Protist, Vol. 170, 125682, November 2019
http://www.elsevier.de/protis

Protist

ORIGINAL PAPER

Taxon-rich Multigene Phylogenetic Analyses Resolve the Phylogenetic Relationship Among Deep-branching Stramenopiles

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Submitted August 20, 2018; Accepted August 28, 2019
Monitoring Editor: Hervé Philippe

Stramenopiles are one of the major eukaryotic assemblages. This group comprises a wide range of species including photosynthetic unicellular and multicellular algae, fungus-like osmotrophic organisms and many free-living phagotrophic flagellates. However, the phylogeny of the Stramenopiles, especially relationships among deep-branching heterotrophs, has not yet been resolved because of a lack of adequate transcriptomic data for representative lineages. In this study, we performed multigene phylogenetic analyses of deep-branching Stramenopiles with improved taxon sampling. We sequenced transcriptomes of three deep-branching Stramenopiles: *Incisomonas marina*, *Pseudophyllomitus vesiculosus* and *Platysulcus tardus*. Phylogenetic analyses using 120 protein-coding genes and 56 taxa indicated that *Pl. tardus* is sister to all other Stramenopiles while *Ps. vesiculosus* is sister to MAST-4 and form a robust clade with the Labyrinthulea. The resolved phylogenetic relationships of deep-branching Stramenopiles provide insights into the ancestral traits of the Stramenopiles.

Key words: Stramenopiles; evolution; phylogeny; transcriptome data; MAST.

Introduction

Stramenopiles are among the largest eukaryotic clades, comprising a phototrophic lineage comprised of unicellular algae and seaweeds, as well as many heterotrophic lineages, including fungus-like osmotrophic organisms, intestinal parasites and diverse free-living phagotrophic flagellates (Cavalier-Smith and Chao 2006; Cavalier-Smith and Scoble 2013; Moriya et al. 2000; Riisberg et al. 2009; Shiratori et al. 2015). Stramenopiles are

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https://doi.org/10.1016/j.protis.2019.125682

currently separated into three phyla: The Ochrophyta, Pseudofungi and Bigyra (Cavalier-Smith and Chao 2006; Cavalier-Smith and Scoble 2013; Leonard et al. 2018; Ruggiero et al. 2015). The Ochrophyta include all phototrophic clades (e.g., Eustigmatophyceae, Phaeophyceae, and Raphidophyceae) and the Pseudofungi include two fungus-like taxa (Oomycetes, Hyphochytrea) plus a clade of free-living flagellates (Bigyromonadea). Monophyly of the Ochrophyta and Pseudofungi is robustly supported by morphological and ultrastructural similarities, as well as molecular phylogenetic analyses using small subunit ribosomal RNA (SSU rRNA) gene (Cavalier-Smith and Chao 2006; Leipe et al. 1996). The non-monophyletic Bigyra include the rest of the deep-branching heterotrophic Stramenopiles (e.g., the Bikosea, Labyrinthulea, and Opalinea); however, the monophyly and the relationships among internal branches are unclear in the SSU rRNA gene phylogenies (Cavalier-Smith and Chao 2006; Cavalier-Smith and Scoble 2013; Ruggiero et al. 2015).

Stramenopiles also include approximately 18 deep-branching environmental lineages termed MAST (MArine STramenopile), that are related to bigyran lineages (Massana et al. 2004, 2009, 2014). Recent phylogenomic analyses using genomic and transcriptomic data have shed light on the ambiguous phylogeny of the Bigyra and indicated that these lineages separate into two subclades, the Opalozoa and the Sagenista (Burki et al. 2016; Derelle et al. 2016; Noguchi et al. 2016). The Opalozoa includes various free-living phagotrophic flagellates (Bikosea, Placididea, and Nanomonadea) and intestinal parasites (Opalinata and *Blastocystis*). The Sagenista include the Labyrinthulea, a clade of phagotrophic and osmotrophic protists with extracellular filaments, and an uncultured environmental lineage MAST-4, whose cellular structure remains unknown. Despite the power of large-scale phylogenomic analyses, the positions of the Opalozoa and Sagenista are not consistent among published analyses, and the phylogenetic validity of the Bigyra remains uncertain (Burki et al. 2016; Derelle et al. 2016; Noguchi et al. 2016).

