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Morphological homoplasy in Japanese *Plocamium* species (Plocamiales, Rhodophyta) inferred from the Rubisco spacer sequence and intracellular acidity

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Among five species of the genus *Plocamium* present in Japan, *P. cartilagineum, P. recurvatum* and *P. telfairiae* show similar blade morphology, and intermediates exist between them. We investigated the diversity and evolutionary relationships of these *Plocamium* species using morphological observations, estimation of intracellular pH and analysis of Rubisco spacer sequences. Although *P. recurvatum* is characterized by the presence of extremely recurved branchlets, most of the specimens examined also have linear or slightly reflexed branchlets in the same thallus. *Plocamium cartilagineum* is not clearly distinguished from *P. telfairiae* by the conventional diagnostic characters such as width of main axes, number of branchlets in alternate series and size of the lowermost branchlet. Intracellular pH of these species is estimated to be 1.2–6.6 and is variable within a species. Three haplotypes in *P. cartilagineum,* two in *P. recurvatum* and eight in *P. telfairiae* are recognized on the basis of the Rubisco spacer sequences, and most of these haplotypes show specific intracellular pH. The molecular phylogenetic analyses demonstrate three major clades in the Japanese *Plocamium* species: *P. cartilagineum,* one haplotype of *P. recurvatum* and four of *P. telfairiae* in one clade (A); *P. ovicorne* and *P. serratulum* in another (clade B); the remaining haplotype of *P. recurvatum* and four haplotypes of *P. telfairiae* in clade C. Clade C contains only the haplotypes showing strong acidity, whereas clade A is composed of both strongly acidic and weakly acidic or neutral haplotypes. These results suggest that morphological homoplasy occurred in *P. recurvatum* and *P. telfairiae* and that physiological properties such as intracellular pH may be informative and contribute to our understanding of the relationship of the Japanese *Plocamium* species.

INTRODUCTION

Plocamium J.V.F. Lamouroux is widely distributed from tropical to cold-temperate seas, and approximately 35 species are recognized in this genus (Wynne 2002). Members of the genus grow on lower littoral to sublittoral rocks, and are characterized by their flat fronds with sympodial and repeatedly pinnate branches with alternating groups of multiple branchlets. The Order Plocamiales was recently established by Saunders & Kraft (1994) for *Plocamium* and its adelphoparasite *Plocamiocolax* Setchell, but there have been few recent attempts to delimitate *Plocamium* species based on detailed morphological comparison and molecular phylogenetic analysis.

Among five *Plocamium* species recognized in Japan (Yoshida *et al.* 2000), *P. ovicorne* Okamura (formerly *P. ovicornis*) and *P. serratulum* Okamura are well characterized by the presence of shorter branchlets opposite the normal branchlet series and the minute tooth-like serrations along the outer margin of the lowermost branchlet, respectively (Okamura 1913, 1923, 1932). However, the other three species, *P. cartilagineum* (Linnaeus) Dixon (Fig. 1), *P. recurvatum* Okamura (Fig. 2) and *P. telfairiae* (Hooker & Harvey) Harvey (Fig. 3), which are classified on the basis of differences in the number and shape of branchlets and in blade width (Figs 4, 5), demonstrate conspicuous variation of these morphological features. In particular, the shape and size of the lowermost branchlet (double arrowheads in Fig. 4) in each alternate series are variable within the same species. *Plocamium recurvatum* is characterized by extremely recurved and uncinated branchlets, but Okamura (1913) observed a specimen intermediate between *P. recurvatum* and *P. telfairiae* (as *P. abnorme* Hooker & Harvey) and described it as *P. abnorme* f. *uncinatum* Okamura (now *P. telfairiae* f. *uncinatum*). On the other hand, Yendo (1915) reported that such uncinated branchlets are often seen in the upper parts of typical *P. telfairiae* specimens, and therefore considered that *P. recurvatum* and *P. telfairiae* f. *uncinatum* are simply variants of *P. telfairiae.*

Plocamium cartilagineum is the most widespread species in the genus, being recorded in North Atlantic, Chile, eastern and western North Pacific, northern Arabian Sea, Australia, New Zealand and Antarctica (Bischoff-Bäsmann & Wiencke 1996; Wynne 2002). This species is characterized by having three to five branchlets in each alternate series and by the slender shape of the lowermost branchlets (Fig. 1), but considerable morphological variation has been reported (Okamura 1913; Kylin 1925; Dawson 1961; Womersley 1971; Gabrielson & Scagel 1989). Okamura (1913) mentioned the difficulty of distinguishing this alga (as *P. leptophyllum* Kützing var. *flexuosum* J. Agardh) from certain Japanese populations of *P. telfairiae* (as *P. abnorme*).

Many species of the Order Desmarestiales and some of the

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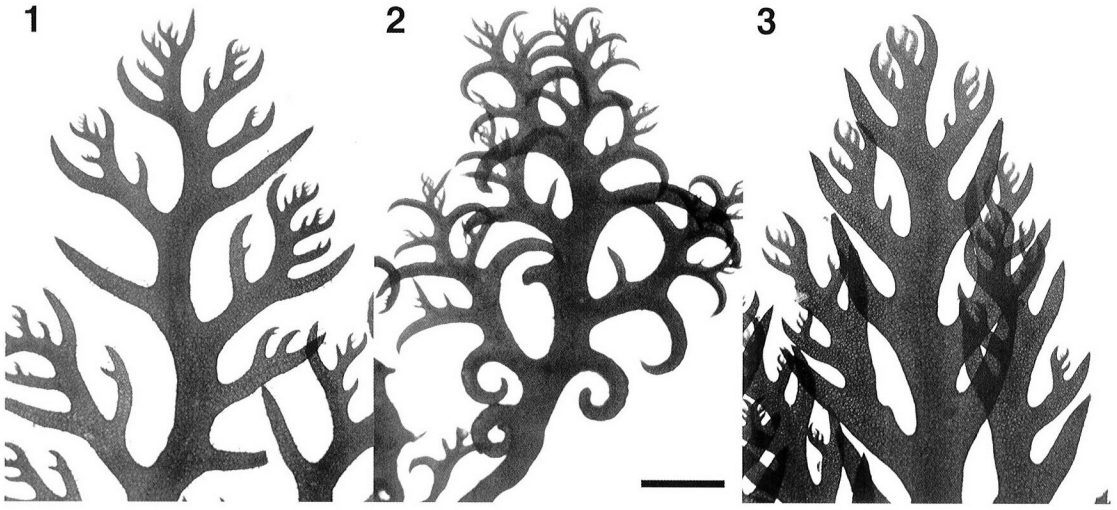


Figs 1–3. Field-collected specimens of *Plocamium* species from Japan. Scale bar = 1 mm.
Fig. 1. *Plocamium cartilagineum* from Anaga, Awaji Island.
Fig. 2. *Plocamium recurvatum* from Choshi, Chiba.
Fig. 3. *Plocamium telfairiae* from Yura, Awaji Island.

