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Taxonomic revision of the genus *Chorda* (Chordaceae, Laminariales) on the basis of sporophyte anatomy and molecular phylogeny

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Chorda kikonaiensis Sasaki & Kawai *sp. nov.* is newly described from Hokkaido, Japan, on the basis of morphological and molecular data. Furthermore, based on the latter, the common *Chorda* species that has been identified as *Chorda filum* in Japan is shown to be independent from the European (true) *Chorda filum* at the species level. Therefore, *Chorda asiatica* Sasaki & Kawai *sp. nov.* is described for the cryptic Japanese taxon. *Chorda kikonaiensis* resembles *C. filum* and *C. asiatica,* but is distinguished by the shorter (0.4–1.3 m) and softer sporophyte, and the thinner cortex composed of fewer (2–4) cells. The independence of this species is further supported by molecular phylogenetic analyses using *rbc*L gene and internal transcribed spacer (ITS) rDNA sequences. *Chorda asiatica* is more variable in morphology (length of erect thallus and number of cell layers composing the cortex) than *C. kikoniensis* and *C. rigida,* and is difficult to distinguish from *C. filum* on the basis of morphology, but is clearly separated from the other species (*C. filum, C. kikoniensis* and *C. rigida*) on the basis of ITS rDNA data. *Chorda filum* is distributed in the Atlantic, whereas *C. asiatica, C. kikonaiensis* and *C. rigida* are distributed in the Pacific Ocean. Furthermore, the presence of one or two additional cryptic species is suggested in the northern Pacific using molecular data. Therefore, it is shown that the genus *Chorda* has considerably higher taxonomic and genetic diversity in the Pacific than in the Atlantic. Although no molecular data are available for other eastern Asian (south-eastern Russian coast, Korea and China) *Chorda* species, on the basis of morphology and geographical distributions, they are likely referable to *C. asiatica.*

KEY WORDS: *Chorda, C. asiatica, C. filum, C. kikoniensis,* rDNA-ITS, Molecular phylogeny, Taxonomy

INTRODUCTION

The genus *Chorda* Stackhouse (Laminariales, Chordaceae) has traditionally included the two species *Chorda filum* (Linnaeus) Stackhouse and *C. tomentosa* Lyngbye. However, Peters (1998) showed that *C. tomentosa* is systematically distant from *C. filum* on the basis of molecular phylogenetic data, and he suggested the use of *Halosiphon* Jaasund 1957 for this species. Later, Kawai & Sasaki (2000) placed the species in the new family Halosiphonaceae, and Kawai & Sasaki (2004) further indicated its close phylogenetic relationship with *Stschapovia flagellaris* A.D. Zinova, Phyllariaceae and Tilopteridaceae, and suggested placing them all in the order Tilopteridales. Kawai *et al.* (2000) newly described *C. rigida* Kawai & Arai from the Sea of Japan on the basis of its more rigid sporophyte morphology, significantly different phenology and adaptation to higher temperature conditions. Furthermore, on the basis of a molecular phylogenetic study using internal transcribed spacer (ITS)-5.8S rDNA and *rbc*L-spacer DNA sequence data, Kawai *et al.* (2000) demonstrated the distinctness of *C. rigida,* as well as the considerable genetic diversity within the Atlantic and Pacific populations of *C. filum.* These findings implied the existence of some cryptic taxa of *Chorda* in the Pacific Ocean, but because of the lack of morphological and phenological data, the authors deferred taxonomic revisions.

In this follow-up of the previous paper (Kawai *et al.* 2000), the taxonomy of Japanese *Chorda* spp. are further examined employing phenological and morphological studies of Japanese populations, as well as molecular phylogenetic analyses using ITS-5.8S rDNA sequences.

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MATERIAL AND METHODS

Phenological and morphological observations

In order to clarify the phenology of the Kikonai populations, monthly sampling was performed at Saraki, Kikonai, Hokkaido, Japan (Fig. 1) on 2 March, 11 April, 26 May, 26 June, 25 July and 29 August 2001.

Specimens preserved in 5% formaldehyde-seawater (Table 1) were used for morphological observations and for the study of anatomical features by light microscopy. For comparisons of the number of cells constituting cortical layers, cross sections were made by hand using a razor blade and 10 sections were measured at the intercalary meristem (except for old plants that had lost their meristem), midway between the meristem and base, and near the holdfast. In addition, the length and width of paraphyses and unilocular sporangia ($n = 10$), and the diameter of the erect thallus ($n = 5$) were measured in cross sections on each plant. Voucher specimens of *C. filum* as listed in Table 2 were also examined for morphological comparisons of the number of cell layers composing the cortex. Microsoft Excel (Microsoft, Redmond, WA, USA) was used for statistical analysis (Student's *t* test) of the data.

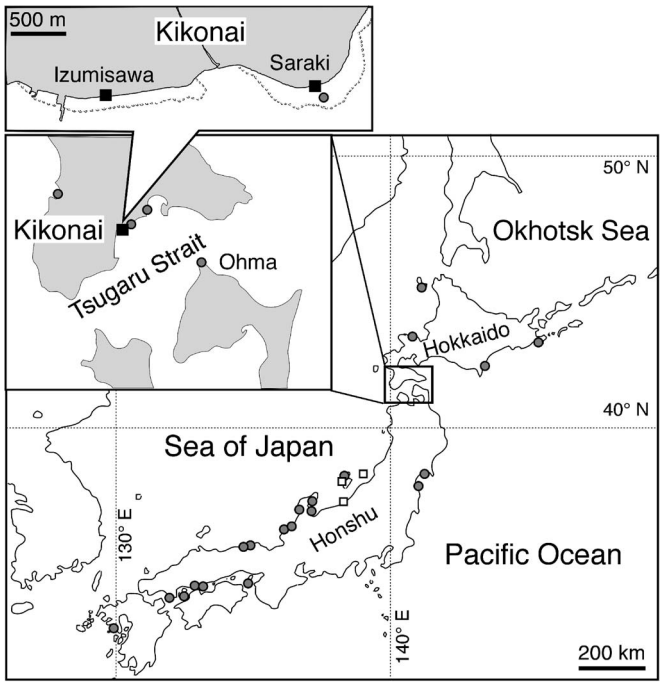


Fig. 1. Geographical distribution of *Chorda asiatica* and *C. kikonaiensis* within Japan. *Chorda asiatica* (●) has a wide distributional range in Japan. *Chorda kikonaiensis* (■) has been collected only at Saraki and Izumisawa, Kikonai, Hokkaido, Japan. (□) shows reported localities of *C. rigida.*

Culture experiments

Unialgal cultures of *C. kikonaiensis* were started from zoospores (unispores) released from unilocular sporangia on erect thalli collected on 11 April 2001 at Saraki, Kikonai, Hokkaido, Japan. The zoospores were pipetted and cultured in polystyrene Petri dishes containing 50 ml PESI medium (Tatewaki 1966). Illumination was with daylight-type white fluorescent lighting of approximately 50 μmol m^{-2} s^{-1} using the temperature gradient culture chambers (TG-100AD, TG-200AD, Nippon Medical & Chemical Instruments, Osaka, Japan). The culture conditions used were as follows: 5°C SD (short day: 8 : 16 h light : dark); 5°C LD (long day: 16 : 8 h light : dark); 10°C SD; 10°C LD; 15°C SD; 15°C LD; 20°C SD; 20°C LD; 25°C SD and 25°C LD.

