

## Taxonomic Note of *Polysiphonia pacifica* (Ceramiales, Rhodophyta) Complex with Focus on Pacific Isolates

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*Polysiphonia pacifica* is rhodomelaceous red algal species that includes five varieties in Pacific Ocean: *P. pacifica* var. *delicatula*, *P. pacifica* var. *distans*, *P. pacifica* var. *determinata*, *P. pacifica* var. *disticha*, and *P. pacifica* var. *gracilis*. We here report morphology and phylogeny of *P. pacifica* to confirm the relationships among previously described varieties as a loan of type specimens from US and to assess phylogenetic relationships of closely related species using plastid protein-coding *rbcl* gene. *Polysiphonia pacifica* is distinguished by having creeping filaments attached by unicellular rhizoids not cut off by cross walls, four pericentral cells, ecorticate, trichoblasts rare, ultimate branchlets attenuate at the tip but not pungent, and tetrasporangia in long straight series in the ultimate branchlets. The protein-coding plastid *rbcl* gene sequence data show that *P. pacifica* is distinctly different from the superficially similar species, *P. morrowii* and *P. stricta*. However, the *rbcl* sequences of *P. pacifica* var. *pacifica* and var. *disticha* are identical though they have morphological variation.

**Key Words:** Ceramiales, morphology, phylogeny, *Polysiphonia pacifica*, *rbcl*, Rhodophyta, taxonomy

### INTRODUCTION

The cosmopolitan genus *Polysiphonia* is widely distributed in most temperate and tropical waters of the world (Abbott 1999). The genus has been one of the largest genera in the Rhodophytes, with approximately 200 species worldwide, and recent monographic studies have divided it into two genera, *Polysiphonia* and *Neosiphonia* (Kim and Lee 1999). *Polysiphonia* is characterized by consisting of prostrate and erect ecorticate axes with four pericentral cells, attaching by unicellular rhizoids in open connection with pericentral cells, lacking vegetative trichoblasts, and branch initials separating at intervals of four or five segments. Spermatangial branches are produced directly from each axial cell and the mature carpogonial branch is four-celled. Tetrasporangia are arranged in straight series on determinate branches (Kim *et al.* 2000). The taxonomy of *Polysiphonia* at the species level, however, is still in confusion because it is extremely variable in morphology (Maggs and Hommersand 1993). In the last five years, molecular analyses have been applied to clarify the taxonomic status of *Polysiphonia*

species (Choi *et al.* 2001; McIvor *et al.* 2001). Kim *et al.* (2004) also employed DNA sequence from the plastid-encoded *rbcl* gene to assess six Korean samples and compared these with data of *P. morrowii* samples from Chile. They suggested that the specimens of *Polysiphonia* sp. from Chile and *P. morrowii* from Korea could be the same species and *P. morrowii* has been introduced recently from the northwest Pacific Ocean to Chile.

*Polysiphonia pacifica* was originally described by Hollenberg (1942) based on collections of Dr. C.L. Anderson at Santa Cruz, USA. Hollenberg (1942) provided a taxonomic treatment as many varieties for the species *P. pacifica* along the entire Pacific coast of North America, that is *P. pacifica* var. *delicatula*, *P. pacifica* var. *distans*, *P. pacifica* var. *determinata*, *P. pacifica* var. *disticha*, and *P. pacifica* var. *gracilis* (Abbott and Hollenberg 1976; Hansen 1977; Scagel *et al.* 1989). The characteristics of *P. pacifica* including all varieties are with creeping filaments attached by unicellular rhizoids, which they are not cut off by cross walls; pericentral cells 4, ecorticate; trichoblasts rare; tetrasporangia in long straight series in the ultimate branchlets; cystocarps conspicuously urceolate; spermatangial branches formed from the entire fruiting branch (Hollenberg 1942). However, several species of the genus *Polysiphonia*, such as *P. stricta* from

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Europe and the Atlantic coast of North America and *P. morrowii* from the northwest Pacific, are closely similar to *P. pacifica* and therefore, their taxonomy is controversial (Hollenberg 1942; Kim *et al.* 2000)

During our collection trip to USA, we collected two varieties of *Polysiphonia pacifica* in the small harbor in Orcas Island, Washington and Seal Rock, Oregon in June 2003. We here observed morphological structures of two varieties, and then determined the phylogenetic affinities of its component lineages among closely related species. We sequenced the plastid protein-coding *rbcl* gene from selected taxa of *Polysiphonia* and *Neosiphonia*. The *rbcl* region was chosen because it is considered to provide optimal resolution for inferring species level phylogenetic relationships within the Ceramiaceae (Cho *et al.* 2003; Yang and Boo 2004). The significance of our phylogenetic and morphological observations for the classification of *Polysiphonia pacifica*'s varieties was discussed.