Order Dictyotales (Phaeophyceae) are known to accumulate highly acidic substances within their vegetative cells (Blinks 1951; Schiff 1962; Sasaki *et al.* 1999, 2004). Molecular analysis using ribosomal DNA internal transcribed spacer (ITS) sequences suggests that this character was acquired only once in the desmarestialean lineage (Peters *et al.* 1997). Our preliminary work revealed that some *Plocamium* species show significantly lower intracellular pH values than other red algae we examined, so this physiological characteristic may demonstrate differences between *Plocamium* species or even within the same species.

In order to delineate the morphologically related species *P. cartilagineum*, *P. recurvatum* and *P. telfairiae*, we performed detailed morphological examination of these species from 40 Japanese populations. We also measured the intracellular pH of these specimens to survey the inter- or intraspecific variation of this physiological character. Furthermore, molecular phylogenetic analysis was carried out to detect genetic variations and infer the evolutionary relationship among the *Plocamium* species from Japan. Large and small subunits of Rubisco (*rbc*L and *rbc*S) are both encoded on the plastid genome and are separated by a short spacer in the Rhodophyta (Valentin & Zetsche 1990; Destombe & Douglas 1991). Because this spacer and its flanking regions of *rbc*L and *rbc*S genes are known to be useful for inferring inter- or intraspecific relationships of red algae (Maggs *et al.* 1992; Goff *et al.* 1994; Kamiya *et al.* 1998; Zuccarello *et al.* 2002), the sequence of this region was determined on 87 specimens of the five *Plocamium* species for molecular phylogenetic analyses.

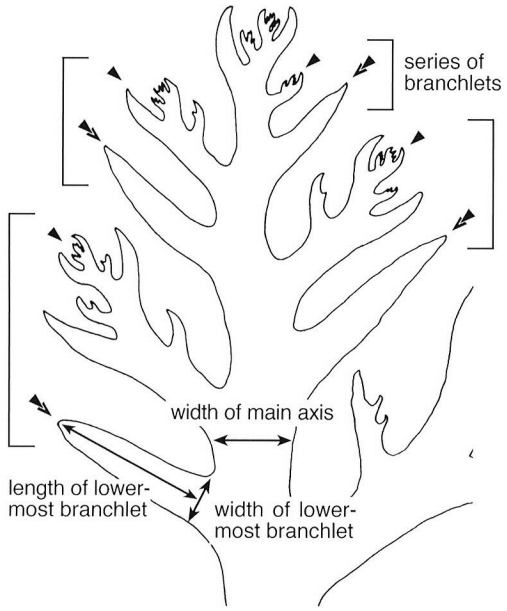


Fig. 4. Drawing of *Plocamium* thallus showing the portion measured in the morphological examinations. Arrowheads and double arrowheads indicate the first- and second-order (= lowermost) branchlets in each alternate series (bracket), respectively.

MATERIAL AND METHODS

Morphological observations

The specimens studied are listed in Table 1 with their collection data. Field-collected materials were put into plastic bottles containing fresh seawater and immediately transported to the laboratory. Some of the fresh specimens were used for pH measurements of cell sap and some were desiccated with silica gels for later DNA extraction, and the rest were pressed for morphological studies.

Upper portions (the terminal *c.* 1 cm) of voucher specimens were used for morphological observations, and the number of branchlets in each alternate series, and shape, length and width of the lowermost branchlets (Fig. 4) were measured on 10 portions of each thallus under light microscopy. Because *P. cartilagineum sensu* Okamura is specifically characterized by having a long and narrow lowermost branchlet, this species was defined in this study by having at least one lowermost

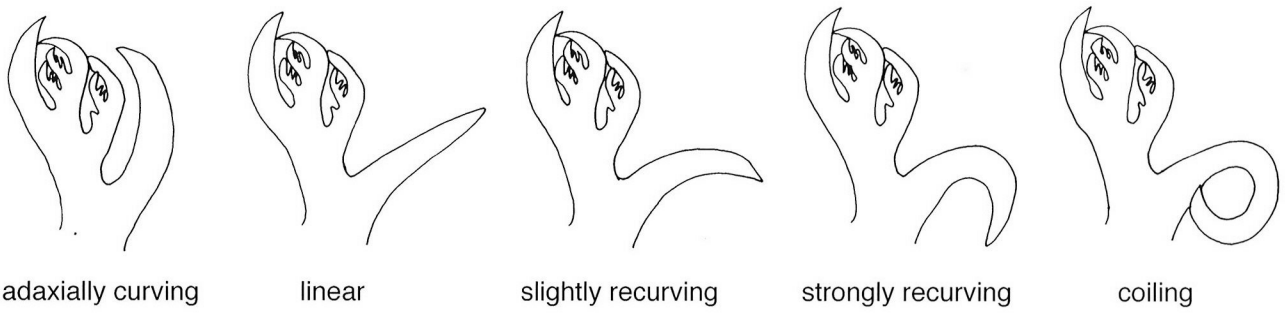


Fig. 5. Definition of five types of lowermost branchlet shape seen in the three *Plocamium* species.

branchlet longer than 2 mm and narrower than 300 μm in a thallus. Curvature of the lowermost branchlet was classified into five types; 'adaxially curving type', 'linear type', 'slightly recurving type', 'strongly recurving type' and 'coiling type' (Fig. 5). *Plocamium recurvatum* was distinguished by the obvious recurvature of its lowermost branchlets (Okamura 1913), so the specimens having one or more 'coiling' lowermost branchlets were identified as *P. recurvatum*. All the other specimens were assigned to *P. telfairiae*. These detailed morphological observations were carried out on 22 specimens of *P. cartilagineum* from eight populations, 46 specimens of *P. recurvatum* from four populations and 197 specimens of *P. telfairiae* from 26 populations (Table 1).

pH measurements of cell sap

Procedures for pH measurements of cell sap and the estimation of intracellular acidity were based on the procedures of McClintock *et al.* (1982) and Sasaki *et al.* (1999). Seawater was thoroughly wiped from the surface of the specimen with filter paper (paper filter No. 1; Advantec, Tokyo, Japan), and then the specimen was washed in 1 M sorbitol solution. After wiping again, 10–100 mg of fresh algal tissue was soaked without agitation in 1 ml distilled water (pH 6.1–6.5) in a 1.5 ml microfuge tube at room temperature for 2 h. The cell sap solution was transferred to a fresh microfuge tube and its acidity was measured using a portable pH meter (B-212 Twin-pH; Horiba, Tokyo, Japan). After the measurements, each sample was weighed after drying at 70°C for 12 h. Intracellular pH was estimated from the pH values of the cell sap based on the following calibration formula of dilution: dilution factor = [distilled water (g) + sample fresh weight (g) × water content]/sample fresh weight (g) × water content. The pH value was measured on 18 specimens of *P. cartilagineum* from four populations, seven specimens of *P. ovicorne* from two populations, 47 specimens of *P. recurvatum* from five populations and 128 specimens of *P. telfairiae* from 26 populations (Table 1). For comparison, five gigartinalean species (*Chondracanthus tenellus, Hypnea flexicaulis, H. japonica, H. variabilis* and *Portieria hornemannii*) were also examined.

