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Shinya Uwai [a] , Chikako Nagasato [b] , Taizo Motomura [a] & Kazuhiro Kogame [b]

[a] Field Science Center for Northern Biosphere, Muroran Marine Station, Hokkaido University, Muroran 051-0003, Japan

[b] Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, 060-0810, Japan

[c] Current address: Kobe University Research Center for Inland Seas, Kobe University, Kobe, 657-8501, Japan E-mail: uwai@kobe-u.ac.jp

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Life history and molecular phylogenetic relationships of *Asterocladon interjectum* sp. nov. (Phaeophyceae)

SHINYA UWAI[1], CHIKAKO NAGASATO[2], TAIZO MOTOMURA[1] AND KAZUHIRO KOGAME[2]

[1]*Field Science Center for Northern Biosphere, Muroran Marine Station, Hokkaido University, Muroran 051-0003, Japan*
[2]*Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, 060-0810, Japan*

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A small prostrate filamentous brown alga, isolated from the warm temperate Pacific coast of Japan, was cultured to investigate its life history, morphology, ultrastructure and molecular phylogenetic position. The isolate had an isomorphic, diphasic life history with anisogamous sexual reproduction. Gametogenesis was regulated by temperature and occurred only at temperatures $\leq$14°C. At higher temperatures, gametophytes reproduced by means of asexual zoids. All swarmers from plurilocular and unilocular zoidangia lacked an eyespot. Both sporophytes and gametophytes had oligostichous filamentous axes, lateral hairs with a basal sheath and no erect thalli. Three to eight oblong chloroplasts, each with a protruding pyrenoid, aggregated to form a stellate chloroplast configuration. All these morphological features suggested that this alga belongs to the genus *Asterocladon*. Plurilocular gametangia produced in an intercalary or terminal position and zoids lacking eyespots, are, however, unlike those of *Asterocladon lobatum*, formerly the only member of the genus. Therefore, we described the alga as a new taxon, *Asterocladon interjectum* sp. nov. Molecular phylogenetic analyses based on the large RUBISCO subunit (*rbc*L) and partial SSU nuclear ribosomal RNA gene sequences showed that *A. interjectum*, *A. lobatum* and *Asteronema rhodochortonoides* together formed a monophyletic clade, sister to the Ectocarpales *sensu lato*. Because the molecular phylogenetic analyses showed *Asteronema* to be paraphyletic, but monophyly of *Asterocladon* spp. and *Asteronema rhodochortonoides* was supported by the absence of cytoplasmic invaginations in pyrenoids, *Asteronema rhodochortonoides* was transferred to *Asterocladon* and an emended generic circumscription of *Asterocladon* was included.

Key words: anisogamy, *Asterocladon interjectum*, *Asterocladon lobatum*, *Asterocladon rhodochortonoides*, *Asteronema*, isomorphic life history, Phaeophyceae, pyrenoid, stellate chloroplast arrangement

Introduction

Like most brown algae, the majority of members of the Ectocarpales *sensu lato* have several discoid chloroplasts per cell. However, two exceptions are known: species of *Ectocarpus* Lyngbye have several ribbon-shaped chloroplasts in each cell, and the scytosiphonacean members have a single cup-shaped chloroplast per cell (Fletcher, 1987; Kawai, 1992). Vegetative cells of the members of many phaeophycean orders, for example, the Dictyotales, Fucales, Desmarestiales, Laminariales, Sphacelariales, and Sporochnales, lack pyrenoids in their chloroplasts (Evans, 1966; Hori, 1971, 1972; Fletcher, 1987; Kawai, 1992). A single protruding pyrenoid is present in the cup-shaped chloroplast of scytosiphonacean species and the disc-shaped chloroplast of species of the Ectocarpales *sensu lato*. Species of the genus *Ectocarpus* have one or several pyrenoids in each chloroplast (Evans, 1966; Hori, 1971, 1972).

In addition to disc- or ribbon-shaped chloroplasts, stellate chloroplasts or configurations of chloroplasts are known for several brown algal groups; *Asteronema* Delépine & Asensi, *Asterocladon* Müller, Parodi *et* Peters, *Bachelotia* Kuckuck ex Hamel and the order Scytothamnales. Members of the Scytothamnales have deeply lobed, stellate chloroplast(s), with a centrally embedded pyrenoid (Delépine *et al*., 1976; Asensi *et al*., 1977; Peters & Clayton, 1998). Species of *Asteronema*, *Bachelotia* and *Asterocladon,* have a stellate arrangement of several chloroplasts (Price, 1973; Delépine & Asensi, 1975; Delépine *et al*., 1976; Magne, 1976; Asensi *et al*., 1977; Womersley, 1987; Müller & Parodi, 1994; Müller *et al*., 1998; Ouriques & Bouzon, 2000). In these genera, each chloroplast is oblong to discoid with a protruding

Correspondence to: Shinya Uwai. Current address: Kobe University Research Center for Inland Seas, Kobe University, Kobe, 657-8501, Japan; e-mail uwai@kobe-u.ac.jp

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pyrenoid; however, several chloroplasts radiate from a proximal pyrenoid-complex, forming a stellate configuration. Genera with a stellate chloroplast-pyrenoid complex have been divided into three lineages based on molecular data (Müller *et al*., 1998; Draisma *et al*., 2001; Peters & Ramírez, 2001; Rousseau *et al*., 2001). *Asteronema ferruginea* (Harvey) Delépine *et* Asensi is related to the Scytothamnales rather than to *Asteronema rhodochortonoides* (Børgesen) Müller *et* Parodi; *A. rhodochortonoides* forms a monophyletic group with *Asterocladon lobatum* Müller, Parodi *et* Peters and the *A. rhodochortonoides*/*A. lobatum* clade is sister to the Ectocarpales *sensu lato*. The phylogenetic position of *Bachelotia antillarum* (Grunow) Gerloff remains unresolved.

In this paper, we describe *Asterocladon interjectum* sp. nov., the second member of the genus based on morphology, ultrastructure and molecular phylogenetic analyses using the large RUBISCO subunit (*rbc*L) gene and combined *rbc*L (plastid) and partial SSU nuclear ribosomal RNA gene sequence data. Some inferences on the phylogeny of brown algae with stellately arranged chloroplasts, and on the evolution of life histories in the Ectocarpales *sensu lato* can be drawn. *A. interjectum* has an isomorphic life history with anisogamous sexual reproduction, which differs from that of *A. lobatum* and its close relative *A. rhodochortonoides*.

Material and methods

Cultures

Unialgal clonal cultures were established by isolating fragments of the filaments into PESI medium (Tatewaki, 1966) containing penicillin G potassium for several days to avoid growth of cyanobacteria. Culture studies were carried out at 10, 14, 18 and 22°C, combined with 8 h:16 h short day (SD) cycle and 16 h:8 h long day (LD) cycle, under fluorescent lamps at 30–50 µmol photons $m^{-2} s^{-1}$. Culture medium was changed every week.

Microspectrofluorometry

Microspectrofluorometry was performed according to the method of Goff & Coleman (1984). Vegetative filaments of a sporophyte and a gametophyte were fixed in chilled Carnoy's fixative (3:1 ethanol:acetic acid) overnight. They were washed in tap water for 30 min and treated for 10 min with 1 M LiCl. After five changes in distilled water, they were stained for about 1 h with 0.1 µg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) in MacIlvaine's buffer at pH 4.2, or 100 µg ml^{-1} Mithramycin A in MacIlvaine's buffer at pH 7.0 with 15 mM $MgCl_2$. Measurements of fluorescent intensity of nuclear DNA were conducted as described by Deshmukhe & Tatewaki (1993).

Electron microscopy

Methods for TEM were similar to those of Motomura & Sakai (1984). Sporophytic and gametophytic thalli were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 3% NaCl, 0.1% $CaCl_2$, and 0.5% caffeine for 30 min at 4°C. Then they were postfixed with 1% OsO_4 in cacodylate buffer (pH 7.2) containing 3% NaCl and 0.1% $CaCl_2$ for 2 h at 4°C. They were dehydrated through a graded acetone series and embedded in Spurr's epoxy resin. Sections were cut using a diamond knife on a Porter-Blum MT-1 ultramicrotome and mounted on formvar-coated slot grids. They were stained with uranyl acetate and lead citrate, and were observed with a Hitachi H-300 electron microscope.

Molecular phylogeny

Total genomic DNA was extracted with the benzylchloride method (Zhu *et al*., 1993). PCR was performed using a GeneAmp™ PCR System 9600 (Applied Biosystems, Foster City, California, USA) under the following conditions: 35 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. PCR products were purified to remove their residual primers using the polyethylene glycol precipitation method and directly sequenced using the ABI PRISM™ BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and a DNA sequencer ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocols. The pairs of primers used for PCR were SR1-SR5, SR4-SR9, and SR8-SR12 (Nakayama *et al*., 1996) for SSU, and PRBF0-PRBR1A, PRBF2-PRBR2, PRBF3-PRBR3A, rbcL3F-RSPR (Kogame *et al*., 1999) and PRBF1 (5′-ATCGAACTCGAATAAAAAGTGA-3′: this study) for *rbc*L. PCR amplifications (35 cycles) were performed using SR1-SR12 and PRBF0 (or PRBF1)-RSPR, both of which produced ca. 1800-bp fragment. Then, using the product of the first PCR as the template, secondary 25-cycle PCR amplifications were performed with each pair of primers, which produced smaller (400–600 bp) fragments. Alignment of sequences was carried out as described by Kogame *et al*. (2001).

