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A NEW BROWN ALGAL ORDER, ISHIGEALES (PHAEOPHYCEAE), ESTABLISHED ON THE BASIS OF PLASTID PROTEIN-CODING *rbc*L, *psa*A, AND *psb*A REGION COMPARISONS[1]

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The brown algal family Ishigeaceae currently includes a single genus, *Ishige* Yendo, with two species. The relationship of the family to other brown algal lineages is less studied in terms of their plastid ultrastructure and molecular phylogeny. We determined the sequences of *rbc*L from four samples of the two *Ishige* species and nine putative relatives and the *psa*A and *psb*A sequences from 37 representatives of the brown algae. Analyses of individual and combined data sets resulted in similar trees; however, the concatenated data gave greater resolution and clade support than each individual gene. In all the phylogenies, the Phaeophyceae was well resolved, the Ectocarpales being placed in a terminal position and the Ishigeaceae ending up in a basal position. From our ultrastructural study, we concluded that the pyrenoid is absent in the Ishigeaceae, despite the presence of a rudimentary pyrenoid in *I. okamurae*. These results suggest that the Ishigeaceae is an early diverging brown lineage. Our molecular and morphological data, therefore, lead us to exclude the Ishigeaceae from the Ectocarpales *s.l.*, which have an elaborate pyrenoid, and to propose its own order Ishigeales ord. nov. The Ishigeales is distinguished by oligostichous structure of thalli, phaeophycean hairs formed within cryptostomata, unilocular sporangia transformed from terminal cortical cells, and plurilocular sporangia lacking sterile terminal cells. This study is the first to document the utility of the *psa*A and *psb*A sequences for brown algae and also the first report on the multigene phylogeny of the Phaeophyceae based on three protein-coding plastid genes.

Key index words: Ishige; **Ishigeaceae; Ishigeales ord. nov.; Phaeophyceae; phylogeny; *psa*A; *psb*A; pyrenoid; *rbc*L; taxonomy; ultrastructure**

Abbreviations: **BS, bootstrap value; LSU, large subunit of rDNA; ML, maximum likelihood; MP, maximum parsimony; *psa*A, PSI P700 chl *a* apoprotein A1 gene; *psb*A, PSII thylakoid protein D1 gene; *rbc*L, large subunit of RUBISCO gene; SH, Shimodaira-Hasegawa; SSU, small subunit of rDNA**

Ishige is a genus of brown algae that contains two species. Both are summer annuals and occur exclusively in the warm waters of the Pacific Ocean (Yendo 1907, Setchell and Gardner 1924, Tseng 1983, Lee et al. 2003). The genus is characterized by cylindrical to foliose thalli, hairs in cryptostomata, uniseriate plurilocular sporangia lacking sterile terminal cells, and an isomorphic life history (Yendo 1907, Ajisaka 1989, Tanaka in Hori 1993, Lee et al. 2003). The genus was based on *I. okamurae*, which was described from two different forms (filiform and foliose forms) collected in Shimoda on the Pacific coast of Japan. The foliose thalli are considered abnormal branches of the filiform type, because the former is commonly epiphytic on the latter. *Ishige okamurae* occurs in the upper intertidal zone along the coasts of Korea (Lee et al. 2003), Japan (Yendo 1907, Yoshida 1998), and China (Tseng 1983). Okamura (in Segawa 1935) described *I. foliacea*, the second member of the genus, based on the foliose type, which has a cortical layer much thinner than that of *I. okamurae*. However, Chihara (1969) showed that *I. foliacea* is a later homonym of *Polyopes sinicola* Setchell et Gardner (1924), revising the name to *I. sinicola* (Setchell et Gardner) Chihara. *Ishige sinicola* occurs as an epiphyte on *I. okamurae* in Korea, Japan, and China (Tseng 1983, Yoshida 1998, Lee et al. 2003), whereas the former occurs alone along the northern coast of the Gulf of California (Setchell and Gardner 1924).

On establishing the genus *Ishige*, Yendo (1907) putatively put it within the family Fucaceae on the grounds of cryptostomata in the foliose form (= *I. sinicola*). However, Okamura (in Segawa 1935) established the monotypic family Ishigeaceae based on the zoospores in the unilocular sporangia of *I. sinicola*, and then Okamura (1936) placed the Ishigeaceae within the Punctariales, because the apical cells of the assimilatory filaments change into unilocular sporangia where zoospores with two lateral flagella are produced. Finally, Arasaki (1943) concluded that the Ishigeaceae belonged to the order Chordariales on the basis of its heteromorphic life history, motile gametes in microscopic gametophytes, and plurilocular sporangia similar to those of some chordarialean species. In contrast, Ajisaka (1989) proposed that the family Ishigeaceae may not belong to the Chordariales, because *I. okamurae* has uniseriate plurilocular sporangia lacking sterile terminal cells, whereas the Chordariales have

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unilocular sporangia formed on the basal or middle part of the assimilatory filaments. Nevertheless, the family Ishigeaceae is still classified in the Chordariales in textbooks of algae (Yoshida 1998).

The presence of a pyrenoid is a diagnostic character for the Phaeophyceae at the ordinal level (Evans 1966, Hori and Ueda 1975, Kawai 1992). This is supported by previous studies using small subunit (SSU), large subunit (LSU), and *rbc*L DNAs (Tan and Druehl 1994, de Reviers and Rousseau 1999, Peters and Ramírez 2001). The Ectocarpales have a large pedunculate pyrenoid with a cap layer in the discoid plastids of vegetative cells (Evans 1966, Hori and Ueda 1975). The Scytothamnales have a pyrenoid in the center of stellate plastids (Peters and Clayton 1998), whereas *Asterocladon*, *Asteronema*, and *Bachelotia*, which are *insertae sedis*, have several terminal pyrenoids in the ribbon-shaped plastids (Müller et al. 1998). In contrast, the pyrenoid is absent in other brown algae (Evans 1966, 1968, Chi 1971, Hori 1971, Hori and Ueda 1975, Henry 1984). The absence/presence of the pyrenoid in the Ishigeaceae has been contentious. Hori (1971) observed a small pyrenoid in the plastids of the vegetative cells of *I. okamurae* from Japan but found no pyrenoids in the plastids of *I. sinicola*. He therefore questioned the absence of the pyrenoid in the latter species because of the congeneric relationship of the two species (Hori 1971, Hori and Ueda 1975).

Rousseau and de Reviers (1999) proposed the broad concept of the order Ectocarpales, including the Chordariales, Dictyosiphonales, Punctariales, and Scytosiphonales and excluding the Ralfsiales and taxa with stellate plastids, based on combined SSU + LSU rDNA sequences. This is corroborated by other DNA phylogenies (de Reviers and Rousseau 1999, Rousseau et al. 2000, 2001, Draisma et al. 2001, 2003, Peters and Ramírez 2001, Cho et al. 2003). de Reviers and Rousseau (1999) placed the Ishigeaceae and 22 other families, formerly assigned to the aforementioned different orders, within the Ectocarpales *s.l.* Then, Peters and Ramírez (2001) reclassified all 23 families into just 5 families, viz. Acinetosporaceae, Adenocystaceae, Chordariaceae, Ectocarpaceae, and Scytosiphonaceae, using DNA data, life history, and plastid structure data. In this classification, the previous chordarialean and dictyosiphonalean families are synonymized with the Chordariaceae (Peters and Ramírez 2001). This forces *Ishige*, the only member of the family Ishigeaceae, to be included in the Chordariaceae *s.l.* Based on partial sequences of SSU rDNA, Lee et al. (2003) suggested that the Ishigeaceae form an independent lineage with some affinity with the early lineage of the Ectocarpales *s.l.* However, the relationship of the family Ishigeaceae to the other brown algal lineages has not been addressed because taxon sampling was limited.

The present study investigated the phylogenetic relationships of the family Ishigeaceae in the class Phaeophyceae by analyses of sequence data derived from three independent protein-coding plastid genes. First, we analyzed the *rbc*L gene from *Ishige* and putative relatives and subsequently compiled an *rbc*L data set that included previously published sequences from GenBank. The *rbc*L gene has frequently been used to study various brown algal groups at a variety of taxonomic levels (Siemer et al. 1998, Draisma et al. 2001, Peters and Ramírez 2001, Cho et al. 2003). The second coding gene we chose was the *psa*A gene, which encodes the PSI P700 chl *a* apoprotein A1 (Morden and Sherwood 2002). To date, *psa*A has been analyzed for only two species, *Fucus vesiculosus* (Pearson et al. 2001) and *Pylaiella littoralis* (Yoon et al. 2002a), in the Phaeophyceae. The third protein-coding gene we used is the *psb*A gene, which encodes the PSII thylakoid protein D1 (Morden and Sherwood 2002). The *psb*A has been analyzed for two species, *Ectocarpus siliculosus* (Winhauer et al. 1991) and *Pylaiella littoralis* (Yoon et al. 2002a), in the Phaeophyceae. Samples of 37 algae including two outgroup taxa, selected from the taxa used in forming the *rbc*L data set described above, were available for the *psa*A and *psb*A analyses. Because *psa*A and *psb*A gene sequences provide better resolution for the deep branches of the red and dinophycean algal phylogenies (Morden and Sherwood 2002, Yoon et al. 2002a, b, Yang and Boo 2004), both genes should be useful for reconstructing the phaeophycean phylogeny at higher ranks.

When multiple independent data sets are available for a phylogenetic study, the investigator uses combined data sets as well as individual data sets for phylogenetic reconstructions. For combing individual data sets, Bull et al. (1993) suggested that congruent data sets only can be combined for phylogenetic analyses, whereas Gatesy et al. (1999a), who advocated a "total evidence" methodology, showed that the combination of incongruent data can increase the resolution and the support within phylogenetic trees, revealing if a "hidden signal" is present in the different data sets. However, advocates of these two approaches agree that use of multiple data sets improve phylogeny estimation (Lavoué et al. 2003, Shimabukuro-Dias et al. 2004). In this study, we follow an empirical strategy, conducting both separate and simultaneous analyses. Together with molecular analyses of the Ishigeaceae, we studied the ultrastructure of plastids in the vegetative cells of *I. okamurae*, the type species of the genus.

MATERIALS AND METHODS

Samples. The starting point for this work was our collection of previously published *rbc*L sequences of the brown algae from GenBank. We compiled *rbc*L sequences from 57 taxa, which consisted of the type genus or representatives of 13 brown algal orders, 4 "ordinal-level taxa" (Choristocarpaceae, Onslowiaceae, Phyllariaceae, and *Asterocladon/Asteronema*) (see Table 2 in Draisma et al. 2003), and 3 outgroup taxa (*Schizocladia ischiensis*, *Tribonema aequale*, and *Phaeothamnion confervicola*). Thirteen *rbc*L sequences we determined here were added to this data set: two samples from each of *Ishige okamurae* and *I. sinicola* and nine other brown algae. Then, *psa*A and *psb*A regions from 37 taxa, including *Tribonema aequale* and *Schizocladia ischiensis* as outgroup taxa, were analyzed. Both *psa*A and *psb*A sequences from *Pylaiella littoralis*

and the *psb*A sequence from *Ectocarpus siliculosus* were downloaded from GenBank (Winhauer et al. 1991, Yoon et al. 2002a). The *psa*A sequence of *Fucus vesiculosus* was not used because of its insufficient length (330 nt, Pearson et al. 2001). The specimens and their corresponding GenBank accession numbers are listed in Table 1.

Analyses of rbc*L,* psa*A, and* psb*A regions*. Total DNA was extracted from approximately 0.01 g of dried thalli ground in liquid nitrogen using a DNeasy Plant Mini Kit (Qiagen Gmbh, Hilden, Germany), according to the manufacturers' instructions, and then dissolved in 150 μL of distilled water. Extracted DNA was stored at −20° C and used to amplify the *rbc*L, *psa*A, and *psb*A regions.