Molecular phylogenetic analyses

The Rubisco spacer and its flanking regions of the *rbc*L and *rbc*S genes were sequenced in *Plocamium cartilagineum* (14 samples from 11 populations), *P. recurvatum* (11 samples from 6 populations), *P. ovicorne* (seven samples from six populations), *P. serratulum* (one sample from one population), *P. telfairiae* (56 samples from 36 populations) and *H. japonica* (one sample from one population) as an out-group. Genomic DNA was extracted from desiccated materials using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracted total DNA was monitored on ethidium bromide–stained 1.0% agarose gels. The Rubisco spacer was amplified using primers containing sequences from the region 54 bp upstream from the 3′ terminus of the *rbc*L gene and complementary to the region 151 bp downstream from the 5′ terminus of the *rbc*S gene (Kamiya *et al.* 1998), 5′-TATACTTCTACAGACACAGCTGA-3′ and AYRTCAAAWAAWGGWARWCCCCA-3′. Polymerase chain reaction (PCR) amplification was carried out with a GeneAmp PCR Cycler 9700 (Applied Biosystems, Foster City, CA, USA) using a TaKaRa Ex Taq reaction kit (Takara Shuzo, Shiga, Japan). Each 25 μl of PCR reaction mixture was composed of 17.4 μl sterile water, 2.5 μl 10× Ex Taq buffer, 2.0 μl of 2.5 mM deoxynucleoside triphosphate (dNTP) mixture, 1 μl of each primer at 0.2 μM, 0.1 μl of TaKaRa Ex Taq polymerase (5 units μl^{-1}) and 1.0 μl of DNA solution containing 10–50 ng genomic DNA. The profile of PCR conditions was as follows: an initial denaturation at 94°C for 1–5 min followed by 30 cycles of denaturation at 94°C for 30–60 s, annealing at 48°C for 30 s, extension at 72°C for 30–60 s and a final extension at 72°C for 7 min. Amplification of the PCR products was checked for correct length, purity and yield on 1% Tris-acetate-EDTA–agarose gels with ethidium bromide. Double-stranded PCR products were sequenced directly using ABI PRISM 310 Genetic Analyzer (Applied Biosystems), following the manufacturer's instructions. The sequences obtained were aligned by eye for phylogenetic analyses.

Maximum-parsimony (MP) trees were constructed with PAUP* 4.0b10 (Swofford 1998), using the branch and bound search option, unordered unweighted characters, and gaps treated as missing data. The program Modeltest version 3.06 (Posada & Crandall 1998) was used to find the model of sequence evolution that best fits the data set by use of a hierarchical likelihood ratio test ($\alpha = 0.05$). When the best sequence evolution model had been determined, maximum likelihood (ML) was performed in PAUP* using the estimated parameters (substitution model, gamma distribution, proportion of invariable sites), with 500 random additions. Distance trees were constructed using the HKY85 distance matrix model (Hasegawa *et al.* 1985) and neighbour-joining (NJ) reconstruction (Saitou & Nei 1987). For bootstrap analysis (Felsen-

Table 1. Japanese specimens used in this study.

Haplo type[1]	Species	Localities	Collection date
CAR1	*Plocamium cartilagineum*	Shiga, Ishikawa	28 Jul. 2001
CAR1	*P. cartilagineum*	Ama, Mihara, Hyogo	31 Oct. 2001
CAR1	*P. cartilagineum*	Anaga, Seidan, Hyogo	31 Oct. 2001; 23 Oct. 2002
CAR1	*P. cartilagineum*	Kurahashi, Hiroshima	30 Jun. 2000
CAR1	*P. cartilagineum*	Kamagari, Hiroshima	9 May 2001
CAR2	*P. cartilagineum*	Kamagari, Hiroshima	9 May 2001
CAR2	*P. cartilagineum*	Shimokamagari, Hiroshima	23 Aug. 2002
CAR2	*P. cartilagineum*	Nojima, Hokudan, Hyogo	14 Aug. 2000
CAR2	*P. cartilagineum*	Oshima, Mugi, Tokushima	22 Jul. 2001
CAR2	*P. cartilagineum*	Shimegaura, Namikata, Ehime	12 Jun. 2000
CAR3	*P. cartilagineum*	Nanao, Ishikawa	7 Aug. 2001
CAR3	*P. cartilagineum*	Shiga, Ishikawa	19 Oct. 2001
CAR3	*P. cartilagineum*	Shimoda, Amakusa, Kumamoto	24 Dec. 2000
OVI4	*P. ovicorne*	Iwafune, Oohara, Chiba	22 May 2000
OVI4	*P. ovicorne*	Tsurishi, Oohara, Chiba	22 May 2000
OVI4	*P. ovicorne*	Shiga, Ishikawa	28 Jul. 2001
OVI4	*P. ovicorne*	Abu, Yuki, Tokushima	24 Jul. 2001
OVI4	*P. ovicorne*	Kushimoto, Wakayama	15 Oct. 2001
OVI4	*P. ovicorne*	Shirahama, Wakayama	16 Oct. 2001
OVI5	*P. ovicorne*	Kushimoto, Wakayama	15 Oct. 2001
REC6	*P. recurvatum*	Terahama, Shizugawa, Miyagi	28 May 2002
REC6	*P. recurvatum*	Sakamoto, Shizugawa, Miyagi	28 May 2002
REC6	*P. recurvatum*	Ooarai, Ibaraki	23 Jul. 2002
REC6	*P. recurvatum*	Tsurishi, Oohara, Chiba	22 May 2000
REC6	*P. recurvatum*	Togawa, Choshi, Chiba	23 May 2000; 19 Mar. 2002
REC6	*P. recurvatum*	Iwafune, Oohara, Chiba	22 May 2000; 19 Mar. 2002
REC7	*P. recurvatum*	Togawa, Choshi, Chiba	19 Mar. 2002; 4 Oct. 2002
REC7	*P. recurvatum*	Ooarai, Ibaraki	23 Jul. 2002
SER8	*P. serratulum*	Shiraho, Ishigaki, Okinawa	26 Aug. 2001
TEL9	*P. telfairiae*	Shiga, Ishikawa	25 May 2001; 19 Oct. 2001
TEL9	*P. telfairiae*	Terahama, Shizugawa, Miyagi	28 May 2002
TEL9	*P. telfairiae*	Sakamoto, Shizugawa, Miyagi	28 May 2002
TEL9	*P. telfairiae*	Shiogama, Miyagi	29 May 2002
TEL9	*P. telfairiae*	Ooarai, Ibaraki	23 Jul. 2002
TEL9	*P. telfairiae*	Togawa, Choshi, Chiba	23 May 2000; 19 Mar. 2002
TEL9	*P. telfairiae*	Yoshio, Katsuura, Chiba	17 May 2000
TEL9	*P. telfairiae*	Iwafune, Oohara, Chiba	22 May 2000; 19 Mar. 2002
TEL9	*P. telfairiae*	Tsurishi, Oohara, Chiba	22 May 2000
TEL9	*P. telfairiae*	Kouyatsu, Tateyama, Chiba	29 Oct. 2002
TEL9	*P. telfairiae*	Moroiso, Miura, Kanagawa	15 Apr. 2002
TEL9	*P. telfairiae*	Ooura, Shimoda, Shizuoka	5 Oct. 2002
TEL9	*P. telfairiae*	Ooura, Takeno, Hyogo	14 Apr. 2001
TEL9	*P. telfairiae*	Imagoura, Imago, Hyogo	12 May 2001
TEL9	*P. telfairiae*	Yura, Sumoto, Hyogo	5, 13 Jul. 2000; 26 Apr. 2001; 2 Oct. 2001
TEL9	*P. telfairiae*	Ama, Mihara, Hyogo	31 Oct. 2001
TEL9	*P. telfairiae*	Anaga, Seidan, Hyogo	14 May 2001; 31 Oct. 2001
TEL9	*P. telfairiae*	Ibota, Touwa, Yamaguchi	16 Apr. 2001
TEL9	*P. telfairiae*	Kamagari, Hiroshima	9 May 2001
TEL9	*P. telfairiae*	Ooshima, Mugi, Tokushima	22 Jul. 2001
TEL9	*P. telfairiae*	Furumugi, Mugi, Tokushima	14 Mar. 2001
TEL9	*P. telfairiae*	Shimegaura, Ehime	12 Jun. 2000
TEL9	*P. telfairiae*	Kamae, Ooita	13 Sep. 2001
TEL9	*P. telfairiae*	Kodomo, Yobuko, Saga	26 Mar. 2001
TEL9	*P. telfairiae*	Misumi, Kumamoto	6 Jun. 2000
TEL10	*P. telfairiae*	Shiga, Ishikawa	19 Oct. 2001
TEL10	*P. telfairiae*	Shiogama, Miyagi	29 May 2002
TEL10	*P. telfairiae*	Miyama, Mie	24 Apr. 2001
TEL10	*P. telfairiae*	Saikasaki, Wakayama	2 Oct. 2000; 11 Jul. 2001
TEL10	*P. telfairiae*	Kushimoto, Wakayama	15 Oct. 2001
TEL10	*P. telfairiae*	Shirahama, Wakayama	16 Oct. 2001
TEL10	*P. telfairiae*	Yura, Sumoto, Hyogo	2 Oct. 2001; 21 Nov. 2002
TEL10	*P. telfairiae*	Anaga, Seidan, Hyogo	23 Oct. 2002
TEL10	*P. telfairiae*	Abu, Yuki, Tokushima	24 Jul. 2001
TEL10	*P. telfairiae*	Kurahashi, Hiroshima	30 Jun. 2000
TEL10	*P. telfairiae*	Ibota, Touwa, Yamaguchi	16 Apr. 2001
TEL10	*P. telfairiae*	Ooshima, Mugi, Tokushima	22 Jul. 2001
TEL10	*P. telfairiae*	Kodomo, Yobuko, Saga	26 Mar. 2001
TEL11	*P. telfairiae*	Sumiyoshi, China, Kagoshima	31 May 2000
TEL11	*P. telfairiae*	Shiraho, Ishigaki, Okinawa	26 Aug. 2001

