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Morphology and molecular phylogeny of *Phaeostrophion irregulare* (Phaeophyceae) with a proposal for Phaeostrophiaceae *fam. nov.*, and a review of Ishigeaceae

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Phaeostrophion irregulare, which is distributed along the Pacific coast of northwestern America, has been placed in the family Punctariaceae, order Dictyosiphonales (or Ectocarpales *sensu lato*), because of anatomical resemblances. On the basis of a previous culture study, the species was assumed to have a direct type of life history, with erect thalli forming unilocular or plurilocular zooidangia. However, the occurrence of a perennial prostrate system (holdfast), the presence of marginal meristematic cells on the holdfast and the lack of pyrenoids in chloroplasts suggest that this taxonomic assignment is questionable. Transmission electron microscopy observation confirmed the absence of pyrenoids in the chloroplasts. Molecular phylogenetic studies using *rbc*L (chloroplast) and partial 18S and 26S ribosomal RNA (nuclear) gene sequences revealed that *Phaeostrophion* is not included in the clade of Ectocarpales *s.l.*, but forms a clade with Sphacelariales which diverged relatively early in the evolution of Phaeophyceae and shares an apical or marginal meristematic manner of growth with them. Members of Ishigeaceae were also shown to have a distant phylogenetic relationship with Chordariales (or Ectocarpales *s.l.*) and diverged early in the phaeophycean lineage. We propose a new family Phaeostrophiaceae to accommodate *Phaeostrophion*, although we suspend taxonomic treatment at the ordinal level.

INTRODUCTION

Phaeostrophion irregulare Setchell & N.L. Gardner (Setchell & Gardner 1924) is a monotypic foliose brown alga that is distributed in the cold water areas along the Pacific coast of North America (Fig. 1). The species grows in colonies on rocks on more or less sheltered coasts frequently influenced by sand (Fig. 2). *Phaeostrophion irregulare* resembles species of *Endarachne* J. Agardh (Scytosiphonaceae, Scytosiphonales) in gross morphology (Fig. 3). However, Setchell & Gardner (1924) originally classified the species in the family Coilodesmaceae, order Chordariales, because the unilocular sporangia are embedded in the peripheral layer. In contrast, later researchers (Mathieson 1967; Abbott & Hollenberg 1976) classified *Phaeostrophion* Setchell & N.L. Gardner in Punctariaceae, Dictyosiphonales (= Punctariales) based on the life history study by Mathieson (1967). Mathieson (1967) reported the occurrence of erect thalli forming unilocular zooidangia, plurilocular zooidangia or both unilocular and plurilocular zooidangia on the same thallus in field material. In his culture study, both unizooids and plurizooids from those zooidangia developed into erect thalli via a plethysmothallus. Crustose holdfasts (basal discs) were perennial and new erect thalli developed on them. Based on these results, Mathieson (1967) assumed a direct type of life history for the species, as had been reported in several species of Dictyosiphonales, and therefore placed *Phaeostrophion* in Dictyosiphonales.

However, the following morphological features of this species suggest a systematic position distant from Chordariales and Dictyosiphonales (or Ectocarpales *sensu lato*, in which both of these orders and Ectocarpales *sensu stricto* and Scytosiphonales are also included): (1) the presence of a perennial (prostrate) basal system; (2) the presence of apical (marginal) meristematic cells in the basal system; and (3) the lack of pyrenoids in the chloroplasts. Unlike *Phaeostrophion*, (1) the basal systems (holdfasts) of members of Chordariales and Dictyosiphonales are generally ephemeral filamentous or pseudoparenchymatous discs and do not develop into perennial and parenchymatous discs (Pedersen 1984; Peters 1987); (2) members of the orders Chordariales and Dictyosiphonales typically exhibit diffuse growth (Fritsch 1945; Bold & Wynne 1985); and (3) members of Ectocarpales *sensu lato* have projected pyrenoids in their chloroplasts (Hori 1971a, b, 1972; Kawai 1992). Although the occurrence of pyrenoids has been reported in *Phaeostrophion* sporelings, the morphology of the pyrenoid depicted by the electron micrograph of the authors (Bourne & Cole 1968, fig. 6) was not conclusive because the cytoplasmic continuity of the presumptive pyrenoid with the chloroplast was not shown, and therefore the nature of this entity is dubious.

The systematic position of *P. irregulare* therefore requires re-examination. In the present study, we re-examined the fine structure of the chloroplast using cultured material and studied the molecular phylogeny using the DNA sequences of the Rubisco large subunit gene (*rbc*L) coded in the chloroplast genome, and the ribosomal DNA (rDNA) (partial 18S and 26S) coded in the nuclear genome. We also consider the phylogeny of Ishigeaceae, which shows some points of resemblance to *Phaeostrophion*.

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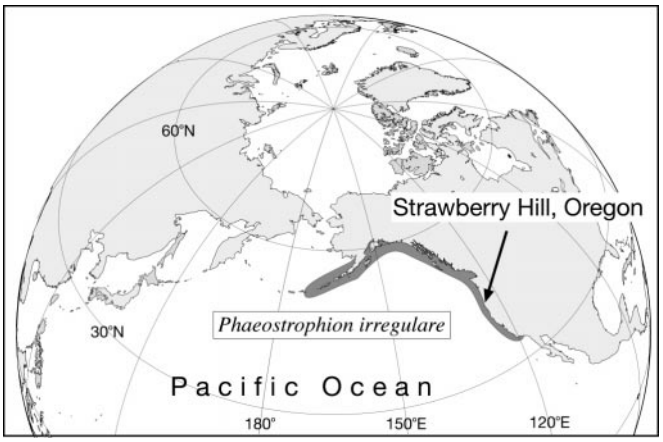

Fig. 1. Geographical distribution of *Phaestrophion irregulare* and the location of the sampling site (Strawberry Hill, Oregon, USA).

MATERIAL AND METHODS

Samples of *P. irregulare* were collected at Strawberry Hill (south of Newport, Oregon, USA; 44°25′N, 124°11′W) on 11 June 1994 (Fig. 1; Table 1). Unialgal cultures were established from the zooids released from unilocular zooidangia on the thalli. Culture material was used for transmission electron microscopy (TEM) observations and molecular phylogenetic study. Cultures were grown in polystyrene Petri dishes containing 50 ml PESI medium (Tatewaki 1966) illuminated by daylight-type white fluorescent lighting of approximately 50 μmol m^{-2} s^{-1} at 15°C long day (16 : 8 h light–dark).

For TEM observations, material was prefixed in 3% glutaraldehyde in 0.1 M cacodylate buffer for 3 h, postfixed in 2% OsO_4 in 0.1 M cacodylate buffer for 1 h, dehydrated in an acetone series and embedded in Spurr's epoxy resin (Spurr 1969), sectioned with a diamond knife, and stained with uranyl acetate and lead citrate. Observations were made using a JEOL 1020 transmission electron microscope (JEOL, Tokyo, Japan).

Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. The material was ground in liquid nitrogen, and approximately 40 mg of algal tissue powder was used. The intactness of extracted total DNA was assessed on ethidium bromide–stained 1.0% agarose gels.