Another remaining problem for the phylogeny of the Bigyra is that available genomic and transcriptomic data do not represent all the major lineages. Although novel deep-branching Stramenopiles that are likely closely related to, or included within, the Bigyra have been described, some of them lack data for large-scale phylogenetic analysis to resolve their position (Cavalier-Smith and Scoble 2013; Gómez et al. 2011; Shiratori et al. 2017). Moreover, except for MAST-3 and 4, most of the deep-branching MAST lineages lack data for multigene phylogenetic analyses.

To better understand the phylogeny and character evolution of Stramenopiles, we perform taxon-rich multigene phylogenetic analyses, including MASTs and species of uncertain phylogenetic position. We sequenced transcriptomes of a recently described deep-branching stramenopile, *Platysulcus tardus,* and two MASTs, *Incisomonas marina* (MAST-3) and *Pseudophyllomitus vesiculosus* (MAST-6) and extended the multigene phylogenetic analyses of the Stramenopiles with the improved taxon sampling. Our new phylogeny provides additional information to understand the character evolution of Stramenopiles.

Results

Dataset Construction

To resolve the phylogeny of deep-branching Stramenopiles, we sequenced transcriptomes of three deep-branching lineages: *Incisomonas marina* (i.e. MAST-3), *Pseudophyllomitus vesiculosus* (i.e. MAST-6), and *Platysulcus tardus* (Platysulcidae). The filtered and assembled sequences yielded 44,382; 116,491; and 31,779 transcript contigs from *I. marina*, *Ps. vesiculosus*, and *Pl. tardus,* respectively. We used the transcriptomic and genomic data of 47 Stramenopiles and 9 outgroups (Alveolates and Rhizaria) from the Marine Microbial Eukaryotes Transcriptome Sequencing Project (MMETSP) and GenBank. This dataset includes representatives from nine classes of Ochrophytes (Bacillariophyceae, Bolidophyceae, Chrysophyceae, Dictyochophyceae, Eustigmatophyceae, Pelagophyceae, Phaeophyceae, Raphidophyceae, and Xanthophyceae) and six classes of heterotrophic Stramenopiles (Bikosea, Placididea, Blastocystea, Labyrinthulea, Bigyromonadea, and Oomycetes). We generated a final dataset of 56 species in 120 gene alignments, containing 34,706 amino acid positions, with missing characters and genes of 16.6% and 12.07%, respectively (Supplementary Material Tables S3–S5, Fig. S1). The average missing characters and genes of three newly sequenced taxa were 13.22% and 7.2%, respectively.

Phylogenetic Results of Stramenopiles

We performed Maximum-Likelihood (ML) analysis under the best-fitting model (LG + C60+F + G) and Bayesian inference (BI) under the site-

