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Improved taxon sampling and multigene phylogeny of unicellular chlamydomonads closely related to the colonial volvocalean lineage Tetrabaenaceae-Goniaceae-Volvocaceae (Volvocales, Chlorophyceae)

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ABSTRACT

In the green algal order Volvocales (Chlorophyceae), flagellate colonial forms have evolved at least four times. One of these colonial lineages, Tetrabaenaceae-Goniaceae-Volvocaceae (TGV), which belongs to the clade *Reinhardtinia*, is closely related to several unicellular chlamydomonads in the genera *Chlamydomonas* and *Vitreochlamys*. However, the unicellular sister of TGV has not been specified. Here, the largest ever 18S *r*RNA phylogenetic tree of *Reinhardtinia* was constructed including several newly isolated chlamydomonads, and a clade (core-*Reinhardtinia*) including 32 unicellular lineages and three colonial families were recognized. Interrelationships within core-*Reinhardtinia* were barely resolved in the tree, and therefore combined 18S-*atp*B-*psa*A-*psa*B-*psb*C-*rbc*L gene phylogenetic analyses were performed with selected representatives of 29 of the 32 unicellular lineages and three colonial families. The 29 unicellular lineages were clustered into five metaclades and an unassigned lineage; the metaclade that includes *Chlamydomonas pila* was resolved, with moderate support, as the sister clade to TGV. To examine possible biases from specific gene(s), long-branch taxa, and the heterogeneous base composition, phylogenetic analyses using several smaller data sets were also performed. Light microscopy of *C. pila* and its relatives indicated that any early steps towards colony evolution appeared after divergence of TGV from the *C. pila* lineage.

1. Introduction

The order Volvocales (Chlorophyceae) includes various forms of both flagellate and coccoid algae, with at least four flagellate colonial lineages having evolved independently: *Pascherina* (Nakada et al., 2010a; Sugasawa et al., 2015a), *Pyrobotrys* (Nakada et al., 2010a; Sugasawa et al., 2015b), *Stephanosphaera* (Buchheim and Chapman, 1991; Buchheim et al., 1994, 2013; Munakata et al., 2016), and Tetrabaenaceae-Goniaceae-Volvocaceae (TGV; e.g., Buchheim and Chapman, 1991; Buchheim et al., 1990, 1994, 1997a, 1997b; Nozaki et al., 2003). Among these, TGV includes simple (consisting of four undifferentiated cells) to complex (consisting of thousands of differentiated cells) colonial forms, and has been extensively studied as a model of early colonial evolution (e.g., Arakaki et al., 2013, 2017; Hallmann, 2011; Herron and Michod, 2008; Herron et al., 2009; Kirk, 1998, 2005; Prochnik et al., 2010). However, the unicellular sister of TGV has not been specified.

Historically, the molecular phylogeny of chlamydomonads (unicellular volvocaleans) has mainly been examined using 18S *r*RNA (e.g., Buchheim et al., 1990, 1996, 1997a, 1997b; Hoham et al., 2002; Nakada et al., 2008, 2016a, 2016b; Pröschold et al., 2001; Yumoto et al., 2013) or ITS regions (Pröschold et al., 2005), while colonial TGV has largely been studied using chloroplast genes (e.g., Nozaki, 2003; Nozaki et al., 1995, 1997, 1999, 2000, 2003, 2006, 2014). Nakada et al. (2016a) constructed an 18S *r*RNA tree including major representatives of both unicellular and colonial forms, and recognized a robust clade (core-*Reinhardtinia*, defined as the most inclusive clade, containing *Volvox carteri* but not *Paulschulzia pseudovolvox*) consisting of 18

Abbreviations: BP, bootstrap proportion; CCAP, Culture Collection of Algae and Protozoa; cp, chloroplast; GV, Goniaceae + Volvocaceae; MC, metaclade; ML, maximum likelihood; NIES, National Institute for Environmental Studies; NJ, neighbor joining; OTU, operational taxonomic unit; PP, posterior probability; SAG, Sammlung von Algenkulturen at the University of Göttingen; TGV, Tetrabaenaceae-Goniaceae-Volvocaceae; UL, unicellular lineage; UTEX, University of Texas at Austin