Polymerase chain reaction (PCR) amplification of the partial 18S and 26S ribosomal RNA (rRNA) genes (18S and 26S rDNAs) and the *rbc*L was carried out with a GeneAmp PCR Cycler 2400 or 9700 (Applied Biosystems, Foster City, CA, USA) using a TaKaRa ExTaq (Takara Shuzo, Shiga, Japan) reaction kit (total volume of 25 μl comprising 2.5 μl 10× ExTaq Buffer, 5.0 μM deoxynucleoside triphosphate mixture, 0.1 μM of each primer, 0.625 units TaKaRa ExTaq and 2.0 μl DNA solution including 0.5–1.0 μg DNA). Primers (Table 2) were designed on the basis of known sequences of the corresponding regions reported for related taxa (Saunders & Druehl 1992; Tan & Druehl 1993, 1996; Kawai *et al.* 1995; Stache-Crain *et al.* 1997 for rDNA; Assali *et al.* 1990; Valentin & Zetsche 1990; Daugbjerg & Andersen 1997; Kogame *et al.* 1999 for *rbc*L). The profile of PCR conditions was as follows: an initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 42°C (rDNA) or 48°C (*rbc*L) for 30 s and 58°C (rDNA) or 54°C (rbcL) for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 7 min. The PCR products were directly sequenced using the Cy5 Auto Cycle Sequencing Kit (Pharmacia Biotech AB, Uppsala, Sweden) and ALF Express DNA sequencer (Pharmacia) or the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) and ABI PRISM 310 Genetic Analyzer (Applied Biosystems), following the manufacturer's instructions. Sequences were preliminarily aligned with reported sequence data of related taxa (Table 1) using the Clustal W computer program (Thompson *et al.* 1994) and then manually aligned for the phylogenetic analyses.

The aligned sequences were subjected to maximum parsimony (MP) analyses in a general heuristic search using PAUP* v. 4.0.2b (Swofford 1999). Twenty random additions of taxa were performed in each heuristic search, using the tree-bisection–reconnection branch-swapping option. Gaps were not taken into account in every analysis. From the same aligned data, two-parameter distances (Kimura 1980) between taxa were estimated and a phylogenetic tree was constructed with the neighbour-joining (NJ) method, using PAUP*. The program Modeltest v. 3.06 (Posada & Crandall 1998) was used to find the model of sequence evolution that best fit the data set by a hierarchical likelihood ratio test (a = 0.05). When the best sequence evolution model had been determined, maximum-likelihood (ML) was performed in PAUP using the estimated parameters (substitution model, gamma distribution, proportion of invariable sites), with three random additions in heuristic search. The robustness of the resulting phylogenies was tested by bootstrap analyses with 1000 (MP and NJ for *rbc*L, 18S and 26S rDNA) or 100 (ML for *rbc*L) re-samplings (Felsenstein 1985).

RESULTS

Marginal meristem and chloroplast morphology

In culture the germlings from unizooids first developed into crustose discs by marginal growth (Figs 4, 5) and then developed erect filaments on the discs. The erect thalli were generally foliose and parenchymatous (Fig. 6) but often

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Figs 2–6. *Phaestrophion irregulare,* habit and chloroplast morphology. Scale bars = 2 μm (Figs 4–6) or 50 mm (Fig. 3).
Fig. 2. Colony (marked with white circles indicated by arrows) at Strawberry Hill, Oregon, USA.
Fig. 3. Habit.
Figs 4, 5. Marginal apical cells of the disc in culture, LM.
Fig. 6. Surface view of cells of erect thallus showing the chloroplasts, LM.
Figs 7–10. *Phaestrophion irregulare,* fine structure of chloroplasts lacking pyrenoids, TEM; germling (Fig. 7), erect thallus (Figs 8–10). Scale bars = 1 μm (Figs 7, 8) or 200 nm (Figs 9, 10).

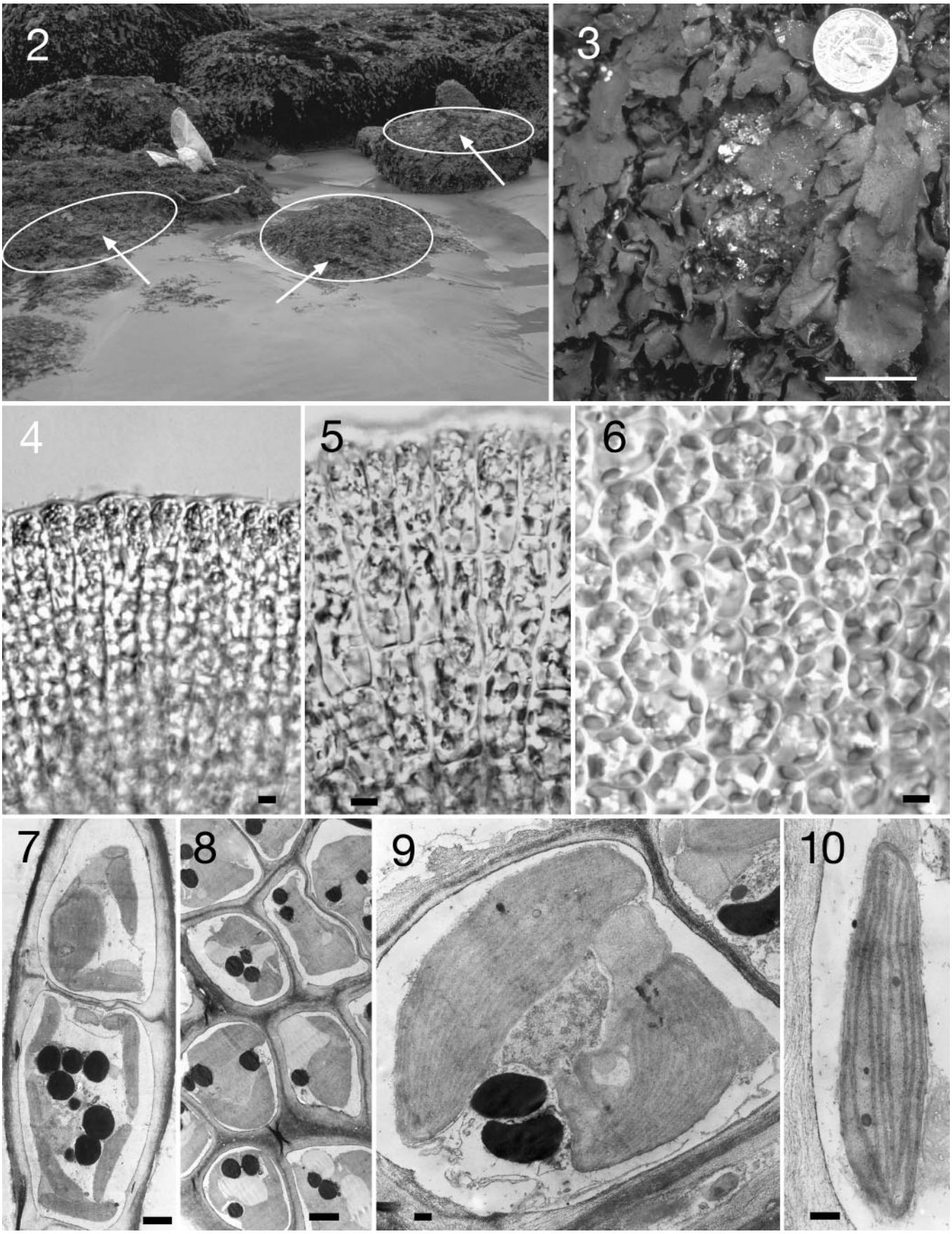
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Table 1. Origin of samples and sequence data used for molecular analyses including their database accession numbers. Accession codes in bold face show newly added sequences from the present study.

