Molecular Phylogenetics and Evolution 168 (2022) 107408

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Providing a phylogenetic framework for trait-based analyses in brown algae: Phylogenomic tree inferred from 32 nuclear protein-coding sequences

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ARTICLE INFO

Keywords:
Ectocarpales
Genomics
Heterokontophytes
Phaeoexplorer
Phaeophyceae
Stramenopiles

ABSTRACT

In the study of the evolution of biological complexity, a reliable phylogenetic framework is needed. Many attempts have been made to resolve phylogenetic relationships between higher groups (i.e., interordinal) of brown algae (Phaeophyceae) based on molecular evidence, but most of these relationships remain unclear. Analyses based on small multi-gene data (including chloroplast, mitochondrial and nuclear sequences) have yielded inconclusive and sometimes contradictory results. To address this problem, we have analyzed 32 nuclear protein-coding sequences in 39 Phaeophycean species belonging to eight orders. The resulting nuclear-based phylogenomic trees provide virtually full support for the phylogenetic relationships within the studied taxa, with few exceptions. The relationships largely confirm phylogenetic trees based on nuclear, chloroplast and mitochondrial sequences, except for the placement of the Sphacelariales with weak bootstrap support. Our study indicates that nuclear protein-coding sequences provide significant support to conclusively resolve phylogenetic relationships among Phaeophyceae, and may be a powerful approach to fully resolve interordinal relationships with increased taxon sampling.

1. Introduction

Brown algae or Phaeophyceae represent a large class of multicellular photosynthetic marine eukaryotes distributed in cold to tropical marine waters with major ecological and economical functions (Bringloe et al., 2020). Brown algae include about 2000 species classified into 340 genera, 59 families and 19 orders (Guiry and Guiry, 2021). In contrast to land plants, our understanding of brown algal evolution remains quite limited (Bringloe et al., 2020). The Phaeoexplorer project (https://phaeoexplorer.sb-roscoff.fr/home/) was initiated in 2016 with the objective of exploring the evolution of biological complexity in brown algae by generating genomic data for a broad range of brown algal species. A fully resolved backbone phylogeny for brown algae is required to investigate questions in evolution, ecology and conservation.

Initial efforts to elucidate phylogenetic relationships among brown algae were based on nuclear ribosomal DNA sequences (Bhattacharya et al., 1992; Druehl et al., 1997; Rousseau and De Reviers, 1999; Tan and Druehl, 1993), followed by studies adding RuBisCO genes (Bailey and Andersen, 1999; De Clerck et al., 2006; Draisma et al., 2001; Peters and Ramírez, 2001; Phillips et al., 2008). More recently, significant attempts were made to produce multi-gene datasets but with a limited number of genes (less than 15), mainly from chloroplast and mitochondrial genomes with nuclear DNA still represented only by ribosomal markers (Bringloe et al., 2020; Cho et al., 2012; Kawai et al., 2015; Lane et al.,

Abbreviations: **BACR**, brown algal crown radiation; **SSDO**, Sphacelariales, Syringodermatales, Dictyotales, Onslowiales.

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https://doi.org/10.1016/j.ympev.2022.107408
Received 18 May 2021; Received in revised form 16 December 2021; Accepted 26 December 2021
Available online 11 January 2022

2007; Silberfeld et al., 2010). While contributing considerably to our understanding of brown algal evolutionary history, these previous phylogenies only partially resolved relationships at some taxonomic levels, in particular for interordinal relationships within the Fucophycideae. Furthermore, in some cases, multi-gene molecular phylogenies based on chloroplast and mitochondrial genes indicated contradictory relationships (Bringloe et al., 2020; Kawai et al., 2013; Kawai et al., 2015; Kawai et al., 2017). Whole genome data are gradually emerging for brown algae (Bringloe et al., 2020), and several studies based on mitochondrial or chloroplast genomes, focused mainly on Laminariales and Fucales members, produced strong evidence supporting the merit of genome data in resolving phylogenetic relationships (e.g., Zhang et al., 2019). However, so far, whole genome data are available for only six of the 19 orders of brown algae (Bringloe et al., 2020). More recently, two studies made use of nuclear transcriptomic data to deduce brown algal evolutionary relationships (Jackson et al., 2017; Sun et al., 2014). The latter study, which was based on 108 orthologous genes from 18 Phaeophyceae species belonging to six orders (i.e., Desmarestiales, Dictyotales, Ectocarpales, Fucales, Ishigeales, Laminariales), produced mixed results (Sun et al., 2014). Overall, phylogenomic trees provided strong to full support for infraordinal relationships and for the relationship between Ectocarpales and Laminariales, but less strong support for other interordinal relationships (e.g., bootstrap (BS) = 55% for Dictyotales-Ishigeales node) (Sun et al., 2014). A second nuclear-transcriptome-based phylogenomic study conducted at the infraordinal level, employed 152 genes from 15 Laminariales species and yielded a nearly fully resolved tree, which enabled relationships between various genera to be revised (Jackson et al., 2017). Family-level relationships were similar to those previously established based on a dataset of eight chloroplast and mitochondrial genes (Kawai et al., 2013). These two transcriptome-based studies effectively resolved lower taxonomic-level phylogenetic relationships (infraordinal), but further studies are needed to improve resolving power at a higher level (i.e., interordinal level).

