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Reassessment of the taxonomic status of *Gelidium subfastigiatum* (Gelidiales, Rhodophyta)

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SUMMARY

The taxonomic relationship between *Gelidium elegans* Kützing and *Gelidium subfastigiatum* Okamura, two morphologically similar species of the red algal genus *Gelidium* (Gelidiaceae) growing in the north-western Pacific, was critically re-examined. *Gelidium subfastigiatum* has been distinguished from *G. elegans* by its more robust thalli, which have antrorse tooth-like branches, although their distinction has been said to be often difficult or impossible. We determined the nuclear encoded internal transcribed spacer 1 (ITS1) for 14 samples from eight populations of this *G. elegans*/*G. subfastigiatum* complex, and two types of ITS1 sequences were found. Analysis of seasonal variations of subterminal portions of major branches revealed that this complex includes two groups: one possessing the type 1 ITS1 sequence and antrorse tooth-like branches that are subterminally thickened and widened during only colder months, and another possessing the type 2 ITS1 sequence and thin and narrow branches throughout the year. These groups should be recognized as separate species; the former is assigned to *G. subfastigiatum* and the latter to *G. elegans*.

Key words: Gelidiales, *Gelidium elegans*, *Gelidium subfastigiatum*, ITS1 sequence, Japan, molecular phylogeny, morphology, Rhodophyta.

INTRODUCTION

The red algal order Gelidiales currently includes eight genera and approximately 140 species (Santelices 1990; Bailey and Freshwater 1997; Shimada *et al*. 1999; Tronchin *et al*. 2002). Our taxonomic studies of Japanese Gelidiales have revealed that five genera and 29 species are recognized in Japanese waters (Shimada and Masuda 1999, 2000; Shimada *et al*. 1999, 2000a, 2000b). *Gelidium* is the largest genus in the Gelidiales and includes approximately 100 species worldwide (Nelson *et al*. 1994). However, there are still several species for which further investigations are necessary to confirm their taxonomic status.

Gelidium subfastigiatum Okamura is one such species. Although Okamura (1934) described the alga as a new species, Inagaki (1933) reported this alga as '*G. subfastigiatum* Okam. mscr.' with a brief diagnosis in Japanese: 'Although the gross morphology and internal structure of thalli are almost similar to those of *Gelidium elegans* Kützing (as *G. amansii* Lamouroux), *G. subfastigiatum* differs from the latter in having branches, which are slightly irregularly divided and are antrorse' (translated). The exact citation of this alga is therefore *G. subfastigiatum* Okamura (in Inagaki 1933). When Okamura (1934) gave the alga a full description, he commented that *G. subfastigiatum* is most similar to *G. elegans* (as *G. amansii*), but the former can be distinguished from the latter by more robust thalli with antrorse tooth-like branches.

Although *G. elegans* is distributed widely in tropical to warm-temperate regions defined by Michanek (1979) in the north-western Pacific (Okamura 1934 from Japan; Fan 1951 from Taiwan; Tseng and Chang 1959 from China; Kang 1966 from Korea; Silvalingam 1977 from Malaysia), *G. subfastigiatum* is endemic to northern Japan (Okamura 1934; Noda 1987), which includes both warm- and cold-temperate regions defined by Michanek (1979). Akatsuka (1982) argued that the robustness of thalli 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*, irrespective of the presence or absence of antrorse tooth-like branches. However, Yoshida (1998) suspended Akatsuka's conclusion.

In this study, we determined the nuclear encoded internal transcribed spacer 1 (ITS1) for 14 samples from eight populations of the *G. elegans*/*G. subfastigiatum* complex. Seasonal variations of subterminal portions of major branches with reference to the antrorse tooth-like branch in this complex were also examined on the basis of periodic samplings at two localities, including the type locality of *G. subfastigiatum*.