The *rbc*L region was amplified and sequenced using the method of Kogame et al. (1999) and Yoon and Boo (1999). Primers PRB-FO, F2, F3, R1A, R2, R3A, RS1, and RS2 were used for most brown algae. For *Cutleria*, *Ishige*, *Sargassum*, and *Sphacelaria*, primer RbcL68F (Draisma et al. 2001), instead of PRB-FO, was used. The same DNA aliquot was used for amplifying the *psa*A and *psb*A regions, and the amplification and sequencing reactions for these regions were the same as those used for *rbc*L. The *psa*A region was amplified and sequenced using primers psaA130F, psaA870F, psaA970R, and psaA1760R (Yoon et al. 2002a). The *psb*A region was amplified using primers psbA-F and psbA-R2 and sequenced using primers psbA-F, psbA-600R, psbA-500F, and psbA-R2 (Yoon et al. 2002a). The PCR products were purified using a High Pure™ PCR Product Purification Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturers' instructions. The sequences of the forward and reverse strands were determined for all taxa using an ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA). The electropherogram output for each sample was edited using the program Sequence Navigator v. 1.0.1 (Applied Biosystems).

All sequences of the *rbc*L gene from 70 taxa (67 brown algae and 3 outgroups) were collated using the multisequence editing program, SeqPup (Gilbert 1995), and aligned by eye to compare our sequences with those published previously (Draisma et al. 2001, Cho et al. 2003). The undefined sequences at the 5 and 3′ ends of the *rbc*L data set were coded as missing data. The *psa*A sequences from 38 taxa (36 brown algae, including previously published sequence of *Pylaiella littoralis*, and 2 outgroups) were also aligned by eye (Table 1). The *psb*A sequences from 39 taxa (37 brown algae, including previously published sequences of *Ectocarpus siliculosus* and *Pylaiella littoralis*, and 2 outgroups) were aligned by eye, as for *rbc*L gene (Table 1). All alignments posed no problems because there were no gaps in each data set of these three protein-coding genes. The alignments are available at Treebase (study accession S1095) under accession numbers M1870 (*psb*A), M1871 (*rbc*L), M1872 (*psa*A), and M1873 (*rbc*L + *psa*A + *psb*A).

Phylogenetic analyses. Four data sets were used for the phylogenetic analyses: 70 taxa for *rbc*L, 39 taxa for *psb*A, and 38 taxa for both the *psa*A and combined *rbc*L + *psa*A + *psb*A data sets. To infer the level of nucleotide saturation of the *rbc*L, *psa*A, and *psb*A sequences, uncorrected *p*-distances were plotted against corrected pairwise distances using the Hasegawa-Kishino-Yano 85 model (Hasegawa et al. 1985) for the first, second, and third codon positions and all positions. We also conducted the partition homogeneity test (incongruence length difference [ILD] test of Farris et al. 1994), implemented in PAUP* 4.0b8 (Swofford 2002). The partition homogeneity test used 1000 replicates, each with 100 random sequence-addition replicates using tree bisection-reconnection (TBR) branch swapping.

Maximum parsimony (MP) trees were constructed for each data set with PAUP* using a heuristic search algorithm with the following settings: 100 random sequence-addition replicates, TBR branch swapping, MulTrees, all characters unordered and unweighted, and branches with a maximum length of zero collapsed to yield polytomies. The bootstrap values (BS) for the resulting nodes were assessed using bootstrapping with 1000 replicates.

For maximum likelihood (ML) and Bayesian analyses, we performed a likelihood ratio test using Modeltest 3.06 (Posada and Crandall 1998) to determine the best available model for individual and combined data sets. For all data sets, the best model was a general time reversible (GTR) model with a gamma correction for among-site variation (Γ) and invariant sites (I). ML analyses (heuristic search with 10 random sequence-addition replicates, TBR branch swapping, and MulTrees on) were performed using the GTR + Γ + I model. BS analysis was conducted by performing replicate ML searches, with two random sequence-addition replicates, using the same search conditions as described above. We succeeded in performing 100 bootstrap replicates for each of individual and combined data sets using two processors. To run simultaneous bootstrap replicates in different processors, we prepared two batch files that differed in their starting random seed number (seed number, 0), each specified to run 50 bootstrap replicates and to save the resulting bootstrap trees into files. A 50% majority rule consensus bootstrap tree was estimated by aggregating and weighting trees accordingly to the number of trees found in each bootstrap replicate, so that the bootstrap replicates had equal weight.

Bayesian phylogenetic analyses were performed using MrBayes 3.0 (Huelsenbeck and Ronquist 2001). Each analysis was initiated from a random starting tree, and the program was set to run four chains of Markov chain Monte Carlo iterations simultaneously for 2,000,000 generations with trees sampled every 100th generation. The likelihood scores stabilized at approximately 50,000 generations, and thus the first 500 trees were burned. For the purpose of comparison with bootstrapping, we chose to consider nodes with Bayesian posterior probabilities (PP) greater than 0.9 (e.g. the node appears in greater than 90% of sampled trees) as being well supported.

The SH test (Shimodaira and Hasegawa 1999) was used to compare statistically alternative phylogenetic hypotheses, focusing on the inclusion of the Ishigeaceae within the Ectocarpales or other putative relatives. The SH test was conducted using PAUP*, with resampling estimated log-likelihood optimization and 10,000 bootstrap replicates.

Morphology of plastids in Ishige okamurae. Apical parts of field-collected thalli were prepared for EM according to the following protocol. Specimens were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) containing 2% NaCl and 0.1% $CaCl_2$ for 3 h at 4° C and then postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h. The apical parts were rinsed with cold distilled water, cut, and transferred into test tubes at 4° C. They were dehydrated in a graded series of ethanol and propylene oxide at 4° C and infiltrated gradually with Spurr's epoxy resin (Spurr 1969). After polymerization of the resin at 70° C for 24 h, serial sections (120–200 sections per sample) were cut with a diamond knife on an Ultracut E ultramicrotome (Reichert-Jung, Germany) and mounted on Formvar-coated slot grids. Thin sections were stained with Reynold's lead citrate (Reynolds 1963) and uranyl acetate and examined with a JEM-1010 transmission electron microscope (JEOL, Ltd., Tokyo, Japan) at the Center for Research Facilities, Chungnam National University.

RESULTS

The rbc*L alignment*. The sequences determined in the present study were 1467 nt long, except for the two *Ishige* species (1325 and 1368 nt), *Cutleria*

TABLE 1. Taxa, collection site or data source, and GenBank accession number of the *rbc*L, *psa*A, and *psb*A sequences.

Taxa	Collection site, date, voucher, or reference of *rbc*L/*psa*A/*psb*A	GenBank accession no. *rbc*L/*psa*A/*psb*A
Phaeophyceae		
Ectocarpales		
Acinetosporaceae		
Geminocarpus austro-georgiae Skottsberg	Peters and Ramírez (2001)	AJ295830/ — / —
Pylaiella littoralis (Linnaeus) Kjellman	Assali et al. (1990)/Yoon et al. (2002a)/*ibid*	X55372/AY119724/AY119760
Adenocystaceae		
Adenocystis utricularis (Bory) Skottsberg	Peters and Ramírez (2001)/Barton, Maxwell Bay, Antarctica, 25 January 2000, PE001/*ibid*	AJ295823/**AY372939**/**AY528824**
Caepidium antarcticum J. Agardh	Peters and Ramírez (2001)	AJ295826/ — / —
Utriculidium durvillei Skottsberg	Peters and Ramírez (2001)	AJ295835/ — / —
Chordariaceae		
Asperococcus fistulosus (Hudson) Hooker	Cho et al. (2003)/Port Erin Bay, Isle of Man, UK, 9 July 2002, PE002/*ibid*	AY095321/**AY372940**/**AY528825**
Chordaria flagelliformis (O. F. Müller) J. Agardh	Cho et al. (2003)/Avacha Bay, Kamchatka, Russia, 24 July 1998, PE003/*ibid*	AY095324/**AY372941**/**AY528826**
Coelocladia arctica Rosenvinge	Siemer et al. (1998)	AF055395/ — / —
Delamarea attenuata (Kjellman) Rosenvinge	Siemer et al. (1998)/Nakhodka, Russia, 22 May 2002, PE004/*ibid*	AF055396/**AY372942**/**AY528827**
Dictyosiphon foeniculaceus (Hudson) Greville	Avacha Bay, Kamchatka, Russia, 28 July 1998, PE005	**AY372973**/**AY372943**/**AY528828**
Elachista fucicola (Velley) Areschoug	Siemer et al. (1998)	AF055398/ — / —
Giraudia sphacelarioides Derbès et Solier	Siemer et al. (1998)	AF055399/ — / —
Hummia onusta (Kützing) Fiore	Siemer et al. (1998)	AF055402/ — / —
Isthmoplea sphaerophora (Harvery) Kjellman	Siemer et al. (1998)	AF055403/ — / —
Myriotrichia clavaeformis Harvey	Siemer et al. (1998)	AF055408/ — / —
Punctaria latifolia Greville	Cho et al. (2003)/Hoedong, Jindo, Korea, 9 March 2001, PE010/*ibid*	AY095322/**AY372948**/**AY528833**
Sphaerotrichia divaricata (C. Agardh) Kylin	Siemer et al. (1998)	AF055412/ — / —
Streblonema maculans (Hamel) South et Tittley	Rousseau and de Reviers (1999)	AY157694/ — / —
Striaria attenuata (Greville) Greville	Siemer et al. (1998)	AF055415/ — / —
Ectocarpaceae		
Ectocarpus sp.	Hoedong, Jindo, Korea, 9 March 2001, PE011	**AY372978**/**AY372949**/**AY528834**
E. siliculosus (Dillwyn) Lyngbye	Winhauer et al. (1991)	—/ —/ X56695
Scytosiphonaceae		
Chnoospora implexa J. Agardh	Kogame et al. (1999)	AB022231/ — / —
Colpomenia sinuosa (Mertens ex Roth) Derbès et Solier in Castagne	Kogame et al. (1999)/Guryongpo, Pohang, Korea, 6 November 2000, PE012/*ibid*	AB022234/**AY372950**/**AY528835**
Hydroclathrus clathratus (C. Agardh) Howe	Kogame et al. (1999)/Tsuyazaki, Fukuoka, Japan, 7 March 1999, PE013/*ibid*	AB022233/**AY372951**/**AY528836**
Myelophycus simplex (Harvey) Papenfuss	Cho et al. (2003)/Daesado, Wando, Korea, 13 June 1999, PE014/*ibid*	AY095320/**AY372952**/**AY528837**
Petalonia fascia (O. F. Müller) Kuntze	Kogame et al. (1999)/Ile de Batz, Roscoff, France, 5 April 2000, PE015/*ibid*	AB022243/**AY372953**/**AY528838**
Rosenvingea intricate (J. Agardh) Boergesen	Kogame et al. (1999)	AB022232/ — / —
Scytosiphon lomentaria (Lyngbye) Link	Kogame et al. (1999)/Seongsan, Jeju, Korea, 22 March 2000, PE016/ *ibid*	AB022238/**AY372954**/**AY528839**
Choristocarpaceae		
Choristocarpus tenellus (Kützing) Zanardini	Draisma et al. (2001)	AJ287862/ — / —
Cutleriales		
Cutleria cylindrica Okamura	Hoedong, Jindo, Korea, 9 March 2001, PC001	**AY372979**/**AY372955**/**AY528840**
C. multifida (J. E. Smith) Greville	Burrowes et al. (2003)	AY157692/ — / —
Zanardinia prototypes (Nardo) Nardo	Burrowes et al. (2003)	AY157693/ — / —
Desmarestiales		
Desmarestia ligulata (Stackhouse) Lamouroux	Draisma et al. (2001)	AJ287848/ — / —
D. viridis (Müller) Lamouroux	Siemer and Pedersen (unpublished data)	AF207799/ — / —
Desmarestia sp.	Penguin Rookery, Maxwell Bay, Antarctica, 11 January 2000, PD001	**AY372980**/**AY372956**/**AY528841**
Himantothallus grandifolius (A. et E. Gepp) Zinova	Draisma et al. (2001)	AJ287850/ — / —
Dictyotales		
Dictyota dichotoma (Hudson) Lamouroux	Draisma et al. (2001)	AJ287852/ — / —
Dictyota sp.	Guryongpo, Pohang, Korea, 15 March 2001, PDI001	**AY422654**/**AY372957**/**AY528842**
Zonaria diesingiana J. Agardh	Ishigaki Island, Okinawa, Japan, 19 January 1998, PDI002	**AJ295823**/**AY372958**/**AY528843**
Fucales		
Ascophyllum nodosum (Linnaeus) Le Jolis	Draisma et al. (2001)/Neeltjejans, Netherlands, 13 August 1997, PF001/*ibid*	AJ287853/**AY372959**/**AY528844**

Table 1. (Contd.)

