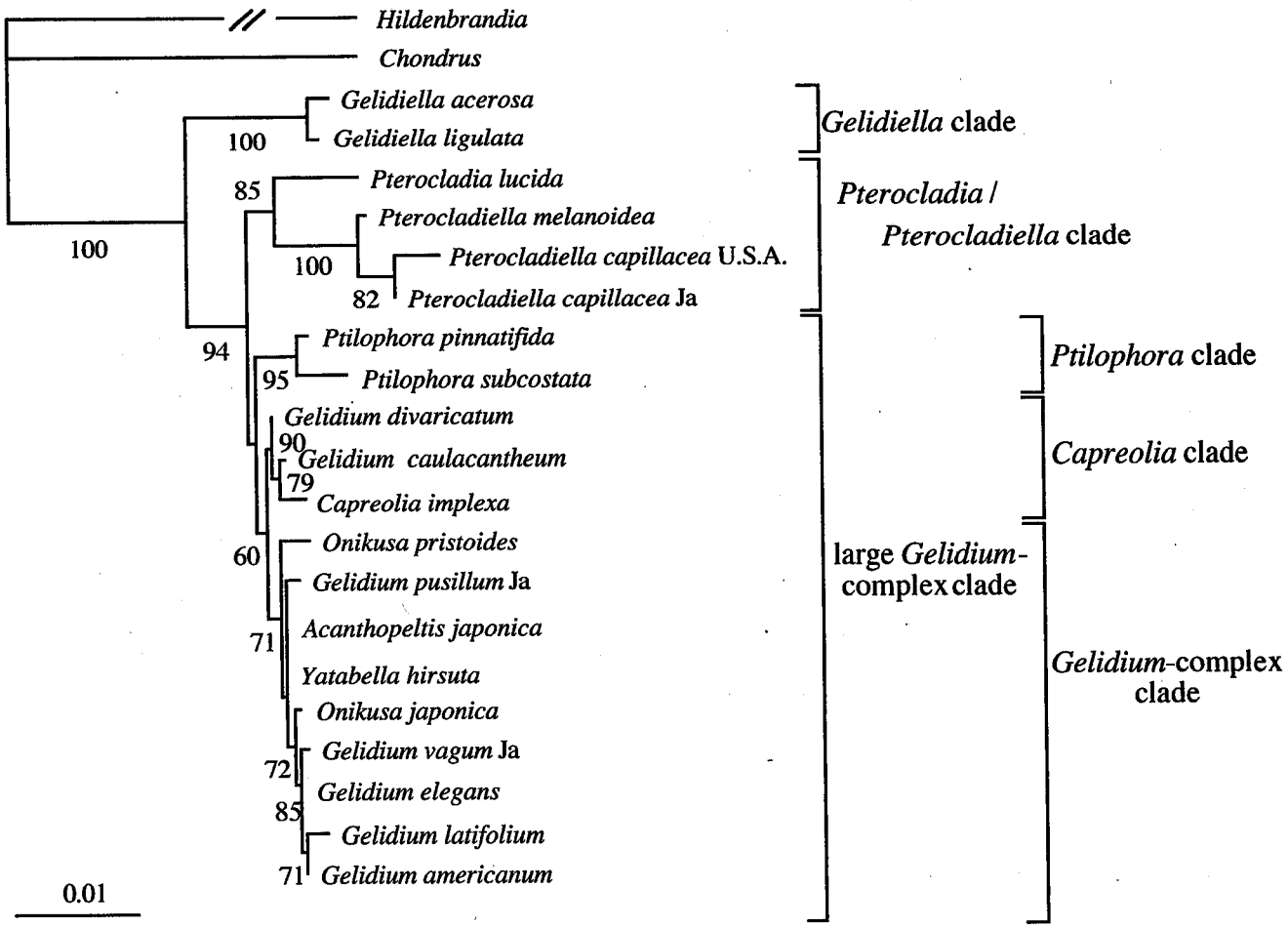


Fig. 1. Phylogenetic tree to elucidate phylogenetic position of several genera in Gelidiales inferred from SSU rDNA sequences with the neighbor-joining (NJ) method based on Kimura's correction using the Clustal W computer program. The numbers indicate bootstrap values (100 replications) greater than 50. Scale bar = 1% divergence.

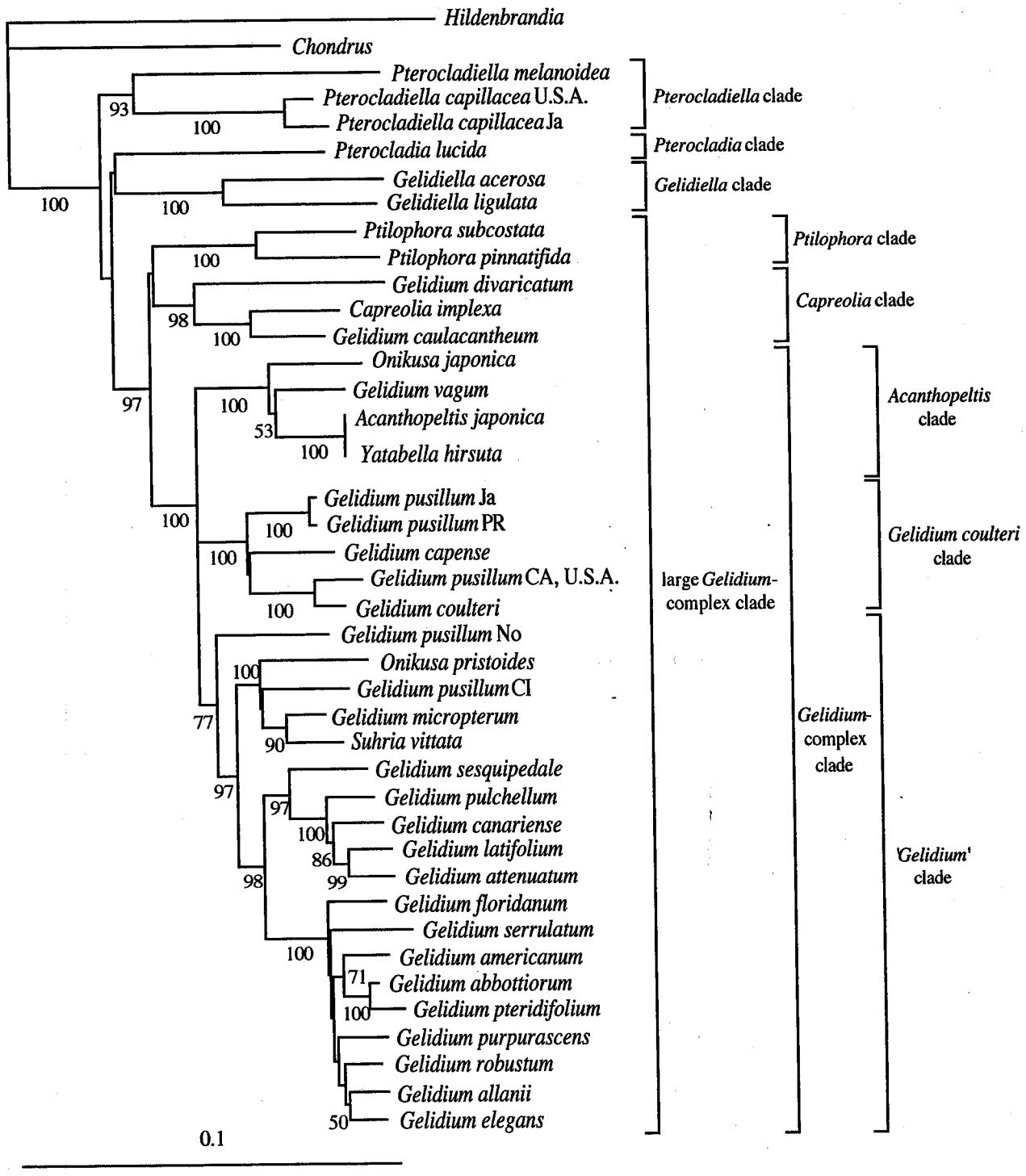


Fig. 2. Phylogenetic tree to elucidate phylogenetic position of several genera and species in Gelidiales inferred from *rbc*L sequences with the neighbor-joining (NJ) method based on Kimura's correction using the Clustal W computer program. The numbers under the branches indicate bootstrap values (100 replications) greater than 50. Scale bar = 10% divergence.

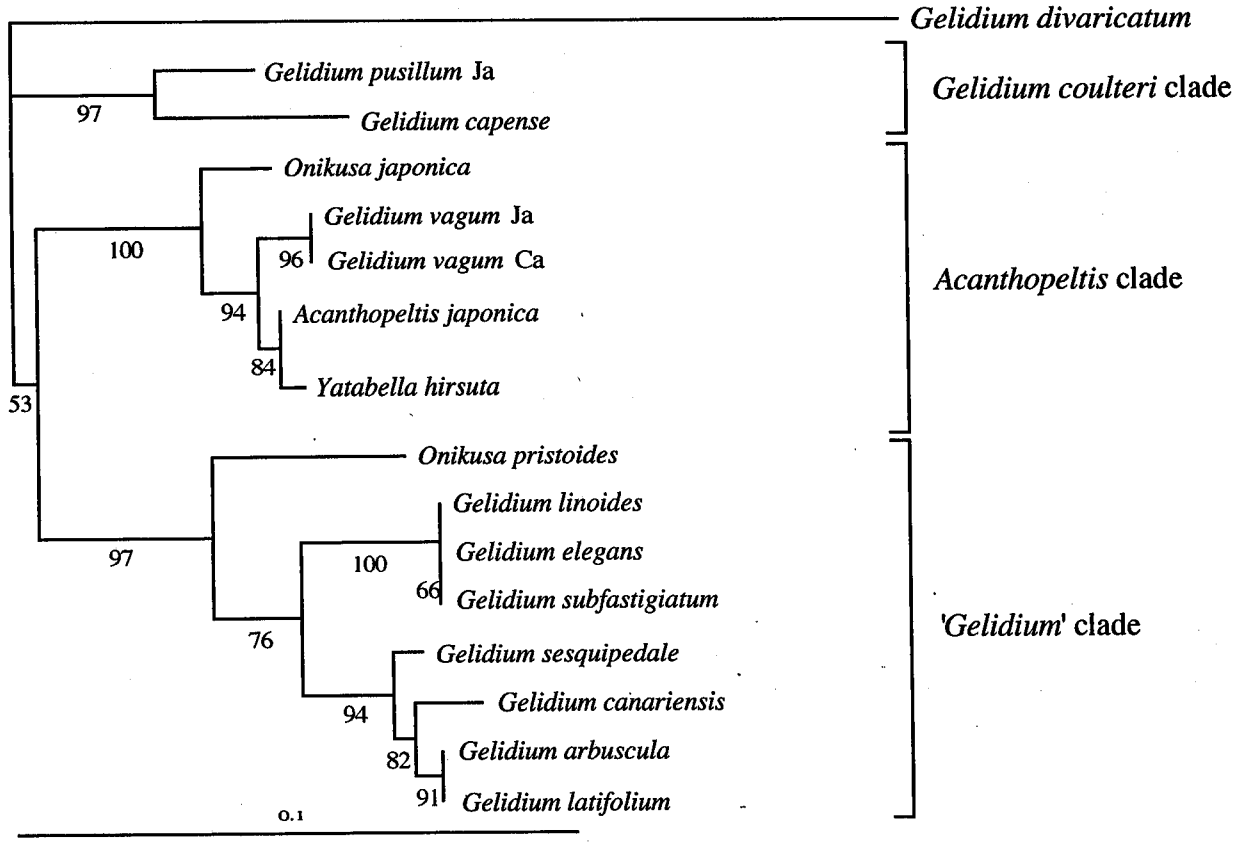
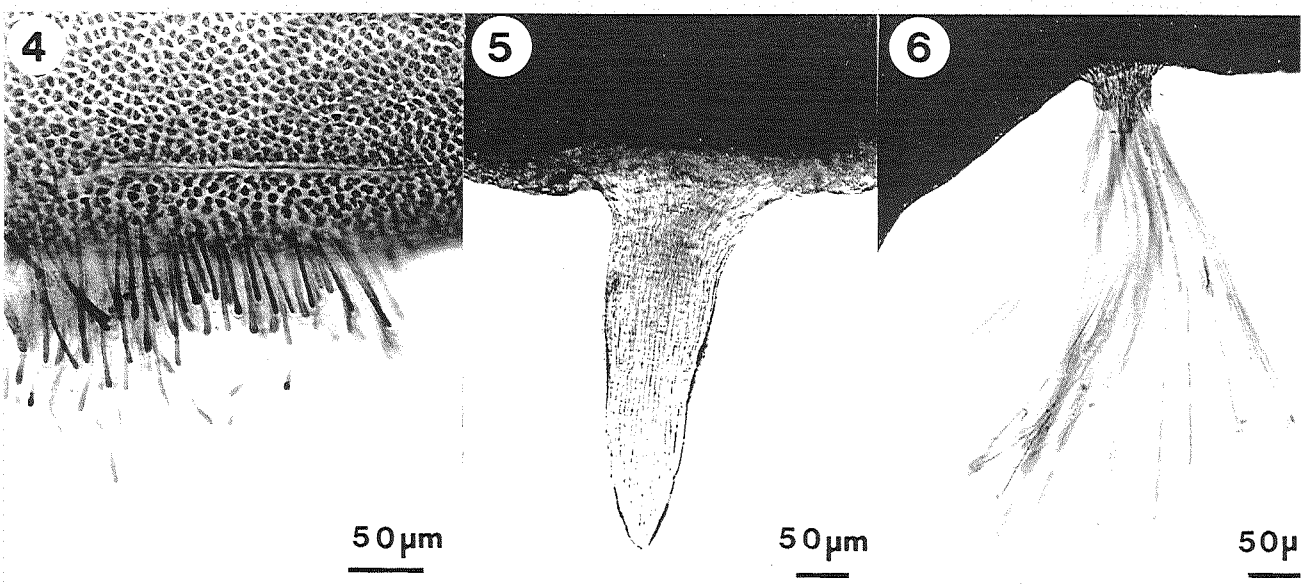


Fig. 3. Phylogenetic tree to elucidate phylogenetic position of species in *Gelidium*-complex clade inferred from ITS1 sequences with the neighbor-joining (NJ) method based on Kimura's correction using the Clustal W computer program. The numbers under the branches indicate bootstrap values (100 replications) greater than 50. Scale bar = 10% divergence.



Figs 4-6. Secondary rhizoidal attachments.
4. Unicellular independent attachment of *Gelidiella acerosa* (field-collected plant, Ginowan, Okinawa Prefecture). 5. Peg-type attachment of *Pterocladiella capillacea* (cultured plant, grown at 20℃ and 16:8 h LD cycle, Shimoda, Shizuoka Prefecture). 6. Brush-type attachment of *Gelidium elegans* (cultured plant, grown at 20℃ and 16:8 h LD cycle, Awaji Is., Hyogo Prefecture).