For the *rbc*L sequence, saturation was checked by comparing the transition/transversion ratio for pairs of sequences against number of transversions, as well as the pairwise uncorrected distance (p-distance) against inferred Tajima-Nei distances (Tajima & Nei, 1984). For combined data, the congruence of two regions, i.e., *rbc*L and partial SSU sequences, was evaluated using the partition homogeneity/incongruence-length difference test (Partition-Homogeneity Test; PHT, Farris *et al*., 1995; Cunningham, 1997) implemented in PAUP* 4.0b10 (Swofford, 2002), before inferring phylogenetic trees (number of replicate 1000, 10 replicate random taxon addition, maxtree 1000).

Phylogenetic trees were inferred from *rbc*L sequences alone, and from the combined *rbc*L and partial SSU data, using minimum evolution (ME), maximum parsimony (MP) and maximum likelihood (ML) methods as implemented in PAUP* 4.0b10 (Swofford, 2002).

The Jukes–Cantor distance (Jukes & Cantor, 1969) was selected for constructing the ME trees according to the guideline in Nei & Kumar (2000). In addition, the ME trees were also inferred using the substitution models in the ML analyses of each data set (see below). For the ME and MP methods, heuristic searches were performed using random orders of sequence addition (100 replicates) with the TBR option. ME was performed using a heuristic search and the distance criterion. Bootstrap analyses (1000 replicates, 10 replicates of sequence additions in each bootstrap replicate) were used to estimate the stability of MP and ME tree topologies. The ML trees were constructed using the general time reversible substitution model (Rodríguez *et al.*, 1990) with the shape parameter of the gamma distribution and proportion of variable sites estimated by MODELTEST ver. 3.06 (Posada & Crandall, 1998) for both data sets. One replicate of sequence additions (as-is) was performed in the ML analyses. Robustness of each lineage in ML trees was estimated using the quartet puzzling method (10,000 replicates, Strimmer & von Haeseler, 1996. See also Cao *et al.*, 1998; Nei & Kumar, 2000). *Choristocarpus tenellus* (Kützing) Zanardini was used as an outgroup in both data sets according to Draisma *et al.* (2001).

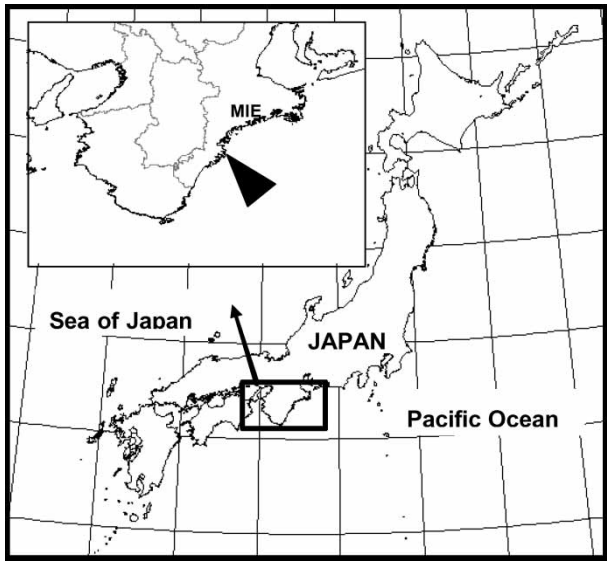


Fig. 1. Map showing the collecting site of *Asterocladon interjectum* Uwai, Nagasato, Motomura *et* Kogame sp. nov. Arrowhead (inset) shows the type locality (Kumano, Mie Prefecture, Japan).

Results

Vegetative filaments of a minute brown alga were collected on 27 March 2001 in Kumano (34°27′N, 136°07′E), Mie Prefecture, Japan (Fig. 1). The filaments were found as endo-epiphytes on cyanobacterial thalli that grew on the red alga, *Grateloupia ramosissima* Okamura.

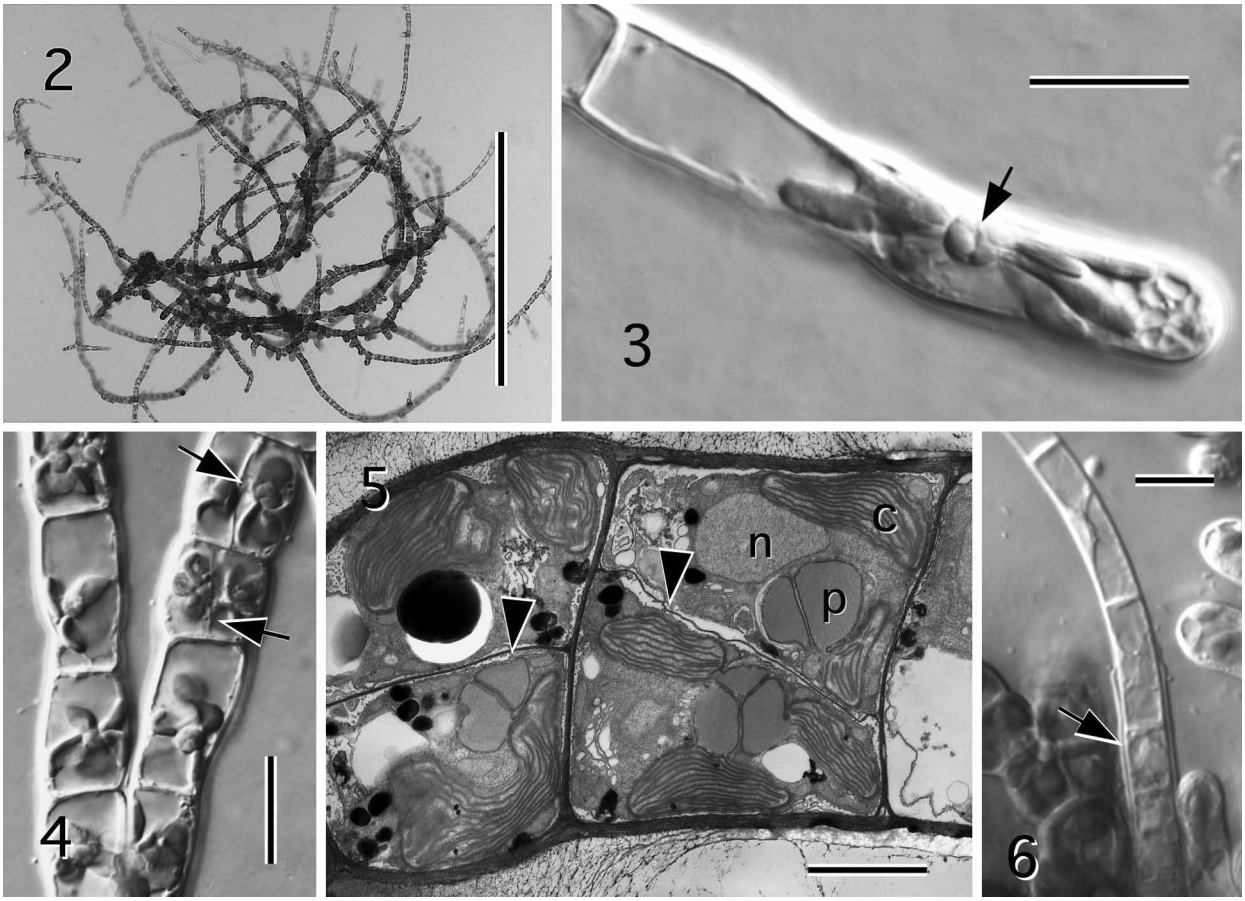
Life history

Cultured thalli were irregularly branched, prostrate filaments (Fig. 2), 1–3 mm long under our conditions. Each filament grew predominantly by divisions of cylindrical apical cells, 5–7 μm in diameter (Fig. 3). In older filaments, each cell was barrel-shaped (Fig. 4) and frequently underwent longitudinal division (Figs 4, 5), which resulted in new laterals or partially oligostichous axes with two to four cells. Thalli remained in contact with the substratum, i.e., creeping filaments with irregular, numerous branches, or grew freely, forming loose tufts of filaments. Erect filaments were not produced. Phaeophycean hairs, 5–9 μm in diameter with a basal sheath, were formed laterally (Fig. 6).

Vegetative cells contained several oblong chloroplasts, each with one protruding pyrenoid (Figs 5, 7). Pyrenoids of several chloroplasts were joined to form a complex from which the thylakoid-bearing parts radiated (Fig. 5), resulting in a single stellate chloroplast arrangement in each cell (Fig. 3). Chloroplasts were located at the periphery of cells. Chloroplast envelope, chloroplast ER and pyrenoid cap membranes were appressed against individual pyrenoids in this pyrenoid complex. Tubular pyrenoid invaginations were not observed. Each pyrenoid was positioned laterally in relation to the thylakoid-membrane orientation.