Taxa	Collection site, date, voucher, or reference of *rbc*L/*psa*A/*psb*A	GenBank accession no. *rbc*L/*psa*A/*psb*A
Fucus vesiculosus Linnaeus	Burrowes et al. (2003)/Coos Bay, Oregon, USA, 16 May 2001, PF002/*ibid*	AY157695/**AY372960**/**AY528845**
Sargassum horneri (Turner) C. Agardh	Seosang, Namhaedo, Korea, 3 November 2002, PF003	**AY372981**/**AY372961**/**AY528846**
Ishigaceae		
Ishige okamurae Yendo	Hanrim, Jeju, Korea, 4 December 2002, PE006	**AY372974**/**AY372944**/**AY528829**
I. okamurae	Kominato, Chiba, Japan, 28 July 2002, PE007	**AY372975**/**AY372945**/**AY528830**
I. sinicola (Setchell et Gardner) Chihara	Hanrim, Jeju, Korea, 4 December 2002, PE008	**AY372976**/**AY372946**/**AY528831**
I. sinicola	Kominato, Chiba, Japan, 28 July 2002, PE009	**AY372977**/**AY372947**/**AY528832**
Laminariales		
Alaria crassifolia Kjellman in Kjellman et Petersen	Hakodate, Hokkaido, Japan, 21 August 1999, PL001	**AY372982**/**AY372962**/**AY528847**
Chorda filum (Linnaeus) Stackhouse	Oshoro, Hokkaido, Japan, 25 April 2002, PL002	**AY372983**/**AY372963**/**AY528848**
Laminaria digitata (Linnaeus) Lamouroux	Port Erin Bay, Isle of Man, UK, 9 July 2000, PL003	**AY372984**/**AY372964**/**AY528849**
Onslowiaceae		
Onslowia endophytica Searles	Draisma et al. (2001)	AJ287864/ — / —
Verosphacela ebrachia Henry	Draisma et al. (2001)	AJ287867/ — / —
Phyllariaceae		
Phyllariopsis brevipes ssp. *brevipes* (C. Agardh) Henry et South	Sasaki et al. (2001)	AB045244/ — / —
Saccorhiza polyschides (Lightfoot) Batters	Sasaki et al. (2001)/Ile Callot, Roscoff, France, September 1999, PP001/*ibid*	AB045256/**AY372965**/**AY528850**
Ralfsiales		
Analipus japonicus (Harvey) Wynne	Cho et al. (2003)/Boiler Bay, Oregon, USA, 16 May 2001, PR001/*ibid*	AY095323/**AY372966**/**AY528851**
Scytothamnales		
Scytothamnus australis (J. Agardh) Hooker et Harvey	Peters and Ramírez (2001)/Scorching Bay, Wellington, New Zealand, 3 August 2001, PS001/*ibid*	AJ295833/**AY372967**/**AY528852**
Splachnidium rugosum (Linnaeus) Greville	Peters and Ramírez (2001)/Scorching Bay, Wellington, New Zealand, 3 August 2001, PS002/*ibid*	AJ295834/**AY372968**/**AY528853**
Sphacelariales		
Alethocladus corymbosus (Dickie) Sauvageau	Draisma et al. (2001)	AJ287860/ — / —
Halopteris filicina (Grateloup) Kützing	Draisma et al. (2001)/Seongsan, Jeju, Korea, DNA from Y. S. Keum/*ibid*	AJ287894/**AY372969**/A**Y528854**
Sphacelaria divaricata Montagne	Draisma et al. (2001)	AJ287889/ — / —
S. divaricata	Seongsan, Jeju, Korea, DNA from Y. S. Keum	**AY372985**/**AY372970**/**AY528855**
Sporochnales		
Carpomitra costata (Stackhouse) Batters	Sasaki et al. (2001)/Munseom, Jeju, Korea, 8 April 2003, PSP001/*ibid*	AB045257/**AY372971**/**AY528856**
Sporochnus radiciformis (B. Brown ex Turner) C. Agardh	Kawai and Sasaki (2000) as *S. scoparuis* Harvey /Cheonbu, Ulreungdo, Korea, 26 August 2003, PSP002/*ibid*	AB037142/**AY528861**/**AY528857**
Syringodermatales		
Microzonia velutina (Harvey) J. Agardh	Burrowes et al. (2003)	AY157697/ — / —
Syringoderma phinneyi Henry et Müller	Draisma et al. (2001)/Moe 2, Culture of Prof. D. G. Müller (Konstanz, Germany)/*ibid*	AJ287868/**AY528862**/**AY528858**
Tilopteridales		
Haplospora globosa Kjellman	Kawai and Sasaki (2000)	AB037138/ — / —
Tilopteris mertensii (Turner in Smith) Kützing	Sasaki et al. (2001)	AB045260/ — / —
Incertae sedis		
Asterocladon lobatum Müller et al.	Peters and Ramírez (2001)	AJ295824/ — / —
Asteronema rhodochortonoides (Boergesen) Müller et Parodi	Peters and Ramírez (2001)	AJ295825/ — / —
Phaeothamniophyceae		
Phaeothamnion confervicola G. Lagerheim	Bailey et al. (1998)	AF064746/ — / —
Schizocladiophyceae		
Schizocladia ischiensis Henry, Okuda et Kawai	Kawai et al. (2003)/CCMP2287/*ibid*	AB085615/**AY528863**/**AY528859**
Tribophyceae		
Tribonema aequale Pascher	Bailey and Andersen (1998)/UTEX 50/*ibid*	AF084611/**AY372972**/**AY528860**

CCMP, Provasoli-Guillard National Center for Culture of Marine Phytoplankton; UTEX, Culture Collection of Algae at the University of Texas at Austin. Bold numbers indicate new sequences published in the present study.

TABLE 2. Nucleotide composition of the *rbc*L, *psa*A, and *psb*A and statistics from MP analyses of the individual and combined data sets including outgroups.

	*rbc*L	*psa*A	*psb*A	Combined
Number of taxa	70	38	39	38
Nucleotides (bp)	1467	1488	885	3840
Base frequency (A/C/G/T)	0.2949/0.1627/0.2203/0.3221	0.2952/0.1564/0.1922/0.3562	0.2467/0.1825/0.2077/0.3632	
Number of transitions/ transversions (Ti/Tv ratio)	191379/200661 (0.95)	74550/73082 (1.02)	29095/23450 (1.24)	
Variable sites (%)	685 (46.7)	661 (44.4)	306 (34.6)	1583 (41.2)
Informative sites (%)	558 (38.0)	578 (38.8)	226 (25.5)	1296 (33.8)
Number of MP trees	2	2	4	1
MP tree length	4062	3387	1021	7243
Consistency index	0.278	0.333	0.378	0.344
Retention index	0.561	0.457	0.554	0.477

cylindrica (1372 nt), *Sargassum horneri* (1292 nt), and *Sphacelaria divaricata* (1383 nt), which were amplified with different primers. The 70 aligned *rbc*L sequences had 685 (46.7%) variable bases and 558 (38.0%) parsimoniously informative sites. There were excesses of adenine (29.49%) and thymine (32.21%) at all codon positions. Transversions were more common than transitions for all codon positions (Ti/Tv = 0.95) (Table 2).

The uncorrected sequence divergence (*p*-distance) values for the *rbc*L region within *Ishige* ranged from 6.77% between *I. okamurae* and *I. sinicola* from Korea to 7.10% between *I. okamurae* and *I. sinicola* from Japan. The samples of *I. okamurae* from Korea and Japan differed by seven nucleotides (0.51% sequence divergence), and there was a difference of two nucleotides (0.15% sequence divergence) between *I. sinicola* from Korea and Japan. *Ishige* differed from other ectocarpalean algae between 14.01% (between *I. okamurae* from Japan and *Dictyosiphon foeniculaceus*) and 16.61% (between *I. sinicola* from Japan and *Pylaiella littoralis*) sequence divergence.

The psa*A alignment*. The sequences determined for the *psa*A region totaled 1488 nt. For the 38 aligned sequences of the *psa*A gene, 661 (44.4%) bases were variable and 578 (38.8%) were parsimoniously informative. There were excesses of adenine and thymine at all codon positions (29.52% and 35.62%, respectively). Transversions and transitions occurred at approximately similar frequencies for all codon positions (Ti/Tv = 1.02) (Table 2).

The sequence divergence for the *psa*A gene within *Ishige* ranged from 8.27% (between *I. okamurae* from Japan and *I. sinicola* from Korea) to 8.74% (between *I. okamurae* Korea and *I. sinicola* from Japan). *Ishige okamurae* from Korea and Japan differed by 11 nt (0.74% sequence divergence), and *I. sinicola* from Korea and Japan differed by 5 nt (0.34% sequence divergence). *Ishige* diverged from other ectocarpalean algae between 16.4% (between *I. sinicola* from Korea and *Dictyosiphon foeniculaceus*) and 18.82% (between *I. okamurae* from Japan and *Adenocystis utricularis*).

The psb*A alignment*. The sequences determined for the *psb*A region totaled 885 bp. For the 39 aligned sequences of the *psb*A gene, 306 (34.6%) bases were variable and 226 (25.5%) were parsimoniously informative. There were excesses of adenine and thymine at all codon positions (24.67% and 36.32%, respectively). Transitions were more common than transversion for all codon positions (Ti/Tv = 1.24) (Table 2).

The sequence divergence for the *psb*A gene within *Ishige* ranged from 6.44% (between *I. okamurae* and *I. sinicola* from Japan) to 6.89% (between *I. okamurae* and *I. sinicola* from Korea). *Ishige okamurae* from Korea and Japan differed by 6 nt (0.68% sequence divergence), and *I. sinicola* from Korea and Japan differed by 2 nt (0.23% sequence divergence). *Ishige* diverged from other ectocarpalean algae between 9.49% (between *I. okamurae* from Korea and *Petalonia fascia*) and 11.86% (between *I. sinicola* from Korea and *Adenocystis utricularis*).

Saturation tests. Saturation tests showed that the first and second codon positions for all three genes were conserved and the third codon positions were the most variable. The scatter plots (not shown) for the *rbc*L, *psa*A, and *psb*A sequences were linear and showed no evidence of multiple hit problems for all three codon positions. Therefore, we included all events for the three codon positions in the phylogenetic analysis.

Partition homogeneity tests. Partition homogeneity tests between *rbc*L and *psa*A were significant ($P = 0.002$, mean of three tests); however, with *Desmarestia* or the kelp genera (*Chorda*, *Alaria*, and *Laminaria*) excluded, the results were not significant ($P = 0.102$ and 0.150, respectively), indicating congruence. The *psb*A data was incongruent with the *psa*A and *rbc*L data sets (at the $P \leq 0.01$ level) even with *Desmarestia* and/or the kelp genera excluded. The *P* value was not changed when outgroup taxa or uninformative sites were excluded. So, we provided all the phylogenetic trees reconstructed from individual and combined data sets.

Phylogenetic relationships. The independent analyses of *rbc*L, *psa*A, *psb*A, and *rbc*L + *psa*A + *psb*A data sets resulted in congruent, though not identical, phylogenetic reconstructions. The statistics for the

MP analyses are compared among individual and combined data sets (Table 2), and the ML trees for all four data sets are shown in Figures 1 through 4.