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Table 1. List of population numbers, sample numbers of unialgal cultures and collection data in a *Gelidium elegans*/*Gelidium subfastigiatum* complex

Population number	Sample number	Locality	Date of collection
1	#16	Yura, Awaji Island, Hyogo Prefecture	16 May 1996
2	#143	Izura, Ibaraki Prefecture	3 April 1998
3	#177	Shiriya, Aomori Prefecture	7 April 1998
4	#45, #47, #48	Oshoro, Hokkaido	6 March 1997
4	#354, #355, #356	Oshoro, Hokkaido	2 September 1999
5	#43	Tomari, Hokkaido	16 April 1997
6	#166	Fukaura, Aomori Prefecture	6 April 1998
7	#157, #161	Oga, Akita Prefecture	6 April 1998
8	#152	Sasakawanagare, Niigata Prefecture	5 April 1998

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

                      210
(type 1):   CAAA.....
(type 2):   CAAA......
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Fig. 1. Alignment of internal transcribed spacer 1 sequences. The numbers on the right refer to nucleotide positions (including gaps). (*), Different position between type 1 and type 2.

MATERIALS AND METHODS

Sampling and molecular analysis

Fourteen unialgal cultures were established from excised tips of branchlets of plants collected from eight local populations in Japan (Table 1). They were grown in PES medium (Provasoli 1968) at 15°C, 16 : 8 h LD with a 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). Methods for total DNA extraction, polymerase chain reaction (PCR) amplification and sequencing of the ITS1 sequences were as described in Shimada *et al*. (1999).

Morphological observations

For examination of the presence or absence of seasonal variations of antrorse tooth-like branches, the following specimens were used: Oshoro, 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, Awaji Island, Hyogo Prefecture (17.iv.1995, 29.vi.1995, 19.x.1995, 30.i.1996, 16.v.1996). We selected three plants from each collection and measured means and standard deviations of the maximum thickness and width in subterminal portions (1–2 mm below the apex) of major branches. Corresponding portions of parental plants for DNA extraction were also measured.

RESULTS

ITS1 sequences

Two different ITS1 sequences, type 1 (204 bp) and type 2 (203 bp), were found within the 14 sequences. Only one difference (gap) has been found in these two types (Fig. 1). The geographic distribution of these ITS1 types is shown in Fig. 2: (type 1) Izura, Shiriya, Oshoro, Tomari and one sample at Oga; and (type 2) Yura, Fukaura, one sample at Oga and Sasakawanagare. All the six samples collected at Oshoro (6.iii.1997, 2.ix.1999) had type 1 ITS1 sequences. Two samples collected at the same site and on the same date at Oga, Akita Prefecture (6.iv.1998) had different sequences: #161 had type 1; and #157 had type 2.

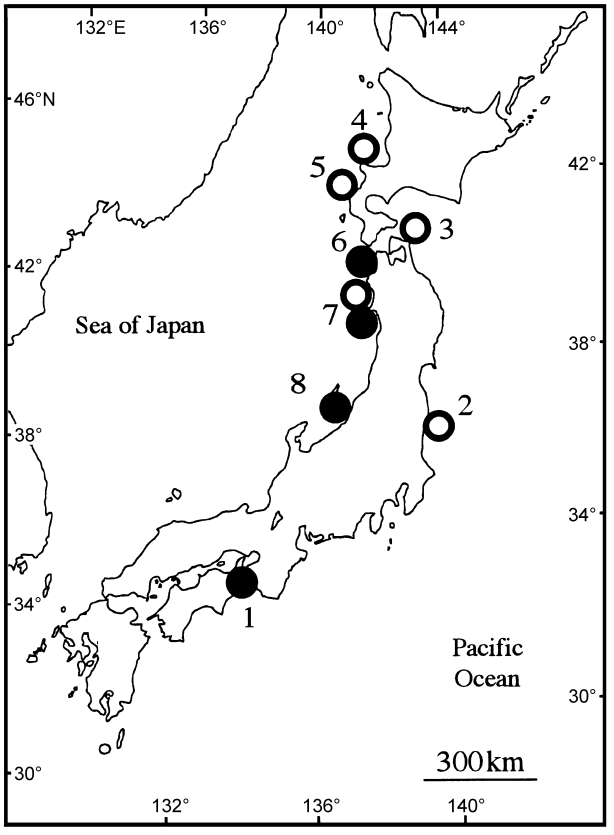


Fig. 2. Map showing populations of the two types of internal transcribed spacer 1 sequences in a *Gelidium elegans*/*Gelidium subfastigiatum* complex. (○), type 1; (●), type 2. The population numbers correspond to those in Table 1.