Table 1. Continued.

Haplo type[1]	Species	Localities	Collection date
TEL12	*P. telfairiae*	Saikasaki, Wakayama	11 Jul. 2001
TEL13	*P. telfairiae*	Kushimoto, Wakayama	15 Oct. 2001
TEL13	*P. telfairiae*	Shirahama, Wakayama	16 Oct. 2001
TEL14	*P. telfairiae*	Kushimoto, Wakayama	15 Oct. 2001
TEL15	*P. telfairiae*	Iwaya, Awaji, Hyogo	4 Apr. 2002
TEL16	*P. telfairiae*	Nanao, Ishikawa	6 Aug. 2001
	Chondracanthus tenellus (Harvey) Hommersand	Yura, Sumoto, Hyogo	23 Oct. 2002
	Hypnea flexicaulis Yamagishi & Masuda	Yura, Sumoto, Hyogo	29 Jul. 2002; 27 Aug. 2002; 12 Sep. 2002; 23 Oct. 2002; 21 Nov. 2002
HJ	*H. japonica* Tanaka	Yura, Sumoto, Hyogo	25 Feb. 2002; 21 Nov. 2002
	H. variabilis Okamura	Iwafune, Oohara, Chiba	19 Mar. 2002
	Portieria hornemannii (Lyngbye) Silva	Yura, Sumoto, Hyogo	29 Jul. 2002; 27 Aug. 2002

[1] Haplotypes are based on the RuBisco spacer sequence. Accession codes recorded in the DDBJ (DNA Data Bank of Japan) are as follows: HJ, AB104690; CAR1, AB104691; CAR2, AB104692; CAR3, AB104693; OVI4, AB104694; OVI5, AB104695; REC6, AB104696; REC7, AB104697; SER8, AB104698; TEL9, AB104699; TEL10, AB104700; TEL11, AB104701; TEL12, AB104702; TEL13, AB104703; TEL14, AB104704; TEL15, AB104705; TEL16, AB104706.

stein 1985), 1000 bootstrap data sets were generated from resampled data for ML, MP and NJ analyses.

RESULTS

Morphological comparison

The mean width and length of the lowermost branchlet were proportional to each other in *P. recurvatum* and *P. telfairiae,* whereas those of *P. cartilagineum* were mostly less than 300 μm in width regardless of their length (Fig. 6). Some specimens of *P. cartilagineum* produced lowermost branchlets shorter than 1.5 mm on average, and these specimens were not always distinguishable from *P. recurvatum* or *P. telfairiae* based on this character. The width of the main axes varied within species, and even within the same individual: 0.4–1.5 mm (0.8 ± 0.08 mm, $\bar{x} \pm s_{\bar{x}}$) in *P. cartilagineum* (n = 22), 0.4–1.4 mm (0.8 ± 0.04 mm) in *P. recurvatum* (n = 46) and 0.4–2.0 mm (1.1 ± 0.02 mm) in *P. telfairiae* (n = 197). There were no significant differences between the three species in the thickness of the main axes and the cell size of cortical and medullary cells (data not shown).