The *rbc*L tree (Fig. 1) showed that all the phaeophycean algae investigated here were strongly monophyletic (100% BS for ML, 92% BS for MP, and PP = 1), having the Schizocladiophyceae as the sister taxon. The basal-most taxon of the Phaeophyceae was the Choristocarpaceae. *Ishige okamurae* and *I. sinicola* from Korea and Japan were strongly monophyletic (100% BS for ML and MP and PP = 1), and the Ishigeaceae was the sister taxon to all other remaining taxa (100% BS for ML, 79% BS for MP, and PP = 1). The Dictyotales, Onslowiaceae, Sphacelariales, and Syringodermatales formed a clade, although the clade was only supported by Bayesian PP. The remaining 11 higher taxa, including the Phyllariaceae and *Asterocladon/Asteronema* group, formed a monophyletic clade (56% BS for ML, 51% BS for MP, and PP = 1). The Ectocarpales, comprising the Acinetosporaceae, Adenocystaceae, Chordariaceae, Ectocarpaceae, and Scytosiphonaceae, was strongly monophyletic (94% BS for ML, 90% BS for MP, and PP = 1) and was placed in a terminal position. The Laminariales and *Asterocladon/Asteronema* group clustered with the Ectocarpales (88% BS for ML, 85% BS for MP, and PP = 1). However, interrelationships of most of other orders and families were not well resolved.

The *psa*A tree (Fig. 2) is similar to the *rbc*L tree in having the monophyletic Phaeophyceae with the Ishigeaceae in a basal position and the Ectocarpales in a terminal position. The clade of the Sphacelariales, Dictyotales, and Syringodermatales was not supported with bootstrap values or Bayesian probabilities. The remaining taxa formed a monophyletic clade (61% BS for ML, 59% BS for MP, and PP = 1). However, the *psa*A tree is different from the *rbc*L tree in having the Desmarestiales as the sister taxon to the Laminariales (66% BS for ML, 75% BS for MP, and PP = 1), and the Laminariales is not monophyletic.

The *psb*A tree (Fig. 3) had a similar topology to the *rbc*L and *psa*A trees in the positions of the Ishigeaceae and the Ectocarpales within the Phaeophyceae. However, the *psb*A tree is different from the *rbc*L and *psa*A trees in having the Syringodermatales separated from the clade of the Dictyotales and Sphacelariales. The latter two orders were not supported with ML and MP bootstrap values but were supported with 0.91 and 0.97 PP, respectively.

Although the tree of the concatenated data set was similar to the *rbc*L tree than the *psa*A and *psb*A trees, all these trees were congruent in having the monophyletic Phaeophyceae with the Ishigeaceae in a basal position and the Ectocarpales in a terminal position (Fig. 4). Most of orders and families included in the present study received higher bootstrap values and Bayesian probabilities than each individual data set. The Dictyotales, Sphacelariales, and Syringodermatales formed a clade supported with 65% BS for ML and 1 PP. The other remaining taxa produced a monophyletic clade (93% BS for ML, 94% BS for MP, and PP = 1). The Laminariales was monophyletic (97% BS for ML, 90% BS for MP, and PP = 1) and was shown to be the sister taxon to the Ectocarpales, but the sister relationship was not supported with bootstrap values. The interrelationships between most of orders and families were not well resolved.

The SH tests using the combined data set showed that the best position for the Ishigeaceae was on the basal branch of the Phaeophyceae. The alternative topologies that force the genus to be placed into a clade within the Chordariaceae, Ectocarpales, Fucales, Sphacelariales, Dictyotales, or Syringodermatales were significantly worse than the best tree ($P < 0.05$, Table 3).

Morphology of plastids and ultrastructure of Ishige okamurae. Thalli of *I. okamurae* (Fig. 5a) contain plastids in the vegetative cells of the assimilatory filaments. They are discoid, and the number is three to four per cell (Fig. 5b). The ultrastructure is typical of other brown algal plastids. No pyrenoid was found in most of the plastids in our samples, but a small exserted pyrenoid was very rarely observed (Fig. 5, c and d).

DISCUSSION

This study presents the first *psa*A and *psb*A phylogenies of the Phaeophyceae based on 38–39 representatives from 13 orders or "ordinal-level families," including two outgroup taxa. The *psa*A and *psb*A phylogenies are compared with the *rbc*L tree from 70 taxa from 17 orders, including 4 ordinal-level taxa and 3 outgroup species. To our knowledge, such a large data set containing three protein-coding plastid genes has not been used for the Phaeophyceae. Although the *rbc*L region is more variable (46.7%) than the *psa*A (44.4%) and *psb*A (34.6%) regions, the *psa*A region contains more informative sites (38.8%) than the *psb*A (25.5%) and *rbc*L (38%) regions. Lower consistency index of the *rbc*L region (0.278) among three genes is probably due to the number of taxa investigated. The result that the consistency index values of the *psa*A (0.333) and *psb*A (0.378) in the present study are lower than those (0.493 in *psa*A and 0.383 in *psb*A) in previous study of red algae (Yang and Boo 2004) may be assigned to taxon sampling of brown algae at the ordinal level than that of red algae at the genus level. A comparison of other phylogenetic signals in individual and combined data sets is given in Table 2.

Recently, several studies pointed out that the results of congruence tests should not be used as criteria for combining individual data sets. The reliability of the congruence tests such as the ILD test has been questioned (Lavoué et al. 2003, Shimabukuro-Dias et al. 2004), and some different data sets, being in conflict over some parts of a tree, may be congruent over other parts (Gatesy et al. 1999b). Although there are no signs of saturation of each gene sequence, the ILD test detected a significant incongruence (at the $P < 0.05$ level)

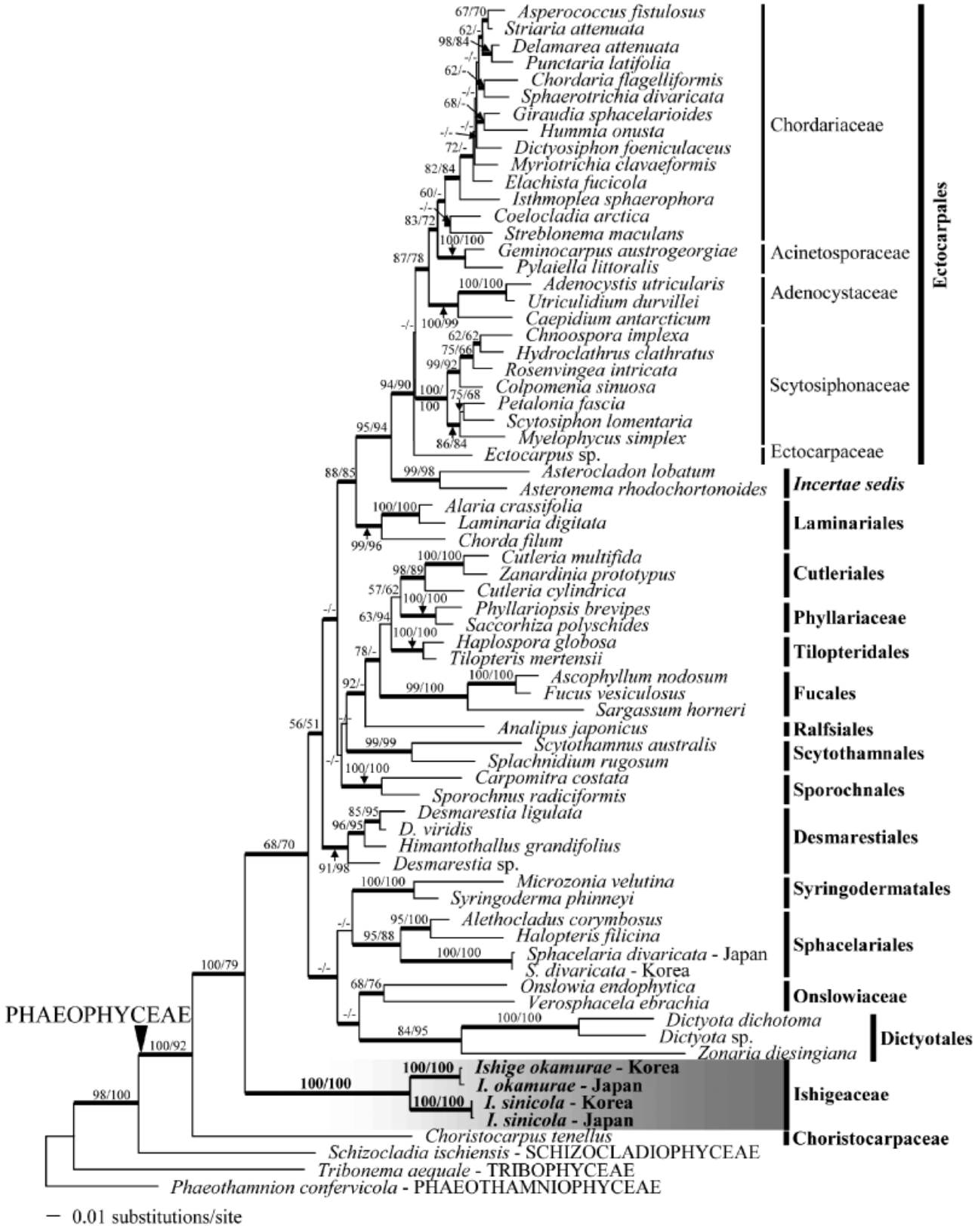

FIG. 1. ML tree for the Ishigeaceae and other phaeophyceaen algae estimated from the *rbc*L sequence data (GTR + Γ + I model, –ln likelihood = 20839.33098; Γ = 0.798767; I = 0.463896; A–C = 1.223060, A–G = 4.104599, A–T = 1.183624, C–G = 2.299422, C–T = 7.838568, G–T = 1). The bootstrap values shown above the branches are from ML/MP methods and dashes indicate <50% support of bootstrap. Thick branches indicate Bayesian posterior probabilities ≥0.9.

