Molecular Phylogeny and Classification of Filamentous Microalgae Belonging to the Family Kornmanniaceae

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Abstract

The class Ulvophyceae is one of the groups of the phylum Chlorophyta. This group includes microalgae and macroalgae like seaweeds. Ulvophycean algae have various morphologies such as unicellular, colonial, filamentous, blade-like, and siphonous. Although most species of the Ulvophyceae grow in marine environment, species that grow in freshwater and terrestrial environment also exist. Moreover, some species have symbiotic relationships with other organisms. Microalgae are less studied taxonomically than macroalgae. In particular, the taxonomic research on branched filamentous microalgae has not made much progress. Although these branched filamentous algal taxonomy were performed based on thallus and cell morphology or reproductive mode, their morphologies are easily changed by cultivate conditions and the fluctuation of morphology is large. Moreover, there also have similar morphology among phylogenetically different species so that this makes difficult their taxonomy. However, these algae are very common in marine, freshwater and terrestrial environments. Accordingly, the development of the taxonomy on branched filamentous algae is important to establish the classification system of green algae. In these backgrounds, Darienko & Pröschold (2017) performed the large-scale taxonomic study on some ulvophycean species including branched filamentous species. They reclassified branched filamentous algae that are confused taxonomically using freshwater and marine strains maintained in public culture collections according to information of 18S rDNA and ITS-2 phylogeny, CBC approach and morphology. Although the framework of taxonomical foundation was established by this study, it had some problems, because they used only strains in culture collection and they mainly focused only on freshwater species, while branched filamentous green algae are found universally in marine

and terrestrial environments.

I have been studied the taxonomy of branched filamentous ulvophycean microalgae growing in marine and terrestrial environments with aim to reclassify and get the robust taxonomy of them. For this aim, I collected and isolated branched filamentous microalgae from several locations, and observed their morphology and performed preliminary phylogenetic analyses. The results showed that many branched filamentous microalgae were the members of the Kornmanniaceae. Therefore, in this study, I aimed to clarify and discuss the diversity, phylogeny, taxonomy and evolution of the Kornmanniaceae. I wish that this study leads to understanding the diversity and evolution of whole Ulvophyceae in the future.

I collected various samples (e.g., water, water sample of washed leaves and rocks, sand, and green shells of door snails) from several locations in Japan and Republic of Palau. These samples were primary cultured using liquid medium of BBM for freshwater samples, or ESM and IMK for seawater samples, and 1.5 % agar BBM or ESM medium containing anti biotics. After 2-3 weeks, unialgal colony was picked up and established unialgal strains. These strains were observed morphological features by light microscope and some strains were also observed ultrastructure by scanning electron microscope and transmission electron microscope. The total DNA was extracted from these strains, and the 18S rDNA and ITS region were amplified by polymerase chain reaction. Amplified products were sequenced and analyzed to construct phylogenetic trees by ML analysis and Bayesian analysis. The sequences of ITS-2 were also analyzed to compare the secondary structure among closely related strains.

In this study, about 85 strains of branched filamentous green algae from marine, freshwater and

door snail shells were established. In preliminary phylogenetic analysis, it was suggested that many strains belonged to the Kornmanniaceae, so that these strains were studied in phylogenetic analyses and morphology in detail. Most of these strains were collected from marine environment but some strains were collected from freshwater and terrestrial environment (include on the shell surface of door snails). It could be said that many branched filamentous microalgae growing marine are belonged to the Kornmanniaceae by this study. These strains have very similar each other in morphology such as the thallus composed of aggregated cell and peripheral radiating filaments. The size and morphology of cells have large fluctuation. Most of cells have one parietal chloroplast, with one pyrenoid. A part of strains was observed reproduction by quadriflagellate zoospores. These characters were similar to *Pseudendoclonium*, *Paulbroadya*, *Lithotrichon* belonging to the Kornmanniaceae and some genera transferred from the Ulvales to Ulotrichales by Darienko & Pröschold (2017). However, the number of branching, length, ratio of cylindrical cell and elliptical cell composing filaments was different among strains. In the result of detailed phylogenetic analysis, these strains were separated at least about 7 linages (it might be corresponded to genus revel). 4 of them were novel linages that never been reported in previous studies. There were some strains having fast evolving sequence of 18S rDNA in *Pseudendoclonium.* Many strains were closely related to *Lithotrichon*. Another 6 strains possessed the similar sequences to the environmental sequence of the photobiont of marine lichen collected from Ascension Island. This photobiont was not examined taxonomically and present study showed that this group was sister to the shell-attached algae discovered in this study (see below). In this study, some shell-attached algal strains were established from several places and several clausiliid species. Interestingly, they showed no CBCs in their ITS-2

secondary structure, so that it was suggested that these strains are the same species. Based on this study I described a new genus and new species, *Annulotesta cochlephila* for the shell-attached alga. This study revealed the hidden diversity of the Kornmanniaceae that was unknown previously. Although the Kornmanniaceae includes some macroalgae such as *Kornmania*, most members of this family are typically branched filamentous microalgae. Moreover, present study indicates that the habitats of the Kornmanniaceae were very diverse, such as marine, freshwater, land, lichen photobiont and surface of clausillid shell. The phylogenetic relationships among these algae suggest that they were originally marine algae and colonized freshwater independently several times. Interestingly, some lineages of the Kornmanniaceae include lichen photobionts, and these lichen symbioses might be the intermediate stages in the evolution of these frequently change of habitats.

Chapter 1: General introduction

1.1. Taxonomy of branched filamentous green algae

The green plants (Viridiplantae, Chloroplastida) form a large clade in eukaryotes. They are basically photosynthetic and have the chloroplast enclosed by two membranes, derived through the primary endosymbiosis with a cyanobacterium (McFadden 2001; Rodriguez-Ezpeleta et al. 2005). The green plants accumulate starch as storage polysaccharide in plastids. In addition, green plants have a unique structure, stellate structure, at the flagellar transition region. The green plants include very diverse organisms including land plants.

The green plants excluding land plants are called green algae. The green algae are very diverse organisms in morphology and ecology (e.g., Bold & Wynne 1978; Leliaert et al. 2012). Some species are more larger than 1 m but some other species are less than 1 μm. They are unicellular flagellate, coccoid, colonial (flagellate, palmelloid, sarcinoid, coenobial etc), unbranched or branched filamentous, parenchymatous, or siphonous. Many green algae inhabit aquatic environments including marine, freshwater or even in salt lakes. In aquatic environments, they are planktonic or benthic. Some green algae are found in terrestrial environments such as soil, rock, wall or tree bark. Furthermore, some green algae engage the symbiotic relationship with other organisms. The most photobionts of lichens are green algae such as *Trebouxia* and *Asterochloris* (Skaloud & Peksa 2010; Thüs et al. 2011). Various organisms such as ciliates, amoebae, hydra, and salamanders harbor unicellular green algae as endosymbionts (Reisser 1984; Kerney et al. 2011; Gomaa et al. 2014; Ishikawa et al. 2016). Some green algae are living on specific animals such as sloths or a certain species of sea snail (Matsuyama et al. 1999; Pantazidou et al. 2006; Graham et al. 2009;

Suutari et al. 2010; Pauli et al. 2014).

The green algae were traditionally classified based on their organization, such as unicellular, colonial, filamentous, or siphonous (see above) (Bold & Wynne 1978). However, ultrastructural studies since 1960's suggested that the traditional classification system based on their organization did not reflect their phylogenetic relationships (e.g., Mattox & Stewart 1984). In addition, molecular phylogenetic studies since the end of 20th century support the phylogenetic hypothesis proposed based on the ultrastructural studies (summarized in Leliaert et al. 2012). These studies indicate that the frequent convergent evolution of organization level occurred in the evolution of green algae. Actually, some green algae classified traditionally in the same order, family, or even genus are now appeared to be distantly related each other. As the result, the traditional classification system of green algae has been discarded and the new classification system including many new classes, orders, and families proposed recently. For example, a new class, the Ulvophyceae was proposed and many new orders, families and genera were included in this class (Table 1).

In the traditional classification systems of green algae, branched filamentous green algae were generally classified in the order Chaetophorales (Bold & Wynne 1978). They are found in various habitats, such as marine, freshwater, and terrestrial environments. However, as many other traditional orders of green algae, the Chaetophorales in traditional sense has been discarded based on ultrastructural and molecular phylogenetic studies (e.g., Mattox & Stewart 1984; Leliaert et al. 2012). The freshwater genus *Coleochaete*, previously classified in the Chaetophorales, is now considered to be closely related to land plants and classified in the own class, the Coleochaetophyceae. Many other freshwater species including *Chaetophora* spp. are now classified

in the Chlorophyceae. Because the *Chaetophora* is the type genus of the Chaetophorales, this order is classified in the Chlorophyceae in the present classification system of green algae. Some marine species such as *Pseudendochlonium* and *Ulvella* spp. have been transferred to the Ulvophyceae. However, unfortunately, the taxonomic studies on the members of the traditional Chaetophorales are insufficient and many species remain in the traditional classification. Because these green algae are very common in marine, freshwater and terrestrial environments, the reorganization of the classification system of them is important not only taxonomically but also ecologically.

Recently, Darienko & Pröschold (2017) studied many branched filamentous green algae especially isolated from freshwater based on morphological and molecular data. In this study, many new genera and species were descrived. They found that many branched filamentous green algae are members of the order Ulotrichales and Ulvales in the class Ulvophyceae. In the Ulvales, the family Kornmanniaceae includes many these green algae.

1.2. Summary of the family Kornmanniaceae Golden & Cole

The family Kornmanniaceae established by Golden & Cole (1986) is one of the groups of the class Ulvophyceae. Genera belonging to the Kornmanniaceae, such as *Blidingia* (marine to freshwater, temperate regions), *Pseudendoclonium* (marine, freshwater, terrestrial and aerophytic, also as lichen phycobiont, globally distributed), *Paulbroadya* (freshwater and marine, terrestrial and phycobiont of marine lichens, temperate and arctic), *Lithotrichon* (phycobiont of freshwater lichens), grow widely in various environments (Škaloud et al. 2018). The thallus of the members belonging to this family is filamentous, tubular or blade shaped (e.g., Golden & Cole 1986; Darienko & Pröschod

2017). Both of microscopic and macroscopic species are described in the Kornmanniaceae. The cells usually have a single parietal or stellate chloroplast, with or without pyrenoid. They are reproduced asexually by biflagellate or quadriflagellate zoospores, or fragmentation of filaments (see Škaloud et al. 2018). In the Kornmanniaceae, six genera are reported in recent studies (*Kornmannia*, *Blidingia*, *Pseudendoclonium*, *Paulbroadya*, *Lithotrichon*, *Halofilum*), and genera *Neostomatella* and *Tellamia* have been reported in AlgaeBase (Guiry & Guiry 2021), as shown in Table 2. The species of the Kornmanniaceae were traditionally classified based on only morphological features. However, the classification system of the family has been changed drastically according to the recent studies using molecular phylogenetic analysis because their morphological features are easily changed and they share similar morphological features. Genus *Dilabifilum* is one such example. This genus was established by Tschermak-Woess (1970) and *D. arthropyreniae* (Vischer & Klement) Tschermak-Woess was the type species in this genus. Tschermak-Woess (1970) described this new genus and species based on the formation of aplanospores and quadriflagellate zoospores. After that, some species were also added to this genus. However, the establishment of genus *Dilabifilum* was questioned because morphological similarity to genus *Pseudendocloniuom*, and the definitive feature of the genus (formation of quadriflagellate zoospores) was not observed in later studies (Bourrelly 1973; Johnson & john 1990). Moreover, some studies using molecular phylogenetic analysis showed that *Dilabilium* was not monophyletic and the part of them formed a clade with some species of *Pseudendoclonium* (Mullins 2007). Recently, Darienko & Pröschod (2017) analyzed phylogenetic relationships of a wide variety of non-marine ulvophyceaean linages using molecular approach (phylogenetic analysis and ITS-2/CBC approach). Thereby, they revealed that some species of

Dilabifilum should be transferred to the genus *Pseudendolonium*, and some new genera (e.g., *Paulvroadya*, *Lithotrichon*) were described for the rest of species of *Dilabifilum*. They also transferred some species *Dilabifilum* to *Halofilum* with *Pirula* species. As these, the drastic taxonomic changes were performed in the Kornmaniaceae based on molecular data.

1.3. ITS-2/CBC (compensatory base changes)

The second Internal Transcriber Spacer (ITS-2) is a fast evolving part of the nuclear-encoded rRNA operon located between the 5.8S and 28S rRNA genes. This region is one of the most frequently used for phylogenetic analysis among closely related organisms, e.g. genus and species levels. Currently, a large amount of sequences were reported and is open to the public under ITS-2 Database (Koetschan et al., 2009; http://its2.bioapps.biozentrum.uni-wuerzburg.de/). ITS-2 secondary structure was reported that it is highly conserved and most of the eukaryote groups (green algae, brown algae, terrestrial plants, flat-worms, mollusks, insects, vertebrates and higher fungi) share the same structure (Coleman 2003). This ITS-2 forms four helices and represent some features: (1) helix II has least one pyrimidine-pyrimidine mismatch (U-U, U-C and C-C), (2) helix I and IV are highly variable in sequence and length rather than helix II, (3) helix III usually much longer than the other helices, and it contains on the 5' side the single most conserved primary sequence, a region of ~20 nucleotides encompassing the YGGY motif (Mai and Coleman 1997, Schultz et al., 2005, Coleman 2003). Nucleotide sequence evolves most rapidly in helix IV, and next most rapidly in helix I among plants and green algae (Coleman 2003).

In the ITS-2 region, the change of a single base in the relatively slowly evolving stem regions

frequently occur although it is difficult to occur because of the necessity to retain the potential for pairing with its opposite nucleotide in the stem. However, it is easily occurred than the occurrence of a double-sided alteration, a change in both positions which pair on opposite sides of the stem (e.g., A-T changed to G-C). This double-sided alteration is called Compensating Base pair Changes (CBCs). In contrast, Hemi-CBC is where only one of the two changes, but still preserves the pairing. Coleman (2000) reported that the presence of CBCs in ITS-2 regions refer to the reproductive isolation using some genus of the Volvocales (a order of Chlorophyta). Müller et al. (2007) compared more than 1300 sequences of ITS-2 using fungi, plants and some animals from GenBank. As a result, they reported that if at least one CBC was observed in helix II or helix III between organisms classified within the same genus, they belong to different species at 93 % probability. However, this probability was able to apply to where the target is fungi and plants because it was included a few sequences of animals. After that, more diversified ITS-2 secondary structure of algae, protozoa, fungi, plants and animals was compared by Coleman (2009), and this study reported that if CBC in conserved region, in the 5'- side of helix III (about 5' 30 bp) has appeared, the clade of organisms defined by that CBC tend to contains at least one, and perhaps a very small number of Z clades, several "biological species" and one or more morphological species. After some revisions, CBCs became to be used as one of the index to identify species boundary. Currently, some definitions: 1)one or more CBC appear in highly conserved region of helix III of ITS-2 or 2)one or more CBC appear among helix I to IV, are applied for treating as different species. However, this the presence or absence of CBC in ITS-2 is sufficient condition but it is not necessary condition. Therefore, attention should be paid when using CBC for identification of species and that will

probably need more research in the future too.

1.4. Symbiosis in class Ulvophyceae

Among algae, many species that have symbiotic relationships between with other organisms reported. These relationships are ecologically diverse such as mutualism, commensalism and parasitism. There are two type of symbiotic relationships; endosymbiosis and ectosymbiosis in which the former shows that symbionts live inside the tissues or cells of the host, the later shows that symbionts live on the surface of the host. Moreover, symbiotic partner for algae also very diverse (e.g., bacteria, protists (including algae), mosses, angiosperms, cladocerans, copepods, isopods, opiliones, sloths, turtles, and whales (Goff 1982; Machado & Vital 2001; Brooks 2004; Lindquist et al. 2005; Graham et al. 2009; Suutari et al. 2010; Totti et al. 2010; Tiffany 2011; Adams et al. 2012; Hilton et al. 2013; Pauli et al. 2014; Bury et al. 2015; Cooper & Smith 2015). The most common examples of this are corals and lichens. These photosynthetic organisms are very diverse and widely distribute on the earth, and play important roles in ecosystems (e. g., Rikkinen 2003; Gordon & Leggat 2010). Moreover, some symbiotic algae have a co-evolutionary relationship with hosts (Kawaida et al. 2013; Ishikawa et al. 2016), so that symbiosis is also important for the evolution and phylogeny.

In the Ulvophyceae, some species of the Cladophorales, Trentepohilales and Ulvales are well known that they have symbiotic relationships with other organisms. The species of the Pithophoraceane (Cladophorales), *Arnoldiella chelonum* (Collins) C.Boedeker, *A. crassa* (W.E.Hoffmann & J.E.Tilden) C.Boedeker and *A. sinensis* (N.L.Gardner) C.Boedeker (transferred from *Basicladia*) were reported that they grow on the carapaces of freshwater turtles (e.g., Garbary

et al. 2007; Škaloud et al. 2018). *Basicladia ramulosa* Ducker is also known that they occur on freshwater turtles (Ducker 1958). *Pseudocladophora conchopheria* (Sakai) Boedeker & Leliaert (Cladophorales, Pseudocladophoraceae) grows only on the shell of living marine snail *Lunella conreensis* (Récluz 1853), and there is no report that *P. conchopheria* grows on other substrates (Matsuyama et al. 1999). According these and the reports that many cladophoralean species were described from surface of rock and some substrate, it is suggested that cladophoralean symbionts grow on other organisms for seeking hard substrates. Some species of the Trentepohliales are plant parasites. One of them, *Cephaleuros* causes algal spots and gives a damage called chlorosis and branch dieback (Brooks, 2004). Moreover, some species of the Trentepohliales (e.g., *Trentepohlia*, *Printzina*) are also known as photobionts of lichens (Hametner et al. 2014). It has been reported that some macroalgae belonging to the Ulvophyceae have mutual interaction with bacteria. For example, true morphogenesis and acceleration of growth in some ulvophycean macroalgae are induced by bacteria (Spoerner et al. 2012; Singh & Reddy 2014). Matsuo et al. (2005) reported the structure of chemical structure of "thallusin" which promote normal morphology in genus *Ulva*. Zoospores of *Ulva* are promoted to attach substrate by acylated homoserine lactone (AHL) generated by bacteria (Joint et al. 2002).

In the Ulvales, some species were described as photobionts of lichens. For example, some linages of *Dilabifilum* (they were transferred to *Psendendoclonium* in recent studies) are associated with lichen forming fungi [e.g., *Hydropunctaria rheitrophila* (Zschacke) Keller, Gueidan & Thüs, *H. maura* (Wahlenb.) Keller, Gueidan & Thüs, *Verrucaria aquatilis* Mudd, *Wahlenbergiella striatula* (Wahlenb.) Gueidan & Thüs] (Thüs et al., 2011). *Halofilum ramosum* Darienko & Pröschold, *Lithotrichon*

pulchrum Darienko & Pröschold, *Paulbroadya petersii* Darienko & Pröschold, *Pseudendoclonium arthopyreniae* (Vischer & Klement) Darienko & Pröschold, *P. commune* Darienko & Pröschold, *P. incrustans* Vischer, *P. submarinum* Wille are also photobionts of lichens [*Hydropunctaria amphibia* (Clemente) Orange, *H. maura*, *Verrucaria aquatilis*, *V. maura* Wahlenberg, *V. mucosa* Wahlenberg, *V. rheitrophila* Zschacke, *Wahlenbergiella striatula*] (Darienko & Pröschold 2017; Gasulla et al. 2019). Like these, many genera and species of the Ulvales associate with some species of fungi and form lichen. In other examples, some species belonging to the Ulvales were described from surface of shell of marine shellfish. *Blidingia dawsonii* (Hollenberg & I.A.Abbott) S.C.Lindstrom, L.A.Hanic & L.Golden grows on the shell of limpet *Tectura scutum* (Rathke) (Lindstrom & Golden 2006). *Tellamia contorta* Batters was found only in periostracum of *Littorina obtusata littoralis* (Linnaeus, 1758) (e.g., Batters 1895a, b; Nielsen & McLachlan 1986) and this phenomenon is similar to *Pseudocladophora conchopheria* (Sakai) Boedeker & Leliaert in the Cladophorales (see above).

1.5. Purpose of this study

Many macroalgae belonging to the Ulvophyceae have been studied in detail previously. However, microalgae, especially species having branched filamentous thalli, are not well understood. These branched filamentous microalgae are universal on the earth and they inhabit most environments. Therefore, it is important to clarify their phylogeny and taxonomy. However, in these algae, most species have a similar morphology, making it very difficult to identify them at the genus and species level and resulted in extensive taxonomic confusion. In the later eras, the reclassification using molecular methods like phylogenetic analysis based on 18S rDNA, ITS-2 sequence and

comparison of ITS-2 secondary structure for ITS-2/CBC approach by Darienko & Pröschold (2017) basically use only culture strains of freshwater species deposited in public collections. Therefore, I applied the similar approach to the branched filamentous green microalgae collected from marine and terrestrial environments. In this process, it was suggested that many culture strains established in this study were the members of the family Kornmanniaceae (Ulvales, Ulvophyceae). Therefore, the purpose of this research is to clarify the diversity, phylogenetical relationship and taxonomy of branched filamentous algae belonging to the Kornmanniaceae. In the future, it is thought that this research will lead to deeper understanding for evolution of the class Ulvophyceae.

Chapter 2: Materials and methods

2.1. Collection, culture establishment, culturing

For collect other branched filamentous Ulvophytes, some samples of water, substrate samples (e.g., soil, fallen leaves and rock in the water) were collected several locations in the Japan and Republic of Palau (Table 4). These samples were primary cultured using liquid medium of BBM (Bischoff and Bold 1963) for freshwater samples, and ESM (Kasai et al. 2009) and IMK (Wako Chemical Co., Ltd., Japan) for seawater samples, and 1.5 % agar BBM or ESM medium containing anti biotics (Ampicillin; 50 µg/ml, Streptomycin; 20 µg/ml, Kanamycin; 20 µg/ml, Amphotericin B; 2.5 µg/ml) at 23 °C under white light of 10–50 µmol photons m-2 s-1 with a 14 : 10 h light : dark cycle. After 2 or 3 weeks, the branched filamentous microalgal strains were established two methods : (1) single cell was isolated using micropipette or individual colonies were isolated from agar plates (2) Several branched filamentous microalgal cells placed from primary cultivated sample to new agar plate with anti biotics. After 2 or 3 weeks, individual colonies were isolated from agar plates or some cells placed in liquid medium from colonies and isolated using micropipette. This step was repeated until uni-algal cultures established. Strain name, collection sites and collection date are shown in Table 2. Each strain was cultivated under same condition as above.

2.2. Light microscopy

Light microscopy of unialgal cultures was carried out using a Zeiss Axio Imager A2 microscope (Zeiss, Oberkochen, Germany) equipped with Nomarski differential interference contrast (DIC) optics and an Olympus IX71 inverted microscope (Olympus, Tokyo, Japan). Microphotographs were

taken with an Olympus DP-71 CCD camera (Olympus, Tokyo, Japan) and a digital single-lens reflex camera (Canon EOS 70D, Canon, Tokyo, Japan).

2.4. Transmission electron microscopy

For transmission electron microscopy (TEM), the cultivated cells of branched filamentous ulvophytes strains BFA102, BFA203 and BFA211 were centrifuged and fixed for 2 h at 4 °C with a mixture of 2.5 % GA in 0.1 M cacodylate buffer (pH 7.2). Fixed cells were washed with 0.2 M cacodylate buffer three times. In post-fixation, BFA102, BFA203 and BFA211 were fixed for 1h at 4 °C with 1 % osmium tetroxide. Fixed cells were washed with 0.2 M cacodylate buffer three times. Dehydration was performed using a graded series of 30 %–100 % ethanol (v/v). After dehydration, cells were placed in a 1:1 mixture of 100 % ethanol and acetone for 10 min and acetone for 10 min twice. The cells were embedded in resin by placement in a 1:1 mixture of acetone and Agar Low Viscosity Resin R1078 (Agar Scientific Ltd., Stansted, U.K.) for 30 min and resin for 1 h, twice. The resin was polymerized by heating at 70 °C for 12 h. For the holotype specimen, resin-embedded cells were mounted on glass slides and polymerized under the same conditions. Ultrathin sections were prepared on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria) and double-stained with 2 % (w/v) uranyl acetate and lead citrate (Sato 1968). Transmission electron microscopy was performed using a Hitachi H-7650 electron microscope (Hitachi High-Technologies Corp., Tokyo, Japan) equipped with a Veleta TEM CCD camera (Olympus Soft Imaging System, Münster, Germany).

2.5. DNA extraction, polymerase chain reaction, and sequencing

For the samples of branched filamentous Ulvophytes, cultivated cells were centrifuged by Low Speed Refrigerated Centrifuge AX-511 (TOMY, Tokyo, Japan) and transferred to 2ml screw cap tube. Then, 300 μL of extraction buffer (NaCl, Tris-HCl, EDTA and MilliQ), small amount of ceramic beads (0.5 mm in diameter), one ceramic beads (5 mm in diameter), 25 μL of 10 % CTAB and 300 μL of chloroform (Sigma-Aldrich, Saint Louis, MO, USA) were added and the tube was vortexed to break the cells by Beads Crusher μT-01 (TAITEC, Saitama, Japan) (2500 oscillations/minute, 5min). The tube was centrifuged 15000 rpm 2 min and supernatant was transferred to 1.5 ml tube. Total DNA was extracted using the DNeasy Plant Mini Kit. The following steps were performed according to the manufacturer's instructions. The 18S rDNA was amplified by polymerase chain reaction (PCR) using KOD One (Toyobo, Osaka, Japan) and TaKaRa EX tap (Takara Bio, Shigma, Japan) with SR1 and SR12 primers (Nakayama et al. 1998). For ITS rDNA, SR10 (Nakayama et al. 1998) and ITS055R primers (Marin et al. 2003) were used. PCR amplifications used KOD One included initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 5 s, and extension at 68 °C for 5 sec. PCR amplifications used TaKaRa EX taq (Takara Bio, Shigma, Japan) included initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 2 min, followed by a final extension at 72 °C for 7 min. PCR products were checked by electrophoresis and excised. Purification of PCR products was carried out using the QIAquick Gel Extraction kit (Qiagen, Science, Valencia, CA). DNA sequencing was carried out using BigDye Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Tokyo, Japan) and a 3130

Genetic Analyzer (Applied Biosystems, Tokyo, Japan) and Applied Biosystems SeqStudio Genetic Analyzer (Applied Biosystems, Tokyo, Japan).