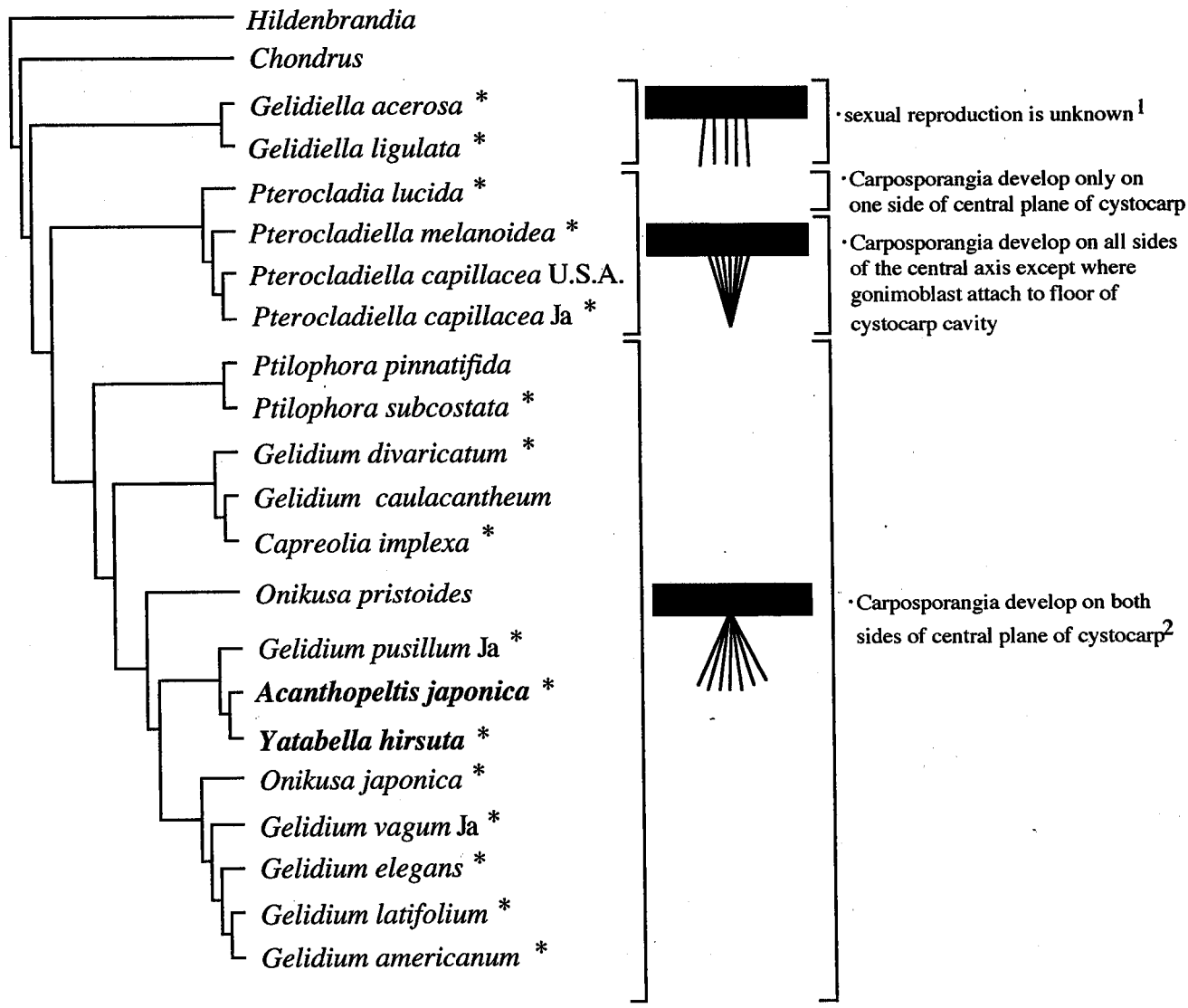
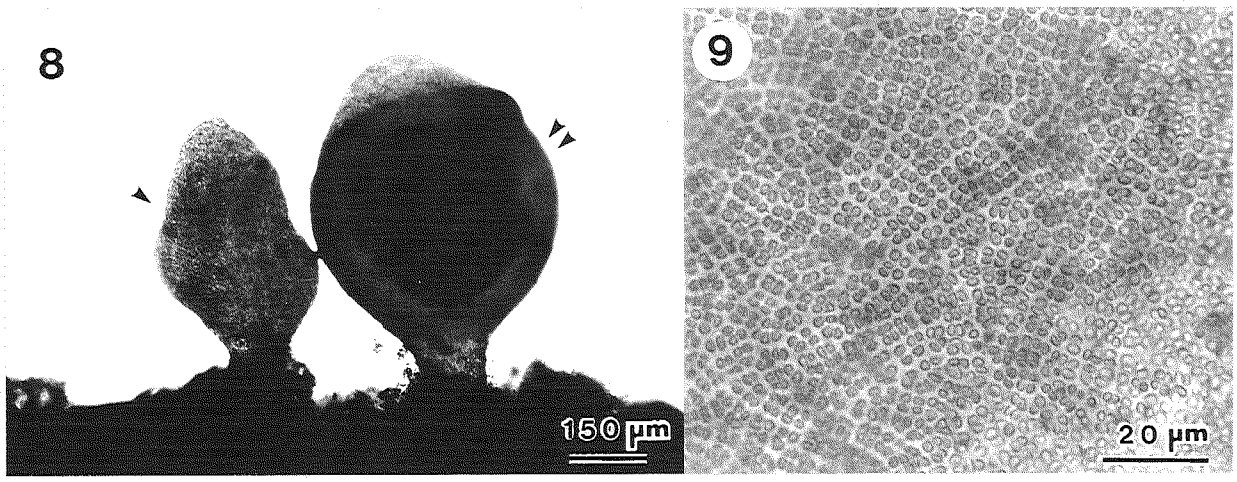


Fig. 7. Correlation of morphological data and SSU tree topology. The types of secondary rhizoidal attachments and developing carposporophyte were used as morphological data.

* The species denoted with asterisk have been observed for its secondary rhizoidal attachment types either by previous researchers or by myself.

1 Only male gametophyte of *Gelidiella acerosa* were reported (Santelices 1997b).

2 Capreolia has no carposporophytic phase (Guiry & Womersley 1993).



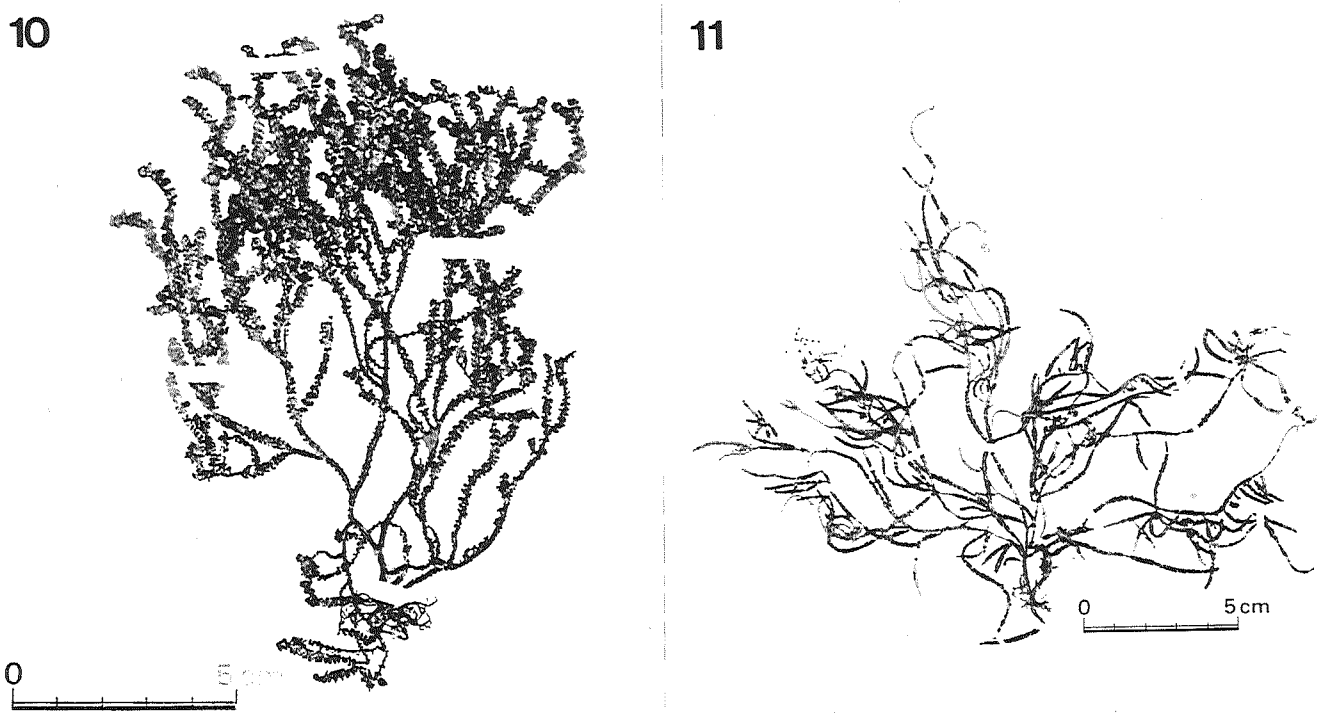
Figs 8-9. Spermatangial sorus of *Yatabella hirsuta*.
8. Spermatangial sorus (arrow head) with cystocarp (double arrow heads).
9. Surface view of spermatangial sorus.

Table 3. List of species used in this study.

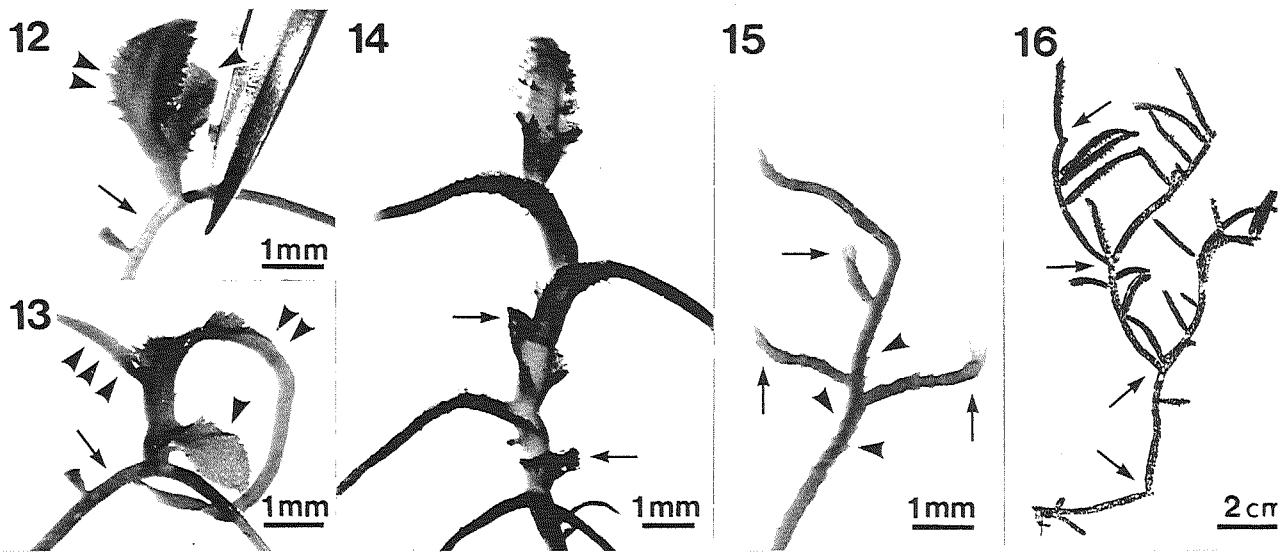
Species	Locality (Date, voucher number in SAP)	accession number		
		SSU	*rbc* L	ITS1
Acanthopeltis japonica Okamura Shi	Shimoda, Shizuoka Prefecture (25.ix.1996, 064829)	AB017664	AB017673	AB017682
Acanthopeltis japonica Okamura Ory	Oryuzako, Miyazaki Prefecture (3.viii.1997, 064830)	AB017665	AB017674	AB017683
Yatabella hirsuta Okamura Ory-1	Oryuzako, Miyazaki Prefecture (11.vii.1996, 064845)	AB017666	AB017675	AB017684
Yatabella hirsuta Okamura Ory-2	Oryuzako, Miyazaki Prefecture (3.viii.1997, 064846)			

Table 4. Pairwise distances (including gaps) between individual plants of *Acanthopeltis* and *Yatabella* from SSU, *rbc* L and ITS1 sequences.

	SSU				*rbc* L				ITS1			
	1	2	3	4	1	2	3	4	1	2	3	4
1 *A. japonica* Shi	-				-				-			
2 *A. japonica* Ory	0	-			1	-			0	-		
3 *Y. hirsuta* Ory-1	0	0	-		0	1	-		2	2	-	
4 *Y. hirsuta* Ory-2	0	0	0	-	0	1	0	-	2	2	0	-



Figs 10, 11. Holotype specimens of *Acanthopeltis japonica* and *Yatabella hirsuta*. 10. *Acanthopeltis japonica* collected at Misaki, Kanagawa Prefecture (iv.1885, TI). 11. *Yatabella hirsuta* collected at Oryuzako, Miyazaki Prefecture (13.vii.1899, herb. Okamura in SAP).



Figs 12-16. Development of erect axes of *Acanthopeltis japonica* (Figs 4-6) and *Yatabella hirsuta* (Figs 7-8).
Figs 12-14. Cultured plants of *Acanthopeltis japonica* grown at 20°C and 16:8 h LD cycle. 12. Erect axis developing from a creeping axis (arrow), the arrowhead indicating the first leaflike structure, the double arrowheads showing the second one (3-month old). 13. The stage after Fig. 12 three weeks showing a creeping axis (arrow), the first leaflike structure (arrowhead), the second one (double arrowheads) and third one (triple arrowheads). 14. Portion of a 7-month-old plant showing leaflike structures repeatedly piled up, the arrows indicating leaflike structures ceasing elongation. 15. 5-month-old cultured plant of *Yatabella hirsuta* grown at 20°C and 16:8 h LD cycle, showing three lateral branches (arrow) and multifid-echinate ramuli (arrowheads). 16. Portion of a herbarium specimen of *Yatabella hirsuta* collected at the type locality (11.vii.1996, SAP 064847) showing overtopped branches (arrow).