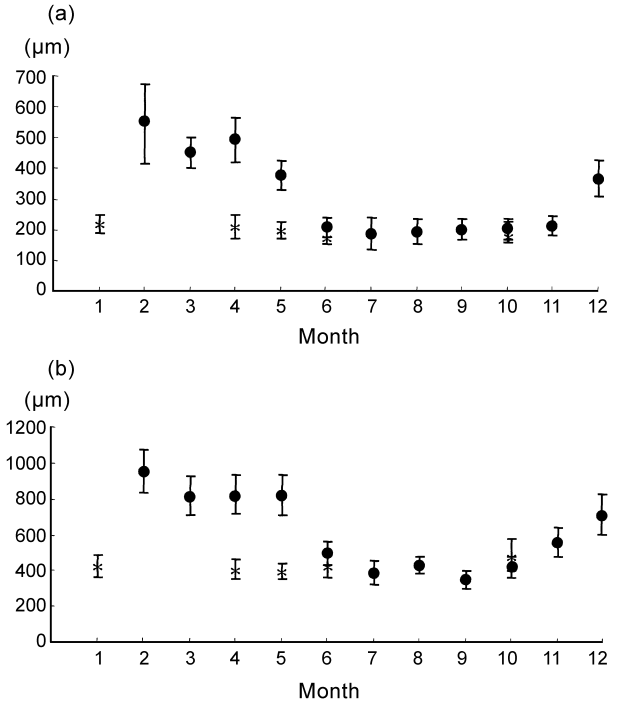


Fig. 3. Means and standard deviations of thickness (a) and width (b) in subterminal portions of major branches of a *Gelidium elegans*/*Gelidium subfastigiatum* complex, which were sampled periodically, are plotted in each population. (●), Oshoro; (*), Yura.

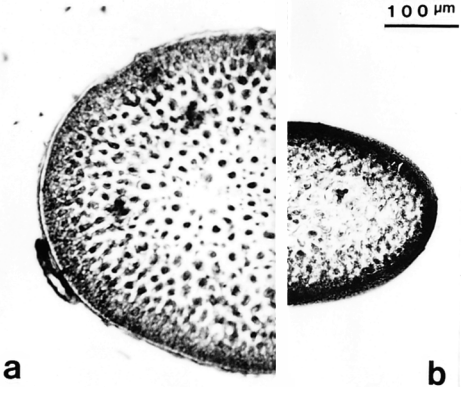
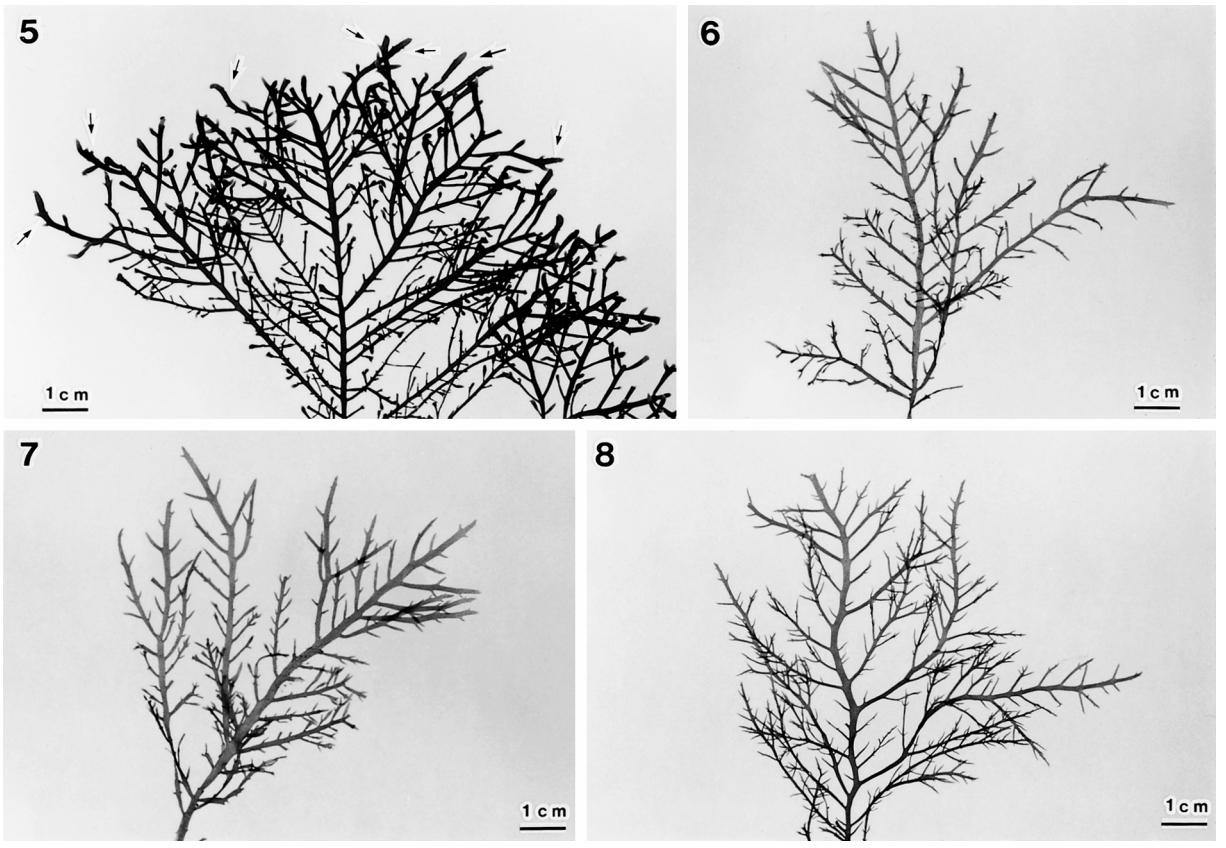


