J. Phycol. **52,** 682–686 (2016)

DOI: 10.1111/jpy.12419

NOTE

MOLECULAR PHYLOGENY OF *ZEACARPA* (RALFSIALES, PHAEOPHYCEAE) PROPOSING A NEW FAMILY ZEACARPACEAE AND ITS TRANSFER TO NEMODERMATALES[1]

Hiroshi Kawai,[2] *Takeaki Hanyuda*

Kobe University Research Center for Inland Seas, 1-1 Rokkodai, Kobe 657-8501, Japan

John Bolton

Department of Biological Sciences and Marine Research Institute, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

and Robert Anderson

Department of Biological Sciences and Marine Research Institute, University of Cape Town, Private Bag X3, Rondebosch 7701, South Africa

Department of Agriculture, Forestry and Fisheries, Private Bag X2, Roggebaai 8012, South Africa

***Zeacarpa leiomorpha* is a crustose brown alga endemic to South Africa. The species has been tentatively placed in Ralfsiaceae, but its ordinal assignment has been uncertain. The molecular phylogeny of brown algae based on concatenated DNA sequences of seven chloroplast and mitochondrial gene sequences (*atp*B, *psa*A, *psa*B, *psb*A, *psb*C, *rbc*L, and *cox*1) of taxa covering most of the orders revealed the most related phylogenetic relationship of *Z. leiomorpha* to *Nemoderma tingitanum* (Nemodermatales) rather than Ralfsiaceae (Ralfsiales). Morphologically, *Zeacarpa* and *Nemoderma* share crustose thallus structure and multiple discoidal chloroplasts without pyrenoids in each cell, however, the formation of lateral unilocular zoidangia in tufts in loose upright filaments in *Zeacarpa* is distinctive in brown algae. Considering the relatively distant genetic divergence between the two taxa, comparable to that among families or orders in representative brown algae, in addition to the above-mentioned unique morphological features, we propose the classification of *Zeacarpa* in a new family Zeacarpaceae in the order Nemodermatales.**

Key index words: **molecular phylogeny; Nemodermatales; Phaeophyceae; *Zeacarpa*; Zeacarpaceae fam. nov.**

The crustose brown alga *Zeacarpa leiomorpha* R.J.Anderson, Simons et J.J.Bolton (Ralfsiaceae; Fig. 1a) was described from South Africa by Anderson et al. (1988). This species is characterized by its unique unilocular zoidangia formed in sori, laterally in tufts, intercalary in loose upright filaments (Fig. 1, b and c). It was first placed in the Ralfsiaceae, however the ordinal position of the family was controversial at the time. Nakamura (1972) established the order Ralfsiales to accommodate brown algal taxa having crustose thalli, an isomorphic life history, discoidal early development of the thallus, and each cell containing a single, plate-shaped chloroplast without pyrenoids. However, the validity of the order has been questioned because a number of taxa exhibiting exceptions to these criteria have been found within the order, and Nelson (1982) suggested classifying them in Ectocarpales. Referring to these discussions, Anderson et al. (1988) tentatively placed *Zeacarpa* in Ralfsiaceae because of its general morphology, but suspended its ordinal assignment.

Later, mainly based on genetic analyses, Lim et al. (2007) suggested the reappraisal of the order Ralfsiales, proposing emendation of the order to contain only species having: (i) discoidal early development of the thallus; (ii) intercalary plurilocular gametangia with sterile terminal cells, and terminal or lateral unilocular zoidangia; and (iii) a crustose phase in the life history. Later, Phillips et al. (2008) suggested a significantly distant phylogenetic relationship of *Nemoderma tingitanum* Schousboe ex Bornet from Ralfsiales and proposed a new order Nemodermatales. However, *Zeacarpa* was not included in these taxonomic revisions, and its molecular phylogeny has not been examined. Furthermore, the placement of *Zeacarpa* in Ralfsiaceae is inappropriate, because of the considerable morphological differences in the loose vegetative thallus constructions and distinctive unilocular zoidangia. Therefore, in order to clarify the ordinal and familial taxonomic position of *Zeacarpa*, we conducted a

