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Two species of the genus *Acinetospora* (Ectocarpales, Phaeophyceae) from Japan: *A. filamentosa* comb. nov. and *A. asiatica* sp. nov.

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Abstract: The brown algal genus *Acinetospora* is characterised by sparsely branched uniseriate filaments, scattered meristematic zones, short laterals ("crampons") and scattered reproductive organs. The morphology and life history of the generitype *A. crinita* have been studied repeatedly, and accounts of the species' highly varied reproductive patterns were assumed to be due to the presence of multiple taxa that were reported under this name. Herein, we attempt to contribute to the taxonomy of the genus by conducting morphological and culture studies on 33 *Acinetospora* samples collected from Japan. We recognised two *Acinetospora* species and propose to name them *A. filamentosa* comb. nov. and *A. asiatica* sp. nov. These two species are distinguished from *A. crinita* by the absence of monosporangia and plurilocular acinetosporangia/megasporangia. *Acinetospora filamentosa* and *A. asiatica* have similar vegetative morphologies but possess different reproductive patterns. The former forms unilocular sporangia on erect filaments and both unilocular sporangia and plurilocular zoidangia on prostrate filaments, while the latter forms plurilocular zoidangia only on both erect and prostrate filaments. Molecular analyses based on *rbc*L and *cox*1 genes supported independence of these species.

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Introduction

Acinetospora (Acinetosporaceae, Ectocarpales) is a genus of filamentous brown algae whose sparsely branched uniseriate filaments form entangled tufts on other seaweeds. This genus was established by Bornet (1891) for plants with plurilocular sporangia (acinetosporangia) that produce large non-motile cells (acinetospores). Later, additional characters were used to circumscribe the genus, which include possessing monosporangia that produce a non-motile spore, scattered meristematic zones and straight to curved short laterals called "crampons" (Sauvageau 1895, 1899, Cardinal 1964, Kim and Lee 1994, Pedersen and Kristiansen 2001). The generitype *A. pusilla* (Griffiths *ex* Harvey) De Toni (1895), which was originally described from the British Isles (Harvey 1841), is currently considered as a taxonomic synonym of *A. crinita* (Carmichael in Harvey) Sauvageau (1899) (Pedersen and Kristiansen 2001). Only two species are currently recognised in this genus: *A. crinita* (type locality: British Isles, Harvey 1833) and *A. nicholsoniae* Hollenberg (1971) (type locality: Santa Catalina Is., California).

Acinetospora crinita is found in warm to cold coastal marine waters, and there are numerous reports on its morphology and life history around the world, such as in Europe (Pedersen and Kristiansen 2001, and included references), the east coast of the United States (Amsler 1984), East Asia (Tanaka and Chihara 1977, Kim and Lee 1994, Kim 2010) and Australia (Clayton 1974, Womersley 1987). This species exhibited different life history patterns among different culture strains and populations (Pedersen and Kristiansen 2001, and included references). For example, Kornmann (1953) reported plurilocular acinetosporangia, unilocular sporangia and plurilocular zoidangia with small and large loculi in German isolates, while Müller (1986) found monosporangia and unilocular

sporangia in an Italian isolate. Meanwhile, Pedersen and Kristiansen (2001) observed that their Danish strain of *A. crinita* formed both monosporangia and plurilocular zoidangia with small loculi or large loculi, but their French strain formed only plurilocular zoidangia (megasporangia) with very large loculi. Pedersen and Kristiansen (2001) also pointed out that there is no clear distinction between plurilocular acinetosporangia and megasporangia before liberation of spores/zoids. In contrast to European isolates, Japanese *A. crinita* produced plurilocular or unilocular sporangia but was not observed to have monosporangia or plurilocular acinetosporangia/megasporangia (Kurogi 1950, Tanaka and Chihara 1977, Kitayama 1996).

Considering these highly variable reproductive patterns, previous workers on *A. crinita* suggested that plants under this name possibly include more than one species (Müller 1986, Pedersen and Kristiansen 2001). However, detailed taxonomic studies are limited and molecular data that can be used to investigate the relationships in *Acinetospora* remain scarce. Herein, we report on morphological, culture and molecular phylogenetic studies to investigate the diversity and relationships of *Acinetospora* from Japan.

Figure 1: Collection localities in Japan for samples of *Acinetospora* used for phylogenetic analyses in this study. Circles, *A. filamentosa*; squares, *A. asiatica*.

Materials and methods

Samples of *Acinetospora* were collected from 11 localities in Japan (Figure 1, Table 1). From the collected samples, pressed specimens were made as voucher herbarium specimens, and unialgal isolates were established by transferring a short fragment of a thallus filament into individual wells of a 48-well plate containing PESI medium (Tatewaki 1966). Four unialgal isolates were established from emerging algae in crude cultures of other seaweeds. Voucher specimens are deposited in the Herbarium of the Faculty of Science, Hokkaido University (SAP112484–112510). Cultures were inoculated in plastic Petri dishes (circular, 90 mm diameter×20 mm depth) containing PESI medium. Culture conditions used were 5°C SD (short day, 8:16 h light:dark regime), 10°C SD, 10°C LD (long day, 16:8 h light:dark regime), 15°C SD, 15°C LD, 20°C SD, 20°C LD and 25°C LD, under 30–50 μmol m^{-2} s^{-1}.

Total genomic DNA was extracted from cultured thalli and purified as previously described by Kogame et al. (1999). The purified DNA was used as template DNA for PCR to amplify the *rbc*L and *cox*1 genes. Primers for PCR and sequencing in *rbc*L gene were PRB-F0, PRB-F2, PRB-F3, PRB-R1A, PRB-R2, PRBR3A and RSPR (Kogame et al. 1999) as well as those designed in the present study (Table 2). The primer pair used for *cox*1 was GazF2 and GazR2 (Lane et al. 2007). PCR was performed using TaKaRa *Ex* Taq DNA Polymerase (TAKARA Bio Inc., Otsu, Japan). Amplification conditions consisted of 1 min at 94°C for denaturation, followed by 40–50 cycles of 30 s at 94°C, 30 s at 52°C and 30 s at 72°C, with a final extension of 5 min at 72°C. PCR was performed with a GeneAmp PCR System 9600 or 9700 (PE Applied Biosystems, Foster City, USA). PCR products were precipitated using PEG (polyethylene glycol=6000, Nakalai Tesque, Kyoto, Japan) and were directly sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 1.1 (PE Applied Biosystems) and an ABI Prism 3130 or 3730 Genetic Analyzer (PE Applied Biosystems), following the manufacturer's protocols.