Lateral unilocular sporangia were produced when the thalli were cultured at 22°C SD (Fig. 8). Neither unilocular nor plurilocular zoidangia were produced in other culture conditions. Unilocular sporangia were ovate, 20–25 μm long by 15–20 μm wide, sessile (Fig. 8), and were formed in clusters of 3–6. The number of spores in each unilocular sporangium could not be counted. Fig. 9 shows an immature unilocular zoidangium on the sporophyte, in which four nuclei can be seen.

Zoids from unilocular zoidangia were pyriform to fusiform, 10–15 μm long by 5–7 μm wide, heterokont with a longer anterior flagellum (Fig. 10). They had 2–3 chloroplasts and lacked an eyespot. Unizoids germinated uni- or bipolarly (Fig. 11) and developed into prostrate filaments with apical cells dividing transversely (Fig. 12). Longitudinal cell division was also observed frequently. Filaments formed prostrate thalli (Fig. 13) that were morphologically similar to sporophytes, except that cells were smaller and globular rather than cylindrical to barrel-shaped (Fig. 13, cf. sporophytic thallus of Fig. 2). Chloroplasts showed the same stellate arrangement as sporophyte cells (Fig. 14). The thalli derived from unizoids produced plurilocular gametangia at 14°C LD and 10°C LD and SD (Figs 15, 16). When they became fertile, cells divided into 2–4 (or more) cells by transverse and longitudinal subdivisions and became globular intercalary plurilocular

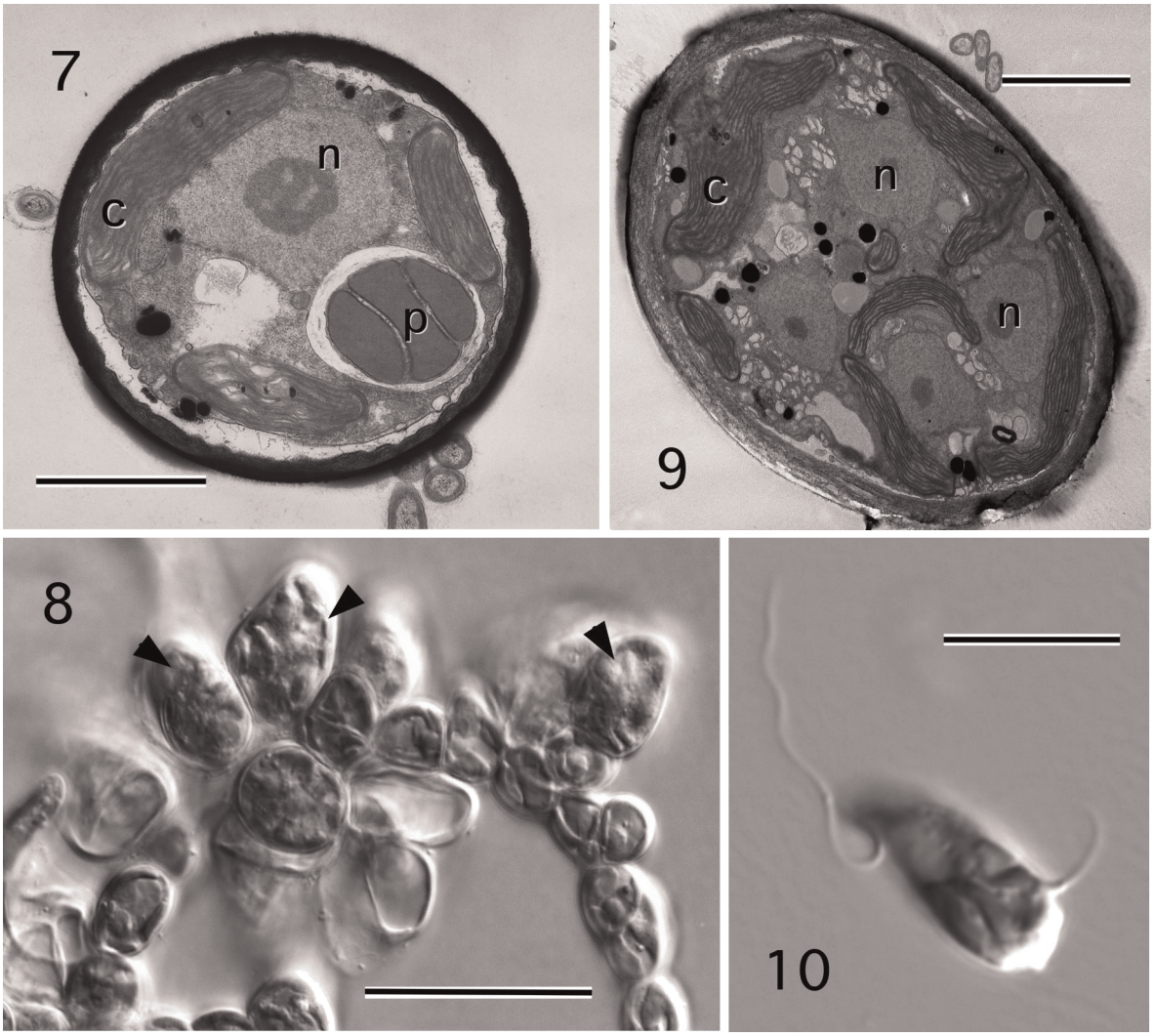


Figs 2–6. Sporophytes of *A. interjectum* in culture. Fig. 2. General habit. Fig. 3. An apical cell showing a stellate chloroplast configuration. Arrow indicates the prominent pyrenoid complex. Fig. 4. Vegetative axes. Arrows indicate longitudinal cell walls. Fig. 5. Longitudinal section of sporophytic filament. Arrowheads indicate thin transverse cell walls. Fig. 6. Lateral hair. Arrow indicates basal sheath. Abbreviations: c: chloroplast, n: nucleus, p: pyrenoid. Scale bars: Fig. 2: 50 μm; Figs 3, 4, 6: 10 μm; Fig. 5: 2 μm.

gametangia (Fig. 15). Uni- or biseriate, branched or linear plurilocular gametangia that contained 2–7 cells were also formed terminally on short laterals (Fig. 16). Plasmogamy was anisogamous; male and female gametes were pyriform to fusiform, laterally heterokont with a longer anterior flagellum, lacking an eyespot (Figs 17, 18). Female gametes were 9–12 μm long by 5–7 μm wide, and contained 2–3 chloroplasts (Fig. 17). Male gametes were 5–7 μm long by 3–5 μm wide, and had 1–2 small chloroplasts (Fig. 18). Both male and female gametes were produced on the same thallus (monoecism). It was difficult to distinguish male and female gametangia, as well as gametangia from vegetative cells before gamete liberation, because both types lacked an eyespot. It was unclear whether male and female gametes were formed in the same plurilocular gametangium or in separate ones. However, male gametangia were slightly lighter in colour than vegetative cells and female gametangia.

Plasmogamy was observed at 14°C LD (Figs 19–22), but was rare. Several male gametes gathered around settled female gametes. The male gametes touched the female with their anterior flagella, before one of the male gametes fused with the female. No odour indicating the presence of pheromone was noticed. Both types of gamete were also observed at 10°C, however, plasmogamy was not observed at this temperature. Zygotes germinated uni- or bipolarly and developed into irregularly branched, oligostichous sporophytes. Unilocular sporangia were produced at 22°C SD on these sporophytes.

At 14°C SD, 18°C LD and SD and 22°C LD and SD, gametophytes only produced large plurizoids, morphologically similar to female gametes. Male gametes were not observed under these conditions. The large plurizoids germinated without fusion, and developed again into filamentous gametophytes bearing both male and female gametangia at lower temperatures (14°C LD, 10°C LD and SD). Under these conditions (14°C LD, 10°C LD and SD) large plurizoids that had not fused with male gametes developed into gametophytes. Germination of unfused male gametes was also observed, although these germlings did not grow beyond a two-celled stage.



Figs 7–10. Sporophytes and unizoid of *A. interjectum* in culture. Fig. 7. Transverse section of sporophytic filament. Pyrenoid aggregation is located at the periphery of the cell. Fig. 8. Unilocular zoidangia (arrowheads). Fig. 9. Immature unilocular sporangium on the sporophyte. Fig. 10. Unizoid. Scale bars: Fig. 7: 2 μm; Fig. 8: 50 μm; Fig. 9: 3 μm; Fig. 10: 10 μm.