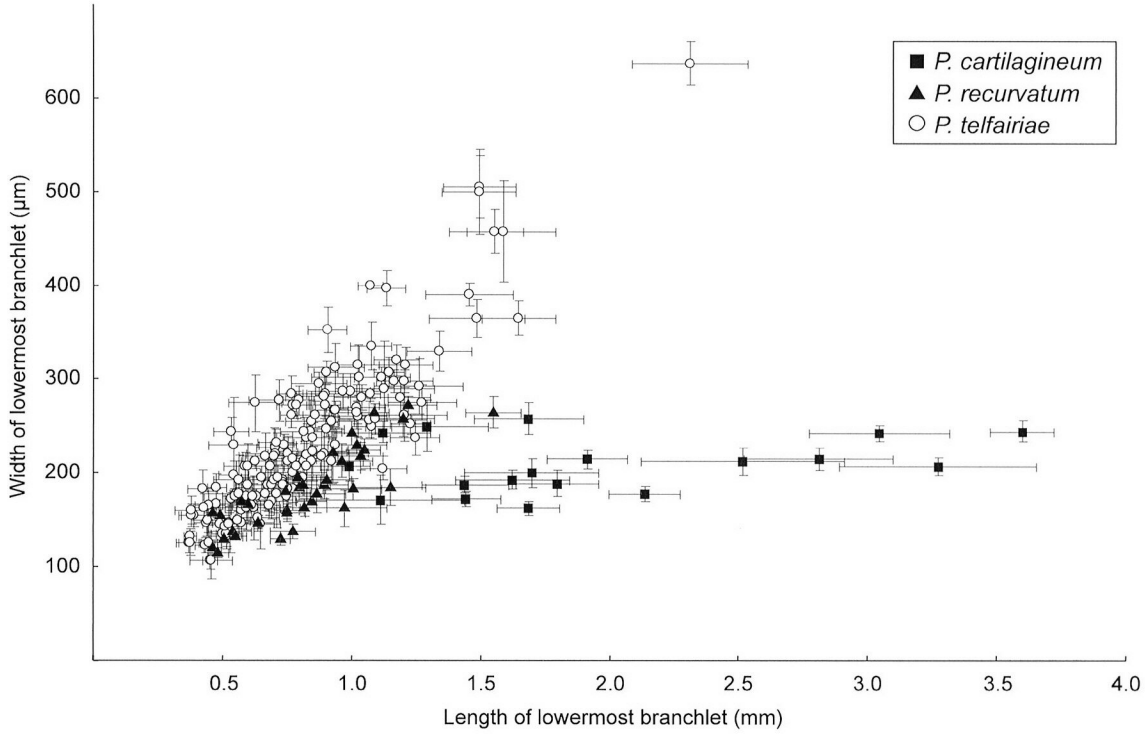


Fig. 6. Variation of width and length of lowermost branchlet in *Plocamium cartilagineum* (n = 22), *P. recurvatum* (n = 46) and *P. telfairiae* (n = 197). Each point and bar represents mean value of 10 branchlets in each specimen and its standard error, respectively.

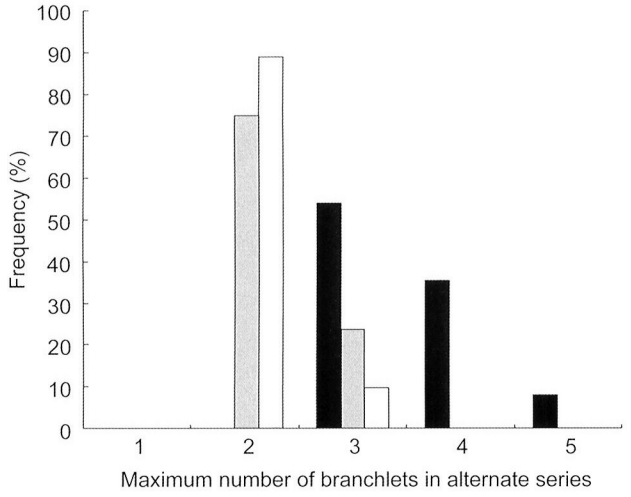


Fig. 7. Comparison of maximum number of branchlets in alternate series within a thallus in the three *Plocamium* species. Black bars, *P. cartilagineum* (n = 22); grey bars, *P. recurvatum* (n = 46); white bars, *P. telfairiae* (n = 197).

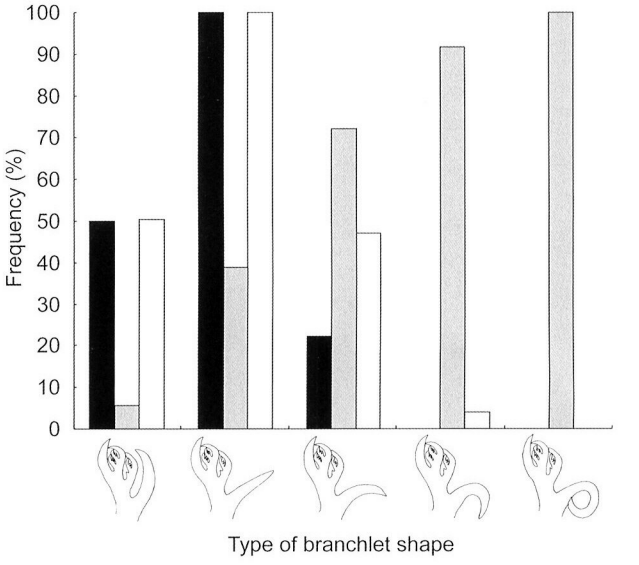


Fig. 8. Frequencies of each type of branchlet shape in the three *Plocamium* species. Black bars, *P. cartilagineum* (n = 22, 209 branchlets); grey bars, *P. recurvatum* (n = 46, 458 branchlets); white bars, *P. telfairiae* (n = 197, 1938 branchlets).

The number of branchlets in each alternate series (Fig. 4) is also an important key character in classifying *Plocamium* species. All specimens of *P. cartilagineum* possessed three or more branchlets in some of the alternate series (Figs 1, 7), but a high proportion of specimens produced two branchlets in a thallus. Nearly 75% of *P. recurvatum* specimens and 90% of *P. telfairiae* specimens had only two branchlets in every alternate series (Figs 2, 3), and the other specimens formed both two and three branchlets.

Ten lowermost branchlets of each specimen were classified into the five types of branchlet shape (Fig. 5), and the frequency of each type was compared in the three species (Fig. 8). The branchlet shape of *P. recurvatum* was diverse within the same thallus, and these branchlets were not always recurved. All specimens of *P. cartilagineum* and *P. telfairiae* examined had linear branchlets, but some of them also produced slightly or strongly recurved branchlets.

pH measurements of cell sap

The values of intracellular pH varied between and within the three *Plocamium* species (Table 2). Of 18 specimens of *P. cartilagineum* examined, only one showed strong acidity (pH 2.7) and the rest were less acidic (pH 4.5–5.6). Of 47 specimens of *P. recurvatum*, 12 specimens were strongly acidic (below pH 3.0), whereas nearly 70% of the specimens of *P. telfairiae* showed strong acidity (128 samples from 18 populations). Intracellular pH of *P. ovicorne* and five gigartinalean species (*Chondracanthus tenellus, Hypnea flexicaulis, H. japonica, H. variabilis* and *Portieria hornemannii*) was 4.1–6.1.

