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Phylogenetic affinities of genera *Acanthopeltis* and *Yatabella* (Gelidiales, Rhodophyta) inferred from molecular analyses

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Phylogenetic affinities of *Acanthopeltis japonica* Okamura and *Yatabella hirsuta* Okamura were determined from nucleotide sequences of the nuclear-encoded small subunit rDNA (SSU), internal transcribed spacer 1 (ITS1), and plastid-encoded large subunit of the ribulose-1,5-bisphosphate carboxylase/oxgenase gene (*rbc*L). We have sequenced an additional nine species of Japanese gelidialean species. Although *Acanthopeltis* and *Yatabella* had been suggested to be derived from separate lineages by researchers who emphasized the difference of growth pattern, they were recognized as a monophyletic group in the SSU, ITS1, and *rbc*L analyses. We have also demonstrated that *Acanthopeltis* and *Yatabella* possess a fundamentally similar type of 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., is proposed. Additional information on phylogenetic relationships within the Gelidiales was obtained. Our phylogenetic analyses of the Gelidiales using the above genes show three major clades, the *Gelidiella* clade that was the earliest diverging group within the order, a *Pterocladia*/*Pterocladiella* clade, and a large *Gelidium*-complex clade. The large *Gelidium*-complex clade is composed of the *Ptilophora* clade, *Capreolia* clade, and *Gelidium*-complex clades. In the *rbc*L and ITS1 trees, the *Gelidium*-complex clade includes three subclades, one of which is recognized for the first time and includes *Acanthopeltis*/*Yatabella*, *Onikusa japonica* (Harvey) Akatsuka, and *Gelidium vagum* Okamura, all distributed in the western and eastern Pacific. The type of secondary rhizoidal attachment, the unicellular independent type, peg type, and brush type, is consistent with the respective three major clades of the Gelidiales, suggesting that this morphological character reflects the phylogeny of this order.

INTRODUCTION

The red algal order Gelidiales currently includes 11 genera and approximately 140 species (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). Of these seven genera, *Acanthopeltis* and *Yatabella* are monotypic and possess unique properties in the Gelidiales with regard to geographical distribution and morphology. The genus *Acanthopeltis*, containing *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*, containing *Y. hirsuta* Okamura (1900), is endemic to southern Japan and has subcylindrical erect axes beset 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 in other vegetative and reproductive features (Okamura 1900, 1901).

Recent molecular analyses of the Gelidiales show that they can provide clues to the phylogeny of this order (Freshwater & Rueness 1994; Freshwater *et al.* 1995; Bailey & Freshwater 1997; Patwary *et al.* 1998). *Acanthopeltis* and *Yatabella*, however, have not been included in these analyses. In this article, we determined nucleotide sequences of the nuclear-encoded small subunit rDNA (SSU) gene, ITS1 sequence, and plastid-encoded *rbc*L gene of *Acanthopeltis* and *Yatabella*. We also have sequenced the SSU in nine species of the Japanese Gelidiales, the *rbc*L in seven species, and the ITS1 in nine species. We reexamined growth patterns of *A. japonica* and *Y. hirsuta* on the basis of type material, laboratory-cultured plants, and herbarium specimens. We also attempted to seek critical morphological characters that are correlated with molecular data of the Gelidiales.

MATERIALS AND METHODS

Sampling and DNA extraction

Total DNA was extracted from 13 unialgal cultured strains of Gelidiales (Table 1), for which voucher specimens were 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 L : D cycle with a photon flux of 15–25 $\mu Em^{-2}s^{-1}$.

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

Species	Locality (date, voucher number in SAP)	DNA	Rhizoid
Acanthopeltis japonica Okamura Shi	Shimoda, Shizuoka Pref. (25 Sep 1996, 064829)	*	*
Acanthopeltis japonica Okamura Ory	Oryuzako, Miyazaki Pref. (3 Aug 1997, 064830)	*	*
Capreolia implexa Guiry et Womersley	Tamarama, Australia (23 Apr 1998, 064831)		*
Gelidiella acerosa (Forsskål) Feldmann et Hamel	Ginowan, Okinawa Pref. (11 Jun 1998, 064832)		*
Gelidiella ligulata Dawson	Miyake Is., Tokyo (14 Jul 1998, 063883)	*	*
Gelidium divaricatum Martens	Nishiizu, Shizuoka Pref. (26 Sep 1996, 064833)	*	*
Gelidium elegans Kützing	Awaji Is., Hyogo Pref. (16 May 1996, 064834)	*	*
Gelidium linoides Kützing	Shimoda, Shizuoka Pref. (25 Sep 1996, 064835)	*	*
Gelidium pacificum Okamura	Enoshima, Kanagawa Pref. (29 Mar 1998, 064836)		*
Gelidium pusillum (Stackhouse) Le Jolis	Awaji Is., Hyogo Pref. (1 Oct 1996, 064837)	*	*
Gelidium subfastigiatum Okamura	Oshoro, Hokkaido (6 Mar 1997, 064838)	*	*
Gelidium vagum Okamura	Jodogahama, Iwate Pref. (11 Jun 1997, 064839)	*	*
Onikusa japonica (Harvey) Akatsuka	Shimoda, Shizuoka Pref. (25 Sep 1996, 064840)	*	*
Pterocladia lucida (Brown et Turner) J. Agardh	Scarborough, Perth (7 Dec 1997, 064841)		*
Pterocladiella capillacea (Gmelin) Santelices et Hommersand	Shimoda, Shizuoka Pref. (25 Sep 1996, 064842)	*	*
Pterocladiella sp.[1]	Sandakan, Malaysia (16 May 1998, 064843)		*
Ptilophora subcostata (Okamura) Norris	Naminoura, Wakayama Pref. (29 Sep 1996, 064844)		*
Yatabella hirsuta Okamura Ory-1	Oryuzako, Miyazaki Pref. (11 Jul 1996, 064845)	*	*
Yatabella hirsuta Okamura Ory-2	Oryuzako, Miyazaki Pref. (3 Aug 1997, 064846)	*	*

[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).

Blotted algal tissue was ground in liquid nitrogen. To remove polysaccharides, the frozen powder was rinsed with washing buffer (Kawahara *et al.* 1995) 3–5 times until the supernatant became colorless, and then UNSET buffer (Garriga *et al.* 1984) was added to the rinsed pellet and this mixture incubated on ice for 40 min. 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 10,000 rpm (7000 $\times g$) for 5 min. The upper aqueous phase was transferred to a new tube, and the extraction repeated three times. CIA (chloroform : isoamyl alcohol = 24 : 1) mixture was added and mixed gently for 10 min. The solution was then centrifuged at 10,000 rpm (7000 $\times g$) for 5 min. Total genomic DNA was precipitated by 0.2 M NaCl in 2.5 vol 99.5% ethanol on ice for 10 min. This was followed by centrifugation at 12,000 rpm (10,000 $\times g$) for 15 min; the pellet was washed with cold 70% ethanol and air-dried. The pellet was redissolved in 50–100 µl of autoclaved distilled water.

