

MOLECULAR PHYLOGENY OF TWO UNUSUAL BROWN ALGAE, *PHAEOSTROPHION IRREGULARE* AND *PLATYSIPHON GLACIALIS*, PROPOSAL OF THE STSCHAPOVIALES ORD. NOV. AND PLATYSIPHONACEAE FAM. NOV., AND A RE-EXAMINATION OF DIVERGENCE TIMES FOR BROWN ALGAL ORDERS¹

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The molecular phylogeny of brown algae was examined using concatenated DNA sequences of seven chloroplast and mitochondrial genes (*atpB*, *psaA*, *psaB*, *psbA*, *psbC*, *rbcL*, and *cox1*). The study was carried out mostly from unialgal cultures; we included *Phaeostrophion irregulare* and *Platysiphon glacialis* because their ordinal taxonomic positions were unclear. Overall, the molecular phylogeny agreed with previously published studies, however, *Platysiphon* clustered with *Halosiphon* and *Stschapovia* and was paraphyletic with the Tilopteridales. *Platysiphon* resembled *Stschapovia* in showing remarkable morphological changes between young and mature thalli. *Platysiphon*, *Halosiphon* and *Stschapovia* also shared parenchymatous, terete, erect thalli with assimilatory filaments in whorls or on the distal end. Based on these results, we proposed a new order Stschapoviales and a new family Platysiphonaceae. We proposed to include *Phaeostrophion* in the Sphacelariales, and we emended the order to include this foliose member. Finally, using basal taxa not included in earlier studies, the origin and divergence times for brown algae were re-investigated. Results showed that the Phaeophyceae branched from Schizocladiphyceae ~260 Ma during the Permian Period. The early diverging brown algae had isomorphic life histories, whereas the derived taxa with heteromorphic life histories evolved 155–110 Ma when they branched from the basal taxa. Based on these

results, we propose that the development of heteromorphic life histories and their success in the temperate and cold-water regions was induced by the development of the remarkable seasonality caused by the breakup of Pangaea. Most brown algal orders had diverged by roughly 60 Ma, around the last mass extinction event during the Cretaceous Period, and therefore a drastic climate change might have triggered the divergence of brown algae.

Key index words: divergence time; molecular phylogeny; Phaeophyceae; *Platysiphon*; Platysiphonaceae fam. nov.; Stschapoviales ord. nov.

Platysiphon glacialis (Rosenvinge) H. Kawai et T. Hanyuda, a little-studied brown alga endemic to the Arctic region, was first described by Wilce (1962) based on vegetative specimens collected at Inglefield Bay, Thule district, West Greenland. There have been few reports on this species since then (Pedersen 1976, Mathieson et al. 2010), and neither the reproductive structures nor the life history have been fully elucidated (Wilce and Bradley 2007). The species is distinctive owing to its laterally expanded blade terminated by a terete portion, both of which have numerous clumps of hair-like assimilatory filaments arranged more or less in whorls on the distal end. On the basis of the gross morphology of juvenile thalli, Wilce (1962) placed the species in the Striariaceae of the Dictyosiphonales (Ectocarpales *s.l.*); however, the absence of pyrenoids suggested a more distant phylogenetic position from Ectocarpales *s.l.* (R.T. Wilce, unpublished data).

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Phaeostrophion irregulare Setchell & N.L. Gardner is another enigmatic species also considered distant from Ectocarpales *s.l.* based on morphology and molecular phylogenetic analysis (Kawai et al. 2005). The alga was placed in its own family Phaeostrophaceae, but the ordinal assignment was suspended because of insufficient resolution in the molecular analysis.

Historically, the divergence time of brown algal orders and families has been unclear because brown algal fossil evidence for evolutionary interpretations is scanty. Their soft tissues are composed of polysaccharides such as alginates, fucoidans and cellulose, and these tissues generally decompose before fossilization can occur. Furthermore, only a few calcified taxa exist (i.e., *Padina* spp. and *Neohousia imbricata* Kraft, G.W. Saunders, I.A. Abbott & Haroun) but they typically grow in exposed habitats where sedimentation is not common. The oldest generally accepted phaeophycean fossil is *Julescranea grandicornis* B.C. Parker & E.Y. Dawson from the Tertiary Period, described from the upper Miocene Epoch; this fossil is intermediate in appearance between the laminarialean genera *Pelagophycus* and *Nereocystis* (Parker and Dawson 1965).

Using RNA sequence data, the divergence time of brown algae from other heterokont lineages (e.g., diatoms) was first estimated to be 150–200 Ma (Lim et al. 1986). Silberfeld et al. (2010) published a time-calibrated molecular phylogenetic tree based on 10 mitochondrial, chloroplast and nuclear gene DNA sequences that inferred a diversification of the brown algal crown groups in the Lower Cretaceous Period; however, their analyses did not include basal taxa such as the Discosporangiales and Ishigeales. Brown and Sorhannus (2010) published a chronogram of the Ochrophyta (Heterokontophyta) using 18S rDNA sequences; their analyses included Schizocladiphyceae, the sister group of Phaeophyceae, as a basal taxon, and their estimated divergence time was ~170–200 Ma.

In this paper, we re-investigated *Platysiphon glacialis* and *Phaeostrophion irregulare* using a seven chloroplast and mitochondrial genes. In addition, we re-examined the brown algal tree of life by including basal taxa, and we re-estimated the divergence times for brown algal orders.

MATERIALS AND METHODS

Culture strains used for DNA extractions were housed in the Kobe University Macroalgal Culture Collection. Strains were grown in polystyrene Petri dishes containing 50 mL PESI medium (Tatewaki 1966) at 10°C and 15°C long day (LD; 16:8 light:dark) conditions illuminated by daylight-type white fluorescent lighting of ~50 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Specimens of *Platysiphon glacialis* were collected by SCUBA from a small bay at the eastern entrance of Ragged Channel, north Baffin Island, Nunavik, Canada, and quickly dried in silica gel. The culture strain codes and origins of the field-collected specimens used for molecular phylogenetic studies are listed in Table S1 in the Supporting Information.