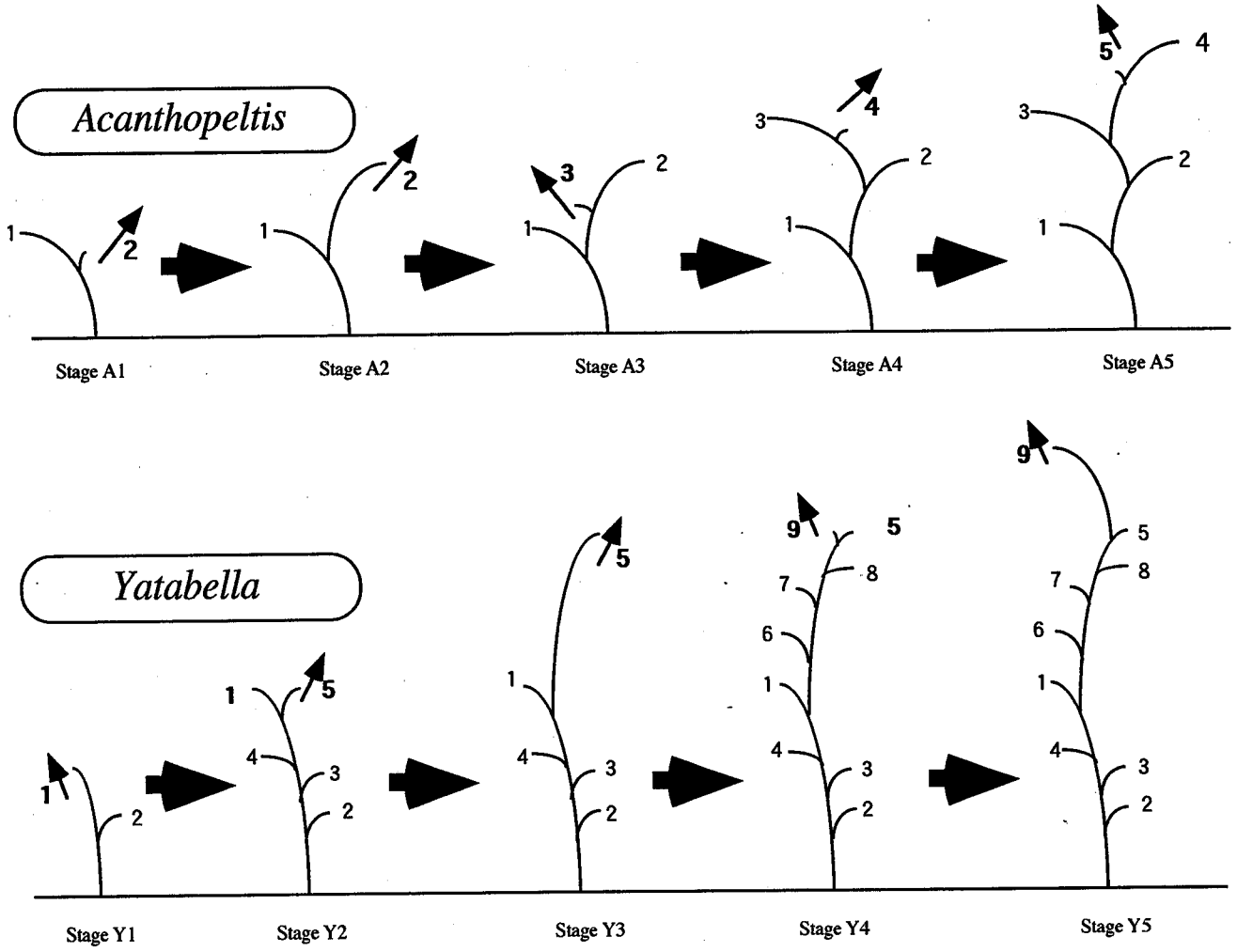


Fig. 17. Schematic illustrations of growth patterns of erect axes in *Acanthopeltis japonica* and *Yatabella hirsuta*. Numerals, sequence of formation of leaflike structures or branches; arrows indicate early stage of elongation of respective leaflike structures or branches. In Yatabella multifid-echinate ramuli are omitted from the figures.

Table 5. List of populations, collection data, sample numbers used for *rbc* L analysis, the number of specimens used for morphological measurements and GenBank/DDBJ accession numbers.

Population No.	Locality & date of collection	Sample No.	No. of measured specimens	Genbank/DDBJ accession number
1	Shimo-Koshiki Is. Kagoshima Pref. (31.vii.1997)	#83	16	AB023840
2	Tomioka, Kumamoto Pref. (30.vii.1997)	#97	5	AB023845
3	Takedatsu, Oita Pref. (4.viii.1997)	#98	5	AB023846
4	Tsuyazaki, Hukuoka Pref. (4.xi.1998)	#274	3	AB023851
5	Kiwado, Yamaguchi Pref. (19.xi.1998)	#303	2	AB023855
6	Hinomisaki, Shimane Pref. (20.xi.1998)	#304[1]	4	AB023856
7	Hinomisaki, Shimane Pref. (20.xi.1998)	#314[1]	1	AB023857
8	Uradomi, Tottori Pref. (21.xi.1998)	#317	6	AB023858
9	Echizen, Hukui Pref. (22.xi.1998)	#278	5	AB023852
10	Unoura, Ishikawa Pref. (7.ix.1998)	#251	2	AB023850
11	Kasashima, Niigata Pref. (17.vi.1997)	#61	4	AB023843
12	Oga, Akita Pref. (19.vi.1997)	#75	5	AB023844
13	Taisei, Hokkaido (22.viii.1997)	#118	4	AB023847
14	Oshoro, Hokkaido (15.ix.1996)	#5	7	AB023841
15	Shiriya, Aomori Pref. (7.iv.1998)	#173	3	AB023848
16	Onahama, Hukushima Pref. (13.vi.1997)	#55	7	AB023842
17	Enoshima, Kanagawa Pref. (5.i.1999)	#320	3	AB023859
18	Hachijo Is., Shizuoka Pref. (9.vii.1998)	#233	4	AB023849
19	Shimoda, Shizuoka Pref. (25.ix.1996, 28 iii.1998)	#7	9	AB017681
20	Hamashima, Mie Pref. (17.xi.1998)	#297	4	AB023854
21	Kushimoto, Wakayama Pref. (18.xi.1998)	#291	4	AB023853

[1] Hinomisaki #304 and Hinomisaki #314 were collected from different populations in the same locality

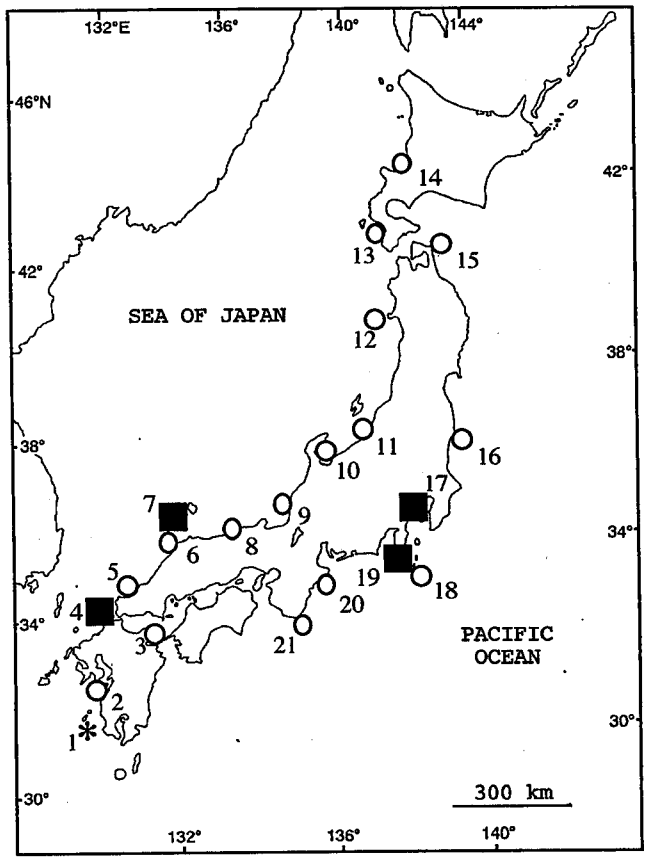


Fig. 18. Map showing populations of the three groups of Japanese *Pterocladia/Pterocladiella* complex revealed in the molecular analysis. * , group 1; ■ , group 2; ○ , group 3. The population numbers correspond to those in Table 5.

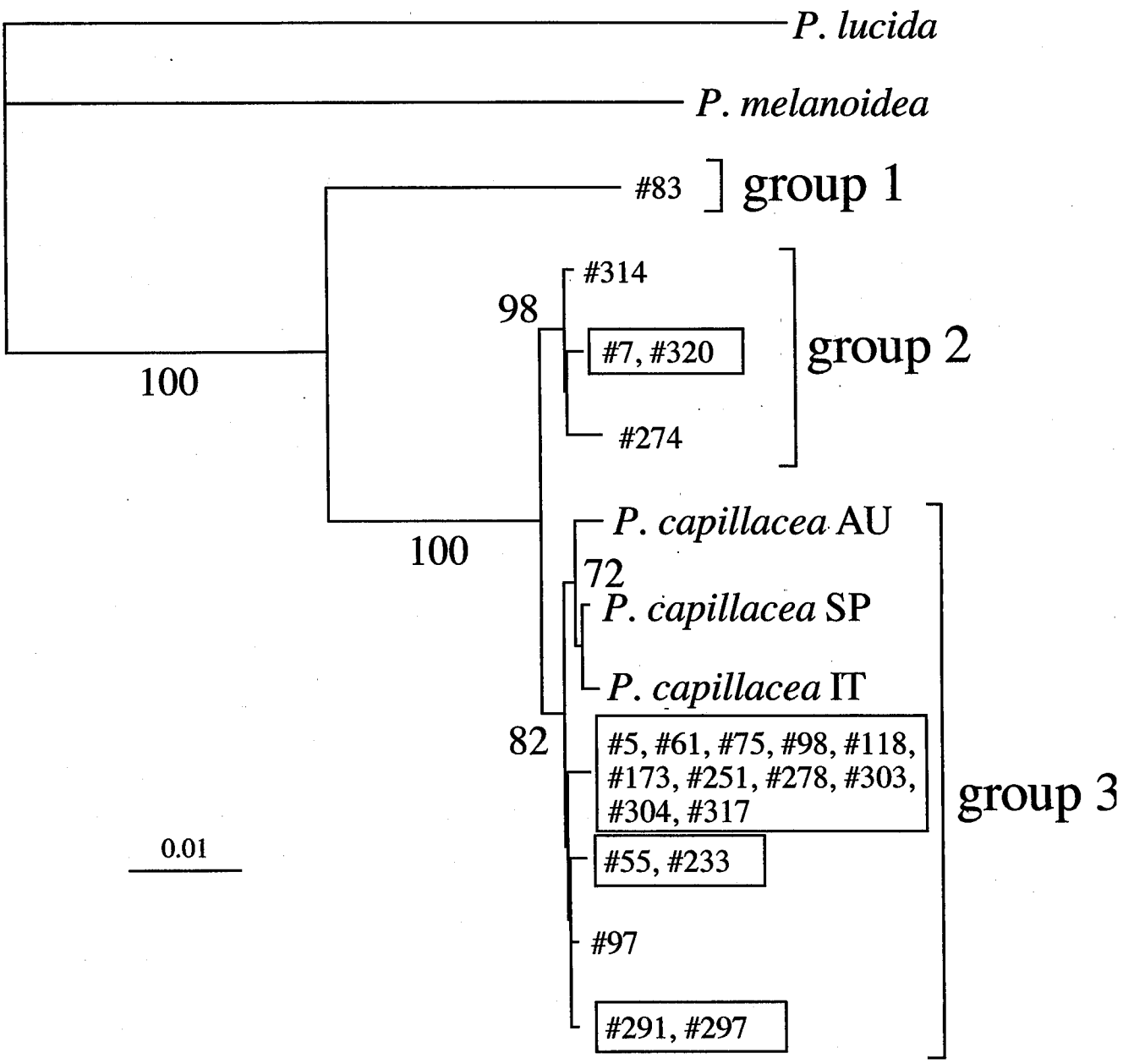
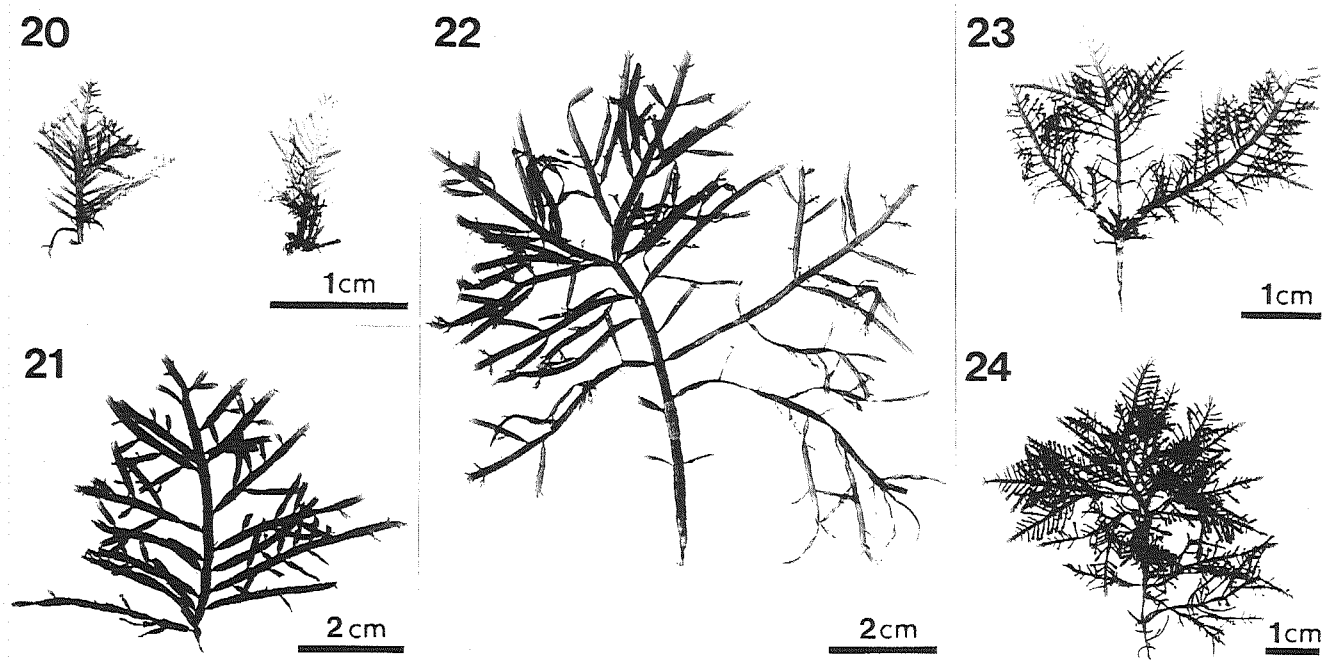


Fig. 19. Phylogenetic tree to elucidate phylogenetic position of Japanese *Pterocladia/Pterocladiella* complex inferred from *rbc*L sequences with the neighbor-joining (NJ) method based on Kimura's correction using the Clustal W computer program. The numbers at each node indicate bootstrap values (100 replications) greater than 70. Identical sequences from multiple populations (sample numbers enclosed in boxes) were represented by only one sequence in the analysis. AU, Australia; SP, Spain; IT, Itary. Scale bar = 1% divergence.



Figs 20-24. Formalin/seawater-preserved specimens of three groups of Japanese *Pterocladia/Pterocladiella* complex.
20. Group 1 plants, collected at the southernmost locality Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture. Figs 21, 22. Group 2 plants. 21. Plant collected at the Pacific coast of central Japan (Enoshima, Kanagawa Prefecture). 22. Plant collected at the Pacific coast of central Japan (Shimoda, Shizuoka Prefecture). Figs 23, 24. Group 3 plants. 23. Plant collected at the northernmost locality, Oshoro, Hokkaido. 24. Plant collected at the Pacific coast of central Japan (Hamashima, Mie Prefecture).