The *rbc*L sequences produced were aligned with previously published sequences of the Ectocarpales, adding two species of *Asterocladon* as outgroup taxa (Peters and Ramírez 2001, Silberfeld et al. 2011). The *cox*1 sequences produced were aligned with published sequences of the *Acinetospora* cluster (Peters et al. 2015), which included a European *A. crinita*. Pairwise sequence differences (p-distances) were calculated in MEGA5.2 (Tamura et al. 2011). Phylogenetic analyses of *rbc*L were performed using Bayesian inference (BI) and maximum likelihood (ML) analyses as implemented in MrBayes 3.2* (Ronquist and

Table 1: Samples of *Acinetospora* from Japan used for phylogenetic analyses in this study.

Taxa	Sample code	Reproductive organ in field-collected sample	Reproductive organ in culture[a]	Collection date	Collection locality in Japan	Voucher specimen No. in SAP	Accession numbers for *rbc*L and *cox1*
A. filamentosa	Tachimachi.100626.3	No	PzP	26 June 2010	Tachimachi Cape, Hakodate, Hokkaido	112507	LC060488
A. filamentosa	Tachimachi.100626.4	No	No	26 June 2010	Tachimachi Cape, Hakodate, Hokkaido	112507	LC060489
A. filamentosa	Iwagasaki.100618.1	UzE	UzPE, PzP	18 June 2010	Iwagasaki, Murakami, Niigata Pref.	112488	LC060490 LC060608[b]
A. filamentosa	Iwagasaki.100618.2	UzE	PzP	18 June 2010	Iwagasaki, Murakami, Niigata Pref.	112488	LC060491 LC060609[b]
A. filamentosa	Yaekojima.101225.1	No	UzPE, PzP	25 December 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112484	LC060492 LC060610[b]
A. filamentosa	Yaekojima.101225.2	No	No	25 December 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112484	LC060493
A. filamentosa	Yaekojima.101225.4	No	UzP, PzP	25 December 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112484	LC060494
A. filamentosa	Yaekojima.101225.5	No	UzPE	25 December 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112484	LC060495
A. filamentosa	Yaekojima.101225.6	No	UzP	25 December 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112484	LC060496
A. filamentosa	Yaekojima.101225.8	No	UzPE	25 December 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112484	LC060497
A. filamentosa	Yaekojima.110129.1	No	No	29 January 2011	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112485	LC060498
A. filamentosa	Oohamacho.110129.3	No	UzPE, PzP	29 January 2011	Oohamacho, Innoshima, Hiroshima Pref.	112486	LC060499
A. filamentosa	Oohamacho.110207.3	No	UzP, PzP	7 February 2011	Oohamacho, Innoshima, Hiroshima Pref.	112487	LC060500
A. filamentosa	Ashikita.100314	UzE	UzP, PzP	14 March 2010	Ashikita, Ashikita-gun, Kumamoto Pref.	–	LC060501 LC060611[b]
A. filamentosa	Senaga.090330.2	–	PzP	30 March 2009	Shenaga, Naha, Okinawa Pref.	–	LC060502
A. filamentosa	Taketomi.010324	–	UzPE	24 March 2001	Taketomi Is., Yaeyama-gun, Okinawa Pref.	–	LC060503
A. filamentosa	Iriomote.080330	–	PzP	30 March 2008	Iriomote Is. Yaeyama-gun, Okinawa Pref.	–	LC060504
A. asiatica	Oshoro.100511	PzE	PzPE	11 May 2010	Oshoro, Otaru, Hokkaido	112508	LC060505
A. asiatica	Oshoro.100615.1	PzE	PzPE	15 June 2010	Oshoro, Otaru, Hokkaido	112509	LC060506 LC060612[b]
A. asiatica	Oshoro.100615.2	PzE	PzP	15 June 2010	Oshoro, Otaru, Hokkaido	112510	LC060507 LC060613[b]
A. asiatica	Muroran.070518	–	PzPE	18 May 2007	Muroran, Hokkaido	–	LC060508
A. asiatica	Muroran.100517	PzE	PzPE	17 May 2010	Muroran, Hokkaido	112505	LC060509
A. asiatica	Shinori.100627	PzE	PzPE	27 June 2010	Shinori, Hakodate, Hokkaido	112506	LC060510
A. asiatica	Yaekojima.100527.1	No	PzPE	27 May 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112493	LC060511 LC060614[b]
A. asiatica	Yaekojima.100527.2	No	PzPE	27 May 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112493	LC060512 LC060615[b]
A. asiatica	Yaekojima.100625.1	No	No	25 June 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112495	LC060513
A. asiatica	Yaekojima.100625.2	No	No	25 June 2010	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112495	LC060514
A. asiatica	Oohamacho.110129.2	No	PzPE	29 January 2011	Oohama-cho, Innoshima, Hiroshima Pref.	112497	LC060515
A. asiatica	Yaekojima.110207.1	No	PzP	7 February 2011	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112498	LC060516
A. asiatica	Oohamacho.110207.2	No	PzPE	7 February 2011	Oohama-cho, Innoshima, Hiroshima Pref.	112499	LC060517

Table 1 (continued)

Taxa	Sample code	Reproductive organ in field-collected sample	Reproductive organ in culture[a]	Collection date	Collection locality in Japan	Voucher specimen No. in SAP	Accession numbers for *rbc*L and *cox1*
A. asiatica	Yaekojima.110419.1	No	PzPE	19 April 2011	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112501	LC060518
A. asiatica	Yaekojima.110419.2	No	PzPE	19 April 2011	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112501	LC060519
A. asiatica	Yaekojima.110617.1	–	PzP	17 June 2011	Yaekojima, Oohama, Innoshima, Hiroshima Pref.	112504	LC060520

PzE, Plurilocular zoidangia on erect filaments; UzE, unilocular sporangia on erect filaments; No, no reproductive organs; -, not checked; Uz, unilocular sporangia; Pz, plurilocular zoidangia; P, on prostrate filaments; E, on erect filaments; No, no reproductive organs observed.
[a]Reproductive organs formed in 20°C long-day conditions.
[b]Accession number for *cox1* sequence.