PCR amplification and sequencing

The total DNA was used as template for the polymerase chain reaction (PCR) (Saiki *et al.* 1988). In this study, we used seven pairs of primers: SR1–SR5, SR4–SR9, and SR8–SR12 for SSU (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′); and TW81–RED5.8R for

ITS1 (Goff *et al.* 1994). The temperature-cycling protocol consisted of an initial denaturation step at 93°C for 1 min, followed by 35 cycles of 30-s denaturation at 94°C, 30-s primer annealing at 55°C, and 45-s extension at 72°C; product then was held at 4°C. The PCR products were sequenced directly using a DNA autosequencer (ABI PRISM, 310 Genetic Analyzer) with dye-terminator method (Nakayama *et al.* 1996).

Sequence analysis

The SSU 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 alignments are available from the first author on request. 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 (Ragan *et al.* 1994) and *rbc*L (Freshwater *et al.* 1994) analyses (Table 2). The distance matrix method was used to construct phylogenetic trees. For the distance matrix method, we used Kimura's two-parameter method (Kimura 1980) to calculate the distance matrix and neighbor-joining 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.

Growth patterns of *A. japonica* and *Y. hirsuta*

We reexamined growth patterns of *A. japonica* and *Y. hirsuta* on the basis of the following specimens: (1) the holotype specimen of *A. japonica* collected at Misaki, Kanagawa Prefecture (April 1885), and deposited in the University Museum, University of Tokyo (TI); the holotype specimen of *Y. hirsuta* collected at Oryuzako, Miyazaki Prefecture (13 July 1899), and deposited in Herbarium Okamura housed in SAP; (2) cultured thalli shown in Table 1; (3) three herbarium specimens of *A. japonica* collected at Shimoda, Shizuoka Prefecture (8 July 1983, SAP 062000), at Akiya, Kanagawa Prefecture (4 March 1988, SAP 060389), and at Enoshima, Kanagawa Prefecture (15 March 1930, SAP 060892); and five herbarium specimens of *Y. hirsuta* collected at the type locality (23 March 1948, SAP 062325; 3 April 1948, SAP 062326; 3 August 1958, SAP 062324; 1 June 1962, SAP 061052; 11 July 1996, SAP 064847).

Secondary rhizoidal attachments and correlation of molecular and morphological data

We observed secondary rhizoidal attachments of 17 species of nine genera in cultured strains and field-collected plants (Table 1). We analyzed the correlation between morphological data, secondary rhizoidal attachments, and phylogenetic trees of the SSU and *rbc*L.

RESULTS

SSU analysis

Twenty-three samples (21 species) were used for the SSU gene (1707 bp) analyses. The phylogenetic tree obtained from neighbor-joining 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* (Schousboe 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 the remaining taxa is referred to as 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 comprising *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 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-two samples (36 species) were used for the *rbc*L gene (1467 bp) analyses. The phylogenetic tree obtained from neighbor-joining 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 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 (*G. coulteri* complex, Freshwater *et al.* 1995) contained *G. pusillum* (Stackhouse) Le Jolis (strains of Japan, California USA, and Puerto Rico), *G. capense* (Gmelin) Silva, and *G. coulteri* Harvey. The *Acanthopeltis* clade contained *Onikusa japonica* (Harvey) Akatsuka, *Gelidium vagum* Okamura, *A. japonica*, and *Y. hirsuta*. The monophyly of this subclade was supported by 100% bootstrap value and *Gelidium vagum* formed a 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, which was included in the '*Gelidium*' clade.

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

Species	Accession number		
	SSU	*rbcL*	*ITS1*
Acanthopeltis japonica Okamura Shi	AB017664	AB017673	AB017682
Acanthopeltis japonica Okamura Ory	AB017665	AB017674	AB017683
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]
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 USA		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
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 USA	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 Ory	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).

ITS1 analysis

Sixteen samples (14 species) in the *Gelidium*-complex clade and *G. divaricatum* as outgroup were used for the ITS1 sequence (252 bp) analyses. The phylogenetic tree was obtained from neighbor-joining analysis (Fig. 3). The three subclades that were described above were also supported by high bootstrap values: 97% for the *G. coulteri* clade, 100% for the *Acanthopeltis* clade, and 97% for the '*Gelidium*' clade. However, no bootstrap support (<52%) was obtained for the topological positions of these subclades. *Acanthopeltis* and *Yatabella* formed a monophyletic group, and *G. vagum* that includes strains from Japan and the Pacific coast of Canada was recognized as the sister group of the *Acanthopeltis*/*Yatabella* group with 94% bootstrap value. *Onikusa japonica* again was not monophyletic with *Onikusa pristoides*. *Gelidium elegans* Kützing, *G. linoides* Kützing, and *G. subfastigiatum* Okamura were recognized as a monophyletic group and formed a sister group to the European species of *Gelidium*, such as *G. sesquipedale* (Clemente) Turner, *G. arbuscula* (Montagne) Børgesen and *G. latifolium* (Greville) Bornet et Thuret.