Fig. 4. Cross sections through major branches of a *Gelidium elegans*/*Gelidium subfastigiatum* complex. (a) Antrorse tooth-like branches of *G. subfastigiatum* (Oshoro, Hokkaido, 16.iv.1997). (b) Major branch of *G. elegans* (Yura, Hyogo Prefecture, 17.iv.1995).

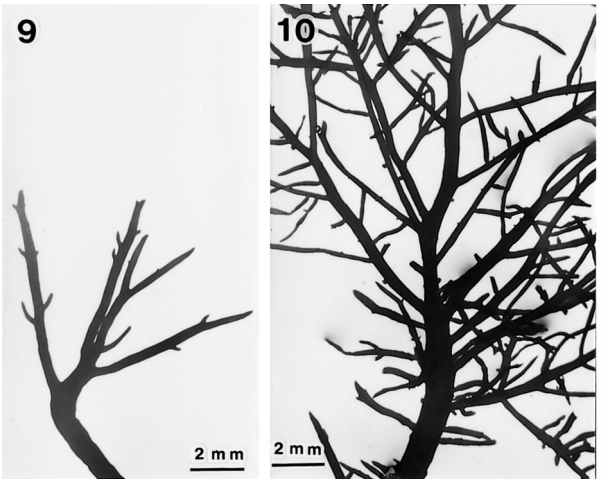
Seasonal variations of subterminal portions of major branches

For analysis of seasonal variations of subterminal portions of major branches of specimens from Oshoro and Yura, means and standard deviations of thickness and width, respectively, were plotted (Fig. 3). Subterminal portions of major branches of the Oshoro population showed a clear seasonal change in thickness. They are thick (380–820 μm) from February to April (Fig. 4a), becoming thinner (300–480 μm) from May onward, reaching 120–300 μm in thickness from June to November, but becoming thicker (300–440 μm) again from December onward. These branches of the Oshoro population showed a similar seasonal change in width. They are wide (660–1140 μm) from February to May (Fig. 5), becoming slender (380–620 μm) from June onward, reaching 280–440 μm in width in July to October (Fig. 6) and then becoming wider (400–680 μm) again from November onward.