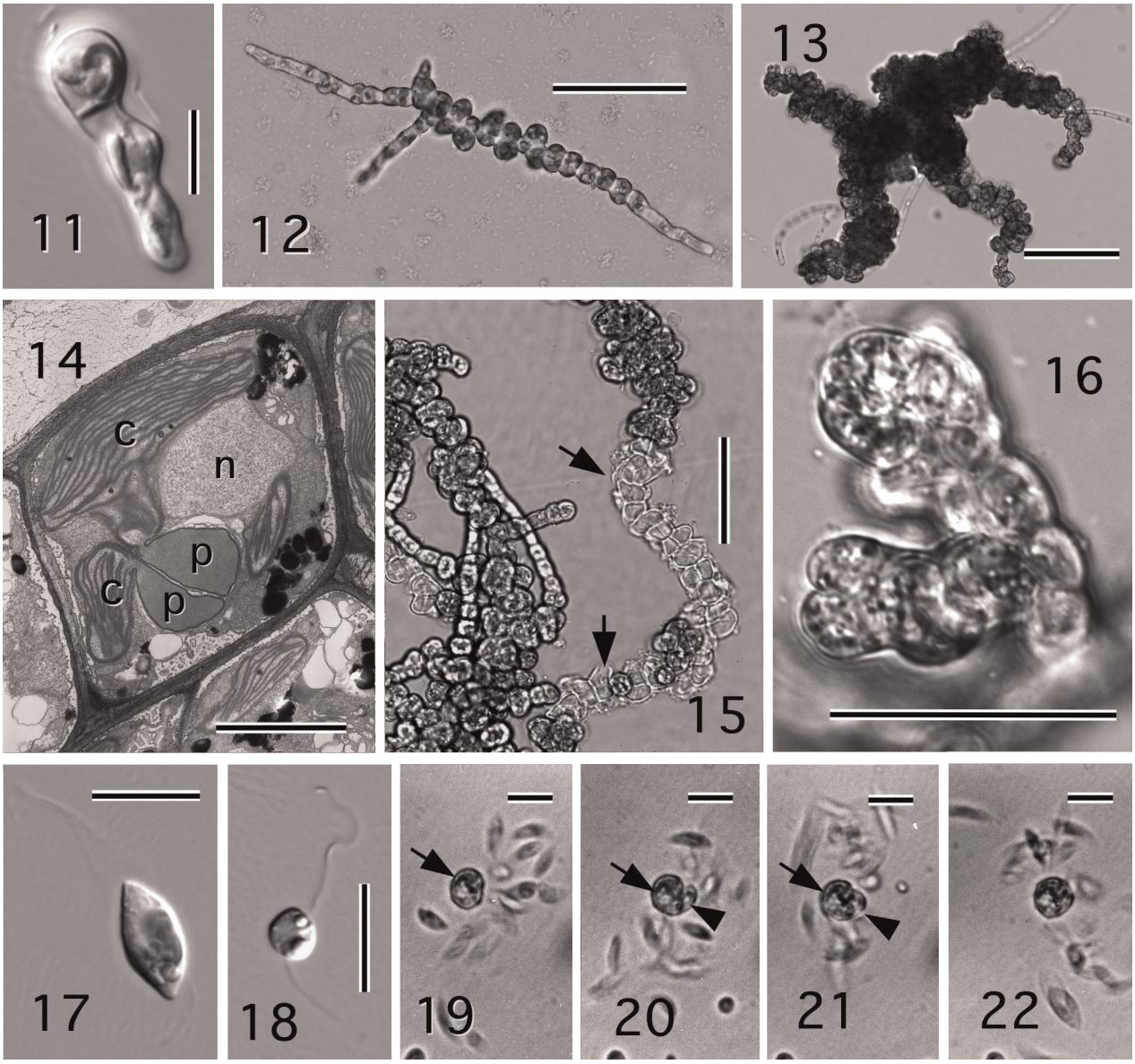
Microspectrofluorometry

Relative fluorescence values of DAPI stained nuclei in vegetative cells of gametophytes ranged from 0.44 to 1.83, with a mean of 1.01 (number of nuclei measured [n] = 96; standard deviation = 0.25). Values for nuclei in vegetative sporophyte cells ranged from 0.65 to 3.17, with a mean of 1.77 (n = 97; standard deviation = 0.43). The results obtained from mithramycin-stained samples were similar.

rbc*L nucleotide sequence analysis*

The *rbc*L sequence (1467 bp) of *A. interjectum* was determined. 1399 bp were compared for 91 phaeophycean taxa (Table 1). Our alignment included six sequences for five species with stellately arranged chloroplasts, of which two were newly determined in the present study: *A. interjectum* and *A. rhodochortonoides* from Japan (specimen in Kogame *et al.*, 2001). Saturation was indicated by the ratio of transition/transversion for pairs of sequences against the number of transversions of those sequences plotted for all three codon positions and for the third codon position. When the values were plotted for the first and second codon positions, saturation was not indicated. Although saturation was indicated, the third codon positions were not excluded as in Draisma *et al.* (2001).

Four trees inferred from *rbc*L sequences (MP, ML, ME based on the JC substitution model [ME-JC] and ME based on the GTR model [ME-GTR]), showed similar topologies (Fig. 23). The ME trees were similar to each other in topology as well as bootstrap values, therefore, the ME-GTR tree was not shown. In all trees, *A. interjectum* grouped with *A. rhodochortonoides* and *A. lobatum* with high support (100% bootstrap in MP and ME-JC trees and 93% quartet-puzzling in ML tree), and this *Asterocladon*/*A. rhodochortonoides* clade was the closest sister clade to the Ectocarpales *sensu lato*, with strong bootstrap or quartet-puzzling support



Figs 11–22. Development and morphology of gametophytes (Figs 11–18) of *A. interjectum* from unizoids, and gamete fertilization (Figs 19–22) in culture. Fig. 11. A one-day-old unizoid germling. Fig. 12. Six-day-old germling. Fig. 13. Fertile gametophyte. Fig. 14. Longitudinal section of gametophytic filament. Note that the pyrenoids are adjoining. Fig. 15. Empty intercalary plurilocular zoidangia (arrows) after gamete liberation. Fig. 16. Terminal plurilocular zoidangia. Fig. 17. Female gamete. Fig. 18. Male gamete. Figs 19–22. Fertilization. Arrows indicate a settled female gamete and arrowheads indicate male gametes fusing with the female gametes. Note that numerous male gametes gather around a settled female gamete. Scale bars: Figs 11, 17–22: 10 μm; Figs 12, 15, 16: 50 μm; Fig. 13: 100 μm; Fig. 14: 2 μm.

(99% in MP, 96% in ME-JC, and 82% in ML). The *Asterocladon*–Ectocarpales clade was sister to one of two laminarialean clades with strong (97% in ME-JC) or moderate (78% in MP) support.

Neither *A. ferruginea* nor *B. antillarum*, two other taxa with stellately arranged chloroplasts, grouped with the *Asterocladon*/*A. rhodochortonoides* clade. The phylogenetic positions of *A. ferruginea* and *B. antillarum* were not clear. *Asteronema ferruginea* and *B. antillarum* tended to group together, as well as with the Scytothamnales (*Scytothamus* and *Splachnidium*) and/or the Sporochnales (*Carpomitra* and *Sporochnus*). However, *A. ferruginea* grouped with the fucalean members in the ME-GTR tree, and *B. antillarum* became monophyletic with the Fucales in the MP trees. No significant bootstrap supports were obtained for those clades.

An MP analysis based on the first and second codon positions of *rbc*L resulted in trees with slightly improved CI/RI/RC values compared with those using all three codon positions (Table 2). However, the resultant trees lost any resolution between the *Asterocladon*/*A. rhodochortonoides* clade and the Ectocarpales *sensu lato* (data not shown). The CI/RI/RC values were also improved in the MP trees based on the SSU data set (Table 2). In the SSU trees, relationships among

Table 1. Taxa included in the present study, with accession numbers of DNA sequences