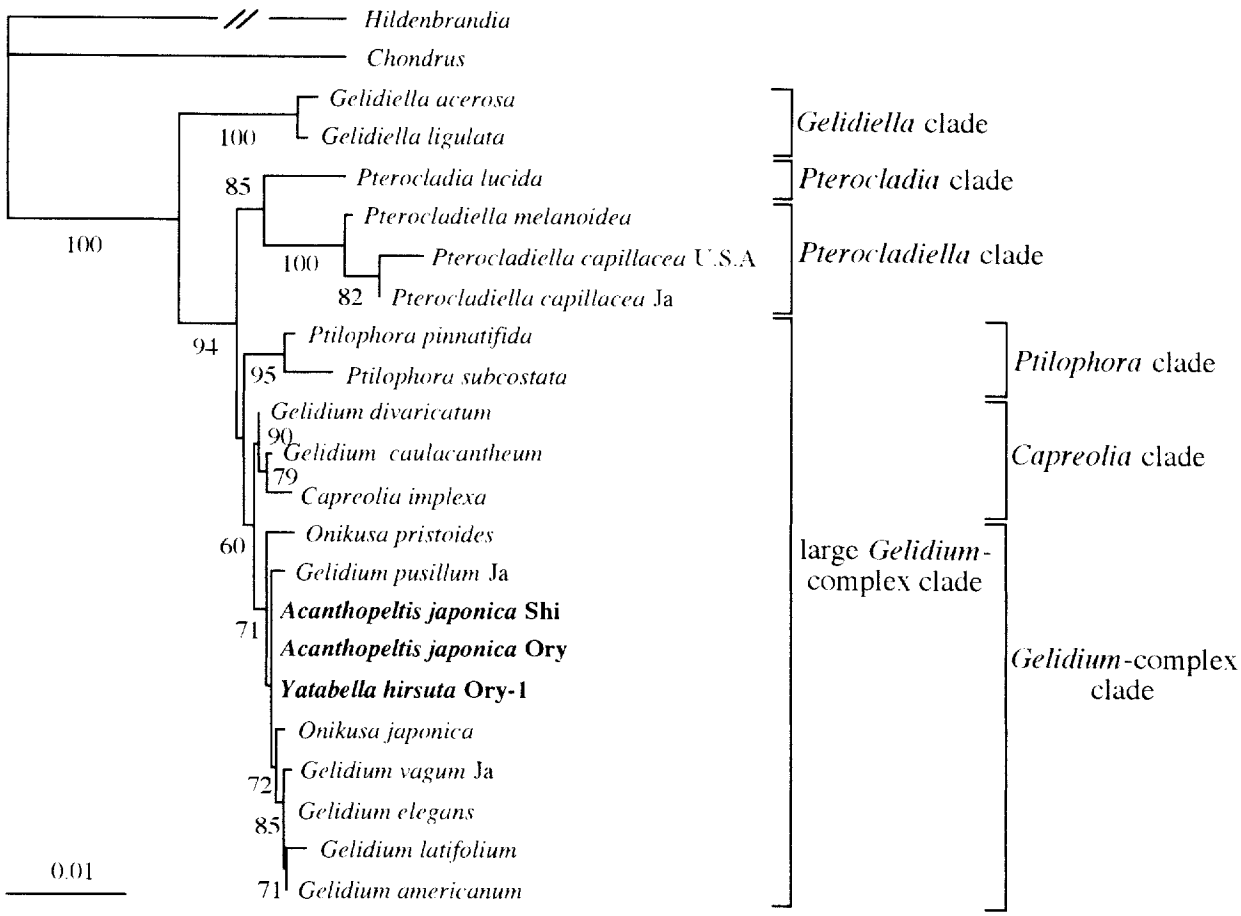


Fig. 1. Phylogenetic tree inferred from SSU sequences with the neighbor-joining method based on Kimura's correction using the Clustal W computer program. The numbers under the branches indicate bootstrap values (100 replications) greater than 50.

Pairwise distances between individual thalli of *Acanthopeltis* and *Yatabella*

The sequence divergence was compared between individuals of *A. japonica* from different localities (Shimoda, Shizuoka Prefecture, 25 September 1996, and Oryuzako, Miyazaki Prefecture, 3 August 1997) and two individuals of *Y. hirsuta* from the same locality (Oryuzako, Miyazaki Prefecture) but collected at different times (11 July 1996 and 3 August 1997). There was no sequence difference in the SSU (1707 bp), no sequence difference (*A. japonica* from Shimoda against two samples of *Y. hirsuta*) or only one substitution (*A. japonica* from Oryuzako against two samples of *Y. hirsuta*) in the *rbc*L (1467 bp) sequences, and only two differences (*A. japonica* against *Y. hirsuta*) in the ITS1 (252 bp) sequences (Table 3). To guard against technical error, we twice extracted DNA from the same individual and twice independently sequenced these genes. No sequence difference was found in these repeated experiments.

Growth patterns of *Acanthopeltis* and *Yatabella*

Growth patterns of erect axes of *Acanthopeltis* and *Yatabella* are shown in Figs 4–9. Isolated apical tips of branchlets of both species grew into creeping axes in laboratory culture. At 20° C these creeping axes formed erect axes after 1–2 mo. The erect axis of *A. japonica* became broad and grew into a leaflike structure, producing proliferations on both surfaces (Fig. 9, stage A1). Then one of the proliferations became broad and grew into a second leaflike structure (Fig. 4) that overtopped the parental leaflike structure (Fig. 9, stage A2). The second leaflike structure also produced proliferations on the surfaces, one of which grew into a third leaflike structure (Figs 5, 9, stage A3). This process was repeated many times, and leaflike structures were piled up in three-dimensions (Figs 6, 9, stages A4, A5).

Yatabella hirsuta produced multifid-echinate ramuli (Fig. 7) oriented in many directions at the surface, and several of them grew into lateral branches (Figs 7, 9). After the production of several branches (Fig. 9, stages Y1, 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. 9, stage Y3, branch 5). This branch (functioning as new axis) also produced a number of multifid-echinate ramuli variously oriented at the surface, and several of them grew into lateral branches (Fig. 9, stage Y4, branches 6–9). The new axis (Y3, branch 5) was also overtopped by one of the branches (Fig. 9, stage Y5, branch 9), and the branch grew into a new axis. Field-collected specimens, including the holotype specimen of *Y. hirsuta*, have several overtopped branches (Fig. 8).