2.6. Phylogenetic analysis

In branched filamentous ulvophytes, the 18S rDNA sequences of 55 ulvophytes from GenBank, 10 sequences of *A. cochlephila* and 44 sequences of branched filamentous ulvophytes strain that established in this study were used. These sequences were aligned using MAFFT v7.409 (Kathoh & Standley 2013) and the gaps and poorly aligned regions of the alignment were manually excluded and checked using MEGA7 (Kumar et al. 2016). In ML analysis, IQ-TREE Ver. 1.6.7 (Nguyen et al. 2015) was used to constructing phylogenetic tree with the TNe+R4 model that decided by automatic estimation in IQ-TREE. Bootstrap values were calculated using the standard nonparametric bootstrap method with 100 replicates. Bayesian analyses were performed using MrBayes v3.2.6 (Ronquist et al. 2012) with the SYM Gamma model. One cold and three heated Markov chain Monte Carlo (MCMC) chains were run for 1,000,000 generations, with sampling every 500 cycles. The initial 250,000 cycles were discarded as burn-in. Bayesian posterior probability was calculated from the remaining trees.

2.7. ITS-2 secondary structure

ITS-2 regions of all strains of branched filamentous ulvophytes strains (BFA102, BFA201, BFA203, BFA205, BFA208, BFA209, BFA223, BFA224, BFA226, BFA227, BFA242, BFA243, BFA244, BFA248 and BFA250) were annotated using a web interface in the ITS-2 database

(Ankenbrand et al. 2015; http://its2.bioapps.biozentrum.uni-wuerzburg.de/). The sequences of ITS-2 were folded as described previously (Matsuzaki et al. 2014) using Centroidfold (Hamada et al. 2009) and RNAfold at the RNAfold WebServer (Gruber et al. 2008; http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi). The sequences of the ITS-2 regions were aligned and the existence of compensatory base changes (CBCs) was confirmed using the program 4SALE version 1.7.1 (Seibel et al. 2006). The ITS-2 secondary structure of all samples were drawn using VARNA version 3.93 (Darty et al. 2009). For the comparison among the strains of branched filamentous ulvophytes strains, the ITS-2/CBC approach was applied following the procedure described by Darienko & Pröschod (2017). The alignments of the conserved region of ITS-2 have been translated into base pair alignment by using a number code for each base pair (1 = A-U; 2 = U-A; 3 = G-C; 4 = C-G; 5 = G•U; 6 = U•G), and the resulting numeric codes of each sequence are given in Figs. 12-16.

Chapter 4: Molecular Phylogeny and Classification of Filamentous Microalgae Belonging to the Family Kornmanniaceae

4.1. Introduction

The Ulvophyceae is one of the classes of green algae. Although many species are distributed in marine environment, some species grow in freshwater, brackish water, and terrestrial environments. Moreover, some species have symbiotic relationships with other organisms. For example, some members of the Trentepohliales form lichens with fungi (Hametner et al. 2014). In the Ulvophyceae, many macroscopic members such as *Ulva* are living in coastal zone around the world. These macroscopic species have been studied for a long time because they are easily collected and closely associated with human life as food or green tide. In contrast, microalgae belonging to the Ulvophyceae especially members having branched filamentous shape have not been studied well in comparison to macroscopic species. These species were traditionally classified in Chaetophorales (Chlorophyta, Chlorophyceae). Green algae having branched filamentous shape have been classified based on morphological features of thalli and cells, and their life styles. However, their morphologies are easily changed by environmental conditions. Moreover, some species that are distantly related phylogenetically each other have similar morphologies, that makes difficult for taxonomic classification of these algae. However, these algae are very common in various environments on the earth. Thus, reclassification of these branched filamentous microalgae is an important and required subject. Recently, Darienko & Pröschold (2017) performed the reclassification study of some filamentous and coccoidal ulvophytes using molecular methods including the phylogenetic analysis using 18S rDNA and ITS-2 sequence, and comparison of ITS-2

secondary structure for ITS-2/CBC (compensatory base changes) approach. Darienko & Pröschold (2017) focused on non-marine species using culture strains deposited in public collections. However, branched filamentous green microalgae are also common in marine environments and new strains need to be established from these environments to understand the true diversity of these algae.

In this study, sampling, establishment of culture strains, observation of morphological features and analyzing of their phylogeny were performed for the classification taxonomy of branched filamentous ulvophytes focused on marine species. In the process, it was suggested that many culture strains belonging to the family Kornmanniaceae (Ulvales, Ulvophyceae). Therefore, the purpose of this research is to clarify the diversity, phylogenetical relationship and taxonomy of branched filamentous algae belonging to the family Kornmanniaceae. In the future, it is thought that this research will lead to deeper understanding for evolution of the class Ulvophyceae.

4.2. Results

4.2.1. Phylogenetic analysis of the 18S rDNA

In this study, 68 strains were sequenced and used to perform the homology retrieval of GenBank using BLAST search. The 44 sequences of these showed high similarity to the sequences of the Ulvales and Ulotrichales. Therefore, construction of datasets were performed using 10 sequences of *Annulotesta cochlephila* and 55 ulvophyte sequences including two Oltmannsiellopsidales sequences (*Oltmannsiellopsis viridis* (P.E.Hargraves & R.L.Steele) M.Chihara & I.Inouye; FN562431, *Neodangemannia microcystis* M.J.Wynne, G.Furnari, Kryvenda & Friedl; AJ416104) and one Scotinosphaerales sequence (*Scotinosphaera austriaca* (Puncochárová) Wujek

& R.H.Thompson; HE860253) as outgroup. Final alignment was containing 109 OTUs with 1594 bases. The phylogenetic tree is shown Fig. 11. The four sequences (BFA109, BFA111, BFA112 and BFA112) were included in the Ulotrichales (Fig. 11). Interestingly, all these four sequences were derived from freshwater strains. The phylogenetic positions of these strains in the Ulotrichales were nor resolved because the OUT sampling of the Ulotrichales was poor in this analysis. Then, the taxonomical and phylogenetic relationships of them were not clear. In order to resolve this, more OTUs are need to be included for the analysis.

Other 38 sequences were included in the Ulvales *sensu* Mattox & Stewart and separated into several lineages. In the family Ulvellaceae, 16 sequences (BFA204, BFA210, BFA213, BFA220, BFA222, BFA232, BFA238, BFA241, BFA246, BFA247, BFA262, BFA267, BFA 270, BFA273, BFA273 and BFA278) were included. 11 sequences were closely related to *Ulvella endozoica* (Goldberg, Makemson & Colley) R.Nielsen, C.J.O'Kelly & B.Wysor (AY205327) and they formed a clade with high statistical supports (BV = 100 %, BPP = 1.00). Other five sequences (BFA204, BFA210, BFA213, BFA232 and BFA238) formed a robust clade with *Ulvella ramosum* (N.L.Gardner) R.Nielsen, C.J.O'Kelly & Wysor (AY205328), *Ulvella repens* (Pringsheim) R.Nielsen, C.J.O'Kelly & B.Wsor (AY303593 and FJ715684) and *Ulvella leptochaete* (Huber) R.Nielsen, C.J.O'Kelly & B.Wysor (JN104106) supported by high statistical supports (BV = 100 %, BPP = 1.00). BFA263 was included in the Ulvaceae (BV = 84 %, BPP = 0.99) and sister to *Ruthnielsenia tenuis* (Kylin) C.J.O'Kelly, B.Wynsor & W.K.Bellows AY454425 (BV = 99 %, BPP = 1.00).

All other 22 sequences were included in the Kornmanniaceae (BV = 100 %, BPP = 1.00). In the

Kornmanniaceae, the sequences determined in this study were separated into two known clades and five novel clades. These clades are named for convenience sake; 1) *Pseudendoclonium* clade, 2) *Annulotesta cochlephila* clade, 3) clade 1, 4) clade 2, 5) clade 3, 6) clade 4, and 7) clade 5 (shown in Fig. 11). Among them, *A. cochlephila* clade was discussed in chapter 3 and omitted in this chapter.

Pseudendoclonium clade included the sequences of BFA102, BFA211, BFA223, BFA224 and BFA239 although the bootstrap support was low (BV = >60 %, BPP = 0.99). In the *Pseudendoclonium* clade, BFA102, BFA211 and BFA239 formed a robust clade with high statistical supports (BV = 100 %, BPP = 1.00). This clade showed a very long blanch length. Amongst those sequences, BFA211 and BFA239 formed a clade (BV = 100 %, BPP = 1.00). BFA223 and BFA224 were closely related to *Pseudendoclonium arthropyreniae* (Vischer & Klement) Darienko & Pröschold MF034609 and two unidentified filamentous ulvophyte sequences (BV = 97 %, BPP = 0.97).

The clade 5 included the sequence of the photobiont of the ascomycete lichen (*Acremonium stroudii* K. Fletcher, F.C. Küpper & P. van West) collected from the splash zone on Ascension Island (Photobiont of ascomycete lichen KM22529) (Fletcher et al. 2017). In addition, BFA203, BFA240, BFA244, BFA249, BFA250 and BFA264, all of these sequenced in this study, were included in the clade 5 (BV = 100 %, BPP = 1.00). This clade was sister to *Annulotesta* clade (BV = 78 %, BPP = 1.00) as reported in chapter 3 and showed relatively long branch length. The clade composed of the clade 5 and *Annulotesta* clade was sister to *Halofilum* with relatively high statistical supports (BV = 80 %, BPP = 1.00).

Although BFA228, the sole member of the clade 1, was sister to *Paulbroadya*, the statistical

supports were very low (BV = > 60 %, BPP = > 0.7). Therefore, the clade 1 represented an independent lineage in the Kornmanniaceae and the phylogenetic position of this clade was uncertain at present. This situation also holds same for BFA243 that was the sole member of the clade 2 and sister to *Kornmannia leptoderma* (Kjellman) Bliding without statistical supports (BV = > 60 %, BPP = > 0.7). BFA243 showed very long branch length in the tree.

The clade 3 included BFA201, BFA205, BFA208, BFA209, BFA226, BFA227, BFA242, BFA245 and BFA248 with two *Lithotrichon* species (BV = 100 %, BPP = 1.00). In addition, the clade 3 also included uncultured ulvophyceae clone Alchichica AL52 5E 36 JN825658, *Tellamia contorta* Batters AF499663 and "*Pseudendoclonium fucicola*" AF499662. However, the detailed taxonomic information of these sequences is unclear. The intrarelationship within the clade 3 was mostly unclear with low statistical supports. BFA261 formed a clade with the clade 3 with relatively high statistical supports (BV = 89 %, BPP = 0.99). This clade was situated at the base of the Kornmanniaceae (BV = 76 %, BPP = 0.99). However, the genetic distance between BFA261 and the clade 3 was relatively large, and the former was treated as independent clade, clade 4, in this study.

4.2.2. ITS-2 secondary structure

In the 18S rDNA phylogeny, some sequences showed close relatedness each other (Fig. 11). Therefore, the investigation of ITS-2 sequence and secondary structure were performed to resolve the relationship among closely related strains. The comparison was performed each clade that showed close relatedness in phylogenetic analysis of 18S rDNA. For folding of ITS-2 sequences, Centroidfold and RNAfold were used and the results of these were edited manually according the

structure in Darienko & Pröschod (2017) as reference. The ITS-2 secondary structure of some strains such as BFA211 (*Pseudendoclonium* clade), BFA239 (*Pseudendoclonium* clade), BFA228 (clade 1), BFA240 (clade 5), BFA245 (clade 3), BFA249 (clade 5), BFA261 (clade 4) and BFA264 (clade 5) could not be constructed because the sequences of ITS-2 region for these strains were not obtained. All constructed ITS-2 secondary structures of strains have three helices (helix I, II, III), but helix IV was not discovered.

In the *Pseudendocolonium* clade, BFA223 and BFA224 were closely related to *Pseudendoclonium arthropyreniae* along with the sequences of unidentified filamentous ulvophytes (AB183573 and AB183574) with strong statistically supports (BV = 97 %, BPP = 0.97) in the 18S rDNA tree. However, genus *Pseudendoclonium* also includes some species having similar 18S rDNA sequence and morphology. Therefore, ITS-2 secondary structures of BFA223 and BFA224 were estimated and compared among species belonging genus *Pseudendoclonium* reported by Darienko & Pröschod (2017) to clarify their relationships. The estimated ITS-2 secondary structure of BFA223 is shown in Fig. 12. The ITS-2 secondary structures of BFA223 and BFA224 were concerted with *P. arthrophyreniae* (SAG467-2) and no compensatory base changes (CBCs) were detected (Fig. 12). CBCs, Hemi CBCs and insertion/deletion were detected among other *Pseudendoclonium* species. The ITS-2 secondary structure of BFA102 (Fig. 13) was markedly different with other species of *Pseudendoclonium* so that, it could not be compared. The ITS-2 secondary structure of BFA211 and BFA239 were not organized because these sequences showed some polymorphism.

The strain BFA243 representing the sole member of a novel linage (clade 2) formed a clade with *Paulbroadya* species with no statistical supports in the 18S rDNA tree (see above). The ITS-2

secondary structure of BFA243 could not compare with other species including *Paulbroadya* (Fig. 14).

BFA201, BFA205, BFA208, BFA209, BFA226, BFA227, BFA242, BFA245, BFA248 were included in the clade 3 with *Lithotrichon* species. The ITS-2 structures of *Lithotrichon pulchrum* Darienko & Pröschold (SAG2038) and *L. fluminensis* (Fritsch) B.W.Liu, Q.H.Wang, S.Y.Li, J.Fang, G.X.Liu & Z.Y.Hu (FACHB-2334) were completely identical each other (Fig. 15). The other strains in the clade 3 were separated into four groups based on the secondary structures of ITS-2 (Fig. 15). Among these groups, BFA208/209/226 possessed no CBC to *Lithotrichon* species. In comparison to this group, BFA205/227/248, BFA242, and BFA201 showed one or two CBCs. In addition, all four groups possessed one to four CBCs each other.

The clade 5 was composed of BFA203, BFA240, BFA244, BFA249, BFA250, BFA264 and previously reported sequence (Photobiont of ascomycete lichen; KM225292). The ITS-2 secondary structure of BFA203, BFA244 and BFA250 were constructed (Fig. 16) but those of other strains could not be constructed because of polymorphism in this region. BFA203 and BFA244 had the same secondary structure of ITS-2. However, in comparison between three strains, only BFA250 had different secondary structure and two CBCs, two hemi CBCs and one insertion/deletion were detected (Fig. 16).

4.2.3. Light microscopy

In this study, many branched filamentous algal strains were established. Because the morphological plasticity was reported in branched filamentous microalgae (e.g., Darienko &

Pröschod 2017), the strains were cultivated in the same condition. Most of them had typically the thalli composed of central aggregated elliptical to spherical cells and peripheral radiating filaments of cylindrical cells (Figs. 17–36). The detailed morphological features of the strains are described below on order of the clades found in the phylogenetic analysis of 18S rDNA (chapter 4.2.1). The morphological features of each strain are summarized in Tables 6–11.

***Pseudendocolonium* clade** (Figs. 17–21)

The *Pseudendoclonium* clade included BFA102, BFA211, BFA223, BFA224 and BFA239 and the morphological features of these strains are summarized in Table 6. All of them had the thallus composed of central aggregated cells and peripheral radiating filaments (Figs. 17–21). The cells in the central aggregated mass were elliptical to spherical in shapes. The sizes of cells were similar between closely related strains in 18S rDNA phylogeny (Table 6). For example, the size of elliptical to spherical cells of BFA223 and BFA224 were 5.8–12.2 µm in diameter (Figs 17, 18). The size of BFA211 and BFA239 was 4.0–7.8µm in diameter (Figs 20, 21) but that of BFA102 was 3.8–10.1µm in diameter (Fig. 19) and the fluctuation of the cell size of BFA102 was slightly bigger than these two strains. Cylindrical cells forming filaments were 7.7–30.1 µm long and 3.3–6.1 µm wide. BFA102 was smallest in the present observation, 6.5–20.5 µm long and 3.0–5.1 µm wide (Fig. 19). On the other hand, the maximum size was measured in BFA223, that was 7.9–78.6 µm long and 3.2–7.2 µm wide (Fig. 17). The cells of all strains had a single parietal chloroplast usually with one pyrenoid (Figs. 17–21). However, some strains possessed multiple pyrenoids; two to three (up to five) in BFAA223 (Fig. 17F), and two in BFA239 (Fig. 21). Cells usually contained small oil

droplets (Figs. 17–21). Filaments were composed of multiseriate elliptical to spherical cells at proximal side and uniseriate spherical to cylindrical cells at terminal side (Figs. 17–21). The degree of branching was various. BFA102, BFA223 and BFA224 sometimes formed sarcinoid packets (Fig. 18E). The cell wall of BFA223 was swollen in old cultures (Fig. 17H). Zoospore was not observed in all strains in this study.

Clade 1 (Fig. 22)

BFA228 was the sole member of the clade 1 and the morphological features of this strain are summarized in Table 7. This strain had a cushion-like thallus composed of aggregated elliptical to spherical cells and radiating filaments (Fig. 22A–D). Although BFA228 produced filaments composed of cylindrical cells, the number of these filaments was small. The size of cylindrical cells was 10.8–28.6 μm long and 3.2–6.1 μm wide. The size of elliptical to spherical cells was 4.8–9.7 μm in diameter. Cells have one parietal chloroplast, with one pyrenoid (Fig. 22D, G). Cells sometime produced small oil droplets (Fig. 22H). The proximal part of filaments frequently branched, and possessed branching initials (Fig. 22E). Zoospores were not observed.

Clade 2 (Fig. 23)

BAF243 was the sole member of the clade 2 and the morphological features of this strain are summarized in Table 8. The thallus of BFA243 was composed of central aggregation of elliptical to spherical cells and peripheral radiating filaments. The filaments were very long and frequently branched at the proximal part (Fig. 23A–C). The size of cylindrical cells was 8.9–27.2 μm long and

4.3–4.9 μm wide. The size of elliptical to spherical cells was 6.26–9.4 μm in diameter. The cells possessed a single parietal chloroplast, with one or two pyrenoids (Fig. 23C–E). Intriguingly, the terminal cylindrical cell of filaments frequently contained a large oil drop (Fig. 23F). Zoospores were not observed.

Clade 3 (Figs. 24–29)

Six strains (BFA201, BFA227, BFA242, BFA208, BFA209 and BFA226) of the clade 3 were observed in light microscopy and the morphological features of these strains are summarized in Table 9. The thallus of these strains was basically composed of central aggregated cells and peripheral radiating filaments (Figs 24–29). However, the thalli of BFA208, BFA209 and BFA226 were mainly composed of aggregated cells and filaments were not well developed (Figs 25–27). In BFA208, BFA209 and BFA226, the length of cylindrical cells in filaments (6.4–13.0 μm) was relatively shorter than those of other strains (5.9–31.8 μm) (Table 9). The cells of these six strains possessed a single parietal chloroplast, with one to three pyrenoids (Figs 24–29). Cells frequently contained small oil droplets (Fig. 28), but they were rare in BFA209 and BFA226. Quadriflagellate zoospores were observed in BFA201 and BFA227 (Figs 24H, 28H) but it was not observed in other strains. Sarcinoid packets were produced in BFA208 and BFA209 (Fig 25C).

Clade 4 (Fig. 30)

BAF261 was the sole member of the clade 4 and the morphological features of this strain are summarized in Table 10. The old thalli were composed of central aggregation of elliptical to spherical

cells, but the peripheral filaments of cylindrical cells were not well developed (Fig. 30B). However, the young thallus formed some branched filaments (Fig. 30A). The size of cylindrical cells was 11.7–40.4 μm long and 4.7–6.7 μm wide and the size of elliptical to spherical cells was 6.9–12.5 μm in diameter. Cells possessed a single parietal chloroplast, with one pyrenoid. Although each cell frequently contained small oil droplets, the terminal cylindrical cell of filaments frequently contained one or some large oil droplets (Fig. 30G, H). Zoospores were not observed.

Clade 5 (Figs. 31–36)

The clade 5 included BFA203, BFA240, BFA244, BFA249, BFA250 and BFA264, and the morphological features of these strains are summarized in Table 11. Although the shape of thallus was various, the typical thallus was composed of aggregated cells and peripheral radiating filaments, but the filaments were not developed in BFA240 (Figs 31–36). The size of cylindrical cells was 7.0–48.2 μm long and 3.1–5.4 μm wide in BFA203, BFA244 and BFA249, but slightly shorter (5.1–23.2 μm long and 2.8–5.2 μm wide) in other strains (Table 11). BFA264 sometimes formed sarcinoid packets (Fig 36G). The cells possessed a single parietal chloroplast, with one to three pyrenoids (Figs 31H, I, 32D, E, 33D–F, 34D, F, 35C–F, 36D, F, G). The cell of BFA250 contained small oil droplets (Fig 35F). The terminal cylindrical cells of filaments of BFA244 (also sometime BFA203) had a large oil drop (Fig 33G), but other strains possessed no or very small oil droplets in the cell. Quadriflagellage zoospores were observed only in BFA203 and BFA244 (Fig. 31J).

4.2.4. Transmission electron microscopy

In the transmission electron microscopy, the ultrastructure of pyrenoid was observed for BFA102, BFA211 (*Pseudendoclonium* clade), and BFA203 (clade 5).

The cell of BFA102, BFA211 and BFA203 contained a single parietal chloroplast (Fig. 37). The pyrenoid matrix was surrounded by some starch sheaths and transversed by a single thylakoid (Fig. 37).

4.3. Discussion

In this study, several novel lineages and new members of known lineages have been discovered in the Kornmanniaceae. The taxonomy of these lineages is discussed below.

***Pseudendoclonium* clade**

Pseudendoclonium was established by N. Wille (1900). The type species of the genus is *P. submarinum* and additional four species are recognized at present (*P. arthropyreniae*, *P. commune* arienko & Pröschold, *P. incrustans* (Vischer) Darienko & Pröschold, and *P. laxum* D.M.John & L.R.Johnson). The species of *Pseudendoclonium* have morphological features such as the brush-like and crust-shape thallus composed of prostrate and erect irregularly branched filaments (e.g., Mullins 2007; Škaloud et al. 2018). Each cell usually has a single, parietal, plate-like chloroplast with one pyrenoid. Some species perform both sexual and asexual reproduction. In asexual reproduction, they produce ovoid, biflagellate or quadriflagellate zoospores or akinetes. This group have been described and recognized based on morphological features. However, it was revealed very confused condition

of their taxonomy by recently phylogenetic analysis because their morphological features are similar to different species (e.g., Darienko & Pröschod 2017) and easily change by growth condition (John & Johnson 1989; Johnson & John 1990).

In this study, I isolated five strains of the *Pseudendoclonium* clade from marine and freshwater environments. In these strains, BFA223 and BFA224 can be identified certainly as *P. arthropyreniae* because of their phylogenetic position in the 18S rDNA tree and the identical secondary structure of ITS-2 (Darienko & Pröschod 2017). This identification is also supported by morphological observations. Although the cell sizes of both strains (especially the cylindrical cells of BFA223) are slightly larger than that reported previously for *P. arthopyreniae*, the size of cells in *Pseudendoclonium* is reported as very changeable according to the culture condition (John & Johnson 1989; Johnson & John 1990).

In this study, a novel subclade is discovered in the *Pseudendoclonium* clade. This novel subclade was composed of BFA102, BFA211 and BF239, and showed a very long branch in the phylogenetic tree. This high evolutional rate is probably the reason of low statistical supports (BV = > 60 %, BPP = > 0.7) for the monophyly of *Pseudendoclonium* clade in this study. Because of the low statistical supports, only the molecular phylogenetic analysis based on 18S rDNA sequences cannot provide the answer for their taxonomical position. However, these three strains have similar morphological features with some species of *Pseudendoclonium*, such as the thallus composed of aggregated cells and branched filaments and the cell with a chloroplast and pyrenoid (John & Johnson 1989, Johnson & John 1990, Darienko & Pröschod 2017). Therefore, it is suggested that these three strains belong to the genus *Pseudendoclonium*. In this novel subclade of *Pseudendoclonium*, BFA211 and BFA239 form

a clade (BV = 100 %, BPP = 1.00) that is sister to BFA102. BFA211 and BFA239 were very closely related in 18S rDNA phylogeny and their morphological features of thallus and cell size were very similar each other. According this, they are probably the same species. Unfortunately, their ITS-2 secondary structure could not be determined in this study because of polymorphism of ITS-2 sequences. There is no distinct difference of morphology between BFA102 and other two strains, but it was phylogenetically difference in 18S rDNA sequence. Moreover, only BFA102 was collected from freshwater environment. These evidences suggest that the novel subclade in the *Pseudendoclonium* clade probably include two new species (represented by BFA102 and BFA211/BFA239 respectively). The estimated ITS-2 secondary structure of BFA102 was apparently different from those of described species of *Pseudendoclonium*.