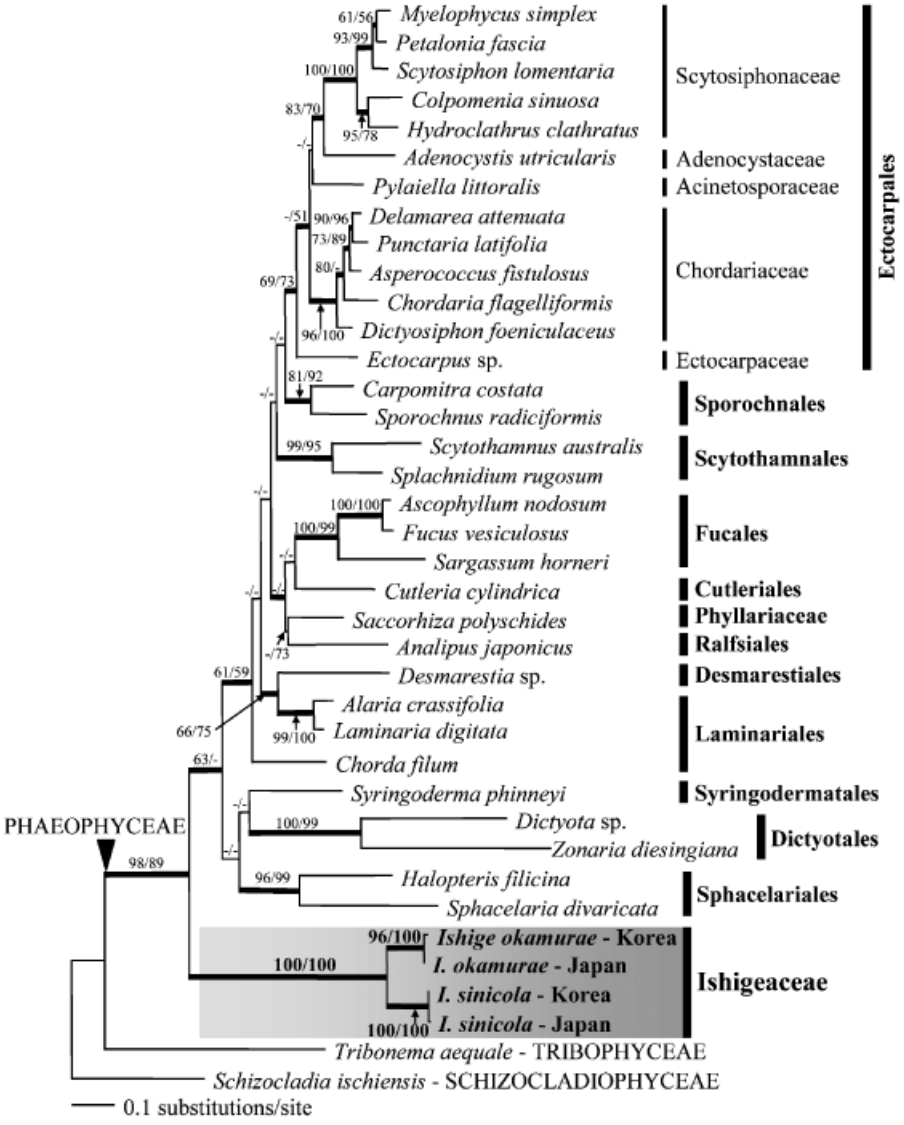

FIG. 2. ML tree for the Ishigeaceae and other phaeophyceaen algae estimated from the *psa*A sequence data (GTR + Γ + I model, –ln likelihood = 16551.60179; Γ = 0.568987; I = 0.492528; A–C = 2.626896, A–G = 4.898248, A–T = 0.072912, C–G = 3.757704, C–T = 12.922112, G–T = 1). The bootstrap values shown above the branches are from ML/MP methods and dashes indicate <50% support of bootstrap. Thick branches indicate Bayesian posterior probabilities ≥0.9.

among the *rbc*L, *psa*A, and *psb*A data sets in the present study. This is a contrast to congruence among the same three genes in the ceramiaceous red algal tribe Griffithsieae (Yang and Boo 2004). However, the combined sequences of the three genes have more resolving power and clade support for all ordinal taxa including the three orders, the Laminariales not resolved in the *psa*A and *psb*A trees and the Dictyotales and Sphacelariales not supported in the *psb*A tree, than the sequences of individual gene. The individual and combined data sets are congruent in phylogenetic positions of the Ishigeaceae and the Ectocarpales within the Phaeophyceae investigated. It appears that incongruence among the three genes is probably attributed to unstable relationships among orders, which are a feature well known in phylogenetic reconstructions of the Phaeophyceae (Draisma et al. 2001, 2003, Rousseau et al. 2001). Our studies show that statistically incongruent data can be combined for a better understanding of brown algal phylogeny, although the *rbc*L, *psa*A, and *psb*A genes possess different phylogenetic information. Our results indicate that the *psa*A and *psb*A genes, both being independent to each other (Morden and Sherwood 2002), are useful for other brown algal phylogeny and have more resolution when combining with other molecular data such as the *rbc*L gene.

In the present study, the Phaeophyceae are well resolved, having the Schizocladiophyceae as sister taxon in the *rbc*L and combined data sets, although the Tribophyceae was the sister to the Phaeophyceae in the *psa*A and *psb*A data sets. Based on trees of individual and combined data sets, the Phaeophyceae consist of two large groups. The "basal group" included the Choristocarpaceae, Ishigeaceae, Dictyotales, Sphacelariales, and Syringodermatales and the "crown" contained the Ectocarpales, Fucales, Laminariales, and other many lineages. These two groups are also reflected in the trees of previous *rbc*L (Draisma et al. 2001, 2003) and LSU + partial SSU data sets (Rousseau et al. 2001). However, because the interrelationships of the

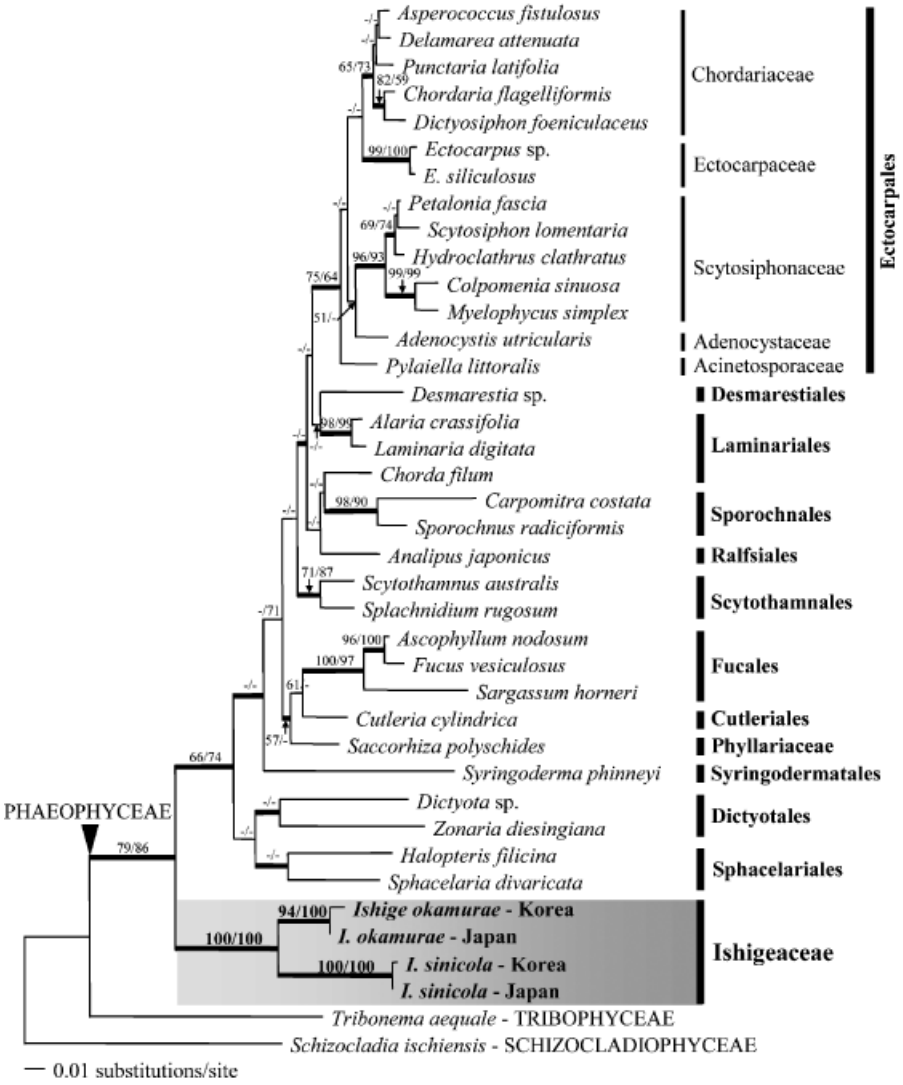

FIG. 3. ML tree for the Ishigeaceae and other phaeophyceaen algae estimated from the *psb*A sequence data (GTR + Γ + I model, –ln likelihood = 5953.44945; Γ = 0.472723; I = 0.402634; A–C = 1.245033, A–G = 9.15766, A–T = 8.589725, C–G = 1.088511, C–T = 23.338746, G–T = 1]. The bootstrap values shown above the branches are from ML/MP methods and dashes indicate <50% support of bootstrap. Thick branches indicate Bayesian posterior probabilities ≥0.9.

orders or families within each of the two groups were not resolved, we focus on the phylogenetic and taxonomic implications of the Ishigeaceae compared with other relative orders.

In all phylogenies reconstructed from three protein-coding genes, the Ectocarpales was placed in a terminal position, whereas the Ishigeaceae ended up in a basal position. The monophyly of the Ectocarpales, which includes the largest number of taxa in our study, is strongly supported and, within the order, the five families established by Peters and Ramírez (2001) are well resolved, although the Chordariaceae appear paraphyletic. The long-held concept of an ancestral Ectocarpales and derived Fucales (van den Hoek et al. 1995) is not supported by our *psa*A and *psb*A gene phylogenies as well as *rbc*L gene, as is seen in previous *rbc*L (Draisma et al. 2001, 2003) and LSU + partial SSU trees (Rousseau et al. 2001).

The main finding of our study is that the family Ishigeaceae does not cluster with members of the Chordariaceae such as *Chordaria flagelliformis* or other taxa of the Ectocarpales. These results contradict the current classifications of Yoshida (1998) that assign the family to the Chordariales (or the Chordariaceae *s.s.*). Our trees confirm that the Ectocarpales *s.l.* (Peters and Ramírez 2001), which is circumscribed by a pedunculated pyrenoid in discoid plastids and mostly a heteromorphic life history (Peters and Ramírez 2001, Draisma et al. 2003), cannot contain the genus *Ishige*. Instead, all analyses of our *rbc*L, *psa*A, *psb*A, and combined data sets indicate that the family Ishigeaceae consistently forms a basal group in the Phaeophyceae. The Ishigeaceae was the basal most in the *psa*A, *psb*A, combined data sets, and SH tests because *Choristocarpus* is not included; however, the Ishigeaceae was the penultimate basal taxon, after the Choristocarpaceae, in the *rbc*L tree. These results suggest that the Ishigeaceae might have diverged early in brown algal evolution. This is the first time that such a hypothesis has been proposed on either morphological or molecular

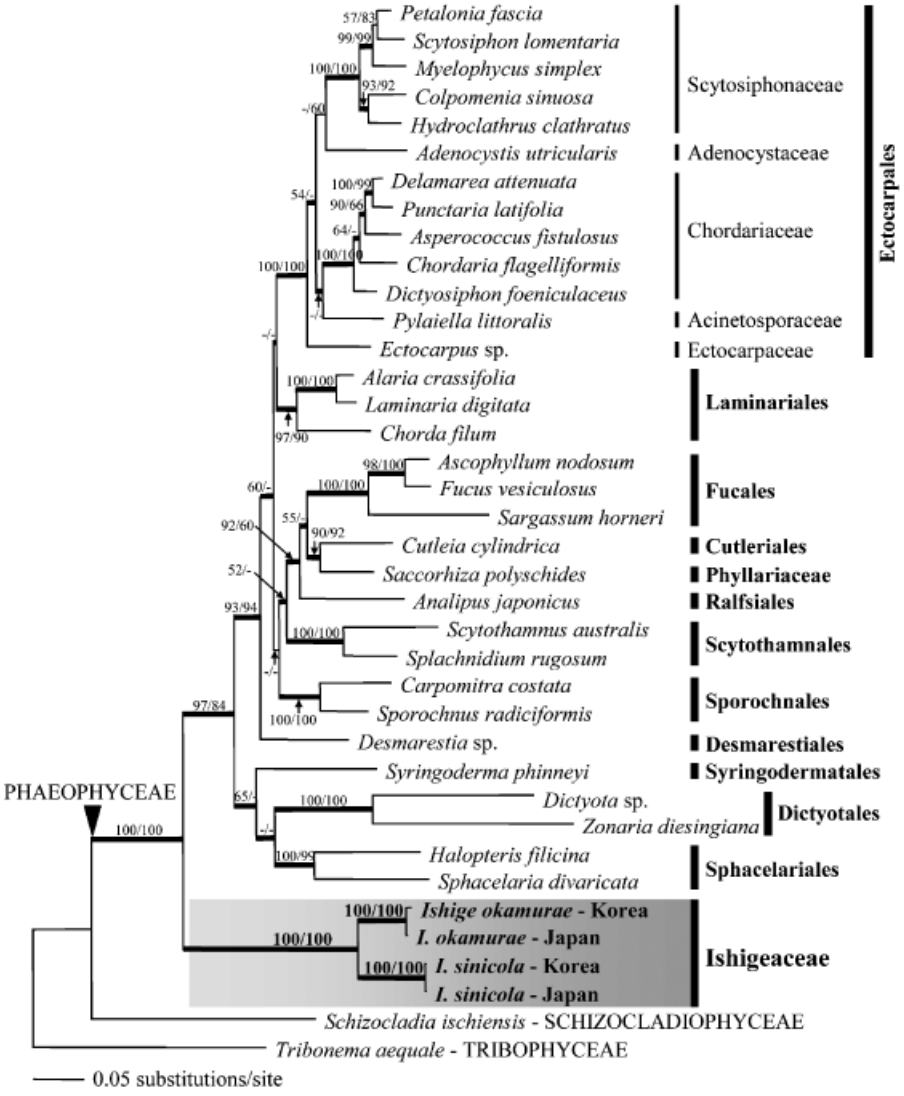

FIG. 4. ML tree for the Ishigeaceae and other phaeophyceaen algae estimated from combined *rbc*L + *psa*A + *psb*A sequence data (GTR + Γ + I model, –ln likelihood = 37313.27150; Γ = 0.756446; I = 0.516176; A–C = 2.052989, A–G = 5.063074, A–T = 1.442179, C–G = 2.684154, C–T = 12.351116, G–T = 1). The bootstrap values shown above the branches are from ML/MP methods and dashes indicate <50% support of bootstrap. Thick branches indicate Bayesian posterior probabilities ≥0.9.

evidence. A closer look on morphological features of the Ishigeaceae that support their probable early divergence is described below.