Field-collected specimens of *A. japonica* (Fig. 10) and *Y.*

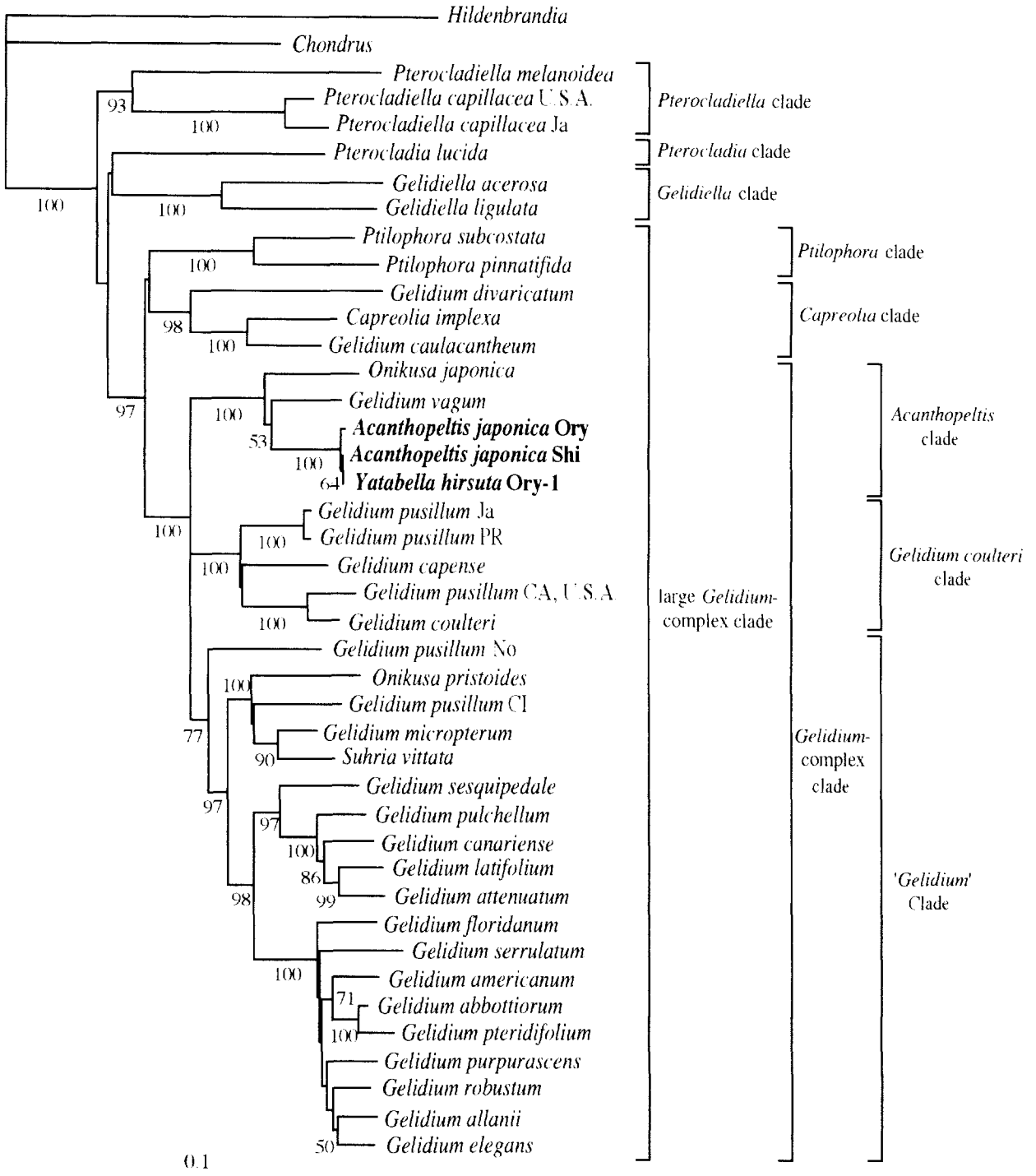


Fig. 2. Phylogenetic tree inferred from *rbc*L sequences with the neighbor-joining method based on Kimura's correction using the Clustal W computer program. The numbers under the branches indicate bootstrap values (100 replications) greater than 50.

hirsuta (Figs 8, 11) are profusely branched. In *A. japonica* profuse branching may result from the formation of leaflike structures apically from similar structures that form a single leaflike structure proximally. The apical leaflike structure grows in a manner similar to that of the proximal one. By contrast, such branching in *Y. hirsuta* is due to the production of branches with sympodial growth.

Secondary rhizoidal attachments and correlation of molecular and morphological data

Similar to Perrone (1994), in the present study, three types of secondary rhizoidal attachments were recognized (Figs 12–14): (1) the unicellular independent type was observed in *Gelidiella acerosa* (Fig. 12) and *Gelidiella ligulata*; (2) the peg

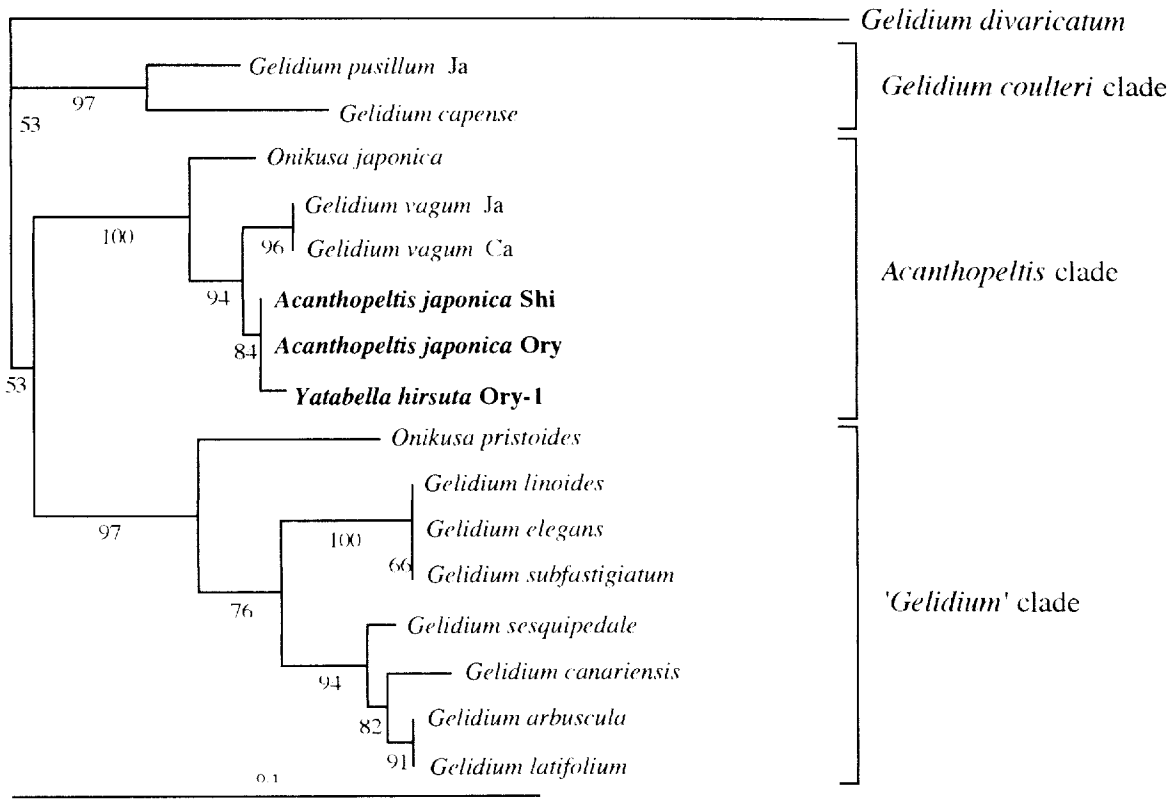


Fig. 3. Phylogenetic tree inferred from ITS1 sequences with the neighbor-joining method based on Kimura's correction using the Clustal W computer program. The numbers under the branches indicate bootstrap values (100 replications) greater than 50.

type was found in *Pterocladia lucida*, *Pterocladiella capillacea* (Fig. 13), and *Pterocladiella* sp.; (3) the brush type was observed in *Ptilophora subcostata*, *Capreolia imprexa*, *A. japonica*, *Y. hirsuta*, *Onikusa japonica*, *Gelidium divaricatum*, *G. pusillum*, *G. elegans* (Fig. 14), *G. linoides*, *G. pacificum*, *G. vagum*, and *G. subfastigiatum*. These types of secondary rhizoidal attachments were correlated with the type of cystocarps (Dawson 1952; Fan 1961; Bailey & Freshwater 1997; Yoshida 1998) and the neighbor-joining tree of the SSU gene in the *Gelidiella* clade and *Pterocladia*/*Pterocladiella* clade (Fig 15), and the *rbc*L gene in the large *Gelidium*-complex clade.