Species (taxonomic position)	Origin	DDBJ[1] accession code for *rbc*L	DDBJ accession code for 18S	DDBJ accession code for 26S rDNA
Phaeophyceae				
Incertae sedis				
Phaeostrophion irregulare Setchell & N.L. Gardner	Present study (Strawberry Hill, Oregon, USA)	**AB117948**	**AB117949**	**AB117950**
Ishigeaceae				
Ishige okamurae Yendo	Present study (Awaji Island, Hyogo, Japan)	**AB117951**		
I. sinicola (Setchell & N.L. Gardner) Chihara	Present study (Awaji Island, Hyogo, Japan)	**AB117952**		
Choristocarpaceae				
Choristocarpus tenellus (Kützing) Zanardini	Draisma *et al.* (2001)	AJ287861	AJ287441	AJ287442
Halosiphonaceae				
Halosiphon tomentosus (Lyngbye) Jaasund	Kawai *et al.* (2001)	AB036136		
H. tomentosus	Tan & Druehl (1996)		L43056	
H. tomentosus	Rousseau & Reviers (1999)			AF071156
Phyllariaceae				
Phyllariopsis brevipes ssp. *brevipes* (C. Agardh) Henry & South	Sasaki *et al.* (2001)	AB045244		
Saccorhiza dermatodea (de la Pylaie) J. Agardh	Sasaki *et al.* (2001)	AB045252		
S. polyschides (Lightfoot) Batters	Sasaki *et al.* (2001)	AB045254		AB045271
S. polyschides	Tan & Druehl (1996)		L43059	
Ascoseirales				
Ascoseira mirabilis Skottsberg	Rousseau *et al.* (2001)		AJ229126	AJ229141
Chordariales (Ectocarpales *sensu lato*)				
Chordaria flagelliformis (O.F. Müller) C. Agardh	Peters & Ramirez (2001)		AJ229129	AJ229129
Sphaerotrichia divaricata (C. Agardh) Kylin	Siemer *et al.* (1998)	AF055412		
Elachista fucicola (Velley) Areschoug	Siemer *et al.* (1998)	AF055398		
Cutleriales				
Cutleria multifida (J.E. Smith) Greville	Rousseau & Reviers (1999)		AF073326	
C. multifida	Rousseau & Reviers (1997)			AF053119
Zanardinia prototypus (Nardo) Nardo	Burrowes *et al.* (2003)	AY157693		
Desmarestiales				
Desmarestia latifrons Kützing	Kawai & Sasaki (2000)	AB037139		
D. ligulata (Lightfoot) J.V. Lamouroux	Tan & Druehl (1996)		L43060	
D. ligulata	Draisma *et al.* (2001)			AJ287434
D. tabacoides Okamura	Kawai & Sasaki (2000)	AB037140		
Desmarestia sp.	Kawai & Sasaki (2000)	AB037141		
Himantothallus grandifolius (A. Gepp & E.S. Gepp) Zinova	Draisma *et al.* (2001)		AJ287432	AJ287433
Dictyosiphonales (Ectocarpales *s.l.*)				
Delamarea attenuata (Kjellman) Rosenvinge	Siemer *et al.* (1998)	AF055396		
Dictyosiphon foeniculaceus (Hudson) Greville	Siemer *et al.* (1998)	AF055397		
D. foeniculaceus	Peters & Burkhardt (1998)		Z99463	
D. foeniculaceus	Müller *et al.* (1998)			AJ229137
Dictyotales				
Dictyota cervicornis Kützing	Draisma *et al.* (2001)	AJ287851	AJ287435	AJ287436
D. dichotoma (Hudson) J.V. Lamouroux	Draisma *et al.* (2001)	AJ287852	AJ287437	AJ287438
Durvillaeales				
Durvillaea potatorum (Labillardière) Areschoug	Rousseau & Reviers (1999)		AF091290	AF091283

Table 1. Continued.

Species (taxonomic position)	Origin	DDBJ[1] accession code for *rbc*L	DDBJ accession code for 18S	DDBJ accession code for 26S rDNA
Ectocarpales *sensu stricto*				
Ectocarpus siliculosus (Dillwyn) Lyngbye	Valentin & Zetsche (1990)	X52503		
Pilayella littoralis (Linnaeus) Kjellman	Assali *et al.* (1990)	X55372		
P. littoralis	Rousseau & Reviers (1999)		AF115434	AF071782
Fucales				
Ascophyllum nodosum (Linnaeus) Le Jolis	Draisma *et al.* (2001)	AJ287853		
Sargassum muticum (Yendo) Fensholt	Draisma *et al.* (2001)	AJ287854		
S. muticum	Rousseau & Reviers (1999)		AF091295	
S. muticum	Rousseau & Reviers (1997)			AF053109
Turbinaria ornata (Turner) J. Agardh	Phillips (1998)	AF076688		
T. turbinata (Linnaeus) O. Kuntze	Rousseau & Reviers (1999)		AF091300	AF091272
Laminariales				
Akkesiphycus lubricum Yamada & Tanaka	Kawai & Sasaki (2000)	AB036038		
Agarum clathratum Dumortier	Kawai *et al.* (2001)	AB035791		
Alaria esculenta (Linnaeus) Greville	Rousseau & Reviers (1999)		AF115427	AF071151
Chorda filum (Linnaeus) Stackhouse	Kawai *et al.* (2001)	AB035786		
C. filum	Boo *et al.* (1999)		AF123585	
C. filum	Rousseau & Reviers (1999)			AF073324
Laminaria ochroleuca de la Pylaie	Rousseau & Reviers (1999)		AF071154	AF091301
Pseudochorda nagaii (Tokida) Inagaki	Kawai *et al.* (2001)	AB035789		
Undaria peterseniana (Kjellman) Okamura	Kawai *et al.* (2001)	AB035794		
Scytosiphonales (Ectocarpales *s.l.*)				
Chnoospora implexa J. Agardh	Kogame *et al.* (1999)	AB022231		
Scytosiphon lomentaria (Lyngbye) Link	Kogame *et al.* (1999)	AB022238		
S. lomentaria	Kawai *et al.* (1995)		D16558	D16558
Scytothamnales				
Asteronema ferruginea (Harvey) Delépine & Asensi	Müller *et al.* (1998)		AJ229134	AJ229114
A. rhodochortonoides (Børgensen) Müller & Parodi	Müller *et al.* (1998)		AJ229117	AJ229135
Scytothamnus australis (J. Agardh) Hooker & Harvey	Peters & Ramirez (2001)	AJ295833		
S. australis	Rousseau & Reviers (1999)		AF073325	AF071780
Splachnidium rugosum (Linnaeus) Greville	Peters & Ramirez (2001)	AJ295834		
S. rugosum	Rousseau & Reviers (1999)		AF073327	AF071781
Sphacelariales				
Alethocladus corymbosus (Dickie) Sauvageau	Draisma *et al.* (2001)	AJ287860	AJ287439	AJ287440
Cladostephus spongiosus (Hudson) C. Agardh	Draisma *et al.* (2001)	AJ287863		
Halopteris filicina (Grateloup) Kützing	Draisma *et al.* (2002)			
Onslowia endophytica Searles	Draisma *et al.* (2001)	AJ287864	AJ287443	AJ287444
Sphacelaria cirrosa (Roth) C. Agardh	Draisma *et al.* (2001)	AJ287865		
S. nana Nägeli *ex* Kützing	Draisma *et al.* (2002)	AJ287875		
S. plumigera Holmes *ex* Hauck	Draisma *et al.* (2002)	AJ287878		
S. rigidula Kützing	Draisma *et al.* (2002)	AJ287885		
S. tribuloides Meneghini	Draisma *et al.* (2002)	AJ287892		
Sphacella subtilissima Reinke	Draisma *et al.* (2002)	AJ287869		
Stypocaulon durum (Ruprecht) Okamura	Draisma *et al.* (2002)	AJ287897		
S. scoparium (Linnaeus) Kützing	Draisma *et al.* (2002)	AJ287898		
Verosphacela ebrachia Henry	Draisma *et al.* (2001)	AJ287867		
Sporochnales				
Carpomitra costata (Stackhouse) Batters	Sasaki *et al.* (2001)	AB045257		
Sporochnus scoparius Harvey	Kawai & Sasaki (2000)	AB037142		

Table 1. Continued.