The objective of the study presented here was twofold: (1) to test the power of nuclear genome-based datasets for resolving inter and infraordinal phylogenetic relationships in brown algae and (2) to provide a reliable phylogenetic framework for trait-based phylogenetic analyses in the context of the Phaeoexplorer project. Maximum likelihood and Bayesian phylogenomic trees were constructed based on the predicted amino-acid sequence data of 32 nuclear protein-coding genes in 39 brown algal species and two outgroup taxa selected in the Phaeoexplorer project.

2. Materials and methods

2.1. Taxa sampling

A total of 39 species of brown algae representing 24 genera, 16 families and eight orders (i.e., Desmarestiales, Discosporangiales, Dictyotales, Ectocarpales s.l., Fucales, Laminariales, Sphacelariales, Tilopteridales), in addition to two outgroup taxa, *Schizocladia ischiensis* E.C. Henry, K.Okuda & H.Kawai (class Schizocladiophyceae) and *Phaeothamnion wetherbeei* R.A.Andersen, L.Graf & H.S.Yoon (class Phaeothamniophyceae), selected in the Phaeoexplorer project, were used in the present study for the phylogenetic reconstructions (Table S1).

2.2. Culture conditions, DNA extraction, genome sequencing and assembly

Genomic DNA was extracted from laboratory-housed culture strains (Roscoff Culture Collection (RCC), Bezhin Rosko Culture Collection, Kobe University Macroalgal Culture Collection (KU-MACC), Culture Collection of Algae at Göttingen University (SAG)) or from specimens collected in the field (Table S1). For species with diplohaplontic life cycles, the gametophyte generation was grown in culture either in 140 mm Petri dishes or in 2–10 L bottles, the latter aerated by bubbling with sterile air. Standard growth conditions were sterile, Provosoli-enriched (Starr and Zeikus, 1993) natural seawater (PES medium) under fluorescent white light (10–30 μM photons/m^2·s) at 13 °C. The freshwater species *Pleurocladia lacustris* A.Braun and *Heribaudiella fluviatilis* (Areschoug) Svedelius were grown in natural seawater that had been diluted to 5% with distilled water (i.e., 95% distilled water/5% seawater) before addition of ES medium (http://sagdb.uni-goettingen.de/culture_media/01%20Basal%20Medium.pdf) micronutrients, at 20 °C for *P. lacustris*. *Phaeothamnion wetherbeei* was grown in MIEB12 (medium 7 in (Schlösser, 1994)). Material for fucoid species was collected on the seashore, and extractions used either dissected meristematic regions or released male gametes. DNA was extracted with either the Nucleospin Plant II midi DNA Extraction Kit (Macherey-Nagel, Düren, Germany) or the OmniPrep Genomic DNA Purification Kit (G Biosciences, St. Louis, MO, USA). DNA quality was assessed using a Qubit fluorometer (Themo Fisher Scientific, Waltham, MA, USA), and fragment length was assessed by migration on a 1% agarose gel for some of the samples. DNA (30–100 ng) was sonicated to a 100–800 bp size range using a Covaris E220 sonicator (Covaris, Woburn, MA, USA). Fragments were end-repaired, 3′-adenylated and Illumina adapters (Bioo Scientific, Austin, TX, USA) were then added using the NEBNext Sample Reagent Set (New England Biolabs, Ipswich, MA, USA). Ligation products were purified using Ampure XP (Beckmann Coulter Genomics, Danvers, MA, USA), and DNA fragments (>200 bp) were PCR amplified using Illumina adapter-specific primers and the KAPA HiFi HotStart polymerase (KapaBiosystems, Wilmington, MA, USA). Libraries were quantified by qPCR using the KAPA Library Quantification Kit for Illumina Libraries (KapaBiosystems), and library profiles were assessed using a DNA High Sensitivity LabChip kit on an Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Some samples were prepared according to the same protocol but with less DNA input (10–25 ng) and using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs). Illumina short read sequence data (150 bp paired end sequence) was generated on either a HiSeq4000 or a NovaSeq6000 platform. The sequence data was first assembled with Megahit (Li et al., 2015), and eukaryotic contigs were separated from the bacterial contigs using a gene detection strategy based on Metagen and Blastp. The bacterial contigs were then used to filter the initial dataset by mapping Illumina reads to the contigs with BWA and then removing these reads, which were not of eukaryotic origin. The set of filtered Illumina reads were then assembled with SPAdes (Bankevich et al., 2012) using the standard protocol.