Molecular phylogenetic analyses

Based on the Rubisco spacer sequences, 16 haplotypes were detected in the five species: three in *P. cartilagineum*, two in *P. ovicorne*, two in *P. recurvatum*, one in *P. serratulum* and eight in *P. telfairiae*. Haplotypes TEL9, TEL10 and TEL16 showed completely identical sequences to haplotypes REC6, CAR1 and CAR3, respectively. The conspecific specimens showing identical DNA sequences were assigned the same haplotype number (Table 1).

The analysed sequences, 269–273 bp in length, had a mean 27.6% G + C content and included 80 variable sites identified among all the samples; 46 of these were phylogenetically informative. Figure 9 shows the strict consensus tree of the two most parsimonious trees (length = 113 steps, consistency index = 0.841, retention index = 0.910), and this topology was basically similar to that of the NJ tree (data not shown). ML estimation (−ln = 874.15) produced the tree topology (Fig. 10) based on the HKY model (Ti:Tv ratio = 2.6301; base frequencies of A = 0.4158, C = 0.1313, G = 0.1311, T = 0.3218; gamma distribution = 0.240; proportion of invariable sites = 0). Consequently, three major clades were recognized (Figs 9, 10): *P. cartilagineum*, one haplotype of *P. recurvatum* and four haplotypes of *P. telfairiae* in clade A; *P. ovicorne* and *P. serratulum* in clade B; and the other one haplotype of *P. recurvatum* and four of *P. telfairiae* in clade C.

DISCUSSION

This is the first report to demonstrate the speciation of *Plocamium* based on the physiological characteristics and molecular sequences as well as morphological differences. Goff *et al.* (1996) sequenced ITS1 and ITS2 of the nuclear ribosomal repeat in four *Plocamium* and two *Plocamiocolax* species, and they revealed that each *Plocamiocolax* species was independently evolved from different *Plocamium* species. We preliminarily sequenced ITS1 and ITS2 of some Japanese *Plocamium* specimens, but the mutation rate of both regions was too fast to align the sequences between the major clades with confidence (data not shown). Fredericq *et al.* (1996) showed molecular phylogenetic trees of five *Plocamium* species using partial sequences of the *rbc*L gene (*c.* 1300 bp), but most of the clades were not supported by high bootstrap values (less

Table 2. Estimated intracellular pH.

Species	n[1]	pH ($\bar{x} \pm s_x$)	pH range
Seawater	15	7.91 ± 0.07	7.5–8.3
Chondracanthus tenellus	4	4.88 ± 0.12	4.5–5.0
Hypnea flexicaulis	18	4.77 ± 0.08	4.1–5.3
H. japonica	10	5.00 ± 0.08	4.7–5.4
H. variabilis	5	5.30 ± 0.04	5.2–5.4
Portieria hornemannii	10	5.00 ± 0.13	4.5–5.8
Plocamium ovicorne	7	5.46 ± 0.12	5.1–6.1
P. cartilagineum (Ama)	1	5.20	5.2
P. cartilagineum (Shiga)	4	4.00 ± 0.44	2.7–4.6
P. cartilagineum (Shimokamagari)	4	5.22 ± 0.13	5.0–5.6
P. cartilagineum (Anaga; 31 Oct. 2001)	5	5.36 ± 0.09	5.1–5.6
P. cartilagineum (Anaga; 23 Oct. 2002)	4	4.67 ± 0.07	4.5–4.8
P. recurvatum (Iwafune)	11	3.73 ± 0.48	1.7–6.1
P. recurvatum (Ooarai)	10	5.37 ± 0.39	2.6–6.6
P. recurvatum (Togawa; 19 Mar. 2002)	12	4.34 ± 0.40	2.0–6.0
P. recurvatum (Togawa; 14 Oct. 2002)	10	4.03 ± 0.52	1.8–6.0
P. recurvatum (Terahama)	3	5.10 ± 0.32	4.6–5.7
P. recurvatum (Sakamoto)	1	3.10	3.1
P. telfairiae (Ama)	5	1.54 ± 0.06	1.4–1.7
P. telfairiae (Igumi)	8	1.75 ± 0.25	1.2–3.3
P. telfairiae (Iwafune)	12	2.76 ± 0.34	1.8–4.9
P. telfairiae (Ooarai)	3	2.60 ± 0.15	2.4–2.9
P. telfairiae (Ooura)	10	2.25 ± 0.41	1.2–4.8
P. telfairiae (Kamae)	2	1.95 ± 0.05	1.9–2.0
P. telfairiae (Kushimoto)	16	5.04 ± 0.23	2.8–6.2
P. telfairiae (Shiogama)	7	2.54 ± 0.49	1.6–5.4
P. telfairiae (Shiga)	8	3.60 ± 0.57	1.4–4.9
P. telfairiae (Shimoda)	1	2.10	2.1
P. telfairiae (Terahara)	1	2.00	2.0
P. telfairiae (Sakamoto)	1	3.10	3.1
P. telfairiae (Shirahama; 16 Oct. 2001)	7	5.77 ± 0.22	4.9–6.6
P. telfairiae (Shirahama; 19 Jun. 2002)	6	5.13 ± 0.23	4.5–6.0
P. telfairiae (Togawa)	3	1.80 ± 0.12	1.6–2.0
P. telfairiae (Iwaya)	1	1.70	1.7
P. telfairiae (Anaga; 31 Oct. 2001)	5	1.70 ± 0.12	1.3–2.0
P. telfairiae (Anaga; 25 Feb. 2002)	11	2.22 ± 0.28	1.3–4.5
P. telfairiae (Misaki)	1	1.40	1.4
P. telfairiae (Yura; 25 Feb. 2002)	10	1.61 ± 0.07	1.3–2.0
P. telfairiae (Yura; 27 Aug. 2002)	10	1.95 ± 0.06	1.7–2.4