Taxon	*rbc*L	SSU (in combined data set)
Adenocystis utricularis (Bory) Skottsberg	AJ295823	AF073322
Agarum clathratum Dumortier	AB035791	
Akkesiphycus lubricum Yamada et Tanaka	AB036038	AB036036
Alaria esculenta (Linnaeus) Greville	AF064745	AF115427
Alethocladus corymbosus (Dickie) Sauvageau	AJ287860	AJ287439
Ascophyllum nodosum (Linnaeus) Le Jolis	AJ287853	AF091297
Asperococcus fistulosus (Hudson) Hook	AF207796	
Asterocladon interjectum Uwai, Nagasato, Motomura et Kogame	AB102866	AB102865
Asterocladon lobatum Müller, Parodi et Peters	AJ295824	AJ229120
Asteronema ferruginea (Harvey) Delépine et Asensi	AJ295818	AJ229114
Asteronema rhodochortonoides (Børgesen) Müller et Parodi	AJ295825	AJ229117
	AB102867[a]	AB056156[a]
Bachelotia antillarum (Grunow) Gerloff	AF207797	AJ229123
Caepidium antarcticum J. Agardh	AJ295826	AJ295827
Carpomitra costata (Stackhouse) Batters	AB045257	Z99445
Chorda filum (Linnaeus) Stackhouse	AB035781	AF073324
Chordaria flagelliformis (Müller) C. Agardh	AF207798	AF073324
Choristocarpus tenellus (Kützing) Zanardini	AJ287862	AJ287441
Chnoospora implexa J. Agardh	AB022231	
Cladostephus spongiosus (Hudson) C. Agardh	AJ287863	AF091298
Coelocladia arctica Rosenvinge	AF055395	
Delamarea attenuata (Kjellman) Rosenvinge	AF055396	
Desmarestia aculeata (Linnaeus) J.V. Lamouroux	AJ287847	Z99451
Desmarestia latifrons Kützing	AB037139	
Desmarestia ligulata (Lightfoot) J.V. Lamouroux	AJ287848	L43060
Desmarestia viridis (Müller) Lamouroux	AJ287849	AJ295828
Dictyosiphon foeniculaceus (Hudson) Greville	AF055397	Z99463
Dictyota cervicornis Kützing	AJ287851	AJ287435
Dictyota dichotoma (Hudson) Greville	AJ287852	AJ287437
Ectocarpus siliculosus (Dillwyn) Lyngbye	X52503	L17015
Eisenia arborea Areschoug	AJ287855	
Elachista fucicola (Velley) Areschoug	AF055398	
Fucus distichus Linnaeus	AF195515	AB011423
Geminocarpus austrogeorgiae Skottsberg	AJ295830	AJ295831
Giraudia sphacelarioides Derbès et Solier	AF055399	
Halosiphon tomentosus (Lyngbye) Jaasund	AB037136	L43056
Haplospora globosa Kjellman	AB037138	AF130712
Hecatonema sp.	AF055400	
Himantothallus grandifolius (A. et E.S. Gepp) Zinova	AJ287850	AJ229110
Hummia onusta (Kützing) Fiore	AF055402	
Isthmoplea sphaerophora (Harvey) Kjellman	AF055403	
Kjellmaniella crassifolia Miyabe	AB035792	AF123577
Laminariocolax tomentosoides (Farlow) Kylin	AF055404	
Leptonematella fasciculata (Reinke) P.C. Silva	AF055405	
Litosiphon pusillus (Lyngbye) Harvey	AF055406	
Macrocystis pyrifera (Linnaeus) C. Agardh	AJ287856	AF115430
Mikrosyphar porphyrae Kuckuck	AF207806	
Myelophycus cavum J. Tanaka et Chihara	AY095319	
Myelophycus simplex (Harvey) Papenfuss	AY095320	
Myrionema strangulans Greville	AF055407	
Myriotrichia clavaeformis Harvey	AF055408	Z99475
Nereocystis luetkeana (Mertens) Postels et Ruprecht	AJ287857	
Onslowia endophytica Searles	AJ287864	AJ287443
Pelagophycus porra (Leman) Setchell	AJ287858	
Petalonia fascia (O.F. Müller) Kuntze	AB022243	AF115431
Petalonia zosterifolia (Reinke) Kuntze	AB022242	
Phaeosiphoniella cryophila R.G. Hooper, E.G. Henry et Kuhlenkamp	AB045259	AF130713
Phaeostroma pustulosum Kuckuck	AF207808	
Phyllariopsis brevipes ssp. *brevipes* (C. Agardh) E.C. Henry et South	AB045245	
Phyllariopsis purpurascens (C. Agardh) E.C. Henry et South	AB045249	
Pogotrichum filiforme Reinke	AF055409	
Pseudochorda gracilis Kawai et Nabata	AB035790	
Punctaria latifolia Greville	AY095322	AF115432
Punctaria plantaginea (Roth) Greville	AF055410	
Pylaiella littoralis (Linnaeus) Kjellman	X55372	AF115434
Saccorhiza dermatodea (de la Pylaie) J. Agardh	AB045252	

(*continued*)

Table 1. Continued

Taxon	*rbc*L	SSU (in combined data set)
Saccorhiza polyschides (Lightfoot) Batters	AB045254	L43059
Sargassum echinocarpum J. Agardh	AF076689	
Sargassum muticum (Yendo) Fensholt	AJ287854	AF091295
Sargassum obtusifolium J. Agardh	AF195516	
Sargassum polyphyllum J. Agardh	AF076690	
Scytosiphon lomentaria (Lyngbye) Link	AB022238	L43066
Scytothamnus australis (J. Agardh) Hooker et Harvey	AJ295833	AF073325
Sorocarpus micromorus (Bory) P.C. Silva	AF055411	
Sphacelaria caespitula Lyngbye	AJ287871	
Sphacelaria cirrosa (Roth) C. Agardh	AJ287865	AF115428
Sphacelaria nana Naegeli ex Kützing	AJ287875	
Sphacelaria plumigera Holmes	AJ287878	
Sphacella subtilissima Reinke	AJ287869	
Sphaerotrichia divaricata (C.Agardh) Kylin	AB066061	
Splachnidium rugosum (Linnaeus) Greville	AJ295834	AF073327
Sporochnus scoparius Harvey	AB037142	
Stictyosiphon soriferus (Reinke) Rosenvinge	AF055413	
Streblonema tenuissimum Hauck	AF055414	
Striaria attenuata (Greville) Greville	AF055415	Z99478
Stypocaulon scoparium (Linnaeus) Kützing	AJ287866	AF091299
Syringoderma phinneyi Henry et Müller	AJ287868	L17017
Thalassiophyllum clathrus (Gmelin) Postels et Ruprecht	AB035793	
Tilopteris mertensii (Turner in Smith) Kützing	AB045260	
Turbinaria ornata (Turner) J. Agardh	AF076688	
Verosphacella ebrachia E.C. Henry	AJ287867	
Undaria peterseniana (Kjellman) Okamura	AB035794	
Utriculidium durvillei (Bory) Skottsberg	AJ295835	AF073321

[a]Samples collected from Japan (Kogame *et al.*, 2001).

the orders became a polytomy although the *Asterocladon*/*A. rhodochortonoides* clade was sister to the Ectocarpales *sensu lato* (data not shown).

Nucleotide sequence analyses of the combined data set

The partial SSU sequence of *A. interjectum* (1761 bp) was determined, of which 439 bp were aligned based on the alignment of Draisma *et al.* (2001) and combined with the *rbc*L sequence. 47 phaeophycean taxa were used in our alignment of the combined data set (Table 1), which was 1848 bp in length. A PHT between *rbc*L and partial SSU yielded a *p*-value of 0.474, which indicated congruence between the partitions. Saturation was not indicated in the partial SSU sequence used in the combined data set, although saturation was indicated in the combined data set due to the *rbc*L sequence. Since the ME-GTR tree was very similar to the ME-JC tree we have not described the former.

In all trees inferred from the combined data set (Fig. 24), *A. interjectum* was monophyletic with *A. lobatum* and *A. rhodochortonoides* (100% in MP and ME-JC, 99% in ML), while elsewhere the phylogenetic position of *A. interjectum* was similar to that inferred from *rbc*L sequences: monophyletic with the Ectocarpales *sensu lato* (100% in MP and ME-JC trees, 95% in ML), and sister to one of two laminarialean clades. *Asteronema ferruginea* grouped with two species of the Scytothamnales with weak (63% in MP) or moderate (74% in ME-JC, 76% in ML) support. *Bachelotia antillarum* became monophyletic with *C. costata*, and this clade was sister to the Scytothamnales/*A. ferruginea* clade in ML and ME-JC trees, but without significant support.

Tree scores and nucleotide differences

Tree scores of both data sets are shown in Table 2. MP trees of both data sets, especially those based on *rbc*L only, had a low consistency index (CI), which suggest high homoplasy in the *rbc*L data set. CI for our *rbc*L data set was lower than that of Draisma *et al.* (2001; table 5, CI = 0.36), as well as that based on our *rbc*L-SSU combined data set. The low CI for our *rbc*L tree was due, at least in part, to the large number of OTUs in each order. The *rbc*L data subset using the same taxon sampling as our combined data set, showed a CI similar to that of the combined data set (Table 2) as well as that of Draisma *et al.* (2001).

Sequence data from *Asterocladon* spp. and *Asteronema* spp. were compared in Table 3. 127 bp differences were found between *A. interjectum* and *A. lobatum* in *rbc*L and 13 bp in SSU. In *rbc*L, *A. rhodochortonoides* was 112–117 bp different from *Asterocladon* spp. and 171–173 bp from *Asteronema ferruginea*.