2.3. Identification of orthologous single-copy genes

Genomes were predicted from *Schizocladia ischiensis* and 23 Phaeophycean species that belong to various orders (Table S1). Predicted genomes were analyzed with OrthoFinder v.2.3.11 (Emms and Kelly, 2015) to detect orthologous single-copy genes. The program detected a total of 32 single-copy genes, which were used in the phylogenetic analyses. Table S2 presents a list of the 32 orthologues in the reference *Ectocarpus* sp.7 strain Ec32 genome. For *Phaeothamnion wetherbeei* and the other 16 species, orthologous amino acid sequences were retrieved using the "tblastn" function of BLAST + 2.6.0 (Camacho et al., 2009).

2.4. Phylogenetic analyses

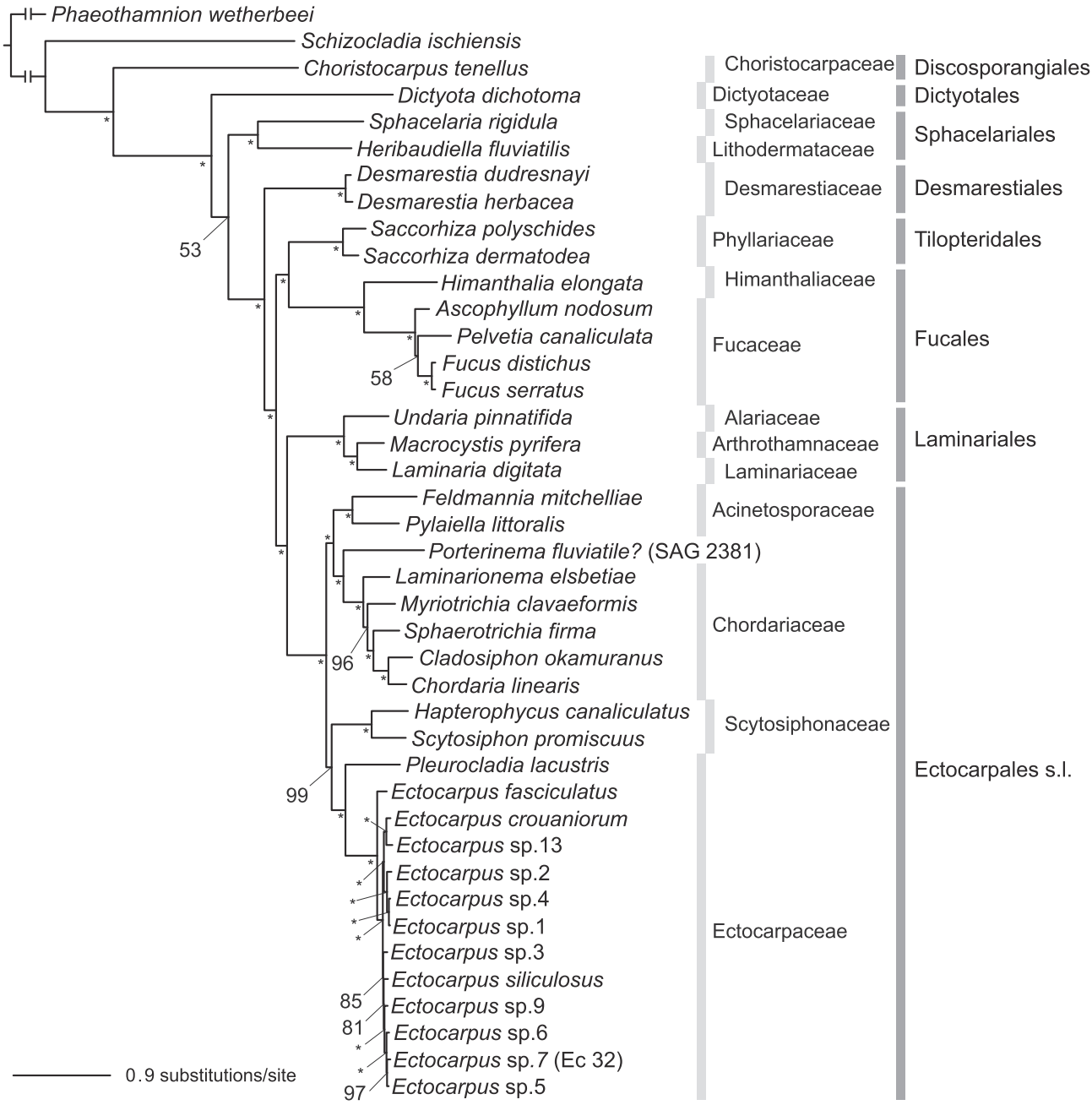
Amino acid sequences of the thirty-two single-copy genes were aligned manually using the alignment software AliView v.1.26 (Larsson, 2014). The aligned sequences were concatenated (41 OTUs, 15,597 amino acids (aa)) and subjected to Maximum likelihood (ML) and Bayesian (BI) analysis. For ML analysis, 10,000 Rapid Bootstrap searches and the subsequent ML search with gamma model were conducted using RAxML v.8.2.9 (Stamatakis, 2014). Bayesian analyses were performed using MrBayes v.3.2.2 (Ronquist et al., 2012). The best-fit evolutionary