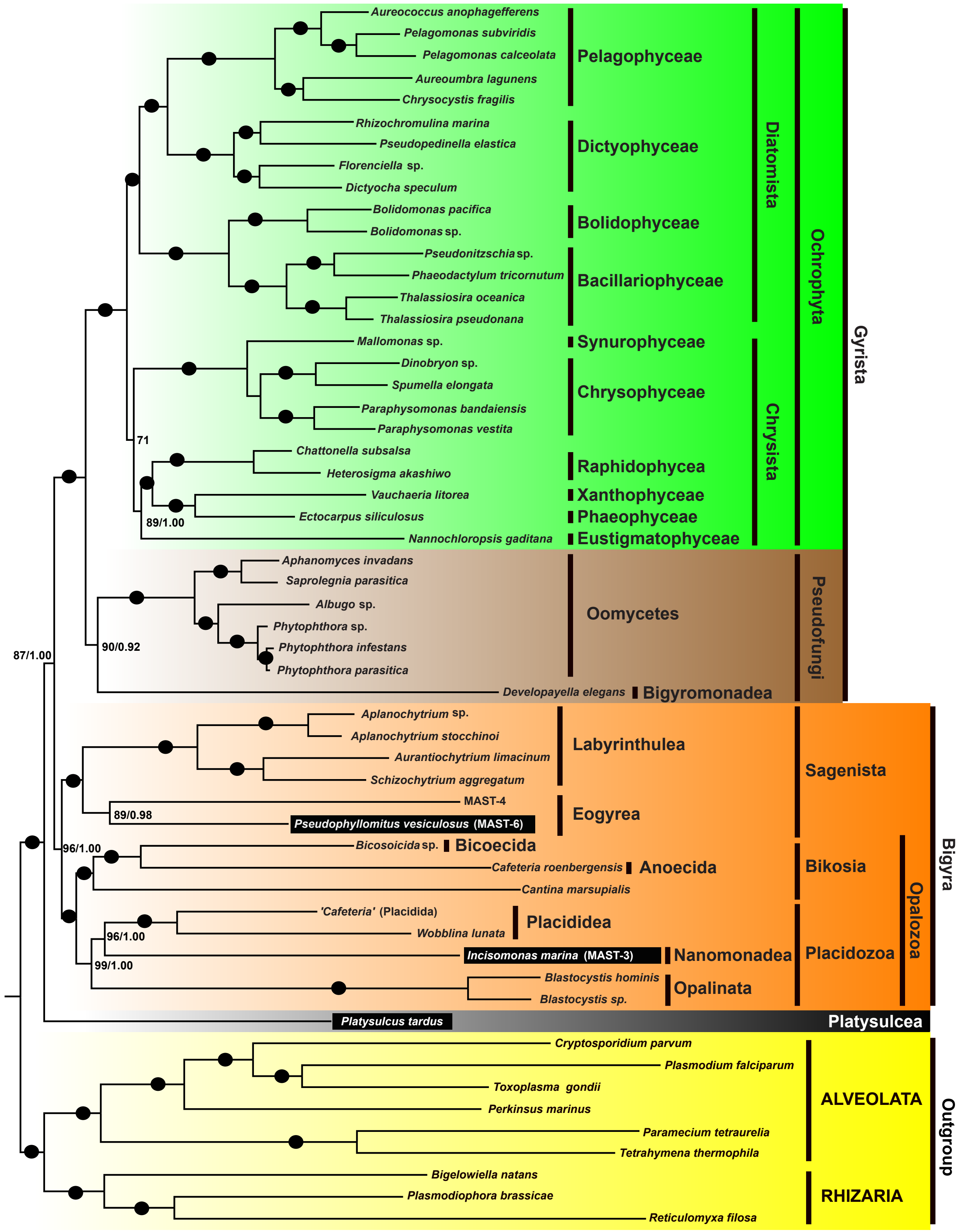

Figure 1. The phylogenetic tree of Stramenopiles inferred from the dataset (56 taxa/120 genes). The topology is Maximum-likelihood (ML) tree under the best-fitting model (LG + C60+F + G) with both ML bootstrap percentage (BP) values and Bayesian posterior probabilities (PP). BP < 70% and PP < 0.92 are omitted from the figure. Black dots correspond to a BP of 100% and a PP of 1.00. The tree is rooted on Alveolata plus Rhizaria.

heterogeneous mixture model (CAT + GTR + G4). The ML tree of the 120-gene dataset recovered the monophyly of Stramenopiles, with ML bootstrap percentage (BP) values of 100% and Bayesian posterior probabilities (PP) of 1.00. Major subclades of Stramenopiles such as the Ochrophyta, the Gyrista (Ochrophyta and Pseudofungi), the Sagenista, and the Opalozoa were also recovered with robust support (Fig. 1). In the Ochrophyta, the Pelagophyceae, Dictyochophyceae, Bacillariophyceae, and Bolidophyceae form a robust clade, making up the Diatomista. However, the monophyly of the Chrysista (i.e., Eustigmatophyceae, Chrysophyceae, Phaeophyceae, Raphidophyceae, Synurophyceae, and Xanthophyceae) is moderately supported exclusively under ML tree (BP = 71%). In the BI tree, the Chrysista are separated into two subclades: a clade comprising the Chrysophyceae and Synurophyceae is positioned as a sister to the Diatomista, and a clade comprising Eustigmatophyceae, Phaeophyceae, Raphidophyceae, and Xanthophyceae branches as a sister lineage of Ochrophyta with strong support (PP = 1.00; Supplementary Material Fig. S1).