Genomic DNA was extracted from the silica gel-dried algal tissue using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. PCR amplifications of the chloroplast *atpB*, *psaA*, *psaB*, *psbA*, *psbC*, *rbcL*, and mitochondrial *cox1* genes were carried out using the KOD FX (ToYoBo, Osaka, Japan) PCR enzyme and the TaKaRa PCR Thermal Cycler Dice (Takara Shuzo, Shiga, Japan). Primers used for PCR and/or sequencing are listed in Table 1. After PEG purification (Lis 1980), PCR products were sequenced using the CE DTCS Quick Start Kit (Beckman Coulter, Fullerton, CA, USA) and the CEQ8000 DNA analysis system (Beckman Coulter) according to the manufacturer's instructions. For the molecular phylogenetic analyses, published and newly determined sequence data of the Phaeophyceae and the Schizocladaceae were used (Table S1). *Schizocladia ischiensis* E.C. Henry, K. Okuda & H. Kawai, which is sister to Phaeophyceae was chosen as the outgroup. Alignments were prepared using the program MAFFT v. 6 (Katoh and Toh 2008) and then manually adjusted prior to phylogenetic analyses.

Concatenated DNA sequences (data set 1: 44OTUs, seven genes, total 9,706 bp) were subjected to maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. The MP tree was constructed in MEGA 5 (Tamura et al. 2011) using Tree-Bisection-Reconnection with a search level of 1 and initial trees by random addition (100,000 reps). To assess support for each clade, bootstrap analyses (Felsenstein 1985) were performed with 1,000 replicates. For ML analysis, we used RAXML GUI v. 1.31 (Silvestro and Michalak 2012) run to conduct 10,000 Rapid Bootstrap searches followed by an ML search, with the GTR + G + I model for each codon position of each gene. In addition, ML analyses were performed using two additional data sets for testing the differences in divergence time depending on the OTUs as follow. In data set 2 (39 OTUs), the basal taxa of Phaeophyceae (*Discosporangium mesarthrocarpum* (Meneghini) Hauck and *Choristocarpus tenellus* (Kützinger) Zanardini (Discosporangiales), *Ishige okamurae* Yendo (Ishigeales), and *Onslowia endophytica* Searles (Onslowiales) and their sister taxon *Schizocladia ischiensis* (Schizocladiphyceae), which were not included in Silberfeld et al. (2010), were removed from data set 1, and a total of 9,706 bp was analyzed for the seven genes as mentioned above. In data set 3 (50 OTUs), the sister taxa of Phaeophyceae/Schizocladiphyceae such as *Rhizosolenia*, *Corethron* (Bacillariophyceae), *Mallomonas*, *Synura* (Synurophyceae), *Tribonema* (Xanthophyceae), and *Phaeothamnion* (Phaeothamniphyceae) were added, and a total of 1,425 bp were analyzed for the *rbcL* gene. In data sets 4–10 (44 OTUs) the following genes were removed sequentially from the seven genes used in data set 1 and each data set analyzed using six genes: data set 4, *-atpB*, 8,535 bp; data set 5: *-psaA*, 7,778 bp; data set 6: *-psaB*, 8,013 bp; data set 7: *-psbA*, 8,735 bp; data set 8: *-psbC*, 8,400 bp; data set 9, *-psaA*, 8,287 bp; data set 10, *-cox1*, 8,488 bp. The comparisons of the ML tree topologies (AU test) were performed using RAXML v. 7.2.8 (Stamatakis 2006) and CONSEL (Shimodaira and Hasegawa 2001). Bayesian analysis was run using MrBayes v. 3.2.2 (Ronquist et al. 2012). With the aid of the Kakusan4 program (Tanabe 2011), the best-fit evolutionary model for each codon position of each gene was determined by comparing different evolutionary models via the corrected Bayesian Information Criterion (Schwarz 1978). The Bayesian analysis was initiated with a random starting tree and four chains of Markov chain Monte Carlo (MCMC) iterations were run simultaneously for 10,000,000 generations, keeping one tree every 100 generations. The first 10,000 trees sampled were discarded as "burn-in," based on the stationarity of ln L as assessed using Tracer v. 1.5 (Rambaut and Drummond 2007). A consensus topology and posterior probability values were calculated from the remaining trees.

Molecular dating analyses for data sets 1–3 were performed using the MCMCTREE program in the PAML 4 package (Yang 2007). The best-scoring ML trees based on the above-

TABLE 1. List of primers used for PCR and sequencing.