Species (taxonomic position)	Origin	DDBJ[1] accession code for *rbc*L	DDBJ accession code for 18S	DDBJ accession code for 26S rDNA
Syringodermatales				
Microzonia velutina (Harvey) J. Agardh	Burrowes *et al.* (2003)	AY157697		
Syringoderma phinneyi Henry & Müller	Draisma *et al.* (2001)	AJ287868		AJ287446
S. phinneyi	Tan & Druehl (1993)		L17017	
Tilopteridales				
Haplospora globosa Kjellman	Kawai & Sasaki (2000)	AB037138		
Phaeosiphoniella cryophila Hooper, Henry & Kuhlenkamp	Sasaki *et al.* (2001)	AB045259		
Tilopteris mertensii (Turner *in* Smith) Kützing	Sasaki *et al.* (2001)	AB045260		
Schizocladiophyceae				
Schizocladia ischiensis Henry, Okuda & Kawai	Kawai *et al.* (2003)	AB085615		
Xanthophyceae				
Tribonema aequale Pascher	Ariztia-Carmona *et al.* (1991)		M55286	
T. aequale	Van Der Auwera & De Wachter (1997)			Y07979

[1] DDBJ, DNA Data Bank of Japan.

remained filamentous. The growth pattern of the foliose thallus appeared to be diffuse, and no obvious meristematic cells were seen in the marginal portion. In contrast, the filamentous erect thallus had apical meristematic cells. The cells of both the discs and the erect thalli contained several disc-shaped chloroplasts. The presence or absence of pyrenoids in the cells was difficult to discern using light microscopy (LM) because of the presence of many lipid body–like granules or physodes (Figs 5, 6).

The TEM observations revealed that neither germlings from the zooids (Fig. 7) nor vegetative cells of the erect thalli (Figs 8–10) had pyrenoids, although the quality of the TEM images was compromised by the very thick cell walls and the presence of many granules, perhaps containing phenolic compounds (Figs 7–9).

The *rbc*L gene sequence analyses

In the analyses based on the taxa (operational taxonomic units – OTUs) covering most of the phaeophycean orders (Figs 11, 12), the phylogenetic position of *Phaeostrophion* varied somewhat among the analyses based on *rbc*L gene sequence data; however, the following features were consistent: (1) *Phaeostrophion* did not cluster with members of the Ectocarpales *sensu lato*; (2) *Phaeostrophion* first clustered with Syringodermatales and Sphacelariales (excluding Choristocarpaceae), although the tree topologies were different between the analyses. In the NJ and ML analyses, *Phaeostrophion* first clustered with Syringodermatales, but with Sphacelariales in the MP analysis. However, the bootstrap support of these branches was weak ($< 50\%$). Nevertheless, the monophyly of the clade including *Phaeostrophion*, Syringodermatales and Sphacelariales was supported by the relatively high bootstrap values (58–81%); and (3) the clade including *Phaeostrophion* diverged relatively early in the evolution of the Phaeophyceae together with Dictyotales and Ishigeaceae, following the divergence of Choristocarpaceae.

In the analyses more focused on basal taxa of the Phaeophyceae, the general tree topologies were basically the same. They only differed in the branching order of the subclade within Sphacelariales clade (Fig. 13). In the analyses, *Phaeostrophion* first grouped with Sphacelariales *sensu stricto* in all of the analyses supported by high (90% in NJ and 92% in ML, Fig. 13) or moderate (67% in MP, tree not shown) bootstrap values. Then the clade consisting of Syringodermatales became sister group of the clade including *Phaeostrophion* and Sphacelariales, although the bootstrap value supporting this branch was not high (58/67% in NJ/ML, Fig. 13; 63% in MP, tree not shown). Ishigeaceae was basal within the phaeophycean lineage together with Choristocarpaceae, but the branching order was not clearly resolved in the present analyses.

Partial 18S and 26S rDNA sequence analyses

The resolution and branching orders of partial 18S and 26S rDNA for elucidating the phylogenetic relationships of *Phaeostrophion* with other phaeophycean taxa were rather limited (Figs 14, 15). The bootstrap values of the branches connecting the taxa were mostly less than 50%, indicating weak support. Nevertheless, the general tree topologies indicating the phylogenetic relationships were consistent: Dictyo-

Table 2. List of primers used for PCR. Annealing positions correspond to the sequences of *Scytosiphon lomentaria* (18S, 5.8S and 25S of rDNA, accession number D16558: Kawai *et al.* 1995) and those of *Ectocarpus siliculosus* (*rbc*L and *rbc*S, accession number X52503: Valentin & Zetsche 1990). Mixtures: D, A + G + T; H, A + C + T; S, C + G; W, A + T; Y, C + T.

Code	F or R[1]	Sequence (5′ to 3′)	Annealing position
LD2	F	TAGTCATACGCTTGTCTCAA	18S (21–40)
LDA	F	CGATTCCGGAGAGGGAGCCTG	18S (377–397)
LDB	F	GTCTGGTGCCAGCAGCCGCGG	18S (558–578)
LDG	F	TAGCATGGAATAATGAGATAG	18S (813–833)
LDD	F	CAGAGGTGAAATTCTTGGAT	18S (914–933)
LD7	F	CTGAAACTTAAAGAAATTGACGG	18S (1145–1167)
LDH	F	CGCACGCGCGCTACACTGATG	18S (1473–1493)
5.8F-1	F	ACGCAGCGAAATGCGATACG	5.8S (47–66)
25F1[2]	F	CCGCTGAATTTAAGCATAT	26S (27–45)
LD4	R	TCAGGCTCCCTCTCCGG	18S (397–377)
LD5	R	CCGCGGCAGCTGGCACCAGAC	18S (578–558)
LD6	R	ATCCAAGAATTTCACCTCTG	18S (933–914)
18R-1	R	CCTTGTTACGACTTCACCTT	18S (1787–1768)
5.8R-1	R	CGTATCGCATTTCGCTGCGT	5.8S (66–47)
26R-1	R	GTTAGTTTCTTTTCCTCCGC	26S (70–51)
25R1[2]	R	CTTGGTCCGTGTTTCAAGAC	26S (635–616)
rbc-F0	F	ATCGAACTCGAATAAAAAGTGA	*rbc*L (20–41)
rbc-F1	F	CGTTACGAATCWGGTG	*rbc*L (43–58)
rbc-F2	F	AGGTTCWCTWGCTAA	*rbc*L (342–356)
PRB-F2[3]	F	TTCCAAGGCCCAGCAACAGGT	*rbc*L (454–474)
rbc-F3	F	CACAACCATTCATGCG	*rbc*L (635–650)
rbc-F4	F	GTAAATGGATGCGTA	*rbc*L (953–967)
rbc-F5	F	ATTTGGTGGTGGTACTATTGG	*rbc*L (1212–1232)
rbc-F6	F	TTAGATTTATGGAAAGATATWAC	*rbc*L (1384–1406)
rbc-R1	R	TTAGCWAGWGAACCT	*rbc*L (356–342)
rbc-R2	R	CGCATGAATGGTTGTG	*rbc*L (650–635)
PRB-R2[3]	R	CCTTTAACCATTAAGGGATC	*rbc*L (1040–1021)
PRB-R3[3]	R	GTAATATCTTTCCATAAATCTAA	*rbc*L (1406–1384)
DPrbcL7[4]	R	AAASHDCCTTGTGTWAGTYTC	*rbc*S (23–3)
RSPR[3]	R	AATAAAGGAAGACCCCATAATTCCCA	*rbc*S (167–142)

[1] F, forward; R, reverse.
[2] Rousseau & Reviers (1997).
[3] Kogame *et al.* (1999).
[4] Daugbjerg & Andersen (1997).

tales and Choristocarpaceae first diverged in the Phaeophyceae, followed by Sphacelariales, *Phaeostrophion* and Syringodermatales. Furthermore, *Phaeostrophion* first clustered with a member of the Sphacelariales (*Alethocladus* Sauvageau), and the relationship was supported by moderate bootstrap support (62% for MP and 75% for NJ).