Both ML and BI trees demonstrates monophyly of the Sagenista and Opalozoa, recovering the Bigyra (Fig. 1). In our phylogeny, Sagenista, *Ps. vesiculosus* (MAST-6) and MAST-4 are monophyletic, with strong support (PP = 0.98, BP = 89%). This relationship is consistent with the proposed clade, Eogyrea, which was originally found in studies of environmental sequences (e.g. MASTs; Cavalier-Smith and Scoble 2013) and more recently formally established as a class following the description of *Ps. vesiculosus* (i.e. MAST-6; Cavalier-Smith 2018). The Opalozoa are separated into the Placidozoa (Opalinata, Nanomonadea, plus Placididea) and a clade comprising the Bikosea and *Cantina marsupialis*. In the Placidozoa, the Nanomonadea (*I. marina*) and the Placididea form a clade with robust support (PP = 1.00, BP = 96 %). The Placididea and the monophyly of the Bikosea and*, Cantina marsupialis* are both robustly supported in our tree (PP = 1.00, BP = 100%; Fig. 1). *Platysulcus tardus* falls sisters to all other Stramenopiles with strong support (PP = 1.00, BP = 87%; Fig. 1).

Stability of Deep-branching Stramenopiles

To evaluate stability of the topology of the deep-branching Stramenopiles, we performed phylogenetic analyses using datasets with iterative removal of fast-evolving sites. For this analysis, we removed 2–20% of fast-evolving amino acid sites from our concatenated alignment. The position of *Pl. tardus* and the monophyly of the Opalozoa and Sagenista are robustly supported across all analyses (Fig. 2). We also constructed an ML tree excluding some fast-evolving taxa (*Cafeteria roenbergensis*, *Blastocystis* sp., *Blastocystis hominis*, and *Cantina marsupialis*), which results in no change in either the position of *Pl. tardus* or the monophyly the Opalozoa and Sagenista. The monophyly of the Bigyra is weakly supported in our tree after removal of fast-evolving taxa (Supplementary Material Fig. S3).

Discussion

Root of Stramenopiles

This study helps to refine the phylogeny of the Stramenopiles using a new taxon-rich dataset that includes transcriptome sequences for three deep lineages. First, we revisit the root of the Stramenopiles. The SSU rRNA gene tree topology of Cavalier-Smith and Scoble (2013) shows that the Stramenopiles are rooted between the Bigyra and the Gyrista, albeit with weak support. Phylogenomic analyses by Burki et al. (2016) and Noguchi et al. (2016) shows that the Opalozoa (Placidiozoa and Bikosea) are the deepest diverging clade within Stramenopiles. In the present study, *Pl. tardus* fall sister to all remaining Stramenopiles, with strong support in both ML and Bayesian analyses (Fig. 1). This position has also supported when fast-evolving sites and taxa are removed (Supplementary Material Fig. S3), suggesting that *Pl. tardus* is the earliest independent branch of the Stramenopiles. Cavalier-Smith (2018) proposed a new class, the Platysulcea, for *Pl. tardus* as a subclade of the Bigyra. In contrast, our results support the establishment of the Platysulcea but not as a subclade of the Bigyra.

The Inferred Ancestral Traits of Stramenopiles

Our multigene phylogenetic analyses identify the deepest node within the Stramenopiles with robust support, which allows us to make inferences about ancestral morphology and ultrastructure within Stramenopiles. Here we focus on three key characters: tubular mastigonemes, transitional helix, and ventral groove. The tubular mastigonemes on the anterior flagellum have been considered as the synapomorphy of Stramenopiles (Cavalier-Smith and Chao 2006). Tubular mastigonemes are common among the Stramenopiles, but are miss-