Code	F/R	Sequence (5'-3')	Annealing position
ycf3-F1P*	F	CAAGCDYTAATAATATWGCTG	<i>ycf3</i> (319-340)
atpB-F1P*	F	GCWAAAAGCNCATGGTGGTGT	<i>atpB</i> (535-554)
atpB-F1.2P*	F	AARGTMGTWGAYTATTAGC	<i>atpB</i> (427-446)
atpB-F1.3P*	F	GTHMGHGNCNATTGCNATGAGTGC	<i>atpB</i> (187-209)
atpB-F2P*	F	GCDGTRGAYCCHTTAGATTCA	<i>atpB</i> (1,057-1,077)
atpB-2R [†]	R	AGCTTGWACAAATCTAAAAATA	<i>atpB</i> (810-789)
atpB-R2P*	R	GCWATAATATCTTGTAAATTC	<i>atpB</i> (1,184-1,165)
atpB-R2.2P*	R	TCKACHACTAADCGRCTTTC	<i>atpB</i> (1,232-1,213)
atpB-R3P*	R	CAVRTAYAAATCRITTCCTTC	<i>atpB</i> (609-589)
atpB-R4P*	R	GCWGRKCKATGRATAGGTAA	<i>atpB</i> (374-355)
atpB-R1P*	R	TTTGCTTTAGMDATWGCTTC	<i>atpB</i> (1,430-1,411)
psaA130F [‡]	F	AACWACWACTTGGATTGGAA	<i>psaA</i> (126-146)
psaA-P2 [§]	R	NCCWGACCAAGMWARACAAC	<i>psaA</i> (639-620)
psaA-P2.2*	R	TCDGCRITTYTGRAACCAYTC	<i>psaA</i> (578-559)
psaA-P3 [§]	R	GCTGGDTRTARTCACGAACC	<i>psaA</i> (1,253-1,233)
psaA-P3.2*	R	GCWGGRTTATARTCRGDACC	<i>psaA</i> (1,232-1,233)
psaA970R [‡]	R	GCYTCTARAATYCTTTCA	<i>psaA</i> (977-959)
psaA970R2*	R	TRCTATGDCCRATNCCCCAA	<i>psaA</i> (958-939)
psaA1760R [‡]	R	CCTCTWCCWGGWCCATCRCAWGG	<i>psaA</i> (1,736-1,714)
psaA1760R2*	R	CCRTCAATACGGRAAWCGGAA	<i>psaA</i> (1,724-1,705)
psaA-F3P*	F	CWGCWGAAYTTTATGGTTCAYC	<i>psaA</i> (1,583-1,603)
psaA-P4 [§]	R	NGATTCAATHARYTCTTGCC	<i>psaA</i> (2,094-2,075)
psaA-P5 [§]	F	CAYCATATHCATGCNTTYAC	<i>psaA</i> (1,600-1,619)
psaA-F1P*	F	ATGCAATCNGAYRTTTGGGG	<i>psaA</i> (1,834-1,853)
psaB-F1P*	F	ATGCAATCNGAYRTTTGGGG	<i>psaB</i> (21-40)
psaB-F3P*	F	WGATGCWCAYMGWCCMCCCTG	<i>psaB</i> (915-934)
psaB-F3.2P*	F	GSTATHGGNCAYAAAYATGAAAGA	<i>psaB</i> (886-908)
psaB-R2P*	R	KAWTGCATACCATGTGTAG	<i>psaB</i> (1,065-1,046)
psaB-R3P*	R	CAATGCCAATARAHHGTAACCC	<i>psaB</i> (1,787-1,766)
psaB-R3.2P*	R	CAATARAHHGTAACCCAWCC	<i>psaB</i> (1,781-1,762)
psaB-R1P*	R	TTCCAGCHGTTGAWGCWATA	<i>psaB</i> (2,194-2,175)
psaB-F4P*	F	CCWTATGCHTTATYATGGCAAAAAGAT	<i>psaB</i> (1,069-1,092)
psbA-F [‡]	F	ATGACTGCTACTTTAGAAAGACG	<i>psbA</i> (1-23)
psbA-F1P*	F	ACCGTTTATACATYGGTTGG	<i>psbA</i> (77-96)
psbA500F [‡]	F	CTCTGATGGWATGCCWYTAGG	<i>psbA</i> (504-524)
psbA600R [‡]	R	CCAAATACACCAGCAACACC	<i>psbA</i> (620-601)
psbA600R2*	R	AWACACCAGCAACACCAGCC	<i>psbA</i> (616-597)
psbA-R1P*	R	TACGYTCRTGCATTACTTCC	<i>psbA</i> (1,003-984)
psbA-R1 [‡]	R	GCTAAATCTARWGGGAAGTTGTG	<i>psbA</i> (1,031-1,009)
psbC-P1.2*	F	CCACGTGGAACCGCYCTTTA	<i>psbC</i> (33-52)
psbC-P1*	F	TAGCTCATGCAGGYWTAATGG	<i>psbC</i> (152-172)
psbC-P3*	R	CTTGCCAAGGTTGRATATCATT	<i>psbC</i> (1,168-1,147)
psbC-P3.2*	R	ATWCCTTTTTTCRAARCCAGC	<i>psbC</i> (1,379-1,360)
psbC-P4*	F	CCGTGAYAAAAAYAAATGAC	<i>psbC</i> (444-464)
psbC-P5*	R	AARAATGGRAAWGAYTCTTC	<i>psbC</i> (431-412)
psbC-P6*	F	GAAGCWTCTCAAKKDCARGC	<i>psbC</i> (925-944)
rbcL-P2 [¶]	F	GAWCGRACCTCGAWTWAAAAGTG	<i>rbcL</i> (19-40)
rbc-R2.5**	R	CCTTCAATAAACACACG	<i>rbcL</i> (587-571)
Ral-R952 [¶]	R	CATACGCATCCATTTACA	<i>rbcL</i> (969-952)
rbcL-P5*	F	CWTAYYTAACAAACWTTCCAAG	<i>rbcL</i> (440-460)
rbcL-P4*	R	AGKTGRTGCATYTGRCCACA	<i>rbcL</i> (1,178-1,159)
rbcL-Rh3 ^{††}	F	TAAAYTCTCARCCDITYATGCG	<i>rbcL</i> (629-650)
rbcL-P1 [¶]	F	GKGTWATTTGTAARTGGATGCG	<i>rbcL</i> (944-965)
rbcL-P3**	F	CARTTYGGWGGWGGTACDATTGG	<i>rbcL</i> (1,210-1,232)
rbcL-P3.2*	F	GARGGTCTGADATTYTACGT	<i>rbcL</i> (1,327-1,347)
rbcL-Pla1	F	TATTCTCGTGCAGTTGGTAG	<i>rbcL</i> (778-797)
rbcL-Pla4	F	ATTTACTAACRCGCTTGTGAC	<i>rbcL</i> (227-246)
rbcL-Pla5	R	AAAGTATTATAGAATCCTCG	<i>rbcL</i> (1,055-1,036)
rbcS-P1 [¶]	R	GGATCATCTGYCCATTCTACAC	<i>rbcS</i> (122-101)
GazF2 ^{‡‡}	F	CCAACCAYAAAGATATWGGTAC	<i>cox1</i> (104-125)
cox1-P1.2*	F	GATHITTYTTATGGTDATGCC	<i>cox1</i> (276-296)
cox1-P2*	R	GGDATAACGDCGHGGCATAACC	<i>cox1</i> (1,400-1,381)
cox1-P3*	R	CNGTAAACATRTGRTGVGCC	<i>cox1</i> (961-942)
cox1-P4*	F	DGCRGCNNTTACNATGTTTG	<i>cox1</i> (1,230-1,249)
cox1-P5*	R	TAATACCNCRCGCAAAAACWGG	<i>cox1</i> (700-679)

(continued)

TABLE 1. (continued)

Code	F/R	Sequence (5'-3')	Annealing position
cox1-P5.2*	R	GCHGTDATTAADACHGACCA	<i>cox1</i> (656-637)
trnI-P1.2*	R	GCTTATCAGGCGTACACTCT	<i>trnI</i> (20-39)
trnI-P1*	R	TTGAACGAWCGVCTTTACGC	<i>trnI</i> (38-57)

*Kawai et al. (2013); †Silberfeld et al. (2010); ‡Yoon et al. (2002); §Kawai et al. (2012); ¶Kawai et al. (2007); **Kawai et al. (2008); ††Hanyuda et al. (2004); †††Lane et al. (2007).