models for each gene were determined on the basis of AIC for ML and BIC for BI using Kakusan4 (Tanabe, 2011) (Table S2). The Bayesian analyses were initiated with a random starting tree, and four chains of Markov chain Monte Carlo 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 log-likelihood as assessed using Tracer v.1.7.1 (Rambaut et al., 2018). A consensus topology and posterior probability values were calculated from the remaining trees.

3. Results

3.1. Data characteristics

We assembled a 41-species dataset based on 32 nuclear genes (Tables S2, S3) from 39 species of Phaeophyceae, belonging to 24 genera, 16 families and eight orders (Desmarestiales, Discosporangiales, Dictyotales, Ectocarpales, Fucales, Lamiariales, Sphacelariales, Tilopteridales), together with one species from the Schizocladiophyceae (*Schizocladia ischiensis*) and one species from the Phaeothamniophyceae (*Phaeothamnion wetherbeei*), which served as outgroups (Table S2). The



- ML tree constructed using RAxML-ng (53 OTUs, 32 genes, 15,597aa, 10,000 replicates of bootstrap analyses).
- Branches with a star indicate bootstrap value 100.

Fig. 1. Maximum likelihood phylogenomic tree based on 39 brown algal taxa and two outgroup species inferred from the amino-acid sequence data of 32 nuclear protein-coding genes. '*' indicates ML bootstrap = 100%.

gene matrix was 95.6% complete and the dataset contained 15,597 aa (Table S2).

3.2. Phylogenetic relationships

ML and BI analyses of the 32-nuclear concatenated genes in the 41-species dataset produced trees with identical topology except for the placement of the Sphacelariales in relation to the Dictyotales (Figs. 1, S1). In both analyses, placement of the Sphacelariales was poorly supported with a BS of 53% and a Bayesian posterior probability (BPP) of 0.59. Eighty-two percent of the 38 brown algal nodes were fully supported in the ML analysis (i.e., 100% BS) and 0.95 BPP in BI analysis (i.e., 1.00 BPP). Five nodes within the genus *Ectocarpus* yielded BS values of 81 to 99%. The seven inter-family relationships presented 99–100% BS and 0.99–1.00 BPP support, and the seven interordinal relationships were fully supported for both BS and ML analyses with the exception of the Sphacelariales. *Porterinema fluviatile* (H.C.Porter) Waern (SAG 2381) positioned among the Ectocarpales as a sister to the Chordariaceae.