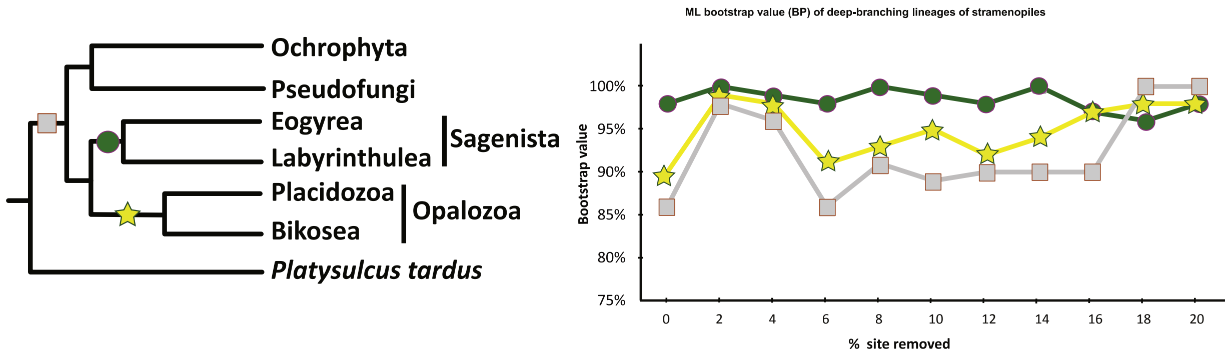

Figure 2. Changes in Maximum-likelihood (ML) bootstrap support among deep-branches of Stramenopiles when fast-evolving sites were removed. In the illustrated tree: the squares, stars, and circles represent the change in the ML bootstrap support. Site rates were estimated from an alignment (56 taxa). Sites were then removed in 2% increments from the alignments of 56 taxa (including *Cafeteria roenbergensis, Blastocystis* sp., *Blastocystis hominis*, and *Cantina marsupialis*). ML bootstrap support for *Platysulcus tardus*, Opalozoa and Sagenista were calculated under the best-fitting model.

ing in some major clades such as the Bikosea (e.g., *Rictus* and *Caecitellus*), the Opalinata (e.g., *Proteromonas*), and the Nanomonadea (e.g., *Incisomonas* and *Solenicola*) (Cavalier-Smith and Scoble 2013; Gómez et al. 2011; O'Kelly and Nerad 1998; Yubuki et al. 2010). Our phylogenetic analyses show that these mastigoneme-lacking flagellates branched as early diverging lineages within Bigyra. The position of mastigoneme-bearing *Platysulcus tardus* as sister to all other Stramenopiles, suggests that the common ancestor of the Stramenopiles possessed tubular mastigonemes, and that lineages such as Bikosea, Opalinata and Nanomonadea have lost this feature. Since Alveolata and Rhizaria lack tubular mastigonemes, it is probable that this feature was acquired in the common ancestor of the Stramenopiles. Alternatively, some phylogenomic analyses support the monophyly of the mastigoneme-bearing telonemids and SAR (Stramenopiles, Alveolata, and Rhizaria; Burki et al. 2012, 2016; Strassert et al. 2018), with the Stramenopiles as a sister group to Alveolata plus Rhizaria (He et al. 2016). Under this topology, it is also most parsimonious that the tubular mastigoneme was present in the common ancestor of SAR and telonemids.

Stramenopiles have various helical structures in their flagellar transitional regions or basal bodies (Anderson 1991; Cavalier-Smith and Chao 2006). A single and double transitional helix has been described in various clades of Ochrophyta (Chrysophyceae, Eustigmatophyceae, and Xanthophyceae); however, some ochrophytes (Bolidophyceae, Phaeophyceae, and Raphidophyceae) lack these structures (Andersen 2004; Cavalier-Smith and Chao 2006; Preisig 1989). In the Placididea and Opalinea, deep-branching within Bigyra, have a double transitional helix in their transitional regions, just above the transitional plate and intrakinetosomal shelves in basal bodies (Cavalier-Smith 1997; Cavalier-Smith and Chao 2006; Moriya et al. 2002; Patterson 1985; Patterson and Delvinquier 1990). In the Bikosea, some flagellates have spiral fibers in their flagellar transitional regions (e.g., *Adriamonas peritocrescens*, *Bicosoeca maris*, and *Boroka karpovii*), or intrakinetosomal shelves in basal bodies (*Rictus lutensis*), while most species lack these structures (Cavalier-Smith and Chao 2006; Karpov et al. 2001; Shiratori et al. 2015; Yubuki et al. 2010). A bell-shaped double helix has been described in the transitional region of the Labyrinthulea (Barr and Allan 1985).

Although helical structures are widely distributed in the Stramenopiles, *Pl. tardus* lacks these structures (Shiratori et al. 2015). Helical structures at the flagellar transitional region are rare in other eukaryotic groups and are reported exclusively in the distantly related euglenoid *Entosiphon* and in a green alga, *Pyramimonas*, and have not been reported in the Rhizaria or Alveolata (Moestrup 1982). As *Platysulcus tardus* falls sister to other Stramenopiles, it is more likely that the transitional helix evolved in the Stramenopiles after *Pl. tardus* branched, rather than *Pl. tardus* having lost the helical structures secondarily.