[1]Received 16 July 2015. Accepted 15 March 2016.
[2]Author for correspondence: e-mail kawai@kobe-u.ac.jp.
Editorial Responsibility: K. Müller (Associate Editor)

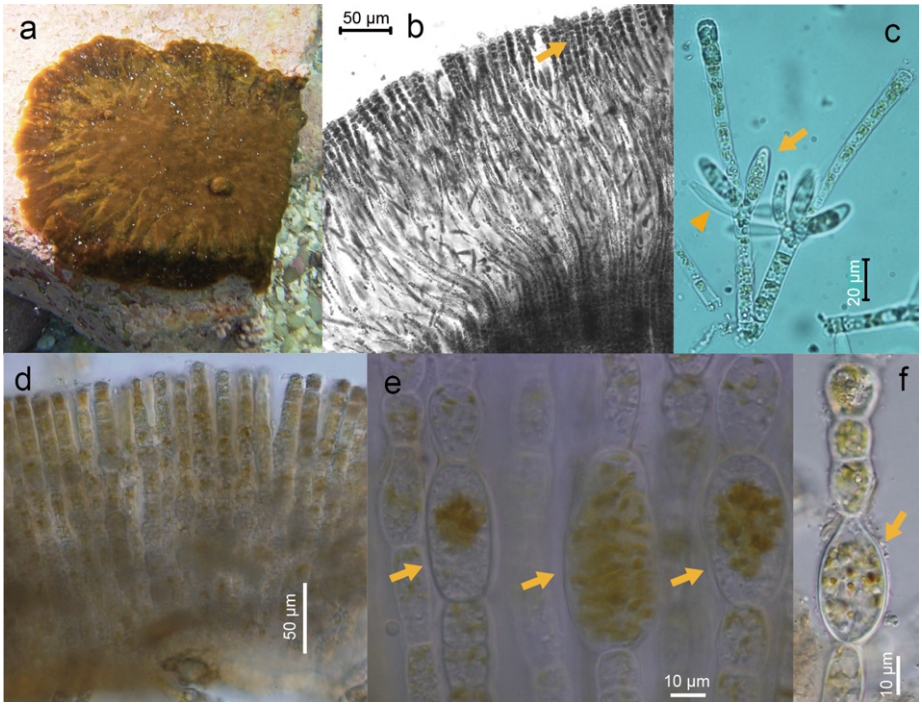

FIG. 1. (a–c) *Zeacarpa leiomorpha*. a. Habit (specimen photographed in September, 2007 at Brandfontein, west of Cape Agulhas, South Africa). (b) Longitudinal section of vegetative thalli (Type specimen, collected by RJA at Hout Bay, South Africa on 13 August, 1986). Arrow shows assimilatory filament (soral filament). (c) Unilocular zoidangium (arrow) formed in groups intercalary in assimilatory filaments. Arrowhead shows emptied locule of unilocular zoidangium. (d–f) *Nemoderma tingitanum*. Collected at Banyuls sur Mer, France by H. Kawai on 13 October, 2014. (d) Longitudinal section of vegetative thallus. (e) Immature intercalary unilocular zoidangia (arrows). Zoidangium in the center is close to maturity and individual zoids can be recognized. (f) Fully mature unilocular zoidangium. Individual zoids provided with eyespot are discernible.

molecular phylogenetic analysis using multiple gene sequences at Kobe University.