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chlamydomonad lineages. Unicellular forms of core-*Reinhardtinia* mainly consist of *Chlamydomonas* and *Vitreochlamys*, and typically possess a *Euchlamydomonas*-type chloroplast (cup-shaped with a single axial-basal pyrenoid; Ettl, 1983). However, interrelationships among core-*Reinhardtinia* were barely resolved using 18S *r*RNA data. The phylogeny among colonial forms, and probably among unicellular forms, too, is better resolved using multigene phylogenies (e.g., Nakada et al., 2016a; Nozaki, 2003; Nozaki et al., 2000, 2003, 2006, 2014), but few unicellular forms have been included in such analyses.

In this study, 18S *r*RNA phylogeny was performed with improved unicellular taxon sampling, focusing on several unsequenced or newly isolated *Chlamydomonas*- and *Vitreochlamys*-like strains with a *Euchlamydomonas*-type chloroplast. Based on the constructed 18S *r*RNA tree, representatives of robustly supported clades or orphan strains with no robust relatives were selected and then combined 18S-*atp*B-*psa*A-*psa*B-*psb*C-*rbc*L phylogenetic analyses of the selected chlamydomonads and TGV were performed. Using light microscopy to examine the close relatives of TGV, the phylogenetic origins of colony-related characteristics are also discussed.

2. Materials and methods

2.1. Culture strains

Chlamydomonas species with a *Euchlamydomonas*-type chloroplast were obtained from the Culture Collection of Algae and Protozoa (CCAP; https://www.ccap.ac.uk/), the Microbial Culture Collection at the National Institute for Environmental Studies (NIES; http://mcc.nies.go.jp/top.jsp), and Sammlung von Algenkulturen at the University of Göttingen (SAG; http://www.unigoettingen.de/en/45175.html). Additional strains (Table S1; deposited in the NIES as NIES-4241–4256, http://mcc.nies.go.jp/) were isolated from rewetted soil samples by using the pipette-washing method (Andersen and Kawachi, 2005; Pringsheim, 1946) or from colonies formed on AF-6 agar plates (Kato, 1982; modified as in Andersen et al. (2005)). Some strains showing prokaryotic contamination were re-isolated using the pipette-washing method (Andersen and Kawachi, 2005; Pringsheim, 1946); the examined substrains are indicated with a subscript (e.g., TgA0901C4$_{p9}$ for a substrain of TgA0901C4).

2.2. DNA sequencing

For DNA extraction, PCR, and sequencing, methods previously described by Nakada et al. (2007, 2010c) were used or Tks Gflex DNA Polymerase (Takara Bio, Shiga) for PCR and/or a FastGene Gel/PCR Extraction Kit (Nippon Genetics, Tokyo) for purification of the PCR-amplified DNA fragments were used. The PCR and sequencing reactions were performed using previously published (Nakada et al., 2007, 2010b; Nakazawa and Nozaki, 2004; Nozaki et al., 1995, 1997, 1999, 2000) and newly designated (Table S2) primers.

Newly obtained sequences used in this study are available from the International Nucleotide Sequence Database Collaboration (http://www.insdc.org) under accession numbers LC380237–LC380264 for 18S rRNA (Fig. 1) and LC380265–LC380395 for chloroplast genes (Table S3). Preliminary 18S phylogeny showed that *Chlamydomonas simplex* SAG 6.93 (LC380241) and SAG 13.79 (LC380242) belong to the clade *Moewusinia* and "*Chlamydomonas subehrenbergii*" SAG 18.89 (LC214871; re-identified as a new species of *Microglena*; Nakada et al., 2018) to the clade *Monadinia*. They were therefore excluded from further analyses.