DISCUSSION

Taxonomic treatment of *Acanthopeltis* and *Yatabella*

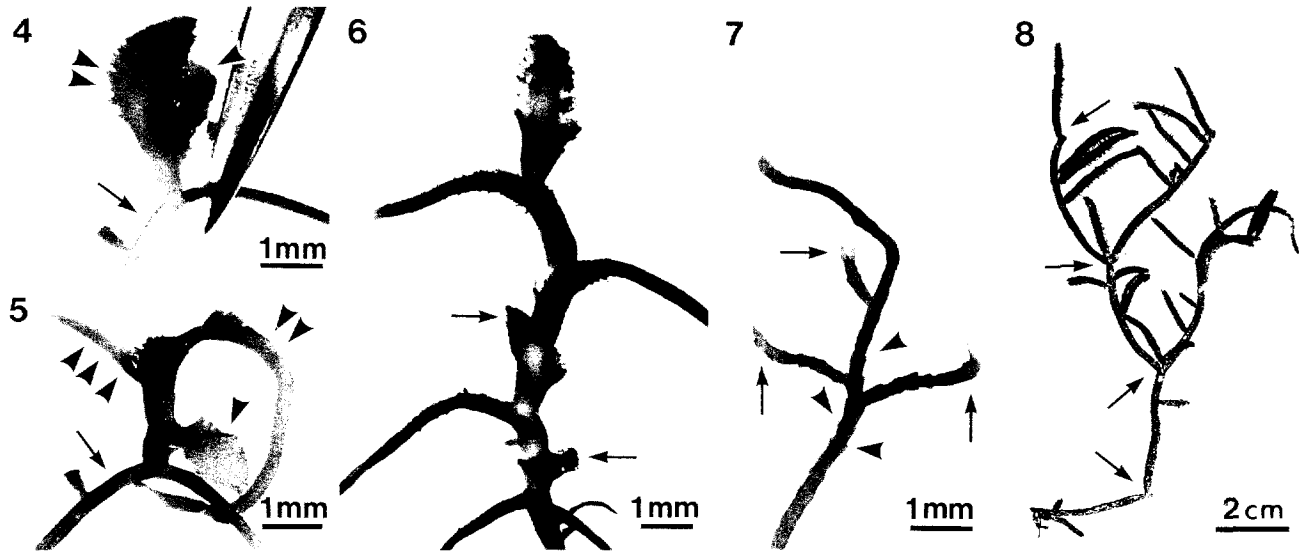
When Okamura (1900) described the genus *Yatabella* with a new species, *Y. hirsuta*, he noted that this species was similar to *A. japonica* in its vegetative and reproductive features. However, the difference in growth patterns, sympodial vs monopodial, led Okamura to separate these two species at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

In the present study we have demonstrated that *Acanthopeltis* and *Yatabella* possess fundamentally similar growth patterns. Sympodial growth and the production of overtopped leaflike structures occurs repeatedly and regularly with short intervals in *Acanthopeltis*, whereas sympodial growth with the formation of overtopped branches occurs infrequently and irregularly in *Yatabella*. In the latter, lateral branches are formed monopodially on a particular branch, and one of these branches formed near the apex of the parental branch shows sympodial growth. We think that Okamura (1900) might have been 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. A similar result was obtained with the SSU

Table 3. Pairwise distances between individual plants of *Acanthopeltis* and *Yatabella* for SSU, *rbc*L, and ITS1 sequences. The lower half of the matrix shows the number of base-pair changes between individual pairs including gaps.

	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 4–9. Development of erect axes of *Acanthopeltis japonica* and *Yatabella hirsuta*.

Figs 4–6. Cultured plants of *A. japonica* grown at 20°C and 16 : 8 h L : D cycle.

Fig. 4. 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).

Fig. 5. Three weeks after the stage shown in Fig. 4, now showing a creeping axis (arrow), the first leaflike structure (arrowhead), the second one (double arrowheads), and the third (triple arrowheads).

Fig. 6. Portion of a 7-month-old plant showing leaflike structures repeatedly piled up, the arrows indicating leaflike structures ceasing elongation.

Figs 7, 8. *Yatabella hirsuta*.

Fig. 7. A 5-month-old cultured plant grown at 20°C and 16 : 8 h L : D cycle, showing three lateral branches (arrow) and multifid-echinate ramuli (arrowheads).

Fig. 8. Portion of a herbarium specimen collected at the type locality (11 July 1996, SAP 064847) showing overtopped branches (arrow).

analysis, but the bootstrap support was low. This is due to the inclusion in the analysis of sequences of taxa that are little divergent from those of *Acanthopeltis* and *Yatabella*. For example, the SSU of *O. japonica* differs from that of *Acanthopeltis* and *Yatabella* by only two bases. The resampled sequences from such taxa often were identical during bootstrap resamplings, and this effect obscured the close relationship of *Acanthopeltis* and *Yatabella*, which possess completely identical sequences (Table 3). As shown in Table 3, there are minor sequence differences of one substitution between these two species in the *rbc*L gene and two substitutions in the ITS1 sequences. These results indicate that *Acanthopeltis* and *Yatabella* are closely related. Taking morphological and molecular closeness into consideration, we think that there is no reason to continue separating these taxa at the generic rank.

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

In Freshwater & Rueness (1994), *rbc*L sequences were generated for multiple samples of *G. pulchellum* (six samples), *G. pusillum* (four samples), and *G. latifolium* (eight 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 nine samples of *Pterocladiella capillacea* showed a range of 0.2–1.4% (Freshwater *et al.* 1995). Compared with 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* suggests conspecificity of these taxa.

There are only two base substitutions in the ITS1 sequence. Such a small amount of sequence divergence in the ITS sequences has been observed within individual species of fungi (Gardes *et al.* 1991), diatoms (Zechman *et al.* 1994), the red alga *Chondrus crispus* Stackhouse (Chopin *et al.* 1996), and between species of 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 morphological and ecological grounds we believe that these entities are not the same species. *Acanthopeltis japonica* and *Yatabella hirsuta* occur sympatrically in Oryuzako, Miyazaki Prefecture, Japan, growing on subtidal rocks. Yet, the morphology of each species can be clearly distinguished in the field, and no intermediate forms have been observed at Oryuzako. Under similar culture conditions, the differences of branch morphology and branching patterns between species are maintained. We have not yet attempted crossing experiments, but these facts strongly suggest that these two entities are not conspecific. In this case, the sequence divergence of 2 bp 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 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 pos-