Clade 1

The clade 1 is a novel lineage found in this study and includes only a single strain, BFA228. The clade 1 was sister to *Paulbroadya* clade composed of *P. petersii* Darienko & Pröschold and *P. prostrata* (Broady & Ingerfeld) Darienko & Pröschold in this phylogenetic analysis, but the statistical support for this sister relationship was low (BV = > 60 %, BPP = > 0.7). Genus *Paulbroadya* was established by Darienko & Pröschod (2017) and the type species is *P. prostrata* that was transferred from genus *Dilabifilum*. This genus includes two species and they share morphological features, such as the thallus having prostrate and erect systems of filaments (Darienko & Pröschod 2017). The prostrate system is composed of rounded cells, but the erect systems is frequently branched uni- or bilaterally. These morphologies of thallus are also similar to the features found in BFA228. Moreover, the characteristics

of cells such as one parietal chloroplast with one pyrenoid and no zoospores are also identical. *P.prostrata* and *P. petersii* have nearly same morphological features but are different in SSU-ITS sequences(Darienko & Pröschod 2017). The ITS-2 sequence and secondary structure of BFA228 was not obtained in this study. However, the phylogenetic position of BFA228 in the 18S rDNA phylogenetic tree suggests that the strain should be treated as new genus and species in the Kornmanniaceae based on the criteria in Darienko & Pröschod (2017). Further study especially the comparison with *Paulbroadya* species to confirm the taxonomic position of BFA228.

Clade 2

As in the case of the clade 1, the clade 2 is a novel lineage found in this study and includes only a single strain, BFA243. The clade 2 was sister to *Kornmannia leptoderma* but the statistically support was low (BV = > 60 %, BPP = > 0.7). In addition, *K. leptoderma* is a macroalga having the erect membranous thallus with a branched holdfast (Weinbergeret al. 2019). Therefore, BFA243 is suggested to be a new genus and species according to morphological features and 18S rDNA phylogeny. The other molecular data such as *rbc*L sequences are reported for some species of the Kornmanniaceae including *K. leptoderma*. Therefore, these molecular data may help to clarify the taxonomic position of BFA243 (and other strains such as BFA228).

Clade 3

In the clade 3, BFA201, BFA205, BFA208, BFA209, BFA226, BFA227, BFA242, BFA245, BFA248, two *Lithotrichon* species, one environmental sequence, *Tellamia controrta* and

"*Pseudendoclonium fucicola*" were included. This environmental sequence, uncultured Ulvophyceae clone Alchichica_AL52_5E_36 (JN825658) was derived from the microbialite collected from 14 m water depth of Alchichica alkaline lake in Mexico, but detailed observation was not applied (Couradeau et al. 2011). The microbialite is one of the sediments composed by microbial mat and benthic biological community inducing to sedimentation of minerals. Many microbialites are carbonate sediments and thought to be formed by cyanobacteria (e.g., Gérard et al. 2013). In the clade 3, the sequence identified as to "*Psudendoclonium fucicola*" was included. *Pseudendoclonium fucicola* was reported as the epibiont of *Fucus inflatus* (= *F. evanescens*) in the west coast of Greenland and it was described as *Ulvella fucicola* Rosenvinge at that time (Rosenvinge 1893). After that, the species was transferred to *Pseudopringsheimia* according to the growth behavior by Wille (1909). At last, *Pseudopringsheimia fucicola* was transferred to *Psedendoclonium* by Nielsen (1980). However, *Pseudendoclonium* in the traditional sense (e.g., Nielsen, 1980) is resolved to be polyphyletic and *P. fucicola* is considered to be not the member of true *Pseudendoclonium* (Mullins 2007). However, the taxonomic position of this alga has not been settled. Moreover, although Darienko & Pröschod (2017) performed the large-scale reclassification of these ulvophycens, this study did not include *P. fucicola* in the phylogenetic analysis. The sequence (AF499663) of alga identified as *Tellamia contorta*, which is thy type species of the genus *Tellamia* is also included in the clade 3. In the original description, *T. contorta* was reported from the periostracum (outermost layer of shells) of yellow periwinkle, *Littorina obtusata* (Batters 1895a). This genus includes two species (*T. contorta* and *T. intricata*), but the difference between these species was obscure and the reevaluation of species definition was required (Norton, 1976). Based on the morphological observation using culture materials, it was

concluded that *T. intricata* is a synonym of *T. contorta* (Nielsen & McLachlan 1986). The establishment of genus *Tellamia* was questioned by Acton (1960), but *T. contorta* was included in the Kornmanniaceae with *P. fucicola* in the molecular phylogenetic studies (Hayden & Waaland 2002). However, *T. contorta* and *P. fucicola* were not used in the phylogenetic analyses of some recent studies (e.g., Darienko & Pröschod 2017; Gasulla et al. 2019; Liu et al. 2019). The phylogenetic positions of *T. contorta* and *P. fucicola* were isolated from all branched filamentous algal strains established in this study, two *Lithotrichon* species, and environmental sequence JN825658. Although the taxonomic position of *T. contorta* and *P. fucicola* is unclear at present, their phylogenetic positions in the 18S rDNA tree suggest that *T. contorta* and *P. fucicola* are members of the same genus to *Lithotrichon*.

All nine strains established in this study (BFA201, BFA205, BFA208, BFA209, BFA226, BFA227, BFA242, BFA245 and BFA248) were closely related to *L. fluminensis* and *L. pulchrum*. *L. fluminensis* (FACHB-2334) was transferred from *Gongrosira* to *Lithotrichon* by Liu et al (2019), but no conclusion was reached whether *L. fluminensis* was synonym of *L. pulchrum* or not because of insufficient specimens and collection information. However, *L. fluminensis* and *L. pulchrum* formed a clade and have closest relationship to each other in the 18S rDNA and ITS phylogeny by Liu et al (2019) and the result of 18S rDNA phylogeny in this study. Therefore, the ITS-2 secondary structure of *L. fuluminensis* was constructed and compared with that of *L. pulchrum* in this study. The result of this analysis indicates that *L. fuluminensis* and *L. pulchrum* have the same secondary structure, and *L. fuluminensis* is probably synonym of *L. pulchrum*. However, *L. fuluminensis* has two different bases in 18S rDNA and one in ITS rDNA (Liu et al. 2019), and additional analysis and comparison are necessary.

The morphological features of all strains closely related to *Lithotrichon* are similar to those of *Lithotrichon* species. Therefore, these strains seem to belong to the genus *Lithotrichon*. However, the species taxonomy of these strains is difficult. Based on the comparison of ITS-2 secondary structure, the strains in the clade 3 can be classified into four groups. The group composed of BFA208, BFA209 and BFA226 has one hemi-CBC but no CBC compared to *L. pulchrum* and *L. fuluminensis*. This feature suggests that BFA208, BFA209 and BFA226 are the same species to *L. pulchurum* and *L. fuluminensis*. However, the more detailed analysis is necessary to reach the taxonomical conclusion because BFA209 and BFA226 were collected from marine environments but BFA208, *L. pulchrum* and *L. fuluminensis* were collected from freshwater environments (*L. pulchurm* was described as the photobiont of a lichen *Verrucaria rheitrophila*). Other groups (BFA205/227/248, BFA242, BFA201) have one to two CBCs to *Lithotrichon* and one to four CBCs each other. This result suggests that these strains contain plural species. However, no distinct morphological features corresponding to the ITS-2 groups are found and the phylogenetic relationship among these groups and *Lithotrichon* species strains was unclear in the 18S rDNA phylogeny. Therefore, further studies are necessary for the species taxonomy of these strains.

Clade 4

The cade 4 is a novel lineage found in this study and includes only a single strain, BFA261. The clade 4 was sister to the clade 3 (see above) in the 18S rDNA phylogenetic analysis, and this sister relationship was supported by moderate statistical supports (BV = 89 %, BPP = 0.99). BFA261 has a thallus composed of the aggregated elliptical to spherical cells and peripheral radiating filaments. This

feature of thallus was similar to other linages including clade 3. However, the cell size of BFA261 was larger than the strains of clade 3. Moreover, although the strains of clade 3 produced oil droplets in cells, BFA261 especially produced large oil droplets especially in their terminal cylindrical cell of filaments. This difference and the phylogenetically distance in the 18S rDNA phylogeny suggest that BFA261 represent a new genus and species in the Kornmanniaceae.

Clade 5

The clade 5 included the sequences from BFA203, BFA240, BFA244, BFA249, BFA250, BFA264 and environmental sequence, Photobiont of ascomycete lichen (KM225292). This environmental sequence KM225292 was originated from marine lichen collected from a narrow seawater blowhole located at Whale Point, Ascension Island (Central South Atlantic Ocean, 7.95°S 14.3°W) (Fletcher et al. 2017). Fletcher et al. (2017) showed that this marine lichen was composed of green alga and *Acremonium stroudii* (Hypocreales, Ascomycota). They described some morphological features of the photobiont, but they could not isolate this green alga. The green algae could grow only with symbiotic fungus and they formed a compound colony. The thallus of this photobiont formed multi-cellular tufts composed of circular to oval (coccoid) cells and filaments of cylindrical cells in liquid medium (Fletcher et al. 2017). These morphological features are similar to those of strains included in the clade 5. However, the strains established in this study especially BFA203, BFA244 and BFA249 have longer cylindrical cells (about 50 µm long) in comparison to the photobiont. The reason of this incongruence may be the measurement of the photobiont cell size by SEM, and the restrain of photobiont cell growth by symbiotic fungi. In the 18S rDNA phylogeny, all sequences formed a clade

and they were nearly identical (except for BFA250). These strains are different morphologically from the sister group of the clade 5, *Annulotesta cochlephila*, in the absence of ring-like structure on the cell wall. Therefore, molecular and morphological features support that these strains and the photobiont of marine lichen in Ascension Island represent a new genus in the Kornmanniaceae.

BFA203 and BFA244 have the same ITS-2 secondary structure and this evidence suggest that these strains are the same species in this clade 5. However, BFA250 has the different secondary structure from these two strains and two CBCs, two hemi CBCs and one insertion/deletion were decided between them. That evidence suggests that BFA250 is a different species from BFA203 and BFA244. Morphological study also suggests that clade 5 includes plural species. BFA203, BFA244 and BFA249 have very long cylindrical cells that are not found in other strains. BFA240 and BFA264, both of which were collected from shells, produce no oil droplets in the cells. Therefore, molecular and morphological studies suggest that the clade 5 contains three species.

Chapter 5: General discussion

5.1. Evolution of organization in the Kornmanniaceae

Many species belonging to the Ulvophyceae has been reported in previous studies so far. Accordingly, ulvophycean species have very various shapes (e.g., unicell, colony, unbranced filaments, branched filaments, leaf shape and coenocytes) (Škaloud et al. 2018). Among these, this study focuses on the branched filamentous microalgae because these algae have been not well studied than macroalgae and the their taxonomy of these algae needs the reclassification. I collected these microalgae from several places including marine, freshwater and terrestrial environments and established many culture strains. In the results of 18S rDNA phylogenetic studies using these strains, I found that some branched filamentous microalgae were included in the Ulvellaceae (Ulvales) or Ulotrichales, but most of these were included in the Kornmanniaceae (Ulvales). In the Kornmanniaceae, nine genera are reported (Škaloud et al. 2018; Guiry & Guiry 2021). Among of these, *Kornmannia* and *Blidingia* are macroalgae (Golden & Cole 1986; Lindstrom & Golden, 2006; Weinbergeret al. 2019). The other all genera have typically the thallus composed of aggregated cells and peripheral radiating filaments (Nielsen & McLachlan 1986; Darienko & Pröschold 2017; Liu et al. 2019). In the 18S rDNA phylogenetic tree, macroscopic genera (*Kornmannia* and *Blidingia*) were not closely related and were not situated at the base of the Kornmanniaceae. This result suggests that the branched filamentous thalli is the ancestral character of the Kornmanniaceae and the formation of macroscopic thalli such as in *Kornmannia* probably occurred independently at least twice from these branched filamentous microalgal conditions.

5.2. Pyrenoid ultrastructure in the Kornmanniaceae

Many species of algae and hornworts have pyrenoid in their chloroplast (Villarreal & Renner 2012). The pyrenoid is the structure accumulated ribulose-1, 5-bisphosphate carboxylase/ oxygenase (RubisCO) that role in carbon fixation in photosynthesis (Holdsworth 1971; Lacoste-Royal & Gibbs 1987). The presence or absence, and structure of pyrenoid are used characteristics for species identification in taxonomy (Hori 1973; Friedl 1989; Ikeda & Takeda 1995). In the Ulvophyceae, the structure and distribution of pyrenoids have been reported by some studies. Hori (1973) observed the ultrastructure of pyrenoids using ten species of *Monostroma* and *Blidingia minima* (Nägeli ex Kützing) Kylin, *Enteromorpha intestinalis* (Linnaeus) Nees (*Ulva intestinalis*), *Ulva* sp., *Casosiphon fulvescens* (C.Agardh) Setchell & N.L.Gardner and *Percursaria percursa* (C.Agardh) Rosenvinge, and found eight type of pyrenoids based on ultrastructure of the pyrenoid matrix, starch sheaths and pattern of the intrapyrenoidal thylakoid.

The pyrenoid ultrastructure of *Pseudendoclonium arthropyreniae* (Vischer & Klement) Darienko & Pröschold (SAG467-2; Fig. 7 in chapter 3), *Annulotesta chclephila* (NN-301; Fig. 6 in chapter 3), BFA102, BFA203 and BFA211 were investigated in this study. All these strains possessed the pyrenoid matrix surrounded by some starch sheaths and transversed by thylakoid membrane. In the most strains, the pyrenoid matrix was transvered by single thylakoid (type I in Hori 1973). However, three separated thylakoids were found in the pyrenoid matrix of *P. arthropyreniae* (type III in Hori 1973). This difference is also associated with the number of starch sheaths surrounding pyrenoid matrix. In previous studies, type I pyrenoid was also reported from *Lithotrichon fluminensis* (Fritsch) B.W.Liu, Q.H.Wang, S.Y.Li, J.Fang, G.X.Liu & Z.Y.Hu (Liu et al. 2019),

Blidingia spp. (Hori 1972; Lindstrom & Golden, 2006). In the Kornmanniaceae, genus *Kornmannia* has unique pyrenoids. Hori (1973) reported that the chloroplasts of *Monostroma zostericola* Tilden (=*K. zostericola*, regarded now as a synonym of *Kornmannia leptoderma* (Kjellman) Bliding) contained the solid body composed of an electron-dense ground material enclosed by a well-defined, double-layered membrane and there were no starch sheaths and transverse thylakoids (type VIII in Hori 1972). Although *Kornmannia* was considered to be closely related to *Blidingia* based on cytology, lifecycle and development of thalli (Bliding 1968; Gayral 1971), Cole (1986) proposed the Kornmanniaceae based on this unique pyrenoid ultrastructure.

Halofilum ramosum Darienko & Pröschold was possesses the pyrenoid transversed by a single thylakoid (type I in Hori 1973) as closely related members such as *A. chochlephila* and BFA203 (Gasulla et al. 2019). However, *H. ramosum* have some plastoglobuli between thylakoid lamellae. Plastoglobuli are plastid lipoprotein particles surrounded by a membrane lipid monolayer (van Wijk & Kessler 2017). Plastoglobuli were not observed in my all strains. These diversities suggest that the ultrastructural feature of pyrenoids is useful for the classification in the Kornmanniaceae, and further studies on members that have not been studied yet (e.g., genus *Paulvroadya* and *Tellamia*) are necessary.

Based on the available data described above, it is suggested that the type I pyrenoid is the ancestral character in the Kornmanniaceae because the phylogenetically diverse genera of this family have the type I pyrenoid. Type I pyrenoid is also very common in the order Ulvales (Hori 1973; Lindstrom & Golden, 2006). Moreover, it is also known that some species of the other orders in the class ulvophyceae such as the Cladophorales and Ulotrihcales also have the type I or similar type

(type II and III) of pyrenoid (Boedeker et al. 2012; Wetherbee & Verbruggen 2016). Therefore, the type I pyrenoid would be the ancestral feature in the Ulvophyceae and secondary changes probably occurred multiple times independently.

5.3. Habitats of the Kornmanniaceae

In this study, the hidden diversity of the Kornmanniaceae is clarified, and new members of the family are described from various environments. Therefore, I plot their habitat information on the phylogenetic tree obtained in this study to consider the evolution of habitats in the Kornmanniaceae (Fig. 38). This analysis suggests that the ancestral habitat of the family is in marine. Furthermore, the most members of the Ulvophyceae are living in marine, and the class is considered to evolve mainly within marine.

Figure 38 suggests that the advance into freshwater environments would occur plural times in the Kornmanniaceae, In the tree, BFA102 (*Pseudendochlonium* clade) and *Lithotrichon fluminensis* were collected from freshwater habitats and cultivated in freshwater media (Liu et al. 2019; this study). In addition, BFA201, BFA208, BFA242, BFA245 (clade 3) and BFA228 (clade 1) were collected from freshwater environments, but were isolated from precultures using seawater medium (ESM). The advance into the freshwater environments from marine is also reported in other families in the Ulvales. For example, the most species of *Ulva* (Ulvaceae) are known to grow in marine but some species such as *Ulva limnetica* K.Ichihara & S.Shimada grow in brackish and freshwater environment (Ichihara et al. 2009; Rybak 2018). This species was collected from the rivers in Ishigaki Island and Yonagunijima Island but it was not found in brackish and marine environment.

However, this species could be cultivated under all salinity condition (Ichihara et al. 2009). The molecular phylogenetic analysis indicated that *U. limnetica* were embedded phylogenetically in marine species. This phylogenetic relationship suggests that linage originally growing in marine would get the ability to adaptat to freshwater environment (Ichihara et al. 2009). In *U. limnetica*, the expression analysis was performed to understand the mechanism of adaptation to freshwater in *Ulva* (Ichihara et al. 2011). Some studies about the adaptation to freshwater environment from marine have been reported using macroalgae such as *U. limnetica*, *U.linza* Linnaeus and *U. prolifera* O.F.Müller (Ichihara et al. 2011; Masakiyo et al. 2016). However, there are no studies about this topic using microalgae. However, microalgae also important ecologically and it is necessary to study the adaptation to freshwater environment in microalgae. Because the colonization into freshwater would occur multiple times in the microalgal members of the Kornmanniaceae, the study using the members of this family based on the genetic information of *U. limnetic* would be useful to understand the adaptation to freshwater environment in the Ulvales.

The photobionts of lichens are common in the Kornmanniaceae (e.g., Thüs et al. 2011; Darienko & Pröschold 2017; Gasulla et al. 2019). In the figure 38, lichen is the second common habitat in this family. Figure 38 suggests that the colonization in fungi (forming lichens) would occur multiple times in the evolution of the Kornmanniaceae. Although lichens live in various environments on the earth, some species grow in the coastal environment that instantaneously or temporarily exposed to the sprays of the seawater (Fletcher et al. 2017; Gasulla et al. 2019). These lichens are called "marine lichens". Interestingly, the most photobiont algae in the Kornmanniaceae are photobionts of marine lichens [excluding *Pseudendoclonium incrustans* (Vischer) Darienko &

Pröschold and *Lithotrichon pulchrum* Darienko & Pröschold]. Because marine lichens grow in the environment that fluctuates intensely in salinity, they have the ability of adaptation to both high and low salinity (Delmail et al. 2013; Gasulla et al. 2019). The environment of intra-lichen might be stable in salinity in comparison to the direct splash zone. However, such environment also might be suitable for the evolution of algae to adopt osmotically different habitats. Therefore, these lichen symbiosis might be a one of the intermediate stages in the colonization on freshwater or land habitats from marine environment.

Many strains of the Kornmanniaceae were collected from substrates in water such as rocks and leaves. Therefore, the most members of the Kornmanniaceae are probably benthic organisms and it is necessary to collect substrate samples for search this group. At present, what type of substrate is preferable for these algae is uncertain. The environmental sequence in the clade 3 (JN825658) was reported from a calcified microbialite (Couradeau et al. 2011). In addition, *Tellamia contorta* (AF499663), *Blidingia dawsonii* and *Annulotesta cochlephila* were collected from the surface of shellfish shells (Batters. 1895a, b, Hao-Jan and Bo-Tang 1984, Nielsen and McLachlan 1986, Lindstrom and Golden, 2006, this study). According to these information, some members of the Kornmanniaceae may prefer calcareous substrates.

5.4. Taxonomy of the Kornmanniaceae

The Kornmanniaceae established by Golden & Cole (1986) is a family of the Ulvales. The family originally included only the genus *Kornmannia*. However, recent studies have added some genera including branched filamentous microalgae (Darienko & Pröschold 2017).

In this study, I collected and isolated many strains of branched filamentous green microalgae from marine, freshwater, and terrestrial (mostly on the shell of door snails) environments. In these strains, many freshwater strains were the members of the Ulotrichales. On the other hand, the most strains from marine were the members of the Ulvales. In the Ulvales, many strains were revealed to be the members of the Kornmanniaceae, but some were the members of the Ulvellaceae (and one from the Ulvaceae). This result in the present study suggests that the Kornmanniaceae is the most important group as marine branched filamentous microalgae in marine environments. In the Ulvellaceae, only two distinct lineages were recognized in this study. However, at least six independent lineages were detected in the Kornmanniaceae. In these six lineages, four of these represent the novel clades that are never reported previously. If the criteria to establish new genera in previous studies (e.g., Darienko & Pröschold 2017) are applied, these four clades can be treated as independent new genera. In these novel clades, the clade 5 had been known only as the photobiont of marine lichen collected from Ascension Island (Fletcher et al. 2017), contained relatively many strains. This evidence suggests that this novel clade, possibly a new genus of the Kornmanniaceae represents a relatively important members in marine environments.

In addition, I found a novel distinct lineage in the genus *Pseudendocloniuom* in this study. This lineage probably includes at least two new species of *Pseudendocloniuom*. The present study also suggests that the genus *Lithotrichon* is relatively common as branched filamentous microalgae in marine coastal environments. I found that the genus includes a genetically diverse species complex that may represent plural species. In addition, the molecular phylogenetic analysis suggests the strains identified as "*Pseudendoclonium fucicola"* and *Tellamia controrta* are closely related to

Lithotrichon. Further studies are necessary to clarify the taxonomy of the clade containing *Lithotrichon* (clade 3 in this study) both in the generic and species level.

The present study also revealed a terrestrial epizoic member in the Kornmanniaceae. This unique alga was described as a new genus and species, *Annulotesta cochlephila*. The species represents a independent lineage in the Kornmanniaceae. Interestingly, the present study suggests the high specificity to the shell of door snails as the substrate of this species. Additional studies on the habitat, life cycle, and relationship to the host snails are necessary to clarify the nature of this unique epizoic alga. The studies on the door snails are also important because all described epizoic algae (*Trichophilus neniae*, *Trypanochloris clausiliae*, and *A. cochlephila*) on land snails have been reported from only door snails (clausiliid land snails) (Lagerheim 1892; Geitler 1935). In addition, the discovery of *A. cochlephila* suggests that the surface of other animals is possible habitat of novel green algae.

The present study is the first large-scale taxonomic study on the branched filamentous green microalgae using many strains collected from marine and epizoic environments. Molecular and morphological analyses on these strains show several novel lineages including *Annulotesta cochlephila* gen. et sp. nov. in the Kornmanniaceae. It means that I clarify the hidden diversity of the Kornmanniaceae based on molecular and morphological features. Further taxonomic studies based on the same strategy to the present one will clarify the true diversity of the branched filamentous green microalgae that are very common in various habitats.

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Tables

Table 1. Current classification of the class Ulvophyceae (edited from Škaloud et al. 2018)

Class	Order	Family
Ulvophyceae	Ulvales Blackman &Tansley	Bolbocoleonaceae O'Kekky & Rinkel
		Cloniophoraceae A. L. Carlile, C. J. O'Kelly & A. R. Sherwood
		Kornmanniaceae Goldel & Cole
		Phaeophilaceae D. F. Chappell, C. J. O'Kekky, L. W. Wilcox & al.
		Ulvellaceae Schmidle
		Ulvaceae J. V. Lamouroux ex Dumortier
		Ctenocladiaceae Borzì
	Ulotrichales Borzì	Ulotichaceae Kützing
		Binucleariaceae Škaloud & Leliaert
		Planophilaceae Škaloud & Leliaert
		Hazeniaceae Škaloud & Leliaert
		Helicodictyaceae Whitford & G. J. Schumacher
		Sarcinofilaceae Škaloud & Leliaert
		Tupiellaceae Škaloud & Leliaert
		Gomontiaceae De Toni
		Monostromataceae Kunieda
		Kraftionemaceae Wetherbee & Verbruggen
		Acrosiphoniaceae Jónsson
	Chlorocystidales Kornmann & Sahling	Chlorocystidaceae Kornmann & Sahling
	Oltmannsiellopsidales T. Nakayama, S. Watanabe & I. Inouye	Oltmannsiellopsidaceae T. Nakayama, S. Watanabe & I. Inouye
	Scotinosphaerales Škaloud et al.	Scotinosphaeraceae Škaloud et al.
	Ignatiales Leliaert & Škaloud	Ignatiaceae Leliaert & Škaloud
	Cladophorales Haeckel	Anadyomenaceae Kützing
		Cladophoraceae Wille
		Okellyaceae Leliaert & Rueness
		Pithophoraceae Wittrock
		Pseudocladophoraceae Boedeker & Leliaert
		Siphonocladaceae F. Schmitz

Table 1 Current classification of the class Ulvophyceae (continue)

Class	Order	Family
		Valoniaceae Kützing
	Bryopsidales J. H. Schaffner	Bryopsidaceae Bory de Saint-Vincent
		Codiaceae Kützing
		Derbesiaceae Hauck
		Caulerpaceae Kützing
		Dichotomosiphonaceae Chadefaud ex G. M. Smith
		Halimedaceae Link
		Pseudocodiaceae L. Hillis-Colinvaux
		Rhipiliaceae O. Dragastan et al.
		Udoteaceae J. Agardh
		Ostreobiaceae P. C. Silva
	Dasycladales Pascher	Dasycladaceae Kützing
		Polyphysaceae Kützing
	Trentepohliales Chadefaud ex R. H. Thompson & D. E. Wujek	Trentepohliaceae Hansgirg

Table 2. Current classification of the family Kornmanniaceae

Family	Genus
Kornmanniaceae	*Blidingia*
	Kornmannia
	Lithotrichon
	Pseudendoclonium
	Halofilum
	Paulbroadya
	Neostromatella
	Tellamia

Table 4. Strains of branched filamentous algae established in this study.