Our molecular phylogenetic analyses used specimens of *Z. leiomorpha* collected from two localities (ca. 1,000 km apart) in the eastern and western South Africa (KU-d13217; Double Mouth, Eastern Cape Province, South Africa on 14 July, 2010, and KU-d13218; False By, Western Cape Province, South Africa on 19 January, 2011, collected by R. Anderson) and *N. tingitanum* collected from the Mediterranean coast (KU-d13284 and KU-d13296; Banyuls sur Mer, France, 13 October, 2014 collected by H. Kawai). Specimens with the sample code KU-d#### in Table S1 in the Supporting Information are deposited in the Kobe University Research Center for Inland Seas, Japan and are available for distribution upon request. Genomic DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications of the chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, and *rbc*L, and mitochondrial *cox*1 genes were carried out using the KOD FX (ToYoBo, Osaka, Japan) PCR enzyme and the PCR Thermal Cycler Dice (Takara Shuzo, Shiga, Japan). Primers used for PCR and sequencing are listed in Table S2 in the Supporting Information. 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, or were sequenced by a DNA sequencing service (FASMAC, Atsugi, Japan). In the preliminary analysis using *rbc*L DNA sequences, it was revealed that *Zeacarpa leimorpha* was phylogenetically close to *N. tingitanum*. Therefore, in order to obtain more robust data for elucidating their phylogentic relationship, we carried out a multigene molecular phylogentic analyses using seven genes as used in Kawai et al. (2015). The molecular phylogenetic analyses used published and newly determined sequence data of the Phaeophyceae (Table S2). *Discosporangium mesarthrocarpum* (Meneghini) Hauck and *Choristocarpus tenellus* Zanardini, located at the most basal position in the Phaeophyceae (Silberfeld et al. 2010, Kawai et al. 2015), were 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 (47OTUs, seven genes, total 9,752 bp) were subjected to maximum likelihood (ML) and Bayesian (BI) analysis. 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 model for each codon position of each

gene. BI 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, Table S3 in the Supporting Information). The BI analysis was initiated with a random starting tree and ran four Markov chains of Monte Carlo iterations simultaneously for 10,000,000 generations, keeping one tree every 100 generations. The first 25,000 trees sampled were discarded as "burn-in" based on the stationarity of ln L as assessed using Tracer v.1.6 (Rambaut and Drummond 2013). A consensus topology and posterior probability values were calculated from the remaining trees.

The molecular phylogeny based on concatenated sequence data of all seven genes (9,752 bp) showed