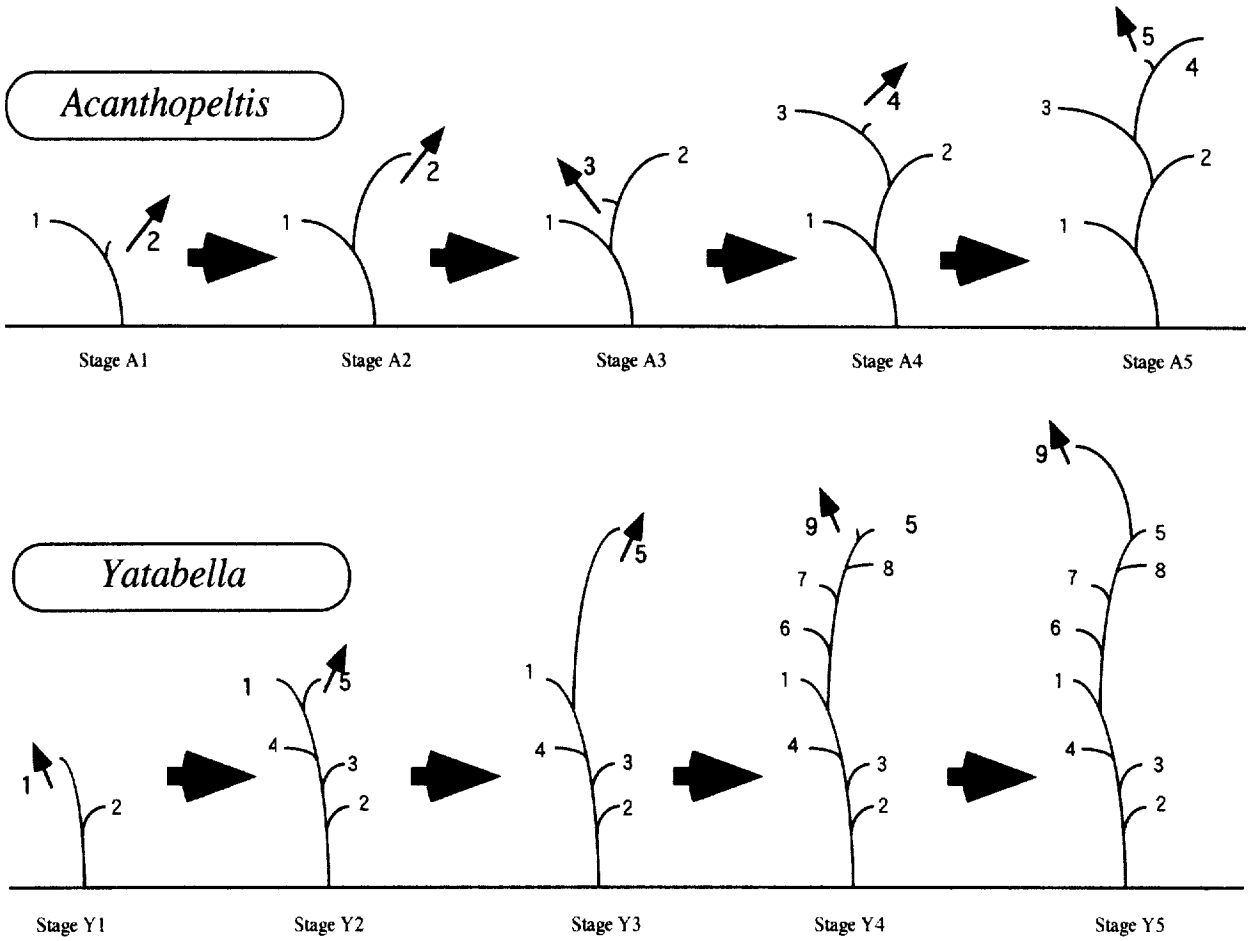


Fig. 9. Schematic illustrations of growth patterns of erect axes in *Acanthopeltis japonica* and *Yatabella hirsuta*. Numerals, sequence of formation of leaflike structures or branches; arrows indicate early stage of elongation of respective leaflike structures or branches. In *Yatabella* the multifid-echinate ramuli are omitted from the figures.

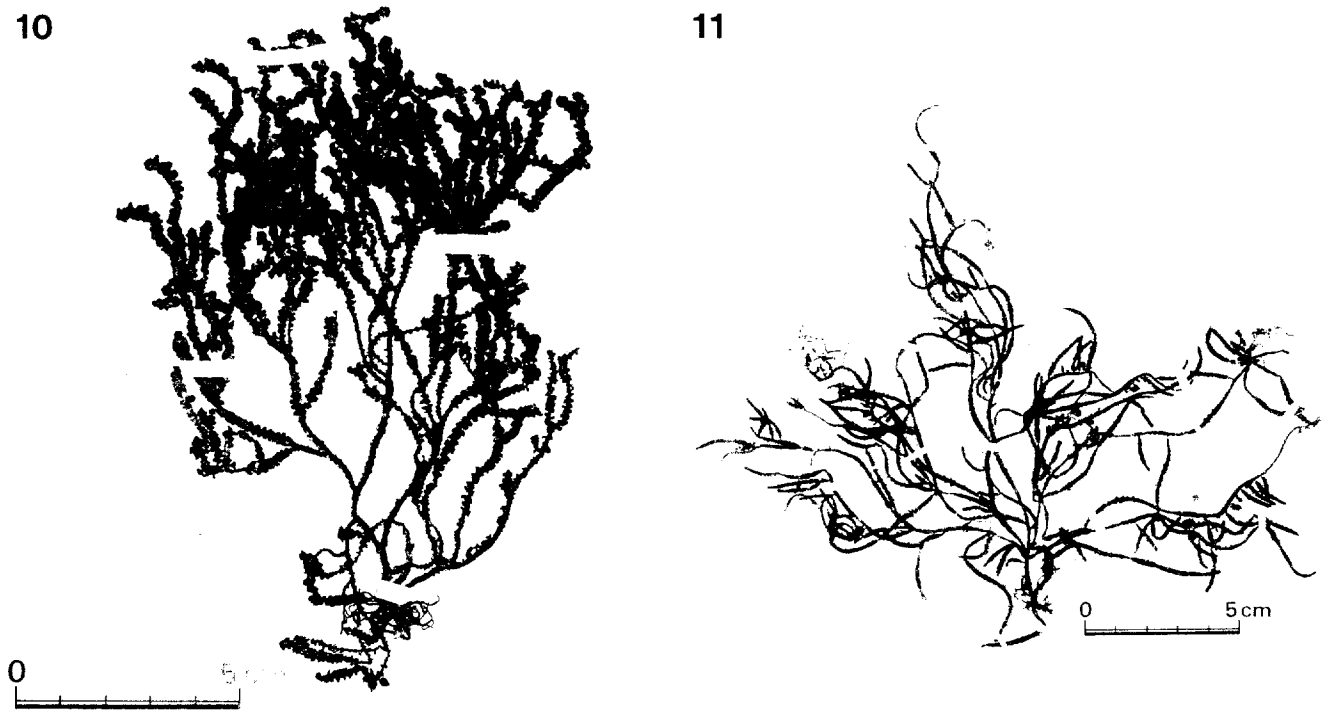
sesses exactly the same sequence as *Y. hirsuta*. Because *A. japonica* and *Y. hirsuta* possess identical sequences in the SSU gene, it is likely that these taxa originally possessed identical sequences in the *rbc*L gene as well. Unless one supposes convergence, it is hard to explain the situation that two species originally had one base difference and became identical in the Shimoda population. We believe that it is likely 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 & Rueness (1994).