	Strain	Locality	Date	Environment
Freshwater	BFA-101	Shimoda City, Shizuoka Pref.	2017.3.18	Surface of pipe in river
	BFA-102	Shimoda City, Shizuoka Pref.	2017.3.18	Surface of rock in puddle
	BFA-103	Kumagaya City, Saitama Pref.	2017.4.13	Water of pond
	BFA-104	Kashiwazaki City, Niigata Pref.	2017.4.1	Water of pond
	BFA-106	Tsukuba City, Ibaraki Pref.	2017.5.25	Water of pond
	BFA-109	Tsukuba City, Ibaraki Pref.	2017.4.22	Water of pond
	BFA-110	Tsukuba City, Ibaraki Pref.	2017.422	Water of pond
	BFA-111	Kumagaya City, Saitama Pref.	2017.4.13	Water of pond
	BFA-112	Tsukuba City, Ibaraki Pref.	2017.4.22	Water of pond
	BFA-113	Kashiwazaki City, Niigata Pref.	2017.4.1	Water of pond
Seawater	BFA-201	Ishigaki City, Okinawa Pref.	2017.2.20	Brackish water sample from the side of Nagura bridge
	BFA-203	Ishigaki City, Okinawa Pref.	2017.2.20	Seawater sample from the side of Nagura bridge
	BFA-204	Ishigaki City, Okinawa Pref.	2017.2.20	Seawater sample from Nagura bay
	BFA-205	Yaeyama District, Okinawa Pref.	2017.2.21	Washed sample of coral in Yubushima island
	BFA-208*	Yaeyama District, Okinawa Pref.	2017.2.21	Washed sample of stone in Shiiragawa river
	BFA-209	Ishigaki City, Okinawa Pref.	2017.2.20	Mud sample in Sakieda bridge
	BFA-210	Ishigaki City, Okinawa Pref.	2017.2.20	Seawater sample from Nagura bay
	BFA-211	Yaeyama District, Okinawa Pref.	2017.2.21	Washed sample of shell in Yubushima island
	BFA-212	Shimoda City, Shizuoka Pref.	2017.3.16	Sample of plankton net
	BFA-213	Yaeyama District, Okinawa Pref.	2017.2.22	Washed sample of coral from the side of Mitarahashi bridge
	BFA-214	Yaeyama District, Okinawa Pref.	2017.2.22	Mud sample in Sonai port
	BFA-215	Yaeyama District, Okinawa Pref.	2017.2.21	Washed sample of brown algae in Funaura port
	BFA-216	Yaeyama District, Okinawa Pref.	2017.2.22	Washed sample of shell from the side of Mitarahashi bridge
	BFA-217	Ishigaki City, Okinawa Pref.	2017.2.20	Seawater sample from beach beside of Inoda port

BFA-218	Yaeyama District, Okinawa Pref.	2017.2.22	Washed sample of shell in Yubushima island
BFA-219	Yaeyama District, Okinawa Pref.	2017.2.22	Mud sample in Sonai port
BFA-220	Ishigaki City, Okinawa Pref.	2017.2.20	Mud sample in Sukuji beach
BFA-221	Yaeyama District, Okinawa Pref.	2017.2.22	Washed sample of coral from the side of Mitarahashi bridge
BFA-222	Yaeyama District, Okinawa Pref.	2017.2.20	Washed sample of coral in Sonai port
BFA-223	Shimoda City, Shizuoka Pref.	2017.3.16	Sample of plankton net
BFA-224	Shimoda City, Shizuoka Pref.	2017.3.16	Shimoda Marine Research Center, University of Tsukuba, Surface of water plumbing
BFA-226	Ishigaki City, Okinawa Pref.	2017.2.20	Washed sample of coral from beach beside of Isobe bridge
BFA-227	Ishigaki City, Okinawa Pref.	2017.2.20	Washed sample of coral from coast in Ohara
BFA-228*	Yaeyama District, Okinawa Pref.	2017.6.8	Water sample of filled ground
BFA-229	Yaeyama District, Okinawa Pref.	2017.2.21	Washed sample of shell in Nakama river
BFA-230	Ishigaki City, Okinawa Pref.	2017.2.20	Washed sample of filamentose green algae in beach beside of Isobe bridge
BFA-231	Yaeyama District, Okinawa Pref.	2017.2.22	Washed sample of coral from the side of Mitarahashi bridge
BFA-232	Yaeyama District, Okinawa Pref.	2017.2.21	Washed sample of leaf in boat embarkation area of Urauchi river
BFA-234	Ishigaki City, Okinawa Pref.	2017.2.20	Washed sample of filamentose green algae in beach beside of Isobe bridge
BFA-236	Ishigaki City, Okinawa Pref.	2017.2.20	Washed sample of coral in beach beside of Isobe bridge
BFA-237	Yaeyama District, Okinawa Pref.	2017.2.21	Washed sample of leaf in boat embarkation area of Urauchi river
BFA-238	Ishigaki City, Okinawa Pref.	2017.2.20	Washed sample of coral from coast in Ohara
BFA-239	Republic of Palau	2017.11.5	Surface of mangroves in Clearlake

BFA-240	Republic of Palau	2017.11.6	Surface of shell from beach in Carp island
BFA-241	Republic of Palau	2017.11.7	Surface of shell at “shell road” in Carp island
BFA-242*	Yaeyama District, Okinawa Pref.	2017.6.12	Washed sample of leaf in Geda river
BFA-243	Republic of Palau	2017.11.5	Washed sample of leaf in Clearlake
BFA-244	Republic of Palau	2017.11.4	Koror, Lake 1, sample of washed leaf
BFA-245*	Republic of Palau	2017.11.4	Airai, stream, sample of washed leaf
BFA-246	Republic of Palau	2017.11.5	Smoky Lake, sample of washed leaf
BFA-247	Republic of Palau	2017.11.5	Smoky Lake, sample of washed mosses
BFA-248	Republic of Palau	2017.11.7	Ulong Island, beach, sand water
BFA-249	Republic of Palau	2017.11.7	Ongael Island, sample of washed seaweeds
BFA-250	Republic of Palau	2017.11.5	Clearlake, sample of washed leaf
BFA-251	Ishigaki City, Okinawa Pref.	2017.2.20	Sand, beach near Isobe bridge,
BFA-252	Republic of Palau	2017.11.4	Washed sample of shell, coast of Airai
BFA-253	Republic of Palau	2017.11.4	Washed sample of leaf, Lake1, Koror
BFA-254	Republic of Palau	2017.11.5	Washed sample of limestone, Smoky Lake
BFA-255	Republic of Palau	2017.11.5	Washed sample of mosses, Smoky Lake
BFA-256	Republic of Palau	2017.11.6	Washed sample of shell, Carp Island
BFA-257	Republic of Palau	2017.11.6	Washed sample of seaweeds, Lake2, Milky Way
BFA-258	Republic of Palau	2017.11.7	Still water of shell, beach, Carp Island
BFA-259	Republic of Palau	2017.11.7	Washed sample of leaf, Carp Island
BFA-260	Republic of Palau	2017.11.7	Washed sample of seaweeds, Ongael
BFA-261	Republic of Palau	2017.11.5	Washed sample of limestone, Smoky Lake
BFA-262	Republic of Palau	2017.11.5	Washed sample of limestone, Smoky Lake
BFA-263	Republic of Palau	2017.11.5	Washed sample of leaf, Smoky Lake
BFA-264	Republic of Palau	2017.11.7	Washed sample of shell, Carp Island

* Collected from freshwater environment, but occurred from seawater medium (ESM).

Table 5. Primer sets used in this study

	Forward primer	Reverse primer
SSU rDNA	SR1 5’ -TACCTGGTTGATCCTGCCAG-3’ Nakayama et al. (1998)	SR12 5’ -CCTTCCGCAGGTTCACCTAG-3’ Nakayama et al. (1998)
	SR4 5 ’-AGCCGCGGTAATTCCAGCT-3’ Nakayama et al. (1998)	SR9 5’ -AACTAAGAACGGCCATGCAG-3’ Nakayama et al. (1998)
ITS rDNA	LR3 TAGTTCACCATCTTTCGGGT	
	SR10 5’ -AGGTCTGTGATGCCCTTAGA-3’ Nakayama et al. (1998)	ITS055R 5’- CTCCTTGGTCCGTGTTTCAAGACGGG-3’ (Marin et al. 2003)

Table 6. Summary of morphological feature of *Pseudendoclonium*

Strain name	BFA102	BFA211	BFA223	BFA224	BFA239
Thallus	Thallus composed cell aggregation and branched filaments	Thallus composed cell aggregation and branched filaments	Thallus composed cell aggregation and branched filaments	Thallus composed cell aggregation and branched filaments	Thallus composed cell aggregation and branched filaments
Cell size (Cylindrical cell)	6.46–20.49 μm long 3.03–5.12 μm wide	7.29–28.29 μm long 3.25–4.25 μm wide	7.88–78.63 μm long 3.16–7.23 μm wide	12.05–30.07 μm long 4.19–6.08 μm wide	7.69–30.09 μm long 4.65–5.21 μm wide
Cell size (Elliptical and spherical cell)	3.78–10.12 μm in diameter	4.02–7.57 μm in diameter	6.03–11.49 μm in diameter	5.76–12.19 μm in diameter	4.67–7.77 μm in diameter
Chloroplast	1 Parietal	1 Parietal	1 Parietal	1 Parietal	1 Parietal
Pyrenoid	1	1	1 Sometime 2 or 3 (Max5)	1	1 or 2
Oil drop	Vegetative cells contain some small oil droplets.	Vegetative cells contain some small oil droplets.	Vegetative cells contain some oil droplets.	Vegetative cells contain some small oil droplets.	Vegetative cells contain some small oil droplets.
Zoospore	Not observed	Not observed	Not observed	Not observed	Not observed
Habitat	Freshwater (terrestrial) Water sampler form rock cavity.	Marine Water sampler of washed shell.	Marine Sample from plankton net.	Marine Seawater waterworks Surface of vinyl hose	Marine Surface of mangroves
Note	Form sarcinoid packets.	Filaments were sometime branched on one side.	Form sarcinoid packets. Cell wall of older cell were swollen. Spherical cells also form filaments. Filaments frequently branched. Filaments were very long	Form sarcinoid packets. Spherical cells also form filaments. Thallus form various shapes.	Proximal side of filaments more branched rather than terminal side.

Table 7. Summary of morphological feature of clade 1

Strain name	BFA228
Thallus	Cushion shape that composed of prostrate and erect filaments, and cell aggregation
Cell size (Cylindrical cell)	10.78–28.56 µm long 3.17–6.09 µm wide
Cell size (Elliptical and spherical cell)	4.76–9.70 µm in diameter
Chloroplast	1 Parietal
Pyrenoid	1
Oil drop	Sometime produce some small oil droplets
Zoospore	Not observed
Habitat	Freshwater (terrestrial) Water sample of filled ground (Occurred from ESM)
Note	Proximal side of filaments were sometime swollen.

Table 8. Summary of morphological feature of clade 2

Strain name	BFA243
Thallus	Thallus composed cell aggregation and branched filaments
Cell size (Cylindrical cell)	8.86–27.22 μm long 4.28–4.88 μm wide
Cell size (Elliptical and spherical cell)	6.26–9.38 μm in diameter
Chloroplast	1 Parietal
Pyrenoid	1 Sometime 2
Oil drop	Terminal cell of filaments contain large oil drop
Zoospore	Not observed
Habitat	Marine Water sample of washed leaf
Note	Filaments were very long. Proximal side of filaments more branched rather than terminal side.

Table 9. Summary of morphological feature of clade

Strain name	BFA201	BFA208	BFA209	BFA226	BFA227	BFA242
Thallus	Thallus composed cell aggregation and peripheral radiating filaments.	Thallus composed cell aggregation of elliptical to spherical cells.	Thallus composed cell aggregation of elliptical to spherical cells.	Thallus composed cell aggregation and branched filaments.	Thallus composed cell aggregation and peripheral radiating filaments.	Thallus composed cell aggregation and peripheral radiating filaments.
Cell size (Cylindrical cell)	5.93–16.09 µm long 3.39–3.97 µm wide	6.69–10.12 µm long 3.55–5.25 µm wide	7.26–12.95 µm long 3.79–5.66 µm wide	6.44–12.13 µm long 3.56–5.46 µm wide	9.62–31.81 µm long 4.08–8.78 µm wide	9.28–18.10 µm long 5.65–7.39 µm wide
Cell size (Elliptical and spherical cell)	3.61–13.00 µm in diameter	3.32–11.48 µm in diameter	3.35–12.89 µm in diameter	4.34–11.82 µm in diameter	5.21–12.23 µm in diameter	6.63–13.24 µm in diameter
Chloroplast	1 Parietal	1 Parietal	1 Parietal	1 Parietal	1 Parietal	1 Parietal
Pyrenoid	1 Sometime 2	1	1 Sometime 2 or 3	1	1	1 Sometime 2
Oil drop	Vegetative cells contain some small oil droplets.	Vegetative cells contain some small oil droplets.	Rarely produce	Rarely produce	Older vegetative cells contain some small oil droplets.	Vegetative cells contain some small oil droplets.
Zoospre	Quadriflagellate zoospores	Not observed	Not observed	Not observed	Quadriflagellate zoospores	Not observed
Habitat	Brackish water sample	Freshwater Washed sample of stone from river (Occurred from ESM)	Marine Mud sample	Marine Washed sample of coral	Marine Washed sample of coral	Freshwater Washed sample of leaf from river (Occurred from ESM)
Note	Filaments were rarely branched. Proximal side of filaments more branched rather than terminal side. Older filaments were composed spherical cells.	Form sarcinoid packets Filaments and cylindrical cells were undeveloped. Filaments were rarely branched.	Form sarcinoid packets. Filaments and cylindrical cells were undeveloped. Filaments were rarely branched.	Filaments frequently branched. Cylindrical cells of filaments were undeveloped and short.	Cell wall of older cell were swollen. Filaments were rarely branched. Sometime branches were produced from center part of cylindrical cells.	Filaments frequently branched.

Table 10. Summary of morphological feature of clade 4

Strain name	BFA261
Thallus	Thallus composed cell aggregation of elliptical to spherical cells and peripheral radiating filaments..
Cell size (Cylindrical cell)	11.74–40.41 μm long 4.66–6.71 μm wide
Cell size (Elliptical and spherical cell)	6.87–12.48 μm in diameter
Chloroplast	1 Parietal
Pyrenoid	1
Oil drop	Vegetative cells contain some small oil droplets. Terminal cell of filaments contain one or some large oil droplets.
Zoospore	Not observed
Habitat	Marine Water sample of washed limestone
Note	Proximal side of filaments more branched rather than terminal side. Chloroplasts of cylindrical cell of filaments were become thin and grow long.

Table 11. Summary of morphological feature of

Strain name	BFA203	BFA240	BFA244	BFA249	BFA250	BFA264
Thallus	Thallus composed cell aggregation and peripheral radiating filaments.	Thallus composed cell aggregation and peripheral radiating filaments.	Thallus composed cell aggregation and peripheral radiating filaments.	Thallus composed cell aggregation and peripheral radiating filaments.	Thallus composed cell aggregation and peripheral radiating filaments.	Older thallus composed cell aggregation. Young thallus produced filaments of cylindrical cell.
Cell size (Cylindrical cell)	7.05–47.62 μm long 3.06–8.21 μm wide	5.14–12.60 μm long 2.75–3.96 μm wide	19.20–48.18 μm long 4.62–5.36 μm wide	9.24–45.11 μm long 3.49–4.77 μm wide	7.93–18.44 μm long 3.05–4.61 μm wide	8.62–23.18 μm long 2.87–5.24 μm wide
Cell size (Elliptical and spherical cell)	4.69–11.31 μm in diameter	3.11–7.12 μm in diameter	4.35–13.18 μm in diameter	4.65–9.28 μm in diameter	4,14–10.41 μm in diameter	3.93–9.81 μm in diameter
Chloroplast	1 Parietal	1 Parietal	1 Parietal	1 Parietal	1 Parietal	1 Parietal
Pyrenoid	1 Sometime 2	1	1 Sometime 2 or 3	1 Sometime 2	1 Sometime 2	1
Oil drop	Rarely produce Terminal cell of filaments somtime contain large oil drop	Not observed	Terminal cell of filaments contain large oil drop	Not observed	Vegetative cells contain some small oil droplets.	Rarely produce
Zoospre	Quadriflagellate zoospores	Not observed	Quadriflagellate zoospores	Not observed	Not observed	Not observed
Habitat	Marine Seawater sample	Marine (beach) Surface of shell	Marine Water sample of washed leaf	Marine Washed seaweeds	Marine Washed leaf	Marine (beach) Washed sample of shell
Note	Filaments were rarely branched. Filaments were very long. Thallus have various shapes.	Filaments were rarely branched. Cylindrical cells were formed but filaments were undeveloped. Each cell sometime easily separate.	Filaments were rarely branched. Sometime branches were produced from center part of cylindrical cells Chloroplasts of cylindrical cell of filaments were become thin and grow long.	Proximal side of filaments more branched rather than terminal side. Filaments were very long.		Form sarcinoid packets

Figures

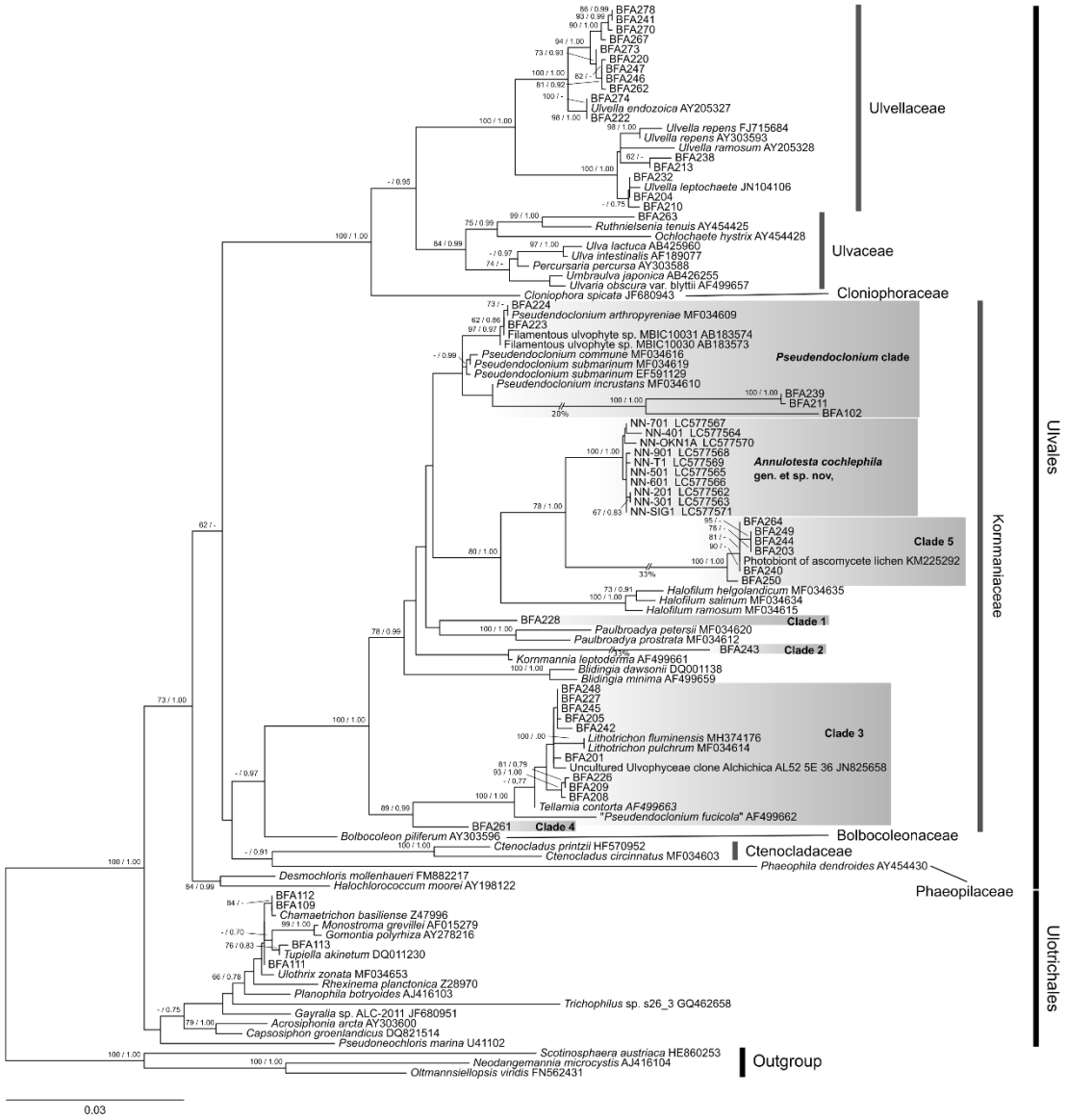
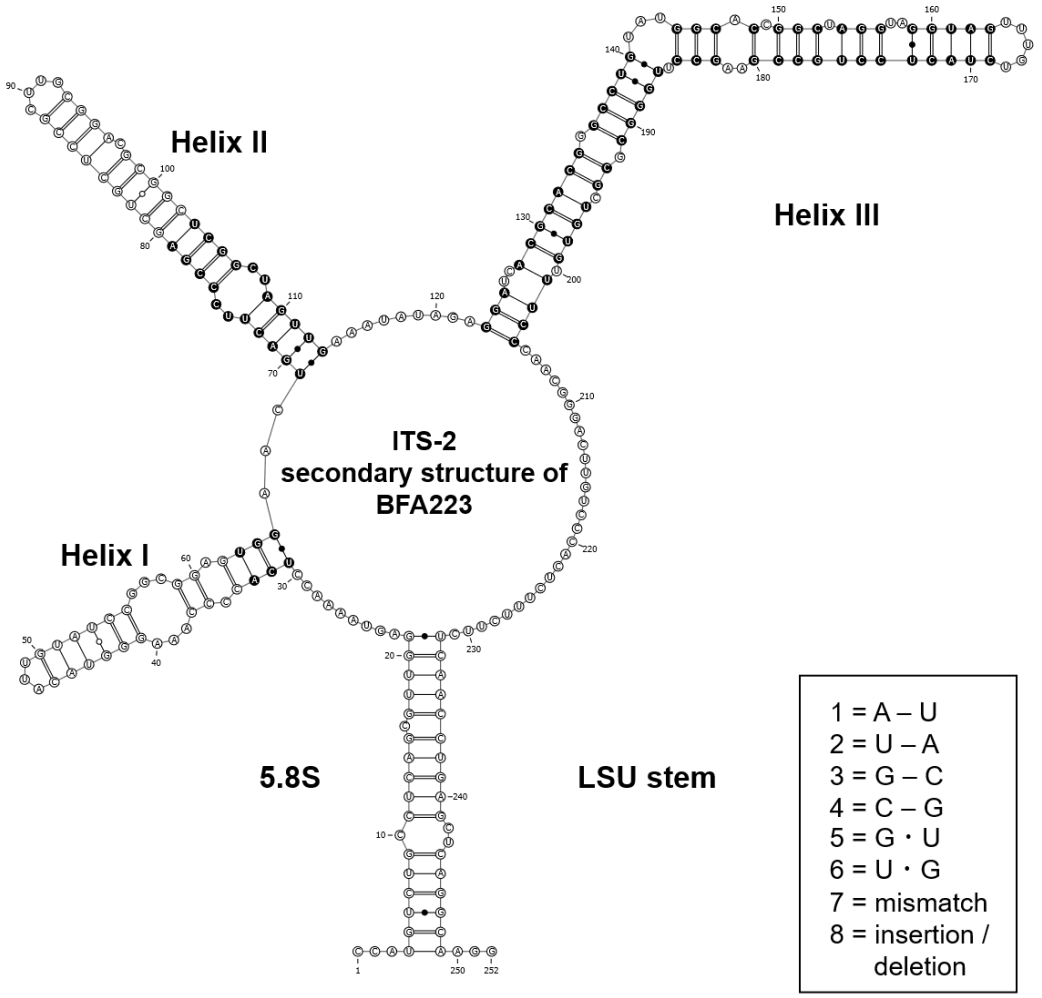