## MATERIALS AND METHODS

### Morphology

Materials of *Polysiphonia pacifica* were collected at East Sound, Orcas Island, Washington, and Seal Rock, Oregon, USA. The samples were preserved in 4% formaldehyde/seawater for morphological observations. Microscopic observations were made from material stained with 1% aqueous aniline blue acidified with 1% HCl. Photographs were taken through an Olympus microscope (Olympus Optical Co., Tokyo, Japan). Some original materials of *P. pacifica* and varieties were observed on loan from the Smithsonian Institution, National Museum of Natural History, Washington DC (US). Digital images were edited and assembled on plates using Photoshop 5.5. Voucher specimens used in this study were deposited in the herbarium of Chungnam National University (CNUK), Daejeon, Korea.

### Analysis of the *rbcl* sequence

Algal material, their collection sites, and the GenBank accession numbers of *rbcl* sequence data were listed in Table 1. Samples were sorted under a dissecting microscope, dried in the air, and preserved with silica gel in the field.

Genomic DNA was extracted from approximately 0.005 g of algal powder ground in liquid nitrogen using a DNeasy Plant Mini Kit (Qiagen) or Invisorb Spin Plant Mini Kit (Invitek), according to the manufacturers' instructions. The *rbcl* region was amplified using

primers F7 - R753 and F645 - RrbcS start and sequenced using primers F7, F645, R753, and RrbcS start (Freshwater and Rueness 1994; Lin *et al.* 2001; Gavio and Fredericq 2002). The PCR products were purified using a High Pure PCR Product Purification Kit (Roche), in accordance with the users' guide. The sequences of the forward and reverse strands were determined for all taxa using an ABI PRISM<sup>TM</sup> 377 DNA Sequencer (Applied Biosystems) at Research Center, Chungnam National University, Daejeon, Korea. We aligned and reconciled complementary chromatograms using Sequence Navigator v. 1.0.1 (Applied Biosystems). The alignment of gene sequence was based on the alignment of the inferred amino acid sequence and was refined by eye. There were no gaps in our alignments of *rbcl*.

Maximum parsimony (MP) tree have been calculated using PAUP\* 4.0b10 (Swofford 2002). Full heuristic search was carried out with 1,000 replicates, random addition sequences of taxa, keeping best trees only, holding 1 trees at each step, tree bisection-reconnection (TBR) branch swapping, collapsed of zero length branches and MULTREES on. Bootstrap values ( $Bt_{MP}$ ) were calculated performing 1,000 replicates with following options selected: heuristic search, TBR branch swapping, collapse of zero length branches, and random sequence addition with one replicate.

Maximum likelihood (ML) analysis was performed using PAUP\* with a general time reversible (GTR) model, with proportion of invariable sites. This model of sequences evolution was chosen based on results from Modeltest v3.6 (Posada and Crandall 1998). The Akaike information criterion (AIC) selected GTR + I model as the best-fitting model for the data. Tree likelihoods were estimated using a heuristic search with 100 random addition sequence replicates, and TBR branch swapping. To test the stability of monophyletic groups, bootstrap ( $Bt_{ML}$ ) analyses were undertaken with 500 replicates.

Bayesian analysis was conducted with MrBayes v.3.0b4 (Ronquist and Huelsenbeck 2003) GTR + I model as the same in ML analysis. The GTR rates and the proportion of invariable sites value were not fixed. For the data matrix, 1.3 million generations were performed with four chains and trees sampled every 100 generations. The burn-in period can be identified graphically by tracking the likelihoods at each generation. After of preliminary analyses, a burn-in period of 300,000 generations was determined to be appropriate for the data. The 10,000 trees sampled at stationarity were used to infer the Bayesian posterior probability (Bp). Majority-rule

**Table 1.** Species studied in the present study

Taxa	Locality	Voucher	Accession Number
<i>Polysiphonia morrowii</i> Harvey	Korea: Gangreung, Anin	P47	AY396030 <sup>1</sup>
	Korea: Namhaedo, Namhaedaegyo	P173	AY396031 <sup>1</sup>
	Korea: Susan, Ungdori	P48	AY958161
	Chile: Punta Arenas, Fuerte Bulnes	P176	AY396029 <sup>1</sup>
	Chile: Punta Arenas, Seno Otway	P177	AY396028 <sup>1</sup>
<i>P. pacifica</i> var. <i>disticha</i> Hollenberg	USA: Oregon, Seal Rock	P193	AY396036 <sup>1</sup>
	USA: Oregon, Seal Rock 2	P194	AY958162
<i>P. pacifica</i> var. <i>pacifica</i> Hollenberg	USA: Washington, Orcas Island	P191	AY396035 <sup>1</sup>
<i>P. stricta</i> (Dillwyn) Greville	England: Yorkshire, Flamborough	P171	AY958167
	Northern Ireland: Moyle, Cushendun	P195	AY958163
	Northern Ireland: Larne, Garron	P196	AY958164
	Northern Ireland: Larne, Garron Pt.	P198	AY958165
	Norway: Møre og Romsdal, Finnøya	P421	AY958166
	Japan: Hokkaido, Oshoro	P77	AY958168
<i>Polysiphonia</i> sp.	Japan: Hokkaido, Denshinhama	P70	AY958169
<i>Neosiphonia harveyi</i> (Bailey) M.S. Kim <i>et al.</i>	-	-	AF342900 <sup>2</sup>
<i>N. japonica</i> (Harvey) M.S. Kim <i>et al.</i> I.K. Lee	-	-	AF342902 <sup>2</sup>
<i>Chondria californica</i> (Collins) Kylin	-	-	AY172578 <sup>3</sup>
<i>Laurencia natalensis</i> Kylin	-	-	AF465816 <sup>4</sup>