DISCUSSION

Among the DNA sequence regions frequently used in molecular phylogenetic analyses of the Phaeophyceae at the present time (i.e. Rubisco genes, 18S and 26S rDNA and their internal transcribed spacer regions), Rubisco genes are considered to have the best resolution for elucidating ordinal- and familial-level phylogenetic relationships (Siemer *et al.* 1998; Draisma *et al.* 2001; Sasaki *et al.* 2001). The 18S and 26S rDNAs are more conserved than Rubisco genes and so are potentially suitable for elucidating ordinal-level phylogeny (Saunders & Druehl 1992; Tan & Druehl 1993; Saunders & Kraft 1995; Rousseau & Reviers 1999). However, 18S and 26S rDNA genes are sometimes reported to show strange molecular phylogenetic relationships, possibly due to the sequence heterogeneities among the multiple copies in the genome (Kawai *et al.* 1995; Boo *et al.* 1999; A. Kato and H. Kawai, unpublished observations) or by saturation in the base substitutions.

In the present study, both *rbc*L and rDNA sequence data analysed by all of the analytical methods (MP, NJ and ML except for rDNA) indicated that *P. irregulare* was phylogenetically distant from Dictyosiphonales (or Ectocarpales *sensu lato*), in which the species had been placed. In the *rbc*L analyses based on the taxa covering most of the phaeophycean orders, it was shown to have closer phylogenetic relationships with basal orders such as Sphacelariales, Syringodermatales, Dictyotales and Choristocarpaceae. Among them, *Phaeostrophion* was closest to the order Sphacelariales, and became sister group of Syringodermatales in the analyses base on those basal taxa.

This result appears reasonable because all these taxa share two basic morphological features: growth by apical meristematic cells and multiple chloroplasts per cell without pyrenoids. It is noteworthy that Sphacelariales also have a perennial basal system as seen in *Phaeostrophion*. These taxa principally show isomorphic life histories, and we surmise that the life history pattern of *Phaeostrophion* could also be isomorphic. Although in Mathieson's culture study (Mathieson 1967), plurilocular zooidangia occurred on the filamentous part of the prostrate thallus, we do not consider these plethysmothalli to be the filamentous gametophytic generation, but rather a malformed erect thallus; both the plurilocular and unilocular zooidangia are formed on a filamentous thallus in a similar manner and therefore the filaments are unlikely to

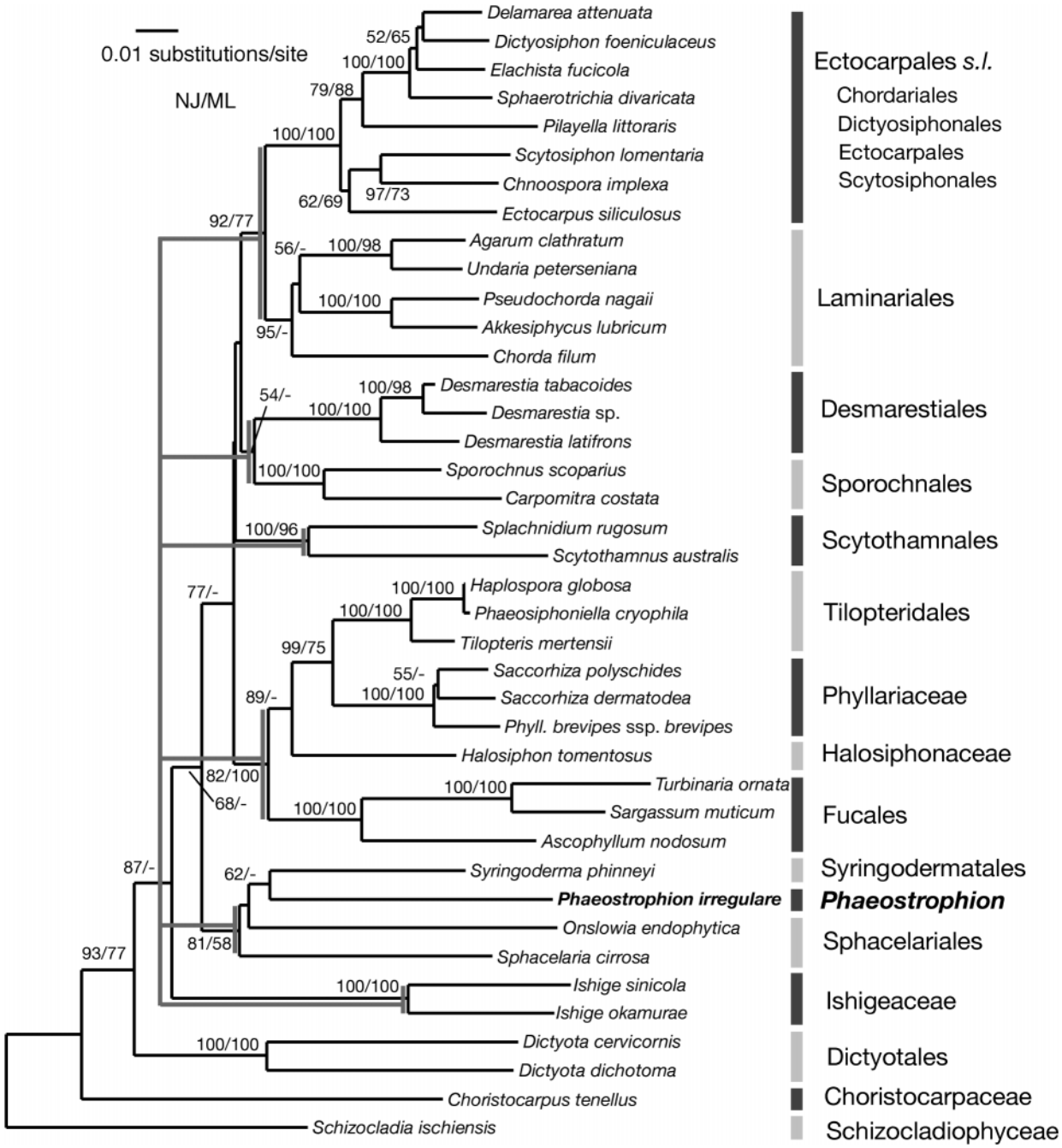

Fig. 11. Molecular phylogenetic tree based on *rbc*L gene sequences (OTUs covering most of the phaeophycean orders) analysed by NJ and ML using the GTR model; proportion of invariable sites (I) = 0.3825; variable sites (G), gamma distribution parameter = 0.6828. The −Ln likelihood was 14,549.78613. The tree shown is based on the NJ tree (indicated by black lines), and the difference between that and the ML analysis is shown as grey lines. Bootstrap values indicate % based on 1000 (NJ) and 100 (ML) replicates. Bootstrap values below 50% are indicated as '-'.

be gametophytes. In contrast, the disc itself did not form any reproductive structures (Mathieson 1967; our own culture results). Therefore, it is possible that *Phaeostrophion* has an isomorphic life history. On the other hand, another phylogenetically related taxon, Syringodermatales, has heteromorphic life histories, although highly diverse patterns of life history are seen in species of *Syringoderma* Levring: *S. phinneyi* Henry & Müller has filamentous gametophytes, whereas the gametophytes are highly reduced and retained on the sporophytic thalli in *S. abyssicola* (Setchell & N.L. Gardner) Levring and *S. floridana* Henry (Henry & Müller 1983; Henry 1984; Kawai & Yamada 1990).