BFA278
BFA241
BFA270
BFA267
BFA273
BFA220
BFA247
BFA246
BFA262
BFA274
Ulvella endozoica AY205327
BFA222
Ulvella repens FJ715684
Ulvella repens AY303593
Ulvella ramosum AY205328
BFA238
BFA213
BFA232
Ulvella leptochaete JN104106
BFA204
BFA210
BFA263
Ruthnielsenia tenuis AY454425
Ochlochaete hystrix AY454428
Ulva lactuca AB425960
Ulva intestinalis AF189077
Percursaria percursa AY303588
Umbraulva japonica AB426255
Ulvaria obscura var. blyttii AF499657
Cloniophora spicata JF680943
BFA224
Pseudendoclonium arthropyreniae MF034609
BFA223
Filamentous ulvophyte sp. MBIC10031 AB183574
Filamentous ulvophyte sp. MBIC10030 AB183573
Pseudendoclonium commune MF034616
Pseudendoclonium submarinum MF034619
Pseudendoclonium submarinum EF591129
Pseudendoclonium incrustans MF034610
BFA239
BFA211
BFA102
NN-701 LC577567
NN-401 LC577564
NN-OKN1A LC577570
NN-901 LC577568
NN-T1 LC577569
NN-501 LC577565
NN-601 LC577566
NN-201 LC577562
NN-301 LC577563
NN-SIG1 LC577571
BFA264
BFA249
BFA244
BFA203
Photobiont of ascomycete lichen KM225292
BFA240
BFA250
Halofilum helgolandicum MF034635
Halofilum salinum MF034634
Halofilum ramosum MF034615
BFA228
Paulbroadya petersii MF034620
Paulbroadya prostrata MF034612
BFA243
Kornmannia leptoderma AF499661
Blidingia dawsonii DQ001138
Blidingia minima AF499659
BFA248
BFA227
BFA245
BFA205
BFA242
Lithotrichon fluminensis MH374176
Lithotrichon pulchrum MF034614
BFA201
Uncultured Ulvophyceae clone Alchichica AL52 5E 36 JN825658
BFA226
BFA209
BFA208
Tellamia contorta AF499663
"Pseudendoclonium fucicola" AF499662
BFA261
Bolbocoleon piliferum AY303596
Ctenocladus printzii HF570952
Ctenocladus circinnatus MF034603
Phaeophila dendroides AY454430
Desmochloris mollenhaueri FM882217
Halochlorococcum moorei AY198122
BFA112
BFA109
Chamaetrichon basiliense Z47996
Monostroma grevillei AF015279
Gomontia polyrhiza AY278216
BFA113
Tupiella akinetum DQ011230
BFA111
Ulothrix zonata MF034653
Rhexinema planctonica Z28970
Planophila botryoides AJ416103
Trichophilus sp. s26_3 GQ462658
Gayralia sp. ALC-2011 JF680951
Acrosiphonia arcta AY303600
Capsosiphon groenlandicus DQ821514
Pseudoneochloris marina U41102
Scotinosphaera austriaca HE860253
Neodangemannia microcystis AJ416104
Oltmannsiellopsis viridis FN562431
Ulvellaceae
Ulvaceae
Cloniophoraceae
Pseudendoclonium clade
Annulotesta cochlephila gen. et sp. nov,
Clade 5
Clade 1
Clade 2
Clade 3
Clade 4
Kornmanniaceae
Bolbocoleonaceae
Ctenocladaceae
Phaeopilaceae
Outgroup
Ulvales
Ulotrichales
0.03

Figure 11

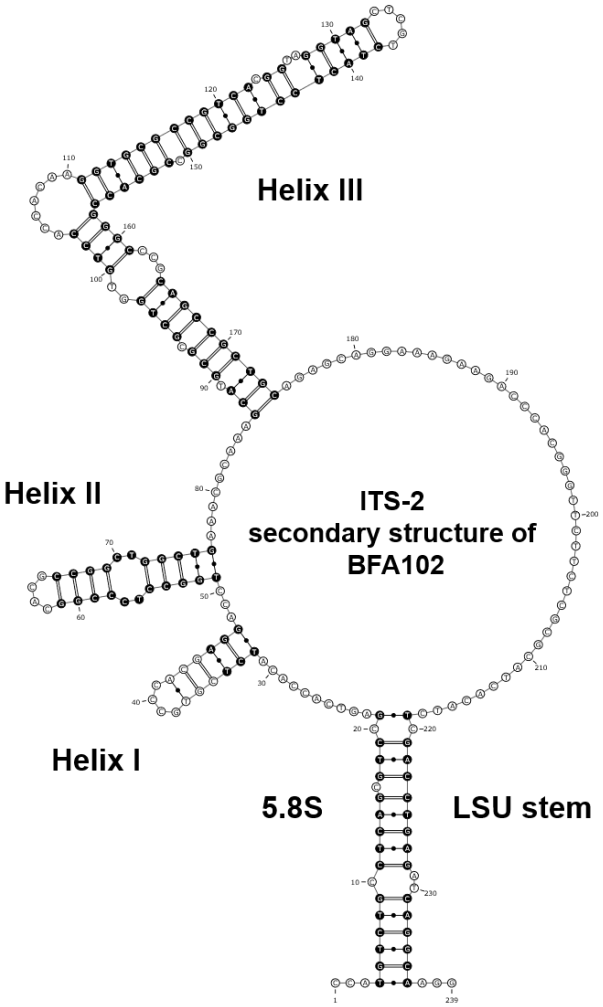
Maximum-likelihood (ML) phylogenetic tree of the Ulvales and Ulotrichales using 1,594 positions from the 18S rDNA sequences (109 OTUs). The TNe+R4 model was chosen as best fit model in ML and Baysian analysis. Values on each node indicate ML bootstrap probabilities (≥ 60 %)/Bayesian posterior probabilities (≥ 0.7).



Strain	5.8S / LSU stem	Helix I	Helix II	Helix III	
SAG 2237	2364234241332435	641	65142774431	331431343433446533443341335321388	*P. submarinum*
ULVO-26	2364234241332435	641	65142774431	331431343433446533443341335321388	
SAG 467-2	2364234241332235	641	65142774431	331145841433446533443341335321388	*P. arthopyreniae*
BFA223	2364234241332235	641	65142774431	331145841433446533443341335321388	
BFA224	2364234241332235	641	65142774431	331145841433446533443341335321388	
CCAP 415/1	2364234241332435	641	65542774431	331645343344655343443342335321342	*P. incrustans*
SAG 2051	2364234241332435	641	65142774431	331434343433446533443341335321388	*P. commune*
ULVO-21	2364234241332435	641	65142774431	331434343433446533443341335321388	
SAG 2236	2364234241332435	641	65142774431	331434343433446533443341335321388	
ULVO-62	2364234241332435	641	65142774431	331434343433446533443341335321388	
CBC	----------●--	---	----------	---●●●--●●●●-●●●●------●---------	
HCBC	----------	---	--●-------	---●-●------●----------	
I / D	----------	---	----------	------●----------●●	

Figure 12.

ITS-2 secondary structure of BFA233 and BFA224 including other species and strain of *Pseudendoclonium* (modified from Darienko & Pröschod 2017). The barcode region was translated into a numeric code as described in the Materials and Methods and is provided for each clone sequences in the box below the structure, as in Darienko & Pröschod (2017). The base changes (CBCs, HCBCs, and Insertions/Deletions) were indicated by dots at the under part.

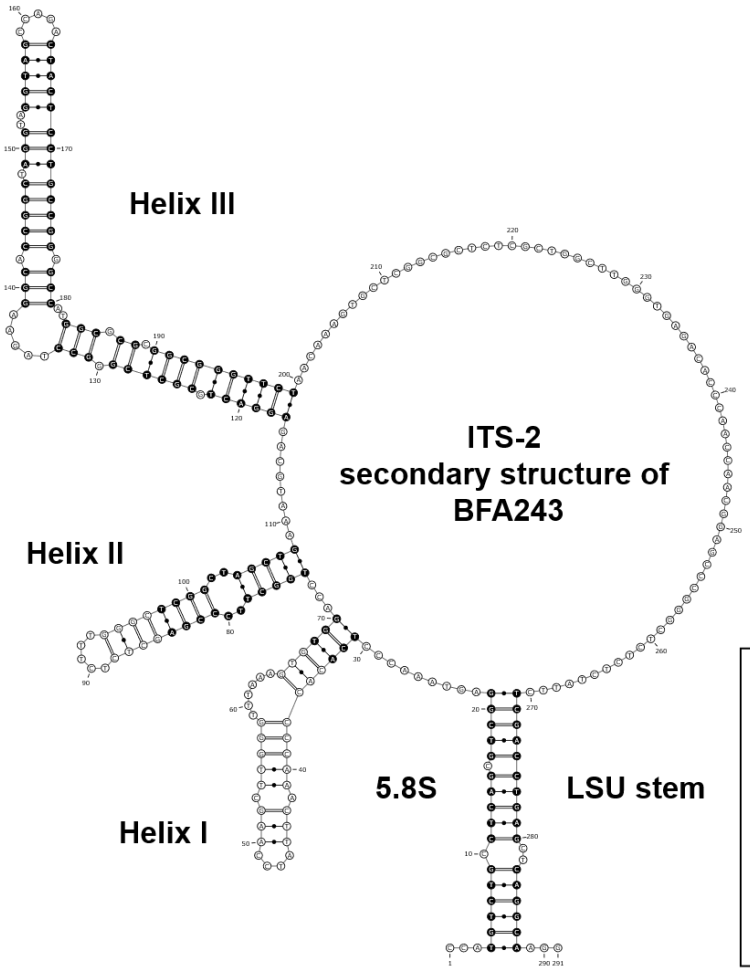


1 = A – U
2 = U – A
3 = G – C
4 = C – G
5 = G・U
6 = U・G
7 = mismatch
8 = insertion / deletion

Strain	5.8S / LSU stem	Helix I	Helix II	Helix III
BFA102	2364234241332475	641	65344774433	341343342336443323434436413353213

Figure 13.

ITS-2 secondary structure of BFA102. The barcode region was translated into a numeric code as described in the Materials and Methods and is provided for each clone sequences in the box below the structure, as in Darienko & Pröschod (2017).

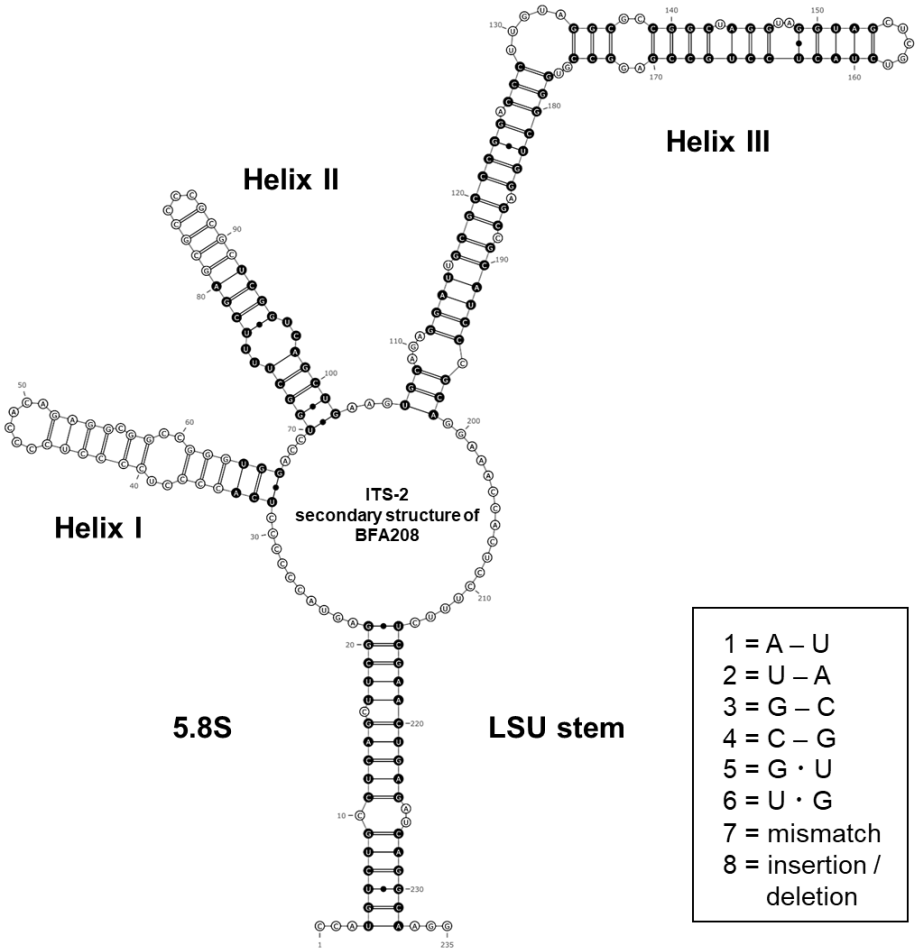


1 = A – U
2 = U – A
3 = G – C
4 = C – G
5 = G・U
6 = U・G
7 = mismatch
8 = insertion / deletion

Strain	5.8S / LSU stem	Helix I	Helix II	Helix III
BFA243	2364234241332435	641	65342774431	135146434643344334443341353213

Figure 14.

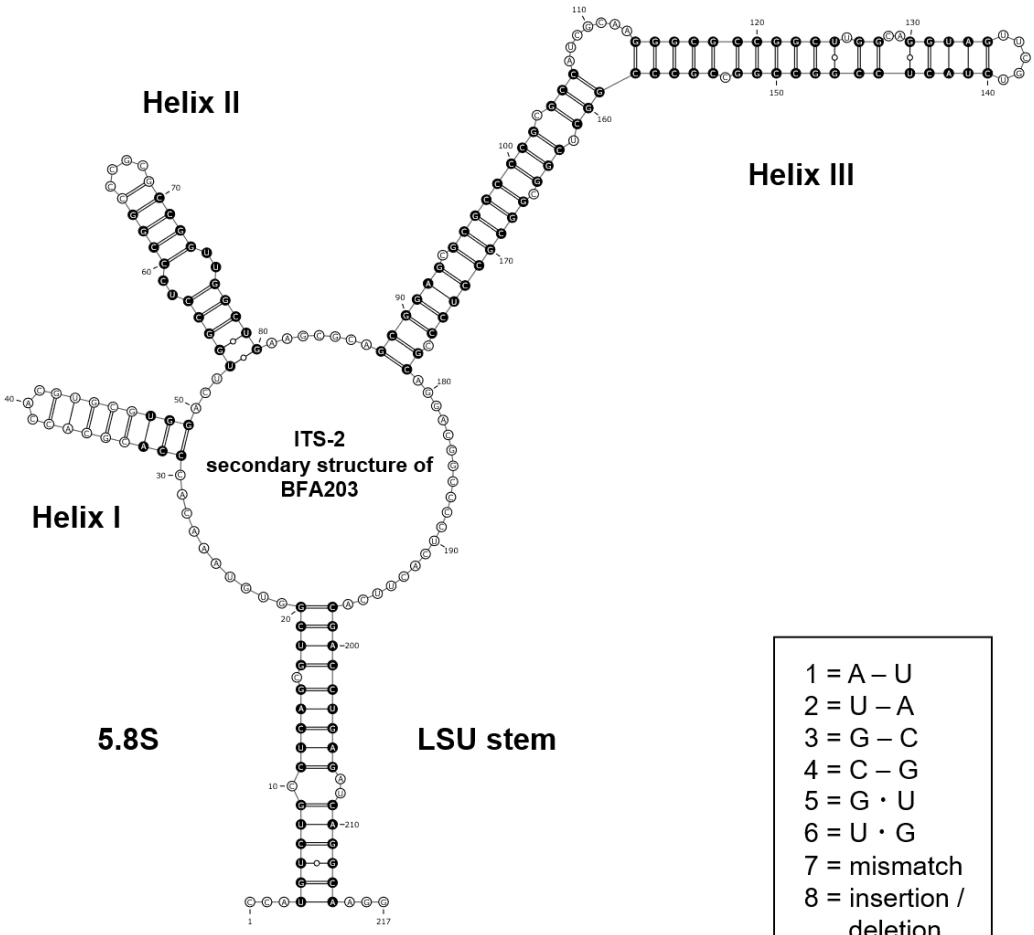
ITS-2 secondary structure of BFA243. The barcode region was translated into a numeric code as described in the Materials and Methods and is provided for each clone sequences in the box below the structure, as in Darienko & Pröschod (2017).



Strain	5.8S / LSU stem	Helix I	Helix II	Helix III	
SAG2038	2364234241322435	641	65342774431	2348331234344453444583348433413353213 8	*Lithotrichon pulchrum*
FACHB-2334	2364234241322435	641	65342774431	2348331234344453444583348433413353213 8	*Lithotrichon fluminensis*
BFA208	2364234241322435	641	65342776431	2348331234344453444883348433413353213 8	
BFA209	2364234241322435	641	65342776431	2348331234344453444883348433413353213 8	
BFA226	2364234241322435	641	65342776431	2348331234344453444883348433413353213 8	
BFA205	2364234241322435	641	65342774431	2388581234344458444883345443413353213 4	
BFA227	2364234241322435	641	65342774431	2348531234384453444543545443413353213 8	
BFA248	2364234241322435	641	65342774431	2348531234384453444543545443413353213 4	
BFA242	2364234241322435	641	65342774431	2341331864544453444583348433413353213 2	
BFA201	2364234241322435	641	65342774431	2843111234344883444843345443413353213 8	
CBC	----------	---	----------	----●●--●----------●---------●	
HCBC	----------	---	--------●---	---●●-----●----------●----------	
I / D	----------	---	----------	-●●●-●-●----●-●●●---●●---●---------●	

Figure 15.

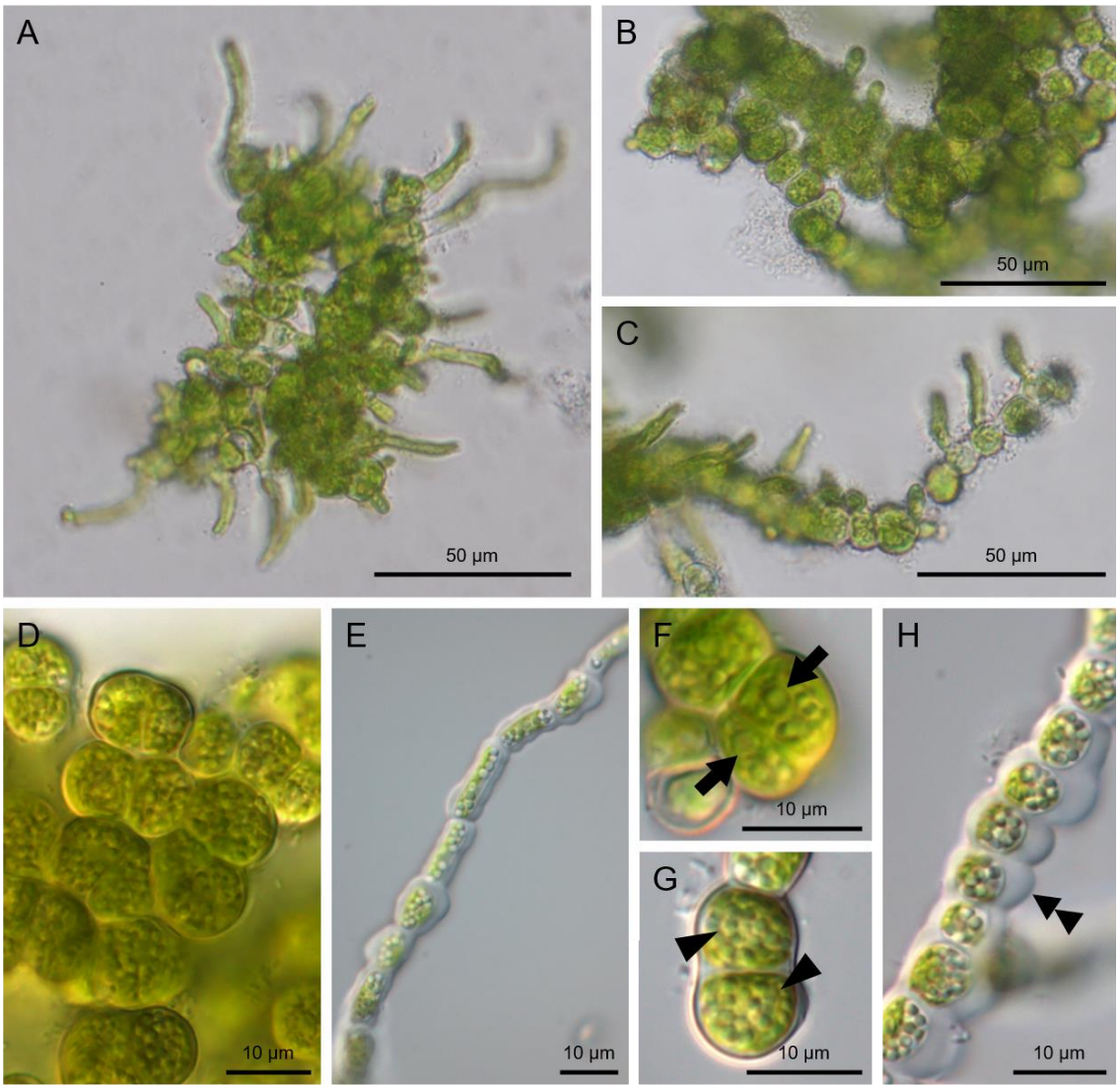
Comparison of ITS-2 secondary structure of clade 3. The secondary structure of BFA208 was shown as representative and other seven strains were shown by number code. The barcode region was translated into a numeric code as described in the Materials and Methods and is provided for each clone sequences in the box below the structure, as in Darienko & Pröschod (2017). The base changes (CBCs, HCBCs, and Insertions/Deletions) were indicated by dots at the under part.



Strain	5.8S / LSU stem	Helix I	Helix II	Helix III
BFA203	236423424133243	441	65344774433	34331334344443834433343443346335321 3
BFA244	236423424133243	441	65344774433	34331334344443834433343443346335321 3
BFA250	236423424133243	641	65344774433	34331334343443434453343443541335321 3
CBC	---------------	---	----------	----------•----------•-------
HCBC	---------------	•--	----------	----------•----------
I / D	---------------	---	----------	----------•----------

Figure 16.

ITS-2 secondary structure of BFA203, BFA244 and BFA250 (clade 5). The barcode region was translated into a numeric code as described in the Materials and Methods and is provided for each clone sequences in the box below the structure, as in Darienko & Pröschod (2017). The base chnges (CBCs, HCBCs and Insertions/Deletins) were indicated by dots at the under part.


A
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B
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G
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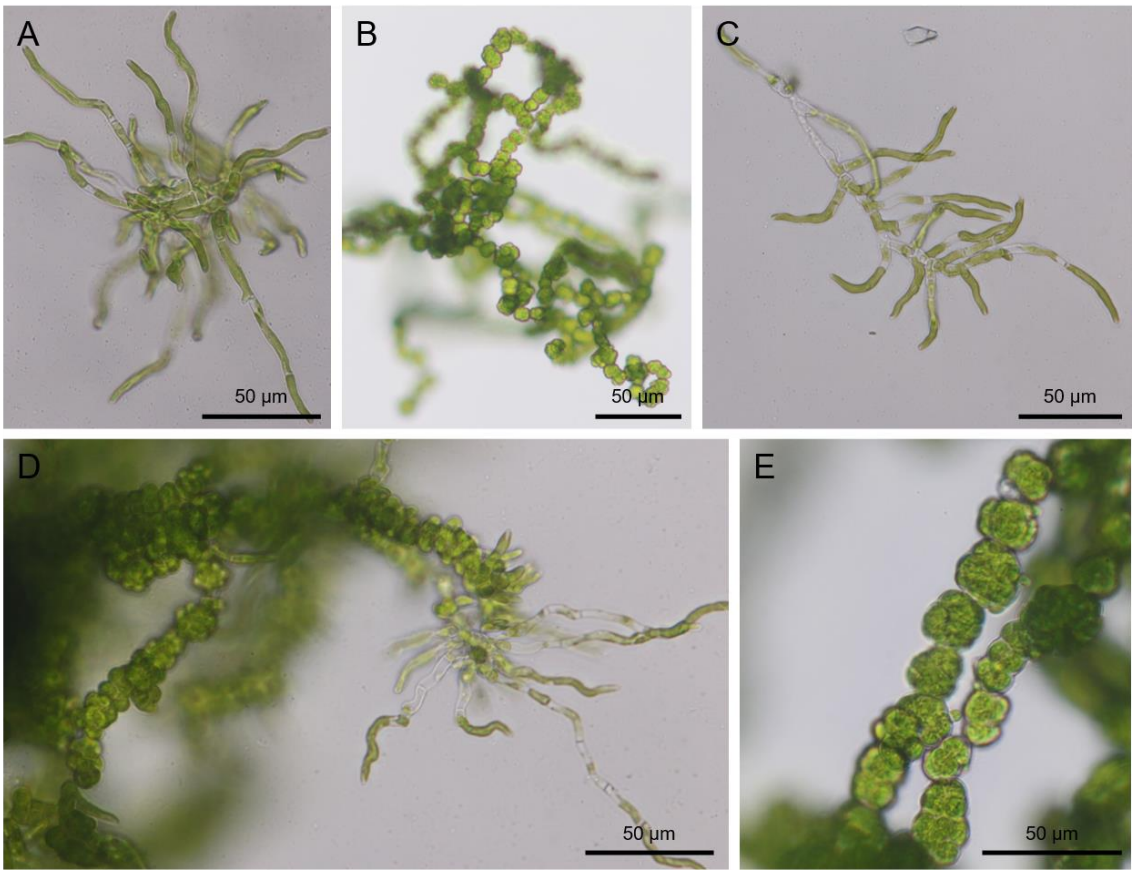
H
10 µm

Figure 17.

Light micrographs of BFA223. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Basal part of filaments composed of multiseriate elliptical to spherical cells. (C) Terminal region of filaments composed uniseriate cylindrical or spherical cells, branching on one side. (D) Elliptical to spherical cells composing the central part of a thallus and basal parts of filaments. (E) Cylindrical cells composing filaments. (F) Pyrenoid in a cell (arrows). (G) Vegetative cells contain some oil droplets (arrowheads). (H) Swollen cell wall of old cells (double arrowhead).


A
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D
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E
50 µm

Figure 18.

Light micrographs of BFA224. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Thallus composed of elliptical to spherical cells. (C) Thallus composed of prostrate filaments. (D) Basal part of filaments were composed of multiseriate elliptical to spherical cells and terminal side of filaments were composed of uniseriate cylindrical cells. (E) Sarcinoid packets.