<sup>1</sup>Kim *et al.* 2004. <sup>2</sup>McIvor *et al.* 2001. <sup>3</sup>Fujii *et al.* unpublished. <sup>4</sup>McIvor *et al.* 2002.

consensus trees were calculated using PAUP\*.

## RESULTS

### Morphology

#### Type specimens (Figs 1-4)

There are two holotypes of *Polysiphonia pacifica* var. *delicatula* and var. *distans* and two isotypes of *P. pacifica* and var. *determinata* in US herbarium.

***P. pacifica* var. *delicatula* (#US-61226, Holotype):** Eight plants are coated with plastic film (Fig. 1). They are all very small (1 cm), delicate and collected from low tide level on pilings of wharf at Monterey, California, in July 20, 1939 by Hollenberg. This plant looks like very similar to *P. atlantica* from the Atlantic coast.

***P. pacifica* var. *distans* (#US-61228, Holotype):** Plant is 12 cm high, dark brownish-red and collected at Carmel Beach, Monterey County, California, in June 22, 1939 by Hollenberg. Plant has inconspicuous prostrate filaments with four pericentral cells and large urceolate cystocarp (Fig. 3). Hollenberg noted in original description that the distinctive features of this variety are the coarse laxly branched filaments, very large cystocarps and tetrasporangia in short series. It looks like a different species from *P. pacifica*.

***P. pacifica* var. *determinata* (#US-61227, Isotype):** The specimen only have an upper part of the tetrasporophyte

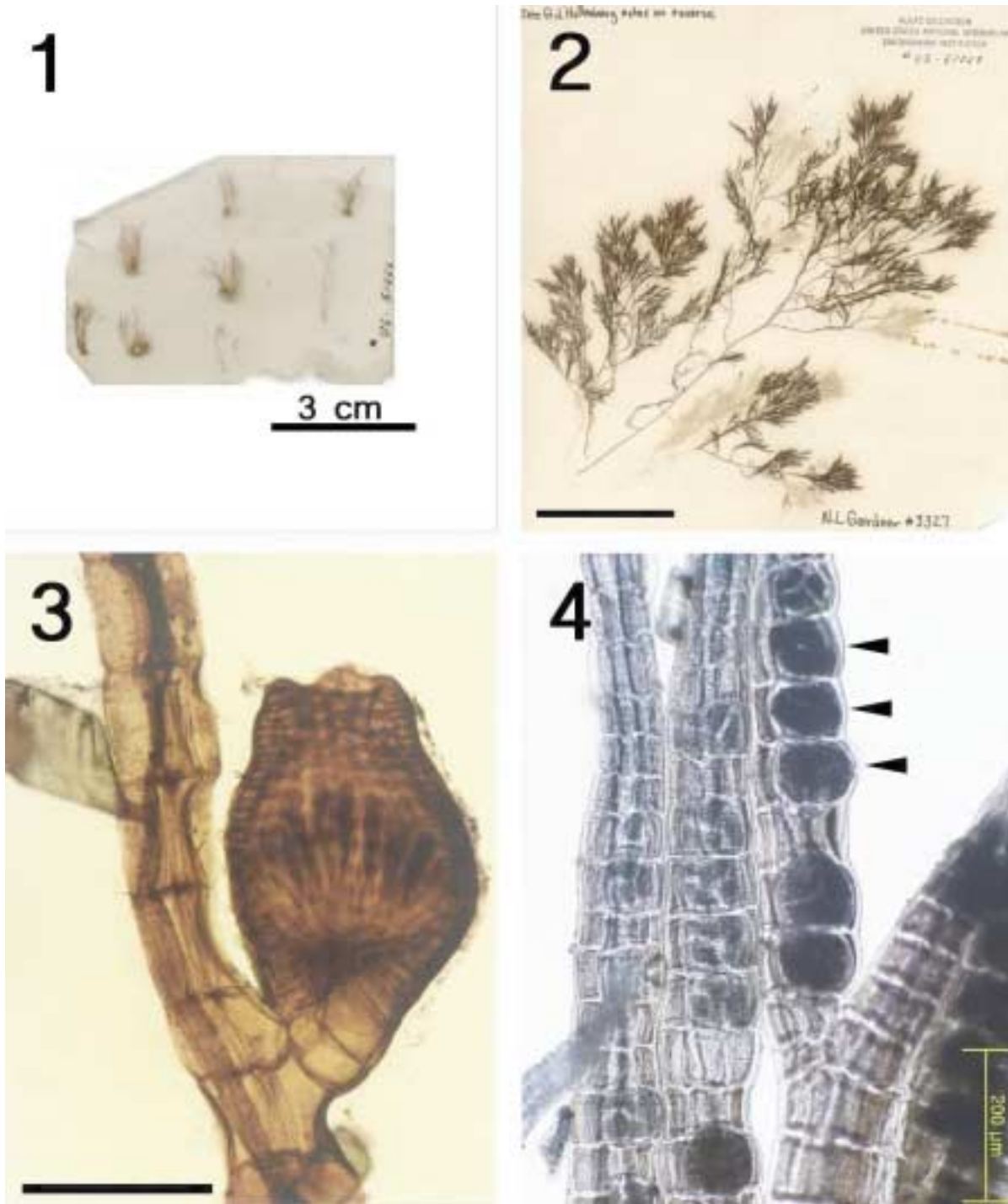
with arranging tetrasporangia in straight series (Fig. 4). Branching is densely and ultimate branchlets are coarse, determinate and dense pencillate tufts (Fig. 2). It is 12 cm high, reddish-black and collected at Pebble Beach, Monterey County, California, in May 1916 by Gardner. It looks like a mature plant of *P. morrowii* from the north-western Pacific Ocean.