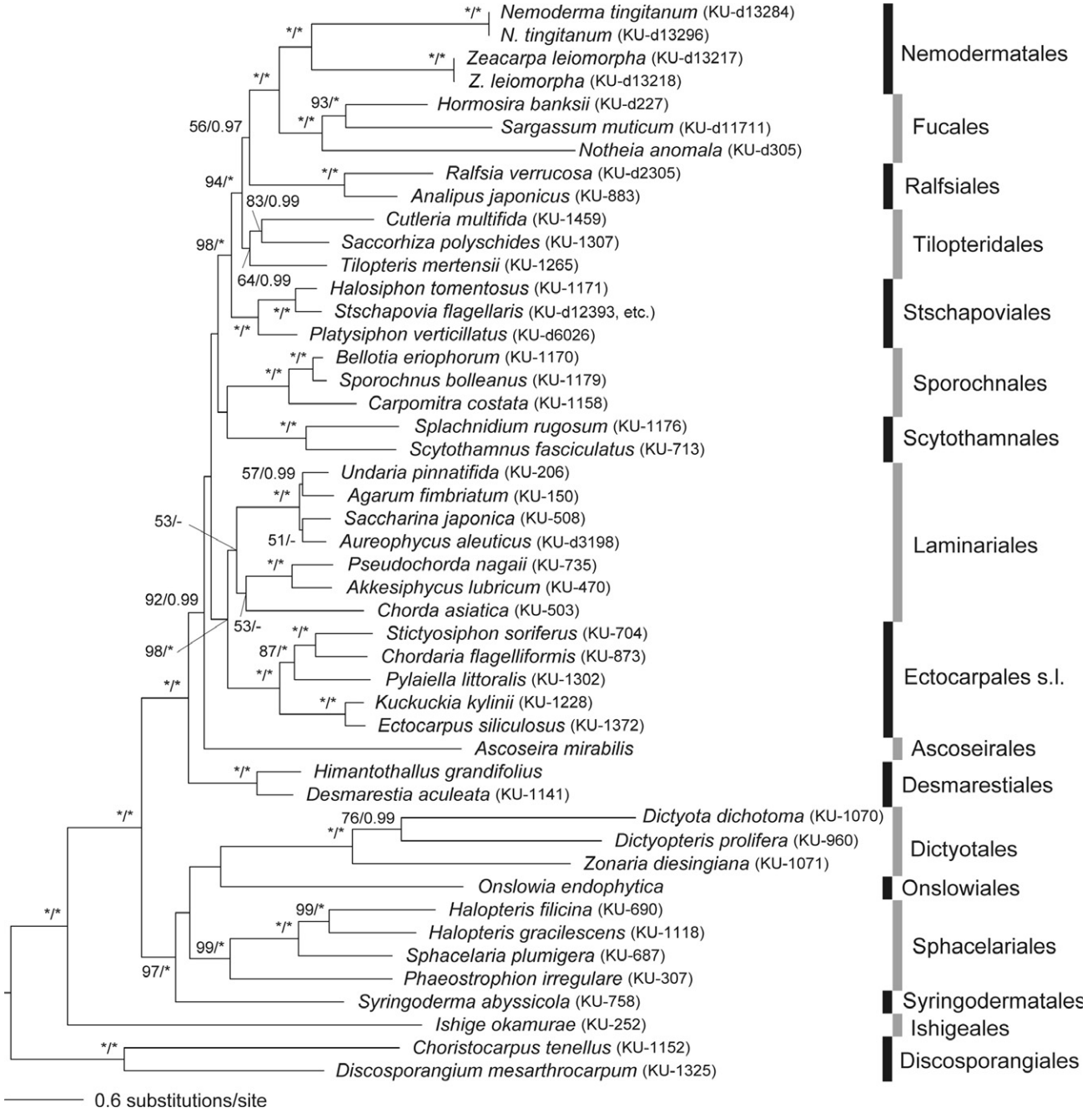

FIG. 2. Maximum likelihood (ML) tree based on the concatenated DNA sequences (chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, and *rbc*L genes, and mitochondrial *cox*I gene). Numbers on the branches indicate bootstrap values from ML analysis (left) and posterior probabilities from Bayesian analysis (right). Asterisk (*) indicates 100 (ML) and 1.00 (Bayes). Only posterior probabilities >0.90 and bootstrap values >50% are shown.

that *Z. leiomorpha* is most related to *N. tingitanum* supported by high bootstrap value (100%), as well as their sister relationship with Fucales (Figs. 2 and S1 in the Supporting Information). The clade consisting of *Nemoderma*/*Zeacarpa* and Fucales was sister to Ralfsiales, although the bootstrap support was low (56%). 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 (Figs. S2–S8 in the Supporting Information). The tree topology of a ML trees (i.e., Fig. S6 removing *psb*C) was identical to that in Fig. 1 (seven genes). In contrast, in the other six ML trees (i.e., Figs. S2–S5, S7–S8), the positions of two to eight taxa were not congruent with those in Figure 1. However, the AU tests indicated that the differences of the tree topologies between these seven ML trees and Figure 1 were not significant (Table S4 in the Supporting Information).

Morphologically, *Zeacarpa* and *Nemoderma* share the morphological features of crustose thallus structure and multiple discoidal chloroplasts without pyrenoids in each cell (Fig. 1, a, e, f). However, while *Nemoderma* forms unilocular zoidangia by the transformation of intercalary cells of upright filaments (Fig. 1, d–f), *Zeacarpa* forms unilocular zoidangia in tufts laterally in the middle of the simple upright filaments, which is unique in brown algae (Fig. 1, e and f). *Nemoderma* is shown to have anisogamous gametangia on the crustose thalli (Bornet 1892) in addition to the above-mentioned intercalary unilocular zoidangia, and hence it is considered to have an isomorphic life history. No gametangia (or plurilocular reproductive organs) have been found in *Zeacarpa*, but it is likely that *Zeacarpa* also has an isomorphic life history as in *Nemoderma*.