Table 2: PCR and sequencing primers designed for *rbc*L gene in this study.

Primer code[a]	Sequence
PRB-ac1 (F)	5′-ACGTTTAGAAGATATGAGAA-3′
PRB-ac3 (F)	5′-GGCAAGAAAGTACGAAATGA-3′
PRB-ac5 (F)	5′-TCACCCAGATGGTATTCAAT-3′
PRB-ac2 (R)	5′-CCTTTAACCATTAAAGGATC-3′
PRB-ac4 (R)	5′-TCTTTCCATAAATCTAAAGC-3′

[a](F): Forward, (R): reverse.

Huelsenbeck 2003) and MEGA5.2, respectively. BI analyses used a GTR+I+G model selected by hLRT and AIC in MrModeltest 2.3 (Nylander 2004) and were performed with four runs of Markov chains with 2 million generations and sampling every 100 generations. The first 25% of trees were discarded as burn-in. ML trees were inferred by the nearest-neighbour-interchange method with a TN93+G+I model, which was selected as the best-fitting model by the BIC in MEGA5.2. In the phylogenetic analyses of *cox*1, Neighbour-Joining (NJ) trees were inferred using Tamura-Nei distance (Tamura and Nei 1993) in MEGA5.2. Bootstrap analyses (Felsenstein 1985) were performed with 500 and 1000 bootstrap pseudoreplicates for ML and NJ analyses, respectively.

Results

Acinetospora filamentosa (Noda) Yaegashi, Uwai *et* Kogame comb. nov. (Figure 2A–G)

Basionym

Ectocarpus filamentosus Noda 1970. Sci. Rep. Niigata Univ., Ser.D 7: 27.

Synonym

Ectocarpus ugoensis Konno in Konno *et* Noda 1974. Sci. Rep. Niigata Univ., Ser. D 11: 80.

Plants are uniseriate, branched filaments, forming entangled tufts to 10 cm or more in length attached to rocks and other seaweeds (e.g. *Sargassum* spp.). Erect filaments are irregularly and sparsely branched at wide to right angles and form short, straight to curved “crampons”. Meristematic zones are scattered, consisting of short cells. Cells of erect filaments are 20–80 μm in length and 18–28 μm in width, containing many discoid chloroplasts with pyrenoids. Phaeophycean hairs are found laterally

or terminally on erect filaments. Unilocular sporangia are spherical to oval, 25–55 μm in length and 25–55 μm in width, sessile or with a pedicel, and are formed on erect filaments.

In samples collected from Iwagasaki, Niigata Pref. and Ashikita, Kumamoto Pref., unilocular sporangia and phaeophycean hairs were observed, and crampons were rare (Figure 2A–C). In contrast, in samples collected from Tachimachi-misaki, Hokkaido and Oohamacho, Hiroshima Pref., unilocular sporangia and phaeophycean hairs were not observed, and crampons were abundant. Plurilocular zoidangia were not found in field-collected samples. Plants were collected in winter from Yaekojima and Oohamacho, Innoshima, Hiroshima Pref., where monthly samplings were conducted from May 2010 to June 2011.

In cultured *Acinetospora filamentosa*, zoids from unilocular sporangia and plurilocular zoidangia showed similar developmental patterns. Settled zoids germinated unipolarly, forming a germ tube. Germlings developed into prostrate filaments, which produced erect filaments and phaeophycean hairs (Figure 2D, E). Prostrate filaments were irregularly branched and their cells were globular (Figure 2D, E). Young erect filaments had a terminal hair. Prostrate filaments formed plurilocular zoidangia (Figure 2D), and erect filaments also formed plurilocular zoidangia but only on the lowermost portion (Figure 2E). Erect filaments grew longer than prostrate filaments and formed scattered meristematic zones and lateral hairs. Cells of erect filaments were 25–75 μm in length and 18–27 μm in width. Plurilocular zoidangia were ectocarpoid, 88–135 μm in length and 30–44 μm in width, and were formed with or without a pedicel (Figure 2D, E) at 10–25°C. Settled zoids from plurilocular zoidangia were round and 8.1–11.0 μm in diameter. Unilocular sporangia were spherical to oval, 52–62 μm in length and 44–62 μm in width, and were formed either sessile or with a pedicel (Figure 2F, G) on prostrate and erect filaments at 10–25°C. At 20°C LD, plants formed reproductive organs 3 weeks after germination. Plants usually produced unilocular sporangia after forming plurilocular zoidangia. In eight strains, unilocular sporangia were not observed on erect filaments while three strains did not produce any reproductive organs (Table 1).

Acinetospora asiatica Yaegashi, Yamagishi *et* Kogame sp. nov. (Figure 3A–H)

Diagnosis

Plants are sparsely branched uniseriate filaments up to 30 cm or more in length, forming entangled tufts on rocks and other seaweeds (e.g. *Sargassum* spp. and *Scytosiphon lomentaria*). Erect filaments have scattered meristematic zones consisting of short cells. Crampons are formed on erect filaments at right angles. Cells of erect filaments are 20–77 μm in length and 18–30 μm in width and contain many discoid chloroplasts. Plurilocular zoidangia are ectocarpoid, 90–135 μm in length and 25–40 μm in width, sessile or with one- or two-celled pedicels.