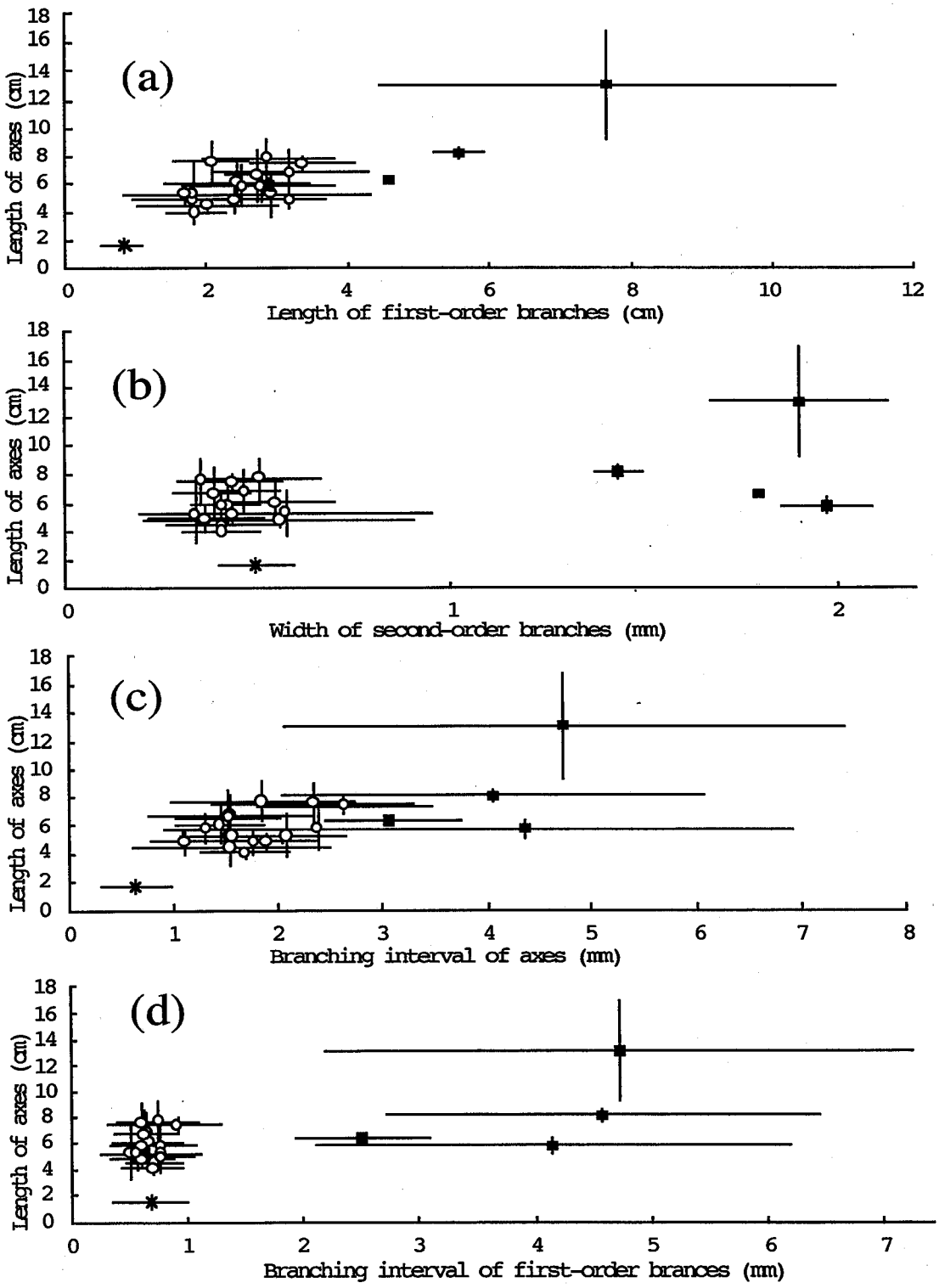
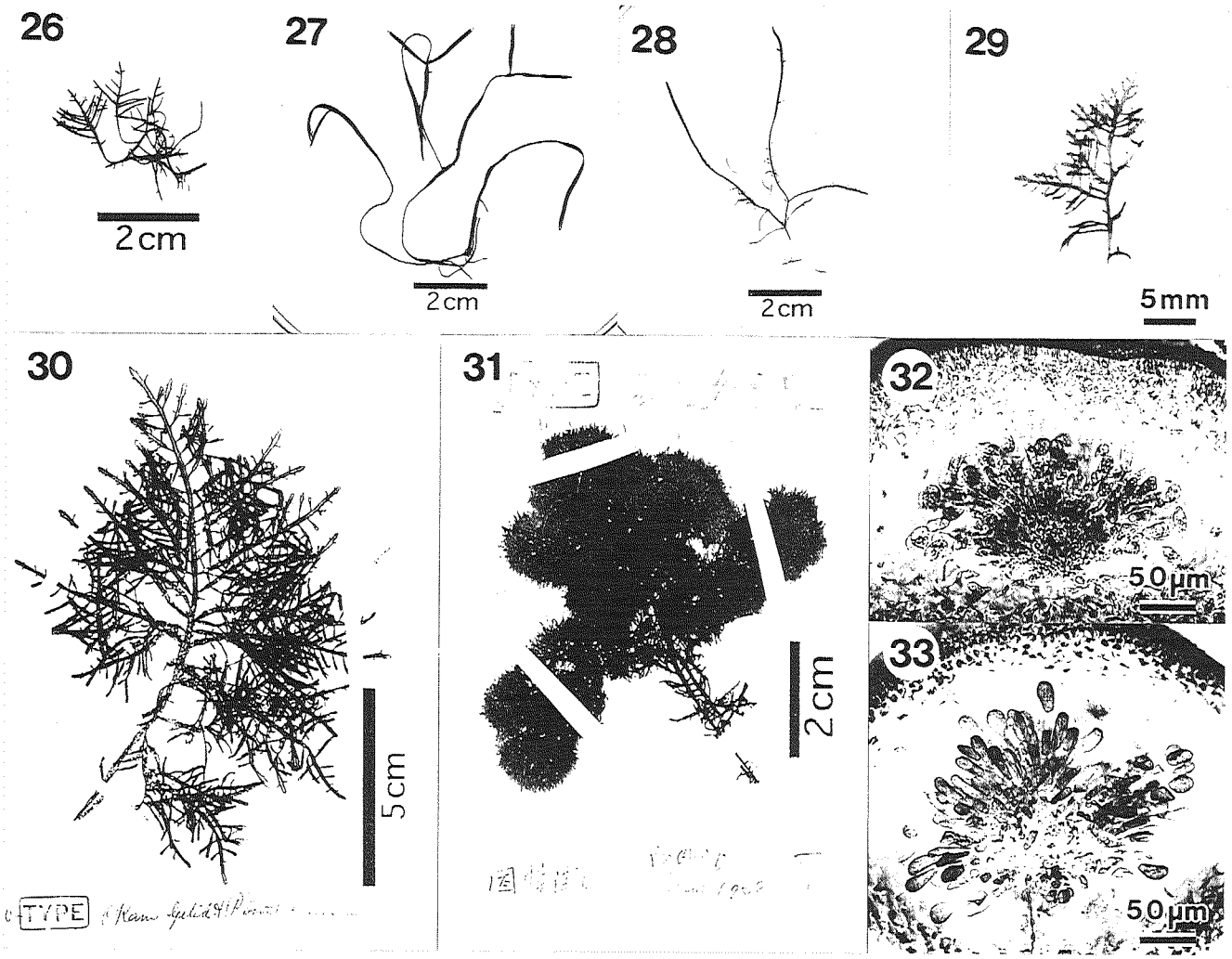


Fig. 25. The means and standard deviations of dimensions in each population of three groups of *Pterocladia/Pterocladiella* complex are plotted in the following combinations: a) length of axes vs. maximum length of first-order branches; b) length of axes vs. maximum width of second-order branches; c) length of axes vs. branching intervals of axes; d) length of axes vs. branching intervals of first-order branches. * the group 1; ■ the group 2; ○ the group 3.



Figs 26-33. Three groups of *Pterocladia/Pterocladiella* complex. Figs 26-28. Five-month-old cultured plants of three groups of Japanese *Pterocladia/Pterocladiella* complex grown at 15°C and 16:8 h LD cycle. 26. Group 1 sample #83 Koshiki Island, Kagoshima Prefecture, showing short axes and branches with short intervals. 27. Group 2 sample #7 Shimoda, Shizuoka Prefecture, showing broad branches with long branching intervals. 28. Group 3 sample #97 Tomioka, Kumamoto Prefecture, showing slender branches that issue ultimate branchlets with short intervals. Figs 29-31. Type material of the *Pterocladia* species described by Okamura. 29. Lectotype specimen of *Pterocladia nana* collected at Yura-jima, Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture (19.vii.1919, Okamura Herb. in SAP). 30. Lectotype specimen of *Pterocladia tenuis* collected at Enoshima, Kanagawa Prefecture (iii.1897, Okamura Herb. in SAP). 31. Lectotype specimen of *Pterocladia densa* collected at Uradomi, Tottori Prefecture (viii.1923, Okamura Herb. in SAP). Figs 32, 33. Transections of cystocarps of *Pterocladiella nana* and *P. tenuis*. Each carposporophyte develops around the axial cell, and the gonimoblast is attached on the one side to the cystocarpic floor, producing chains of carposporangia on the remaining three sides. 32. *Pterocladiella nana* (Koshiki Is., 31.vii.1997). 33. *Pterocladiella tenuis* (Shimoda, 25.ix.1996).

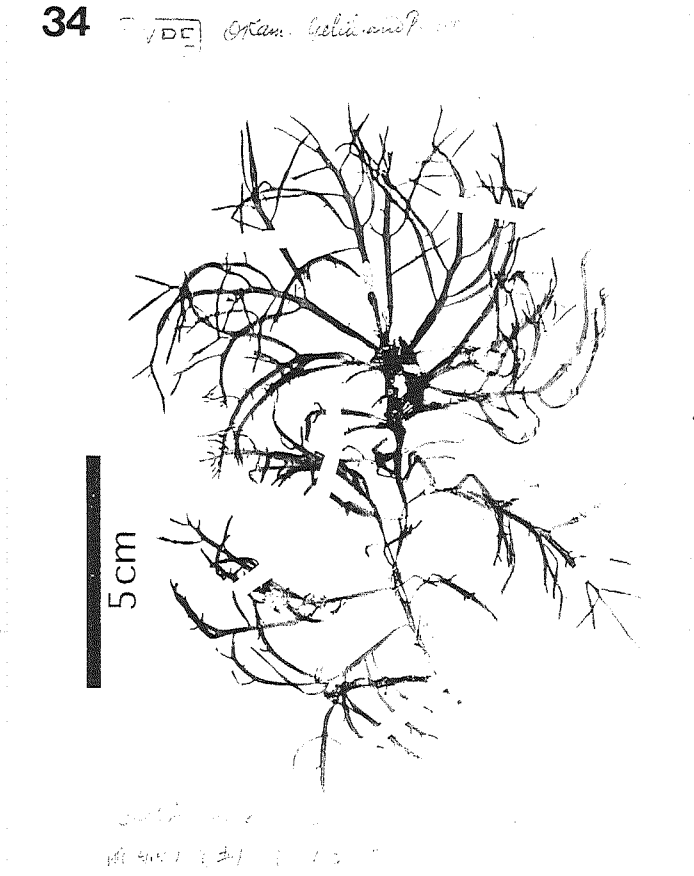


Fig. 34. Lectotype specimen of *Gelidium decumbensum* collected at Enoshima, Kanagawa Prefecture (v.1933, Okamura Herb. in SAP).

Table 6. List of population number, sample number of unialgal culture and collection data in a *Gelidium elegans/G. subfastigiatum* complex.

Population No.	Sample No.	Locality & date of collection
1	#89	Cape of Sata, Kagoshima Pref. (3.viii.1997)
2	#16	Yura, Awaji Is., Hyogo Pref. (16.v.1996)
3	#10	Kushimoto, Wakayama Pref. (29.ix.1996)
4	#24	Hamashima, Mie Pref. (28.ix.1996)
5	#20	Shimoda, Shizuoka Pref. (25.ix.1996)
6	#13	Tateyama, Chiba Pref. (26.iii.1996)
7	#143	Izura, Ibaragi Pref. (3.iv.1998)
8	#57	Kitayamazaki, Iwate Pref. (11.vii.1997)
9	#60	Hachinohe, Aomori Pref. (10.vii.1997)
10	#177	Shiriya, Aomori Pref. (7.iv.1998)
11	#45, #47, #48	Oshoro, Hokkaido (6.iii.1997)
11	#354, #355, #356	Oshoro, Hokkaido (2.ix.1999)
12	#43	Tomari, Hokkaido (16.iv.1997)
13	#73	Tappi, Aomori Pref. (21.vi.1997)
14	#157, #161	Oga, Akita Pref. (6.iv.1998)
15	#67	Atsumi, Yamagata Pref. (18.vi.1997)
16	#152	Sasakawanagare, Niigata Pref. (5.iv.1998)
17	#253	Shitsumi, Fukui Pref. (6.ix.1998)
18	#306	Hinomisaki, Shimane Pref. (20.xi.1998)
19	#309	Kiwado, Yamaguchi Pref. (19.xi.1998)
20	#92	Tsuyazaki, Hukuoka Pref. (26.vii.1997)

Table 7. Means and standard deviations of the maximum width and thickness in 10 second-order branches (1–2 mm below the apex) of parental plants of cultures of a *Gelidium elegans/G. subfastigiatum* complex collected from December to May. Each sample number corresponds to that shown in Table 6.

Type of ITS1 sequence	Sample No.	Width (μm)	Thickness (μm)
type 1	#13	174 (±23.19)	438 (±37.06)
type 1	#16	200 (±21.08)	362 (±43.67)
type 1	#152	194 (±18.97)	458 (±77.43)
type 1	#157	216 (±30.98)	396 (±39.78)
type 2	#43	476 (±60.96)	930 (±112.84)
type 2	#45	474 (±47.19)	862 (±103.04)
type 2	#47	440 (±50.77)	750 (±97.18)
type 2	#48	442 (±52.87)	850 (±107.60)
type 2	#143	426 (±35.34)	900 (±170.23)
type 2	#161	508 (±91.51)	986 (±165.47)
type 2	#177	396 (±57.97)	878 (±187.25)

Table 8. Tolerance of lower temperature (3°C and 8:16 h LD cycle with the photon flux of 30-40 $\mu Em^{-2}s^{-1}$ for three months) in 21 cultured strains of a *Gelidium elegans/G. subfantigiatum* complex.