Zeacarpa shares some characteristic morphological features with *Nemoderma* as mentioned above (i.e., general thallus structure, chloroplast morphology) and show some similarity in the position of unilocular zoidangia (intercalary vs. lateral in the middle of upright filaments), but the lateral tufts of unilocular zoidangia in *Zeacarpa* are distinctive. Futhermore, these show relatively distant genetic divergence (i.e., 23.7%–13.8%, 13.9%–14.0%, 15.1%, 5.1%–5.2%, 10.3%, 12.3%–12.5% and 22.1% in *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, *rbc*L, and *cox*1 genes, respectively). The sequence divergence between *Zeacarpa* and *Nemoderma* in *rbc*L (12.3%–12.5%) is comparable to those among families in Discosporangiales, Sphacelariales and Ralfsiales, and is considerably higher than those in Fucales, Laminariales, and Tilopteridales (Table S5 in the Supporting Information). In conclusion, since the placement of *Zeacarpa* in Ralfsiaceae is inappropriate, we consider that it is appropriate to classify *Zeacarpa* in an independent family. We therefore propose the new family Zeacarpaceae to accommodate *Zeacarpa* in Nemodermatales.

Zeacarpaceae. H.Kawai, T.Hanyuda, R.J.Anderson & J.J.Bolton **fam. nov**

Type: *Z. leiomorpha* R.J.Anderson, Simons & J.J.Bolton 1988: 320, figs 2–8.

DESCRIPTION AND DIAGNOSIS

Thallus a pseudoparenchymatous crust consisting of filaments; vegetative filaments closely coherent, at first prostrate in one or a few layers, thereafter curving upward gradually to become erect; distal cells provided with many discoid chloroplasts without pyrenoids; reproductive tissue exserted above the vegetative surface in the form of sori; sorus consisting of free, mucilaginous, uniseriate, unbranched filaments; assimilatory filaments within the sorus, one or more intercalary cells bearing unilocular sporangia in acropetal sequence from a single lateral process; plurilocular zoidangia unknown; vegetative hairs absent.

We are grateful to Dr. Eric Henry for providing useful comments, Dr. Takeo Horiguchi for providing the opportunity to undertake this collaboration, and Drs Bruno Reviers, Florence Rousseau, Pascal Conan and Pascal Romans for their help in colleting *N. tingitanum*. A part of this work was supported by the JSPS Grants-in-Aid for Scientific Research (nos'. 22370034 and 25291087) to H. K.

Anderson, R. J., Simons, R. H. & Bolton, J. J. 1988. *Zeacarpa leiomorpha* (Ralfsiaceae), a new genus and species of crustose marine Phaeophyceae from southern Africa. *Phycologia* 27:319–26.

Bornet, E. 1892. Les algues de P. K. A. Schousboe. *Mémoires de la Société des Sciences Naturelles et Mathématiques de Cherbourg* 28:165–376, pls I-III.

Katoh, K. & Toh, H. 2008. Recent developments in the MAFFT multiple sequence alignment program. *Brief Bioinform.* 9:286–98.

Kawai, H., Hanyuda, T., Draisma, S. G. A., Wilce, R. T. & Andersen, R. A. 2015. Molecular phylogeny of two unusual brown algae, *Phaeostrophion irregulare* and *Platysiphon verticillatus*, proposal of the Stschapoviales ord. nov. and Platysiphonaceae fam. nov., and a re-examination of divergence times for brown algal orders. *J. Phycol.* 51:918–28.

Lim, P. E., Sakaguchi, M., Hanyuda, T., Kogame, K., Phang, S. M. & Kawai, H. 2007. Molecular phylogeny of crustose brown algae (Ralfsiales, Phaeophyceae) inferred from *rbc*L sequences resulting in proposal for Neoralfsiaceae fam.nov. *Phycologia* 46:456–6.