***P. pacifica* (#US-066798, Isotype):** There are a photo of holotype and a slide (#US-2814). Because there are very small pieces of branch segments, we could not find any good information of the species using the slide.

We have not gotten any kind of the type specimens of *P. pacifica* var. *disticha* and var. *gracilis*.

#### *Polysiphonia pacifica* (Figs 5-8)

Plants are 5-7 cm high (Fig. 5). Plants have inconspicuous prostrate filaments attached by unicellular rhizoids, which they are not cut off by cross walls from the lower pericentral cells. Erect branches are ecorticate, with four pericentral cells and little-branched especially below (Fig. 7). Branching patterns are alternate, 100-200  $\mu$ m in diameter and corymbose at the tips. The ultimate branchlets are attenuating at the tip but without sharply pointed apex (Fig. 6). Trichoblasts and scar-cells are exceedingly rare. Tetrasporangia are 70  $\mu$ m diameter in long straight series in the ultimate branchlets (Fig. 8). Female and male gametophytes could not collect in this study.

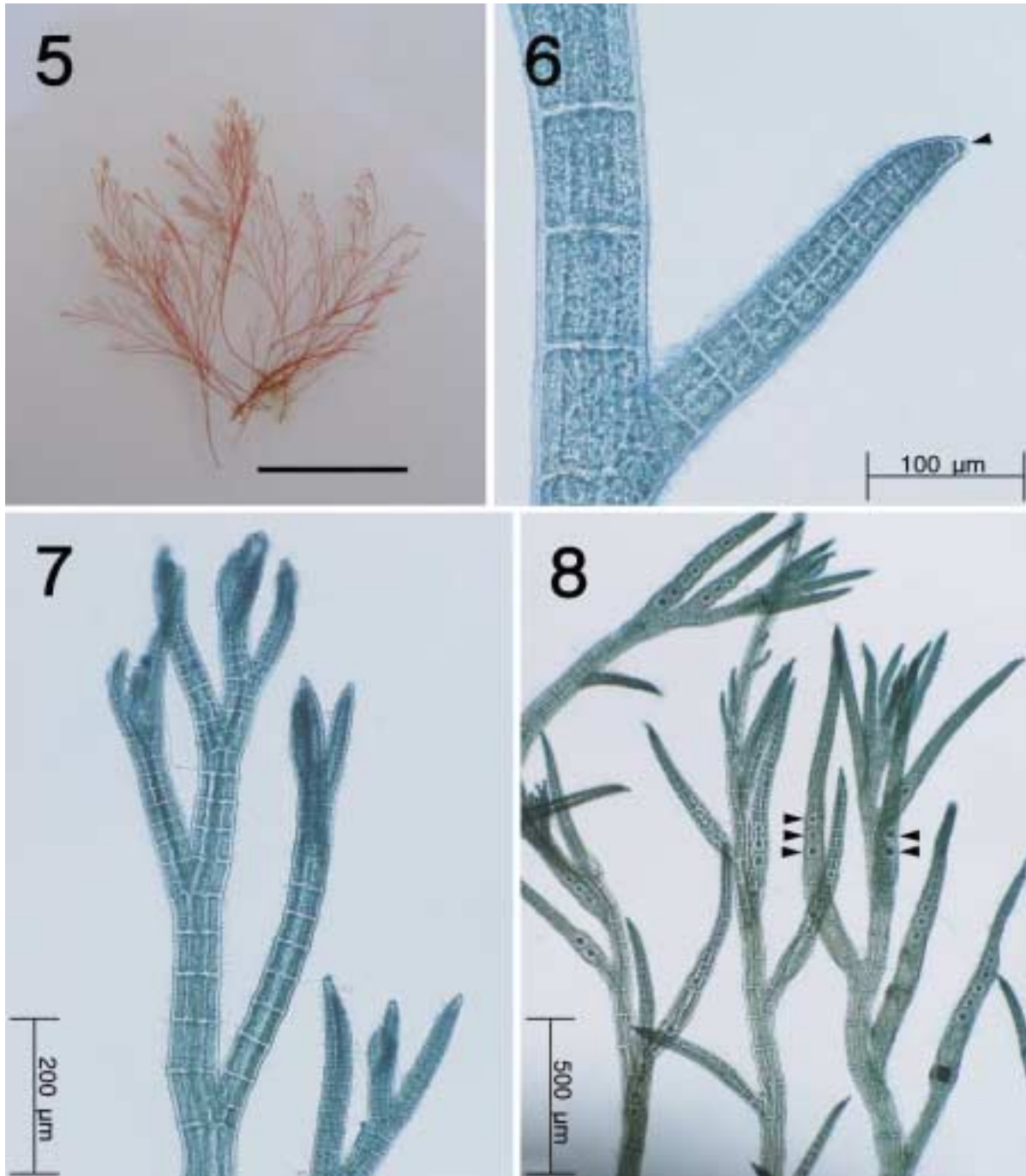


**Figs 1-4.** Type specimens of *Polysiphonia pacifica* Hollenberg. **Fig. 1.** Holotype of *P. pacifica* var. *delicatula* from Monterey, California, July 20, 1939 by Hollenberg. **Fig. 2.** Iso-type of *P. pacifica* var. *determinata* collected at Pebble Beach, Monterey County, California, May 1916 by Gardner. Scale bar = 3 cm. **Fig. 3.** Shape of cystocarp from holotype's microslide (#US-1236) of *P. pacifica* var. *distans* collected at Carmel Beach, Monterey County, California, June 22, 1939 by Hollenberg. Scale bar = 300  $\mu$ m. **Fig. 4.** Tetrasporangia (arrowheads) plants of *P. pacifica* var. *determinata* from isotype specimen.

Plants are growing on wood in the littoral zone at the small harbor of East Sound, Orcas Island, Washington, USA.

***Polysiphonia pacifica* var. *disticha* (Figs 9-12)**

Plants are 2-3 cm high, ecorticate with four pericentral cells and attached by rhizoids at the rocks (Fig. 9). The pattern of branches is spirally arranged in alternating



**Figs 5-8.** *Polysiphonia pacifica* Hollenberg collected at Orcas Island, Washington, USA. **Fig. 5.** Habit of thalli growing on the wood of harbor in littoral zone. Scale bar = 2.5 cm. **Fig. 6.** Branch having four pericentral cells and no sharply pointed cell in the apex (arrowhead). **Fig. 7.** Branching pattern of apex in main axis. **Fig. 8.** Habit of tetrasporangia plants in straight arrangement (arrowheads).