Annealing positions correspond to the sequences of *Ectocarpus siliculosus* (*yef3*, *atpB*, *psaA*, *psaB*, *psbA*, *psbC*, *rbL*, and *rbC*, FP102296; Le Corguillé et al. 2009; *cox1* and *trnI*, FP885846; J.M. Cock unpublished).

mentioned three data sets in this study were used for divergence time estimation. The ML estimates of branch lengths were obtained using the BASEML (in PAML) programs under the GTR + G substitution models for the data set (concatenated DNA sequences separated into three codon partitions). Two priors, the overall substitution rate (rgene gamma) and rate-drift parameter (sigma2 gamma), were set at G (1, 25) and G (1, 4.5) for data sets 1 and 2, and G (1, 20) and G (1, 4.5) for data set 3, using the strict molecular clock assumption with 195.5 Ma (data set 1 and 3) or 155 Ma (data set 2). The former (195.5 Ma) was the mean of the estimated divergence time of Shizocladiphyceae and Phaeophyceae in Brown and Sorhannus (2010) and the latter (155 Ma) was the estimated origin of the Phaeophyceae by Medlin et al. (1997). The independent rates model (Rannala and Yang 2007) was used to specify the prior of rates among internal nodes (clock = 2 in MCMCTREE). Three nodes in the tree were constrained in geological time based on knowledge from the fossil record and prior molecular clock analyses. For data set 1, the maximum (267 Ma) and the minimum (124 Ma) time constraints, which were the estimated divergence times of the Shizocladiphyceae-Phaeophyceae clade (Brown and Sorhannus 2010), were chosen for the most basal node on our tree. On the basis of the fossil record (Parker and Dawson 1965, Rajanikanth 1989), we defined the lower boundaries at 13 and 99.6 Ma for the stem nodes of Sargassaceae and Dictyotales, respectively. For the data set 2, the maximum (185 Ma) and the minimum (125 Ma) time constraints for the root of the tree, which were based on the estimated origin of the Phaeophyceae (155 Ma, Medlin et al. 1997) and a standard deviation of 30 Ma, were used in the analysis, as well as the lower boundaries at 13 and 99.6 Ma for Sargassaceae and Dictyotales, respectively. These settings for data set 2 were similar to that of Silberfeld et al. (2010). For data set 3, based on the fossil record and diatom biomarker, two calibration constraints (93–90 Ma: *Rhizosolenia-Corethron* divergence based on the estimated time of the abrupt increase in the C₂₅ HBI alkene (Sinninghe Damste et al. 2004), 49–40 Ma: first appearance of *Synura uvella* Ehrenberg and *Mallomonas insignis* Penard in the fossil record (Silver and Wolfe 2007) were used in the analysis, as well as the lower boundaries at 99.6 Ma for Dictyotales. MCMC approximation with a burn-in period of 50,000 cycles was run, and sampled every 50 cycles to create a total of 100,000 samples. The effective sample sizes of all parameters were greater than 200 and judged to be sufficient by Tracer v.1.5.

RESULTS

Molecular phylogenetic analyses. The molecular phylogenetic tree by ML (Fig. 1) and MP (Fig. S1 in the Supporting Information) analyses based on the concatenated sequences of seven genes (9,706 bp) resulted in similar tree topologies for most of the

major nodes with high statistical support: i.e., Discosporangiales was the most basal order in the Phaeophyceae with the Ishigeales the next branching lineage. *Phaeostrophion* was a sister group to the Sphacelariales, and *Platysiphon* clustered with *Halosiphon* and *Stschapovia*. The phylogenetic positions of Onslowiales and Syringodermatales differed between the two analyses. Onslowiales branched next to Ishigeales in the ML tree but was sister to Dictyotales in the MP tree although the statistical support was low. In the ML tree, the large clade of more derived orders had Desmarestiales basal, and Laminariales and Ectocarpales forming a well-supported clade. Tilopteriales, composed of *Cutleria*, *Saccorhiza* and *Tilopteris*, were distinct from the monophyletic clade of *Halosiphon*, *Platysiphon* and *Stschapovia* in both ML and Bayesian trees. The Bayesian tree supported the monophyly of most orders by high posterior probabilities (Fig. 2).

The time trees inferred from three data sets are presented in Figure 3, Figures S2 and S3 in the Supporting Information. Estimated divergence times for the major nodes in Figure 3 and Figure S2 were summarized in Table 2. The branching of the Phaeophyceae and Schizocladiphyceae from their common ancestor (node 1) was estimated to have occurred in late Permian Period (mean age 258.0 Ma in Fig. 3 and 268.5 Ma in Fig. S3). Estimated divergence times of comparable nodes between Figure 3 and Figure S2 were similar, and the time differences were 0.3–8.5 Ma (Table 2).

In order to evaluate the effect of the discrepancies between the phylogenetic signals of each gene, we assessed the effects of removing one gene from the seven genes used in data set 1 (data sets 4–10). The topologies of two ML trees (i.e., Figs. S4 and S5 in the Supporting Information) were the same as in Figure 1. In contrast, in the other five ML trees (Figs. S6–S10 in the Supporting Information), the positions of two to eight different taxa were not congruent with those in Figure 1. The AU tests indicated that the differences in the tree topologies between these five ML trees and Figure 1 were not significant.

DISCUSSION

Molecular phylogeny of Phaeophyceae. The overall phylogenetic relationships of representative brown algal orders revealed by the concatenated sequence