Holotype

SAP112509 (Figure 3A, collected on 15 June 2010) deposited in the Herbarium (SAP), the Faculty of Science, Hokkaido University, Sapporo, Japan.

Isotypes

SAP112510-112512 deposited in SAP.

Type locality

Oshoro (43°12′39″ N, 140°51′35″ E), Otaru, Hokkaido, Japan.

In samples collected from Oshoro, Shinori and Muroran, Hokkaido, scattered meristematic zones, crampons and plurilocular zoidangia were observed (Figure 3B–E). Plurilocular zoidangia were not observed, however, in samples collected from Oohamacho, Innoshima, Hiroshima Pref. In Oshoro, plants were collected in May and June but were not found in April and August. In Innoshima, plants were found from January to June. Unilocular sporangia were not found in any of the samples.

In culture, zoids from plurilocular zoidangia germinated unipolarly, forming a germ tube, and developed into branched prostrate filaments (Figure 3F). Cells of prostrate filaments became globular, while cells of erect filaments were cylindrical (Figure 3F, G). Prostrate filaments formed erect filaments which tapered slightly to a pseudohair or a hair with short cells (like those of meristems) near their base and longer pale cells in the upper portion. Plurilocular zoidangia were formed on prostrate filaments and the lowermost portion of young erect filaments (Figure 3G) at 10–20°C, 2–3 weeks after germination. Erect filaments grew longer than prostrate filaments and formed plurilocular zoidangia (Figure 3H) and scattered meristems. Cells of erect filaments were 23–78 μm in length and 20–32 μm in width. Heterokont zoids from

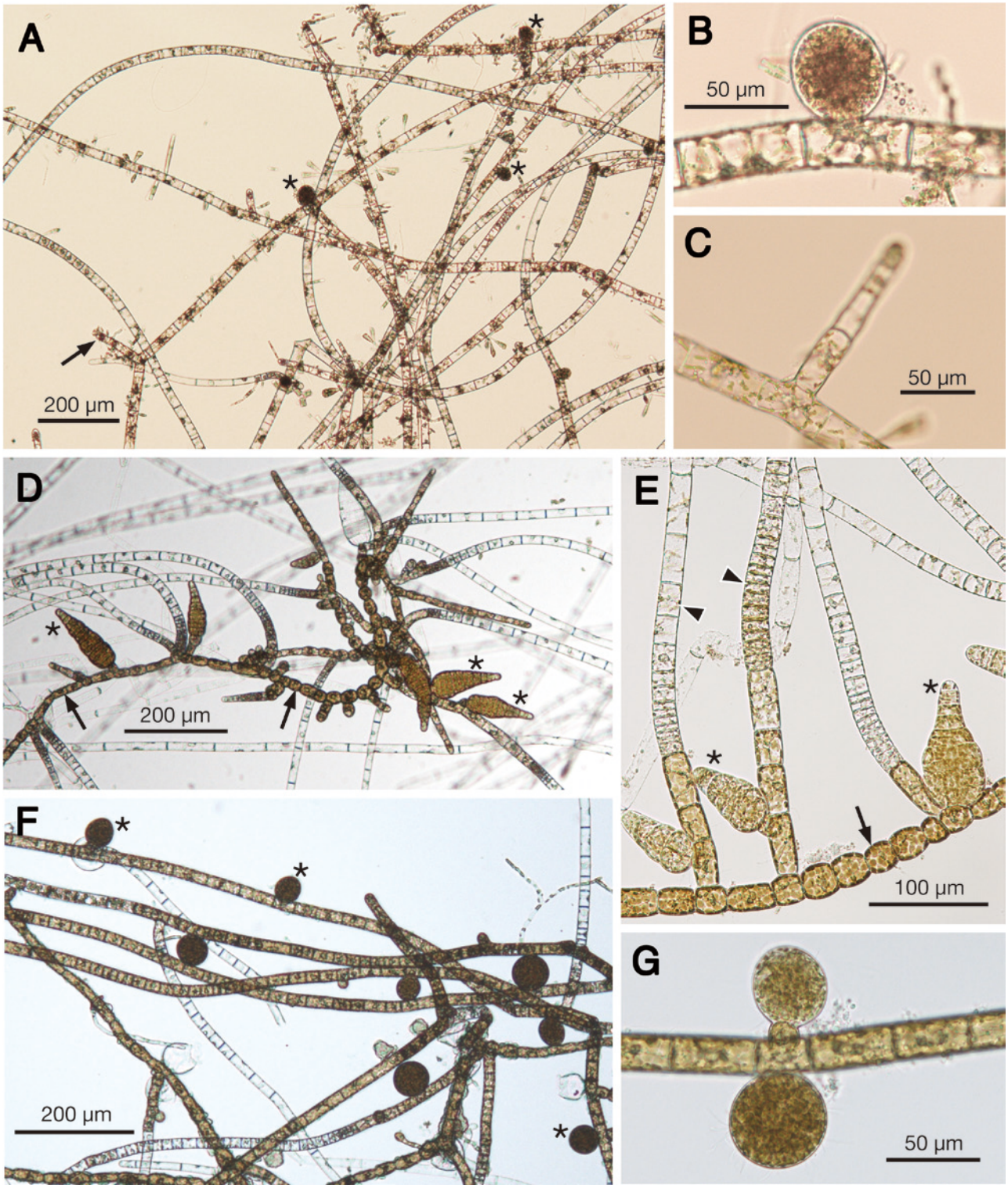

Figure 2: *Acinetospora filamentosa*. A–C. Field-collected thalli. A. Erect filaments with crampons (arrow) and unilocular sporangia (asterisks). B. Unilocular sporangium. C. Crampon. D–G. Cultured thalli (20°C, long day). D. Young prostrate filaments (arrows) forming plurilocular zoidangia (asterisks). E. Prostrate filament (arrow), young erect filaments (arrowheads) and plurilocular zoidangia (asterisks). F. Erect filaments forming unilocular sporangia (asterisks). G. Unilocular sporangia. A–G: 18 June 2010, Iwagasaki, Niigata Pref.