In the Oshoro population, the subterminal portions of major branches are conspicuously thick and wide from December to April and have one to four small serrations, so that these branches (Fig. 5, arrows) are constricted beneath the widened part and equivalent to antrorse tooth-like branches defined by Okamura (1934). The serrations of antrorse tooth-like branches grow into new lateral branches in June (Fig. 9), and each antrorse tooth-like branch becomes a parental axis of lateral branches. These new lateral branches are not subterminally thickened and widened, and continue to grow during warmer months in a manner similar to that of the Yura population mentioned below. Each parental axis (which was formerly an antrorse tooth-like branch) of the new lateral branches becomes



Figs 5–8. Thallus habits of a *Gelidium elegans*/*Gelidium subfastigiatum* complex in different seasons. Formalin/seawater-preserved specimens. 5. Thallus with antrorse tooth-like branches (arrows) (Oshoro, Hokkaido, 16.iv.1997). 6. Thallus with only narrow branches (Oshoro, Hokkaido, 13.x.1995). 7. Thallus with only narrow branches (Yura, Hyogo Prefecture, 17.iv.1995). 8. Thallus with only narrow branches (Yura, Hyogo Prefecture, 19.x.1995).



Figs 9,10. Thallus habits of the Oshoro population from summer to autumn in a *Gelidium elegans*/*Gelidium subfastigiatum* complex. Formalin/seawater-preserved specimens. 9. Thallus collected in June. 10. Thallus collected in October.

indistinguishable from its lower region in October (Fig. 10), as its constricted part and the lower region also become thickened and widened.

Corresponding branches of the Yura population showed no significant seasonal variation in thickness and width. They are 140–280 μm in thickness and 320–620 μm in width (Figs 4b,7,8) throughout the year. Fertile branches of the Yura population fall down in autumn and winter and the remaining portions (scars of the fertile branches) become thicker and produce one or more proliferations from the distal end in spring. Although such branches 'minus their apices' are more thickened and widened than the distally formed proliferations, they are dissimilar in lacking serrations of the Oshoro population, which grow into new lateral branches later.

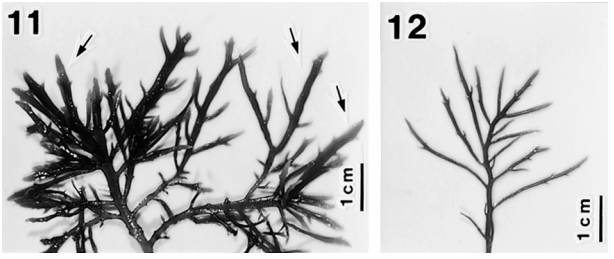
Morphology of subterminal portions of major branches of parental plants of DNA analysis

Eleven parental plants from the DNA analysis, which were collected at eight localities (Table 1), were examined for the morphology of their subterminal portions of major branches. The means and standard deviations of thickness and width are shown in Table 2. There are two groups that are clearly distinguishable by thickness and width. Thickened and widened branches were

Table 2. Means and standard deviations of the maximum thickness and width in major branches of parental plants of DNA extraction of a *Gelidium elegans/Gelidium subfastigiatum* complex collected from December to May

Type of ITS1 sequence	Sample number†	Population number	Thickness (μm)	Width (μm)
Type 1	#43	5	476 ± 60.96	930 ± 112.84
Type 1	#45	4	474 ± 47.19	862 ± 103.04
Type 1	#47	4	440 ± 50.77	750 ± 97.18
Type 1	#48	4	442 ± 52.87	850 ± 107.60
Type 1	#143	2	426 ± 35.34	900 ± 170.23
Type 1	#161	7	508 ± 91.51	986 ± 165.47
Type 1	#177	3	396 ± 57.97	878 ± 187.25
Type 2	#16	1	200 ± 21.08	362 ± 43.67
Type 2	#152	8	194 ± 18.97	458 ± 77.43
Type 2	#157	7	216 ± 30.98	396 ± 39.78
Type 2	#166	6	174 ± 21.19	328 ± 65.96

†Each sample number corresponds to that shown in Table 1. ITS1, internal transcribed spacer 1.