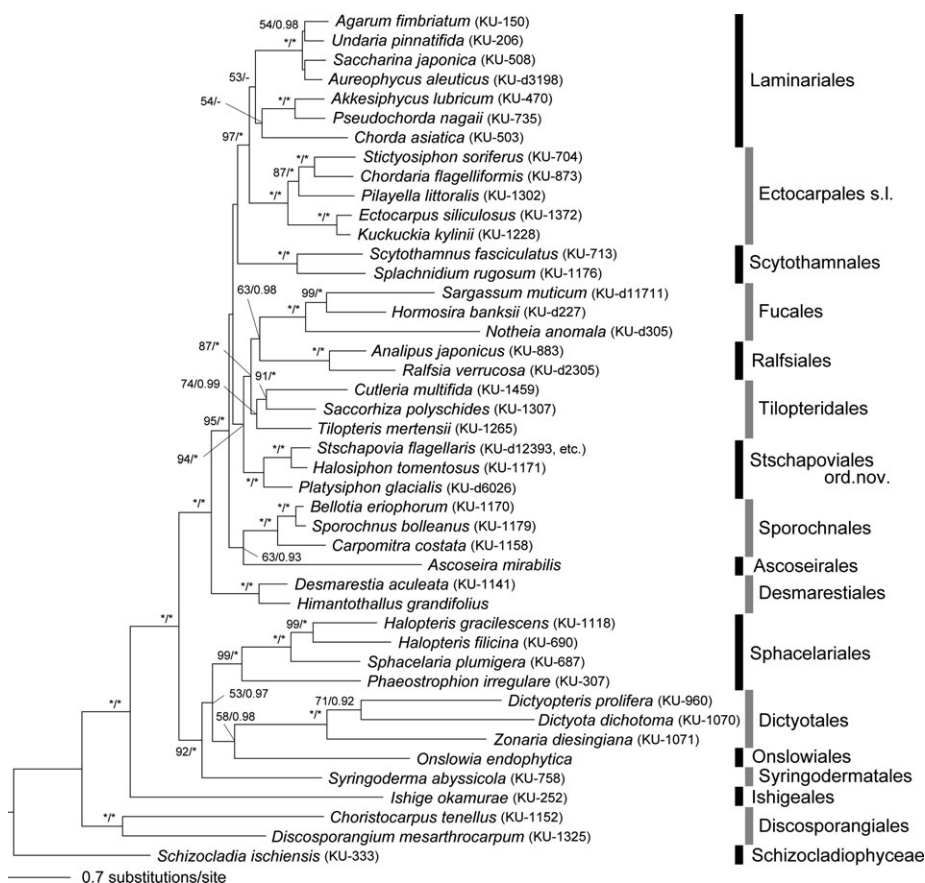


FIG. 1. Maximum likelihood (ML) tree based on the concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psaB*, *psbA*, *psbC*, and *rbcL* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from ML analysis (left) and posterior probabilities from Bayesian analysis (right). Asterisk (*) indicates 100 (ML) and 1.00 (Bayes). Only posterior probabilities >0.90 and bootstrap values >50% are shown.

data of the seven chloroplast and mitochondrial genes generally agreed with previous studies using multigene sequences (Phillips et al. 2008, Silberfeld et al. 2010). In general those taxa with isomorphic life histories (i.e., Discosporangiales, Ishigeales, Onslowiales, Phaeostrophionaceae, Sphacelariales, and Dictyotales) were basal, except the heteromorphic Syringodermatales (Henry and Müller 1983, Henry 1984, Kawai and Yamada 1990), and those principally having heteromorphic life histories or secondarily lacking an alternation of generations in their life histories (i.e., Desmarestiales, Scytothamnales, Ectocarpales, Laminariales, Ascoseirales, Sporocnales, Tilopteridales, and Fucales) formed a more derived clade, with mostly isomorphic Ralfsiales being the exception. However, *Heteroralfsia saxicola* (Okamura & Yamada) H. Kawai has a heteromorphic life history (Kawai 1989).

In the present analyses, *Platysiphon glacialis* was sister to the clade of *Halosiphon tomentosus* and *Stschapovia flagellaris* in all of the ML, MP, and Bayesian trees. In the MP analysis, the *Halosiphon/Platysiphon/Stschapovia* clade clustered with the Tilopteridales clade composed of *Cutleria*, *Saccorhiza* and *Tilopteris*, but the bootstrap support was low (<50%). Conversely, in the ML and Bayesian trees the *Halosiphon/Platysiphon/Stschapovia* clade was not monophyletic with Tilopteridales, but was sister to the clade includ-

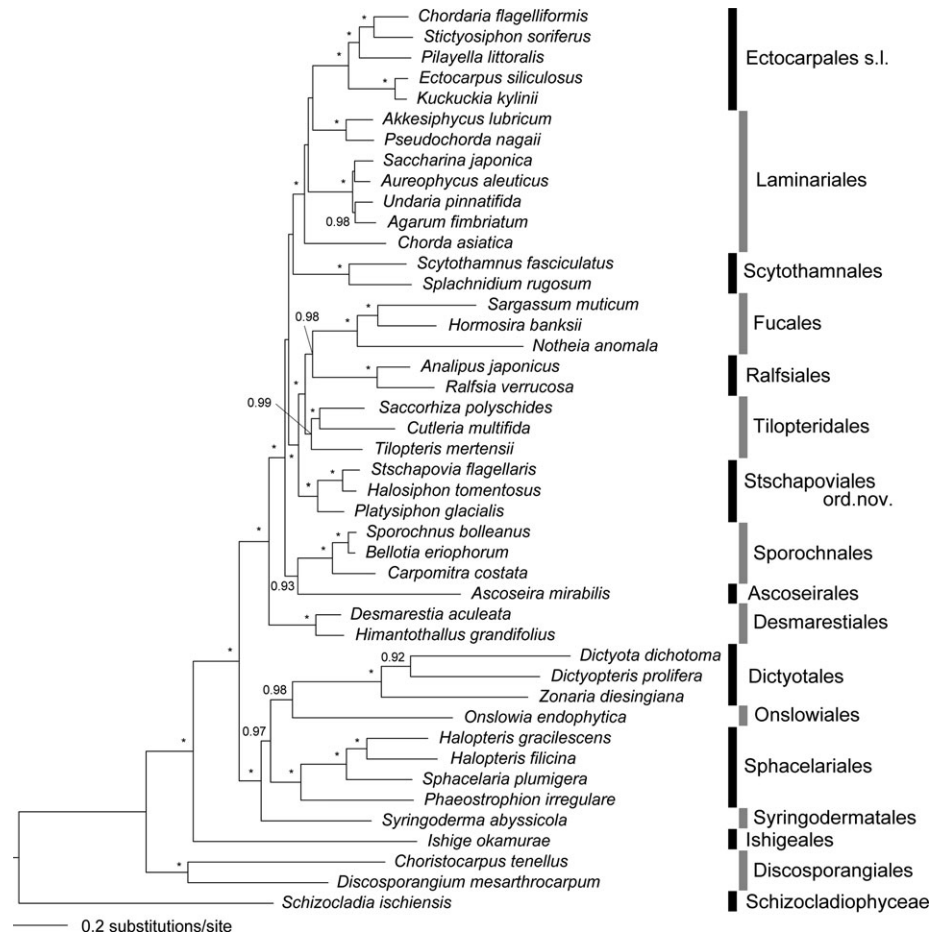
ing Fucales, Ralfsiales, and Tilopteridales supported by a moderate bootstrap values.