A cup-like feeding apparatus has been described in several bikosean and chrysophycean species (Moestrup and Andersen 1991; O'Kelly and Patterson 1996). These feeding cups are formed by sub-divisions of the microtubular root 2 (R2) (Yubuki and Leander 2013). In contrast, *Pl. tar-*

dus possesses a wide longitudinal ventral groove that is distinct from other Stramenopiles. The ventral groove is widely distributed among eukaryotes, such as the Alveolata (*Colponema*) (Tikhonenkov et al. 2014), Rhizaria (e.g., Thaumatomonadida, *Ventrifissura*, and *Cryothecomonas*) (Howe et al. 2011), collodictyonids (*Collodictyon*, *Diphylleia*, and *Sulcomonas*) (Brugerolle 2006; Brugerolle et al. 2002), and excavates (e.g., *Jakoba*, *Trimastix*, and *Carpediemonas*) (Simpson 2003). The widespread presence of a longitudinal ventral groove among diverse lineages suggests that it is probably one of the ancestral traits of eukaryotes (Yubuki et al. 2013). Considering the phylogenetic position of *Pl. tardus*, it is possible that the ancestral Stramenopiles possessed a ventral groove and its loss or change to feeding cups occurred after the divergence of *Pl. tardus*.

Diversity of the Eogyrea

Cavalier-Smith (2018) proposed a new class, the Eogyrea and order Eogyrida, which include MAST-4, -6, -7, -8, - 9, -10, and -11, based on the description of *Ps. vesiculosus*, the sole described species of these MAST lineages (Shiratori et al. 2017). However, monophyly of the Eogyrea was occasionally not fully recovered or well-supported in previous ribosomal rRNA gene phylogenetic analyses (Cavalier-Smith and Scoble 2013; Massana et al. 2014). Our phylogenetic analyses indicate the robust monophyly of MAST-4 and -6 (Fig. 1). Thus, the Eogyrea concept would be valid, at least for these two MASTs, as a comprehensive test of this hypothesis requires data from representatives of remaining eogyrean MASTs.

Our results and those of previous studies on MAST-4 and -6 indicated that the Eogyrea could be a very diverse lineage. *Pseudophyllomitus*, the representative of MAST-6, is one of the largest flagellates among the deep-branching Stramenopiles, except for the parasitic opalinates (e.g., *Opalina* and *Proteromonas*). The members of *Pseudophyllomitus* are benthic and feed on eukaryotic algae (Piwosz and Pernthaler 2010; Shiratori et al. 2017). In contrast to MAST-6, MAST-4 is suggested to comprise picoplanktonic flagellates, that are abundant in surface seawater and are considered to be bacterivorous, representing around 9% of total heterotrophic flagellates (Cheung et al. 2013). Except for MAST-9, the other “eogyrean” MASTs are considered to be small planktonic bacterivorous flagellates like MAST-4. However, a size-fractioned environmental DNA survey suggested that MAST-8 have larger cells than MAST-4 (Massana et al. 2014). MAST-9 showed ribogroup-specific various habitat preferences. Most sequences in ribogroups MAST-9c and MAST-9d are detected from marine sediments and anoxic seawater, respectively, whereas MAST-9a sequences are found in both oxic seawater and marine sediments (Massana et al. 2014). If these MASTs are truly members of the eogyrean lineage, the Eogyrea could be another interesting diverse group of Stramenopiles.

Overall Topology of the Stramenopiles

Besides the ancestral characters of the Stramenopiles, this study also allows discussion of the overall tree topology of the Stramenopiles. Derelle et al. (2016) proposed two Ochrophyta subgroups, Chrysista and Diatomista based on their phylogenomic analysis. In our phylogenetic analyses, the monophyly of the Diatomista is robustly supported as in Derelle et al. (2016), while the monophyly of Chrysista is only moderately supported (71%) exclusively in the ML tree (Fig. 1). Although the tree presented by the Derelle et al. (2016) provided robustness of the Chrysista monophyly using a large gene dataset, it lacks the Eustigmatophyceae taxon and presented only a few of representatives of the deep-branching lineages. The trees presented by Burki et al. (2016) and Noguchi et al. (2016) lack support about the monophyly or paraphyly of Chrysista, suggesting that the existing sequence data are not sufficient to resolve the phylogenetic relationship of the Chrysista.

