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Molecular markers from different genomic compartments reveal cryptic diversity within glaucophyte species

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ABSTRACT

Glaucophytes are the least studied of the three major Archaeplastida (Plantae *sensu lato*) lineages. It has been largely recognized that comprehensive investigations of glaucophyte genetic and species diversity will shed light on the early evolution of photosynthetic eukaryotes. Here we used molecular phylogenetics and genetic distance estimations of diverse molecular markers to explore strain and species diversity within the glaucophyte genera *Cyanophora* and *Glaucocystis*. Single gene and concatenated maximum likelihood analyses of markers from three different genetic compartments consistently recovered similar intrageneric genetic groups. Distance analyses of plastid (*psb*A and *rbc*L) and mitochondrial (*cob* and *cox*1) genes, and the nuclear internal transcribed spacer (ITS) region, revealed substantial genetic divergence between some *Cyanophora paradoxa* and *Glaucocystis nostochinearum* strains. The genetic distances estimated between some glaucophyte strains currently considered the same species are similar or greater than divergence values calculated between different species in other unicellular algae, such as certain green algae and diatoms. The analyzed molecular markers are prospective candidates for future studies of species diversity in glaucophytes. Overall, our results unveil previously unrecognized cryptic diversity within *Cyanophora* and *Glaucocystis* species.

1. Introduction

The glaucophytes (Glaucophyta Skuja, 1954, or Glaucocystophyta; Kies and Kremer, 1986), green plants (land plants and green algae; Viridiplantae Cavalier-Smith, 1981) and red algae (Rhodophyta Wettstein, 1922) are the three groups of photosynthetic eukaryotes bearing primary plastids formally united in the "supergroup" Archaeplastida (Adl et al., 2005, 2012). The Archaeplastida are monophyletic in diverse molecular analyses, supporting the hypothesis for the unique origin of primary plastids as a result of endosymbiosis between a heterotrophic eukaryote and a photosynthetic cyanobacterium (Burki et al., 2008; Hackett et al., 2007; McFadden and van Dooren, 2004; Palmer, 2003; Price et al., 2012; Rodríguez-Ezpeleta et al., 2005). However, conflicting phylogenetic results have been interpreted as evidence of separate origins (Howe et al., 2008; Stiller, 2003). Understanding the origin and diversity of the Archaeplastida lineages is fundamental to comprehending the evolution of eukaryotes as primary producers (Bhattacharya et al., 2004; Keeling, 2010; Reyes-Prieto et al., 2007).

Genomic information from Viridiplantae is relatively abundant and the amount of red algal data has increased substantially in recent times (Bhattacharya et al., 2013; Collén et al., 2013; Matsuzaki et al., 2004; Schönknecht et al., 2013). However, glaucophyte genomic data is currently very limited with only one published genome (Price et al., 2012). Additionally, species diversity within glaucophytes is poorly understood, as are the phylogenetic relationships among the different genera. This limited sampling and understudy of glaucophytes restricts our ability to discern between different hypotheses regarding the evolution of primary photosynthetic eukaryotes, such as the presumed unique origin of Archaeplastida and the internal relationships between the three major lineages (Burki et al., 2012; Chan et al., 2011; Reyes-Prieto and Bhattacharya, 2007). Further, limited taxon sampling may bias phylogenetic inferences towards erroneous monophyletic associations (Heath et al., 2008; Hedtke et al., 2006).

Glaucophytes are freshwater unicellular or colonial organisms bearing blue-green plastids historically referred as "cyanelles" or