Lis, J. T. 1980. Fractionation of DNA fragments by polyethylene glycol induced precipitation. *Meth. Enzymol.* 65:347–53.

Nakamura, Y. 1972. A proposal on the classification of the Phaeophyta. *In* Abbott, I. A. & Kurogi, M. [Eds.] *Contributions to the Systematics of the Benthic Marine Algae of the North Pacific.* Japanese Society of Phycology, Kobe, pp. 147–56.

Nelson, W. A. 1982. A critical review of the order Ralfsiales, Ralfsiaceae and the taxonomic position of *Analipus japonicus* (Harv.) Wynne (Phaeophyta). *Br. Phycol. J.* 17:311–20.

Phillips, N., Burrowes, R., Rousseau, F., de Reviers, B. & Saunders, G. W. 2008. Resolving evolutionary relationships among the brown algae using chloroplast and nuclear genes. *J. Phycol.* 44:394–405.

Rambaut, A. & Drummond, A. J. 2013. Tracer v.1.6. Available at http://tree.bio.ed.ac.uk/software/tracer/ (accessed February 4, 2016).

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeci, J. P. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61:539–42.

Schwarz, G. 1978. Estimating the dimension of a model. *Ann. Stat.* 6:461–4.

Silberfeld, T., Leigh, J. W., Verbruggen, H., Cruaud, C., de Revier, B. & Rousseau, F. 2010. A multi-locus time-calibrated phylogeny of the brown algae (Heterokonta, Ochrophyta, Phaeophyceae): investigating the evolutionary nature of the "brown algal crown radiation". *Mol. Phyl. Evol.* 56:659–74.

Silvestro, D. & Michalak, I. 2012. raxmlGUI: a graphical front-end for RAxML. *Org. Divers. Evol.* 12:335–7.

Tanabe, A. S. 2011. Kakusan4 and Aminosan: two programs for comparing nonpartitioned, proportional and separate models for combined molecular phylogenetic analyses of multilocus sequence data. *Mol. Ecol. Res.* 11:914–21.

Supporting Information

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

Figure S1. Bayesian consensus tree based on concatenated DNA sequences (chloroplast *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, and *rbc*L genes, and mitochondrial *cox*1 gene). Numbers on branches indicate bootstrap values from Baysian analysis. Only posterior probabilities >0.90 are shown and asterisk (*) indicates 1.00.

Figure S2. Maximum-likelihood (ML) tree based on concatenated DNA sequences (chloroplast *psa*A, *psa*B, *psb*A, *psb*C, and *rbc*L genes, and mitochondrial *cox*1 gene). Numbers on the branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S3. Maximum-likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atp*B, *psa*B, *psb*A, *psb*C, and *rbc*L genes, and mitochondrial *cox*1 gene). Numbers on the branches indicate bootstrap values from ML analysis. Only bootstrap values >50% are shown.

Figure S4. Maximum-likelihood (ML) tree based on concatenated DNA sequences (chloroplast *atp*B, *psa*A, *psb*A, *psb*C, and *rbc*L genes, and mitochondrial *cox*1 gene). Numbers on the 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 *atp*B, *psa*A, *psa*B, *psb*C, and *rbc*L genes, and mitochondrial *cox*1 gene). Numbers on the 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 *atp*B, *psa*A, *psa*B, *psb*A, and *rbc*L genes, and mitochondrial *cox*1 gene). Numbers on the 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 *atp*B, *psa*A, *psa*B, *psb*A, and *psbC* genes, and mitochondrial *cox*1 gene). Numbers on the 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 *atp*B, *psa*A, *psa*B, *psb*A, *psb*C, and *rbc*L genes). Numbers on the 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 database accession numbers.

Table S2. List of primers used for PCR and sequencing.

Table S3. Selected models for the Bayesian analysis.

Table S4. Results of approximately unbiased (AU) tests.

Table S5. Sequence divergence (p-distance) of *rbc*L gene among families of representative brown algal orders.