Molecular phylogenetic analysis

Field-collected specimens (rapidly desiccated in silica gel), as well as the specimens grown in culture were used for DNA extractions (Table 3). The silica gel–dried specimens used for the molecular study are deposited at the Kobe University Research Center for Inland Seas. Approximately 40 mg of algal tissue powder ground in liquid nitrogen was used for genomic DNA extractions, which were performed using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification of the nuclear rDNA including ITS1, 5.8S and ITS2 regions was carried out using GeneAmp PCR Systems 2400 and 9700 (Applied Biosystems, Foster City, CA, USA) and a TaKaRa Ex Taq (Takara Shuzo, Shiga, Japan) reaction kit (total reaction volume of 25 μl was composed of 2.5 μl of 10× Ex Taq Buffer, 5.0 μM dNTP mixture, 0.1 μM of each primer, 0.625 units TaKaRa Ex Taq and 2.0 μl of DNA solution including 0.5–1.0 μg DNA). Primers were designed on the basis of known sequences of the corresponding regions reported for related taxa (Saunders & Druehl 1992; Tan & Druehl 1993, 1996; Kawai *et al.* 1995; Stache-Crain *et al.* 1997). Sequence of primers used in this analysis were forward primers, 18F-1 (5′-AAGGTGAAGTCGTAACAAGG-3′) and 5.8F-1 (5′-ACGCAGCGAAATGCGATACG-3′); reverse primers, 5.8R-1 (5′-CGTATCGCATTTCGCTGCGT-3′) and 26R-1 (5′-GTTAGTTTCTTTTCCTCCGC-3′). The profile of PCR conditions was as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 54°C for 30 s, extension at 72°C for 30 s; and a final extension at 72°C for 7 min. PCR products were directly sequenced using the BigDye Terminator Cycle Sequencing Reaction Kit (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

The Clustal W program (Thompson *et al.* 1994) was used for preliminary DNA sequence alignment, followed by manual adjustment. The aligned sequences were subjected to maximum parsimony (MP) analyses in a general heuristic search using PAUP v.4.0b3a (Swofford 1999). Twenty random taxon additions were performed in each heuristic search with the Goloboff fit criterion (k = 2), using the TBR branch swapping option. Gaps were not taken into account in the MP analysis. Because the number of near-identical sequences in ITS alignment precluded complete analysis in a reasonable amount of time, the maximum tree option was set at 5000. From the same alignment, Kimura two-parameter (Kimura 1980), Kimura three-parameter, Jukes-Cantor, F81, F84, Tajima-Nei, HKY85, and Tamura-Nei distances between taxa were estimated, and a phylogenetic tree was constructed with the neighbour-joining (NJ) method, using PAUP*. The program Modeltest v.3.06 (Posada & Crandall 1998) was used to find the model of sequence evolution that best fit the data set by a hierarchical likelihood ratio test (hLRT) (a = 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 10 random additions in a heuristic search.

The robustness of the resulting phylogenies was tested by bootstrap analysis (Felsenstein 1985). Character deletions per jackknife replicate were set at 37%.

In the analysis using ITS sequences, *Pseudochorda nagaii* (Tokida) Inagaki and *P. gracilis* Kawai & Nabata (Pseudochordaceae, Laminariales), *Laminaria diabolica* Miyabe and *L. yendoana* Miyabe (Laminariaceae, Laminariales) were used as outgroups, on the basis of results from *rbc*L (Kawai *et al.* 2000; Sasaki *et al.* 2001) and 18S sequence data (Boo *et al.* 1999).

RESULTS

Habit and phenology of *C. kikonaiensis*

Sporophytes of *Chorda kikonaiensis sp. nov.* collected at Izumisawa and Saraki in Kikonai (41.42°N, 140.27°E), Hok-

Table 1. Origin of liquid-preserved specimens of Japanese *Chorda* spp. used in anatomical analyses.

Locality	Collection date (collector/no. of specimens)
Chorda asiatica	
Rishiri Island, Hokkaido	19 Jul. 1991 (H. Kawai/11)
Oshoro, Hokkaido	18 Jun. 1982 (T. Kubo/6), 5 May 1987 (T. Yoshida/9), 25 Apr. 1988 (H. Kawai/7), 11 Jul. 1988 (Y. Yattori/14), 31 May 2000 (A. Murakami/10)
Esashi, Hokkaido	24 Jul. 2001 (H. Sasaki/1)
Toubetsu, Kamiiso, Hokkaido	25 May 2001 (H. Sasaki/11)
Saraki, Kikonai, Hokkaido	26 Jun. 2001 (H. Sasaki/4), 25 Jul. 2001 (H. Sasaki/12)
Nemura, Hokkaido	14 Jul. 2000 (H. Kawai/10)
Ohma, Aomori	1988 (T. Kitayama/4)
Shichigahama, Miyagi	20 May 1984 (H. Kawai/3)
Aikawa, Sado Island, Niigata	14 Jun. 1993 (S. Arai/13)
Nanao, Ishikawa	May 2001 (S. Arai/3)
Hakui, Ishikawa	May 2001 (S. Arai/4)
Fukuura, Ishikawa	31 May 1993 (S. Arai/17)
Koshino, Fukui	22 Jun. 2001 (J.H.Oak/3)
Ohi, Fukui	23 Jul. 1993 (Y. Maeda/6)
Takahama, Fukui	29 Apr. 1993 (Y. Maeda/7), 7 Jun. 1993 (Y. Maeda/4), 5 Jul. 1993 (Y. Maeda/11), 28 Jun. 2000 (H. Kawai/3)
Aidani, Hyogo	15 Jul. 1993 (H. Kawai/5)
Imagoura, Hyogo	9 Apr. 2000 (H. Sasaki/20), 14 May 2000 (H. Sasaki/19), 13 Mar. 2001 (H. Sasaki/15), 15 Apr. 2001 (H. Sasaki/15), 12 May 2001 (H. Sasaki/12), 29 Jun. 2001 (H. Sasaki/7)
Ama, Awaji Island, Hyoto	12 Jul. 2000 (A. Murakami/5)
Takashima, Ehime	14 Jun. 1987 (H. Kawai/11)
Futashima, Nagasaki	14 May 1991 (S. Arai/9)
Chorda kikonaiensis	
Saraki, Kikonai, Hokkaido	19 Mar. 1987 (K. Kogame/17), 21 Apr. 1987 (H. Kawai/18), 24 Jul. 1988 (Y. Yattori/12), 5 Jul. 1998 (H. Akioka/13), 4 Jul. 2000 (H. Kawai/14), 2 Mar. 2001 (H. Sasaki/1), 11 Apr. 2001 (H. Sasaki/15), 26 May 2001 (H. Sasaki/12), 26 Jun. 2001 (H. Sasaki/15), 25 Jul. 2001 (H. Sasaki/16)
Izumisawa, Kikonai, Hokkaido	26 May 2001 (H. Sasaki/10), 25 Jul. 2001 (H. Sasaki/10)
Chorda rigida	
Awashima Island, Niigata	4 Aug. 1991 (S. Arai/8)
Futami, Sado Island, Niigata	16 Oct. 1991 (S. Arai/12)
Kashiwazaki, Niigata	22 Sep. 1990 (S. Arai/13), 26 Oct. 1990 (S. Arai/10)
Nanao, Ishikawa	7 Aug. 1992 (S. Arai/5)

kaido, Japan (Fig. 1), grew on upper intertidal rocks of a relatively sheltered, somewhat muddy, wide flat rock bed. At this locality the population of *C. asiatica sp. nov.* (= Japanese *C. filum,* see below) typically grew in upper subtidal on rocks. Sporophytes of *C. kikonaiensis* grew in sparse tufts or solitary from a small discoid holdfast 1.0–2.5 mm in diameter (Fig. 2). The erect thalli were simple, unbranched, cord-shaped, and medium to light brown (Fig. 2). Young sporophytes, retaining the intact distal portion of the intercalary meristem (Figs 3, 4), were collected in early March. They retained the intercalary meristem throughout the spring. The sporophytes reached their maximum size (normally up to 0.8 m, but sometimes attaining 1.3 m in length, and 2.5 mm in diameter) during April and May, became fertile in April, and disappeared in July–August. This species resembles *C. asiatica,* but is distinguished by shorter and thinner sporophytes with a superficial resemblance to *Scytosiphon lomentaria* (Lyngbye) Link, which is also common in this habitat.

Morphology and life history in culture of *C. kikonaiensis*

The sporophytes of *C. kikonaiensis* had the same basic anatomical features as other *Chorda* species (*C. asiatica, C. filum* and *C. rigida*). Young sporophytes had a solid intercalary meristem composed of small meristematic cells (Figs 3, 4). Phaeophycean hairs were abundant in the meristematic region (Fig. 4), and scattered in other thallus parts. In the longitudinal section the sporophytes were composed of medullary trumpet-shaped hyphae, a cortical cell layer, and epidermal cells (except at the meristem). The cortical layer was composed of two to five cells (Figs 5, 6), and measured 100–150 μm thick in the middle part of the thallus (Fig. 5), and 130–180 μm in the basal part (Fig. 6). The thickness and the number of cells composing the cortical layer were relatively stable throughout the seasons. Unicellular paraphyses without mucilaginous appendages developed from epidermal cells in early spring (Fig. 7). When mature, cylindrical to oblong unilocular sporangia were formed among the paraphyses (Fig. 8). The holdfast was composed of densely packed rhizoidal filaments issuing from the lower part of the erect thalli (Figs 9, 10).