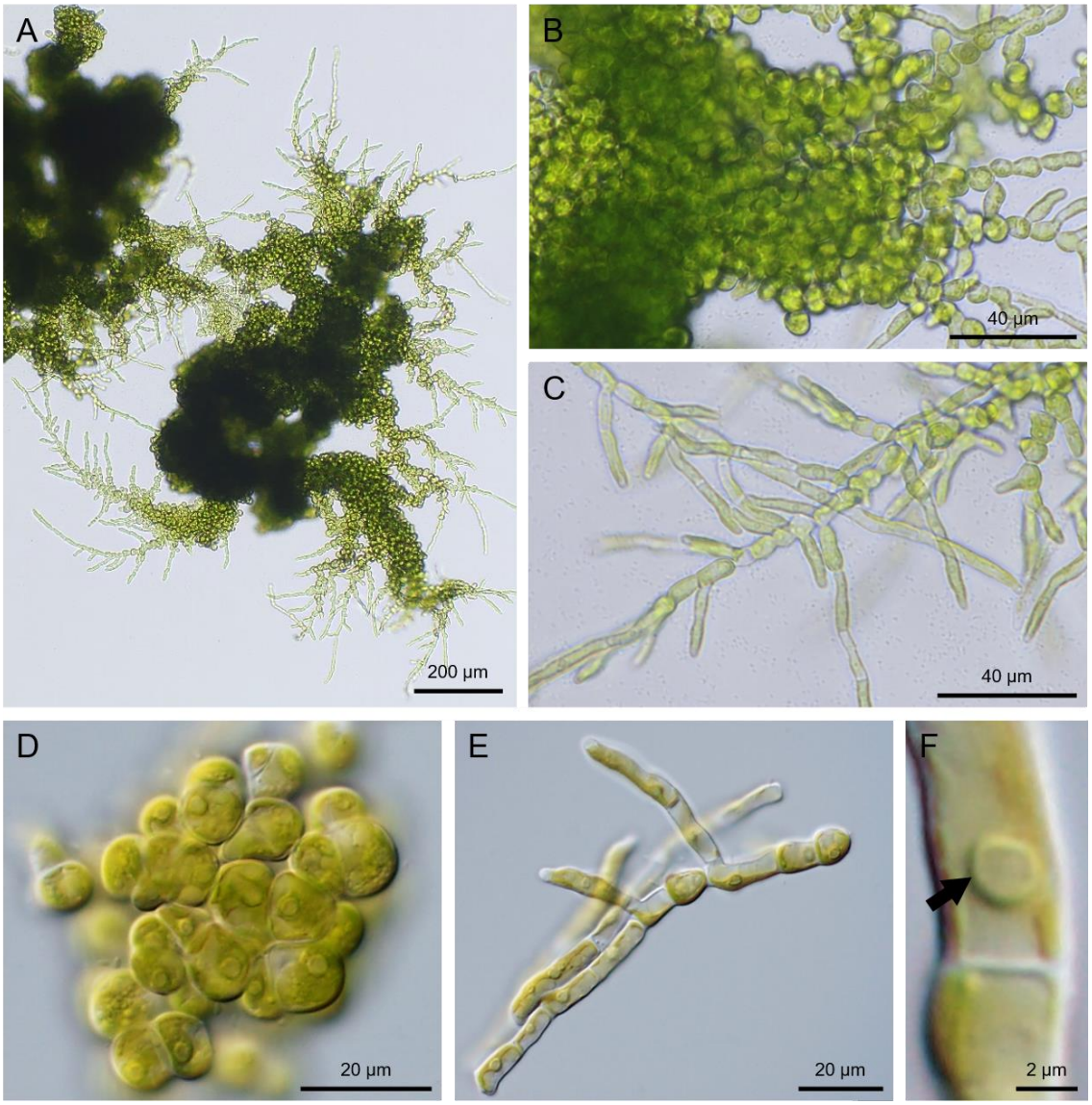
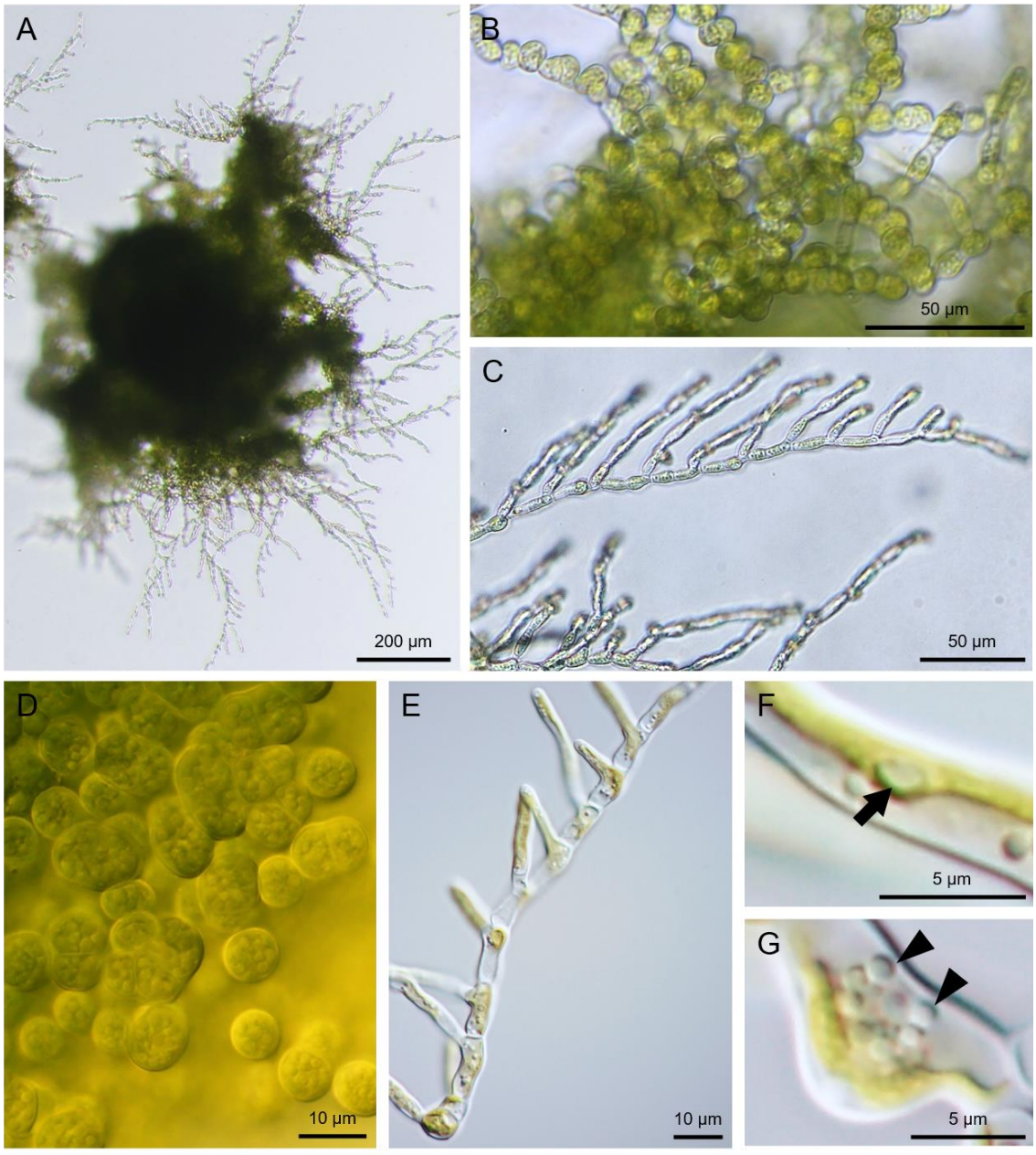

A
200 µm
B
40 µm
C
40 µm
D
20 µm
E
20 µm
F
2 µm

Figure 19.

Light micrographs of BFA102. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Basal part of filaments composed of multiseriate elliptical to spherical cells. (C) Terminal region of filaments branched on both sides and composed uniseriate cylindrical cells. (D) Elliptical to spherical cells composing the central part of a thallus and basal part of filaments. (E) Cylindrical cells composing filaments. (F) Pyrenoid in a cell (arrows).


A
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B
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C
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D
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F
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G
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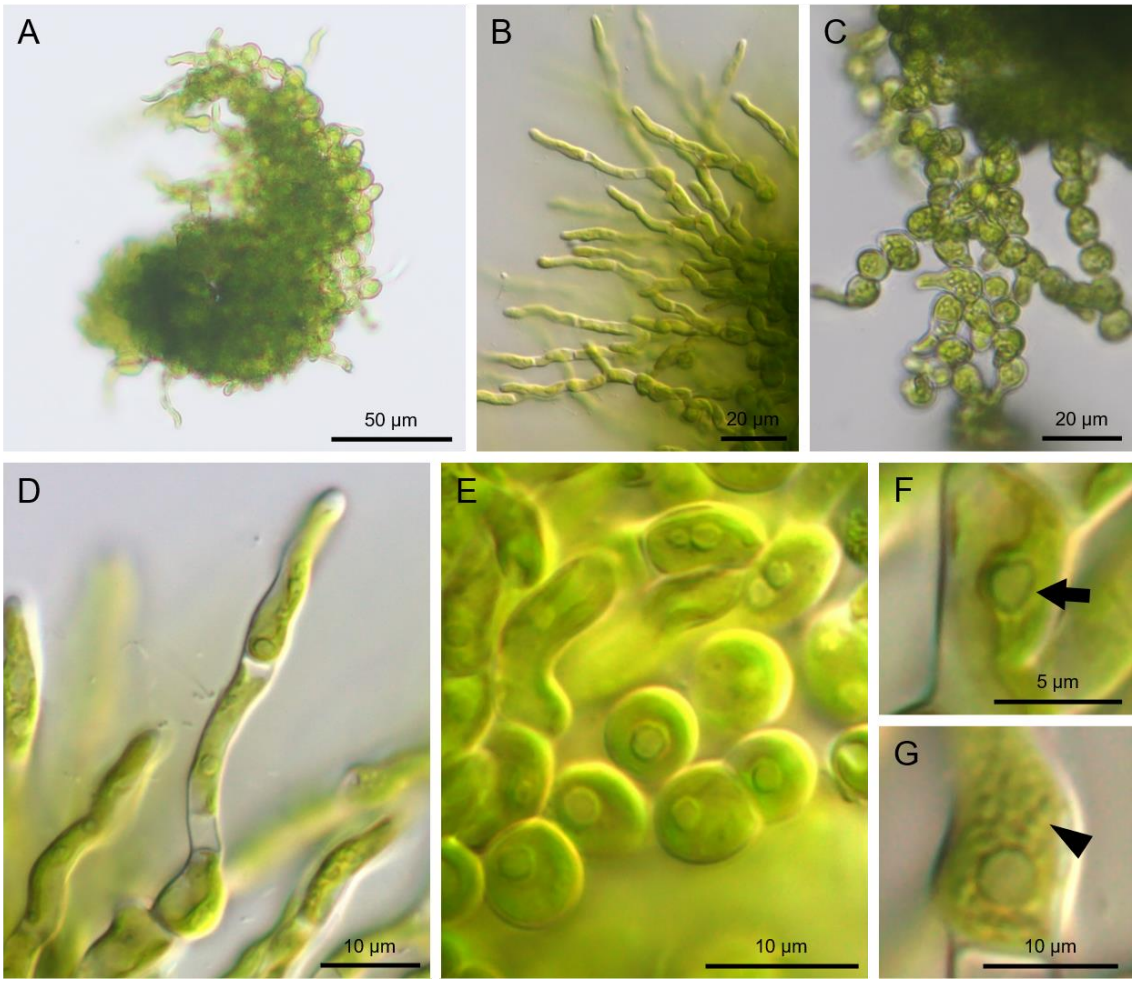
Figure 20.

Light micrographs of BFA211. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Basal parts of filaments composed of multiseriate elliptical to spherical cells. (C) Terminal region of filaments composed uniseriate cylindrical cells, branching on one side. (D) Elliptical to spherical cells composing the central part of a thallus and basal part of filaments. (E) Cylindrical cells composing filaments. (F) Pyrenoid in a cell (arrow). (G) Oil droplets in a cell (arrowheads).


A
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B
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C
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D
10 µm
E
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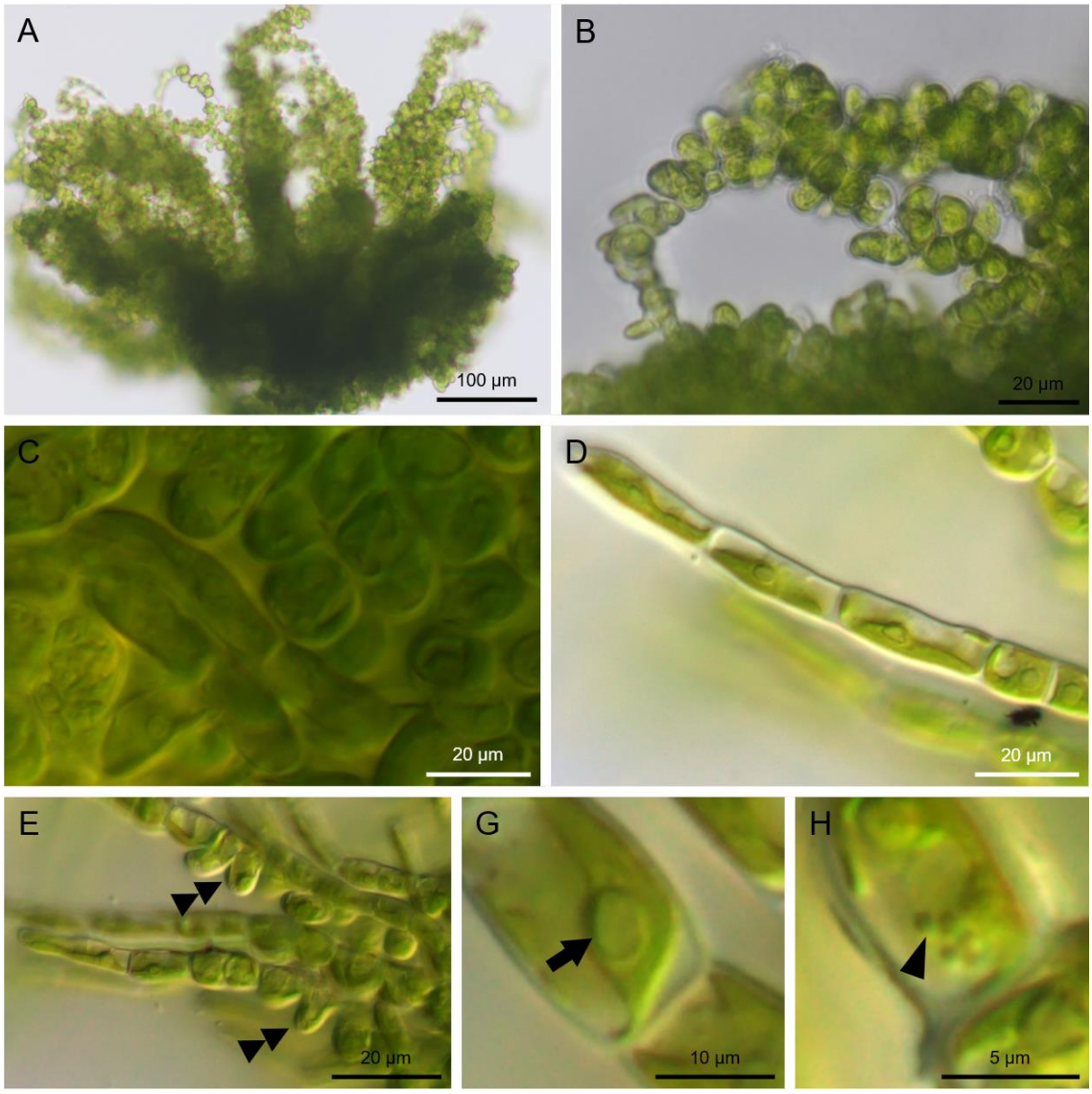
F
5 µm
G
10 µm

Figure 21.

Light micrographs of BFA239. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Branched filaments composed of cylindrical cells. (C) Basal part of filaments composed of spherical cells. (D) Cylindrical cells composing filaments. (E) Elliptical to spherical cells composing the central part of a thallus and basal part of filaments. (F) Pyrenoid in a cell (arrow). (G) Oil droplets in a cell (arrowhead).


A
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B
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C
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G
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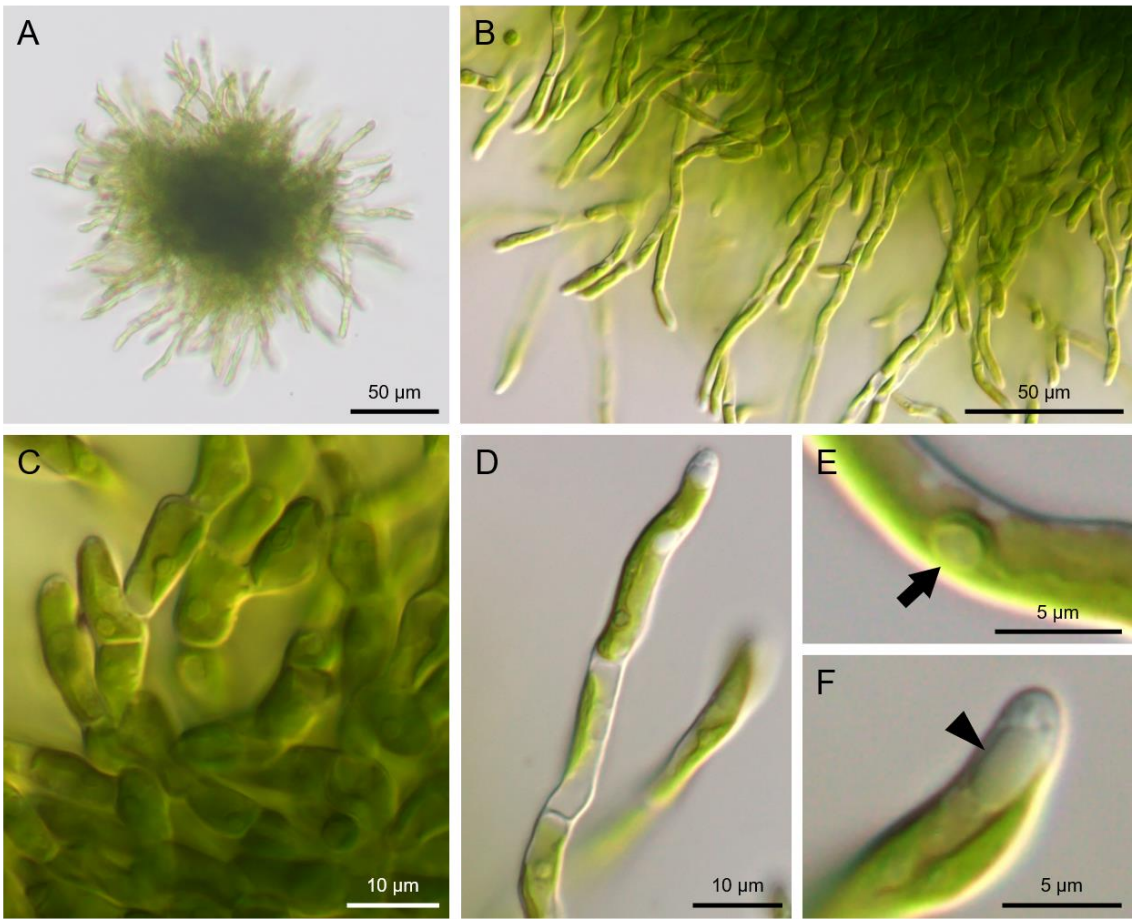
H
5 µm

Figure 22.

Light micrographs of BFA228. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Filaments composed of multiseriate elliptical to spherical cells in addition to cylindrical cells. (C) Elliptical to spherical cells composing the central part of a thallus and basal part of filaments. (D) Cylindrical cells composing filaments. (E) Basal part of filaments with swollen cells (double arrowheads). (F) Pyrenoid in a cell (arrow). (G) Oil droplets in a cell (arrowheads).


A
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E
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F
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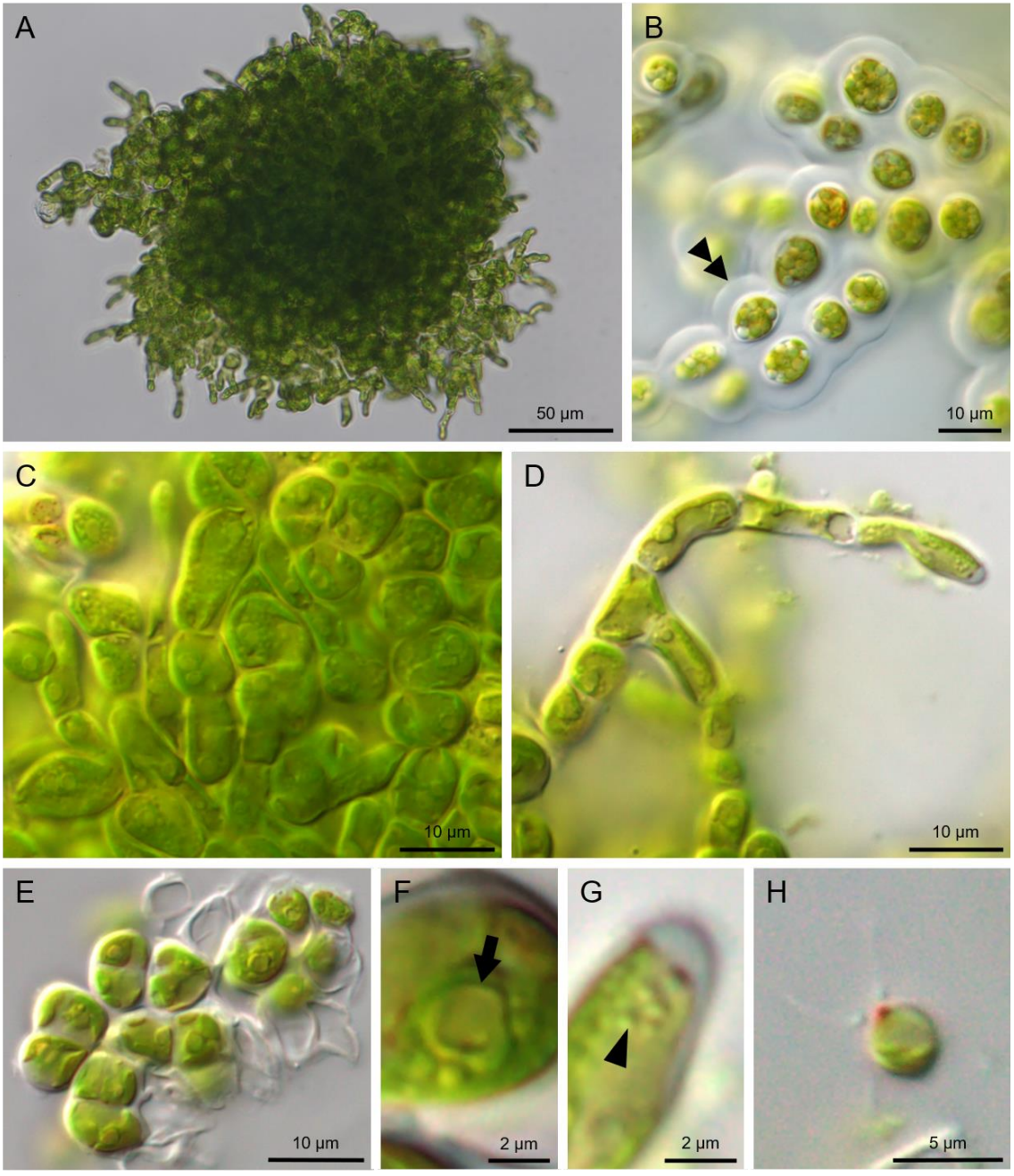
Figure 23.

Light micrographs of BFA243. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Very long filaments composed of uniseriate cylindrical cells. (C) Elliptical to spherical cells composing the central part of a thallus and the basal part of filaments. (D) Cylindrical cells composing filaments. (E) Pyrenoid in a cell (arrow). (G) Large oil drop in the cylindrical cell of filaments at terminal side (arrowhead).


A
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B
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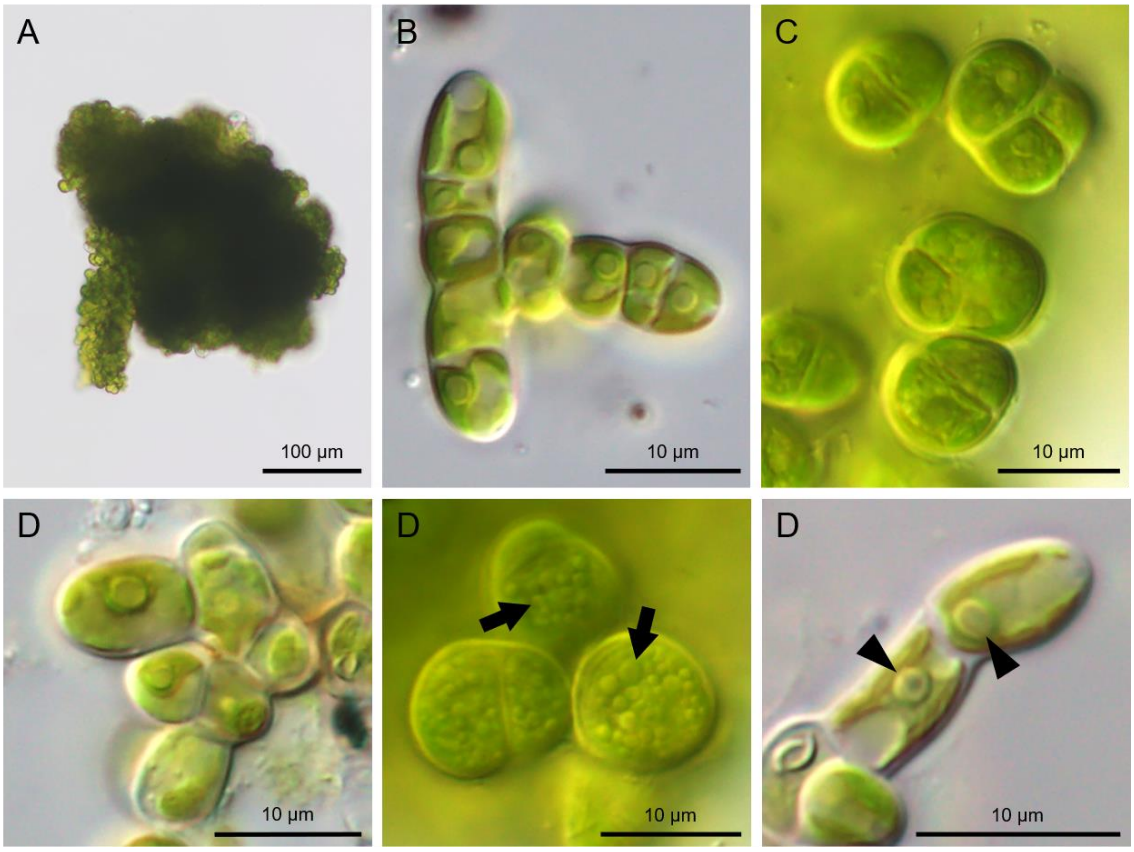
C
10 µm
D
10 µm
E
10 µm
F
2 µm
G
2 µm
H
5 µm

Figure 24.