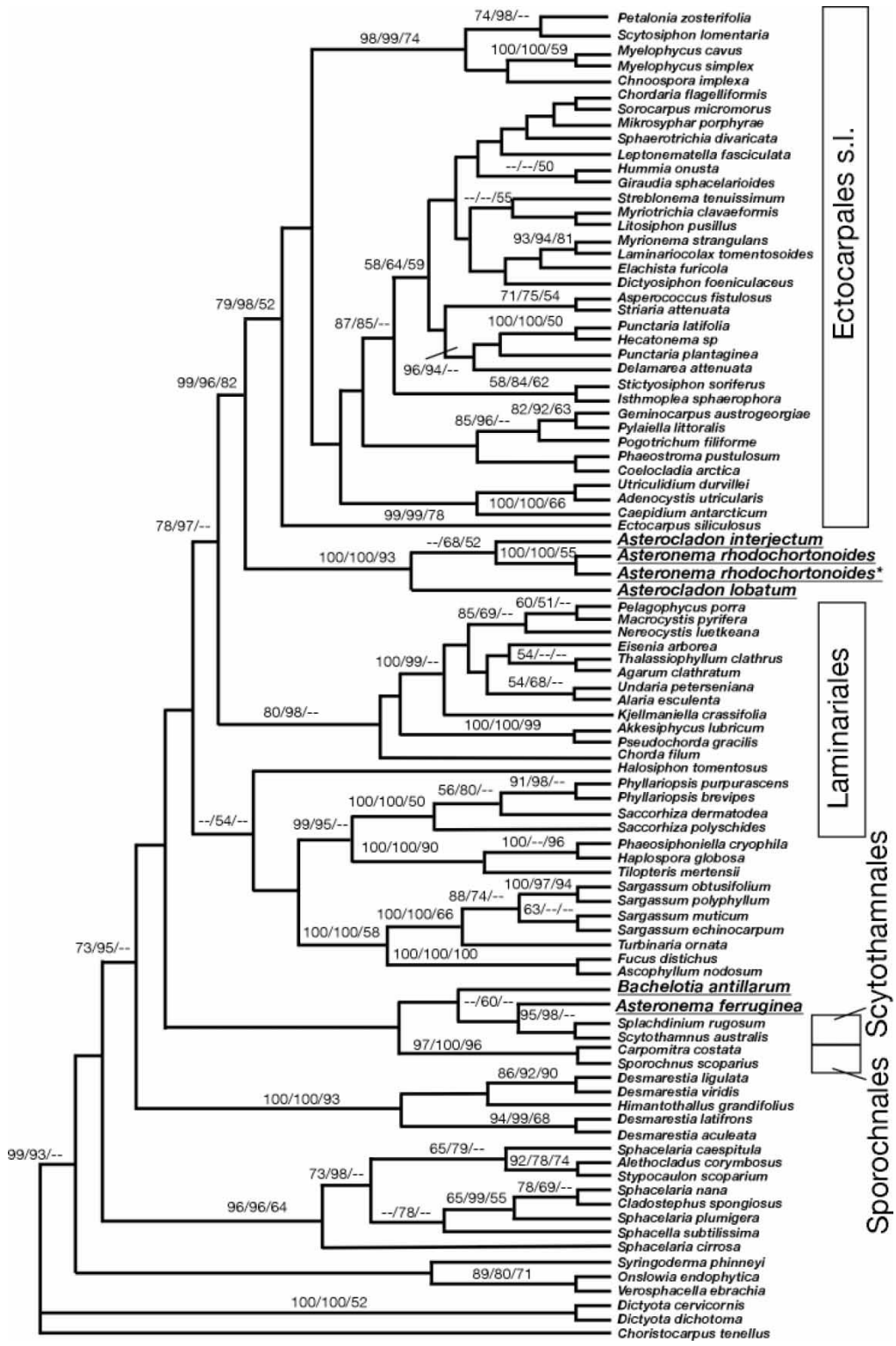


Fig. 23. ML tree based on the *rbc*L gene sequences. Numbers at the nodes represent bootstrap values (>50%, 1000 replicates) of the MP tree (first), ME-JC tree (second) and quartet-puzzling values of ML tree (third, >50%, 1000 replicates). Species that have stellate chloroplast arrangements are represented by underlined letters, and orders closely related to OTUs with stellately arranged chloroplasts are indicated.

Species description

Asterocladon interjectum Uwai, Nagasato, Motomura et Kogame, sp. nov. (Figs 2–22)

DIAGNOSIS: *Thalli filamentosa et prostrata. Quoque axes mono- ad oligostichos. Cellae cum 2–8 chloroplastis oblongis aggregatis una et radiatis ex pyrenoidibus proximalibus. Zoosporangia unilocularia 20–25 μm longa et 15–20 μm lata, sessilia, ovata ad elliptica, lateralia in axibus. Unizoosporae destitutae stigmate. Gametangia multilocularia intercalaria vel terminalia in*

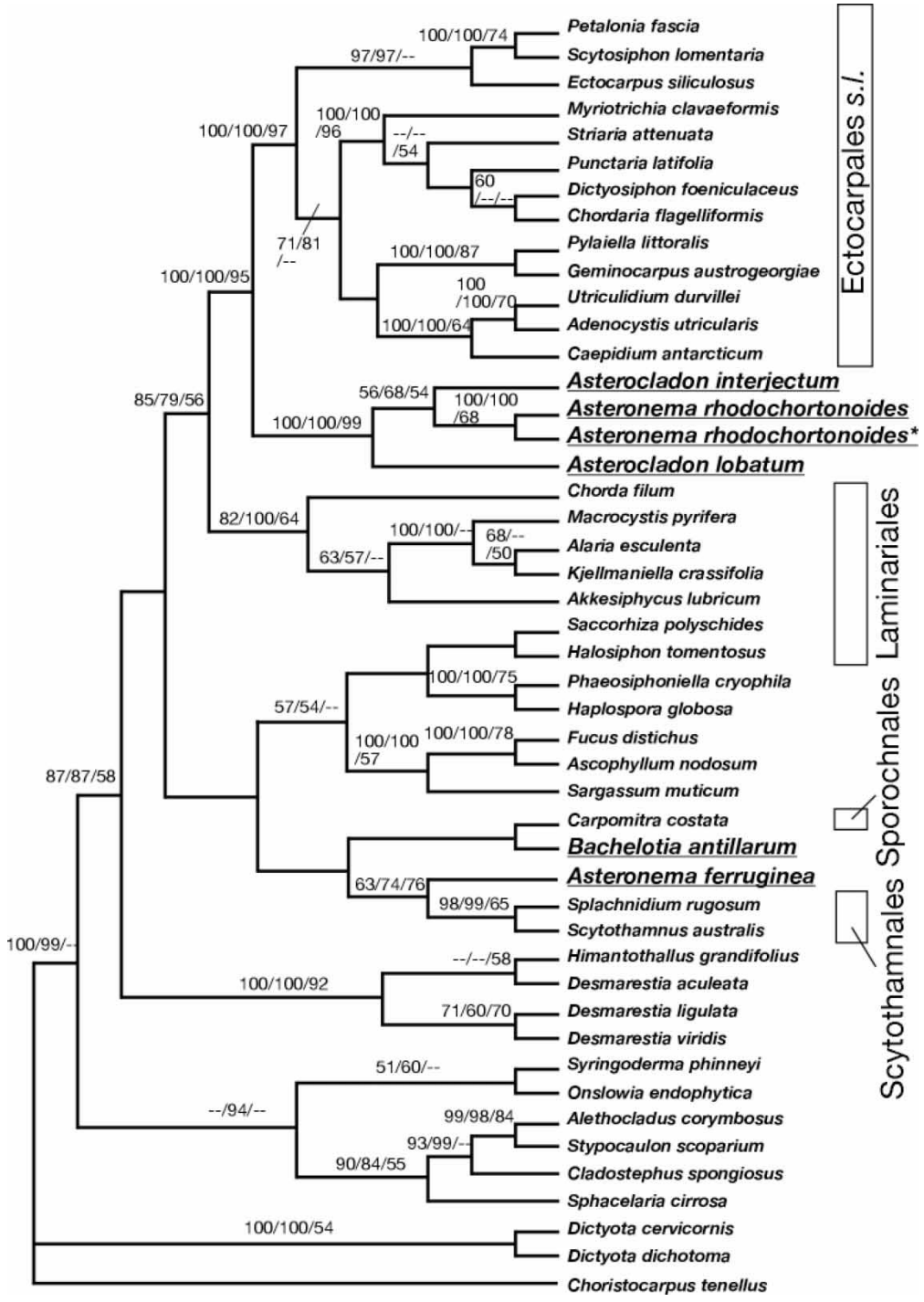


Fig. 24. ML tree constructed from the *rbc*L + SSU combined sequences. Numbers at the node represent bootstrap values (>50%, 1,000 replicates) of the MP tree (first), ME-JC tree (second) and quartet-puzzling values of ML tree (third, >50%, 1,000 replicates). Species that have stellate chloroplast arrangements are represented by underlined letters, and orders closely related to OTUs with stellately arranged chloroplasts are indicated.

axibus. *Reproduction sexualis anisogamica*. *Gametae destitutae stigmate*.

Thalli are filamentous and prostrate. Axes are mono- or oligostichous. Cells contain 2–8 oblong chloroplasts aggregating together and radiating from proximal pyrenoids. Unilocular zoidangia are 20–25 μm long by 15–20 μm wide, sessile, ovate to elliptical, laterally formed on axes. Unizoids lack an eyespot. Plurilocular gametangia are intercalary or terminal in axes. Sexual reproduction is anisogamous. Gametes lack an eyespot.

ETYMOLOGY: Referring to the manner plurilocular zoidangia formation.

HOLOTYPE: Microslide (SAP095046) from the unialgal clonal culture (from Kumano, Mie Prefecture, Japan, 27 March 2001), deposited in the Herbarium of the Graduate school of Science, Hokkaido University (SAP).