Phylogenetic position of *Acanthopeltis/Yatabella*

The phylogenetic position of *Acanthopeltis/Yatabella* in the Gelidiales was consistent in both *rbc*L and ITS1 analyses. *Acanthopeltis/Yatabella*, *G. vagum*, and *O. japonica* were shown to be a monophyletic clade (*Acanthopeltis* clade) with 100% bootstrap values, and *G. vagum* formed a sister group of *Acanthopeltis/Yatabella*. However, SSU analysis showed *G. vagum* and *Acanthopeltis/Yatabella* to be in different lineages. In the SSU analysis, a low sequence divergence was detected among members of the *Gelidium*-complex clade (only three bases change between *G. vagum* and *Acanthopeltis/Yatabella*), and the tree might not reflect the true phylogeny with this clade. Taking into consideration the results of *rbc*L and ITS1 analysis (100% bootstrap values), we conclude that *G. vagum* is included in the *Acanthopeltis* clade. Although there is no clear morphological evidence to support close affinities of *G. vagum* and *Acanthopeltis/Yatabella*, the close relationship between *O. japonica* and *Acanthopeltis/Yatabella* is morphologically supported by stiffly cartilaginous thalli, thickened axes, and the presence of numerous proliferations on margins and surfaces (Yoshida 1998; our unpublished observations).

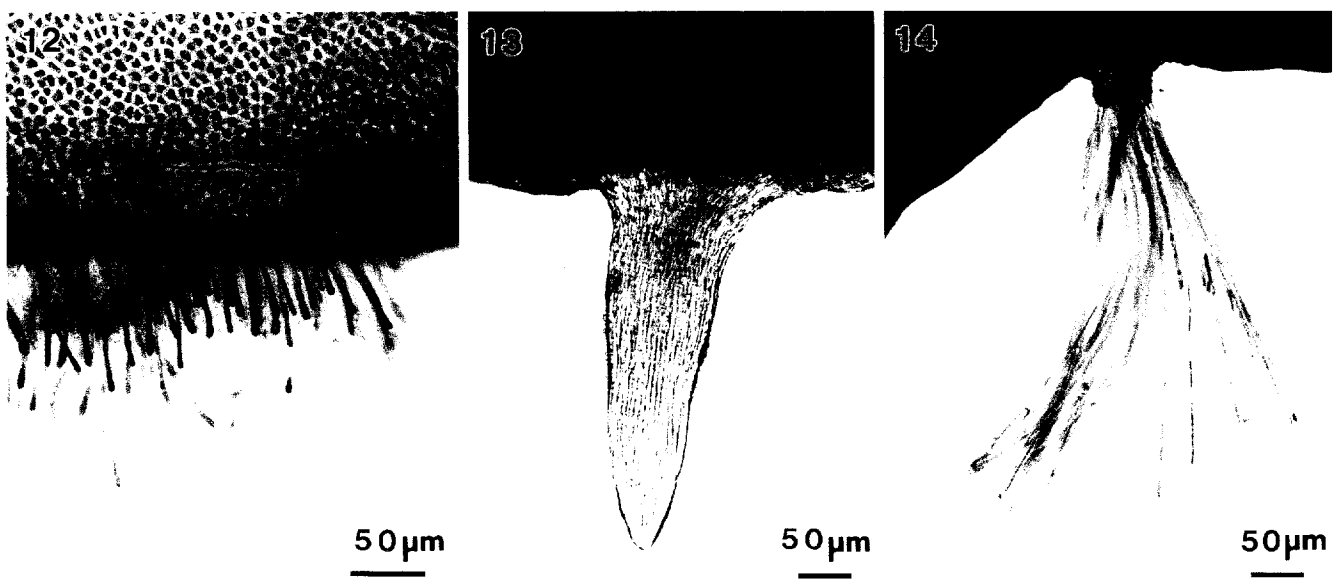
Additional information on the phylogeny of the Gelidiales

Results of the molecular analyses in this study were mostly congruent with those of previous reports (Freshwater *et al.* 1995; Bailey & Freshwater 1997; Patwary *et al.* 1998). Several points were clarified and additional information was obtained by including the 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 and *rbc*L analyses), *Pterocladia/Pterocladiella* clade (SSU 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



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

bootstrap value in the SSU analysis; (3) the large *Gelidium*-complex clade contained three clades, the *Ptilophora* clade, *Capreolia* clade, and *Gelidium*-complex clade (SSU and *rbc*L analyses); (4) the *Gelidium*-complex clade includes three subclades, the *G. coulteri* clade (*G. coulteri* complex, Freshwater *et al.* 1995), the *Acanthopeltis* clade that is recognized for the first time and contains *O. japonica, G. vagum, A. japonica,* and *Y. hirsuta,* all of which are distributed in the western and



Figs 12–14. Secondary rhizoidal attachments.
Fig. 12. Unicellular independent attachment of *Gelidiella acerosa* (field-collected plant, Ginowan, Okinawa Prefecture).
Fig. 13. Peg-type attachment of *Pterocladiella capillacea* (cultured plant, grown at 20°C and 16 : 8 h L : D cycle, Shimoda, Shizuoka Prefecture).
Fig. 14. Brush-type attachment of *Gelidium elegans* (cultured plant, grown at 20°C and 16 : 8 h L : D cycle, Awaji Island, Hyogo Prefecture).