Light micrographs of BFA201. (A) Thallus composed of cell aggregation and peripheral radiating filaments. (B) Bloated cell wall of filaments (double arrowhead) composed of elliptical cells in old thallus. (C) Elliptical to spherical cells composing the central part of a thallus. (D) Cylindrical cells composing filaments radially extending from a thallus. (E) Cells forming sarcinoid packets. (F) Pyrenoid in a cell (arrows). (G) Cell containing many oil droplets (arrowhead). (H) Quadriflagellate zoospore.


A
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B
10 µm
C
10 µm
D
10 µm
D
10 µm
D
10 µm

Figure 25.

Light micrographs of BFA208. (A) Thallus composed of cell aggregation of elliptical to spherical cells. (B) Filaments composed of short cylindrical cells and elliptical to spherical cells. (C) Sarcinoid packets. (D) Elliptical to spherical cells composing the central part of a thallus. (D) Cells containing many oil droplets (arrows). (E) Pyrenoid in cells (arrowheads).

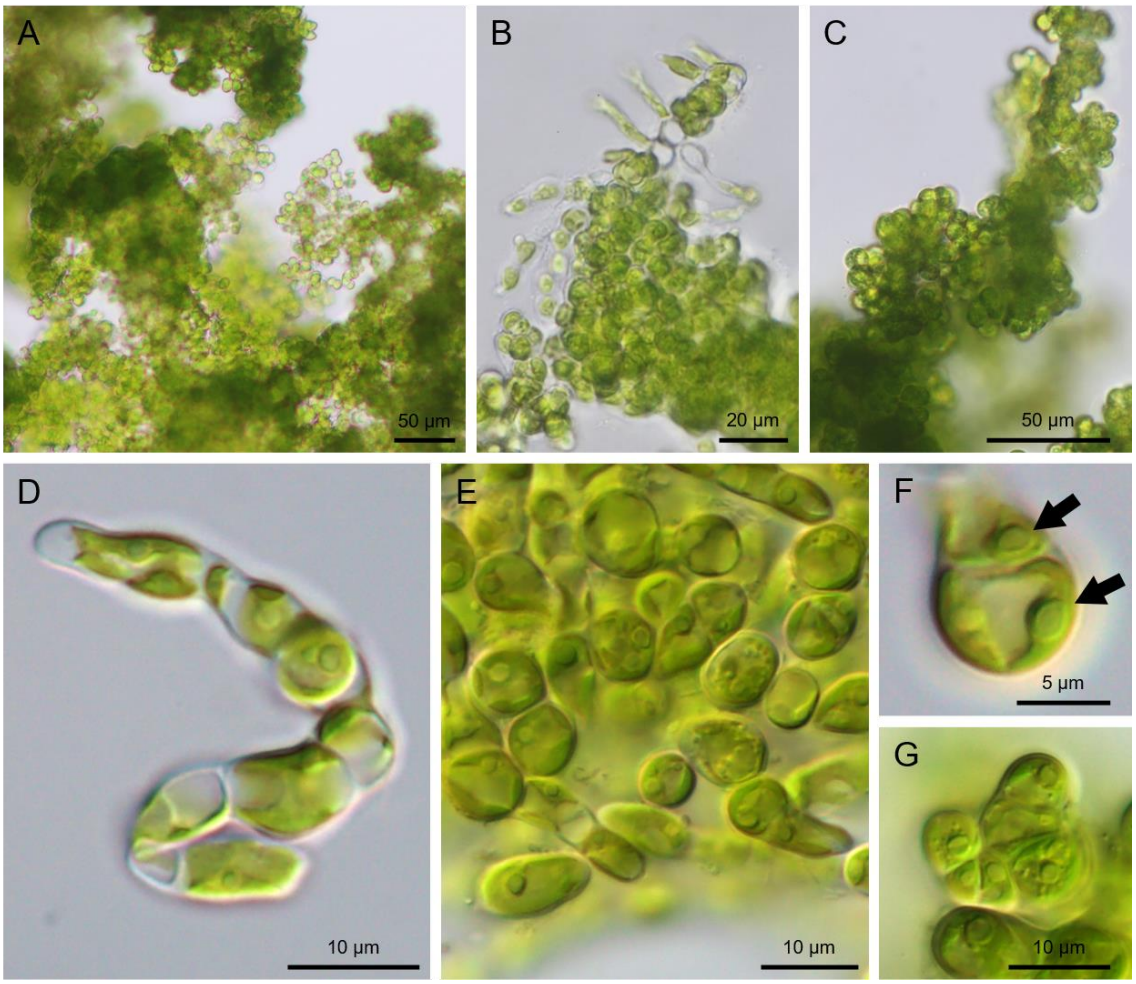
A
50 µm
B
20 µm
C
50 µm
D
10 µm
E
10 µm
F
5 µm
G
10 µm

Figure 26.

Light micrographs of BFA209. (A) Thallus composed of aggregated elliptical to spherical cells. (B) The part of thallus showing filaments composed of cylindrical cells. (C) Proximal part of filaments composed of multiseriate elliptical to spherical cells. (D) Cylindrical cells of filaments. (E) Elliptical to spherical cells composing the central part of a thallus. (F) Pyrenoid in a cell (arrows). (E) Sarcinoid packets.

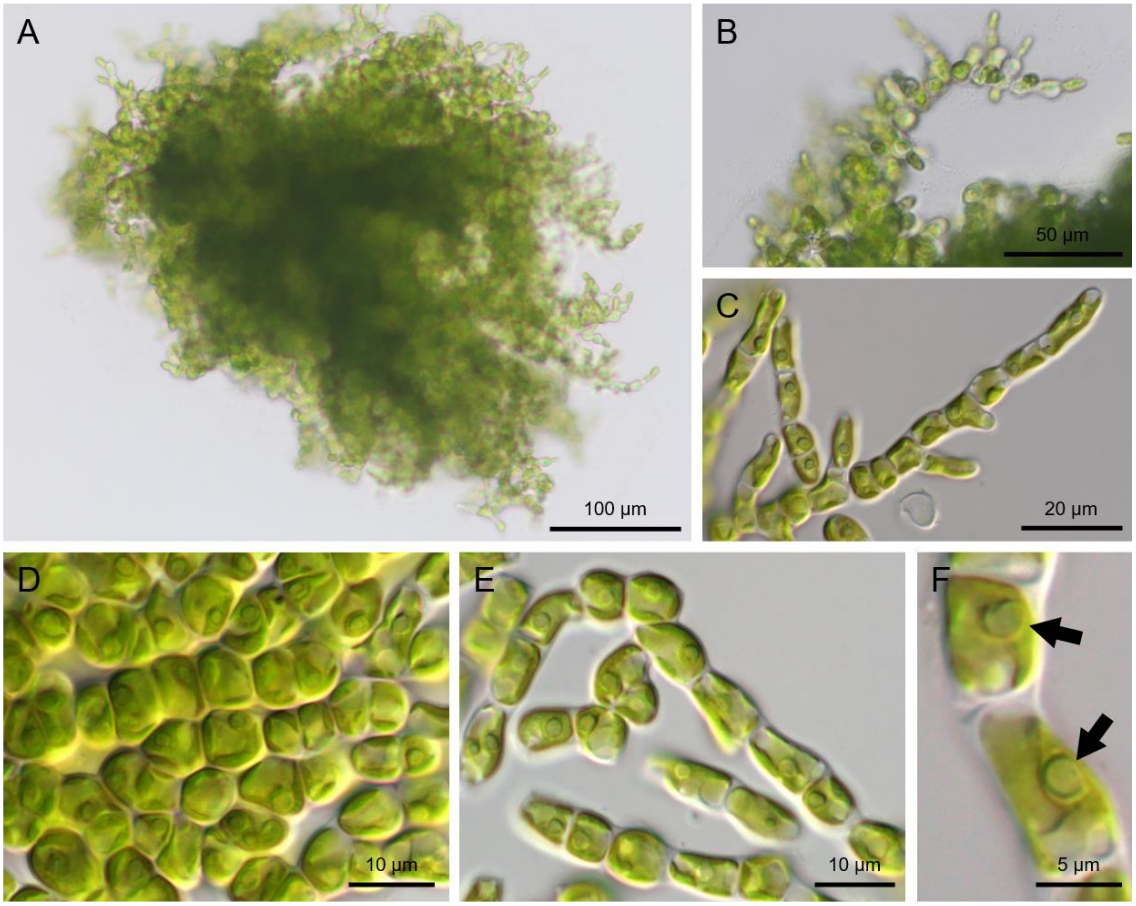
A
100 µm
B
50 µm
C
20 µm
D
10 µm
E
10 µm
F
5 µm

Figure 27.

Light micrographs of BFA226. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating filaments. (B) Filaments composed of short cylindrical cells and multiseriate elliptical to spherical cells. (C) Filaments composed of uniseriate cylindrical cells. (D) Elliptical to spherical cells composing the central part of a thallus. (E) Short cylindrical cells of filaments. (F) Pyrenoid in a cell (arrows).

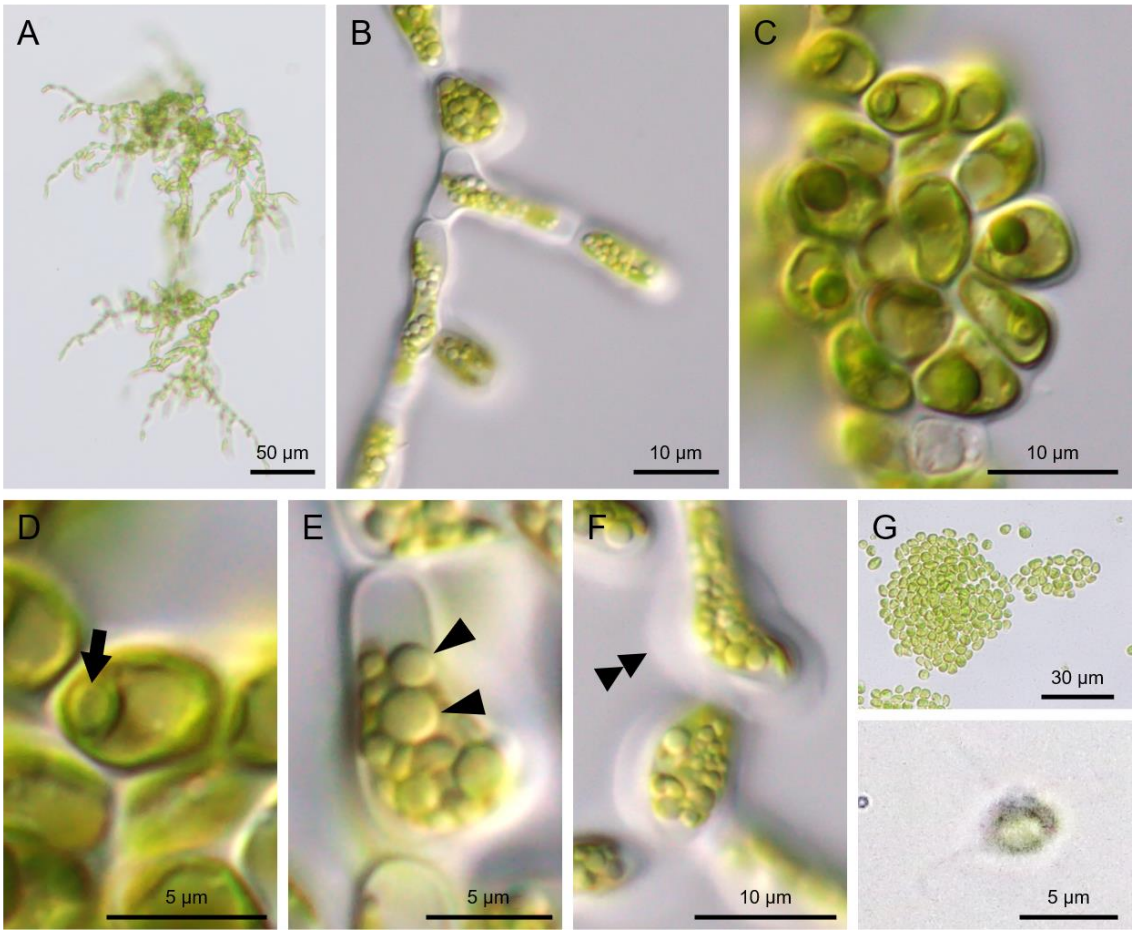
A
50 µm
B
10 µm
C
10 µm
D
5 µm
E
5 µm
F
10 µm
G
30 µm
5 µm

Figure 28.

Light micrographs of BFA227. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating filaments. (B) Filaments composed of cylindrical cell and elliptical to spherical cells. (C) Elliptical to spherical cells composing the central part of a thallus. (D) Pyrenoid in a cell (arrows). (E) Oil droplets in a cell (arrowheads). (F) Swollen cell wall of older cells (double arrowhead). (G) Separate elliptical to spherical cells. (H) Quadriflagellate zoospore.

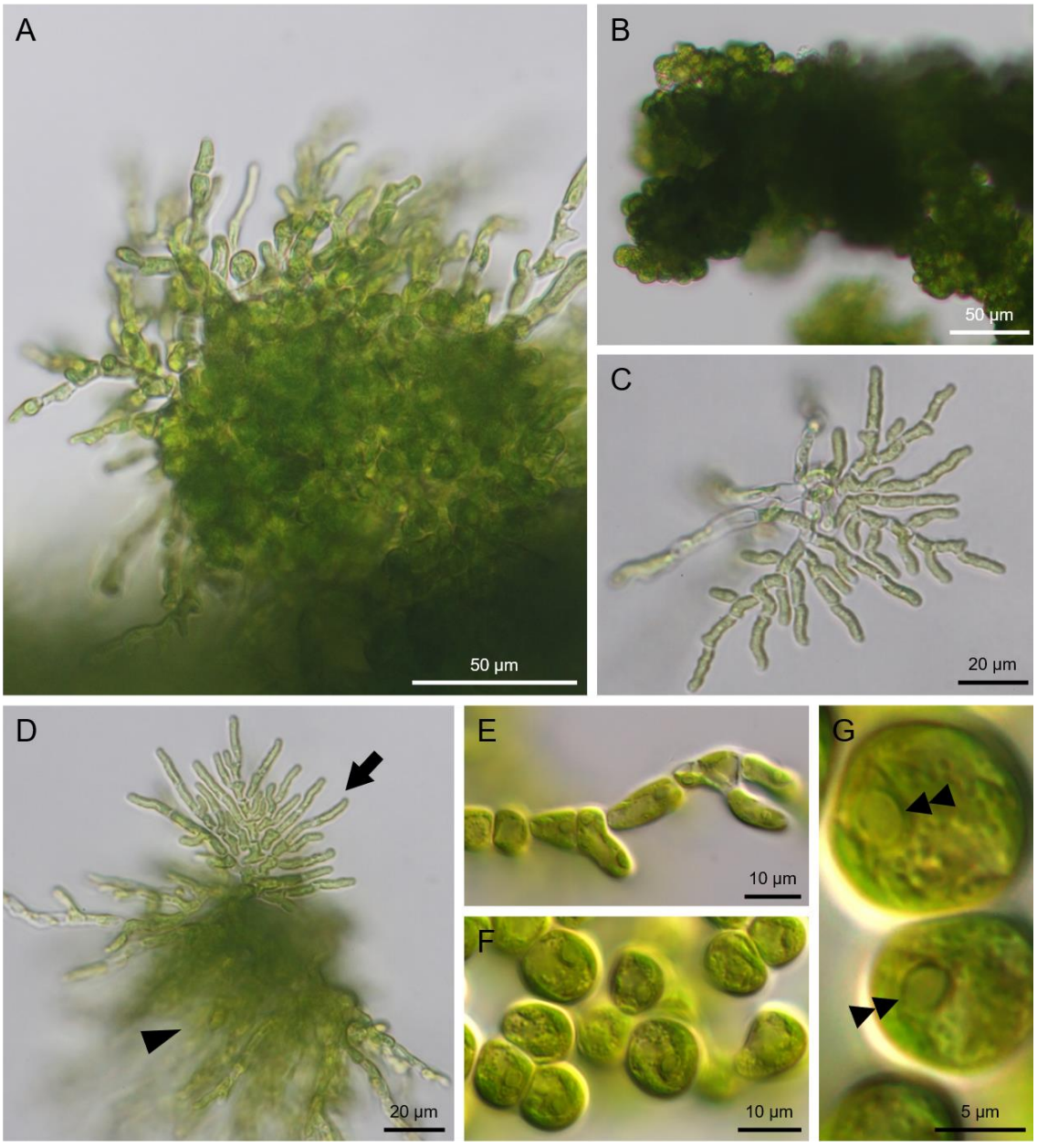
A
50 µm
B
50 µm
C
20 µm
D
20 µm
E
10 µm
F
10 µm
G
5 µm

Figure 29.

Light micrographs of BFA242. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating filaments. (B) Proximal part of filaments composed of multiseriate elliptical to spherical cells. (C) Prostrate system of branched filaments. (D) Thallus composed of prostrate parts (arrow) and erect parts (arrowhead). (E) Cylindrical cells of filaments. (F) Elliptical to spherical cells composing the central part of a thallus. (D) Pyrenoid in a cell (double arrowheads).

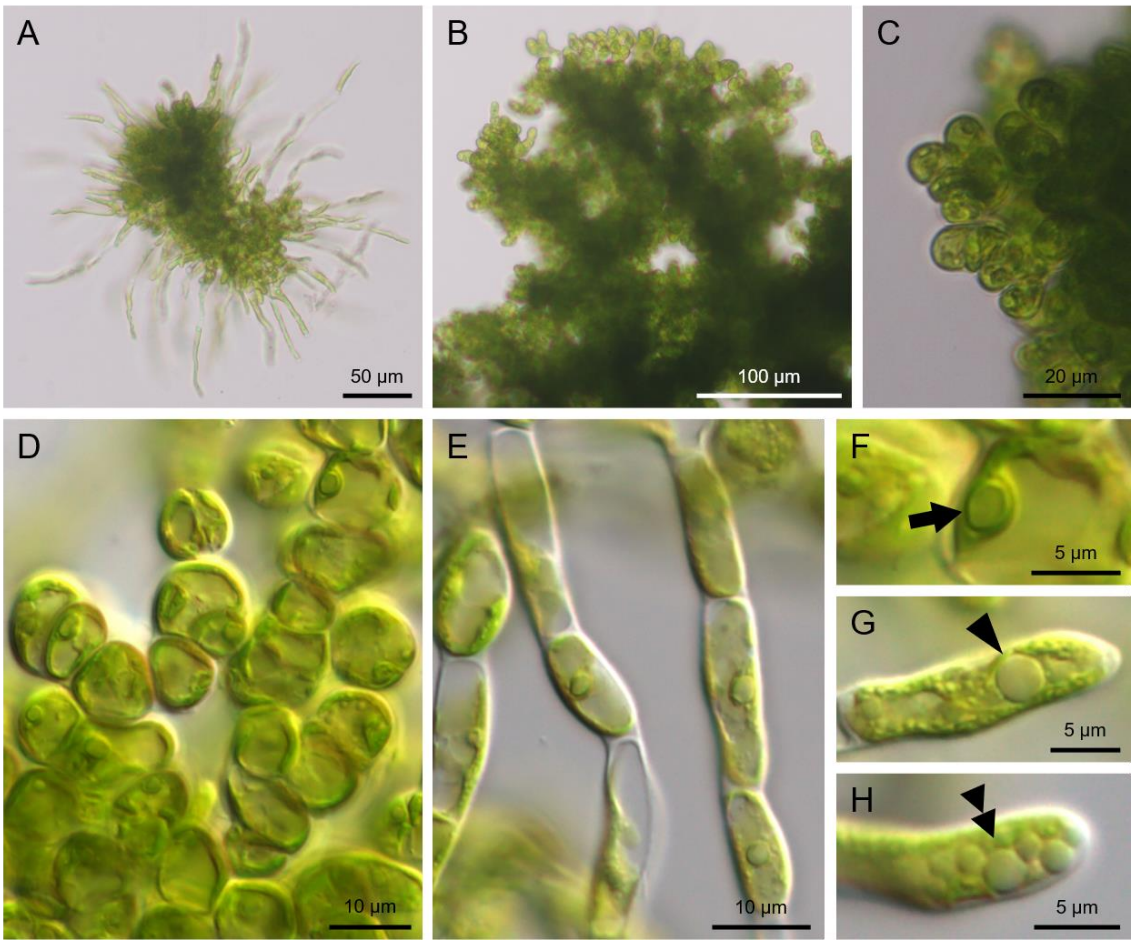
A
50 µm
B
100 µm
C
20 µm
D
10 µm
E
10 µm
F
5 µm
G
5 µm
H
5 µm

Figure 30.

Light micrographs of BFA261. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating filaments. (B) Thallus without filaments. (C) Enlarged image of a thallus without filament, composed of elliptical to spherical cells. (D) Elliptical to spherical cells composing the central part of a thallus. (E) Cylindrical cells of filaments. (F) Pyrenoid in a cell (arrow). (G) Large oil drop in the cylindrical cell of terminal part of filament. (H) Cylindrical cells of terminal part of filaments possessing large oil droplets.

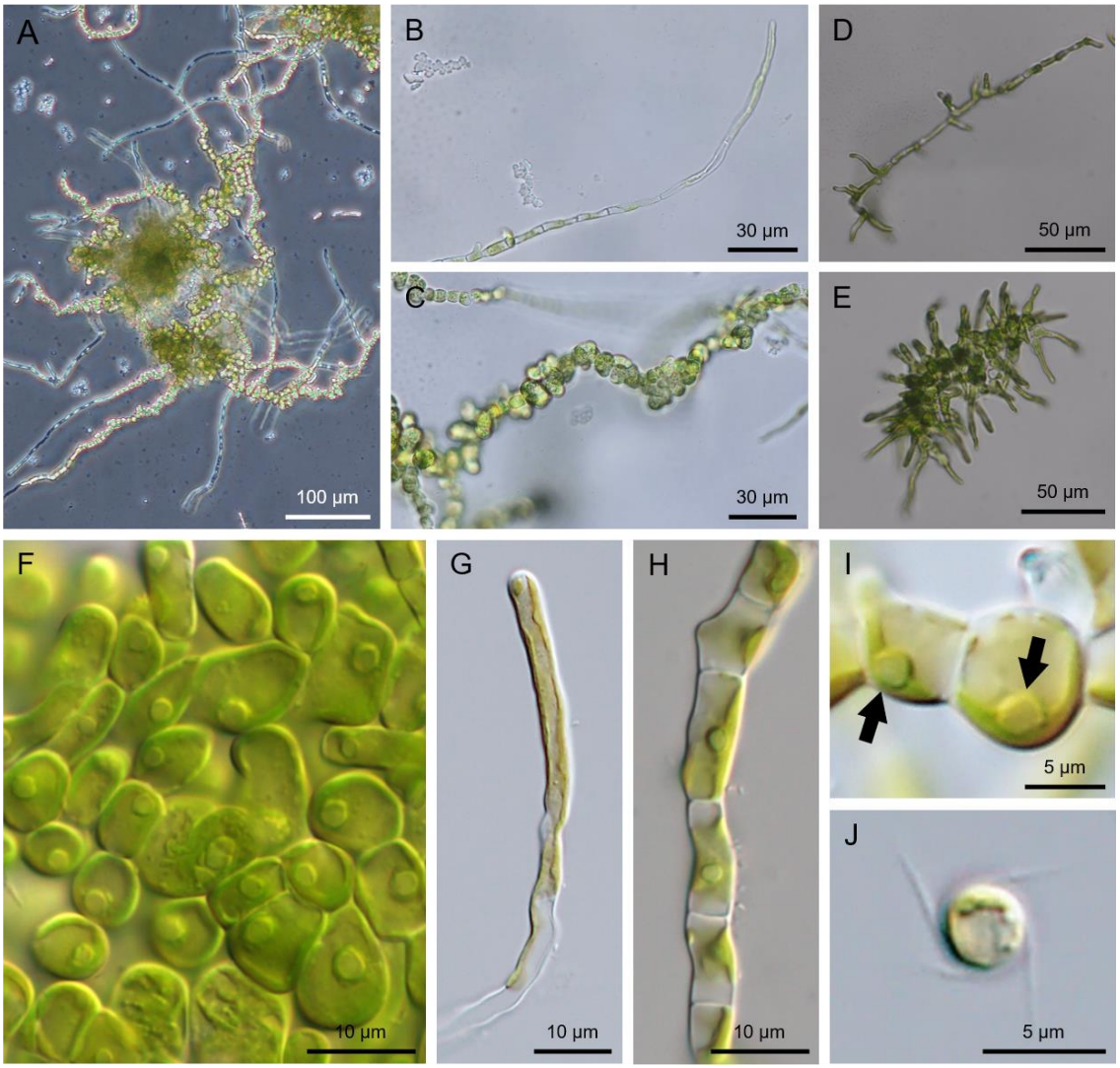
A
100 µm
B
30 µm
C
30 µm
D
50 µm
E
50 µm
F
10 µm
G
10 µm
H
10 µm
I
5 µm
J
5 µm

Figure 31.