The presence and absence of pyrenoids is a relatively distinctive character at the ordinal level (Kawai 1992; Reviers & Rousseau 1999). Because of the notion, generally accepted before the accumulation of molecular phylogenetic data, that Ectocarpales are the most primitive members of the Phaeophyceae because they have prominent pyrenoids, the presence

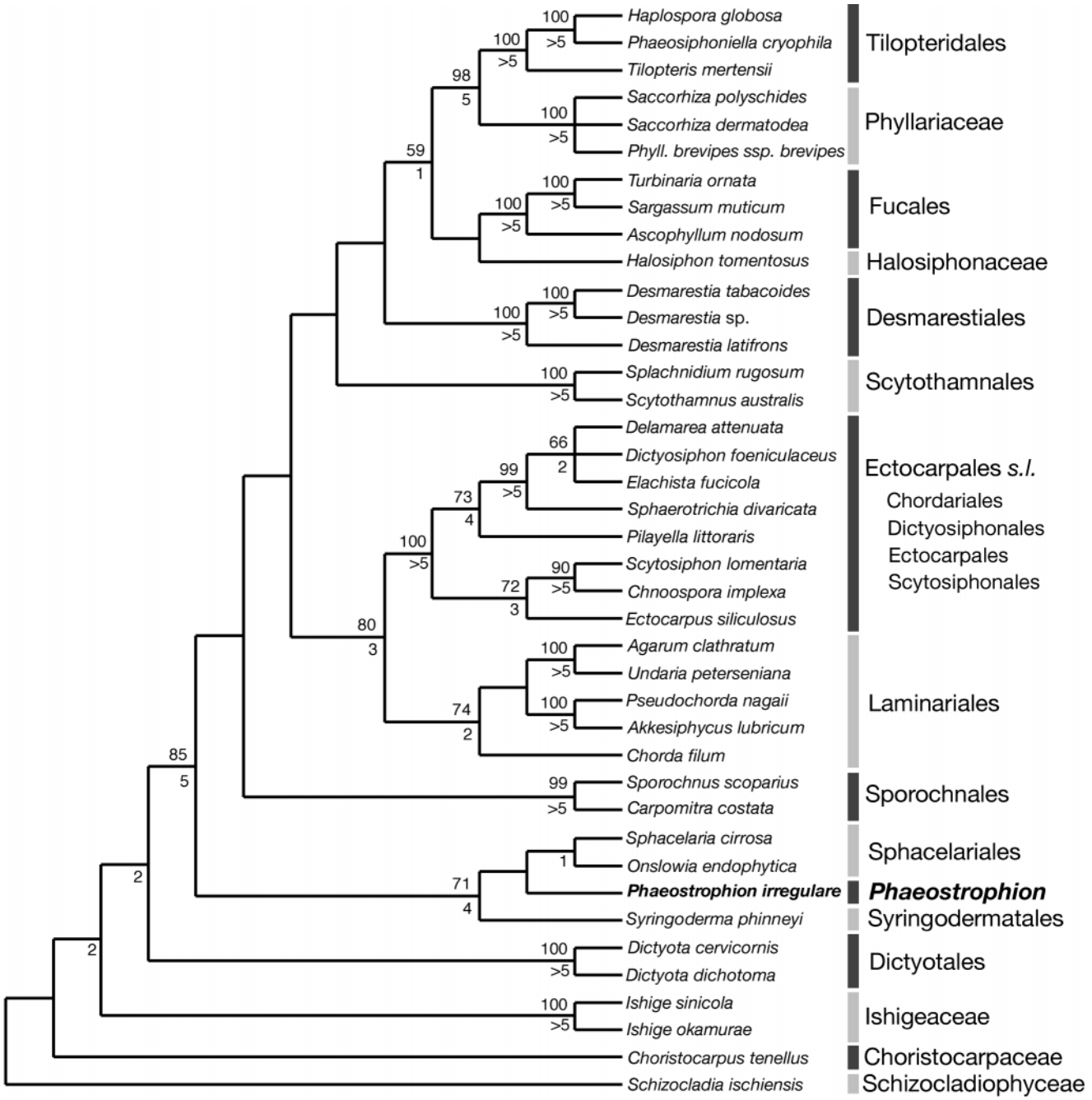

Fig. 12. Molecular phylogenetic tree based on *rbc*L gene sequences analysed by MP. A strict consensus tree of four equally parsimonious trees with 2733 steps was obtained with a consistency index of 0.3648 and a retention index of 0.5162. Bootstrap values (over a branch) indicate % based on 1000 replicates. Bootstrap values below 50% are not indicated. Decay indices are shown below the branch, but not shown when nil.

of pyrenoids was believed to be a primitive feature in the class (Hori & Ueda 1975; Asensi *et al.* 1977). The occurrence of pyrenoids in some members of Xanthophyceae or Chrysophyceae, which were believed to be the closest ancestors of Phaeophyceae among known taxa, also appeared to support this notion (Kawai 1992). By contrast, molecular phylogenetic data have indicated that the order Ectocarpales *sensu lato*, the members of which have prominent pyrenoids, is relatively recently derived in the Phaeophyceae and, furthermore, that those taxa that are considered to have diverged early in the phaeophycean lineage lack pyrenoids (e.g. Dictyotales, Sphacelariales). In addition, Schizocladiophyceae, which was recently described and suggested to be the closest relative of Phaeophyceae (Kawai *et al.* 2003), lacks pyrenoids. Therefore, based on current knowledge, the absence of pyrenoids appears to be a primitive character in the phaeophycean lineage.