Sample No.	tolerance of low temperature	Type of ITS1 sequence
#10	×	type 1
#13	×	type 1
#16	×	type 1
#20	×	type 1
#24	×	type 1
#89	×	type 1
#152	×	type 1
#157	×	type 1
#309	×	type 1
#43	○	type 2
#45	○	type 2
#57	○	type 2
#60	○	type 2
#67	○	type 2
#73	○	type 2
#92	○	type 2
#143	○	type 2
#161	○	type 2
#177	○	type 2
#253	○	type 2
#306	○	type 2

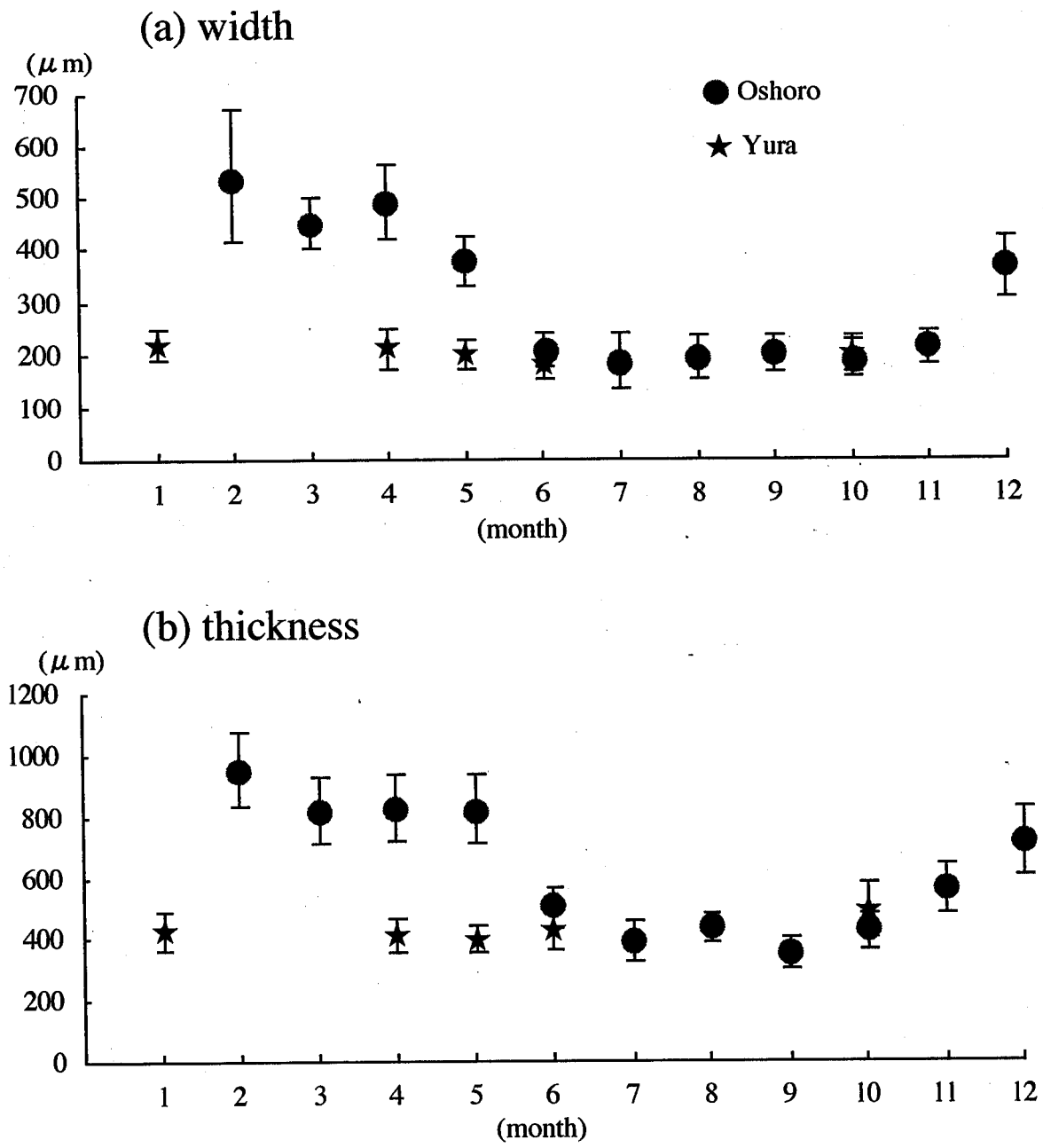
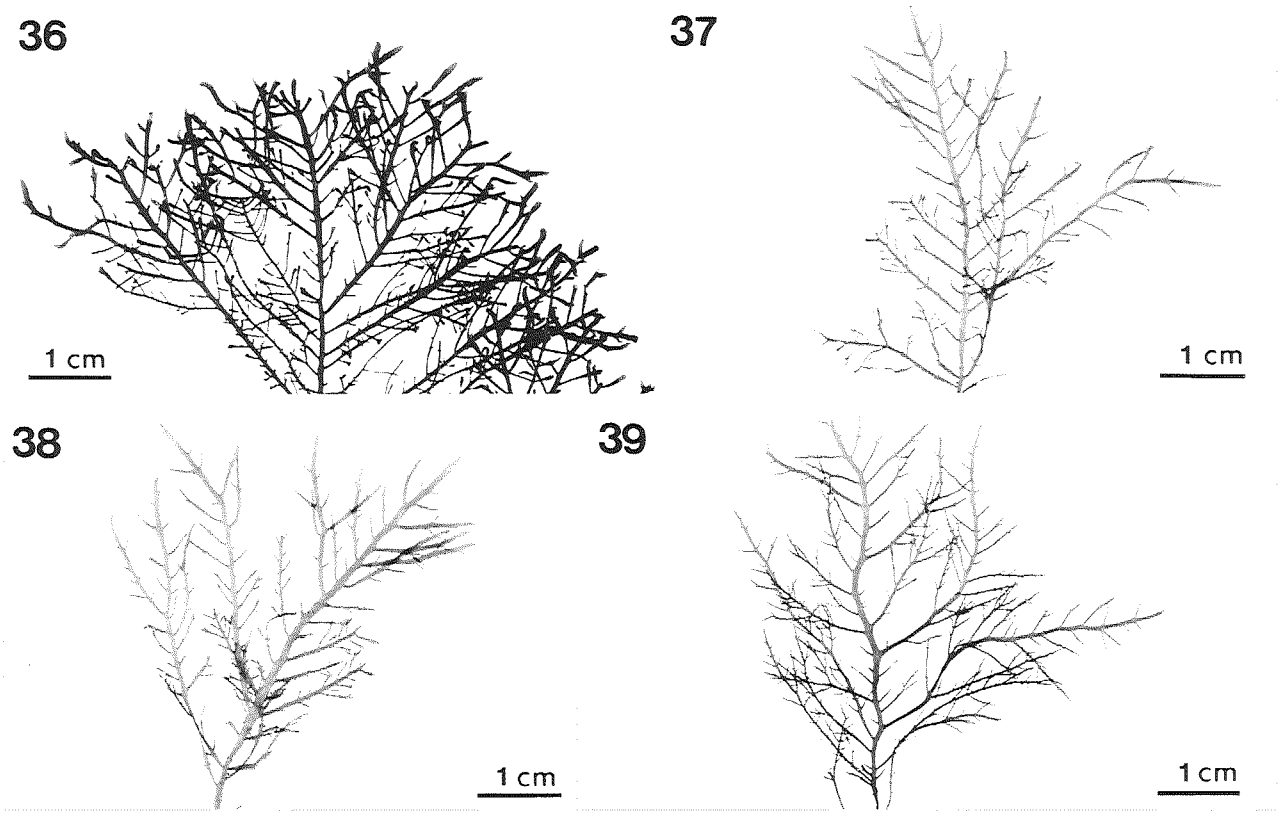


Fig. 35. Means and standard deviations of widths and thicknesses in seasonally swollen branches of a *Gelidium elegans/G. subfastigiatum* complex are plotted in each population (● Oshoro, ★ Yura), respectively.



Figs 36-39. Formalin/seawater-preserved specimens of a *Gelidium elegans/G. subfantigiatum* complex. 36. Plant collected at Oshoro, Hokkaido (16.iv.1997). 37. Plant collected at Oshoro, Hokkaido (13.x.1995). 38. Plant collected at Yura, Hyogo Prefecture (17.iv.1995). 39. Plant collected at Yura, Hyogo Prefecture (19.x.1995).

```
                                                                      50
(type 1):    AGAAAAAACT ATCATTTTGA TTTAAAAACA TATATAGTAT TTTAGAGCCG
(type 2):    AGAAAAAACT ATCATTTTGA TTTAAAAACA TATATAGTAT TTTAGAGCCG

                                                                     100
(type 1):    AAGATTCTGT TTTCTGTGCT CCTATTCTGT TTTTAAATCA TTTGATATTG
(type 2):    AAGATTCTGT TTTCTGTGCT CCTATTCTGT TTTTAAATCA TTTGATATTG

                                                                     150
(type 1):    TTTTTAATGT TTCGTGCTCA AATTCAATCC ACTTTTTTAT TGTTTTTAAT
(type 2):    TTTTTAATGT TTCGTGCTCA AATTCAATCC ACTTTTTTAT TGTTTTTAAT

                                    *                                200
(type 1):    ATTAAACTAC TTTTATTTTT TTT-ATGTTA TTCTTGACAA AACTAAGAAA
(type 2):    ATTAAACTAC TTTTATTTTT TTTTATGTTA TTCTTGACAA AACTAAGAAA

                     210
(type 1):    CAAA......
(type 2):    CAAA......
```

Fig. 40. Alignment of ITS1 sequences. Number on right refer to nucleotide positions (including gaps): * a gap (incertion/deletion) between type 1 and type 2.

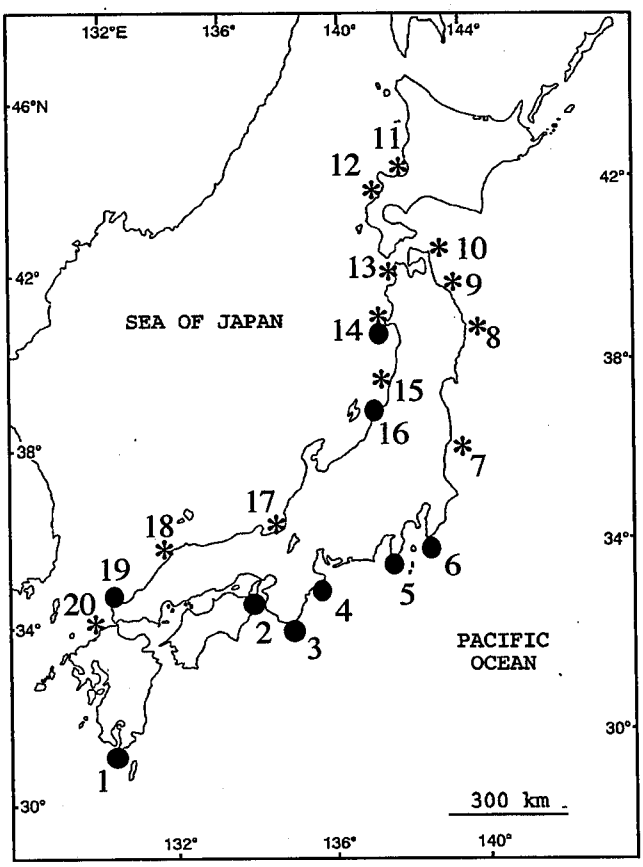


Fig. 41. Map showing populations of the two types of ITS1 sequences in *G. elegans/G. subfastigiatum* complex revealed in the molecular analysis. * type 1; ● type 2. The population numbers correspond to those in Table 6.

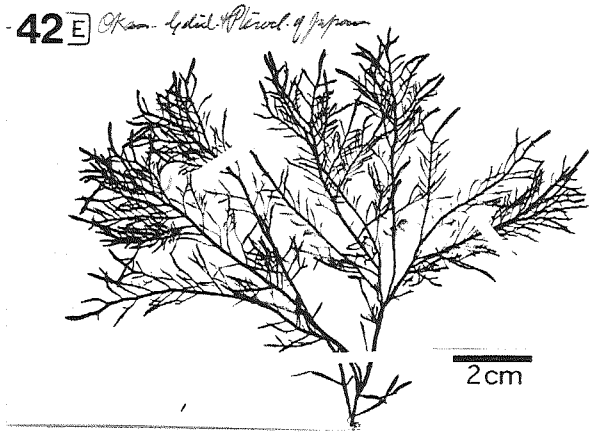


Fig. 42. Lectotype specimen of *Gelidium subfastigiatum* collected at Oshoro, Hokkaido (iii.1920, Okamura Herb. in SAP).

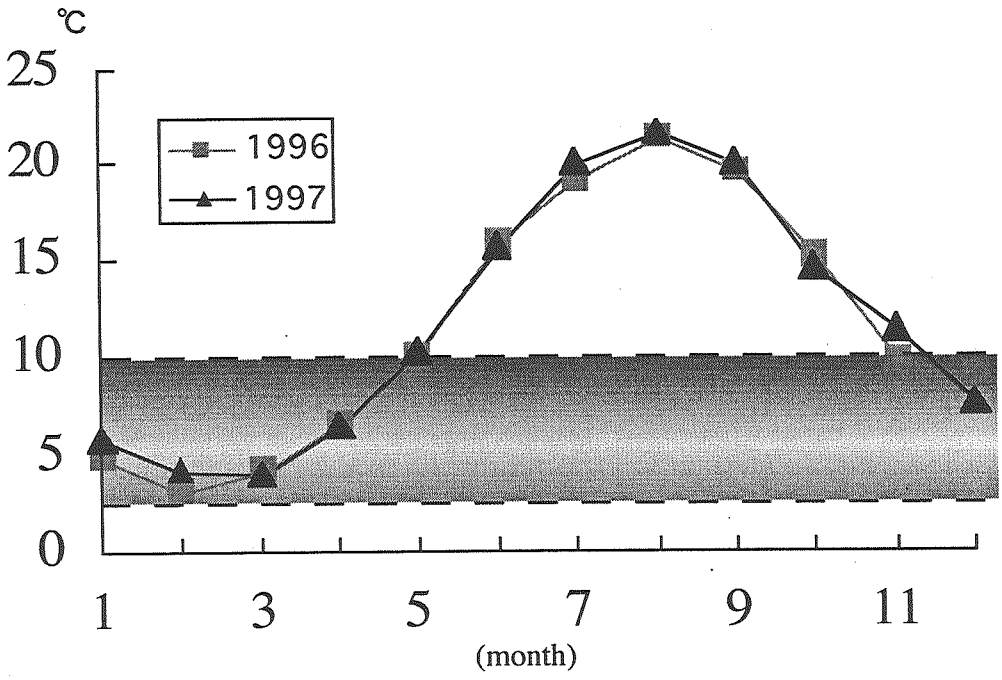


Fig. 43. The monthly mean temperature of seawater at Oshoro, Hokkaido from January 1996 to December 1997. They recorded every morning (8:00) at 1 meter under the sea. Widened and thickened branches can be observed in Oshoro population at gray zone.