In culture, *C. kikonaiensis* showed the oogamous heteromorphic life history pattern common to *C. filum* and *C. rigida.* Zoospores released from the unilocular sporangia (Fig. 11) germinated by forming a germ tube (Fig. 12) and developed into sexually dimorphic dioecious gametophytes. The gametophytes matured at 5°C both under SD and LD conditions. Male gametophytic filaments were 8–10 μm in diameter (Fig. 13), forming terminal antheridia (Fig. 14). Female gametophytic filaments were 14–17 μm in diameter (Fig. 13), and formed oogonia (Fig. 15). Zygotes developed into sporophytes (Fig. 16). Rhizoidal filaments issued from the basal portion of sporophytes, and terminal and lateral hairs occurred in young sporophyes (Fig. 17). The cells of the sporophytes contained many disk-shaped chloroplasts without pyrenoids (Fig. 17). The cortical layer of cultured sporophytes was composed of two to four cell layers (Fig. 18). Sporophytes of *C. kikonaiensis* grew well at 2, 5, 10 and 15°C but did not grow at 20 and 25°C. In contrast, the sporophytes of *C. asiatica* grew at 5, 10 and 15°C, but did not grow at 2°C. *Chorda rigida* grew in the range of 2 to 20°C.

Anatomical comparisons of sporophytes

Comparisons using the liquid-preserved specimens collected from Japan (Table 1) showed that *C. kikonaiensis* has a cortex composed of fewer cell layers than *C. asiatica* (Figs 19–21) and *C. rigida,* and consequently, the sporophyte had a softer texture (Fig. 22).

Table 2. List of additional specimens of *Chorda filum* and closely related taxa used for anatomical studies, and the number of cells composing the cortical layer.

Locality	Collection date	Collector	No. of specimens	Cell no. of cortical layer
Portsmouth, UK	26 Aug. 1999	H. Kawai	7	6.0 ± 0.5
Elby Point, Isle of Man, UK	25 Aug. 1999	H. Kawai	2	6.2 ± 0.4
Port Erin, Isle of Man, UK	24 Aug. 1999	H. Kawai	5	6.5 ± 0.6
Roscoff, France	21 Dec. 1972	T. Yoshida	1	9.7 ± 0.7
	30 Jun. 1973	T. Yoshida	2	8.7 ± 0.7
Bohuslan, Rattholmen, Fiskebakskil, Sweden	16 Aug. 1894	H.G. Simmons	1	6.8 ± 0.8
Sverige, Bohusland, Bonden, Sweden	18 Jul. 1946	T. Levring	1	8.3 ± 0.5
Frederikshaven, Denmark	Jul. 1929	Unknown	1	5.8 ± 0.4
Orafsfjord, Iceland	19 Aug. 1999	H. Kawai	6	5.9 ± 0.7
Reykjavik, Iceland	22 Aug. 1999	H. Kawai	3	6.3 ± 0.5
St Lawrence Island, Bering Sea, USA	6 Aug. 1996	H. Kawai	1	3.5 ± 0.5
Bishop's Beach, AK, USA	12 Jul. 1987	S. Lindstrom	1	5.1 ± 0.6
Puget Sound, WA, USA	30 Jul. 1993	T. Mumford	1	4.3 ± 0.5
Yuzhnaya Glubokaya Bay, Bering Sea, Russia (1)	30 Aug. 1988	O. Selivanova	1	3.8 ± 0.4
Petropavlovsk, Kamchatka, Russia (2)	24 Aug. 1988	O. Selivanova	1	3.8 ± 0.4
Dairen, China	27 Jul. 1937	Y. Yamada	1	6.9 ± 0.3

On the basis of comparisons using additional voucher specimens (Table 2), the average number of cells composing the cortical layer was 6.5 ± 1.2 (ranging from 5.8 to 9.7) in Atlantic *C. filum* and 4.4 ± 1.3 (ranging from 3.5 to 6.9) in Pacific *C. filum* excluding *C. asiatica.* Comparisons of major anatomical features of the sporophytes (average number of cell layers and the thickness at the meristem, middle and basal portions) among *C. kikonaiensis, C. asiatica,* Atlantic *C. filum* and *C. rigida* are shown in Table 4. The significance of the differences in these measurements among the four taxa was confirmed by a *t* test ($P < 0.001$).

Molecular phylogenetic analysis

As shown in Table 3, the length of the ITS1 and ITS2 region sequences varied considerably depending on the taxa (ITS1/ITS2): 285–290 base pairs (bp)/265–266 bp (*C. kikonaiensis*); 268–271 bp/266–275 bp (Atlantic *C. filum*); 274–280 bp/259–267 bp (*C. asiatica*); 259–289 bp/255–269 bp (Pacific *C. filum*); 358–360 bp/301–303 bp (*C. rigida*). The sequence divergence within the genus *Chorda* was 12.5%. Divergences within taxa were 1.5% (*C. kikonaiensis*), 3.0% (Atlantic *C. filum*), 1.9% (*C. asiatica*), 7.8% (Pacific *C. filum* excluding *C. asiatica*), and 3.0% (*C. rigida*), respectively. Divergence between *C. kikonaiensis* and the Atlantic *C. filum* was 9.3%, between *C. kikonaiensis* and *C. asiatica* 6.3%, between *C. kikonaiensis* and the Pacific *C. filum* 5.4%, between the Atlantic *C. filum* and the Pacific *C. filum* 8.5%, and between the Atlantic *C. filum* and *C. asiatica* 7.6%, respectively. Among the sequence data indicated in Table 3, considerably similar sequences are classified in Table 5.

The aligned ITS + 5.8S rDNA sequence data included 1054 sites in total. There were 265 (MP and NJ data set) or 243 (ML data set) parsimony-informative nucleotide positions. In the molecular trees, *C. kikonaiensis* first formed a clade with the specimens from northwestern America (CF-Pug from Puget Sound, WA, USA) and Kamchatka (CF-Pet1 from Petropavlovsk, Russia) (Figs 23, 24; clade B-1, B-2). In contrast, the clade of North Atlantic *C. filum* specimens, including those from France (CF-Ros, CF-San), Norway (CF-Ber, CF-Lon, CF-NyA), Iceland (CF-Ora, CF-Rey), Denmark (CF-Aas1, CF-Aas2, CF-Dis1, CF-Dis2), Britain (CF-IME, CF-IMP, CF-Por) and north-eastern Canada (CF-NFL) formed a clade with other northern Pacific specimens from Kamchatka (CF-Pet2) and the Bering Sea (CF-StL), although the bootstrap supports were not strong (Figs 23, 24; clade A-1, A-2). The clade of *C. rigida* branched first in NJ (Fig. 23) and ML (Fig. 24) trees followed by the clade of *C. asiatica* (clade C), which was sister of the clade A-1, A-2, B-1 and B-2. In contrast, in the MP tree (not shown) *C. rigida* first clustered with *C. asiatica* and the they were a sister of the B-1/B-2 clade.

DISCUSSION

Although molecular phylogenetic analyses of the type specimen of *C. filum* have not been performed, it is probable that the Atlantic population (clade A-1 in Figs 23, 24) corresponds to true *C. filum,* because all of the specimens from a wide range of European and north-eastern Canada localities showed close phylogenetic relationships in the ITS rDNA sequence data. The monophyly of the Atlantic population was clearly supported by all of the analyses and by high bootstrap values (Figs 23, 24). This result was also supported by our previous study using *rbc*L gene sequences, although the number of specimens examined were limited (Kawai *et al.* 2000). The sporophyte morphology of the specimens examined basically agreed with the original description of the species (Stackhouse 1797), and descriptions of the species in later publications (Reinke 1892; South & Burrows 1967). Therefore, there is little doubt that the Atlantic specimens examined in the present study represent true *Chorda filum.* Based on this conclusion, we consider that the clades comprising the Pacific populations include two to four undescribed species (corresponding to clades A-2, B-1, B-2, and C in Figs 23, 24) in addition to *C. rigida* (clade D in Figs 23, 24).