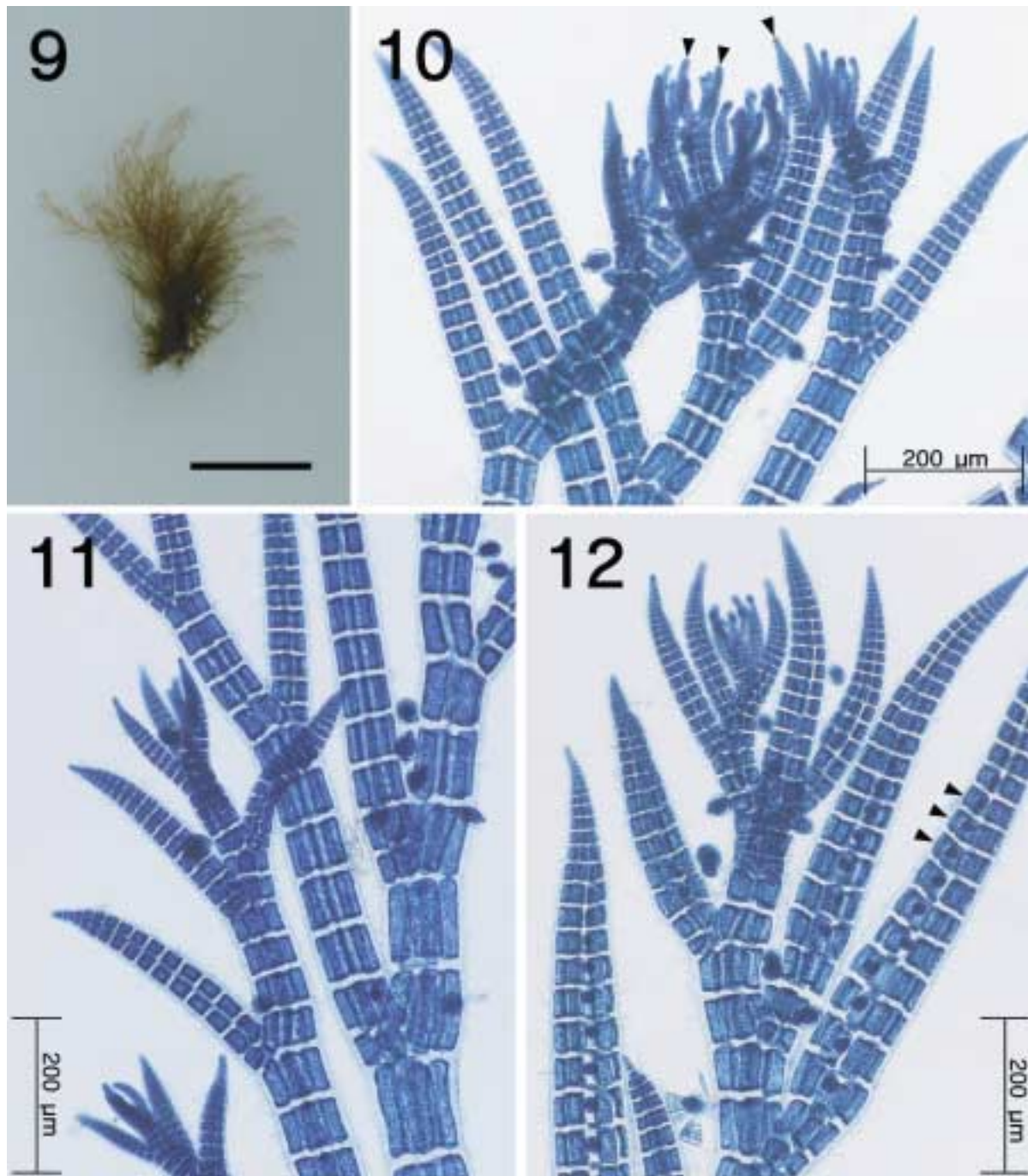
pairs every 2-3 segments throughout the length of all branches (Fig. 10). The ultimate branchlets are determinate, incurved and attenuate at the tip but not pungent (Fig. 11). Trichoblasts are absent. Tetrasporangia are 60  $\mu\text{m}$  diameter in long straight series (Fig. 12). Female and male gametophytes could not collect in this study. Plants are occurring mostly in the upper littoral zone in

exposed place, Seal Rock, Oregon, USA.

#### Phylogeny of plastid protein-coding *rbcL* gene

The 1401 nucleotides (95.5% of full length, 1467 nt) of nine *Polysiphonia rbcL* were newly determined and ten of those were downloaded from GenBank. In all, nineteen *rbcL* sequences were aligned including two outgroups,