It is noteworthy that Ishigeaceae was not included in the clade of Ectocarpales *sensu lato*, and appears to have diverged relatively early in the phaeophycean lineage. The family Ishigeaceae (Segawa 1935), including *Ishige okamurae* Yendo and *I. sinicola* (Setchell & N.L. Gardner) Chihara, has been

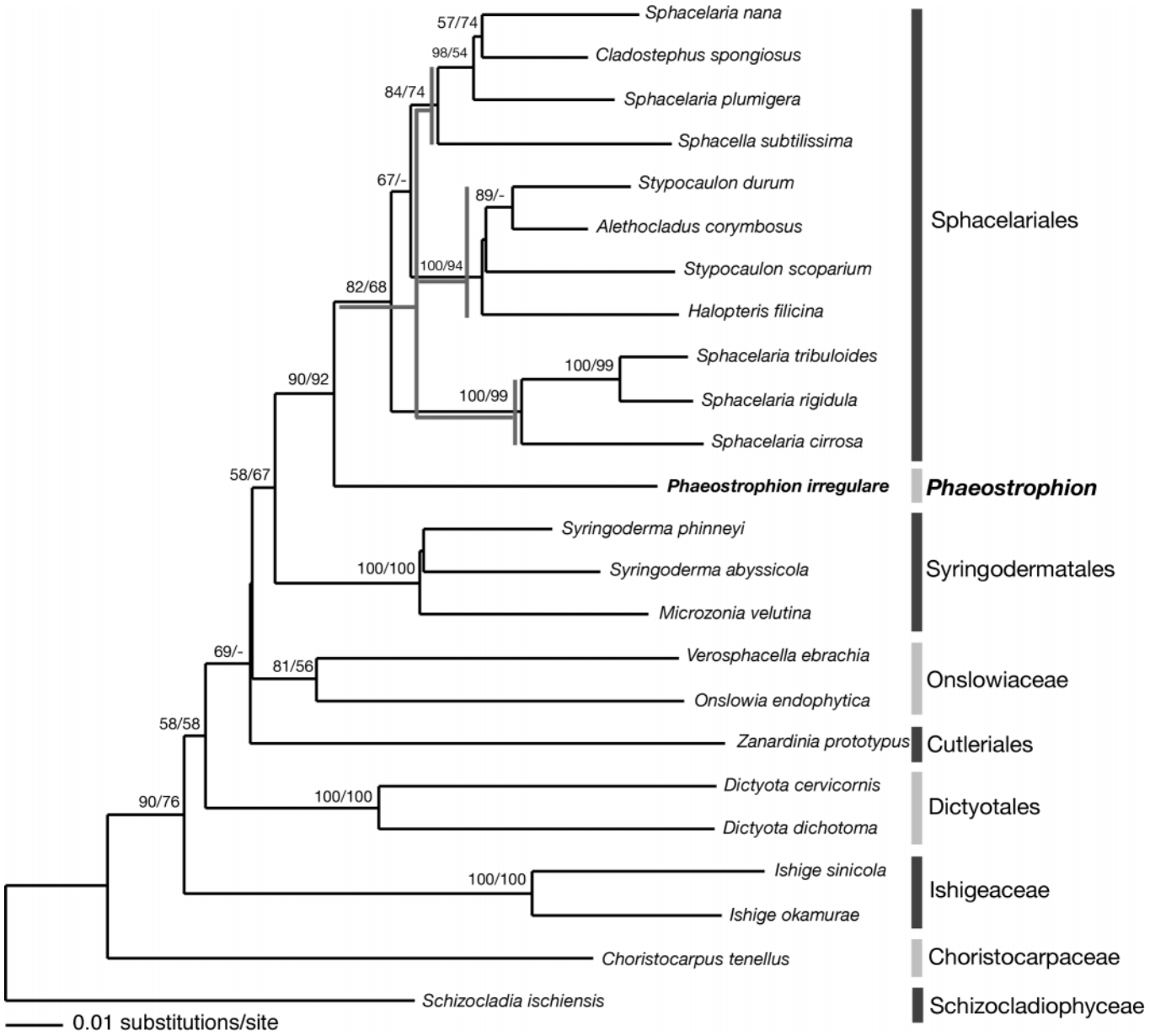

Fig. 13. Molecular phylogenetic tree based on *rbc*L gene sequences (OTUs based on basal orders in the phaeophycean lineage) analysed by NJ and ML methods using the GTR model; proportion of invariable sites (I) = 0.4442; variable sites (G), gamma distribution parameter = 1.0044. The −Ln likelihood was 10,227.06679. The tree shown is based on the NJ tree (indicated by black lines), and the difference between it and the ML analysis is shown as grey lines. Bootstrap values indicate % based on 1000 (NJ) and 100 (ML) replicates. Bootstrap values below 50% are indicated as '-'.

placed in the order Chordariales. Arasaki (1943) reported a heteromorphic life history in *I. sinicola* (= *I. foliacea* Okamura) based on culture results in which the unizooids released from the erect thallus developed into microthalli forming plurilocular gametangia. The released gametes underwent sexual fusion and developed into creeping filaments before forming upright filaments that Arasaki assumed to be the sporophytic thallus. By contrast, in *I. okamurae,* Ajisaka (1989) reported the occurrence of erect thalli forming plurilocular sporangia, in addition to those forming unilocular sporangia. In his culture study, the plurizooids released from the erect thalli developed into a disc and then formed an erect thallus. Although the life history has not been completed in culture in *I. okamurae,* Tanaka (1993) assumed an isomorphic life history in this species, referring to unpublished data reporting that unizooids from the unilocular sporangia developed directly into an erect thallus (E. Tsuruoka, personal communication).

Hori (1971a) reported the occurrence of a small pyrenoid in *I. okamurae,* whereas pyrenoids were absent in *I. sinicola.* Considering the more recent culture studies on *I. okamurae* (Ajisaka 1989; Tanaka 1993) and the results of Hori (1971a), the conclusion of Arasaki (1943) that assumed a heteromorphic life history in *I. sinicola* is doubtful; the filamentous gametophytes that he observed could be some ectocarpalean