We suspend taxonomic conclusions for clades A-2 and B-1, because the number of samples studied for morphology and molecular analyses are too limited. These clades may repre-

Table 3. Origin of sample and sequence data used for molecular phylogenetic analysis, including their database accession numbers.

Species (taxonomic position)	Collection site (source)	Origin	Specimen code	ITS1 (bp)	ITS2 (bp)	DDBJ[1] accession no. for ITS-5.8S rDNA
Chordaceae						
Chorda kikonaiensis Sasaki & Kawai *sp. nov.*	Saraki, Kikonai, Hokkaido, Japan (1)	[Kawai *et al.* 2000]	CK-Kik1	290	266	AB035760
C. kikonaiensis	Saraki, Kikonai, Hokkaido, Japan (2)	[Kawai *et al.* 2000]	CK-Kik2	288	265	AB035761
C. kikonaiensis	Saraki, Kikonai, Hokkaido, Japan (3)	[Kawai *et al.* 2000]	CK-Kik3	289	266	AB035762
C. kikonaiensis	Saraki, Kikonai, Hokkaido, Japan (4)	Field specimen (silica gel)	CK-Kik4	285	266	AB197758
C. kikonaiensis	Saraki, Kikonai, Hokkaido, Japan (5)	Field specimen (silica gel)	CK-Kik5	285	266	AB197759
C. filum (Linnaeus) Stackhouse	Roscoff, Brittany, France	[Kawai *et al.* 2000]	CF-Ros	268	275	AB035748
C. filum	Santec, Brittany, France	[Peters 1998]	CF-San	270	266	Z98585, Z98586
C. filum	Portsmouth, UK	[Kawai *et al.* 2000]	CF-Por	271	272	AB035750
C. filum	Elby point, Isle of Man, UK	[Kawai *et al.* 2000]	CF-IME	270	275	AB035751
C. filum	Port Erin, Isle of Man, UK	[Kawai *et al.* 2000]	CF-IMP	270	274	AB035752
C. filum	Bergen, Norway	[Kawai *et al.* 2000]	CF-Ber	270	273	AB035749
C. filum	Reykjavik, Iceland	[Kawai *et al.* 2000]	CF-Rey	270	274	AB035753
C. filum	Orafsfjord, Iceland	[Kawai *et al.* 2000]	CF-Ora	270	275	AB035754
C. filum	Ny-Alesund, Spitsbergen, Norway	Field specimen (silica gel)	CF-NyA	270	274	AB197760
C. filum	Longyearbyen, Spitsbergen, Norway	Field specimen (silica gel)	CF-Lon	270	274	AB197761
C. filum	Asiaat, Greenland, Denmark (1)	Field specimen (silica gel)	CF-Aas1	271	274	AB197762
C. filum	Asiaat, Greenland, Denmark (2)	Field specimen (silica gel)	CF-Aas2	271	274	AB197763
C. filum	Disko Island, Greenland, Denmark (1)	Field specimen (silica gel)	CF-Dis1	271	274	AB197764
C. filum	Disko Island, Greenland, Denmark (2)	Field specimen (silica gel)	CF-Dis2	270	274	AB197765
C. filum	Newfoundland, Canada	[Kawai *et al.* 2000]	CF-NFL	271	261	AB035755
C. filum ?	St Lawrence Island, Bering Sea, USA	[Kawai *et al.* 2000]	CF-StL	259	259	AB035756
C. filum ?	Puget Sound, WA, USA	[Kawai *et al.* 2000]	CF-Pug	289	269	AB035757
C. filum ?	Petropavlovsk, Kamchatka, Russia (1)	[Kawai *et al.* 2000]	CF-Pet1	285	264	AB035758
C. filum ?	Petropavlovsk, Kamchatka, Russia (2)	[Kawai *et al.* 2000]	CF-Pet2	262	255	AB035759
C. asiatica Sasaki & Kawai *sp. nov.*	Oshoro, Hokkaido, Japan (1)	[Kawai *et al.* 2000]	CA-Osh1	277	259	AB035763
C. asiatica	Oshoro, Hokkaido, Japan (2)	[Kawai *et al.* 2000]	CA-Osh2	280	262	AB035764
C. asiatica	Oshoro, Hokkaido, Japan (3)	[Kawai *et al.* 2000]	CA-Osh3	276	261	AB035765
C. asiatica	Esashi, Hokkaido, Japan	Field specimen (silica gel)	CA-Esa	276	260	AB197766
C. asiatica	Saraki, Kikonai, Hokkaido, Japan (1)	Field specimen (silica gel)	CA-Kik1	276	260	AB197767
C. asiatica	Saraki, Kikonai, Hokkaido, Japan (2)	Field specimen (silica gel)	CA-Kik2	276	260	AB197768
C. asiatica	Sakkari, Kikonai, Hokkaido, Japan	Field specimen (silica gel)	CA-Kik3	276	260	AB197769
C. asiatica	Moheji, Kamiiso, Hokkaido, Japan (1)	[Yotsukura et al. 1999]	CA-Kam1	279	259	AB022815, AB022816
C. asiatica	Moheji, Kamiiso, Hokkaido, Japan (2)	[Kawai *et al.* 2000]	CA-Kam2	276	260	AB035766
C. asiatica	Akkeshi, Hokkaido, Japan	Field specimen (voucher)	CA-Akk	277	260	AB197770
C. asiatica	Ohma, Aomori, Japan	Field specimen (type specimen: SAP101396)	CA-Ohm	276	—	AB263977
C. asiatica	Ohzuchi, Miyagi, Japan	[Kawai *et al.* 2000]	CA-Ohz	280	260	AB035767
C. asiatica	Aikawa, Niigata, Japan	[Kawai *et al.* 2000]	CA-Aik	277	260	AB035768
C. asiatica	Fukuura, Ishikawa, Japan	[Kawai *et al.* 2000]	CA-Fuk	277	260	AB035769
C. asiatica	Hakui, Ishikawa, Japan	[Kawai *et al.* 2000]	CA-Hak	279	260	AB035770
C. asiatica	Takahama, Fukui, Japan	[Kawai *et al.* 2000]	CA-Tak	276	261	AB035771
C. asiatica	Imagoura, Kasumi, Hyogo, Japan	[Kawai *et al.* 2000]	CA-Ima	278	261	AB035772
C. asiatica	Ama, Awaji Island, Hyogo, Japan	Field specimen (silica gel)	CA-Ama	274	264	AB197771
C. asiatica	Sasajima, Hiroshima, Japan	[Kawai *et al.* 2000]	CA-Sas	276	267	AB035773
C. asiatica	Mukaishima, Hiroshima, Japan	[Kawai *et al.* 2000]	CA-Muk	275	264	AB035774

Table 3. Continued

Species (taxonomic position)	Collection site (source)	Origin	Specimen code	ITS1 (bp)	ITS2 (bp)	DDBJ[1] accession no. for ITS-5.8S rDNA
C. asiatica	Heigun Island, Yamaguchi, Japan	Culture (H. Kawai)	CA-Mis	277	262	AB197772
C. asiatica	Futashima, Nagasaki, Japan	[Kawai *et al.* 2000]	CA-Fug	278	266	AB035775
Chorda rigida Kawai & Arai	Futami, Sado Island, Niigata, Japan (1)	[Kawai *et al.* 2000]	CR-Sad1	358	303	AB035776
C. rigida	Futami, Sado Island, Niigata, Japan (2)	Field specimen (frozen)	CR-Sad2	360	301	AB197773
C. rigida	Kashiwazaki, Niigata, Japan	[Kawai *et al.* 2000]	CR-Kas	360	302	AB035777
C. rigida	Nanao, Ishikawa, Japan	[Kawai *et al.* 2000]	CR-Nan	359	302	AB035778
Pseudochordaceae						
Pseudochorda gracilis Kawai & Nabata	Isoya, Hokkaido, Japan	[Kawai *et al.* 2000]		331	280	AB035780
P. nagii (Tokida) Inagaki	Hanasaki, Hokkaido, Japan	[Kawai *et al.* 2000]		313	293	AB035779
Laminariaceae						
Laminaria diabolica Miyabe		[Yotsukura *et al.* 1999]		236	256	AB022795, AB022796
L. yendoana Miyabe		[Yotsukura *et al.* 1999]		241	262	AB022807, AB022808