Light micrographs of BFA203. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating very long filaments. (B) Terminal part of filaments composed of uniseriate cylindrical cells. (C) Proximal part of filaments composed of multiseriate elliptical to spherical cells. (D) Young thallus composed of only cylindrical cells. (E) Young thallus composed of cell aggregation and branched filaments. (F) Elliptical to spherical cells composing the central part of a thallus. (G) Cylindrical cells at the tip of filaments. (H) Cylindrical cells at the middle of filaments. (I) Pyrenoids in cells (arrows). (J) Quadriflagellate zoospore.

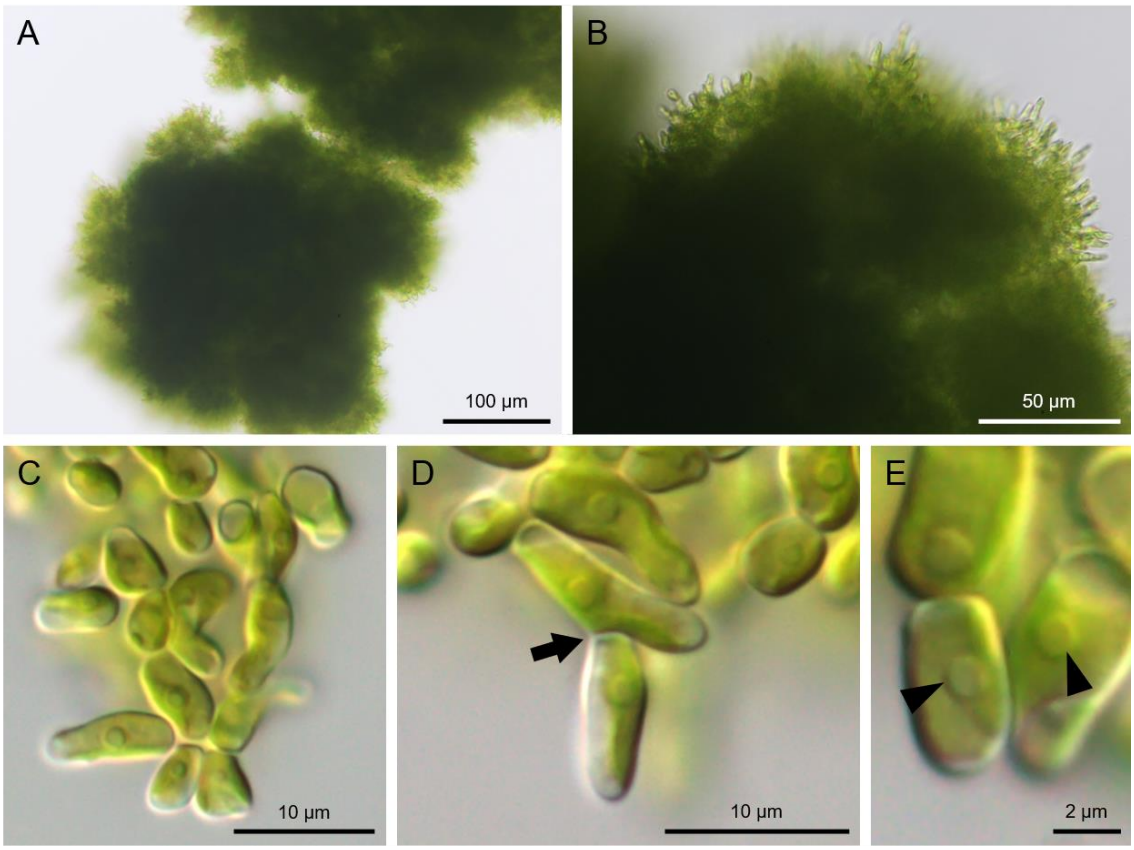
A
100 µm
B
50 µm
C
10 µm
D
10 µm
E
2 µm

Figure 32.

Light micrographs of BFA240. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating filaments. (B) Peripheral part of thallus forming many filaments. (C) Elliptical to spherical cells composing the central part of a thallus. (D) Cylindrical cells of filaments. (E) Pyrenoids in cells (arrows).

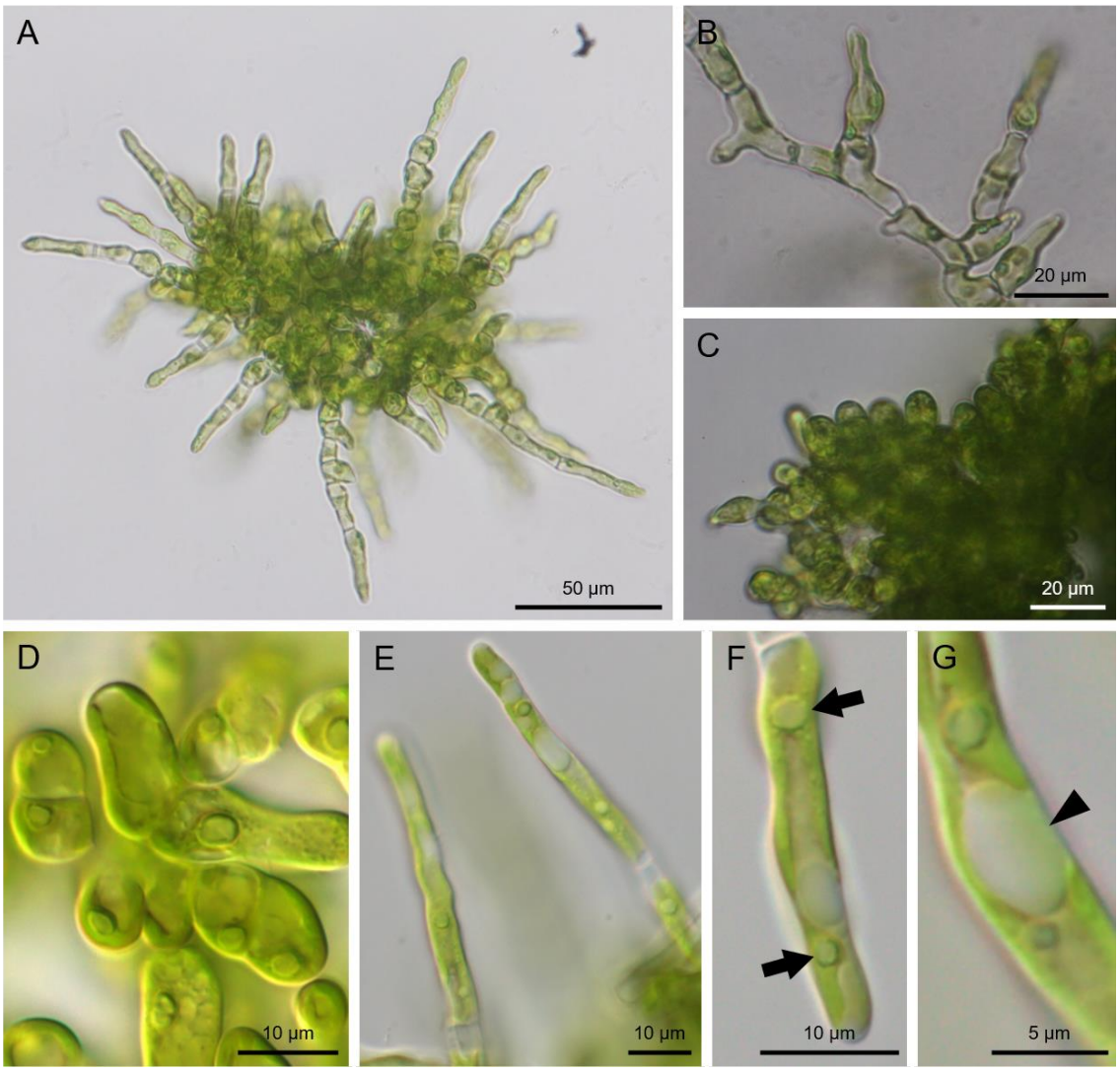
A
50 µm
B
20 µm
C
20 µm
D
10 µm
E
10 µm
F
10 µm
G
5 µm

Figure 33.

Light micrographs of BFA244. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating filaments. (B) Terminal part of filaments composed of uniseriate cylindrical cells. (C) Proximal part of filaments composed of multiseriate elliptical to spherical cells. (D) Elliptical to spherical cells composing the central part of a thallus. (E) Cylindrical cells of filaments. (F) Pyrenoids in a cell (arrows). (G) Lage oil drop in the terminal cell of filament.

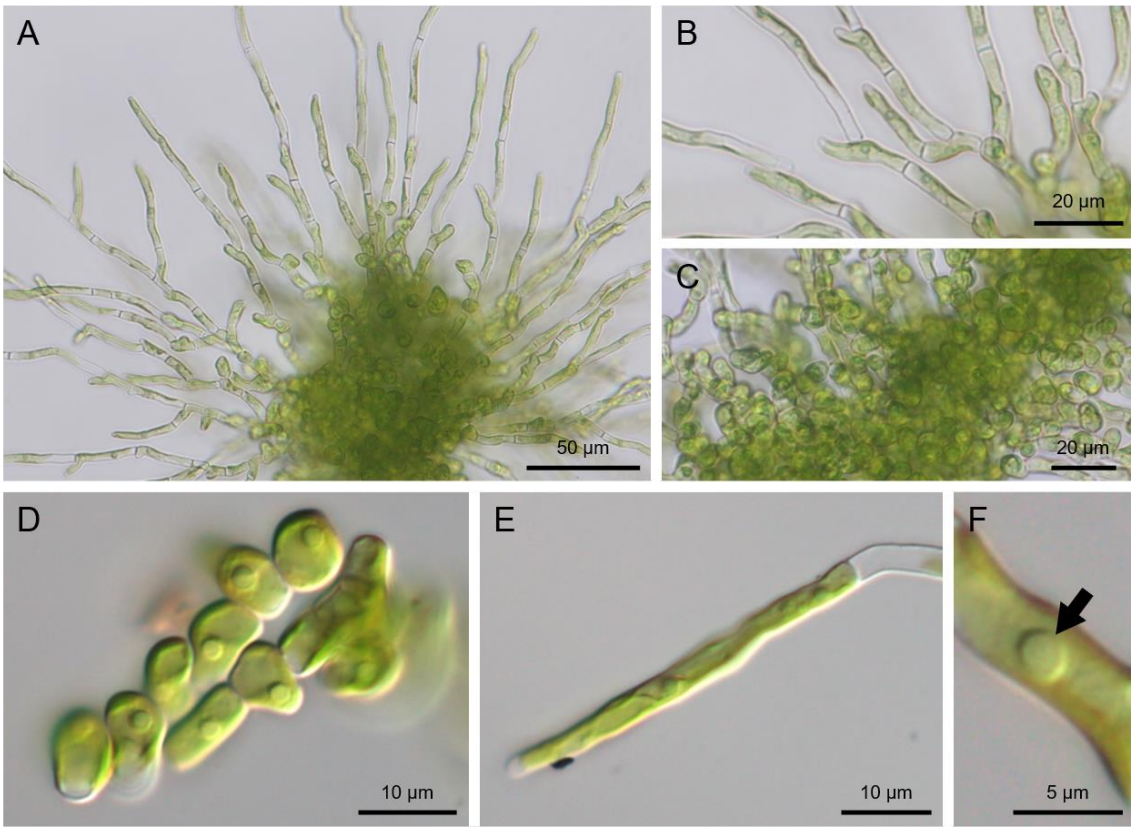
A
50 µm
B
20 µm
C
20 µm
D
10 µm
E
10 µm
F
5 µm

Figure 34.

Light micrographs of BFA249. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating long filaments. (B) Terminal part of filaments composed of uniseriate cylindrical cells. (C) Proximal part of filaments composed of multiseriate elliptical to spherical cells. (D) Elliptical to spherical cells composing the central part of a thallus. (E) Cylindrical cells of filaments. (E) Pyrenoid in a cell (arrows).

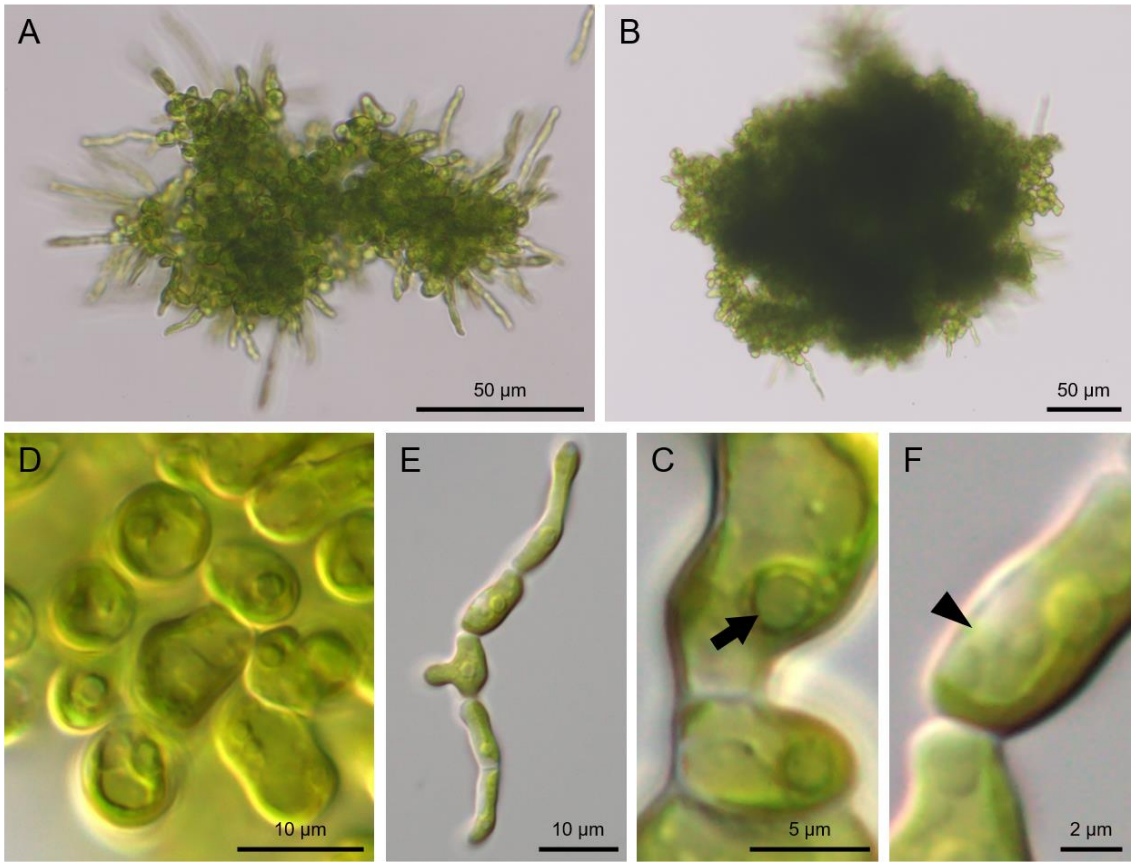
A
50 µm
B
50 µm
D
10 µm
E
10 µm
C
5 µm
F
2 µm

Figure 35.

Light micrographs of BFA250. (A) Thallus composed of aggregated elliptical to spherical cells and peripheral radiating filaments. (B) Thallus with few filaments. (C) Elliptical to spherical cells composing the central part of a thallus. (E) Cylindrical cells of filaments. (E) Pyrenoid in a cell (arrows). (F) Oil droplets in a cell.

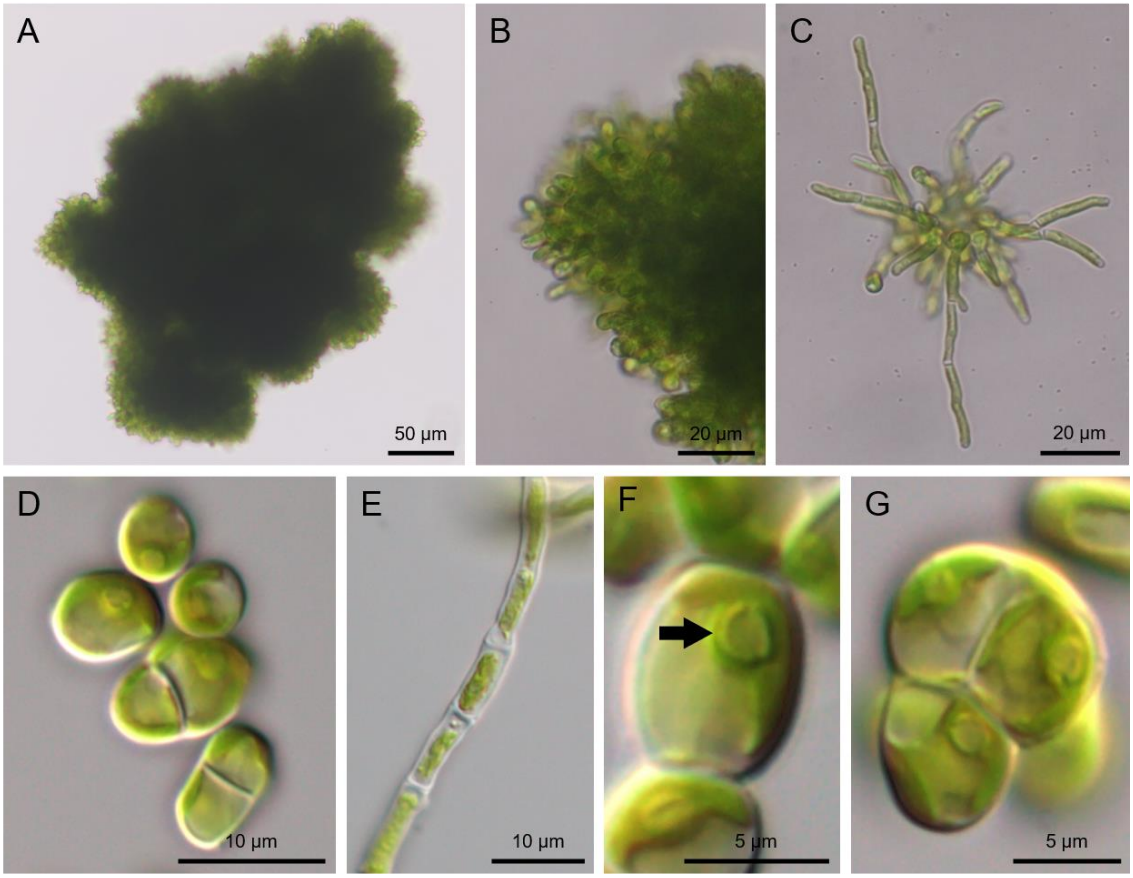
A
50 µm
B
20 µm
C
20 µm
D
10 µm
E
10 µm
F
5 µm
G
5 µm

Figure 36.

Light micrographs of BFA264. (A) Older thallus composed of aggregated elliptical to spherical cells. (B) Enlarged image of the peripheral part of old thallus composed of elliptical to spherical cells. (C) Young thallus produced filaments of cylindrical cells. (D) Elliptical to spherical cells composing the central part of a thallus. (E) Cylindrical cells of filaments. (E) Pyrenoid in a cell (arrows). (F) Sarcinoid packets.

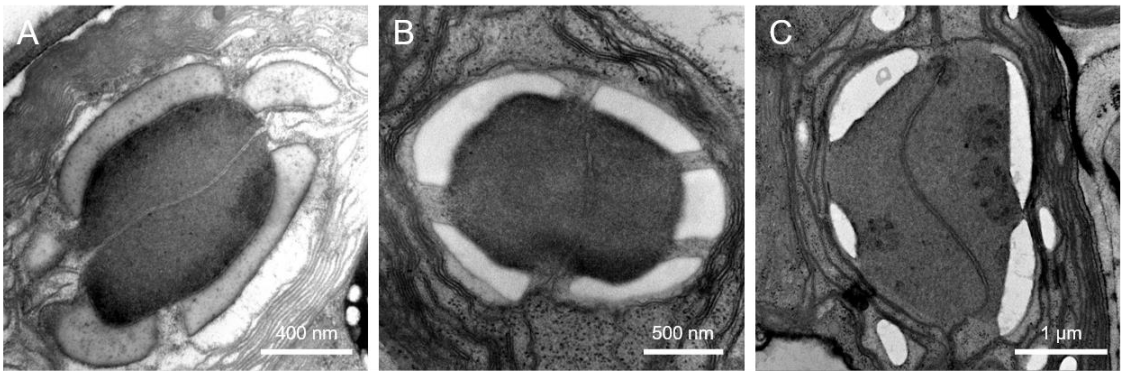
A
400 nm
B
500 nm
C
1 µm

Figure 37.

Transmission electron micrographs of pyrenoid of three strains. The pyrenoid matrix of all three strains were surrounded by several starch sheaths and transversed by single thylakoid membrane. (**A)** Ultrastructure structure of pyrenoid of BFA102. (B) Ultrastructure structure of pyrenoid of BFA211. (C) Ultrastructure structure of pyrenoid of BFA203.

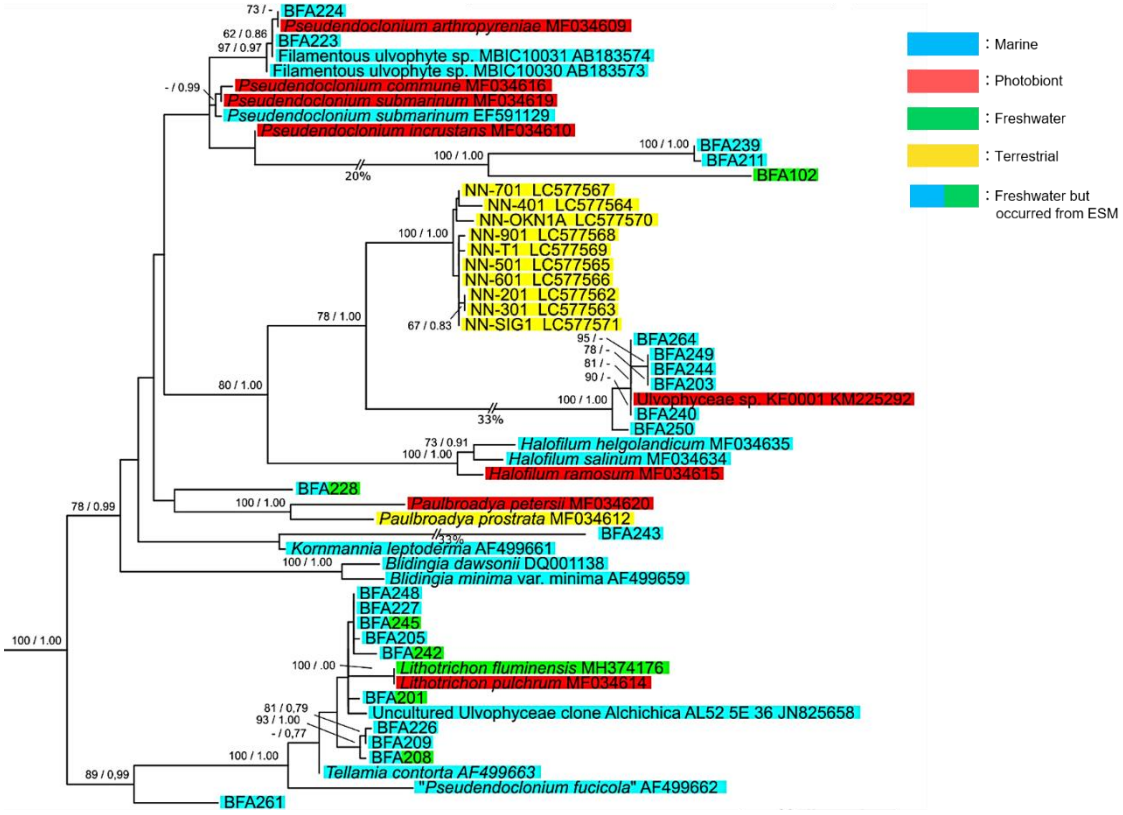
: Marine
: Photobiont
: Freshwater
: Terrestrial
: Freshwater but occurred from ESM
BFA224
Pseudendoclonium arthropyreniae MF034609
BFA223
Filamentous ulvophyte sp. MBIC10031 AB183574
Filamentous ulvophyte sp. MBIC10030 AB183573
Pseudendoclonium commune MF034616
Pseudendoclonium submarinum MF034619
Pseudendoclonium submarinum EF591129
Pseudendoclonium incrustans MF034610
BFA239
BFA211
BFA102
NN-701 LC577567
NN-401 LC577564
NN-OKN1A LC577570
NN-901 LC577568
NN-T1 LC577569
NN-501 LC577565
NN-601 LC577566
NN-201 LC577562
NN-301 LC577563
NN-SIG1 LC577571
BFA264
BFA249
BFA244
BFA203
Ulvophyceae sp. KF0001 KM225292
BFA240
BFA250
Halofilum helgolandicum MF034635
Halofilum salinum MF034634
Halofilum ramosum MF034615
BFA228
Paulbroadya petersii MF034620
Paulbroadya prostrata MF034612
BFA243
Kornmannia leptoderma AF499661
Blidingia dawsonii DQ001138
Blidingia minima var. minima AF499659
BFA248
BFA227
BFA245
BFA205
BFA242
Lithotrichon fluminensis MH374176
Lithotrichon pulchrum MF034614
BFA201
Uncultured Ulvophyceae clone Alchichica AL52 5E 36 JN825658
BFA226
BFA209
BFA208
Tellamia contorta AF499663
"Pseudendoclonium fucicola" AF499662
BFA261

Figure 38.

Habitat and phylogeny of Kornmmaniaceae edited Fig. 11. Names of OTUs were colored by each habitat that collected samples. Strains of *Annulotesta cochlephila* was collected from shell of land snail but they include terrestrial environment here. Blue: marine, Red: photobiont of lichen, Green: freshwater, Yellow: terrestrial, Blue and Green: freshwater but sample occurred from ESM (seawater medium).