2.3. Phylogenetic analyses

The newly obtained gene sequences were unambiguously aligned with the 18S *r*RNA and 18S-*atp*B-*psa*A-*psa*B-*psb*C-*rbc*L alignments used in Nakada et al. (2008) and Nakada et al. (2016a), respectively, and putative group I and II introns (Cech, 1988) were excluded. For the 18S *r*RNA analyses, the root of the clade *Reinhardtinia* was placed on the branch leading to *Heterochlamydomonas* according to Nakada et al. (2008), and analyses of core-*Reinhardtinia* were rooted with the outgroup including *Lobomonas francei*, *L. monstruosa*, *Paulschulzia pseudovolvox*, *Vitreochlamys fluviatilis*, and *V. nekrassovii*.

Base composition homogeneity among taxa was tested with 18S *r*RNA and each codon position of the protein coding genes using the chi-squared test ($p < 0.05$) with PAUP* 4.0b10 (Swofford, 2002). Each *p*-distance between outgroup and ingroup taxa was also calculated using PAUP* 4.0b10 (Swofford, 2002).

Each data set for Bayesian inference was divided into four partitions: 18S *r*RNA genes (18S), and first, second, and third codon positions of the protein-coding chloroplast genes (cp1–cp3), and then an evolutionary model of each partition was selected using MrModeltest 2.2 (Nylander, 2004). The partition models were unlinked in each analysis. Bayesian phylogenetic analyses were performed using MrBayes 3.2 (Ronquist et al., 2012). Convergences of Markov chain Monte Carlo iterations were evaluated based on the average standard deviation of split frequencies for every 1,000,000 generations, discarding the first 25% as burn-in, and the iterations were automatically stopped when the average standard deviations were below 0.01, indicating convergence. Consequently, 1,000,000–26,000,000 generations of iterations were performed for each data set.

Maximum likelihood (ML) bootstrap analyses (Felsenstein, 1985) were performed using RAxML 8.2.7 (Stamatakis, 2014; 1000 replications) with the GTR + I + CAT (18S, full-data, position-trimmed) or GTR + I + G (taxon-trimmed, relative-position) model. Neighbor joining (NJ) bootstrap analyses were performed using PAUP* 4.0b10 (Swofford, 2002; 1000 replications using Jukes and Cantor distances).

2.4. Light microscopy

Vegetative cells were observed using 7-day-old cultures in AF-6 medium (Kato, 1982; modified as in Andersen et al., 2005), and cell divisions were observed using 6-day-old cultures in AF-6 medium (for SAG 39.72, NIES-437, and SAG 25.72) or 3-day-old cultures in MG medium (for SAG 12.93; Ichimura, 1973) in the dark period. All cultures were maintained at 20 °C under a light/dark cycle of 14:10 provided by cool white fluorescent lamps at approximately 30–100 μmol photons $m^{-2} \cdot s^{-1}$. Light and fluorescence microscopy were carried out using a Leica DM2500 microscope equipped with Nomarski interference optics (Leica, Wetzlar, Germany) and an Olympus DP71 digital camera (Olympus, Tokyo, Japan).

3. Results

3.1. 18S gene phylogeny

In the Bayesian tree of 18S *r*RNA (Fig. 1, S1), 32 unicellular lineages (ULs) were recognized within core-*Reinhardtinia*: 13 robustly supported unicellular clades (≥0.99 posterior probability [PP] and 68–100% bootstrap proportions [BPs]; UL-3–7, -11, -18, -19, -21, -24, -25, -28, and -30) and 19 orphan unicellular lineages (UL-1, -2, -8–10, -12–17, -20, -22, -23, -26, -27, -29, -31, and -32) whose unicellular relatives were not robustly resolved (< 0.95 PP and 90% BP at most). Of the recognized lineages, three (UL-1, -6, and -23) were not available from major culture collections such as CCAP, NIES, SAG, or the Culture Collection of Algae at the University of Texas, Austin (UTEX; https://utex.org/), and were therefore excluded from the multigene analyses.

Interrelationships among the unicellular and colonial lineages were barely resolved (< 0.95 PP) within core-*Reinhardtinia*, except for "*C. reinhardtii*" JinCheon-1 (UL-1), which was confirmed as the sister to *Eudorina illinoisensis* (Volvocaceae; 0.99 PP and 99% BPs). Monophyly of Tetrabaenaceae was supported (1.00 PP and 100% BPs), but that of Goniaceae and that of Volvocaceae were not recovered.

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