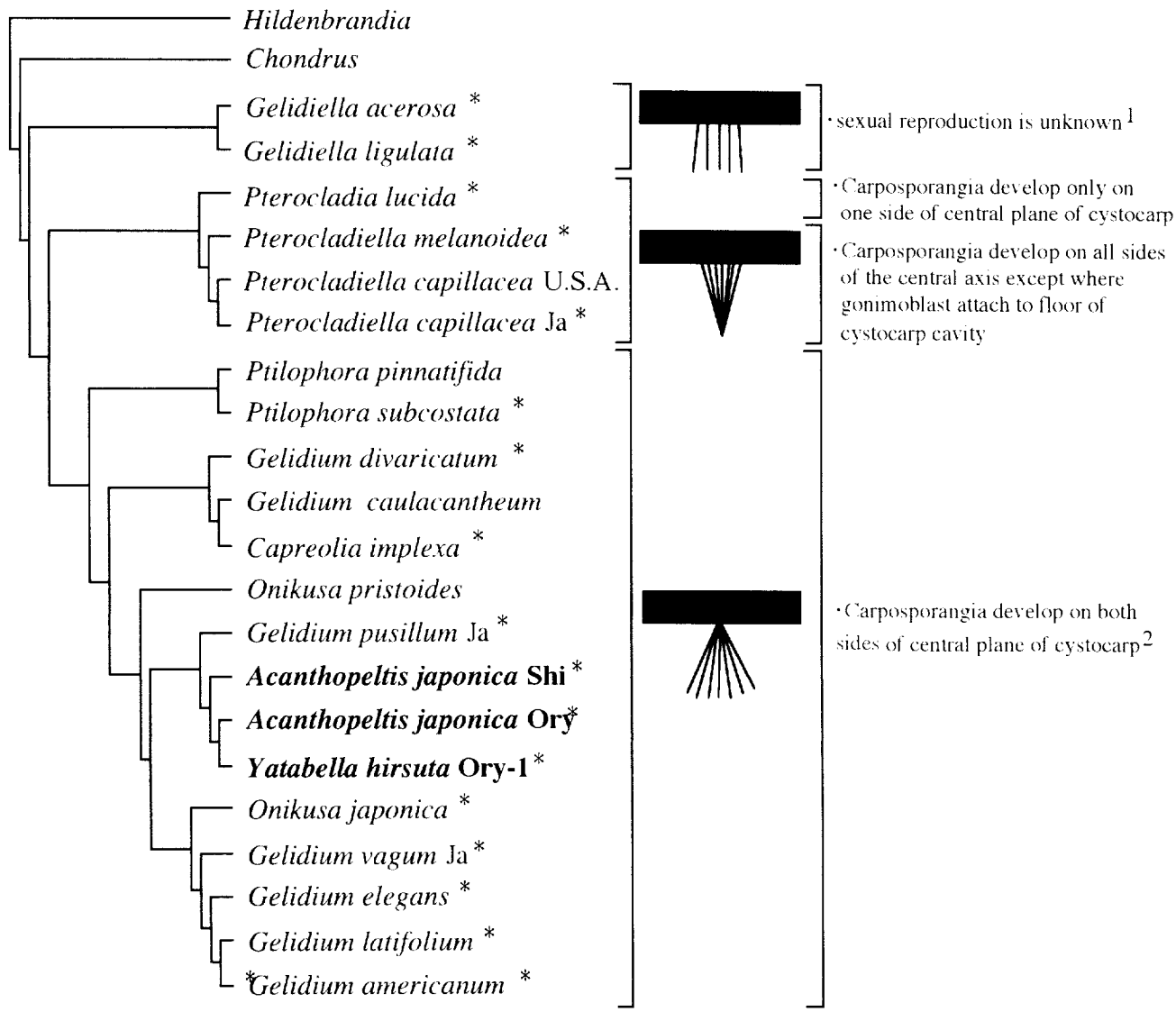


Fig. 15. Correlation of morphological data and SSU tree topology. The types of secondary rhizoidal attachments and developing carposporophyte were used as morphological data.
* Secondary rhizoidal attachment type determined previously or in this study.
[1] Only male gametophytes of *Gelidiella acerosa* were reported (Santelices 1997).
[2] *Capreolia* has no carposporophytic phase (Guiry & Womersley 1993).

eastern Pacific, and the '*Gelidium*' clade that is divisible into four groups, the Indo-Pacific/Caribbean *Gelidium* complex, European *Gelidium* complex, *Suhria* clade, and European *Gelidium pusillum* clade as reported previously (Freshwater *et al.* 1995) (*rbc*L and ITS1 analyses); (5) *O. japonica* was not monophyletic with the type species of the genus, *O. pristoides*, which was part of the *Suhria* clade (Freshwater *et al.* 1995) (*rbc*L and ITS1 analyses); (6) a Japanese strain of *G. pusillum* was included in the *Gelidium coulteri* clade (*rbc*L and ITS1 analyses). There is a tendency that better bootstrap support is obtained for the analysis of early branches in the SSU, whereas 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 is more conserved than the *rbc*L (Bailey & Freshwater 1997). Therefore the SSU is not suitable for the recently diverged taxa.

Freshwater *et al.* (1995) have suggested that certain genera, species, and populations form groups based on geographic distribution. This statement is confirmed in this study, as shown in the findings 4–6 described above. *Gelidium pusillum* has been separated into three clades in the molecular analyses (Freshwater *et al.* 1995). In this study, a Japanese strain of *G. pusillum* was included in the *G. coulteri* clade that contains the Pacific/Caribbean strains of *G. pusillum*, and this clade was separated from European or Eastern Atlantic strains of *G. pusillum*. It is obvious that sequencing of specimens from the type locality (Sidmouth, Devon, 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*. However, Rodríguez & Santelices (1988) and Santelices (1990) pointed out that such features are not sufficient to separate these two genera. In the previous molecular analysis (Freshwater *et al.* 1995), *Onikusa pristoides* was included in the *Suhria* clade and revival of the name *Suhria pristoides* (Turner) J. Agardh was suggested. We have demonstrated that *O. japonica* is not closely related to *O. pristoides*. *Onikusa pristoides* is closely related to *Suhria* as suggested by Freshwater *et al.* (1995), whereas *O. japonica* has close affinity with the *Acanthopeltis* clade. It might be appropriate to treat *O. japonica* as an independent genus. However, more information on morphology of *O. japonica* is needed prior to formal taxonomic action.

As Freshwater *et al.* (1995) reported, the *Capreolia* and *Suhria* clades contain other *Gelidium* species, but the taxonomic treatment of these species requires more morphological data.

Secondary rhizoidal attachments

Based on Fig. 15 (SSU tree), we draw the following conclusions: (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). From the *rbc*L tree we can conclude that (4) *Ptilophora, Capreolia, Acanthopeltis/Yatabella, Onikusa,* and *Gelidium,* members of the large *Gelidium*-complex clade, 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).

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

In the *rbc*L analysis, affinities of *Pterocladia* and *Pterocladiella* have not been resolved clearly, as indicated by low bootstrap values. However, congruence of morphological characteristics and the SSU tree suggests monophyly of *Pterocladia* and *Pterocladiella* and robustness of the SSU tree, at least in this position. Although the genera *Pterocladia* and *Pterocladiella* have been shown to share the same type of secondary rhizoidal attachments, they have different patterns of female reproductive morphology and carposporophyte development (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), which suggests that the species belongs to either *Pterocladia* or *Pterocladiella* rather than to *Gelidiella* or *Gelidium* (Norris 1992).

Concluding remarks and taxonomic proposal

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

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

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

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