From the ultrastructural details of the vegetative cells of *I. okamurae* from Korea, it is clear that most cells do not have a pyrenoid or vary rarely have a pyrenoid. The pyrenoid is very small, exserted (Fig. 5, c and d), and similar to that of *I. okamurae* from Japan (Fig. 12 in Hori 1971). The small exserted pyrenoid of the species is considered a relatively rudimentary type when compared with the elaborate pedunculate pyrenoid of the Ectocarpales (Evans 1968). *Ishige sinicola* also lacks a pyrenoid (Hori 1971, Hori and Ueda 1975). Therefore, we conclude that the Ishigeaceae lack a pyrenoid in plastid. We suspect that Hori (1971) might wonder at the absence of a pyrenoid in *Ishige*, believing it to belong to the Chordariales, which have pyrenoids. The vegetative cells of the Dictyotales, Fucales, Laminariales, and Sphacelariales also lack pyrenoids, although a rudimentary form occurs in their gametes or zoospores (Evans 1966, Chi 1971). Therefore, the presence of the "true" pyrenoid (not a "rudimentary" one in which the exact nature is not demonstrated and perhaps not homologous) is apomorphic in the Phaeophyceae. Our results do not accord with the view of Evans (1966, 1968) that the presence of the pyrenoid is plesiomorphic. However, because the occurrence and development of the pyrenoid varies with the life history stage and physiological conditions (Bourne and Cole 1968), further ultrastructural studies of the plastids of various brown algae are required before the phylogenetic significance of the pyrenoid can be substantiated.

The two species of Ishigeaceae grow via small apical cells (Fig. 1 in Lee et al. 2003). Apical growth occurs in all other members of the basal group, such as the Choristocarpaceae, Dictyotales, Sphacelariales, Onslowiaceae, and Syringodermatales, which have an apical cell, a group of apical cells, or marginal cells cutting off segments proximally (Prud'homme van Reine

TABLE 3. Results of the Shimodaira-Hasegawa tests used to evaluate alternative hypotheses of the Ishigeaceae position in the phylogeny of the *psa*A + *psb*A + *rbc*L data set (see Fig. 4).

Hypotheses tested	–ln *L*	Difference –ln *L*	*P*
Basal position	37313.27150	best	
Chordariaceae	37415.16034	101.88883	0.0000[a]
Ectocarpales	37366.85999	53.58849	0.0020[a]
Fucales	37395.40131	82.12981	0.0000[a]
Sphacelariales	37368.18768	54.91617	0.0118[a]
Dictyotales	37366.71234	53.44084	0.0143[a]
Syringodermatales	37359.43507	46.16357	0.0193[a]

[a]Significant at 0.05 level.

1982, Henry 1984, Bold and Wynne 1985). We agree with Draisma et al. (2001, 2003) and Rousseau et al. (2001) that apical growth is plesiomorphic. In this context, it is interesting to speculate whether apical growth in spermatochnacean ectocarpoids, such as *Chordariopsis*, *Spermatochnus*, and *Stilophora* (Fritsch 1945), is plesiomorphic or convergent. However, the apical growth in the Fucales may be derived in that the apical meristem is composed of one or more cells that can divide in several directions, generating three-dimensional thalli (Graham and Wilcox 2000). In other crown group algae, thalli grow for trichothallic, intercalary, or marginal meristems (Bold and Wynne 1985, Graham and Wilcox 2000).

The internal tissues of the thallus of the *Ishige* species consist of cortex and medulla; the cortex is composed of assimilatory filaments and pseudoparenchymatous tissue (Ajisaka 1989, Figs. 5 and 11 in Lee et al. 2003), and the medulla contains colorless hypheal cells connected to adjacent hypheal cells or directly to cortical cells (Ajisaka 1989, Tanaka in Hori 1993, Lee et al. 2003). This oligostichous organization is similar to the haplostichous structure of uniseriate filament of the Choristocarpaceae (Fritsch 1945). Both haplostichous and polystichous structures are found in the Sphacelariales (Fritsch 1945, Prud'homme van Reine 1982). However, the Dictyotales and Syringodermatales have polystichous organization alone (Fritsch 1945, Henry 1984, Draisma et al. 2003). Therefore, a series of evolutionary changes may have led from haplostichous structures to polystichous structures in the internal organization of the thalli of the basal brown algae. However, it appears that the internal organization of the thallus might have evolved independently several times among the crown group of the brown algae (Draisma et al. 2003).

One of the most distinctive characters of the Ishigeaceae is the presence of the cryptostomata on the surface of thalli, which are well illustrated in previous studies (Fig. 8 in Yendo 1907, Figs. 2 and 10 in Lee et al. 2003). Phaeophycean hairs originate from cells in the medulla. In the presence of cryptostomata, the Ishigeaceae is similar to crown groups, such as the Fucales, Scytothamnales, and *Adenocystis* of the Ectocarpales (Clayton 1984, 1985). However, the scytothamnalean cryptostomata mature into conceptacles containing unilocular sporangia, whereas the fucalean cryptostomata do not form gametangial branches. There are a variety of other genera of the Ectocarpales, such as *Colpomenia*, *Chnoospora*, and *Leathesia*, in which there are groups of phaeophycean hairs growing from shallow epithermal pits (Fritsch 1945, Clayton 1984). Our molecular phylogenies based on the *rbc*L, *psa*A, and *psb*A genes place taxa with cryptostomata, viz. Ishigeaceae, Scytothamnales, and Fucales, in different clades. *Adenocystis* formed a clade independent from the scytosiphonacean genera, despite their belonging to the Ectocarpales. These results demonstrate that the cryptostomata in the family Ishigeaceae is a homoplastic character that misled Yendo (1907) into classifying the genus in the family Fucaceae. At the present state of knowledge, the occurrence of cryptostomata in the Phaeophyceae is evolutionarily enigmatic. On the other hand, the phaeophycean hairs are present on branches in the Sphacelariales (Prud'homme van Reine 1982) or are associated with sporangial sori in the Dictyotales (Bold and Wynne 1985), whereas they are absent in the Choristocarpaceae, Onslowiaceae, and Syringodermatales (Henry 1984, 1987, Womersley 1987, Draisma and Prud'homme van Reine 2001). Our multigene trees do not provide phylogenetic correlation between the cryptostomata and the phaeophycean hairs.

The unilocular sporangia of *I. okamurae* are produced from the outermost cortical cells (Tanaka in Hori 1993, Lee et al. 2003). The plurilocular sporangia of the species originate from the assimilatory filaments, are uniseriate, and lack sterile terminal cells (Ajisaka 1989, Tanaka in Hori 1993, Lee et al. 2003). Both unilocular and plurilocular sporangia occur in the thalli of *I. sinicola* from the south coast of Korea (Lee et al. 2003). The observations of Ajisaka (1989) and Lee et al. (2003) contradict those of Arasaki (1943) that the plurilocular sporangia of *I. sinicola* in culture were gametangia and similar to those of certain species of the Chordariales, in which the unilocular sporangia are always formed in the basal or middle part of the assimilatory filaments, and both types of sporangia are present on the sporophytes of some species (Inagaki 1958, Ajisaka 1989, Yoshida 1998). However, the Dictyotales have meiosporic tetrasporangia (Bold and Wynne 1985), and the Choristocarpaceae, Onslowiaceae, and Sphacelariales have unilocular zoidangia, plurilocular zoidangia, or multicellular propagules (Prud'homme van Reine 1982).

Ajisaka (1989) reported that plurispores of *I. okamurae*, released from the plurilocular sporangia, develop directly into typical filamentous thalli through a pseudoparenchymatous prostrate disc, which is sterile and functions as a perennial holdfast system. A similar perennial basal system is found in the Sphacelariales, including the Choristocarpaceae (Fritsch 1945). Perennial systems are found in *Ralfsia verrucosa* (Areschoug) Areschoug, *Desmarestia aculeata* (Linnaeus) Lamouroux, and the *Aglaozonia*-stage of *Cutleria monoica*

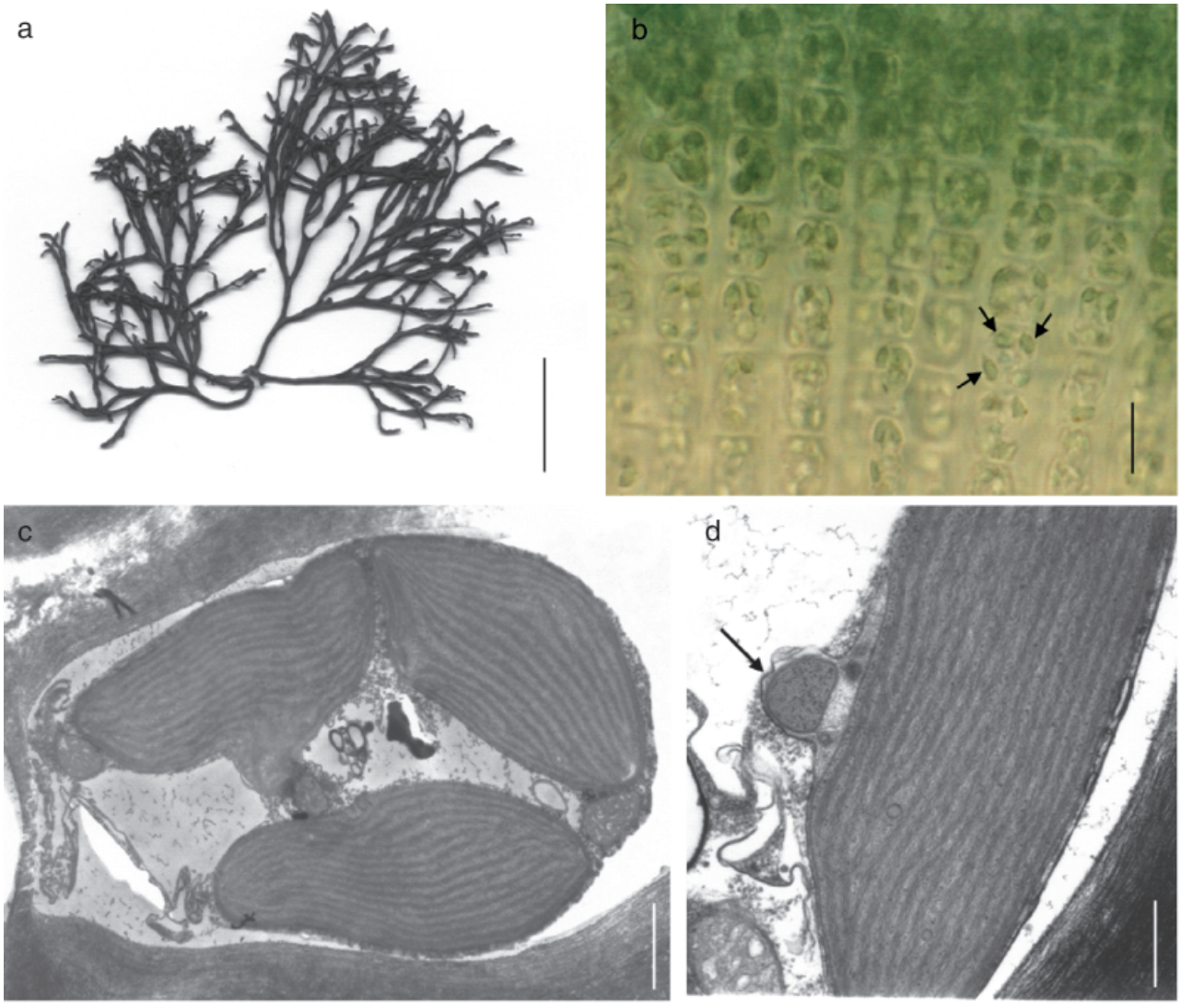

FIG. 5. Thallus and plastid of *Ishige okamurae*. (a) A herbarium specimen collected on 28 June 2001 in Geojedo, Korea. Scale bar, 2 cm. (b) Discoid plastids (arrows) in assimilatory cell. Scale bar, 10 μm. (c) Ultrastructure of a plastid, showing thylakoids, without pyrenoid. Scale bar, 1 μm. (d) A small exserted pyrenoid (arrow) in a plastid. Scale bar, 0.4 μm.