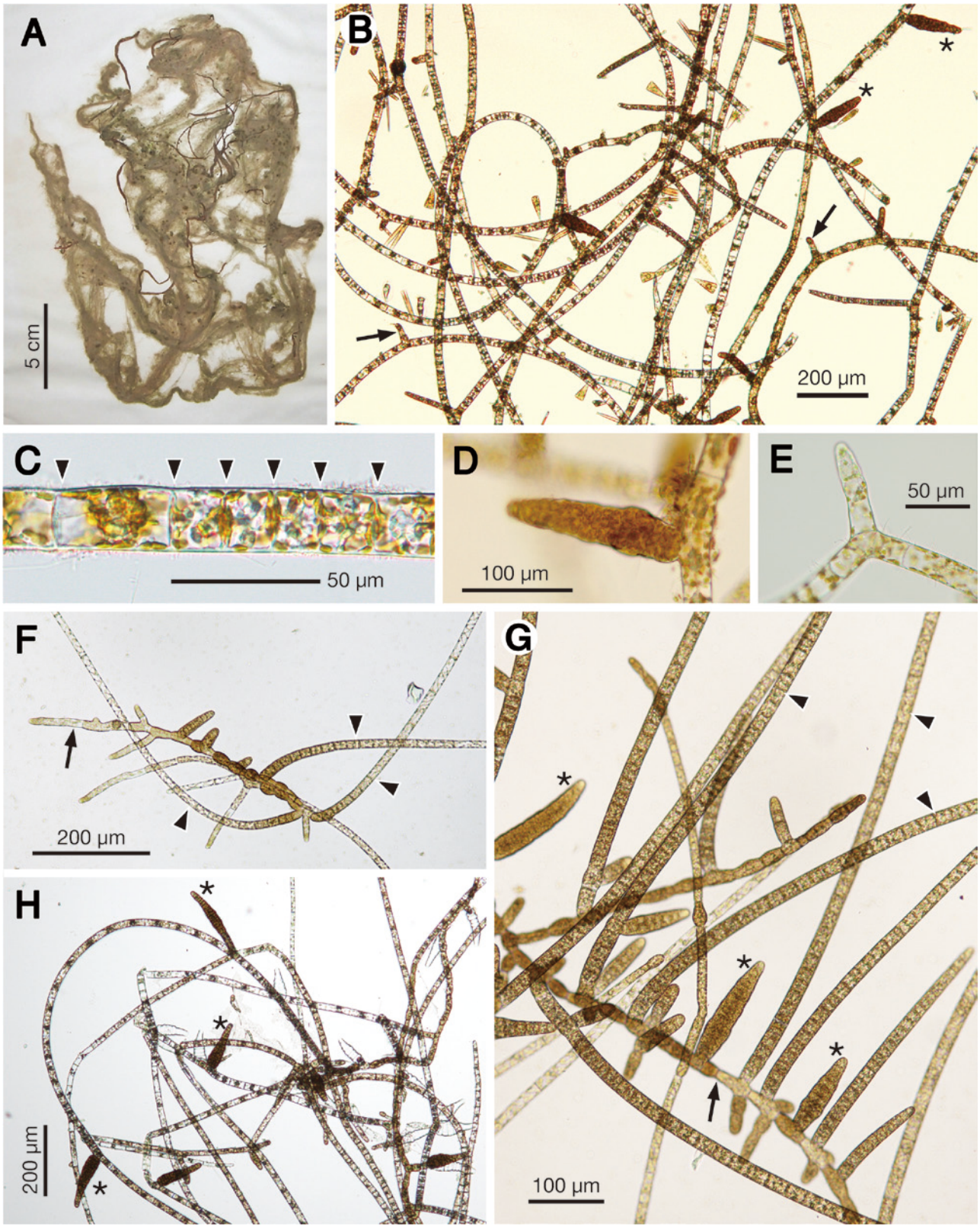

Figure 3: *Acinetospora asiatica*. A. Holotype (SAP112509). B–E. Field-collected thalli. B. Erect filaments forming plurilocular zoidangia (asterisks) and crampons (arrows). C. Meristem in erect filament. Arrowheads indicate boundaries between cells. D. Plurilocular zoidangium. E. Crampon. F–H. Cultured thalli (20°C, long day). F. Prostrate filament (arrow) producing erect filaments (arrowheads). G. Prostrate filaments (arrow) producing erect filaments (arrowheads) and plurilocular zoidangia (asterisks). H. Erect filaments producing plurilocular zoidangia (asterisks). A, B, D–H: 15 June 2010, Oshoro, Otaru, Hokkaido. C: 27 June 2010, Shinori, Hakodate, Hokkaido.

plurilocular zoidangia possessed an eyespot. Settled zoids from plurilocular zoidangia were round and 9.3–10.8 µm in diameter. Unilocular sporangia were not found in any culture condition. In two strains, no reproductive organs were formed at all (Table 1).

Molecular analyses

*Rbc*L sequences were determined for *Acinetospora filamentosa* (17 samples) and *A. asiatica* (16 samples). Alignment length was 1476 bp. BI and ML trees were similar and highly supported clades corresponded between the trees. Samples of *A. filamentosa* formed a fully supported clade, which was sister to the European sample of *A. crinita* (Figure 4). Samples of *A. asiatica* clustered with full support, and formed a clade with *Feldmannia irregularis* (Kützing) Hamel and *Hincksia* sp. The latter clade was sister to the *A. filamentosa*-*A. crinita* clade, and both clades were included in the Acinetosporaceae clade. Sequence differences (p-distances) between *A. filamentosa* and *A. crinita* were 3.0–3.3%, those between *A. asiatica* and *A. crinita* were 4.6–4.7%, and those between *A. filamentosa* and *A. asiatica* were 4.0–4.6%.