Phaeostrophion irregulare was thought to be distant from Ectocarpales s.l. both in the morphology and molecular phylogeny based on *rbcL* and partial 18S and 26S rDNA sequences (Kawai et al. 2005). Thereby, the authors proposed to treat Phaeostrophionaceae in its own family, but an ordinal assignment was not made. In the present analyses, *Phaeostrophion* formed a clade with the Sphacelariales. Although they have different thallus constructions (thin terete or filamentous vs. foliose), they share the occurrence of an apical meristematic cell(s), chloroplast morphology, and isomorphic life history (Kawai et al. 2005). *Bodanella lauterborni* W. Zimmermann, which made a clade with *Phaeostrophion* in the *rbcL* analyses of Phillips et al. (2008), was not included in the present analyses. At present, we find no distinctive phenotypic characters except for the foliose thallus constructions to exclude *Phaeostrophion* from Sphacelariales and, we tentatively place the Phaeostrophionaceae in the Sphacelariales.

Platysiphonaceae H. Kawai, T. Hanyuda et R. T. Wilce **fam. nov.**

Typus: *Platysiphon glacialis* (Rosenvinge) H. Kawai et T. Hanyuda **comb. nov.** *Basionym:* *Punctaria glacialis* Rosenvinge 1910. In: Rosenvinge, L. K. 1910. On the marine algae from North-East Greenland (N. of 76. N.

FIG. 2. Bayesian consensus tree based on concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psaB*, *psbA*, *psbC*, and *rbcl* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from Bayesian analysis. Only posterior probabilities >0.90 are shown and asterisk (*) indicates 1.00.



lat.) collected by 'Danmark-Expedition' Medd. Gr.nl. 43: 118, figs 6,7.

Description: Erect thalli simple, terete, parenchymatous with multicellular hair-like filaments at the tip and on surfaces of distal portions, later becoming flattened and blade-like basally; cells containing many discoid chloroplasts without pyrenoids.

Stschapoviales H. Kawai ord. nov.

Typus: *Stschapovia flagellaris* A. D. Zinova 1954: 242, figs 1–6. In: Zinova, A. D. (1954). Novye smetstvo, rod i vid u burykh vodoroslej. *Trudy Botanicheskogo Instituta Akademii Nauk SSSR, Ser. 2, Sporovye Rasteniya* 9:223–44.

Description: Erect thalli simple, terete, parenchymatous with multicellular hair-like filaments at the tip and on surfaces of distal portions. Erect thalli may become thicker or flattened and blade-like basally and form reproductive structures on the surface. Cells containing many discoid chloroplasts without pyrenoids. Life history heteromorphic or without alternation of generations.

All members of the *Halosiphon/Platysiphon/Stschapovia* clade have rather narrow geographic distributions in the cold-water regions of the Northern Hemisphere that are normally or occasionally influenced by frozen surface seawater. They also share several morphological features, including parenchy-

matous, terete, erect thalli at least in early stages of development, no obvious growth zones or apical cells, and multicellular hair-like filaments (assimilatory filaments) that are arranged in whorls (Wilce 1962, Kawai and Sasaki 2004). It is noteworthy that *Stschapovia* shows developmental processes in erect thalli similar to *Platysiphon*. Thalli of *Stschapovia* are first thin throughout, but then the proximal portion of the thallus becomes thickened (flattened in *Platysiphon*); the reproductive structures form only on thickened portions (Wilce 1962, Kawai and Sasaki 2004, Kawai et al. 2015). Multicellular assimilatory filaments are abundant on juvenile terete thalli, but they are absent on proximal thickened portions (Kawai and Sasaki 2004, Kawai et al. 2015).

The life history and reproductive structures of the Stschapoviales are diverse. *Halosiphon* is heteromorphic, alternating between a macroscopic sporophyte that forms unilocular zoidangia and a microscopic, oogamous gametophyte that is similar to the Laminariales and Phyllariaceae (Tilopteridales) (Maier 1984, Kawai 2014). *Stschapovia* is not yet fully characterized, but uniquely it lacks an alternation of generations. *Stschapovia* reproduces by monospore-like cells although sperm-like flagellated cells are formed, resembling *Tilopteris* and *Phaeosiphoniella* (Tilopteridales) (Kuhlenkamp and Müller 1985,