Figs 11,12. Two form of thalli of a *Gelidium elegans/Gelidium subfastigiatum* complex collected at Oga, Akita Prefecture, Japan (6 April 1998). Formalin/seawater-preserved specimens. 11. Thallus with antrorse tooth-like branches (arrows) (#161). 12. Thallus with narrow branches (#157).

observed in Izura #143 (380–500 μm in thickness and 720–1200 μm in width), Shiriya #177 (300–480 μm in thickness and 720–1220 μm in width), three samples at Oshoro #45, #47 and #48 (380–580 μm in thickness and 640–1020 μm in width), Tomari #43 (360–580 μm in thickness and 740–1100 μm in width) and one in Oga #161 (360–660 μm in thickness and 820–1300 μm in width). Thin and narrow branches were found in Yura #16 (180–240 μm in thickness and 300–440 μm in width), Fukaura #166 (140–200 μm in thickness and 260–450 μm in width), one in Oga #157 (160–260 μm in thickness and 320–460 μm in width) and Sasakawanagare #152 (160–220 μm in thickness and 340–600 μm in width). Two samples collected at the same site and on the same date at Oga, Akita Prefecture (6.iv.1998) had different types of branches: #161 had thickened and widened branches (Fig. 11); and #157 had thin and narrow branches (Fig. 12).

We also examined whether there is a correlation between the morphology of subterminal portions of major branches and the type of ITS1 sequence (Table 2). Samples with type 1 ITS1 sequences have subterminally thickened and widened branches, whereas samples with type 2 ITS1 sequences possess thin and narrow branches.

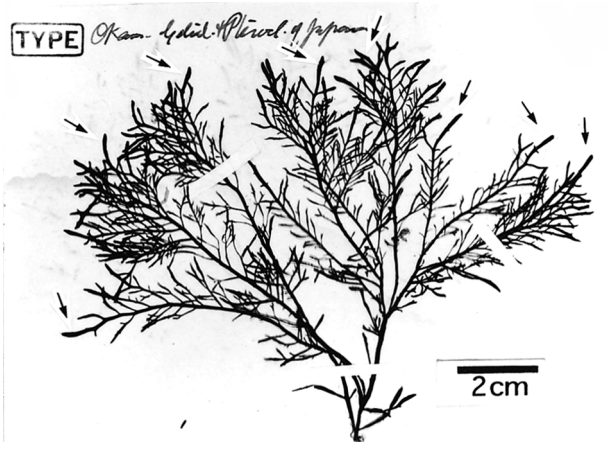


Fig. 13. Lectotype specimen of *Gelidium subfastigiatum* collected at Oshoro, Hokkaido (iii. 1920, Okamura Herbarium in the Herbarium of Graduate School of Science, Hokkaido University, Sapporo, Japan); arrows indicate antrorse tooth-like branches.

DISCUSSION

Analysis of seasonal variations of subterminal portions of major branches between specimens from Oshoro (type locality of *G. subfastigiatum*) and Yura revealed that subterminally thickened and widened branches, which correspond to Okamura's (1934) antrorse tooth-like branches, were observed only from December to April in the Oshoro population. The lectotype specimen of *G. subfastigiatum* (Fig. 13), which was illustrated by Okamura (1934; the upper specimen of pl. 24), was collected at Oshoro in March 1920, and clearly indicates the presence of such branches (Fig. 13, arrows). However, these antrorse tooth-like branches cannot be observed in the type illustration of *G. elegans* (Kützing 1868; pl. 52, fig. c), and all specimens of Yura that agree well with the authentic *G. elegans* (determined by K. Okamura as *G. amansii*), whose branches showed no seasonal variation (thin and narrow throughout the year).

The presence of antrorse branches in *G. subfastigiatum* is restricted to colder seasons when seawater temperatures

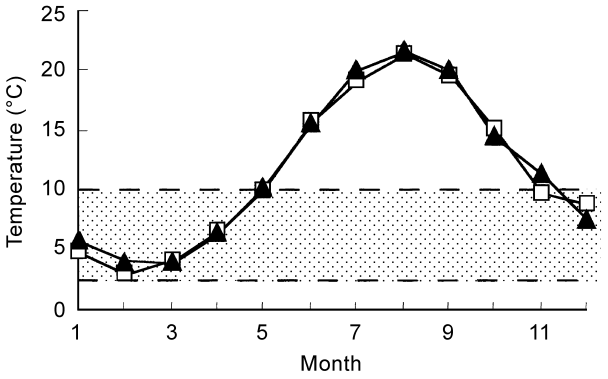


Fig. 14. Monthly means of seawater temperatures at Oshoro, Hokkaido, from January 1996 to December 1997, recorded every morning (8:00) at 1 m depth. Antrorse tooth-like branches can be observed in the Oshoro population at the gray zone. (□), 1996; (▲), 1997.

are 3–10°C at Oshoro (Fig. 14). We carried out culture experiments to examine the effect of seawater temperatures on the branch morphology. However, our cultured plants of *G. elegans* and *G. subfastigiatum* did not show the normal morphology; they grew into prostrate axes or branches, which did not produce erect axes or branches. Thus, we could not get reliable data.