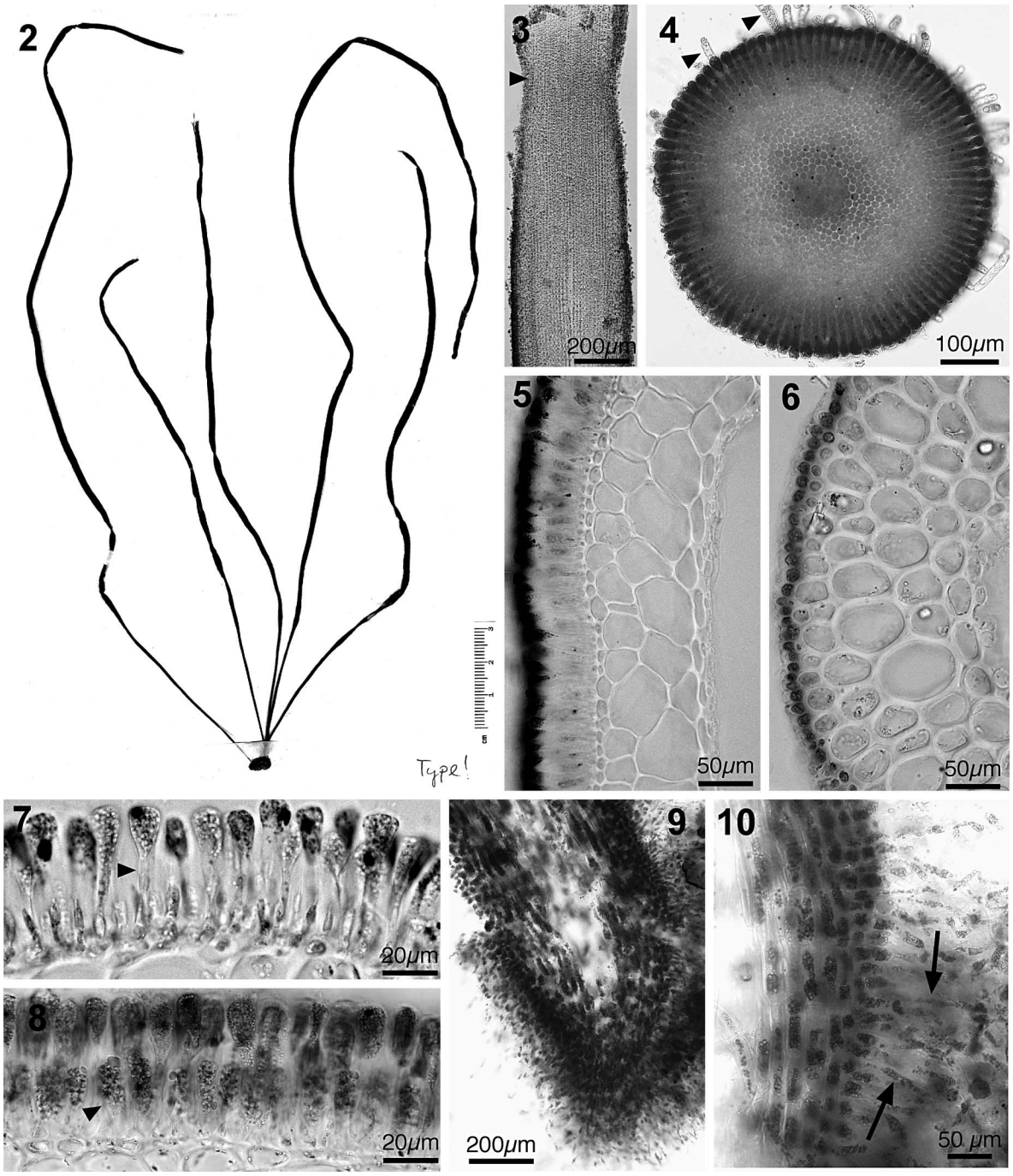
[1] DDBJ, DNA Data Bank of Japan.

sent geographical variants or distinct taxa, but they need more sampling for this to be resolved. The independence of clade C (common *Chorda* species in Japan, which has been identified as *C. filum* by previous researchers; e.g. Okamura 1901; Nagai 1940; Tokida 1954; Kogame & Kawai 1996; Yoshida *et al.* 2005) at the species level is supported by high bootstrap values, and therefore establishes *C. asiatica sp. nov.* Regarding clade B-2, in spite of its apparent paraphyletic status due to the presence of B-1, we propose the establishment of a new species *Chorda kikonaiensis sp. nov.* at least to accommodate the specimens composing the clade B-2 from Hokkaido, Japan. Because the number of specimens examined for the clade B-1 is too small considering the very wide distributional ranges representing the clade (Kamchatka and north-eastern Pacific America), we reserve the taxonomic conclusion on the specimens until future studies based on more specimens from the area, and more detailed examinations using additional gene sequences of reasonable resolutions solve the problem.

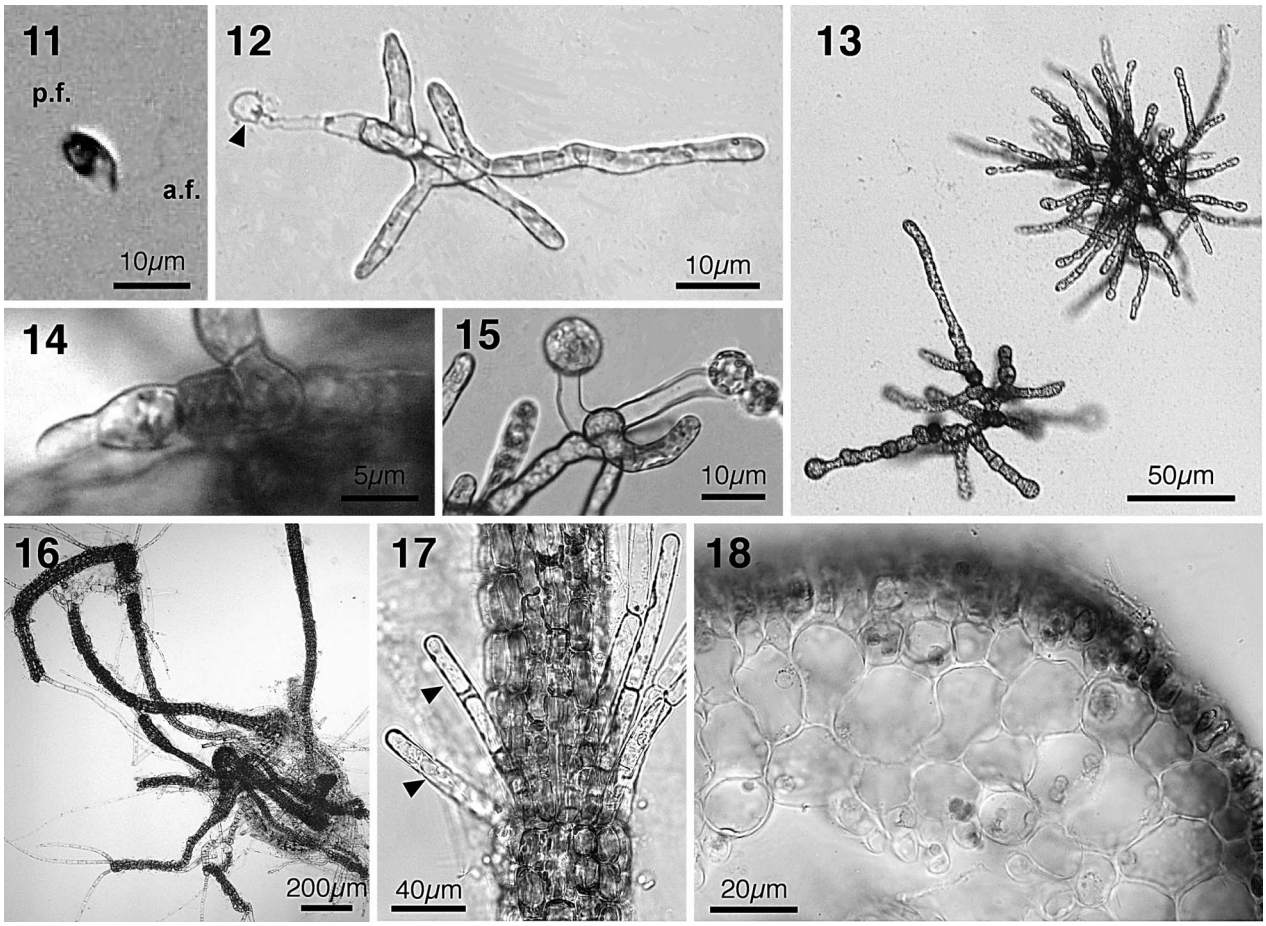
Chorda kikonaiensis resembles *C. filum, C. asiatica* and *C. rigida* with regard to basic morphological features of the sporophyte and the life history including the morphology of gametophytes (Reinke 1892; Kylin 1918; South & Burrows 1967; Kogame & Kawai 1996; Lee & Oh 1998; Kawai *et al.* 2000). However, the morphology of cortical cell layers was considerably different for *C. asiatica* and *C. rigida.* Comparisons of the three taxa distributed in Japan indicate that *C. asiatica* has a cortex of four to eight cell layers, whereas *C. kikonaiensis* has two to five cell layers in the middle and basal part of the sporophytes. *Chorda rigida* has a denser cortex consisting of 8–13 cells. Their morphological distinctiveness in the sporophyte anatomy is clearly indicated in Fig. 22. The number of cells constituting the cortical layer of *C. kikonaiensis* is considerably lower, and therefore, the thallus is thinner and softer. The number of cells composing the cortical layer is predetermined in the meristem, because the horizontal cells of the cortical layers below and above the meristem cease dividing after their differentiation (Kogame & Kawai 1996). Therefore, this character is considered to be rather stable irrespective of environmental conditions, and can be used as a reliable taxonomic character (Kawai *et al.* 2000). Moreover, the number of cells composing the cortical layer was shown to be similar in culture to the field-collected plants (Kawai *et al.* 2000).