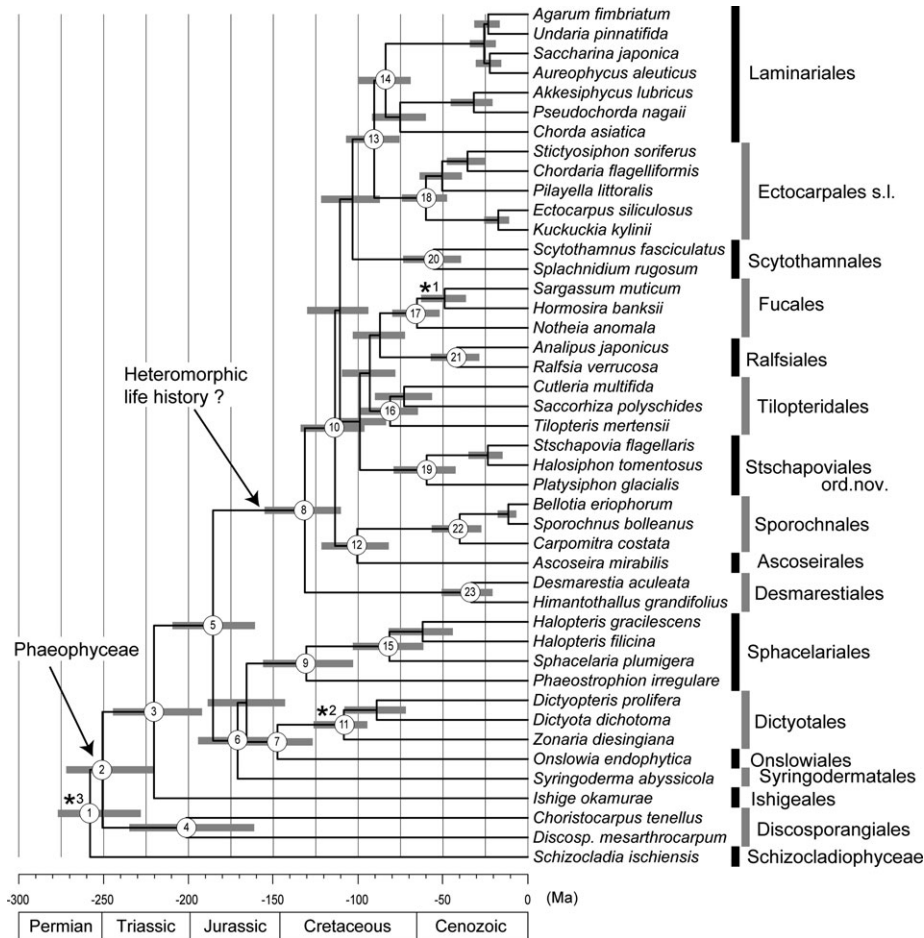


FIG. 3. Time tree derived from relaxed molecular clock method implemented in MCMCTREE in PAML 4.7 (Yang 2007). Horizontal bars indicate 95% credible intervals of divergence time estimates. Asterisks on nodes correspond to calibration points. Asterisks 1 and 2 indicate calibration points with fossils (Parker and Dawson 1965 and Rajanikanth 1989, respectively), and minimum time constraints used for nodes were 13 and 99.6 Ma, respectively. Asterisk 3 shows calibration point based on previous molecular clock study (Brown and Sorhannus 2010), and maximum (267 Ma) and minimum (124 Ma) time constraints used on node. Estimated ages and their 95% credible intervals are listed in Table 2 with node numbers.

Hooper et al. 1988, Kawai and Sasaki 2004). *Platysiphon* life stages are also incompletely known, but flagellated cells are provably not released, and sexual reproductive cells resemble *Stschapovia* and tilopteridacean species (Kawai et al. 2015). The similarity of Stschapoviales and Tilopteridales reproductive features are likely an adaptation to ice coverage in winter, and hence they share these features by convergent evolution.

Among the three monotypic families comprising the clade including *Platysiphon*, Halosiphonaceae (type: *Halosiphon tomentosus* (Lyngbye) Jaasund 1957), Platysiphonaceae (type: *Platysiphon glacialis* (Rosenvinge) H. Kawai et T. Hanyuda 2015), and Stschapoviaceae (type: *Stschapovia flagellaris* Zinova 1954), *Stschapovia* has nomenclatural priority. Therefore, we proposed establishing a new order, Stschapoviales, to accommodate the three families.

Evolutionary divergence times. Regarding the evolutionary divergence time for the Phaeophyceae, Silberfeld et al. (2010) proposed a chronogram based on the Bayesian molecular phylogenetic tree of the concatenated DNA sequences of 10 genes (i.e., mitochondrial *cox1*, *cox3*, *nad1*, *nad4*, *atp9*, chloroplast *rbcL*, *psaA*, *psbA*, *atpB*, and nuclear 26S rDNA) analyzed using the BEAST program. They used three calibration points based on brown algal fossil

records (i.e., *Padina* of Dictyotales, Sargassaceae of Fucales, and *Nereocystis/Pelagophycus*-like species of Laminariales). Furthermore, they followed the estimation of Medlin et al. (1997) for the divergence time of Phaeophyceae to be 155 Ma, although this was solely based on a molecular clock model. The analyses by Silberfeld et al. (2010), which focused on the crown taxa, did not include the basal taxa of the Phaeophyceae or the sister taxon. On the other hand, Brown and Sorhannus (2010) reported a chronogram of the Ochrophyta (Heterokontophyta) based on 18S rDNA sequences, analyzed using the BEAST program, and calibrated principally by diverse microalgal fossils (e.g., diatoms, Haptophyta, etc.) and red algae. When discussing their analyses, they suggested a divergence time for the Schizocladiphyceae and Phaeophyceae as ~172–186 Ma in the exponential age priors and as 180–198 Ma in the lognormal age priors (Brown and Sorhannus 2010).

In our analyses, which included the basal groups Discosporangiales and Ishigeales as well as the sister taxon (Schizocladiphyceae) of Phaeophyceae (Fig. 3), the divergence time of Phaeophyceae from Schizocladiphyceae was estimated to be ~260 Ma in the Permian Period, which was somewhat earlier than the estimation by Brown and Sorhannus

TABLE 2. Comparisons of estimated divergence time in this study (data set 1 and 2) and previous studies; estimated divergence time are expressed in Ma as posterior mean \pm 95% credible interval. Node numbers in this table correspond to those shown in Figure 3.

Node	This study (data set 1)	This study (data set 2)	Silberfeld et al. (2010)	Brown and Sorhannus (2010)*
1	258.0 (228.1–277.0)	–	–	196 (131–267) 186 (124–253)
2	250.7 (220.4–272.0)	–	–	~160 (~100–225) [†] ~155 (~95–210) [†]
3	220.3 (192.0–244.5)	–	–	–
4	201.3 (161.3–234.8)	–	–	–
5	185.5 (160.8–209.4)	177.0 (156.6–187.9)	~180 (~150–215) [†]	–
6	171.0 (147.8–194.3)	172.3 (151.6–185.3)	~170 (~140–205) [†]	–
7	147.6 (126.8–169.7)	–	–	–
8	131.4 (110.2–155.2)	128.2 (108.8–147.4)	128.9 (98.7–162.0)	–
9	130.6 (103.0–156.0)	129.0 (104.1–149.9)	–	–
10	113.7 (96.3–133.9)	112.2 (96.0–129.4)	118.7 (89.5–148.4)	–
11	108.4 (94.6–126.3)	108.8 (95.4–124.9)	~110 (~100–130) [†]	~55 (~20–90) [†] ~50 (~20–80) [†]
12	100.6 (82.0–121.6)	93.1 (89.3–121.5)	–	–
13	90.5 (75.6–107.1)	94.6 (79.0–110.4)	98.0 (72.2–125.0)	–
14	83.8 (69.1–100.3)	87.1 (71.5–103.0)	84.4 (59.9–110.4)	–
15	81.6 (61.6–103.2)	81.3 (61.9–101.4)	–	–
16	81.2 (64.8–98.4)	80.7 (65.0–96.4)	85.3 (59.4–111.7)	–
17	65.3 (52.0–79.9)	64.6 (51.6–78.4)	73.7 (54.6–96.0)	–
18	60.1 (47.6–74.2)	61.0 (48.2–75.0)	69.7 (51.4–89.3)	–
19	59.6 (42.6–79.1)	57.7 (41.2–76.1)	–	–
20	55.5 (39.4–73.5)	55.3 (39.2–73.0)	54.9 (33.1–79.4)	–
21	41.8 (28.6–57.3)	41.7 (28.3–57.2)	62.0 (37.5–89.7)	–
22	40.2 (27.4–56.7)	38.7 (26.5–54.1)	38.6 (22.0–55.5)	–
23	33.3 (20.8–50.8)	33.9 (20.9–52.2)	52.7 (34.4–73.7)	–

*Upper and lower divergence times were estimated by (broad) lognormal and (tight) exponential calibration sets, respectively.