Partial *cox*1 sequences (658 bp) were determined for *A. filamentosa* (four samples) and *A. asiatica* (four samples). In the NJ tree of *cox*1 (Figure 5), the two species of *Acinetospora* from Japan as well as *A. crinita* from Europe (Greece and Brittany, France) formed three separate clades, each with full support. The clade of *A. asiatica* consisted only of Japanese samples while the *A. filamentosa* clade included one unidentified sample from Greece (LM995369). Sequence differences (p-distances) were 11.3–16.3% among the three species and <2.7% within each species.

Discussion

Acinetospora filamentosa and *A. asiatica* can be attributed to the genus *Acinetospora* based on having sparsely branched erect thalli, crampons and scattered meristematic zones. *Acinetospora crinita* exhibits highly varied reproductive patterns, such as producing plurilocular acinetosporangia/megasporangia, plurilocular zoidangia with large or small loculi, unilocular sporangia or monosporangia (Bornet 1891, Sauvageau 1899, Kornmann 1953, Müller 1986, Pedersen and Kristiansen 2001). In *A. crinita,* spores released from plurilocular acinetosporangia or megasporangia were large, approximately 20 µm in diameter (Bornet 1891), and zoids released from plurilocular zoidangia with large loculi were 12×12 µm (Pedersen and Kristiansen 2001). Meanwhile, settled zoids from plurilocular zoidangia of Japanese *Acinetospora* were smaller, 8.1–11.0 µm in diameter. Further, monosporangia were not found in Japanese *Acinetospora*. These differences in reproductive organs (Table 3) suggest that *A. filamentosa* and *A. asiatica* are different species from *A. crinita*. The morphological differences we observed were also supported by our molecular data since these two species showed differences from European *A. crinita* of 3.0–4.7% in *rbc*L and of 11.3–15.8% in *cox*1. These sequence differences are large enough for distinguishing ectocarpacean species (Siemer et al. 1998, Peters et al. 2015). Further, the Greek samples of *A. crinita* used in *cox*1 analyses were observed to produce monospores (Peters, pers.com.).

Moreover, both *A. filamentosa* and *A. asiatica* differ from *A. nicholsoniae* in their vegetative morphology, as *A. nicholsoniae* has larger erect filament cells and no crampons (Hollenberg 1971). Field-collected samples of *A. filamentosa* were observed to possess unilocular sporangia on erect filaments and *A. asiatica* found in nature formed only plurilocular zoidangia on its erect filaments. Meanwhile, *A. nicholsoniae* produces both unilocular and plurilocular zoidangia on its erect filaments (Hollenberg 1971) (Table 3).

Although the vegetative morphologies of *A. filamentosa* and *A. asiatica* are similar, they are also distinguished from each other by the type of reproductive organs they produce on erect filaments. Only unilocular sporangia were found in field-collected *A. filamentosa* while only plurilocular zoidangia were observed in *A. asiatica*. Our cultured *A. filamentosa* also formed plurilocular zoidangia on its prostrate filaments and at the basal portion of its erect filaments, but we did not find plurilocular zoidangia in field-collected samples. Our inability to find plurilocular zoidangia in field-collected *A. filamentosa* samples may be attributed to their entangled habit, which renders the basal portion of the thalli challenging to observe. In addition, we did not find *A. asiatica* with unilocular sporangia in either our field-collected or our cultured material. Separation of *A. filamentosa* and *A. asiatica* was also supported by our *rbc*L and *cox*1 analyses in which the sequence differences between them were 4.0–4.6% and 15.2–16.3%, respectively. Thus, we also describe herein a new species, *A. asiatica*, based on the abovementioned morphological and genetic differences.

The basionym of *A. filamentosa* is *Ectocarpus filamentosus* Noda (1970), which, together with *E. ugoensis* Konno in Konno and Noda (1974), was considered by