Ollivier (Fritsch 1945). Because the latter three taxa were not included in the present study, they are beyond the scope of our discussion. Tanaka (in Hori 1993) demonstrated that *I. okamurae* has an isomorphic life cycle, consisting of sporophytes that form plurilocular sporangia and gametophytes bearing unilocular sporangia. In the same chapter, Tanaka doubted the heteromorphic life history of *I. sinicola* observed by Arasaki (1943). Both plurilocular and unilocular sporangia occurred in erect thalli of *I. okamurae* (Figs. 7 and 8 in Lee et al. 2003) and *I. sinicola* (Figs. 15 and 16 in Lee et al. 2003) during a year-round collection from Namhaedo on the south coast of Korea. All these studies led us to conclude that the family Ishigeaceae has an isomorphic life history, with macroscopic sporophytes and gametophytes. Isomorphic life cycles have been reported for the Dictyotales, Sphacelariales, and Onslowiaceae (Prud'homme van Reine 1982, Henry 1987, van den Hoek et al. 1995, Draisma and Prud'homme van Reine 2001). In the Choristocarpaceae, thalli with either uni- or plurilocular sporangia look similar and could also indicate an isomorphic life history (Fritsch 1945, Burrowes et al. 2003). However, the Syringodermatales have a heteromorphic life history (Henry 1984). Although some members of the Scytosiphonaceae have an isomorphic type (Cho et al. 2003), the Ectocarpales and other brown algae have heteromorphic life histories or single diploid generations (Graham and Wilcox 2000).

Interestingly, both species of *Ishige* are distributed in the warmer waters of the Pacific Ocean (Yendo 1907, Setchell and Gardner 1924, Tseng 1983, Lee et al. 2003). *Choristocarpus tenellus* of the Choristocarpaceae inhabits the Mediterranean Sea (Fritsch 1945). The Dicytotales are very diverse in tropical and subtropical waters (Bold and Wynne 1985), and the Sphacelariales

TABLE 4. A taxonomic comparison of the Ishigeaceae with other basal brown algae that have symplesiomorphic characters such as apical growth and discoid pyrenoidless plastids.

	Ishigeaceae	Choristocarpaceae	Onslowiaceae	Sphacelariales	Dictyotales	Syringodermatales
Thallus construction of sporophytes	Oligostichous	Haplostichous	Oligostichous	Haplostichous or polystichous	Polystichous	Oligostichous
Perennial disc	Present	Present	Present?	Present	Rarely present	Absent
Cryptostomata	Present	Absent	Absent	Absent	Absent	Absent
Phaeophycean hairs	Within cryptostomata	Absent	Absent	On branches	Associated with reproductive organs	Absent
Propagules	Absent	With a large apical cell	Without a large apical cell	Without a large apical cell	Absent	Absent
Plurilocular sporangia	From cortical layer of gametophytes	On branches of gametophytes	On branches of gametophytes	On branches of gametophytes	From the surface cells of gametophytes	From the surface cells of gametophytes
Unilocular sporangia	Many spores, from the cortical layer of sporophytes	Many spores, on branches of sporophytes	Many spores, on branches of sporophytes	Many spores, on branches of sporophytes	Tetraspores, from the surface cells of sporophytes	Bi-, tetraspores, from the surface cells of sporophytes
Gametes	Isogametes	Isogametes	Anisogametes	Mostly iso- and anisogametes	Egg and spermatozoids	Isogametes
Life cycle	Isomorphic	Probably isomorphic	Isomorphic	Mostly isomorphic	Isomorphic	Heteromorphic
Pheromone	Not detected	Not detected	Not detected	Ectocarpene/ hormosirene, desmarestene	Dictyotene, multifidene	Viridene

Data from Fritsch (1945), Prud'homme van Reine (1982), Henry (1984, 1987), Womersley (1987), Ajisaka (1989), Tanaka (in Hori 1993), Draisma and Prud'homme van Reine (2001), Pohnert and Boland (2002), and Lee at al (2003).

and Syringodermatales are distributed from tropical to Antarctic waters (Prud'homme van Reine 1982, Henry 1984). The distributions of living taxa of these basal groups speculate that the brown algae may have originated in tropical waters during a geological period with warm climatic conditions, whereas most brown algae in the crown group appear to exhibit the greatest diversity in terms of species and morphology in cold waters (Bold and Wynne 1985). The Paleozoic may have been the period of the earliest brown algae, as suggested by Clayton (1984), or the Mesozoic, which roughly corresponds to 155 million years ago based on rDNA SSU sequence divergence (Medlin et al. 1997) or approximately 200 million years ago based on 5S rRNA sequences (Lim et al. 1986).

Our molecular study shows conclusively that the family Ishigeaceae produces a basal brown algal group with the Choristocarpaceae, Dictyotales, Sphacelariales, Onslowiaceae, and Syringodermatales. These early diverging brown algae have apical growth and pyrenoidless discoid plastids as morphological symplesiomorphy. In addition, the Ishigeaceae is similar to the Dictyotales, Sphacelariales, Onslowiaceae, and probably Choristocarpaceae in its isomorphic life history. However, our *rbc*L, *psa*A, and *psb*A phylogenies indicate that the family Ishigeaceae makes an independent lineage from the early diverging brown algae in having oligostichous organization of thallus, cryptostomata, and uni- and plurilocular sporangia within cortical layer (Table 4). Our studies on the three protein coding plastid genes and ultrastructure of plastids do not confirm the current classification of the family Ishigeaceae within the Ectocarpales *s.l.* or Chordariaceae *s. str.* Although we did not examine the Ascoseirales, which is only order of the Phaeophyceae not included in the present study because of no collection, it does not appear that the inclusion of the taxon would change our finding that the Ishigeaceae is a distinct basal group clearly separated from other brown algal orders or families. The unique molecular and ultrastructural evidence that defines the Ishigeaceae as an early diverging but independent brown algal group is best expressed by placement of the family in a separate order, Ishigeales ord. nov.

Ishigeales ord. nov. G. Y. Cho et Boo

Diagnosis: Novus ordo Phaeophycearum. Plantae isomorphicae, ad 20 cm altae, ex haptero parvo aut basi crustosa extenta crescentes, epiphyticae vel epilithicae. Augmen per cellulas apicales. Frondes ramosae teretes vel foliosae, medulla corticeque instructae. Cortex pseudoparenchymatus, filamentis assimilantibus. Cellulae plastides aliquot discoideos sine pyrenoide continentes. Medulla cum hyphis. Pili phaeophycei caespitosi, e cryptostomatibus orientes. Sporangia unilocularia e cellulis corticalibus transformatis facta, terminalia. Sporangia plurilocularia e filamentis assimilantibus transformatis facta, uniseriata, sine cellula terminali.

Type family: Ishigeaceae Okamura in Segawa with the same characteristics as the order.

Type genus: Ishige Yendo

New order of Phaeophyceae. Plants isomorphic, to 20 cm high, growing from a small holdfast, or an extended crustose base, epiphytic or epilithic. Growth from apical cells. Fronds branched, terete, or foliose, with medulla and cortex. Cortex pseudoparenchymatous, with assimilatory filaments. Cells containing several discoid plastids without pyrenoids. Medulla with hyphae. Phaeophycean hairs clustered, growing from cryptostomata. Unilocular sporangia transformed from cortical cells, terminal. Plurilocular sporangia transformed from assimilatory filaments, uniseriate, lacking a terminal cell.

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Ajisaka, T. 1989. Plurilocular sporangia and the development of plurispores in *Ishige okamurae* Yendo (Phaeophyceae) from the Kada coast, Wakayama Prefecture in Japan. *Jpn. J. Phycol.* 37:17–22.

Arasaki, S. 1943. On the life history of *Ishige foliacea* Okamura. *Bot. Mag. Tokyo* 57:34–41.

Assali, N. E., Mache, R. & de Göer, S. L. 1990. Evidence for a composite phylogenetic origin of the plastid genome of the brown alga *Pylaiella littoralis* (L.) Kjellm. *Plant Mol. Biol.* 15:307–15.

Bailey, J. C. & Andersen, R. A. 1998. Phylogenetic relationships among nine species of the Xanthophyceae inferred from *rbc*L and 18S rRNA gene sequences. *Phycologia* 37:458–66.

Bailey, J. C., Bidigare, R. R., Christensen, S. J. & Andersen, R. A. 1998. Phaeothamniophyceae classis nova: a new lineage of Chromophytes based upon photosynthetic pigments, *rbc*L sequence analysis and ultrastructure. *Protist* 149:245–63.

Bold, H. C. & Wynne, M. J. 1985. *Introduction to the Algae: Structure and Reproduction*. Prentice Hall, Englewood Cliffs, NJ, 720 pp.

Bourne, V. L. & Cole, K. 1968. Some observations on the fine structure of the marine brown alga *Phaeostrophion irregulare*. *Can. J. Bot.* 46:1369–79.

Bull, J. J., Huelsenbeck, J. P., Cunningham, C. W., Swofford, D. L. & Waddell, P. J. 1993. Partitioning and combining data in phylogenetic analysis. *Syst. Biol.* 42:384–97.

Burrowes, R., Rousseau, F., Müller, D. G. & de Reviers, B. 2003. Taxonomic placement of *Microzonia* (Phaeophyceae) in Syringodermatales based on *rbc*L and 28S nrDNA sequences. *Cryptog. Algol.* 24:63–73.

Chi, E. Y. 1971. Brown algal pyrenoids: brief report. *Protoplasma* 72:101–4.

Chihara, M. 1969. *Pseudogloiophloea okamurai* (Setchell) comb. nov. and *Ishige sinicola* (Setchell and Gardner) comb. nov. *Bull. Jpn. Soc. Phycol.* 17:1–4.

Cho, T. O., Cho, G. Y., Yoon, H. S., Boo, S. M. & Lee, W. J. 2003. New records of *Myelophycus cavus* (Scytosiphonaceae, Phaeophyceae) in Korea and the taxonomic position of the genus on the basis of a plastid DNA phylogeny. *Nova Hedw.* 76:381–97.

Clayton, M. N. 1984. Evolution of the Phaeophyta with particular reference to the Fucales. *Prog. Phycol. Res.* 3:11–46.

Clayton, M. N. 1985. A critical investigation of the vegetative anatomy, growth and taxonomic affinities of *Adenocystis, Scytothamnus*, and *Splachnidium* (Phaeophyta). *Br. Phycol. J.* 20:285–96.

de Reviers, B. & Rousseau, F. 1999. Towards a new classification of the brown algae. *Prog. Phycol. Res.* 13:107–201.