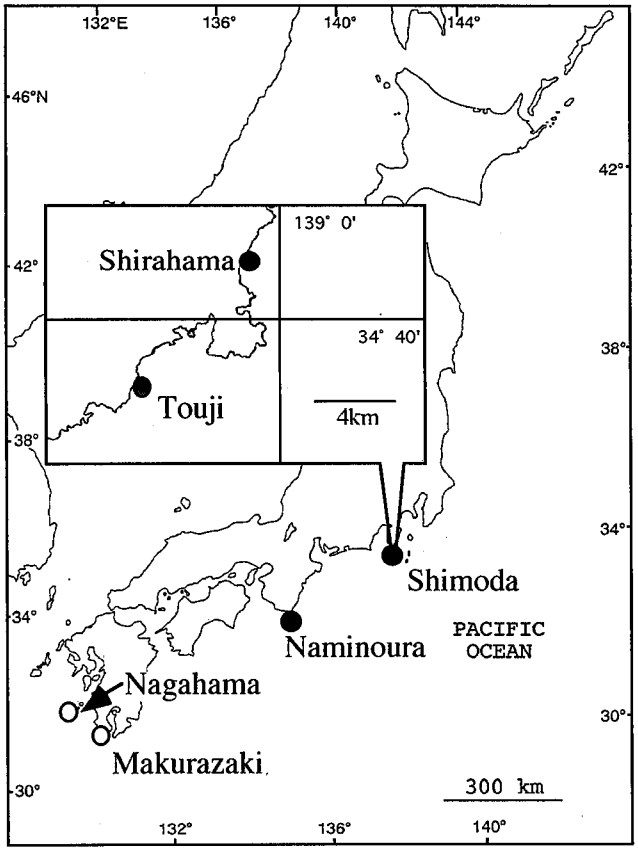
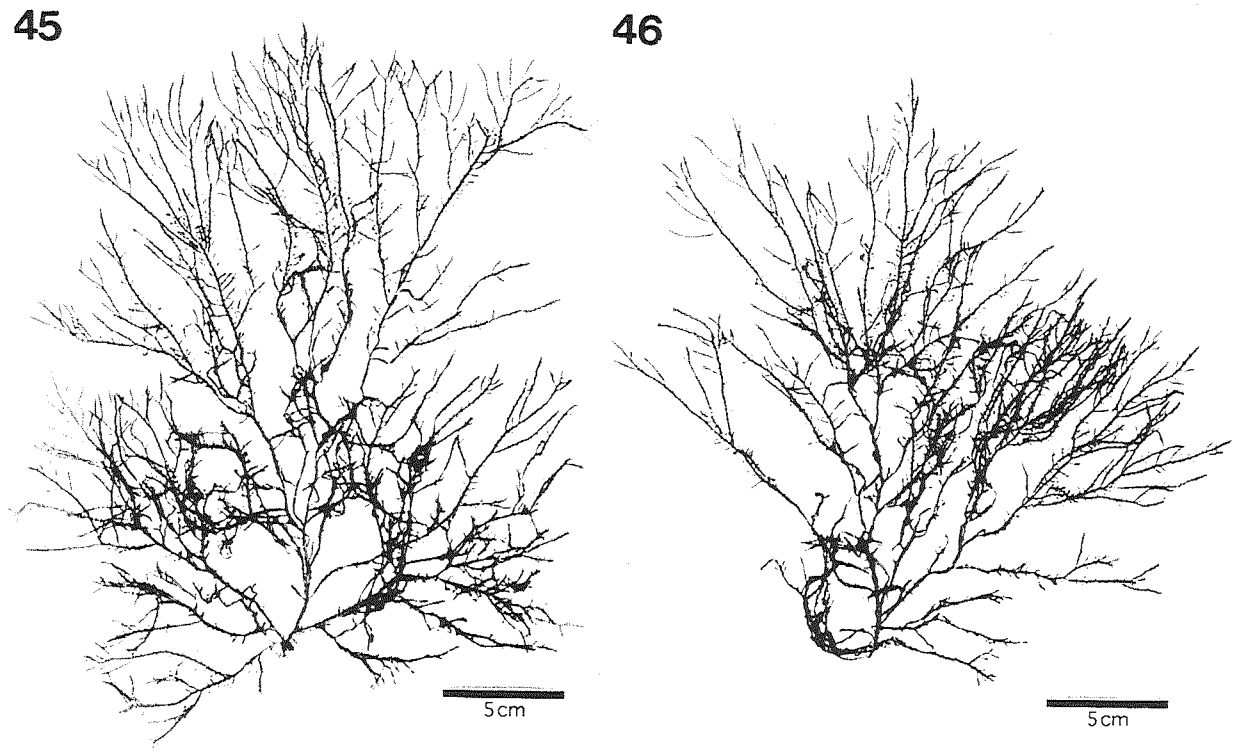
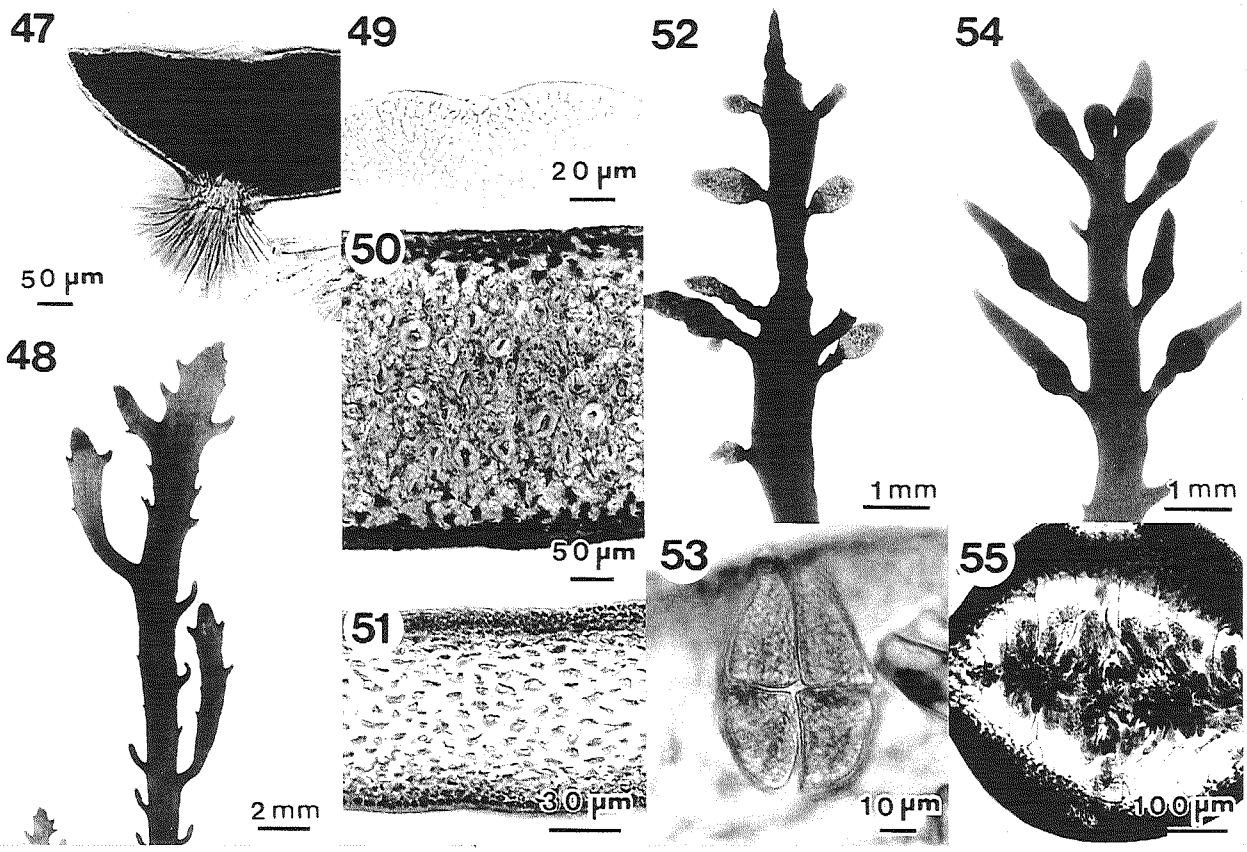


Fig. 44. Map showing collection localities of *G. tenuifolium* (●) and *G. koshikianum* (○) used in this study.

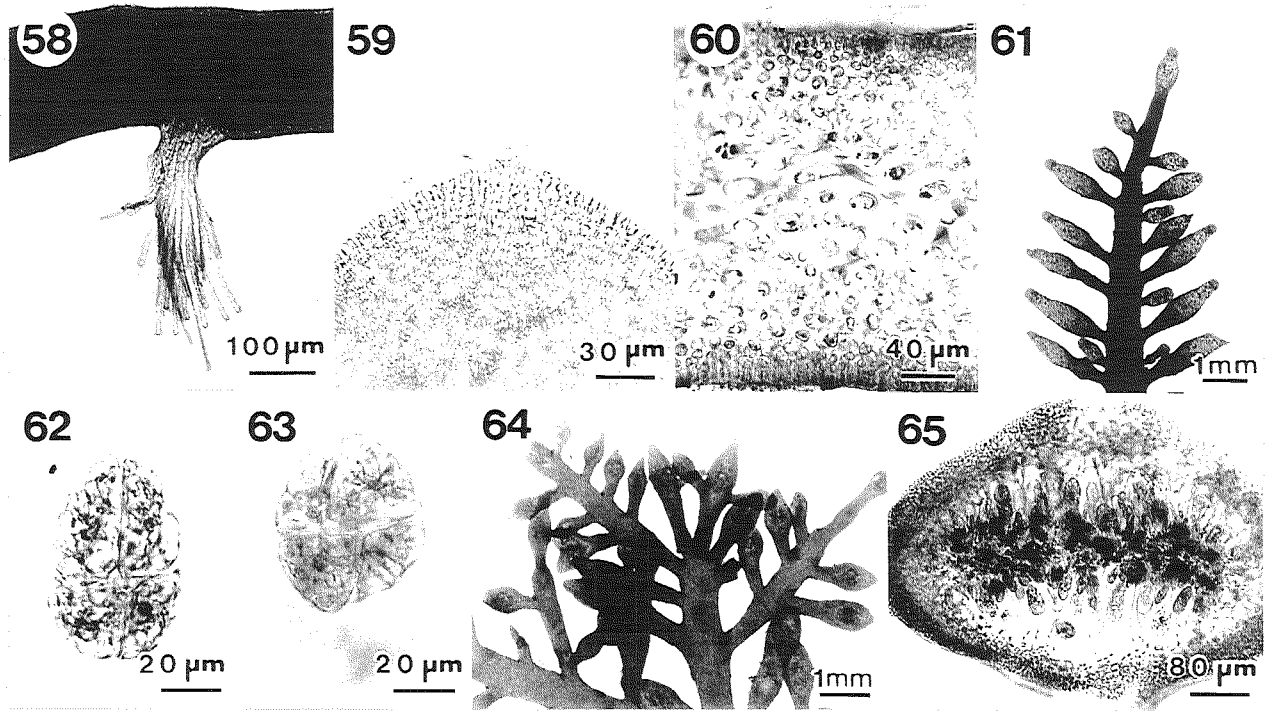


Figs 45, 46. Type specimen of *Gelidium tenuifolium.*
45. Holotype specimen collected at Shirahama, Shimoda, Shizuoka Prefecture (28.iii.1998; SAP #070868). 46. Isotype specimen collected at Shirahama, Shimoda, Shizuoka Prefecture (28.iii.1998; SAP #070869).



Figs 47-55. *Gelidium tenuifolium.*
47. Brush type of secondary rhizoidal attachments (cultured plant grown at 15°C, 16:8 h L:D for one month). 48. Upper portion of a plant, showing long, broad indeterminate branches and short simple determinate branchlets. 49. Dome-shaped apical cell in the apical depression. 50. Transverse section of a basal portion of the axis, showing a few large cells and numerous rhizines in the medulla. 51. Transverse section of the distal portion of a branch. 52. Tetrasporangial branches. 53. Cruciately divided tetrasporangium. 54. Cystocarpic branches. 55. Transverse section of a bilocular cystocarp with a distinct ostiole in each side.

Figs 56, 57. Type specimen of *Gelidium koshikianum.*
56. Holotype collected at Nagahama, Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture (2.viii.1997; SAP #070874). 57. Isotype collected at Nagahama, Shimo-Koshiki Island, Koshiki Islands, Kagoshima Prefecture (2.viii.1997; SAP #070875).



Figs 58-65. *Gelidium koshikianum.*
58. Brush type of secondary rhizoidal attachments (cultured plant grown at 15°C, 16:8 h L:D for one month). 59. Dome-shaped apical cell at the apex of a branch. 60. Transverse section of a first-order branch showing abundant rhizines in the inner cortex and rare in the central medulla. 61. Tetrasporangial branches. 62. Cruciately divided tetrasporangium. 63. Tetrasporangium showing an intermediate division between a cruciate and decussate manner. 64. Cystocarpic branches. 65. Transverse section of a bilocular cystocarp with a distinct ostiole in each side.

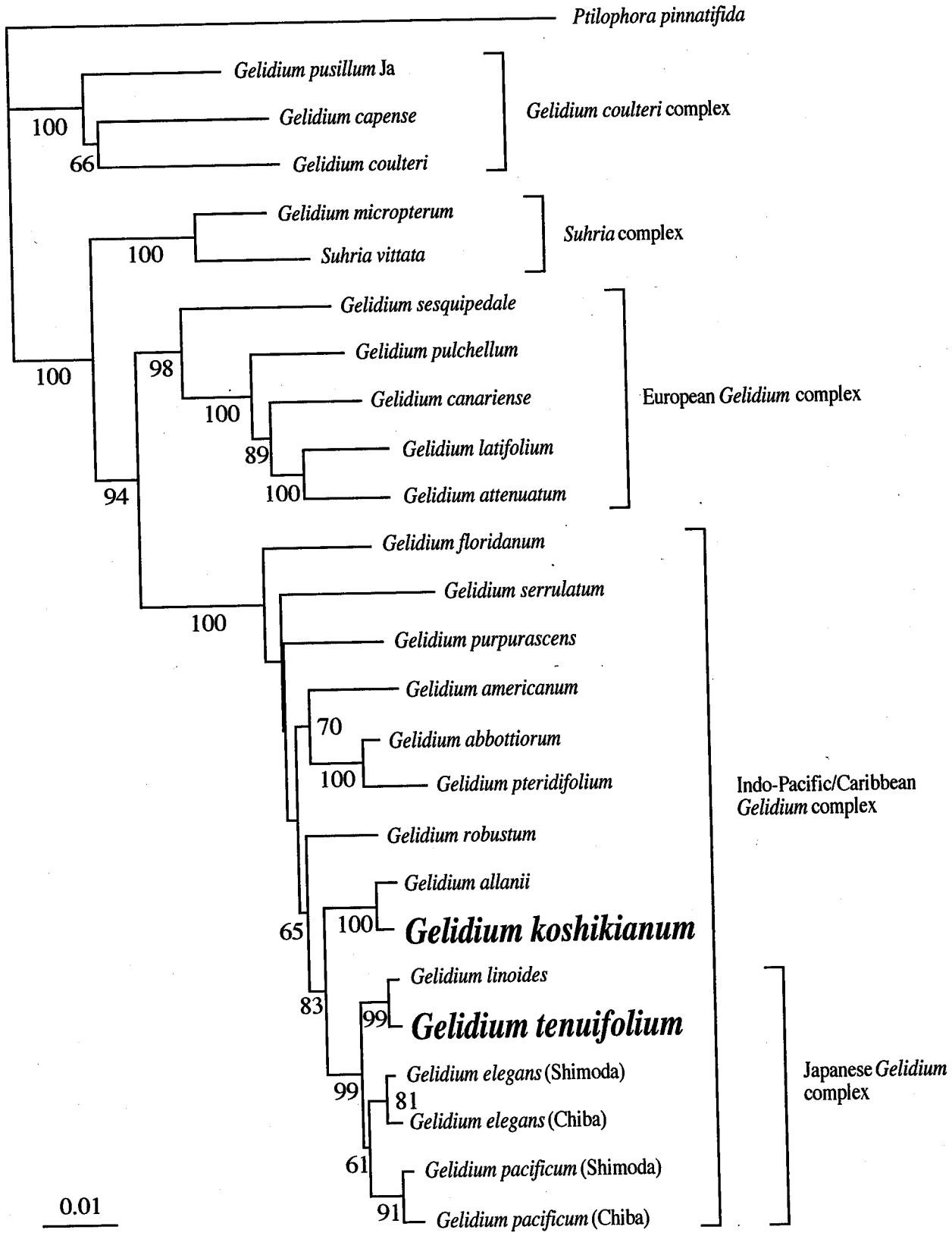


Fig. 66. Phylogenetic tree to elcidate phylogenetic position of two new species of *Gelidium* inferred from *rbc*L sequences with the neighbor-joining (NJ) method based on Kimura's correction using the Clustal W computer program. The numbers under the branches indicate bootstrap values (100 replications) greater than 60. Scale bar = 1% divergence.