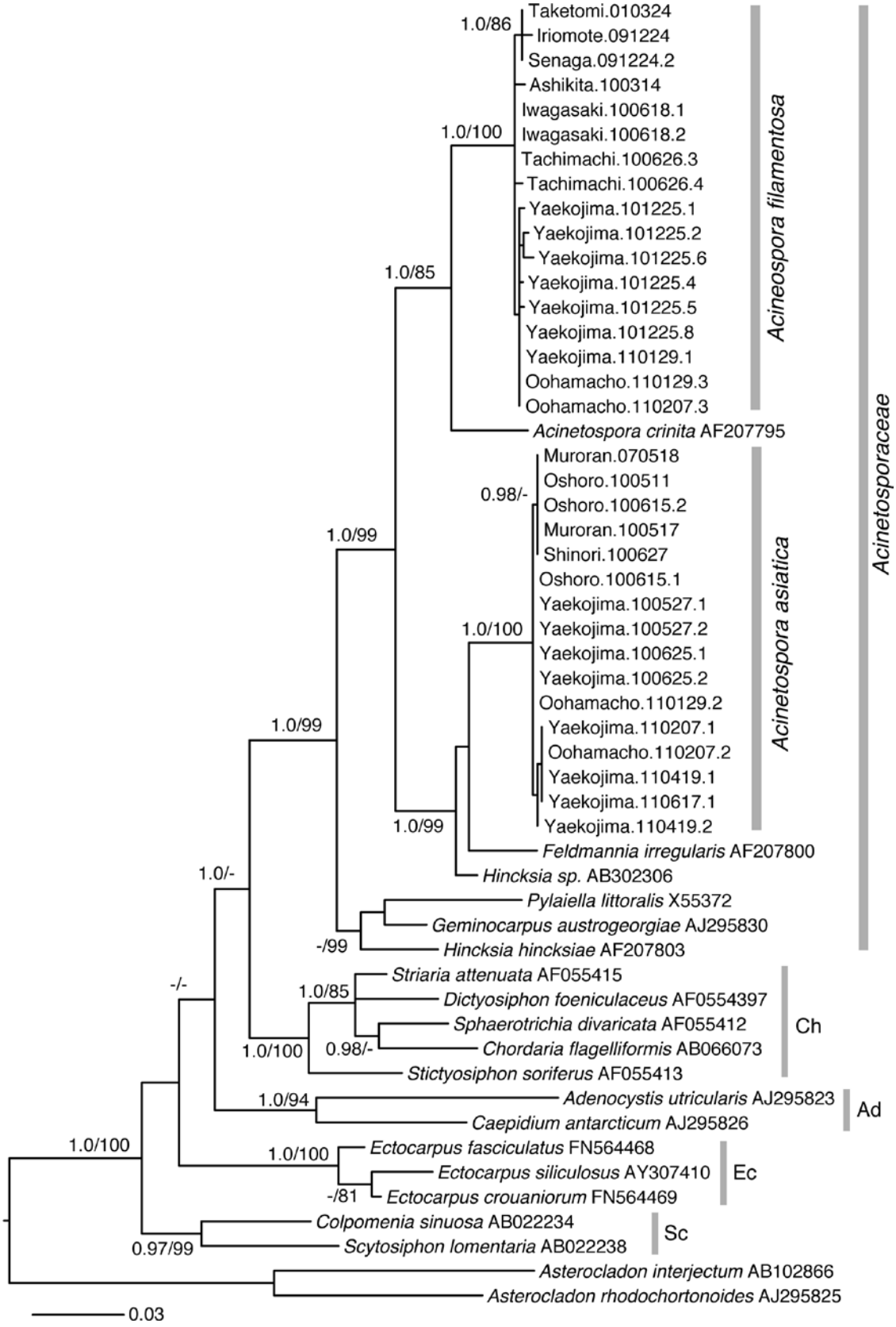

Figure 4: Bayesian tree of Ectocarpales inferred from *rbc*L sequences. Posterior probabilities (>0.95) and bootstrap percentages (>80) from maximum likelihood analysis are indicated near branches. "Ch" indicates Chordariaceae, "Ad" Adenocystaceae, "Ec" Ectocarpaceae and "Sc" Scytosiphonaceae. *Asterocladon* species are outgroup taxa. Scale bar refers to substitutions per site.

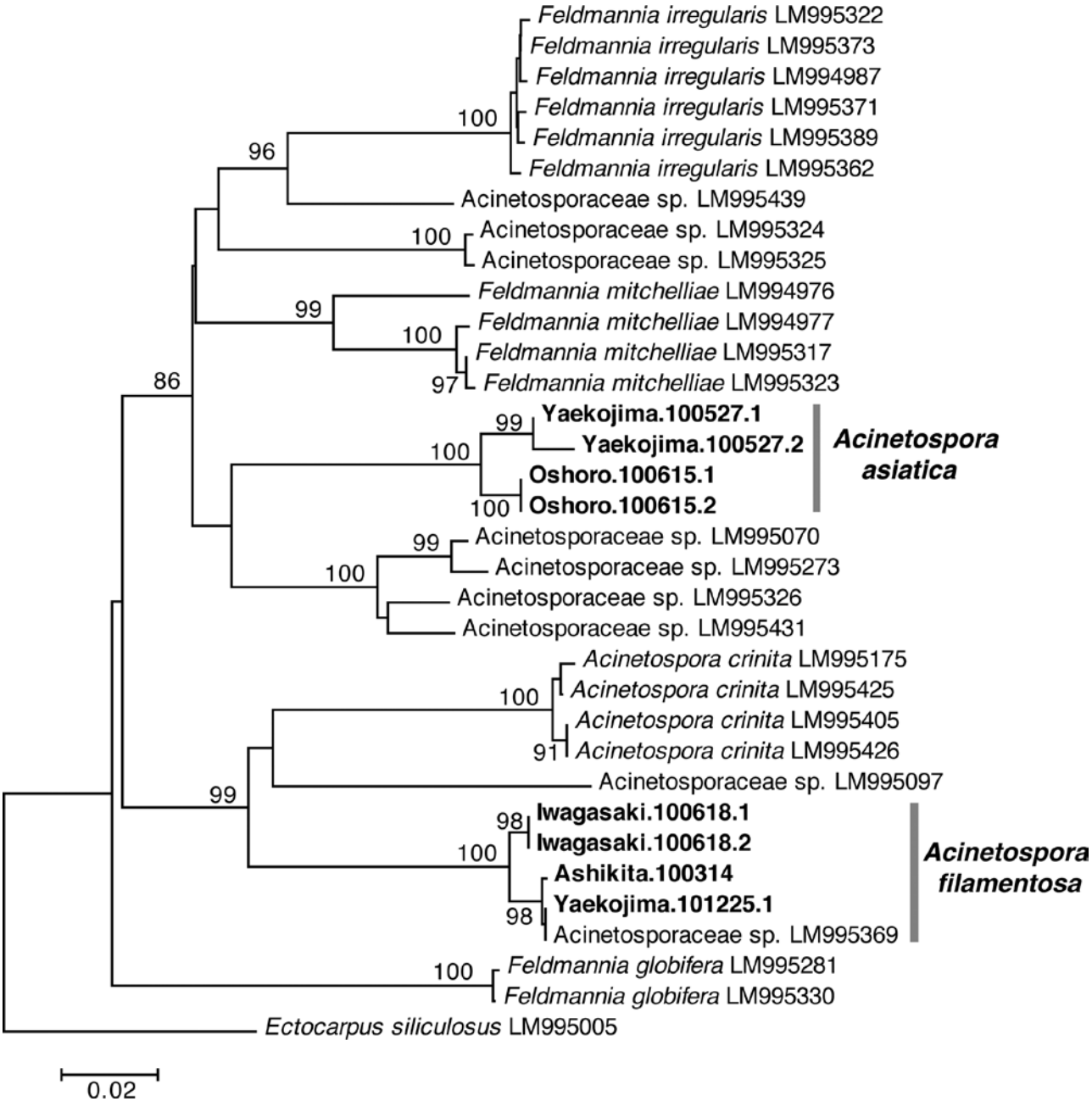

Figure 5: Neighbour-joining tree of *Acinetospora* and related species inferred from *cox*1 sequences. Taxa in boldface were sequences determined in the present study. Bootstrap percentages (>80) are indicated near branches. *Ectocarpus siliculosus* is an outgroup. Scale bar refers to substitutions per site.