Within the Placidozoa, the proposed sister relationship between the Nanomonadea and the Opalinata presented by Derelle et al. (2016) is not recovered in our tree. Instead, the Nanomonadea form a clade with the Placididea (Fig. 1). This topology has been consistently supported, even after removing fast-evolving sites or taxa (Fig. 2, Supplementary Material Fig. S3). Although this suggests that the Nanomonadea-Placididea clade in our tree is not caused by LBA, more taxon-rich multigene phylogenetic analyses are required to resolve their ambiguity. In the Bikosea, a recently described species, *C. marsupialis*, has not included in the Bikosea in the SSU rRNA gene tree presented by Yubuki et al. (2015). However, our tree shows that *C. marsupialis* is a sister species to other Bikosea species, which agrees with the results of Noguchi et al. (2016). Thus, we believe that *C. marsupialis* should be treated as a member of the Bikosea.

Both ML and Bayesian trees from this study recovered the monophyly of the Bigyra which is composed of Sagenista and Opalozoa (Fig. 1).

Monophyly of the Bigyra is also shown in our tree after removing fast-evolving taxa, albeit with limited support (Supplementary Material Fig. S3). Our tree topology is congruent with that of Derelle et al. (2016) as they too recovered the monophyly of the Bigyra in both the Bayesian and ML trees after removal of divergent placidozoan lineage. However, the phylogenomic analyses by Noguchi et al. (2016), support the paraphyly of the Bigyra with 80% and 79% BP. These inconsistent results suggest that the monophyly of the Bigyra remains unresolved, awaiting additional sequence data from other deep-branching Stramenopiles.

Methods

Cultures and RNA extraction: A culture of *Incisomonas marina* (CCAP 977/1) was obtained from the Culture Collection of Algae and Protozoa (CCAP), and cultures of *Platysulcus tardus* (NIES-3720) and *Pseudophyllomitus vesiculosus* (NIES-4114), which had been established in our laboratory (Shiratori et al. 2015, 2017), were used from our private collection. All three species were cultured in 1000 ml culture flasks with ESM medium (Kasai et al. 2009). *Pseudophyllomitus vesiculosus* (MAST-6) was inoculated with *Phaeodyctylum tricornutum* (CCAP1055/1) as a feed. The growth conditions for the selected strains were 20 °C under a 14-h light/10-h dark cycle. One-week-old cultures were scraped from culture flasks, collected in Eppendorf tubes, and centrifuged at 5,000 rpm. Total RNAs were extracted from these cultures using the Trizol reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. This yielded 5.5 μg, 7.7 μg, and 6.6 μg of total RNA from *I. marina*, *Ps. vesiculosus*, and *Pl. tardus* respectively.

Transcriptome data analyses of selected cultures: The cDNA library construction, paired-end sequencing (100 and 125 bp per reads) with HiSeq2000 and HiSeq2500, and HiSeq control Software 2.0.12.0 (Illumina, Inc., San Diego, CA, USA) were performed at Eurofins Genomics (Tokyo, Japan). The raw sequence reads from *I. marina*, *Ps. vesiculosus*, and *Pl. tardus* comprised 79,900,544; 36,635,518; and 73,712,130 reads, respectively. These sequenced reads were filtered using Trimmomatic v.3.0 (Bolger et al. 2014) to remove adaptors and low-quality bases. The filtered sequences were then assembled using Trinity assembler (Grabherr et al. 2011) with an accurate option and yielded 44,382; 116,491; and 31,779 transcript contigs from *I. marina*, *Ps. vesiculosus*, and *Pl. tardus*, respectively. The raw transcriptome sequences were deposited to GenBank as DRA008754 (*I. marina*), DRA008755 (*Ps. vesiculosus*), and DRA008753 (*Pl. tardus*). The assembled transcripts were deposited to FigShare database (https://doi.org/10.6084/m9.figshare.9209831.v1).