4. Discussion

Morphology and gene-based studies of higher-level brown algal phylogeny have a long history (Kawai et al., 2015; Silberfeld et al., 2010; Sun et al., 2014; De Reviers et al., 2007; Draisma et al., 2001). However, many higher-level relationships remain problematic, even with the major improvements that have resulted from multi-gene methodologies (Bringloe et al., 2020; Silberfeld et al., 2010). Recent studies have demonstrated the value of studies based on multi-gene data from organellar (Cui et al., 2019; Liu and Pang, 2015a, b; Liu et al., 2019, 2020; Wu et al., 2021; Zhang et al., 2019) and nuclear genomes (Sun et al., 2014) for solving higher-level relationships in the Phaeophyceae. The current study was based on higher ordinal coverage of protein-coding nuclear genes to assess the resolving power of protein-coding nuclear genes in brown algae at this taxonomic level.

The approach used for the current study significantly improves on previous analyses. With the exception of Sphacelariales, interordinal relationships were fully resolved in the current study. Sun et al. (2014) used a similar approach to resolve interordinal relationships, but it is difficult to compare our results with this earlier study, as it was based on different nuclear genes and taxon sampling. However, some conclusions can still be drawn. First, our study included a smaller number of nuclear genes (32 vs. 108 genes), but comprised a higher number of taxa (39 vs. 18 species, 24 vs. 10 genera, 16 vs. seven families, and eight vs. six orders) than that of Sun et al. (2014). The strong branch support obtained by our phylogenetic analyses indicates that the nuclear genes selected in this study are sufficient to generate a robust molecular higher-level phylogeny, although the number of genes are lower than that used by Sun et al. (2014). Testing the quantity of sequence data required for strong node support (e.g., Wortley et al., 2005) will facilitate targeted inclusion of additional taxa in future studies. Second, a better taxonomic representativity of the different orders seems to increase node robustness in our study. Five of the orders analyzed by Sun et al. (2014) were also analyzed in our study, but we did not include any members of the Ishigeales. Another important difference with Sun et al. (2014) concerns the choice of outgroup. In their study, they used a distant outgroup, i.e., the diatom *Phaeodactylum tricornutum* (Bacillariophyceae), which caused a very long branch and may have affected the ingroup taxa relationships and caused lower bootstrap supports (Li et al., 2014), while we used closely related outgroups, i.e., Scizocladiophyceae and Phaeothamniophyceae, which is recommended, when distantly related ingroup taxa are poorly sampled (Li et al., 2014), as it is the case in Sun et al. (2014) and our studies. The taxa analyzed by Sun et al. (2014) were biased towards Fucales and Ectocarpales, and our study mainly towards Ectocarpales. The Sun et al. (2014) analysis included four brown algal crown radiation (BACR) orders and our study five. In line with previous multi-gene studies, both studies conclusively resolved the sister relationship between the Ectocarpales and the Laminariales. The Sun et al. (2014) analysis did not fully resolve relationships among the BACR orders (Desmarestiales, Ectocarpales, Fucales, Laminariales), but interordinal relationships were fully resolved in our analyses (Desmarestiales, Ectocarpales, Fucales, Laminariales, Tilopteridales). Our analyses conclusively positioned the Desmarestiales as first divergence within the BACR with almost full support (BS = 99%, BPP = 1.00). This stands in contrast with previous nuclear- and mitochondrial-based analyses (Liu and Pang, 2015a, b; Liu et al., 2019; Sun et al., 2014), which positioned the Fucales basally in relation to the Desmarestiales. Nevertheless, in the mitochondrial-based analyses (Liu and Pang, 2015a, b; Liu et al., 2019), the ordinal taxon coverage was very limited, and in the nuclear-based analysis (Sun et al., 2014), placement of the Desmarestiales and the Fucales was supported by lower BS values of 95% and 90%, respectively. These low support values in Sun et al. (2014) may be explained by the long branch of the outgroup. Overall, these results indicate a beneficial effect of increased taxon sampling for phylogenetic inference in brown algae using protein-coding nuclear data. Since our dataset only encompassed eight of the 19 Phaeophyceae orders, we assume that increasing taxon sampling may further resolve the placement of Sphacelariales