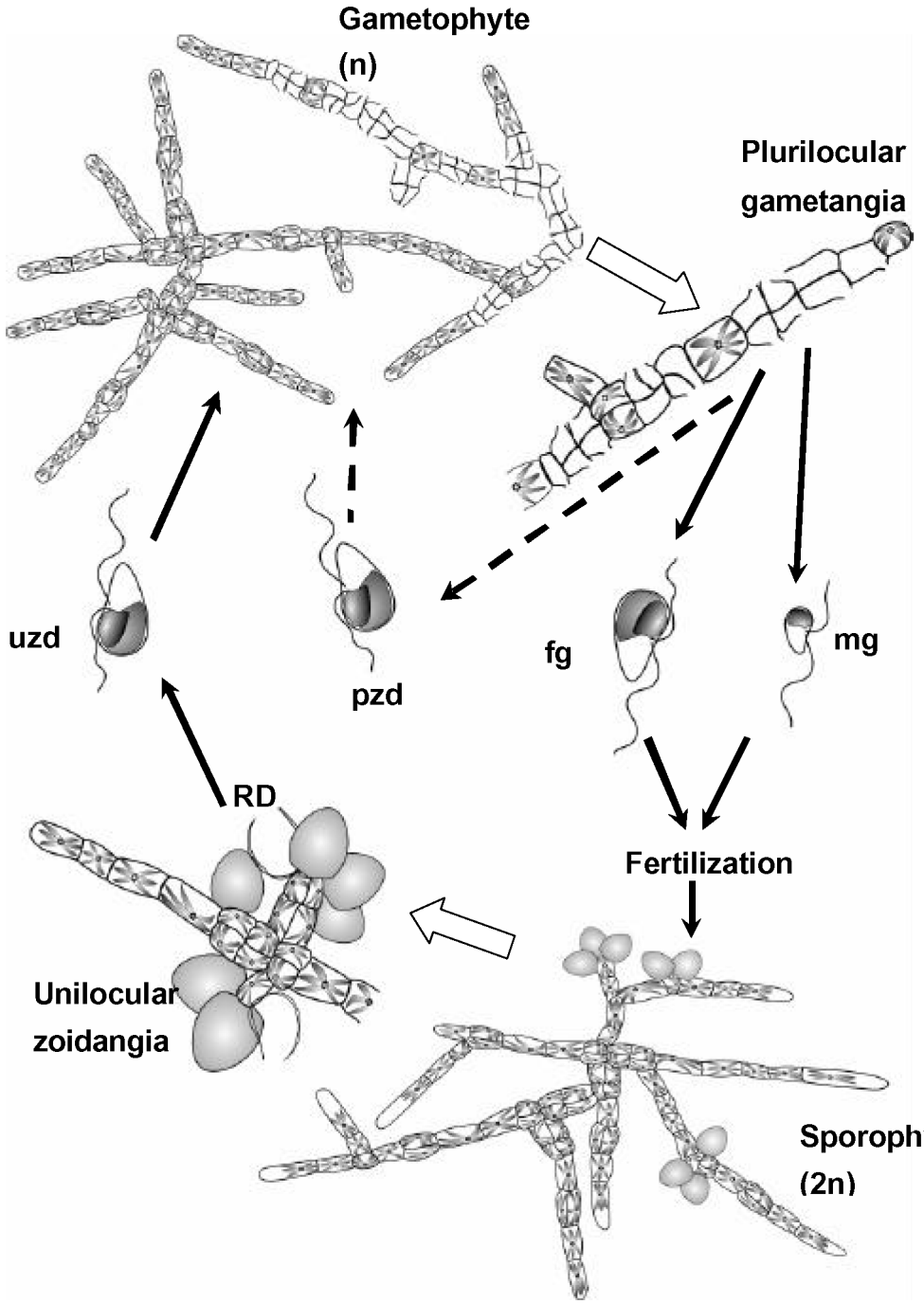


Fig. 25. Diagrammatic summary of the life history of *A. interjectum* in culture. Abbreviations: fg: female gamete; mg: male gamete; pzd: plurizoid; RD: reduction division; uzd: unizoid. Broken line: asexual reproduction by large plurizoids resembling female gamete.

ISOTYPE: Same material as the holotype, microslides (SAP095047, 095048) deposited in SAP.

Discussion

Our isolate has a stellate arrangement of chloroplasts similar to that in *Asteronema*, *Bachelotia* and *Asterocladon*. Morphological characters, such as oligostichous filaments with predominantly apical growth, lack of erect filaments and the presence of phaeophycean hairs with a basal sheath, strongly suggest that our isolate belongs to the genus *Asterocladon* (Müller *et al.*, 1998). However, our isolate is different from *A. lobatum*, the only other member of the genus (Müller *et al.*, 1998), in the morphology of plurilocular zoidangia and the absence of an eyespot in all swarmers. *A. lobatum* has plurilocular zoidangia formed on laterals, which are multi-lobed and conical in shape. In our isolate, plurilocular zoidangia are formed terminally on laterals, or are intercalary (Table 4). Furthermore, the *rbc*L sequence

Table 2. Comparison of tree scores for each data set

	*rbc*L	combined (*rbc*L+SSU)	SSU	*rbc*L (47 OTU)	*rbc*L (first + second position)
Number of OTU	92	47	47	47	92
Length	1399	1848 (1399 + 449)	449	1399	932
Variable site	679	714	104	610	254
MP					
Informative site	533	560	80	480	141
Number of MPTs	32	3	1000[b]	1	93212
Tree length	4049	3130	266	2845	782
CI/RI/RC[a]	0.268/0.625/0.168	0.352/0.549/0.193	0.605/0.806/0.488	0.331/0.519/0.172	0.404/0.709/0.287
ML					
−ln likelihood	21 570.98038	17 328.57589	–	–	–
Model	GTR + G (0.7254) + I (0.4363)	GTR + G (0.6839) + I (0.4763)	–	–	–

[a]CI: consistency index, RI: retention index, RC: rescaled consistency index. [b]Due to maxtree setting = 10,000.

Table 3. Number of nucleotide differences in *rbc*L (below diagonal) and SSU (above diagonal) between isolates

	Asteronema rhodochortonoides	*A. rhodochortonoides* (Japan)	*Asterocladon interjectum*	*A. lobatum*	*Asteronema ferruginea*
Asteronema rhodochortonoides	–	2	18	20	28
A. rhodochortonoides (Japan)	2	–	18	20	28
Asterocladon interjectum	114	112	–	13	21
A. lobatum	117	117	127	–	22
Asteronema ferruginea	173	171	161	167	–

difference between *A. interjectum* and *A. lobatum* is 127 bp, which is far larger than that between other congeneric phaeophyceaen species. In our alignment, interspecific *rbc*L nucleotide differences are 8–50 bp in the genus *Sargassum*, and 23–32 bp in *Desmarestia*. Based on these molecular and morphological differences, we consider our isolate to be a new species, distinct from *A. lobatum*.

Paraphyly of the genus *Asteronema* has been reported previously (Draisma *et al*., 2001; Peters & Ramírez, 2001; Rousseau *et al*., 2001) but was also shown in this study. Reviers & Rousseau (1999) discussed the difference in chloroplast ultrastructure between the generitype *Asteronema australe* Delépine *et* Asensi and *A. rhodochortonoides*. The pyrenoid of *A. australe* has cytoplasmic invaginations (Delépine *et al*., 1976; Asensi *et al*., 1977), whereas that of *A. rhodochortonoides* does not (Müller & Parodi, 1994). *A. australe* was thought to be conspecific with *A. ferruginea* (Delépine & Asensi, 1975; Womersley, 1987; Müller & Parodi, 1994). The differences in pyrenoid ultrastructure also indicate paraphyly of the genus. Cytoplasmic invaginations could not be observed in *A. interjectum* or in *A. lobatum* (Müller *et al*., 1998; figs 10, 11). The phylogenetic significance of cytoplasmic invagination in the brown algal lineage is unclear at present. Such structures were reported for *Petroderma maculiforme* (Wollny) Kuckuck (Asensi *et al*., 1977), but the close relationship between *A. ferruginea* and the Scytothamnales in our molecular trees indicates that cytoplasmic invagination is a synapomorphic character of this lineage compared with the *Asterocladon*/*A. rhodochortonoides* clade.