Furthermore, some ecological differences between *C. kikonaiensis* and *C. asiatica* were recognized. In *C. filum* and *C. asiatica,* the distal portion of sporophytes above the meristem becomes gradually lost as elongation progresses, and no meristem is found in sporophytes that have reached their maximum length (South & Burrows 1967; Kogame & Kawai 1996). In contrast, in *C. kikonaiensis* the meristem is often retained even in fertile sporophytes. The growth and maturation season of *C. kikonaiensis* in the field is earlier than that of *C. asiatica,* consistent with the adaptation to low water temperatures by *C. kikonaiensis* demonstrated under our culture conditions.

In the molecular phylogenetic analyses, the independence of *C. kikonaiensis* was supported by ITS sequence analysis, although the taxonomic status of the related specimens from Kamchatka and north-eastern Pacific America to the species remains unsolved. *Chorda kikonaiensis* was genetically distant from any other Japanese *Chorda* species from various local-



Figs 2–10. Field-collected sporophytes of *Chorda kikonaiensis sp. nov.*
Fig. 2. Type specimen of *C. kikonaiensis* (fertile sporophyte collected on 11 April 2001 at Kikonai, Hakodate, Hokkaido by H. Sasaki; SAP100626).
Fig. 3. Longitudinal section of meristematic region (arrowhead).
Fig. 4. Cross section near meristem. Arrowheads indicate phaeophycean hairs.
Fig. 5. Middle part of sporophyte with cortical layer in cross section.
Fig. 6. Basal part of sporophyte in cross section.
Fig. 7. Unicellular paraphyses (arrowhead) without mucilage caps and initial stage of unilocular sporangia.
Fig. 8. Mature unilocular sporangia (arrowhead) among paraphyses.
Fig. 9. Longitudinal section of lowermost portion of sporophyte and rhizoidal filaments composing basal holdfast.
Fig. 10. Longitudinal section of lowermost portion of sporophyte showing rhizoids (arrows) descending from the meristodermal cells.

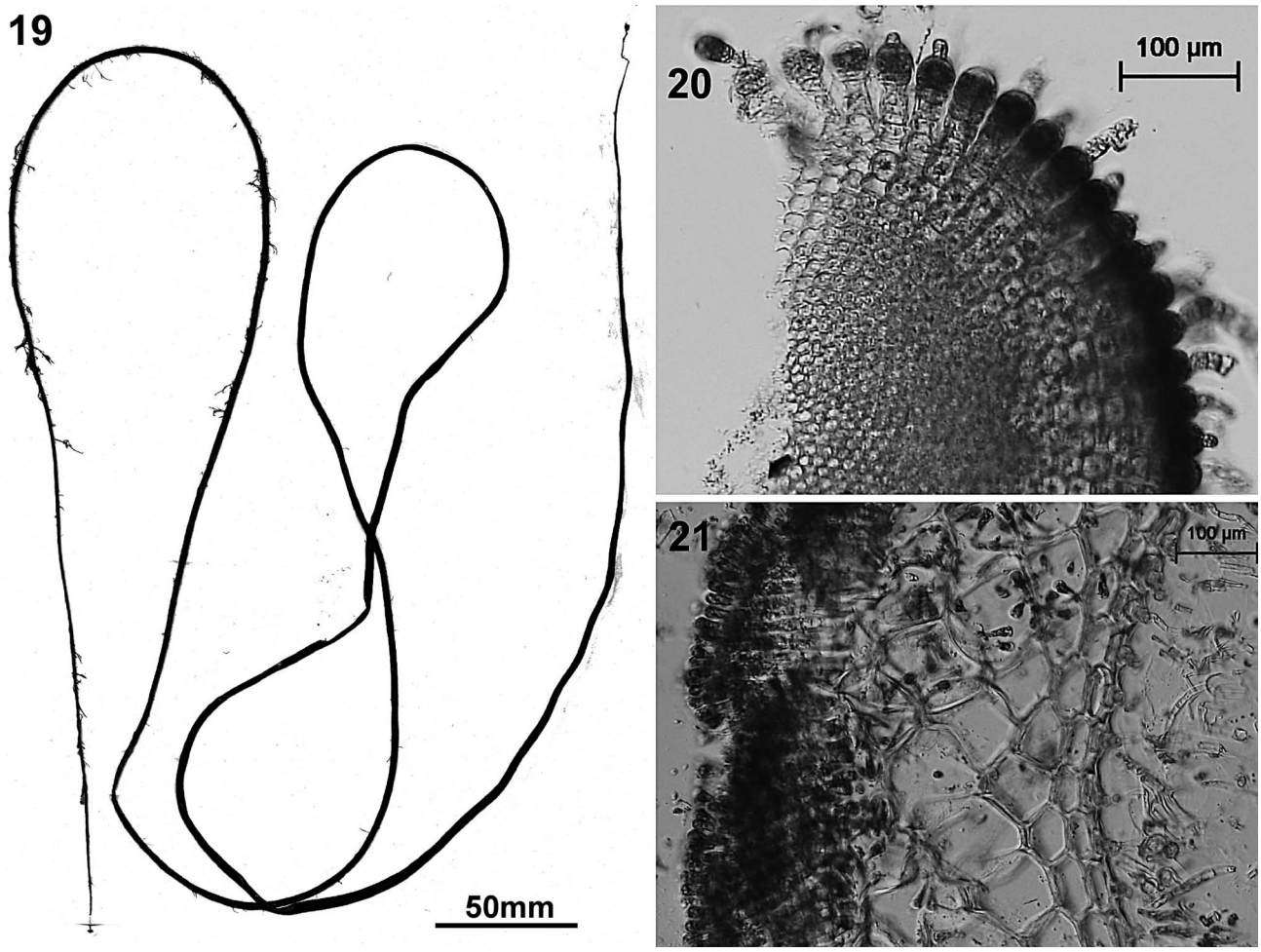


Figs 11–18. *Chorda kikonaiensis sp. nov.* in culture.
Fig. 11. Released zoospore. a.f., anterior flagellum. p.f., posterior flagellum.
Fig. 12. Germling of a gametophyte with evacuated original spore (arrowhead).
Fig. 13. Male (top, right) and female gametophytes (bottom, left).
Fig. 14. Antheridia on a male gametophyte.
Fig. 15. Female gametopyte with egg attached on the release pore of the oogonium.
Figs 16–17. Young sporophytes with phaeophycean hairs (arrowhead in Fig. 17).
Fig. 18. Cross section of the middle part of a young sporophyte in culture displaying the cortical layer.

ities. In the molecular tree based on the ITS region, the CF-Pet1 (Kamchatka) + CF-Pug (north-west America) clustered with the clade of *C. kikonaiensis.* The specimens from Puget Sound and Petropavlovsk had a cortex of three to five cell layers, similar to *C. kikonaiensis.* However, the number of specimens examined is very limited at present, although the genetic divergences among the taxa were rather large. Therefore, we reserve taxonomic placement of these taxa until further analyses are available.

