Molecular phylogeny of Zeacarpa (Ralfsiales, Phaeophyceae) proposing a new family Zeacarpaceae and its transfer to Nemodermatales

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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 (atpB, psaA, psaB, psbA, psbC, rbcL, and coxl) 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.

1Received 16 July 2015. Accepted 15 March 2016.
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Editorial Responsibility: K. Müller (Associate Editor)
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 *atpB*, *psaA*, *psaB*, *psbA*, *psbC*, and *rbcL*, and mitochondrial *cox1* 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 *rbcL* DNA sequences, it was revealed that *Zeacarpa leiomorpha* was phylogenetically close to *N. tingitanum*. Therefore, in order to obtain more robust data for elucidating their phylogenetic relationship, we carried out a multigene molecular phylogenetic 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 (Kato et al. 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 Kakusan 4 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 atpB, psaA, psaB, psbA, psbC, and rbcL genes, and mitochondrial cox1 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 *psbC*) 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).

We are grateful to Dr. Eric Henry for providing us with comments. Dr. Takeo Horiguchi for providing us with the opportunity to undertake this collaboration, and Drs Bruno Reviers, Florence Rousseau, Pascal Conan and Pascal Romans for their help in collecting *N. tingitanum*. A part of this work was supported by the JSPS Grants-in-Aid for Scientific Research (nos. 22570034 and 25291087) to H. K.


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 atpB, psaA, psaB, psbA, psbC, and rbcL genes, and mitochondrial cox1 gene). Numbers on the branches indicate bootstrap values from Bayesian 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 psaA, psaB, psbA, psbC, and rbcL genes, and mitochondrial cox1 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 atpB, psaA, psaB, psbA, psbC, and rbcL genes, and mitochondrial cox1 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 atpB, psaA, psaB, psbA, psbC, and rbcL genes, and mitochondrial cox1 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 atpB, psaA, psaB, psbA, psbC, and rbcL genes, and mitochondrial cox1 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 atpB, psaA, psaB, psbA, and rbcL genes, and mitochondrial cox1 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 atpB, psaA, psaB, psbA, and psbC genes, and mitochondrial cox1 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 atpB, psaA, psaB, psbA, psbC, and rbcL 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 rbcL gene among families of representative brown algal orders.