[†]Detailed data were not shown in Silberfeld et al. (2010) or Brown and Sorhannus (2010), so values were measured with the eye from Figure 2 (Silberfeld et al. 2010) or Figures S1 and S2 (Brown and Sorhannus 2010).

(2010), perhaps due to the influence of the time constraints for the fossil *Padina*. The evolutionary success of Phaeophyceae may be attributed to the development of elaborate multicellular thalli with complex life histories. For achieving the multicellular thalli with tissue differentiation, one of the key elements was the development of cytoplasmic connections (plasmodesmata) allowing the transport of photosynthetic products and regulation signals. Although some heterokonts phylogenetically close to Phaeophyceae, such as Xanthophyceae and Schizocladophyceae, also form multicellular thalli, they lack plasmodesmata. By evolving multicellularity with tissue differentiation, Phaeophyceae could obtain higher tolerance to harsh environmental conditions (i.e., desiccation, osmotic shock, and temperature stresses). These adaptations allowed the brown algae to survive the mass extinction during the Permian-Triassic extinction event, and they diversified subsequently.

Within Phaeophyceae, compared to the chronogram by Silberfeld et al. (2010), the divergence time in our analyses generally shifted to more recent times (~7.5 Ma for the clades that diverged later than Discosporangiales and Ishigeales; Table 2). Basal taxa of brown algae generally share an isomorphic life history and apical growth (i.e., Discosporangiales, Ishigeales, Onslowiales, Dicytiales, and Sphacelariales). Taxa with diffuse and intercalary growth as well as a

heteromorphic life history diverged later from the basal lineage, and the divergence time of such taxa (e.g., Desmarestiales, Sporochneales, Laminariales, Ectocarpales, etc.) was estimated to be 155–110 Ma. The environmental factors that resulted in the evolution of heteromorphic life histories is still unclear, but global climate changes and considerable tectonic activities, such as the breakup of the Pangaea during the period resulting in colder seas and seasonality, may have played a role. The fact that taxa with isomorphic life histories, such as Dictyotales, are more common in the tropical and subtropical regions, whereas taxa with heteromorphic life histories, such as Desmarestiales and Laminariales, are more common in temperate and cold-water regions suggest that heteromorphic life histories are adapted for the survival in the areas where considerable seasonal changes occur in water temperature and transparency. Therefore, considering the estimated divergence time of the derived phaeophycean taxa and the tectonic events, we may hypothesize that the development of heteromorphic life histories in Phaeophyceae and their success in the temperate and cold-water regions was induced by the development of the remarkable seasonality caused by the breakup of Pangaea. Of course such seasonality could have been present before the formation of Pangaea, but Phaeophyceae did not exist prior to Pangaea.

Most brown algal orders had diverged by roughly 60 Ma, around the last mass extinction event during the Cretaceous (70–65 Ma; Schulte et al. 2010, Renne et al. 2013). Therefore, a drastic climate change might have triggered the divergence of brown algae, although the mechanisms causing mass extinctions of marine biodiversity is controversial (Alegret et al. 2012).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Maximum parsimony (MP) tree based on concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psaB*, *psbA*, *psbC*, and *rbcl* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S2. Time tree derived from relaxed molecular clock method implemented in MCMCTREE in PAML 4.7 (Yang 2007) for data set 2. Horizontal bars indicate 95% credible intervals of divergence time estimates. Asterisks on nodes correspond to the calibration points. Asterisks 1 and 2 indicate calibration points with fossils (Parker and Dawson 1965 and Rajanikanth 1989, respectively), and minimum time constraints used for nodes were 13 and 99.6 Ma, respectively. Asterisk 3 shows calibration point based on previous molecular clock study (Medlin et al. 1997), and maximum (185 Ma) and minimum (125 Ma) time constraints used on node. Estimated ages and their 95% credible intervals are listed in Table 3 with node numbers.

Figure S3. Timetree derived from the relaxed molecular clock method implemented in MCMCTREE in PAML 4.7 (Yang 2007) for the data set 3. Horizontal bars indicate 95% credible intervals of the divergence time estimates. Asterisks on the nodes correspond to the calibration points. Asterisk 1 shows the calibration point with fossils (Rajanikanth 1989), and the minimum time constraints on the node was 99.6 Ma. Asterisks 2 and 3 indicate the calibration points based on the fossil and diatom biomarker (Sinninghe Damste et al. 2004 and Silver and Wolfe 2007, respectively), and the time constraints used for the nodes were 49–40 Ma and 93–90 Ma, respectively.

Figure S4. Maximum likelihood (ML) tree based on concatenated DNA sequences (chloroplast *psaA*, *psaB*, *psbA*, *psbC*, and *rbcl* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S5. Maximum likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psbA*, *psbC*, and *rbcl* genes, and mitochondrial *cox1* gene). Numbers on branches

indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S6. Maximum likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atpB*, *psaB*, *psbA*, *psbC*, and *rbcl* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S7. Maximum likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psaB*, *psbA*, and *rbcl* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S8. Maximum likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psaB*, *psbA*, and *rbcl* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S9. Maximum likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psaB*, *psbA*, and *psbC* genes, and mitochondrial *cox1* gene). Numbers on branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S10. Maximum likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atpB*, *psaA*, *psaB*, *psbA*, *psbC*, and *rbcl* genes). Numbers on branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Table S1. Origin of samples and sequence data used for molecular analyses, including their DDBJ/GenBank accession numbers.