It is important to interpret morphological differences in combination with other features, such as geographic distribution and molecular analyses, when one decides whether morphological differences indicate interspecific discriminations or represent intraspecific differentiation (Shimada *et al*. 2000a; Wang *et al*. 2000; Uwai *et al*. 2001). The production of antrorse tooth-like branches, which is a diagnostic feature of *G. subfastigiatum*, was also observed in specimens from other localities (#43, #143, #161 and #177) that were collected from December to May. One sample at Oga (#161) has such branches, but another sample at Oga (#157) collected on the same day possesses thin and narrow branches just like the other three specimens (#16, #152 and #166). Two types of branches, therefore, can be distinguished clearly in the field (Oga, Akita Prefecture), where individual plants with different types occur sympatrically. Furthermore, the types of ITS1 sequences were found to correspond to these two types of branches: one with type 1 ITS1 sequence with antrorse tooth-like branches, and another with type 2 ITS1 sequence with thin and narrow branches. The congruence of morphological and molecular data suggests that two different entities are present in our studied area.

Only one difference (gap) has been found in the ITS1 sequences between these two entities. Such a close range of sequence divergence in the ITS sequences has been observed within individual species in fungi (Gardes *et al*. 1991), diatoms (Zechman *et al*. 1994), the red alga *Chondrus crispus* Stackhouse (Chopin *et al*. 1996), between different species in the brown algal genus *Fucus* (Leclerc *et al*. 1998) and in Gelidiales (Shimada *et al*. 1999). As Bird *et al*. (1992) noted, the taxonomic significance of molecular sequence divergence must be evaluated on a case-by-case basis. In this case, the sequence divergence falls into either inter- or intraspecific differentiation of other taxa, as shown above. The two entities occur sympatrically at Oga, Akita Prefecture, growing on close subtidal rocks. Yet, the morphology of each entity can be distinguished clearly in the field, which suggests the presence of reproductive isolation between the two entities. Although we have not been able to conduct crossing experiments yet, the above-mentioned data indicate that *G. subfastigiatum* is an independent species that can be distinguished from the closely related species *G. elegans* by an apomorphy, the presence of antrorse tooth-like branches during colder months. Such a subtle (but significant) morphological difference and one gap difference in the ITS1 sequences strongly suggest that evolutionary divergence between *G. subfastigiatum* and *G. elegans* was relatively recent. In the north-western Pacific, many species of *Gelidium* are distributed in tropical and warm-temperate regions (Santelices and Stewart 1985) and only a few species, including *G. subfastigiatum* and *Gelidium vagum* Okamura are found in cold-temperate regions (Okamura 1934; Noda 1987). This distribution pattern of *Gelidium* indicates that a diverse species of the genus might have evolved in warmer regions and adapted in such environments, and only a few species, which might have been able to adapt to colder waters, might have migrated into cold-temperate regions. The preference of colder waters over warmer ones may be a further apomorphy of *G. subfastigiatum*.

Norris (1990) demonstrated clearly the differences between genuine *G. amansii* and the alga that had been called *G. amansii* in Japan. The lectotype specimen of *G. amansii* from Madagascar possesses cylindrical to compressed axes and subdichotomous branching with up to second-order branches, of which tips are gradually attenuate, whereas Japanese specimens of '*G. amansii*' have compressed to flattened axes and pinnate branching with up to fourth-order branches, of which tips are abruptly attenuate, and it was proposed to use *G. elegans* for the Japanese alga. However, Santelices (1994) claimed Norris' conclusion: morphological and anatomical differences between the type materials and representative Japanese specimens of *G. amansii* are too small to warrant more than one species. The taxonomic relationship between *G. amansii* and *G. elegans* should be clarified by further critical studies, including molecular phylogenetic analysis. Whether *G. amansii* and *G. elegans* are different species or not, *G. subfastigiatum* is distinguished from both algae by the presence of antrorse tooth-like branches.

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