Draisma, S. G. A., Peters, A. F. & Fletcher, R. L. 2003. Evolution and taxonomy in the Phaeophyceae: effects of the molecular age on brown algal systematics. *In* Norton, T. A. [Ed.] *Out of the Past: Collected Reviews to Celebrate the Jubilee of the British Phycological Society*. British Phycology Society, Belfast, pp. 87–102.

Draisma, S. G. A. & Prud'homme van Reine, W. F. 2001. Onlsowiaceae fam.nov. (Phaeophyceae). *J. Phycol.* 37:647–9.

Draisma, S. G. A., Prud'homme van Reine, W. F., Stam, W. T. & Olsen, J. L. 2001. A reassessment of phylogenetic relationships within the Phaeophyceae based on RUBISCO large subunit and ribosomal DNA sequences. *J. Phycol.* 37:586–603.

Evans, L. V. 1966. Distribution of pyrenoids among some brown algae. *J. Cell Sci.* 1:449–54.

Evans, L. V. 1968. Chloroplast morphology and fine structure in British fucoids. *New Phytol.* 67:173–8.

Farris, J. S., Källersjö, M., Kluge, A. G. & Bult, C. 1994. Testing significance of incongruence. *Cladistics* 10:315–9.

Fritsch, F. E. 1945. *The Structure and Reproduction of the Algae. II. Foreword, Phaeophyceae, Rhodophyceae, Myxophyceae*. University Press, Cambridge, 939 pp.

Gatesy, J., Milinkovitch, M., Waddell, V. & Stanhope, M. 1999a. Stability of cladistic relationships between Cetacea and higher-level Artiodactyl taxa. *Syst. Biol.* 48:6–20.

Gatesy, J., O'Grady, P. & Baker, R. H. 1999b. Correlation among data sets in simultaneous analysis: hidden support for phylogenetic relationships among higher level artiodactyl taxa. *Cladistics* 15:271–313.

Gilbert, D. G. 1995. *SeqPup, a Biological Sequence Editor and Analysis Program for Macintosh Computers*. Biology Department, Indiana University, Bloomington.

Graham, L. E. & Wilcox, L. W. 2000. *Algae*. Prentice Hall, NJ, 640 pp.

Hasegawa, M., Kishino, H. & Yano, T. A. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. *J. Mol. Evol.* 22:160–74.

Henry, E. C. 1984. Syringodermatales ord. nov. and *Syringoderma floridana* sp. nov. (Phaeophyceae). *Phycologia* 23:419–26.

Henry, E. C. 1987. Morphology and life histories of *Onslowia bahamensis* sp. nov. and *Verosphacela ebrachia* gen. et sp. nov., with a reassessment of the Choristocarpaceae (Sphacelariales, Phaeophyceae). *Phycologia* 26:182–91.

Hori, T. 1971. Survey of pyrenoid distribution in brown algae. *Bot. Mag. Tokyo* 84:231–42.

Hori, T. 1993. *An Illustrated Atlas of the Life History of Algae. Vol. 2. Brown and Red Algae*. Uchida Rokakuho, Tokyo, 345 pp.

Hori, T. & Ueda, R. 1975. The fine structure of algal chloroplasts and algal phylogeny. *In* Tokida, J. & Hirose, H. [Eds.] *Advance of Phycology in Japan*. Dr. W. Junk, The Hague, pp. 11–42.

Huelsenbeck, J. P. & Ronquist, F. 2001. MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* 17:754–5.

Inagaki, K. 1958. A systematic study of the order Chordariales from Japan and its vicinity. *Sci. Pap. Inst. Algol. Res. Hokkaido Univ.* 4:87–197.

Kawai, H. 1992. A summary of the morphology of chloroplasts and flagellated cells in the Phaeophyceae. *Korean J. Phycol.* 7: 33–43.

Kawai, H., Maeba, S., Sasaki, H., Okuda, K. & Henry, E. C. 2003. *Schizocladia ischiensis*: a new filamentous marine chromophyte belonging to a new class, Schizocladiophyceae. *Protist* 154: 211–28.

Kawai, H. & Sasaki, H. 2000. Molecular phylogeny of the brown algal genera *Akkesiphycus* and *Halosiphon* (Laminariales), resulting in the circumscription of the new families Akkesiphycaceae and Halosiphonaceae. *Phycologia* 39:416–28.

Kogame, K., Horiguchi, T. & Masuda, M. 1999. Phylogeny of the order Scytosiphonales (Phaeophyceae) based on DNA sequences of *rbc*L, partial *rbc*S, and partial LSU nrDNA. *Phycologia* 38:496–502.

Lavoué, S., Sullivan, J. P. & Hopkins, C. D. 2003. Phylogenetic utility of the first two introns of the S7 ribosomal protein gene in African electric fishes (Mormyroidea: Teleostei) and congruence with other molecular markers. *Biol. J. Linn. Soc.* 78:273–92.

Lee, E. Y., Lee, I. K. & Choi, H. G. 2003. Morphology and nuclear small-subunit rDNA sequences of *Ishige* (Ishigeaceae, Phaeophyceae) and its phylogenetic relationship among selected brown algal orders. *Bot. Mar.* 46:193–201.

Lim, B. L., Kawai, H., Hori, H. & Ozawa, S. 1986. Molecular evolution of 5S ribosomal RNA from red and brown algae. *Jpn. J. Genet.* 61:169–76.

Medlin, L. K., Kooistra, W. H. C. F., Potter, D., Saunders, G. W. & Andersen, R. A. 1997. Phylogenetic relationships of the "golden algae" (haptophytes, heterokont chromophytes) and their plastids. *Plant Syst. Evol.* 11(suppl):187–216.

Morden, C. W. & Sherwood, A. R. 2002. Continued evolutionary surprises among dinoflagellates. *Proc. Natl. Acad. Sci. USA* 99:11558–60.

Müller, D. G., Parodi, E. R. & Peters, A. F. 1998. *Asterocladon lobatum* gen. et sp. nov., a new brown alga with stellate chloroplast arrangement, and its systematic position judged from nuclear rDNA sequences. *Phycologia* 37:425–32.

Okamura, K. 1936. *Nihon Kaiso-shi*. Uchida Rokakuho, Tokyo, 964 pp.

Pearson, G., Serrão, E. & Cancela, M. L. 2001. Suppression subtractive hybridization for studying gene expression during aerial exposure and desiccation in fucoid algae. *Eur. J. Phycol.* 36:359–66.

Peters, A. F. & Clayton, M. N. 1998. Molecular and morphological investigation of three brown algal genera with stellate plastids: evidence for Scytothamnales ord. nov. (Phaeophyceae). *Phycologia* 37:106–13.

Peters, A. F. & Ramírez, M. E. 2001. Molecular phylogeny of small brown algae, with special reference to the systematic position of *Caepidium antarcticum* (Adenocystaceae, Ectocarpales). *Cryptog. Algol.* 22:187–200.

Pohnert, G. & Boland, W. 2002. The oxylipin chemistry of attraction and defense in brown algae. *Nat. Prod. Rep.* 19:108–22.

Posada, D. & Crandall, K. A. 1998. Modeltest: Testing the model of DNA substitution. *Bioinformatics* 14:817–8.

Prud'homme van Reine, W. F. 1982. *A Taxonomic Revision of the European Sphacelariaceae (Sphacelariales, Phaeophyceae)*. Leiden University Press, Leiden, 293 pp.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell. Biol.* 17:208–12.

Rousseau, F., Burrowes, R., Peters, A. F., Kuhlenkamp, R. & de Reviers, B. 2001. A comprehensive phylogeny of the Phaeophyceae based on nrDNA sequences resolves the earliest divergences. *C. R. Acad. Sci. Paris Sci. Life Sci.* 324:305–19.

Rousseau, F. & de Reviers, B. 1999. Circumscription of the order Ectocarpales (Phaeophyceae): bibliographical synthesis and molecular evidence. *Cryptog. Algol.* 20:5–18.

Rousseau, F., de Reviers, B., Leclerc, M.-C., Asensi, A. & Delépine, D. 2000. Adenocystaceae fam. nov. (Phaeophyceae), a new family based on morphological and molecular evidence. *Eur. J. Phycol.* 35:35–40.

Sasaki, H., Flores-Moya, A., Henry, E. C., Müller, D. G. & Kawai, H. 2001. Molecular phylogeny of Phyllariaceae, Halosiphonaceae and Tilopteridales (Phaeophyceae). *Phycologia* 40:123–34.

Segawa, S. 1935. On the marine algae of Susaki, Prov. Idzu and its vicinity. *Sci. Pap. Inst. Alg. Res., Fac. Sci., Hokkaido Univ.* 1:59–90.

Setchell, W. A. & Gardner, N. L. 1924. New marine algae from the Gulf of California. *Proc. Calif. Acad. Sci.* 12:659–949.

Shimabukuro-Dias, C. K., Oliviera, C., Reis, R. E. & Foresti, F. 2004. Molecular phylogeny of the armored catfish family Callichthyidae (Ostariophysi, Siluriformes). *Mol. Phylogenet. Evol.* 32:152–63.

Shimodaira, H. & Hasegawa, M. 1999. Multiple comparisons of log-likelihoods with applications to phylogenetic inference. *Mol. Biol. Evol.* 16:1114–6.

Siemer, B. L., Stam, W. T., Olsen, J. L. & Pedersen, P. M. 1998. Phylogenetic relationships of the brown algal orders Ectocarpales, Chordariales, Dictyosiphonales, and Tilopteridales (Phaeophyceae) based on RUBISCO large subunit and spacer sequences. *J. Phycol.* 34:1038–48.

Spurr, A. R. 1969. A low-viscosity epoxy resin-embedding medium for electron microscopy. *J. Ultrastr. Res.* 26:31–43.

Swofford, D. L. 2002. *PAUP* Phylogenetic Analysis Using Parsimony (* And Other Methods)*. Version 4.0b8. Sinauer, Sunderland, MA.

Tan, I. H. & Druehl, L. D. 1994. A molecular analysis of *Analipus* and *Ralfsia* (Phaeophyceae) suggests the order Ectocarpales is polyphyletic. *J. Phycol.* 30:721–9.

Tseng, C. K. 1983. *Common Seaweeds of China*. Science Press, Beijing, 316 pp.

van den Hoek, C., Mann, D. G. & Jahns, H. M. 1995. *Algae, An Introduction to Phycology.* Cambridge University Press, Cambridge, 627 pp.

Winhauer, T., Jager, S., Valentin, K. & Zetsche, K. 1991. Structural similarities between *psb*A gene from red and brown algae. *Curr. Genet.* 20:177–80.

Womersley, H. B. S. 1987. *The Marine Benthic Flora of Southern Australia. Part II*. South Australia Government, Adelaide, 484 pp.

Yang, E. C. & Boo, S. M. 2004. Evidence for two independent lineages of *Griffithsia* (Ceramiaceae, Rhodophyta) based on plastid protein-coding *psa*A, *psb*A, and *rbc*L gene sequences. *Mol. Phylogen. Evol.* 31:680–8.

Yendo, K. 1907. The Fucaceae of Japan. *J. Coll. Sci. Tokyo Univ.* 21:1–174.

Yoon, H. S. & Boo, S. M. 1999. Phylogeny of Alariaceae (Phaeophyta) with the special reference to *Undaria* based on sequences of the RuBisCo spacer region. *Hydrobiologia* 398/399:47–55.

Yoon, H. S., Hackett, J. D. & Bhattacharya, D. 2002a. A single origin of the peridinin- and fucoxanthin-containing plastids in dinoflagellates through tertiary endosymbiosis. *Proc. Natl. Acad. Sci. USA* 99:11724–9.

Yoon, H. S., Hackett, J. D., Pinto, G. & Bhattacharya, D. 2002b. A single, ancient origin of chromist plastids. *Proc. Natl. Acad. Sci. USA* 99:15507–12.

Yoshida, T. 1998. *Marine Algae of Japan*. Uchida Rokakuho, Tokyo, 1222 pp.