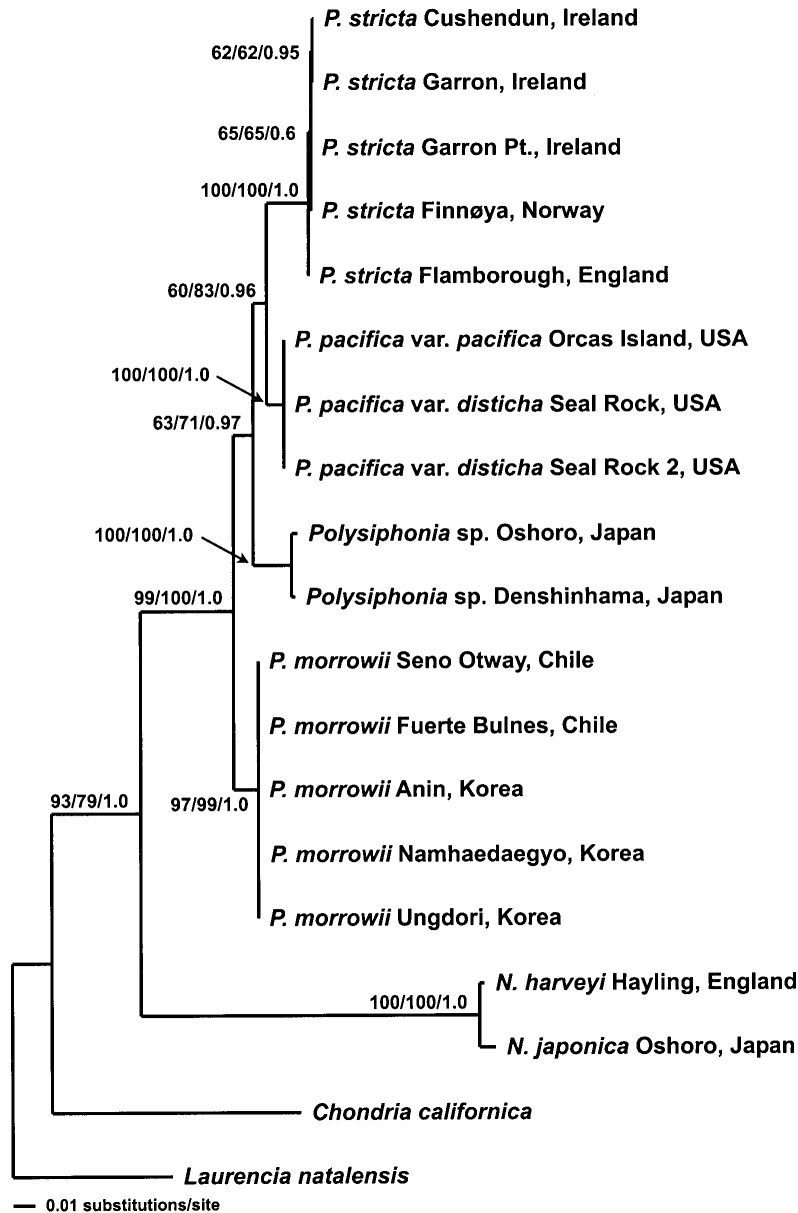


**Figs 9-12.** *Polysiphonia pacifica* var. *disticha* Hollenberg collected at Seal Rock, Oregon, USA. **Fig. 9.** Habit of thalli occurring in the upper littoral zone in exposed places. Scale bar = 1 cm **Fig. 10.** Branch having corymbose at the tips and domed apical cells (arrowheads). **Fig. 11.** Branching showing pseudodichotomous to alternate. **Fig. 12.** Habit of tetrasporangia plants in straight arrangement (arrowheads).

*Chondria californica* and *Laurencia natalensis*; 1074 positions were constant, 327 positions (23.3%) were variable and 230 positions (16.4%) were parsimony informative. Base composition of the gene was slightly AT-biased (63.3%). Transitions were more abundant than transversion (Ti/Tv ratio = 2.84).

Three vouchers belonging to *Polysiphonia pacifica* show

identical *rbcL* sequences. Whereas, each populations of *P. morrowii* and *P. stricta* have a few sequence differences. The *rbcL* sequences from the five samples of *P. morrowii* differed by 1 nucleotides (average p distance = 0.03%) and five of *P. stricta* differed by from 0 to 3 nucleotides (average p distance = 0.1%). Pairwise sequence divergence comparison among three *Polysiphonia* species



**Fig. 13.** Maximum likelihood tree ( $-\ln L = 4691.2951$ ) for *Polysiphonia pacifica* and relatives estimated from the *rbcl* sequences using GTR model ( $R_{AC} = 1.6328$ ,  $R_{AG} = 5.3065$ ,  $R_{AT} = 6.9822$ ,  $R_{CG} = 0.9028$ ,  $R_{CT} = 28.3746$ ,  $R_{GT} = 1$ ) with proportion of invariable sites ( $I = 0.6306$ ) and empirical base frequencies ( $\pi_A = 0.3286$ ,  $\pi_C = 0.1620$ ,  $\pi_G = 0.2053$ ,  $\pi_T = 0.3041$ ). The support values of nodes are from 500 bootstrap resamplings with the ML method using GTR + I model, 1,000 MP bootstrap resamplings, and Bayesian posterior probability from 1,300,000 generations with GTR + I model.

ranged from 3.17% between *P. pacifica* and *P. morrowii* to 2.6% between *P. pacifica* and *P. stricta*. *Neosiphonia japonica* differed from *P. stricta* by 174 nucleotides or 14% sequence divergence.

Three *rbcl* trees constructed using MP, ML and Bayesian analyses showed identical topology (Fig. 13). The closest relationship of *P. pacifica* and *P. stricta* was supported by relatively strong Bt<sub>MP</sub> (83%) and Bp (0.96), despite its decline in Bt<sub>ML</sub> (60%). The monophyly of *P. pacifica* and that of *P. stricta* were supported by maxi-

mum support values (100% for Bt<sub>ML</sub> and Bt<sub>MP</sub> and 1.0 Bp), respectively. The monophyly of *P. morrowii* was supported by strong support values (97% for Bt<sub>ML</sub>, 99% for Bt<sub>MP</sub>, and 1.0 for Bp). *Polysiphonia* sp. from Oshoro and Denshinhama, Japan was a sister of *P. pacifica*/*P. stricta* clade and supported (63% for Bt<sub>ML</sub>, 71% for Bt<sub>MP</sub>, and 0.97 Bp). The genus *Polysiphonia* produced a strong supported clade (99% for Bt<sub>ML</sub>, and maximum for the Bt<sub>MP</sub> and Bp).