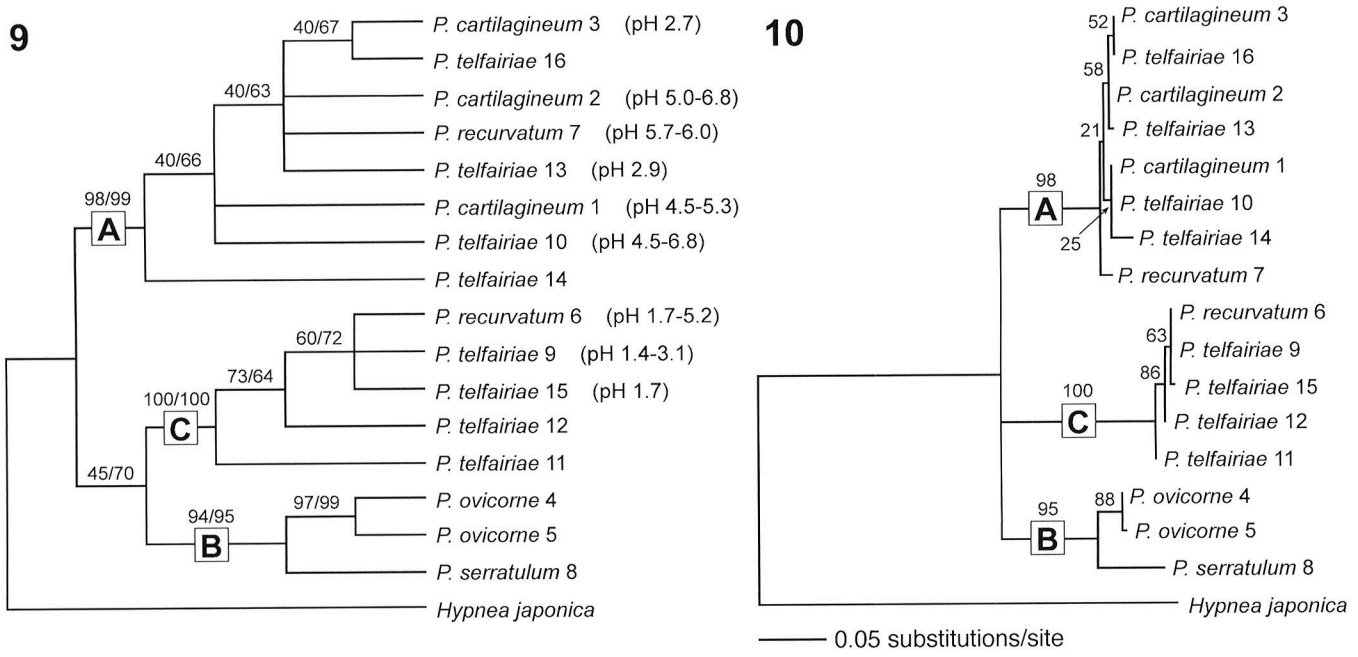
[1] Number of specimens examined in this study.

than 70%). This study confirmed that the Rubisco spacer and its flanking regions, which are only less than 300 bp long in total, are useful to clarify the genetic diversity among the closely related species, although the relationship of the three major clades is still unresolved (Figs 9, 10).

Although *P. ovicorne* is morphologically very different from *P. serratulum* in the presence of shorter branchlets opposite the normal branchlet series, the absence of the minute tooth-like serrations along the outer margin of the lowermost branchlet and the presence of three to five branchlets in each alternate series (Okamura 1913, 1923, 1932), their close relationship is demonstrated in the three phylogenetic analyses (Figs 9, 10). In contrast, *P. cartilagineum* and *P. ovicorne* share a common character in the branchlet number, but they did not occur in the same clade in either tree. The *rbc*L data (Fredericq *et al.* 1996) also suggest that branching pattern, branchlet number and shape of lowermost branchlet changed several times in the *Plocamium* lineage. Extension of the molecular phylogenetic analysis to more species is required for understanding the morphological evolution in this genus.

Ambiguous delimitation of *P. cartilagineum, P. recurvatum* and *P. telfairiae* was confirmed with our detailed morphological examination, which showed great variation of the size, shape and number of branchlets. We tried to alter the morphological key characters to identify each of the three species in several ways, but there was no clear-cut definition to distinguish them (data not shown). Although Okamura (1913) classified the specimens having slightly recurved branchlets as *P. telfairiae* f. *uncinatum,* such branchlets were observed in 46% of the specimens of *P. telfairiae,* 74% of *P. recurvatum* and 22% of *P. cartilagineum* examined in this study (Fig. 8). Because of such continuous variations in this character, there is some doubt about the taxonomic independence of *P. recurvatum* and *P. telfairiae* f. *uncinatum.* This suspicion is confirmed by the present molecular analysis, which demonstrates the polyphyletic relationship of *P. recurvatum* and the identical sequence of the Rubisco spacer region between some specimens of *P. recurvatum* and *P. telfairiae.* The specimens having strongly recurved branchlets often attach to the basal parts of seagrasses (species of *Phyllospadix* Hooker) in the wave-exposed upper sublittoral zone, and this particular environment may influence the formation of branchlet recurvature.

Plocamium cartilagineum is generally characterized by its forming three or more branchlets in alternating pectinate series (Womersley 1971, 1994; Abbott & Hollenberg 1976; Dix-



Figs 9, 10. Molecular phylogenetic trees of the Japanese *Plocamium* species inferred from the Rubisco spacer and its flanking regions of *rbc*L and *rbc*S genes. The numbers following the species names indicate the haplotype listed in Table 1.

Fig. 9. Strict consensus tree of two most parsimonious trees. Parsimony-derived (left) and distance-derived (right) bootstrap values are given above branches (1000 replicates each).

Fig. 10. Topology of the ML tree. Likelihood-derived bootstrap values are given above branches (1000 replicates).

on & Irvine 1977; South & Adams 1979; Gabrielson & Scagel 1989). Okamura (1913) also distinguished the Japanese *P. cartilagineum* (as *P. leptophyllum* var. *flexuosum*) from other species on the basis of its production of three to five branchlets and its slender gross morphology. In this study, however, two branchlets are frequently observed in the Japanese *P. cartilagineum*, and three branchlets are occasionally seen in some specimens of *P. recurvatum* and *P. telfairiae* (Fig. 7). These data imply that the branchlet number in alternating pectinate series is not an appropriate character to identify the Japanese *P. cartilagineum*. Considerable morphological variations of *P. cartilagineum* have been pointed out between the worldwide populations (Okamura 1913; Kylin 1925; Dawson 1961; Womersley 1971; Gabrielson & Scagel 1989), and genetic diversity has also been reported among them. Goff *et al.* (1996) sequenced ITS1 and ITS2 regions in four *Plocamium* species and demonstrated the polyphyletic relationship of *P. cartilagineum* from Antarctica, California and the UK. The partial *rbc*L sequence data indicate that *P. cartilagineum* from Antarctica, California, Chile and Ireland did not make a clade with each other (Fredericq *et al.* 1996). It is necessary therefore to examine the identity of this widespread species, including the Japanese specimens.

Reproductive structures are varied among *Plocamium* species, such as the position of cystocarps and spermatangia, and the branching and clustering pattern of tetrasporangial stichidia (Womersley 1971). In *P. cartilagineum*, tetrasporangial stichidia extend in a series along the margins of main branches and branchlets (Womersley 1971; Gabrielson & Scagel 1989), whereas *P. telfairiae* forms distinct, compact clusters of stichidia (Okamura 1913). In this study, we were not able to collect enough material to compare the reproductive structures, so further examination of these morphological traits is required to reclassify the Japanese *Plocamium* species.

We did not measure the intracellular pH of every haplotype, but the present data suggest that this physiological characteristic is phylogenetically informative. Most of the haplotypes examined in clade C exhibit strong acidity (Fig. 9). Haplotype REC6 in this clade shows variability in the acidity (pH 1.7–5.2), but it is unknown whether such variability is associated with genetic divergence, environmental differences or algal developmental stages. In contrast, many specimens belonging to clade A are weakly acidic or neutral, except for haplotypes CAR3 and TEL13, whose intracellular pH is below 3.0. This means that the ability to accumulate the acidic substance was acquired more than once in the *Plocamium* lineage. The evolution of this physiological property may be elucidated by more extensive examination of pH in other haplotypes and by higher-resolution molecular data.