Japanese species of *Chorda* are apparently distinct from *C. filum* distributed in the Atlantic on the basis of molecular phylogenetic analyses. Atlantic *C. filum* has large morphological variations with respect to the cortical layer anatomy: the cortex is composed of 4–10 cell layers (Kylin 1918; South and Burrows 1967; Russell 1985; Kawai *et al.* 2000; present study). Although morphological difference between the Atlantic and the Japanese *Chorda* species remains unclear, we propose the separation of Japanese *Chorda* species from the Atlantic *C. filum,* and the establishment of *Chorda asiatica.* Furthermore, the taxonomic relationships of the Pacific populations represented by CF-StL and CF-Pet2, which have closer phylogenetic relationships with Atlantic populations than all other Pacific populations, is still unclear. *Chorda filum* also has been reported from continental north-east Asia: Russia (Perestenko 1980), Korea (Lee & Oh 1998) and China (Tseng 1983). No specimens from these regions have been examined for molecular analyses in the present study; however, judging from the geographical distributions and the sporophyte morphology they are likely to be assigned to *C. asiatica.*

Of the four clearly recognized species of *Chorda, C. asiatica, C. rigida* and *C. kikonaiensis* are distributed only in the north-west Pacific (Kawai *et al.* 2000; present work). In addition, the genetic diversity within *C. asiatica* is greater than that of Atlantic *C. filum,* although the data for *C. asiatica* is based only on Japanese specimens. This suggest that the species of *Chorda* originated and diverged in the Pacific Ocean, and migrated into the Atlantic. The notion that the Laminariales originated in the Pacific Ocean, based on the rich diversity of laminarialean taxa in the Pacific and the limited distribution of the Pseudochordaceae (Kawai & Kurogi 1985; Kawai & Nabata 1990; Lüning & Tom Dieck 1990) and Ak-



Figs 19–21. *Chorda asiatica sp. nov.* SAP101396. Collected on 15 June 2006 at Ohma, Aomori Prefecture, Japan, by H. Kawai. Slides used for the photographs are deposited with the type specimen in SAP.
Fig. 19. Habit of Type specimen.
Fig. 20. Cross section through meristem.
Fig. 21. Cross section through middle part of the thallus.

kesiphycaceae (Kawai 1986; Kawai & Sasaki 2000) to this area may also strengthen this idea.

Chorda kikonaiensis Sasaki & Kawai *sp. nov.*

Sporophyton macroscopicum epilithicum littorale, solitarium vel sparsim, simplex filiforme, 0.4–0.5 (–1.3) m longum et ad 2.5 mm diametro, fulvescens, parenchymatosum cavum, cum hypha, cortice, epiderme, pilis, paraphysibus unicellularibus sine pileolibus, et hapteris discoideum rhizoideis. Cortex dilutum, ad 2–4 cellula crassum. Sporangium uniloculare sessile, anguste ovatum. Gametophyton minutum, oogamum, dioecium, dimorphum. Antheridium solitarium. Cellulae sporophyticae et gametophyticae cum chloroplastis numerosis discoideis sine pyrenoidibus.

Quoad fabricam ad *Chordae filum* accedit, sed ab ea differt essentialiter flaccide thalli, etiam in maturitate cum meristema intercalare. Ordines in atomis genericis dictis *rbc*L et ITS rDNA propris/ae. Descriptio sequentiae geneticae ITS rDNA AB197758.

HOLOTYPUS: SAP100626. 2001.4.11. Kikonai, Hokkaido, Japan (Fig. 2).

ETYMOLOGY: The specific epithet originates from the type locality of the species.

Sporophyte macroscopic, epilithic, intertidal, solitary or sparse, simple, cord-shaped, 0.4–0.5 (–1.3) m in length and up to 2.5 mm in diameter, medium brown, parenchymatous, hollow, composed of hypha, cortex, epidermis, and hairs, unicellular paraphyses without mucilagenous cap, and disc-shaped rhizoidal holdfast. Cortex thin composed of 2–4 cells. Unilocular sporangia sessile and narrowly ovate. Microscopic gametophytes dioecious, sexually dimorphic, oogamous. Antheridium solitary. Sporophytic as well as gametophytic cells containing many disc-shaped chloroplasts without pyrenoid.

This species is similar to *Chorda filum* in habit, but it differs essentially by the softer thallus with persistence of the intercalary meristem even at maturity. Nucleotide sequences of *rbc*L and ITS rDNA distinctive (ITS rDNA sequence AB197758).

Chorda asiatica Sasaki & Kawai *sp. nov.*

Sporophyton macroscopicum epilithicum sublittorale, solitarium vel sparsim, simplex filiforme, 1.0–2.0 (–5.0) m longum et circa 1.5–3.5 mm diametro, fulvescens vel fuligineum, parenchymatosum cavum, cum hypha, cortice, epiderme, pilis, paraphysibus unicellularibus sine pileolibus, et hapteris discoideum rhizoideis. Cortex densum, ad 4–8 cellula crassum. Sporangium uniloculare sessile, anguste ovatum. Gametophyton minutum, oogamum, dioecium, dimorphum. Antheridium solitarium. Cellulae sporophyticae et gametophyticae cum chloroplastis numerosis discoideis sine pyreno-

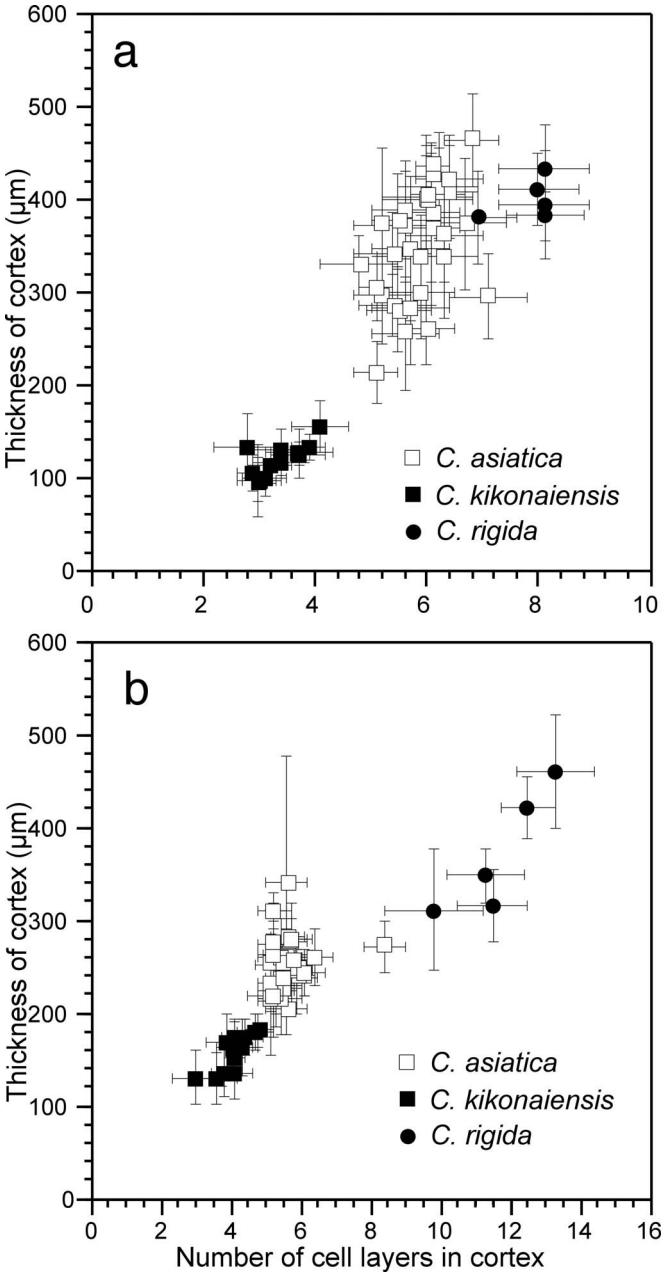


Fig. 22. Cell number and thickness of the cortex for *Chorda kikonaiensis* (■), Japanese *C. filum* (= *C. asiatica*) (□) and *C. rigida* (●) in middle portion (a) and basal portion (b) of sporophytes. S.D. is indicated with cross bars.