Interordinal relationships retrieved in this study were nearly identical to those established by Bringloe et al. (2020) based on 12 plastid, mitochondrial and nuclear markers (18S, 5.8S, 28S, *atp*B, *psb*A, *psa*B, *psa*A, *rbc*L, *psb*C, *cox*1, *cox*3, *nad*1), with the exception of the relationship between the Dictyotales and the Sphacelariales. The phylogenetic position of the Sphacelariales, associated to the monophyly of the Dictyotophycidae, was found to be unstable in previous studies. The weak support and unstable position of the Sphacelariales in most studies is possibly associated with incongruence among markers, with a weak phylogenetic signal and/or methodological bias. We assume that adding the missing orders for the Sphacelariales, Syringodermatales, Dictyotales, Onslowiales (SSDO) clade (i.e., Onslowiales and Syringodermatales) may help improving the resolution.

Our dataset included five out of the seven families of Ectocarpales, and relationships within Ectocarpales *sensu lato* were fully resolved. The Acinetosporaceae were positioned as a sister group to the Chordariaceae. Familial relationships within the Laminariales were fully supported and identical to the most up-to-date phylogeny of this order based on genomic data (Starko et al., 2019). For *Porterinema fluviatile,* it should be noted that strain SAG 2381 differs genetically from strain SAG 124.79 (McCauley and Wehr, 2007), which are both from the Culture Collection of Algae at Göttingen University (SAG). SAG 2381 was positioned within the Ectocarpales in this study, while SAG 124.79 was positioned basally among the brown algae (Draisma et al., 2010; McCauley and Wehr, 2007). Molecular and morphological analyses of the culture strains of SAG 2381, SAG 124.79, and *Pilinia rimosa* collected from various localities worldwide indicated that SAG 2381 is the true *P. fluviatile* displaying the characteristic plurilocular zoidangia, whereas SAG 124.79 is in fact *P. rimosa* (Kawai et al., 2021).

5. Conclusions

In conclusion, this study provides a valuable phylogenetic framework for the Phaeoexplorer project, which focuses on the study of the evolution of biological complexity in brown algae, and further evidence supporting the resolving power of protein-coding nuclear genes at interordinal level in brown algae, demonstrating the interest of nuclear multi-gene analysis as a complementary tool to analysis of mitochondrial and chloroplast organellar genome data. Since the current study only included eight of the 19 orders of brown algae, much effort is still needed to provide a comprehensive phylogeny robustly resolving interordinal relationships in brown algae. By comparing our results with previous studies, taxonomic sampling appears to be a major element in resolving interordinal relationships. In the data vs. taxa trade-off, it would therefore be better to give priority to taxonomic sampling. Given

the satisfactory results based on the 32 nuclear genes included in the present study, we recommend that future studies make use of the same set of genes as a standard approach using whole-genome sequencing data.

CRediT authorship contribution statement

Shingo Akita: Conceptualization, Formal analysis, Investigation, Methodology, Data curation, Visualization, Writing – review & editing. **Christophe Vieira:** Conceptualization, Formal analysis, Investigation, Methodology, Data curation, Visualization, Writing – original draft. **Takeaki Hanyuda:** Formal analysis, Writing – review & editing. **Florence Rousseau:** Writing – review & editing. **Corinne Cruaud:** Formal analysis. **Arnaud Couloux:** Formal analysis. **Svenja Heesch:** Formal analysis, Writing – review & editing. **J. Mark Cock:** Resources, Writing – review & editing, Funding acquisition, Project administration. **Hiroshi Kawai:** Conceptualization, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank the following colleagues for contributions to data production and treatment: Akira Peters (Behzin Rosco), Zofia Nehr, Olivier Godfroy, Delphine Scornet, Susana Coelho, Loraine Guéguen, Erwan Corre, Chloé Jollivet (Station Biologique de Roscoff), France Denoeud (Genoscope). We are grateful to Line Le Gall for her helpful comments on the manuscript. The authors thank the Japan Society for the Promotion of Science (JSPS) for financial support to SA and CV.

Funding

This work was supported by the France Génomique National infrastructure project Phaeoexplorer (ANR-10-INBS-09), the ERC grant SEXSEA (638240) and the JSPS Grant-in-Aid for Scientific Research (19F19796).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2022.107408.

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