Yoshida (1998) as a synonym of *A. crinita*. The type localities of *E. filamentosus* and *E. ugoensis* are Iwagasaki, Niigata Pref. and Takinoma, Akita Pref., Japan, respectively; both areas face the Sea of Japan and they are approximately 250 km apart. Our samples collected from Iwagasaki resembled *E. filamentosus* and *E. ugoensis* in having short laterals and unilocular sporangia, but differed from *A. crinita* in having neither monosporangia nor plurilocular acinetosporangia. In our molecular analyses, the samples from Iwagasaki were placed in the clade of Japanese *Acinetospora* samples with unilocular sporangia. Therefore, we propose the new combination *A. filamentosa* as name for the clade. *Ectocarpus ugoensis* was distinguished from *E. filamentosus* by having spherical unilocular sporangia, which is different from the ellipsoid unilocular sporangia of the latter (Konno and Noda 1974). However, we observed spherical to ellipsoid unilocular sporangia in our *A. filamentosa* culture, suggesting

Table 3: Morphological comparison among species of *Acinetospora*.

	A. filamentosa	*A. asiatica*	*A. crinita*	*A. nicholsoniae*
Diameter of erect filaments (μm)	18–28	18–30	20–30	30–45
Meristems	Scattered	Scattered	Scattered	Scattered
Crampons	Present	Present	Present	Absent
Plurilocular zoidangia	Present on prostrate filaments and lowermost portion of erect filaments	Present on prostrate and erect filaments	Acinetosporangia, megasporangia, plurilocular zoidangia with small or large loculi on erect and prostrate filaments	Present on erect filaments
Unilocular sporangia	Present on prostrate and erect filaments	Absent	Present on erect filaments	Present on erect filaments
Monosporangia	Absent	Absent	Present	Absent
Reference	This study	This study	Kornmann (1953), Müller (1986), Pedersen and Kristiansen (2001)	Hollenberg (1971)

that the difference in the shape of sporangia is not appropriate to separate these species. Therefore, we consider *E. ugoensis* as a synonym of *A. filamentosa*.

The majority of field-collected thalli of *A. filamentosa* and *A. asiatica* had no reproductive organs on their erect filaments (Table 1) and were, therefore, difficult to identify based on morphology. Both species were found in Innoshima, Seto Inland Sea and Hakodate, Hokkaido, suggesting that their distributions overlap. In contrast, records of only *A. filamentosa* in Okinawa (southern islands of Japan) suggest that its distribution extends to warmer regions than that of *A. asiatica*. In the previous accounts of *A. crinita* from Japan (Kurogi 1950, Tanaka and Chihara 1977, Kitayama 1996), plurilocular acinetosporangia/megasporangia and monosporangia were not reported, so that Japanese *A. crinita* likely belonged to *A. filamentosa* or *A. asiatica*. Distributions of both species in areas other than Japan are unknown, but a *cox*1 sequence of "Acinetosporaceae sp." from Greece was identical to that of a Japanese sample of *A. filamentosa*, suggesting a wider distribution of this species.

Acinetospora was regarded to be so closely related to *Feldmannia* and *Hincksia* that Kornmann (1953) and Knoepffler-Péguy (1974) considered *A. crinita* as a phase in the life history of *Feldmannia* or *Hincksia* (as *Giffordia*) based on their culture studies. In contrast, Amsler and Kapraun (1985) suggested that morphological similarities among these three genera are artefacts of incomplete development and mentioned that they should be retained as separate genera. Kim and Lee (1994) and Pedersen and Kristiansen (2001) clearly defined the three genera, using characters of crampons, meristems and the position of sporangia, against the opinions of Kornmann (1953) and Knoepffler-Péguy (1974). However, in the *cox*1 analyses of Peters et al. (2015), which included the type species of the three genera [*Feldmannia lebelii* (Areschoug *ex* P.L. Crouan *et* H.M. Crouan) G. Hamel, *Hincksia hincksiae* (Harvey) P.C. Silva (=*Hincksia ramulosa* J.E. Gray) and *A. crinita*], *Feldmannia* and *Hincksia* did not separate into distinct clades. Moreover, in our molecular analyses based on *rbc*L and *cox*1 sequences, *Acinetospora* species did not form a single clade; rather, the generated trees suggested that *A. asiatica* is more closely related to *Feldmannia irregularis*, *F. mitchelliae* (Harvey) H.-S. Kim and *Hincksia* sp. than to *A. crinita*. These molecular results suggest that taxonomic revision of these three genera is required. Although *A. asiatica* was closely related to *Feldmannia* spp. and *Hincksia* sp., this species was distantly related to the type species of *Feldmannia* and *Hincksia*, which were positioned in the *Pylaiella-Hincksia-Feldmannia* (PHF) group in the *cox*1 analyses by Peters et al.

(2015). In addition, *A. asiatica* was more closely related to the type species of *Acinetospora* than to the type species of either *Feldmannia* or *Hincksia*. As we have indicated earlier, the existing taxonomic problems within *Feldmannia*, *Hincksia* and *Acinetospora* have long been debated, and the need to revise their generic concepts is apparent. Significant progress can be made by conducting further studies on the Acinetosporaceae to settle and clearly delimit the different taxa especially at the generic level. Therefore, at this moment, we attribute *A. asiatica* to its genus based on morphological features.

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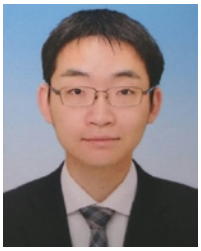

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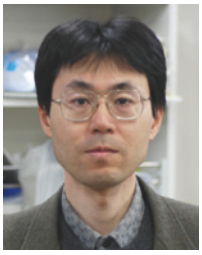

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