Table 5. Similar sequences presented in Fig. 23.[1]

Species	Clade	Specimen code
Chorda filum	Clade A-1	CF-Ros, CF-San, **CF-Por**, CF-IME, CF-IMP, **CF-Ber, CF-Rey,** CF-Ora, CF-NyA, **CF-Lon, CF-Aas1,** CF-Aas2, **CF-Dis1**, CF-Dis2, **CF-NFL**
C. filum?	Clade A-2	**CF-StL, CF-Pet2**
C. filum?	Clade B-1	**CF-Pug, CF-Pet1**
C. kikonaiensis	Clade B-2	**CK-Kik1,** CK-Kik2, CK-Kik3, CK-Kik4, **CK-Kik5**
C. asiatica	Clade C	**CA-Osh1,** CA-Osh2, CA-Osh3, **CA-Esa, CA-Kik1,** CA-Kik2, CA-Kik3, CA-Ohm, CA-Kam1, CA-KaSad1, CR-Sad2, **CR-Kas, CR-Nan**

[1] CA-Ohm (type specimen) whose ITS1 region sequence was identical to CA-Esa is not included in the phylogenetic tree. Only the specimens shown in bold are used in Fig. 24 to represent each clade.

idibus. Ordines in atomis genericis dictis *rbc*L et ITS rDNA propris/ae. Descriptio sequentiae geneticae ITS1 rDNA AB263977.

HOLOTYPUS: SAP101396. 2006.6.15. Ohma, Aomori Prefecture, Japan (Fig. 19).

Sporophyte macroscopic, epilithic, subtidal, solitary or sparse, simple, cord-shaped, 1.0–2.0 (–5.0) m in length and 1.5–3.5 mm in diameter, medium brown to dark brown, parenchymatous, hollow, composed of hypha, cortex, epidermis, and hairs, unicellular paraphyses without mucilagenous cap, and disc-shaped rhizoidal holdfast. Cortex dense composed of 4–8 cells. Unilocular sporangia sessile and narrowly ovate. Microscopic gametophytes dioecious, sexually dimorphic, oogamous. Antheridium solitary. Sporophytic as well as gametophytic cells containing many disc-shaped chloroplasts without pyrenoid. Nucleotide sequences of *rbc*L and ITS rDNA distinctive (ITS1 rDNA sequence AB263977).

ETYMOLOGY: The specific epithet originates from the distributional range of the species.

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Table 4. Comparisons of sporophyte anatomy among the four species of *Chorda.*

	C. kikonaiensis	*C. asiatica*	Atlantic *C. filum*	*C. rigida*
Meristem				
Average no. of cell layers	14.6 ± 1.6	18.1 ± 2.3	10.9 ± 0.7	26.0 ± 2.1
Average thickness	220.1 ± 38.7	284.4 ± 51.7	167.4 ± 22.7	391.2 ± 45.3
Middle portion				
Average no. of cells composing cortical cell layers	3.3 ± 0.6	5.9 ± 0.8	4.5 ± 0.6	7.8 ± 0.9
Average thickness of cortical cell layers	118.3 ± 30.1	343.3 ± 81.4	157.4 ± 35.8	400.4 ± 50.1
Basal portion				
Average no. of cells composing cortical cell layers	4.1 ± 0.7	5.7 ± 0.9	4.2 ± 0.6	11.5 ± 1.6
Average thickness of cortical cell layers	159.7 ± 32.2	250.1 ± 40.4	131.6 ± 26.0	358.8 ± 73.5

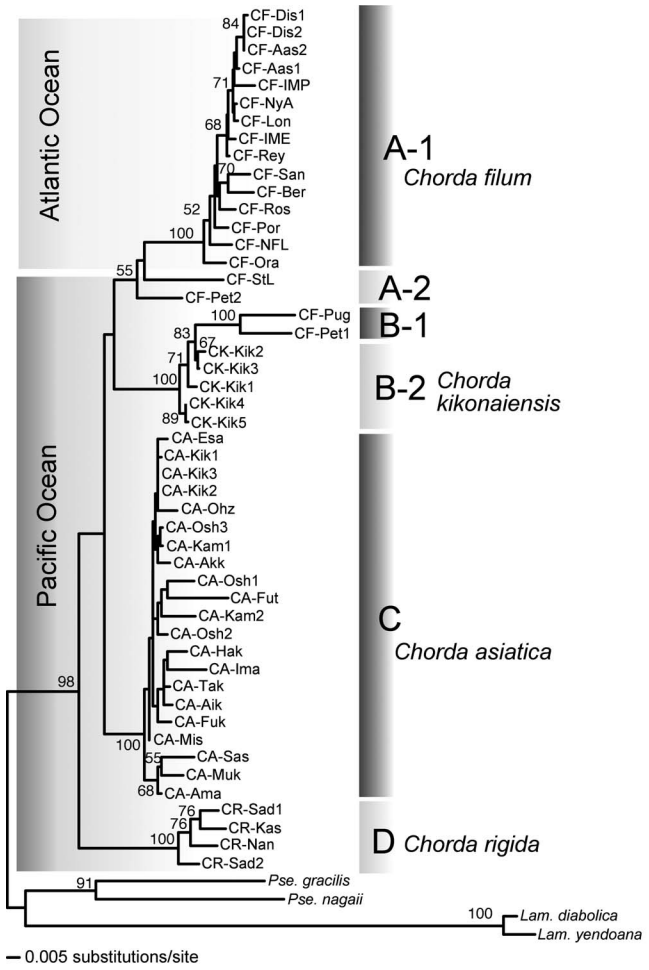

Fig. 23. Neighbour-joining tree based on rDNA ITS1, 5.8S and ITS2 sequences. Bootstrap values (> 50%) are based on 1000 replicates.

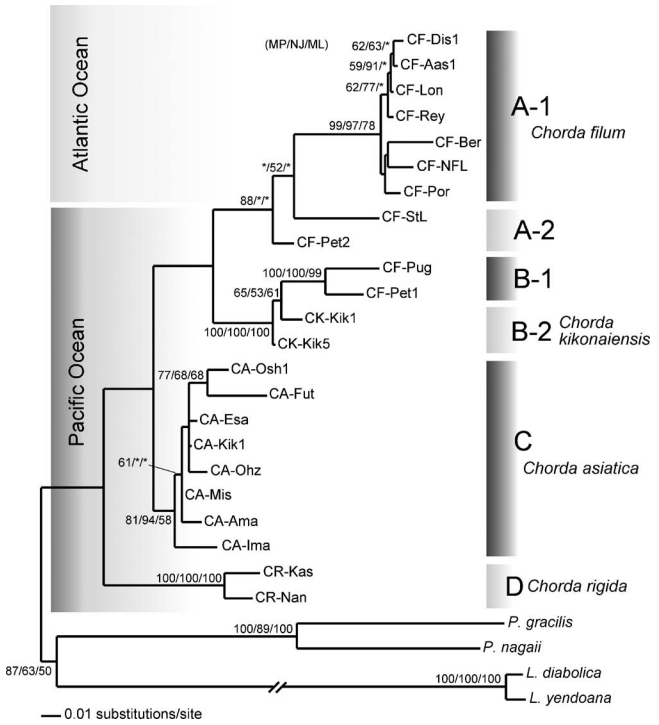

Fig. 24. Maximum likelihood (ML) tree (−ln L = 4636.7684) based on rDNA ITS1, 5.8S and ITS2 sequences. Modeltest determined the optimal evolutionary model as the TVM + G model. Bootstrap values (> 50%) of MP (left) and neighbour-joining (middle) based on 1000 replicates, and ML (right) based on 500 replicates. * Shows that the value is lower than 50, or the node is not supported in the analysis.

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