Sequence data collection: Besides our three newly sequenced transcriptomes from this study, we collected 73 eukaryotic genome and transcriptomes including 47 Stramenopiles taxa from the Marine Microbial Transcriptome Sequencing Project (MMETSP) and genome data from the National Center for Biotechnology Information (NCBI) were obtained. To maintain the taxonomic evenness from all the eukaryotic lineages, we included representatives of the other eukaryotes besides SAR (i.e., Archaeplastida, Opisthokonta, Amoebozoa, Fungi, cryptophytes, and haptophytes). These sequence data were assembled and translated into amino acids using the Trinity assembler v2.2.0 (Grabherr et al. 2011) and TRANSDECODER package (http://transdecoder.github.io/), respectively.

We selected 120 conserved genes from Yabuki et al. (2014) and Cavalier-Smith et al. (2015). List of taxa and genes are in the Supplementary Material Table S1 and S2. We screened the 120 genes from 73 genome and transcriptomes by BlastP search using *Homo sapiens* sequences as a query (E-value < 1e-20). The resulted single gene alignments were then aligned with MAFFT using a Linsi algorithm (Katoh and Standley 2013) and ambiguously aligned positions were curated manually. The identification of and removal of possible paralogous and laterally transferred sequences were performed as follow: Single gene trees were constructed from each alignment by using IQ-TREE v.1.3.0 under best fitting model (Nguyen et al. 2015), with 1000 replicates of ultrafast bootstrap (UFboot) (Minh et al. 2013). Then, we manually identified and removed the sequences that grouped with different phylum with more than 60% bootstrap value from each single gene trees. Also, we removed *Ps. vesiculosus* sequences that were identical to *Phaeodactylum tricornutum* sequences (Bowler et al. 2008). Single gene trees (https://doi.org/10.6084/m9.figshare.9252965), single gene alignments (https://doi.org/10.6084/m9.figshare.9252968), and concatenated alignment (https://doi.org/10.6084/m9.figshare.9252992) were deposited in the FigShare database. Finally, we generated a 120-gene dataset comprising 47 Stramenopiles, six Alveolates, and three rhizarians. The total amino acid position was 34,706 amino acid positions with average missing characters of 16.6% (Supplementary Material Tables S3–S5).

Phylogenetic analysis: ML tree reconstruction was performed using IQ-TREE v.1.3.0 (Nguyen et al. 2015). The model that best fitted the data was determined using IQ-TREE based on Bayesian information criterion. The best-fitting model available under the ML analysis was LG + C60+F + G (Supplementary Material Table S6). ML trees were estimated under this model and the non-parametric bootstrap with 100 replicates (Felsenstein 1985) were estimated under LG + C60+F + G+PMSF model. For Bayesian analysis, LG + C60 and CAT + GTR + G4 models were compared by a model cross-validation using PhyloBayes 4.1 (Lartillot et al. 2009) and the CAT + GTR + G4 showed better score than the LG + C60 (Supplementary Material Table S7). Therefore, Bayesian analyses were performed using PhyloBayes-MPI v.3.4 under CAT + GTR + G4 model. Two independent Markov Chain Monte Carlo chains were run for at least 2,000 generations. Consensus posterior probabilities were calculated from saved tress after discarding the first 300 trees as burn-in (maxdiff = 0.051). Bayesian inference was performed using the site-heterogeneous mixture model. Consensus topology and posterior probability values were calculated from the saved tree.

Site removal experiment: Fast evolving sites were removed using a tree-independent approach; positions of the concatenated matrix were ranked according to their conservation value, as calculated using Trimal (Capella-Gutierrez et al. 2009), and 2–20% wer e removed. The ML tree was reconstructed after each removal (IQ-TREE analyses, UFboot, 1000 replicates) and the node value was calculated from the resulting ML tree.

Acknowledgements

We thank Dr. Goro Tanifugi (National Museum of Nature and Science, Japan) for providing the sequence data of *Developayella elegans*, *Cantina marsupialis*, and *Wobblia lunata*. Also, we thank Professor Dr. Laura A. Katz (Department of Biological Sciences, Smith College, Northampton, MA, United States) for checking the English. This work was supported by JSPS KAKENHI (grant numbers 13J00587, 23405013) and the fund of National Bioresource Project (grant number 15Km0210030;0004).

Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.protis.2019.125682.

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