The intracellular strong acidity has already been demonstrated in some seaweeds. Several species of *Desmarestia* J.V.F. Lamouroux are characterized by the presence of high concentrations of sulphuric acid in the vacuoles with a pH below 1.0 (Schiff 1962; McClintock *et al.* 1982; Sasaki *et al.* 1999), and a molecular phylogenetic analysis using ITS regions revealed the monophyletic relationship of the 14 *Desmarestia* species having sulphuric acid vacuoles (Peters *et al.* 1997). In Dictyotaceae, estimated intracellular pH was below 1.0 in three species of *Dictyopteris* J.V.F. Lamouroux, two species of *Spatoglossum* Kützing and one species of *Zonaria* C. Agardh (Sasaki *et al.* 1999, 2004), although the evolutionary relationship of the acidic dictyotacean species is still unresolved (Lee & Bae 2002; Hoshina *et al.* 2004). In red algae, fresh samples of *Chondria macrocarpa* Hooker & Harvey de-

compose when washed in tap water, turning highly acidic (pH less than 1.0) and becoming pink mush (Furneaux & Stevenson 1990). Nelson & Falshaw (1999) reported that the deterioration seen in herbarium specimens of *Gigartina* Stackhouse and *Callophyllis* Trevisan is caused by sulphuric acid, which is below pH 1.0. It is considered that these acidic substances are produced through autocatalytic hydrolysis of sulphate half-ester groups associated with polysaccharides such as xylogalactan sulphate and carrageenan (Furneaux & Stevenson 1990; Nelson & Falshaw 1999). In the case of the acidic *Plocamium,* the estimated intracellular pH value is never below 1.0, and they do not decompose on desiccation or immersion in freshwater. Considering the distant relationship of these algae, the mechanism to concentrate hydrogen ions within cells has certainly evolved several times in the red algal lineage.

It is well known that *Plocamium* species contain a large number of halogenated monoterpenes, whose structures typically contain 1–6 bromine or chlorine atoms, or both (Fenical 1975). Many chemical studies on *Plocamium* species have shown the qualitative and quantitative variations of halogenated monoterpene composition between conspecific populations (Crews *et al.* 1977; Mynderse & Faulkner 1978; Yoshida *et al.* 1981; San-Martín & Rovirosa 1986; Rovirosa *et al.* 1988; König *et al.* 1999). Crews *et al.* (1977) found that at least three distinct chemical forms of *P. violaceum* Farlow exist in Pacific North American populations and that these chemical differences are invariant with respect to life history or season. Two chemotypes were also reported from six populations of *P. cartilagineum* along 1600 km of central Chilean coast (San-Martín & Rovirosa 1986). In Japan, Yoshida *et al.* (1981) identified seven polyhalogenated monoterpene compounds from two populations of *P. telfairiae* and detected an obviously different component of these secondary metabolites between the two populations. Such chemical variations may be associated with the evolutionary divergence and physiological differentiation of the Japanese *Plocamium* species that were demonstrated in this study.

Although high accumulation of acidic substances within vegetative cells has been recognized for a long time in desmarestiacean and dictyotacean algae, the physiological or ecological significance of the high intracellular acidity is still uncertain. *Desmarestia* had a low palatability to *Parechinus* Mortensen (Anderson & Velimirov 1982), whereas *Aplysia kurodai* Baba (sea hare) demonstrated a lower preference for *Pachydictyon coriaceum* (Holmes) Okamura (nonacidic) than for *Dictyopteris prolifera* (Okamura) Okamura (acidic) (Nagahama & Shin 1998). *Plocamium cartilagineum* (as *P. leptophyllum*) collected from Toyama Bay, Japan, were consumed less by the marine herbivorous gastropod *Turbo cornutus* Lightfoot than other macroalgae (Sakata *et al.* 1991). That study also found that a halogenated monoterpene (aplysiaterpenoid A) extracted from *P. cartilagineum* functions as a feeding inhibitor against not only *T. cornutus* but also other herbivores, such as *Haliotis discus* Reeve (abalone), *Omphalius pfeifferi* Dunker (top shell) and *Strongylocentrotus intermedius* Agassiz (sea urchin). In this study, we examined *P. cartilagineum* from Ishikawa Prefecture, adjacent to Toyama Bay, and found two haplotypes showing different acidities: pH 4.5 in CAR1 and pH 2.7 in CAR3. Feeding studies using these haplotypes may resolve the question of whether high accumulation of acidic substances is associated with low palatability for these herbivores.

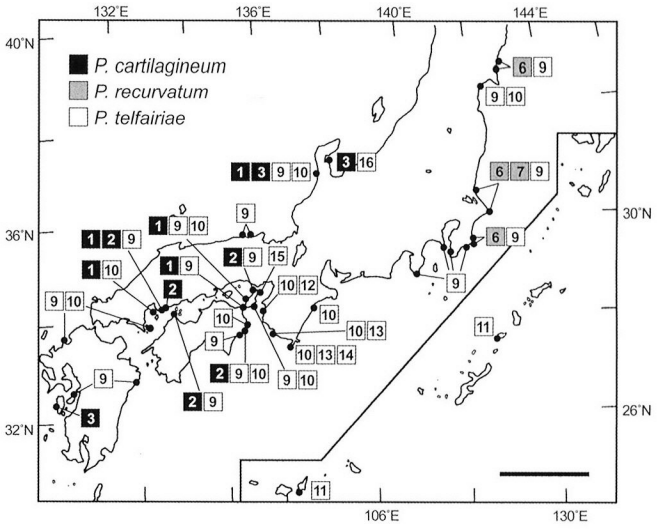


Fig. 11. Map showing the collecting sites for each haplotype. Scale bar = 200 km.

Distribution range and frequency differ between the haplotypes (Fig. 11). TEL9 and TEL10 show wide distribution and appear together in some populations. There are also some endemic haplotypes that are collected from only one site, such as TEL12, TEL14, TEL15 and TEL16. Because genetic distance of most haplotypes is not proportional to their geographic distances (data not shown), *Plocamium* species may have high capability to disperse over long distances. However, there are only a few haplotypes showing widespread distribution, and interhaplotypic competition may be so severe that many haplotypes cannot expand their distribution.

In conclusion, the Japanese specimens of the *P. cartilagineum–P. recurvatum–P. telfairiae* complex are not clearly distinguished by the present morphological comparison or by physiological examination. The molecular phylogenetic results do not support the conventional classification of the Japanese *Plocamium* species, so it is necessary to reclassify these algae on the basis of their evolutionary relationships. The estimated intracellular acidity seems to be useful for assessing their relationship, but more detailed examination is necessary to reveal variation of this characteristic between and within populations and that possibly caused by differences in developmental stages or by environmental factors. We are now proceeding with further analyses of intracellular concentrations of various ions and with detailed examination of morphological plasticity using cultured strains for a better understanding of the diversity of these species complexes.

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