The generic circumscription of *Asterocladon* clearly separates this genus from *Asteronema* (especially *A. rhodochortonoides*), however, these genera could not be clearly separated in our molecular phylogenetic trees. As mentioned above, two lineages of *Asteronema*, i.e. *A. ferruginea* and *A. rhodochortonoides*, can be distinguished on ultrastructural morphology, and *A. rhodochortonoides* shared ultrastructural features with *Asterocladon* spp. Our molecular data did not place *A. rhodochortonoides* within *Asterocladon*; the data only showed a polytomy among these species, or suggested the paraphyly of *Asterocladon* with weak bootstrap support. However, *A. rhodochortonoides* could be transferred to *Asterocladon* based on ultrastructural features, if the generic circumscription of *Asterocladon* was emended. Morphological features of field material of *Asterocladon* spp. are not clearly defined and Müller *et al*. (1998) described the genus and its generitype, *A. lobatum*, from cultured material only. Our material was an endo-epiphytic filament in cyanobacterial thalli, making

Table 4. Comparison of morphological characters among brown algal species with stellately arranged chloroplasts

	Asterocladon interjectum	*Asterocladon lobatum*	*Asteronema rhodochortonoides*	*Asteronema australe* (=*ferruginea*)	*Asteronema breviarticulatum*	*Bachelotia antillarum*
Pyrenoid	Lateral	Lateral	Lateral	Lateral	Unknown[a]	Terminal
Pyrenoid invagination	Absent	Absent	Absent	Present	Absent	Absent
Thallus	Oligostichous	Oligostichous	Uniseriate	Uniseriate	Uniseriate	Uniseriate
Phaeophycean hairs	Present	Present	Absent	Present	Absent	Absent
Erect filament	Absent	Absent	Present	Present	Present	Present
Life history	Isomorphic	Direct	Direct	Unknown	Unknown	Unknown
Unilocular zoidangia	Lateral sessile	Unknown	Unknown	Lateral pedicellate	Unknown	Intercalary
Plurilocular zoidangia	Intercalary or terminal	Lateral/multilobed	Lateral/elliptical	Lateral or terminal/conical	Lateral/elliptical	Intercalary
References	Present study	Müller *et al*., 1998	Müller & Parodi, 1994; Kogame *et al*., 2001	Delépine *et al*., 1976 (as *A. australe*); Asensi *et al*., 1977 (as *A. australe*); Womersley, 1987; Reviers & Rousseau, 1999.	Ouriques & Bouzon, 2000	Price, 1973; Magne, 1976; Asensi *et al*., 1977 (as *A. australe*); Reviers & Rousseau, 1999.

[a]It is difficult to distinguish whether the pyrenoid of *A. breviarticulatum* is lateral or terminal from figures in Ouriques & Bouzon (2000).

it difficult to characterize its morphology, especially with respect to the absence of erect filaments or the presence of hairs. Therefore, the significance of these microscopic characters for generic circumscription is unclear at present. The genus *Asteronema*, i.e., *A. australe* (=*A. ferruginea*), and *Asterocladon* should be distinguished by ultrastructural morphology (i.e., cytoplasmic invaginations in pyrenoids) rather than microscopic features, such as oligostichous filaments, lack of erect filaments and the presence of phaeophycean hairs. In conclusion, we consider that the appropriate taxonomic position of *A. rhodochortonoides* is in *Asterocladon*, and we transfer *A. rhodochortonoides* to *Asterocladon* with an emended generic description (see below). Based on its pyrenoid ultrastructure, *Asteronema breviarticulatum* (J. Agardh) Ouriques & Bouzon (Ouriques & Bouzon, 2000) is also a possible member of *Asterocladon*. However, we have not transferred this species into *Asterocladon* because its phylogenetic position has not yet been clarified using molecular phylogenetic analyses.

Stellately arranged chloroplasts are also known in *B. antillarum*, in which several oblong chloroplasts are joined at their pyrenoids to form a stellate chloroplast-pyrenoid complex, similar to that of *Asteronema* and *Asterocladon*. However, in *Bachelotia* the pyrenoid is terminal in relation to the chloroplast thylakoid orientation (Magne, 1976; Asensi *et al*., 1977; Reviers & Rousseau, 1999). Pyrenoids protrude laterally in *Asteronema* (*A. australe*, *A. rhodochortonoides*; Müller & Parodi, 1994; Reviers & Rousseau, 1999) and *Asterocladon* (Müller *et al*., 1998; and this study). No cytoplasmic invagination was reported for *B. antillarum*. Although the phylogenetic position of *B. antillarum* is unclear at present, and we cannot therefore conclude whether the stellately arranged chloroplasts of *A. ferruginea* and *B. antillarum* are monophyletic or not, three types of stellately arranged chloroplasts (excluding the stellate shaped scytothamnalean chloroplast) can be recognized in the brown algae. Stellate chloroplast arrangements have also been reported in two species of euglenophycean genus *Eutreptiella* (Walne *et al*., 1986). In *Eutreptiella eupharyngea* Møstrup *et* Norris and *Eutreptiella braarudii* Throndsen, several oblong chloroplasts join at the protruding terminal pyrenoids and radiate from the central pyrenoid complex (Walne *et al*., 1986). These euglenophycean stellately arranged chloroplasts are very similar to those in the brown algal genera. Therefore, stellate chloroplast arrangements are considered to have evolved independently in different algal lineages, including several (at least two) brown algal lineages.

All zoids of *A. interjectum* lacked eyespots. Although unilocular zoidangia have not been reported in *A. lobatum* (Müller *et al*., 1998) and *A. rhodochortonoides* (Müller & Parodi 1994; Kogame *et al*., 2001), which are closely related to *A. interjectum*, plurizoids of these species have an eyespot (Müller *et al*., 1998; Kogame *et al*., 2001). Spermatozoids lack eyespots in several groups of oogamous brown algae (Müller & Lüthe, 1981; Henry & Cole, 1982*a*; Müller & Meel, 1982; Motomura *et al*., 1985; Müller *et al*., 1985; Peters & Müller, 1986; Wiencke & Clayton, 1990; Kawai, 1992). However, the absence of eyespots in zoids from unilocular zoidangia is known only for the Laminariaceae, Alariaceae and Lessoniaceae (Laminariales, Henry & Cole, 1982*b*; Kawai, 1992), *Chordaria linearis* (Hooker *et* Harvey) Cotton (Ectocarpales *sensu lato*, Peters, 1992) and *Ascoseira mirabilis* Skottsberg (Ascoseirales, Müller *et al*., 1990), although an eyespot was observed in the latter by Clayton (1987). According to our molecular phylogeny, *A. interjectum* is not so distantly related to the Laminariales and *Chordaria flagelliformis* (it may be a close relative of *C. linearis*). However, it is monophyletic with *A. rhodochortonoides* and *A. lobatum*, rather than with species without eyespots. It is possible that eyespots of unizoids have been lost independently several times in the phaeophycean lineage.

Asterocladon interjectum has an isomorphic and diphasic life history, with alternating diploid sporophytes and haploid gametophytes sharing similar morphology (Fig. 7). Sexual fusion occurs between a large female gamete and a small male gamete (anisogamy), both of which are formed on the same thallus (monoecism). Sexual reproduction has not been reported in brown algal taxa with stellately arranged chloroplasts (as opposed to stellate in shape), that is, species of *Asterocladon*, *Asteronema* and *Bachelotia*. Although Delépine & Asensi (1975) counted 32 chromosomes in thalli bearing unilocular sporangia, and 16 in thalli originating from unizoids, suggesting a diphasic life cycle with meiosis in unilocular sporangia, they did not observe sexual fusion directly. Thalli bearing unilocular sporangia resemble thalli with plurilocular sporangia in *A. australe*, which suggests an isomorphic diphasic life cycle in this species. However, this species is widely separated from *Asterocladon* spp. in our molecular trees. Only plurizoidangia are known in *A. lobatum* and *A. rhodochortonoides*. Müller *et al*. (1998) discussed how the simple direct life history of *A. lobatum* could have originated from a truncated life history: unizoidangial (originally sporophytic) thalli became extinct and non-sexual plurizoidangial (originally gametophytic) thalli evolved independently. Our observation that plurizoids resembling

female gametes repeat the gametophytic phase without sexual fusion, supports their speculation. Since *A. interjectum* shows an isomorphic life history, it is possible that a morphologically similar sporophytic generation occurs in the life histories of *A. lobatum* and *A. rhodochortonoides*. Furthermore, the molecular phylogenetic position of the *Asterocladon* clade, i.e. closest sister to the Ectocarpales *sensu lato*, renders an isomorphic life history ancestral in the Ectocarpales *sensu lato,* although sexual stages of *A. interjectum* have some unique features (no eyespot, monoecism). Peters & Ramírez (2001) could not determine whether an isomorphic or heteromorphic life history is ancestral in the Ectocarpales *sensu lato* because sexuality has not been reported in the closest sister group (*A. rhodochortonoides* and *A. lobatum*) of the Ectocarpales *sensu lato* in their molecular trees.

Asterocladon D. G. Müller, Parodi et Peters (1998, p. 430) emend. Uwai, Nagasato, Motomura et Kogame.
Filamentous brown algal thallus with apical growth, with or without lateral hairs and erect filaments. Longitudinal cell divisions present or absent. Cells contain several chloroplasts in one to several stellate arrangements, and their pyrenoids aggregate to form a spherical body near the nucleus. Cytoplasmic invaginations in a pyrenoid absent.

Asterocladon rhodochortonoides (Børgesen) Uwai, Nagasato, Motomura et Kogame, comb. nov.
Basionym: *Ectocarpus rhodochortonoides* Børgesen, Dansk Botanisk Arkiv 2: 170 (1914).
Synonym: *Asteronema rhodochortonoides* (Børgesen) D.G. Müller et Parodi, Phycologia 33: 473 (1994).

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