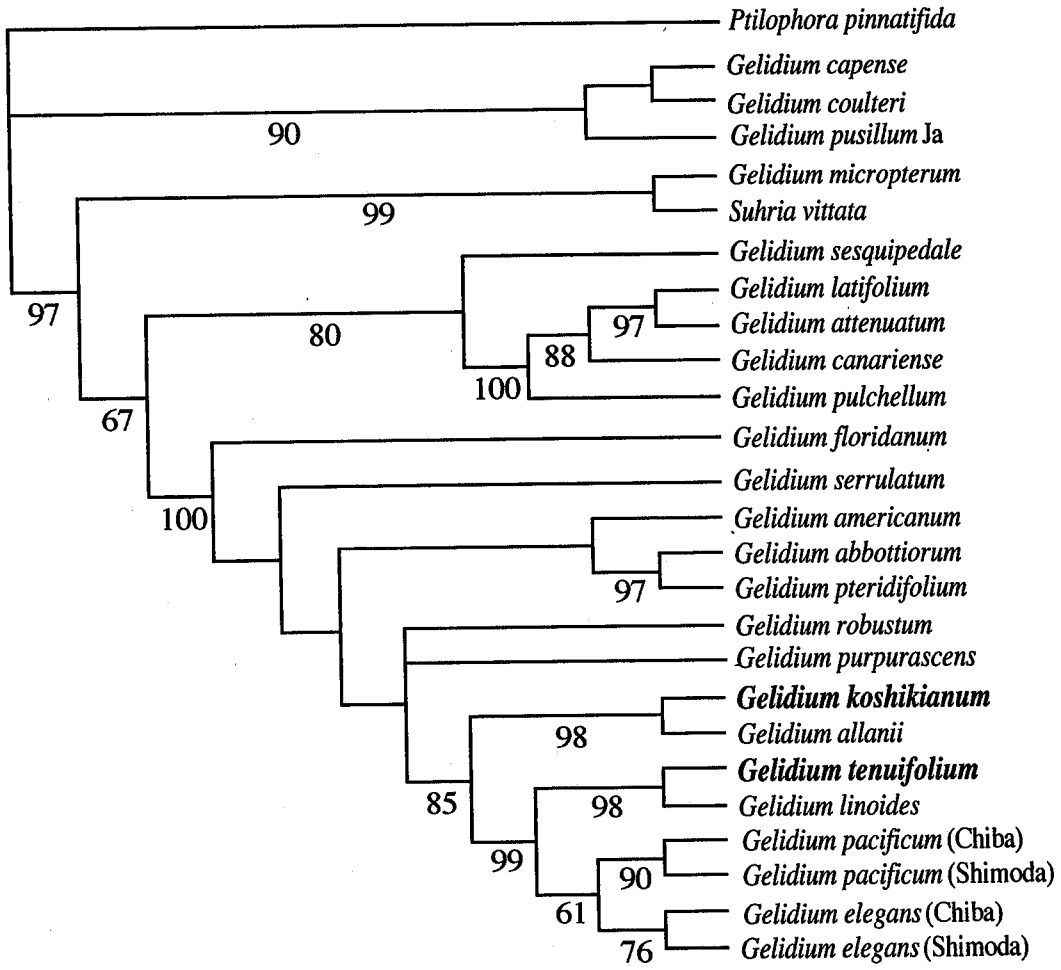


Fig. 67. Phylogenetic tree to elcidate phylogenetic position of two new species of *Gelidium* inferred from *rbc*L sequences with the maximum parsimony (MP) method using PAUP program. The numbers under the branches indicate bootstrap values (100 replications) greater than 60.

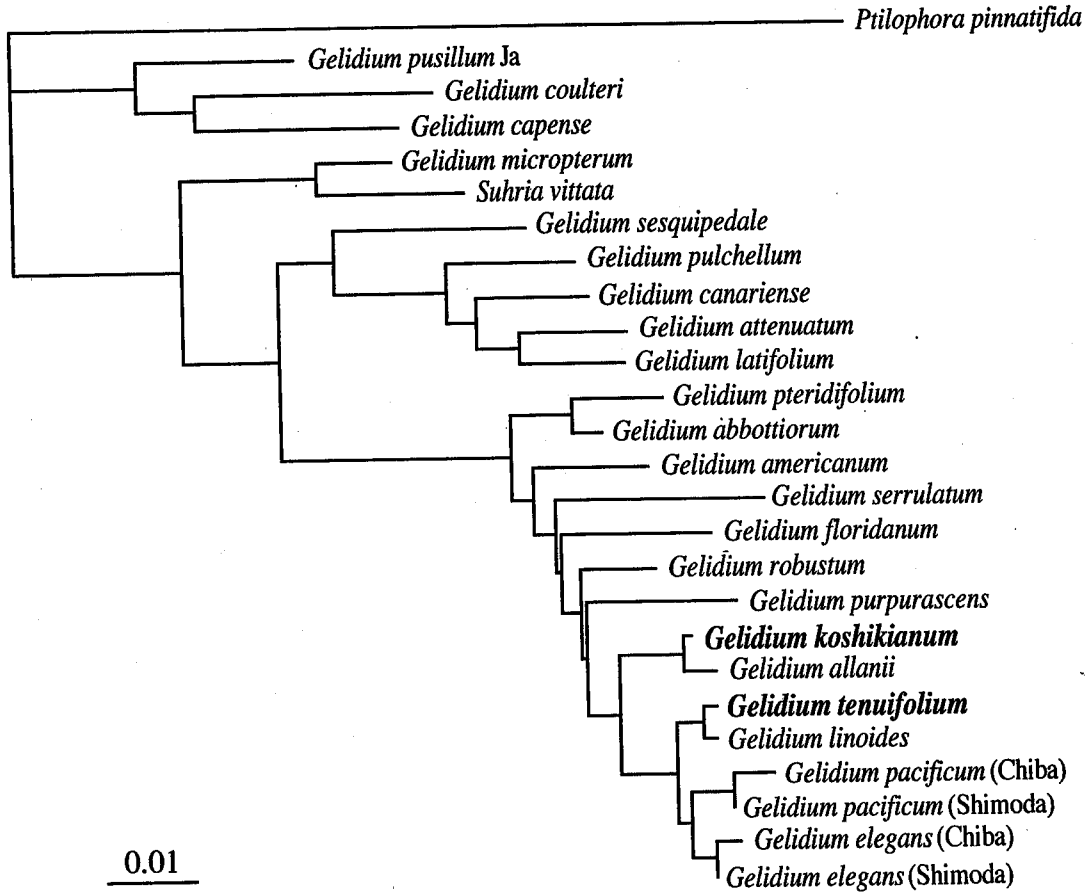


Fig. 68. Phylogenetic tree to elcidate phylogenetic position of two new species of *Gelidium* inferred from *rbc*L sequences with the maximum likelihood (ML) method using fastDNAml program. Scale bar = 1% divergence.

Table 9. A comparison of closely related six species of *Gelidium*

	G. tenuifolium	*G. linoides* *	*G. elegans* *	*G. pacificum* *	*G. koshikianum*	*G. allanii* †
Thallus length	15-30 cm	25-30 cm	10-30 cm	15-35 cm	5-8 cm	Up to 13 cm
Thallus color	Purplish to brownish red	Purplish to brownish red	Dark red	Dark red	Orange to purplish red	Purplish to dark red
Upper portion of branches	Wide, flattened and thin	Linear, flattened and thin	Subterete to compressed	Subterete to compressed	Subterete to compressed	Subterete to compressed
Apex of branches	Obtuse	Acute	Acute	Acute	Acute	Acute
Apical cell	At depression	At apex	At apex	At apex	At apex	At apex
Branchlets	Determinate	Determinate	Indeterminate	Indeterminate	Determinate	Indeterminate
Midlib of axes	Absent	Absent	Absent	Slight	Absent	Absent
Width of axes	1.4-2.0 mm	Up to 2.0 mm	Up to 2.0 mm	Up to 2.5 mm	Up to 2.5 mm	Up to 0.5 mm
Width of branches	Up to 2.0 mm	0.2-0.5 mm	Up to 1.5 mm	Up to 2.0 mm	Up to 1.6 mm	???
Tetrasporangia	Short determunate branchlets	Short determunate branchlets	Indeterminate branchlets	Assembled branchlets	Short determunate branchlets	Branchlets with sterile margins

*Data from Okamura (1934), but he did not distinguish axes and branches, so that we measured width of axes and branches of *G. linoides*, *G. elegans* from 'Type' materials that were illustrated in Okamura's later publication (Okamura 1934) and lectotype specimen of *G. pacificum*.

†Data from Chapman (1969) and Nelson *et al*. (1994)

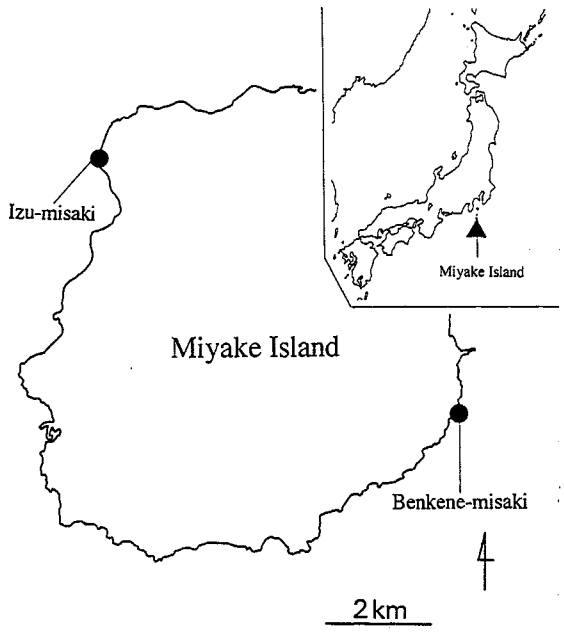


Fig. 69. Map showing the locations on Miyake Island where *Gelidiella ligulata* was collected.

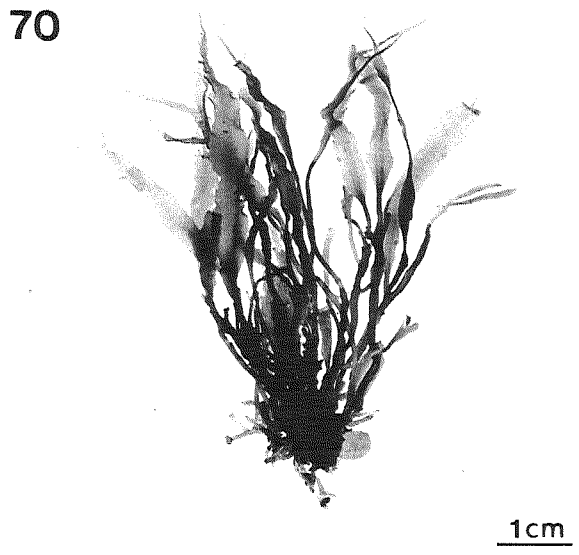
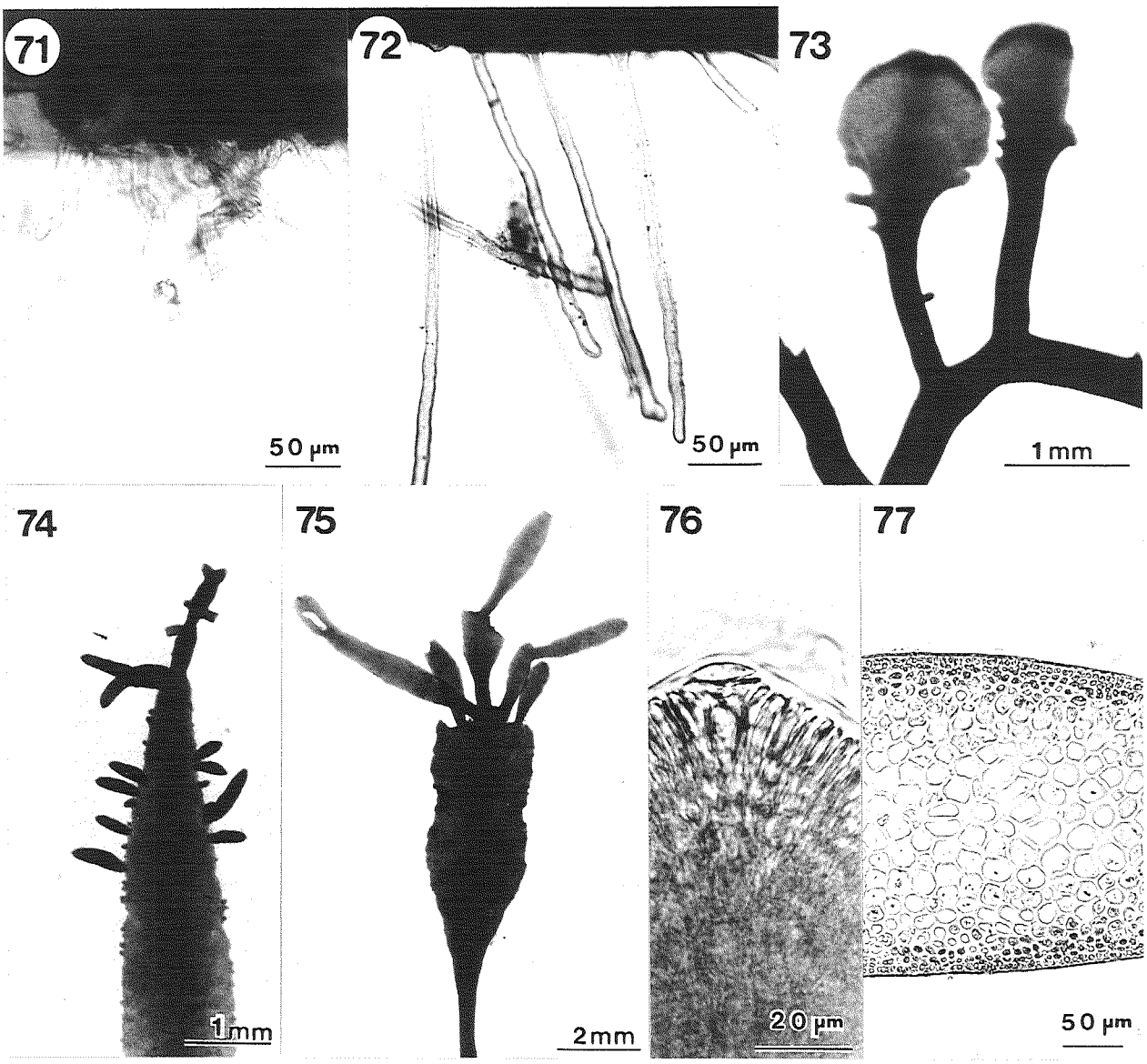
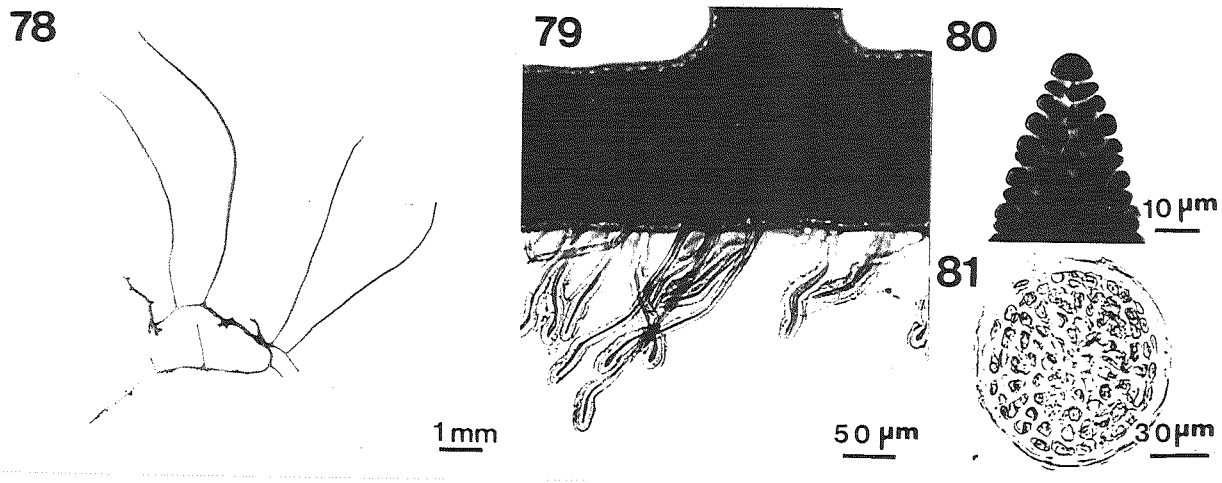