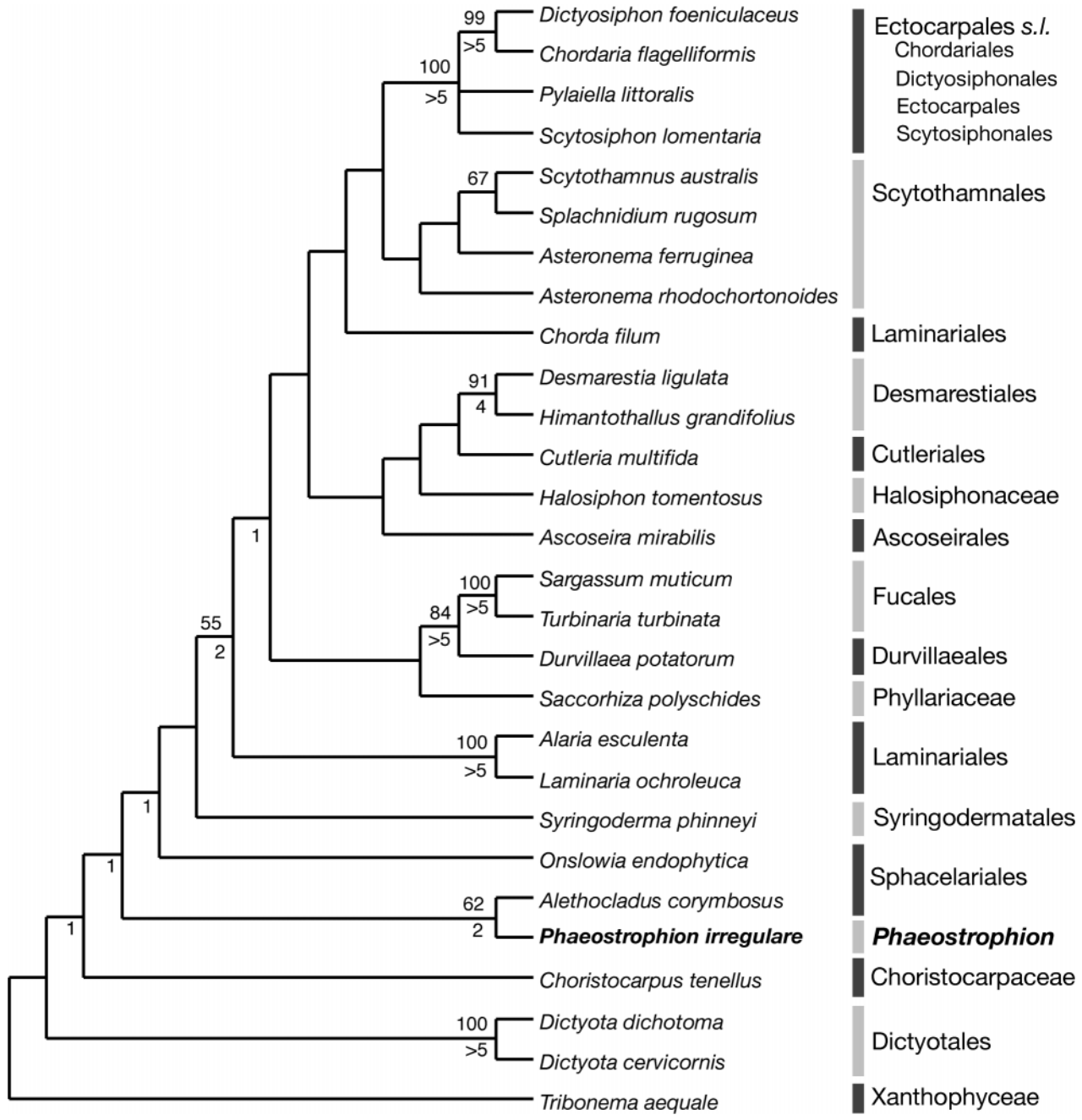

Fig. 14. Molecular phylogenetic tree based on partial 18S and 26S rDNA sequences analysed by MP. In the analysis, two equally parsimonious trees of 1160 steps were obtained with a consistency index of 0.5457 and an retention index of 0.5134. Bootstrap values (over the branch) indicate % based on 1000 replicates. Bootstrap values below 50% are not indicated. Decay indices are shown below the branch, but not shown when nil.

contaminants, because the chloroplasts of the filamentous cells illustrated in Arasaki's culture work have projecting pyrenoids, whereas those in Hori's TEM observations do not (Hori 1971a). Therefore, we consider that Ishigeaceae has essentially isomorphic life histories in common with Dictyotales and Sphacelariales (and possibly also with *Phaeostrophion*), which have close phylogenetic relationships.

Based on our results, we propose the establishment of Phaeostrophiaceae *fam. nov.* to accommodate *P. irregulare*, although we suspend taxonomic treatment at the ordinal level.

Phaeostrophiaceae fam. nov.

Thallus ligulatus solidus polystromaticus, per discum perennem affixus. Sporangia et uni- et plurilocularia in thallis erectis facta, in strato cellularum peripherali inclusa, in thallis separatis vel interdum in eodem thallo mixta. Chloroplasti multi discoidei sine pyrenoidibus in omni cellula. Sequentiae geneticae respectu *rbc*L atque rRNA

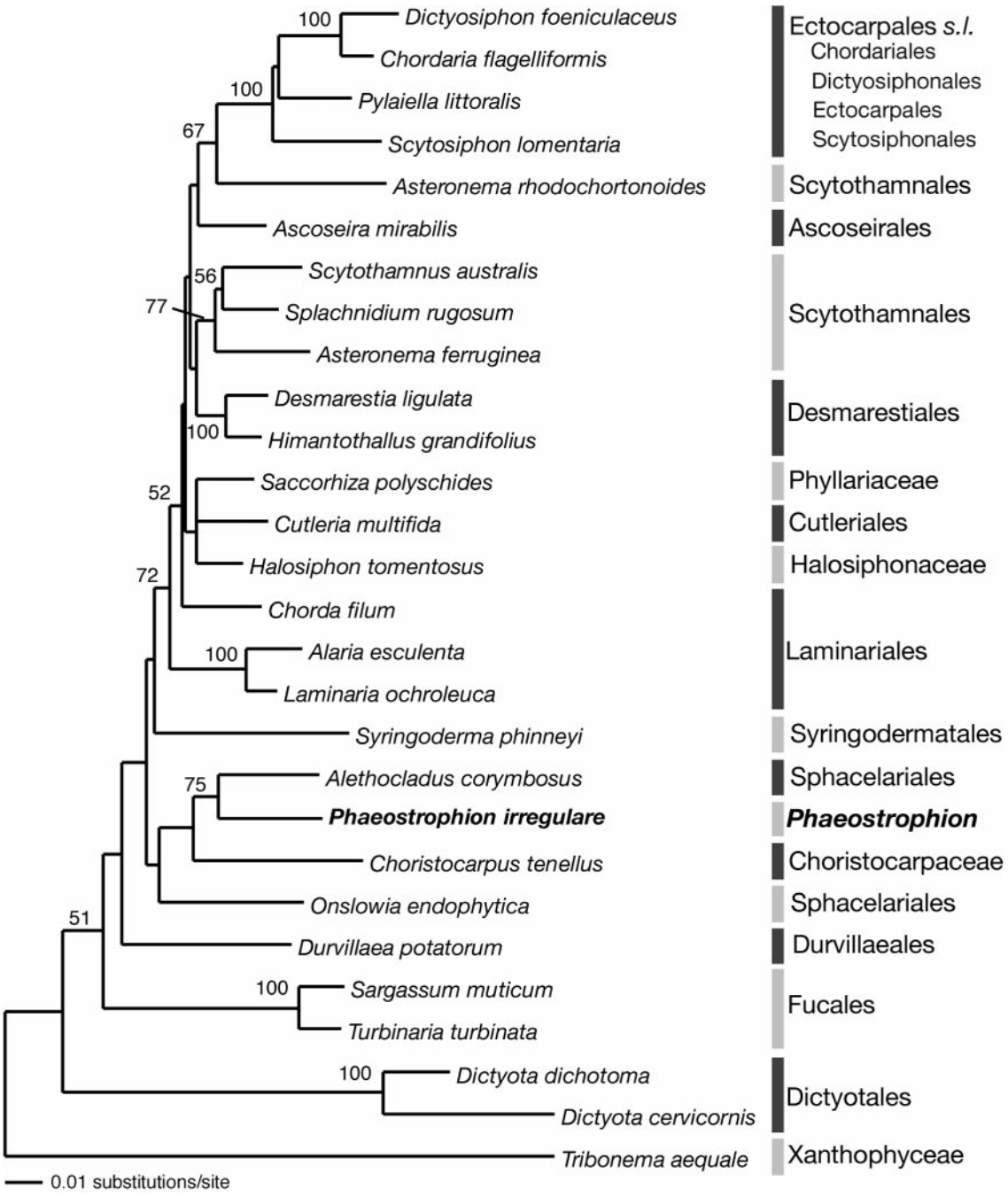

Fig. 15. Molecular phylogenetic tree based on partial 18S and 26S rDNA sequences analysed by NJ. Bootstrap values indicate % based on 1000 replicates. Bootstrap values below 50% are not indicated.

[rARN] et 18S et 26S peculiares (sequentia *rbc*L AB117948, rDNA [rADN] 18S AB117949, rDNA [rADN] AB117950).

Thallus ligulate, solid, polystromatic, attached by a perennial disc. Uni- and plurilocular sporangia formed on erect thallus, embedded in the peripheral layer of cells, in separate thalli or sometimes mixed on one thallus. Many discoid chloroplasts without pyrenoids in each cell. Orders of nucleotides of genes for *rbc*L and both 18S and 26S rRNA distinctive (*rbc*L gene sequence AB117948; 18S rDNA AB117949; 26S rDNA AB117950).

GENUS TYPE: *Phaeostrophion* Setchell & N.L. Gardner (Setchell & Gardner 1924), designated here.

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