## DISCUSSION

*Polysiphonia pacifica* is closely related with *P. morrowii* from northwest Pacific and *P. stricta* (as *P. urceolata*) common in Europe and along the Atlantic coast of North America. They are all characterized by the following features: (i) rhizoid does not cut off by cross walls from the pericentral cells; (ii) antheridial branch arises from the entire trichoblast primordium; (iii) trichoblasts are scarce or lacking; and (iv) tetrasporangia are arranged in straight series on determinate branches. Although they are superficially very similar to *P. pacifica*, but differs in several ways. The sterile tip of spermatangial branch is generally much shorter (1-3) in *P. pacifica*, while *P. stricta* and *P. morrowii* are having 2-5 and 5-8 sterile tip of spermatangial branch, respectively (Hollenberg 1942; Kim *et al.* 1994, 2000). *P. pacifica* also is in general somewhat more robust, variable plant and trichoblasts are rare than *P. stricta* (Hollenberg 1942). In addition *P. pacifica* is different from *P. morrowii* in lacking sharply pointed apex of ultimate branchlets and the axillary adventitious endogenous branchlets (Kudo and Masuda 1992).

This is the first document to report morphology and phylogeny of *Polysiphonia pacifica* since its original description by Hollenberg (1942). We collected two varieties of the species, *P. pacifica*, during our collection trip to Washington and Oregon, USA. One material used in this study is from the small harbor of East Sound, Orcas Islands, where is the type locality of *P. pacifica* var. *gracilis*. It had been collected by Gardner dredged in Deer Harbor, Orcas Island, in July 1910. The plant collected by us, however, was growing on the wood of harbor in littoral zone. We identified this plant as *P. pacifica*, not a *P. pacifica* var. *gracilis* because of the different tidal level for collecting place and the very similar features of general descriptions. The other is from Seal Rock, Oregon and this is *P. pacifica* var. *disticha* because of incurved determinate branchlets.

Hollenberg (1942) described five varieties of *P. pacifica*, namely *P. pacifica* var. *delicatula*, *P. pacifica* var. *disticha*, *P. pacifica* var. *determinata*, *P. pacifica* var. *gracilis*, and *P. pacifica* var. *distans*. These varieties were classified based on morphological characters, such as height of plant, delicate or robust, length of ultimate branchlets, branching pattern, quantity of branches, and color of plant. The distinctive features of each variety are following that: 1) *P. pacifica* var. *delicatula* is very small and delicate, mostly 2 cm high, 2) *P. pacifica* var. *disticha* has relatively short and

rigid determinate branchlets and its main axes usually covered with determinate branchlets, 3) *P. pacifica* var. *determinata* has long naked portions below of main axes and branching usually dense, 4) *P. pacifica* var. *gracilis* is distinguished by its slender laxly branched habit and main axes are distinct to the tips which are usually not exceeded by the upper branchlets, 5) *P. pacifica* var. *distans* has indeterminate ultimate branchlets, the coarse laxly branched filaments, very large cystocarps, and tetrasporangia in short series.

To confirm the specific entity, we borrowed some original materials of *P. pacifica* from the Smithsonian Institution, National Museum of Natural History, Washington DC (US). We investigated two holotypes of *Polysiphonia pacifica* var. *delicatula* and var. *distans* and two isotypes of *P. pacifica* var. *pacifica* and var. *determinata*. As a result of the observations of type specimens, *Polysiphonia pacifica* var. *delicatula* and var. *distans* look like different species from *P. pacifica*. And *P. pacifica* var. *determinata* could also be a mature plant of the different species, *P. morrowii*. But we have not gotten any type specimens of *P. pacifica* var. *disticha* and var. *gracilis*.

It is expected that protein-coding *rbcl* sequences were identical between two varieties's specimens of *P. pacifica* from different locations in USA. Although two varieties of *P. pacifica* was described by Hollenberg based on some different morphological features, they are exactly same in the *rbcl*. The distinctness of *P. pacifica* is strongly supported by phylogenetic analyses inferred from *rbcl* sequences. Our molecular data reveals two distinct clades; one is containing *P. pacifica* from USA, *Polysiphonia* sp. from Japan, northwest Pacific and *P. stricta* from England, the other grouping is *P. morrowii* from Korea, the Pacific Ocean and the introduced sample to Chile forming a very distinct group with little genetic variation. Atlantic *P. stricta* forms a distinct lineage that is a sister relationship to *P. pacifica*. It is sufficient sequence divergence between *P. pacifica* and similarly looking species, *P. stricta* and *P. morrowii*.

In summary, *Polysiphonia pacifica* is the typical species, having prostrate filaments attached by unicellular rhizoids not cut off by cross walls, four pericentral cells, ecorticate, trichoblasts and scar-cells rare, ultimate branchlets attenuate at the tip but not pungent, and tetrasporangia in long straight series in the ultimate branchlets. The protein-coding plastid *rbcl* gene sequence data suggest that *P. pacifica* is distinctly different from the similar species, *P. morrowii* and *P. stricta*. However, the *rbcl* sequences of *P. pacifica*, var. *pacifica*



and var. *disticha* are identical though they have morphological variation.

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