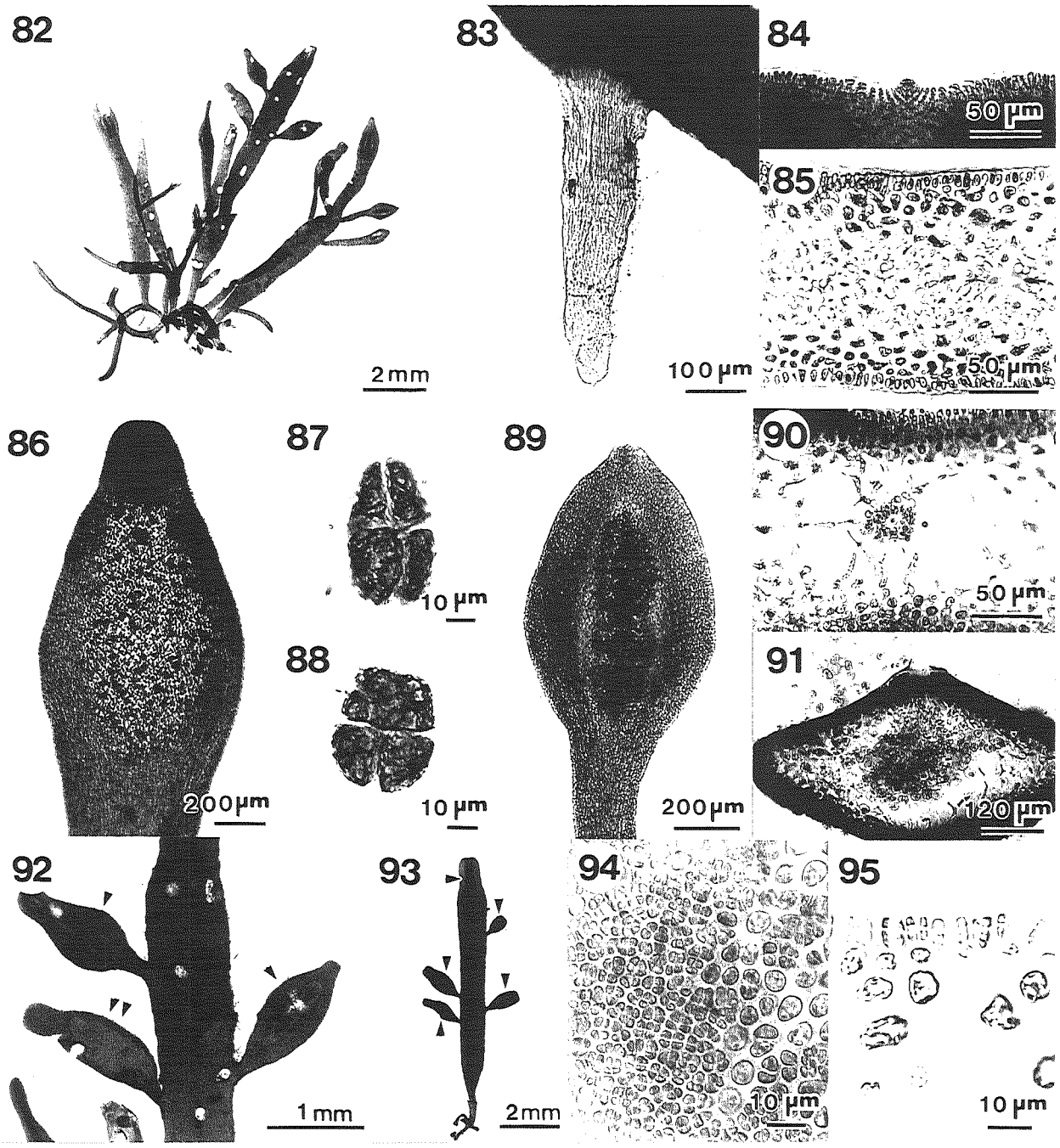
Fig. 70. Formalin/seawater-preserved specimen of *Gelidiella ligulata* collected at Izu-misaki, Miyake Island.



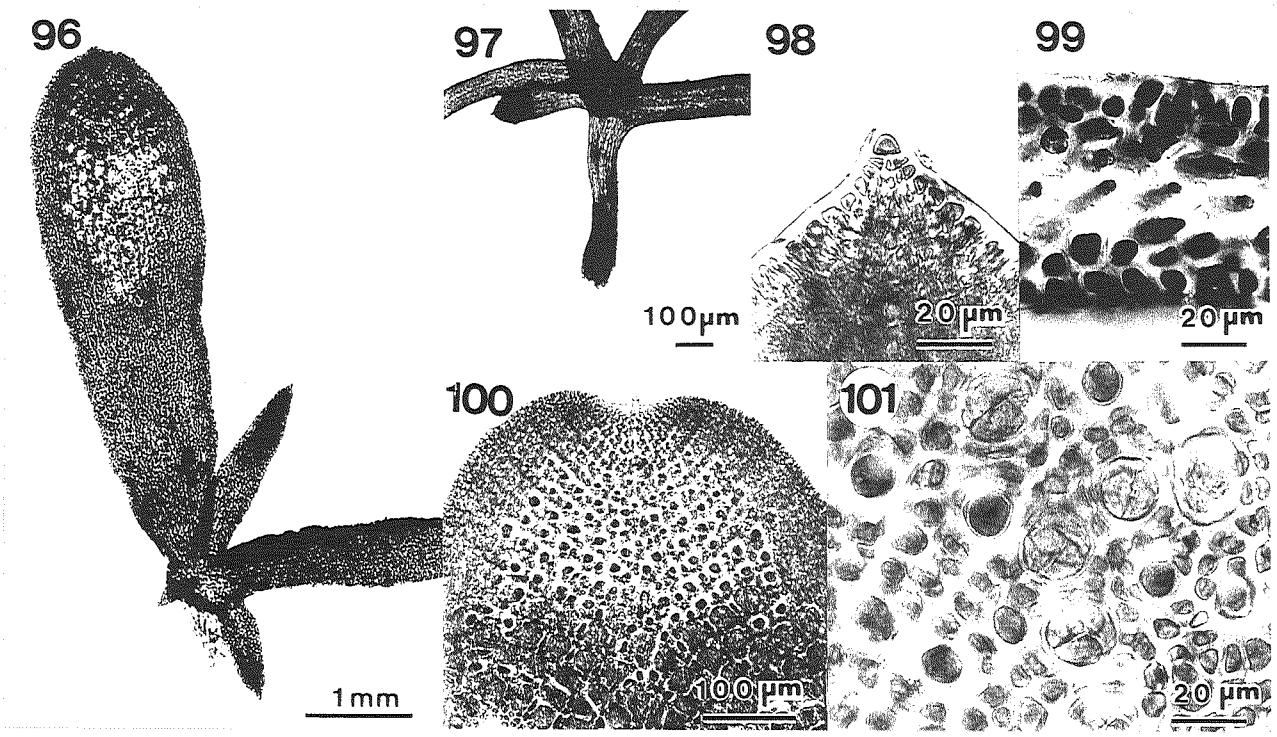
Figs 71-77. *Gelidiella ligulata* collected at Izu-misaki. Formalin/seawater-preserved material unless otherwise indicated. 71. Secondary rhizoidal attachments. 72. Secondary rhizoidal attachments (cultured plant grown at 20°C, 16:8 h L:D for one month). 73. Young fan-shaped blades issued from a creeping axis. 74. Uppermost portion of a erect blade forming branches irregularly to pinnately. 75. Proliferations from an injured (perhaps grazed) end of a blade. 76. Dome-shaped apical cell at the apex of a creeping axis. 77. Transverse section of a blade showing the absence of rhizines in the cortex and medulla.



Figs 78-81. *Gelidiella pannosa.*
78. Formalin/seawater-preserved specimen showing erect axes produced on a creeping axis. 79. Independent type of secondary rhizoidal attachments developing from a creeping axis (cultured plant grown at 20°C, 16:8 h L:D for one month). 80. Dome-shaped apical cell at the apex of an erect axis. 81. Transverse section of an erect axis showing the absence of rhizines in the cortex and medulla.



Figs 82-95. *Pterocladiella caerulescens.*
82. Formalin/seawater-preserved specimen. 83. Peg type of secondary rhizoidal attachments (cultured plant grown at 20°C, 16:8 h L:D for one month). 84. Dome-shaped apical cell in the apical depression of a first-order branch. 85. Transverse section of the middle portion of a first-order branch. 86. Tetrasporangial branch. 87. Cruciately divided tetrasporangium. 88. Decussately divided tetrasporangium. 89. Cystocarpic branch. 90. Transverse section of a young female branchlet showing nutritive filaments which grow centripetally and form a virtually solid cylinder around the central axis. 91. Transverse section of a cystocarp with one ostiole in the upper side. 92. Cystocarpic (arrowheads) and spermatangial (double arrowhead) branchlets. 93. Spermatangial sori (arrowheads) occurring on the spermatangial erect axis. 94. Surface view of a spermatangial sorus 95. Transverse section of a spermatangial sorus.



Figs 96-101. *Pterocladiella caloglossoides.*
96. Formalin/seawater-preserved specimen. 97. Peg type of secondary rhizoidal attachments in a field-collected plant. 98. Dome-shaped apical cell at the apex of an erect axis. 99. Transverse section of the middle portion of an erect axis showing a single row of medullary cells. 100. Surface view of a tetrasporangial sorus showing transverse rows of tetrasporangia. 101. Surface view of a tetrasporangial sorus showing cruciately or decussately divided tetrasporangia.

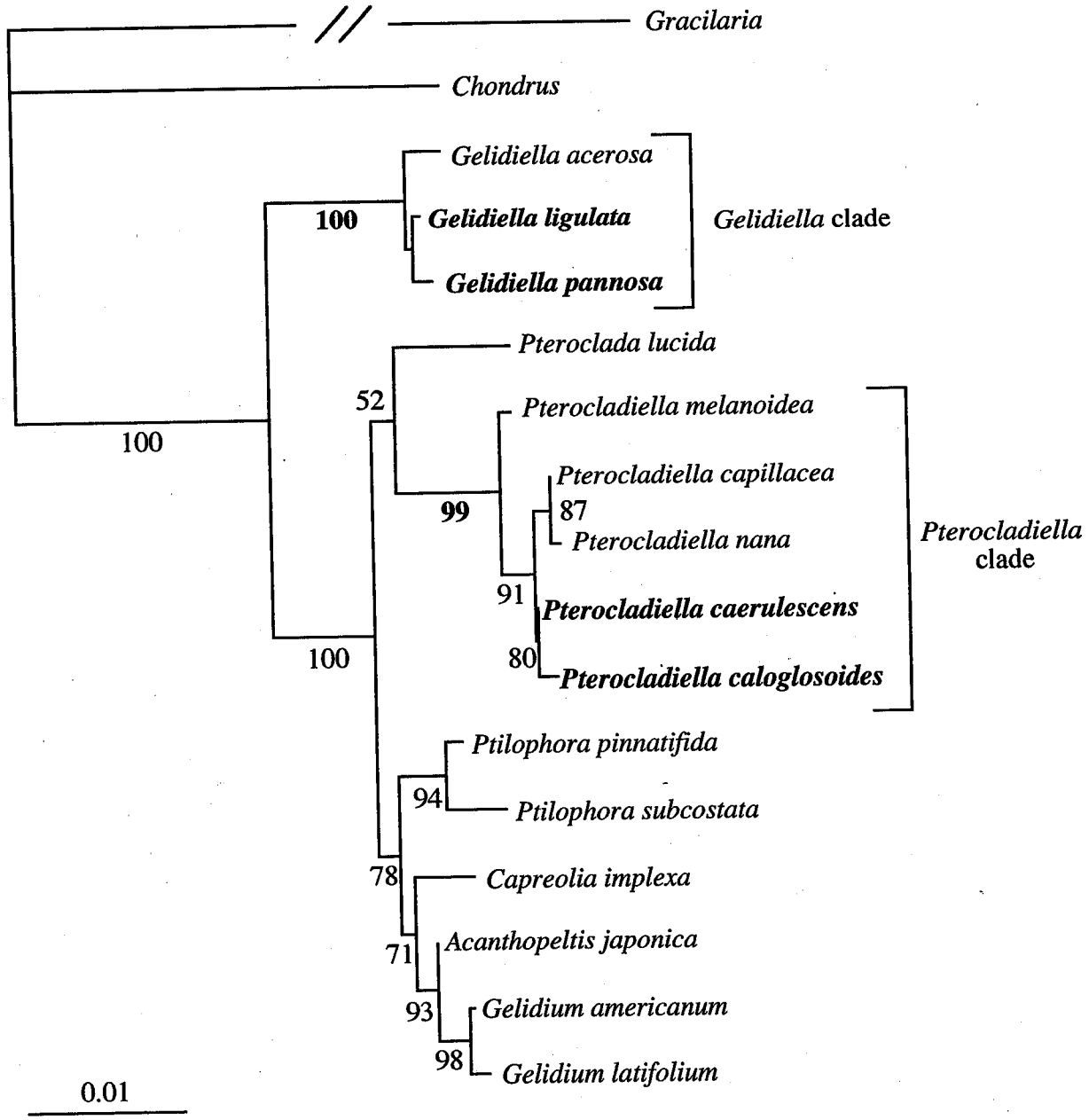


Fig. 102. Phylogenetic tree to elcidate phylogenetic position of newly found gelidialean species in Japanese waters inferred from SSU sequences with the neighbor-joining (NJ) method based on Kimura's correction using the Clustal W computer program. The numbers under the branches indicate bootstrap values (100 replications) greater than 50. Scale bar = 1% divergence.