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"muroplasts" (Hall and Claus, 1963; Löffelhardt and Bohnert, 2004). These plastids are characterized by both a remnant peptidoglycan cell wall between the two plastid membranes, and by carboxysome-like structures localized in the organelle stroma (Löffelhardt and Bohnert, 2004). Both plastid structures are presumably ancestral traits derived from their cyanobacterial relatives and conserved in glaucophytes during the evolution of the group. Several molecular phylogenetic studies using single gene (Bhattacharya and Schmidt, 1997; Bhattacharya et al., 1995; Helmchen et al., 1995; Marin et al., 1998; Maruyama et al., 2008; Yang et al., 2011) and multiple-gene (Burki et al., 2008; Hackett et al., 2007; Reeb et al., 2009; Rodríguez-Ezpeleta et al., 2005) data sets have shown that glaucophytes form a monophyletic group. However, despite the importance of glaucophytes in understanding early evolution of photosynthetic eukaryotes, morphological and molecular studies investigating diversity within this algal group are scarce. Genome-scale comparative analyses have included sequence data from only two taxa: *Cyanophora paradoxa* (strain CCMP 329) and *Glaucocystis nostochinearum* (strain UTEX 64) (Burki et al., 2012; Price et al., 2012; Rodríguez-Ezpeleta et al., 2005) and although dozens of glaucophyte strains are available in algal culture collections, only few have been studied. The vast majority of available glaucophyte isolates have no sequence data in public databases, and so the extent of genetic variation at genus and species levels is unknown.

To distinguish between alternative scenarios regarding the diversification history of Archaeplastida, we require comprehensive studies of diversity within the glaucophytes, and generation of genomic data from a broad glaucophyte taxon sampling. In this study we address a basic question: what is the level of genetic diversity within glaucophyte genera and species? We use both molecular phylogenetics and DNA barcode-like methods to investigate levels of genetic divergence between 17 glaucophyte strains from different culture collections, and four novel *Glaucocystis* isolates from North America and Korea.

2. Material and methods

2.1. Glaucophyte strains and cell cultures

A total of 17 glaucophyte strains were obtained from the Algal Culture Collection of the University of Göttingen (SAG, Germany), the Microbial Culture Collection at the National Institute for Environmental Studies (NIES, Japan), and The Culture Collection of Algae of the University of Texas (UTEX, USA) (supplemental Table S1). The analyzed strains included three *Cyanophora* species (5 strains total), two *Cyanoptyche gloeocystis* strains, one *Gloeochaete wittrockiana* isolate and 9 strains originally identified as *Glaucocystis nostochinearum*. Additionally, four novel *Glaucocystis* sp. strains were collected using a 20 μm mesh plankton net (Bokyeong co., Pusan, Korea) from a pond at Boothbay Railway Village, Boothbay Maine, USA (strain BBH), Sportsmans Lake, Friday Harbor, Washington, USA (strain HS30), and Samhogi, Taean, Chungnam, Korea (strains KRJ1 and KRJ2). Individual cells were isolated under an inverted microscope (Leica DMI 3000B, Wetzlar, Germany) using mouth-picking techniques. Cell cultures from single isolates were established and maintained in liquid DY-V culture media (Andersen et al., 1997) at 18 °C on a 14/10 h light/dark cycle.

2.2. DNA extraction, gene amplification and sequencing

Cells were harvested at stationary phase by centrifugation for 15 min at 1800–2000*g*. We extracted total DNA from all strains following the protocol for fresh plant tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Sequences of different molecular markers were amplified by PCR. A region of the plastid gene *psb*A (D1 protein of the photosystem II) was amplified with different combinations of the forward primers psbAF1, psbAFn2 or psbAF91 with the reverse primer psbAR2 (Yoon et al., 2002) using a denaturation step at 94 °C for 4 min, then 38 cycles of 94 °C for 1 min, 46–50 °C for 30 s and 72 °C for 2 min, concluding with a 10 min extension at 72 °C. Partial sequence of the mitochondrial gene *cob* (apocytochrome *b*) was amplified using alternative combinations of the forward cobF81 and cobF199 primers and the cobR1054 and cob1084 reverse counterparts. The protocol for *cob* amplification comprised an initial denaturation step at 98 °C for 40 s, then 31 cycles of 98 °C for 10 s, 48 °C for 30 s and 62 °C for 30 s, with a final extension of 10 min at 72 °C. The nuclear internal transcribed spacer (ITS) region was amplified using the primer combination P1 and G4 (see Saunders and Moore, 2013) for regular PCR or nested reactions with the primer pair GlaITSF1 and GlaITSR1. Conditions for ITS amplification comprised an initial denaturation step at 94 °C for 4 min, followed by 38 cycles of 94 °C for 1 min, 45 °C for 45 s and 72 °C for 2 min with a final extension of 10 min at 72 °C. Additionally, we attempted amplification of the 5′ region of the mitochondrial gene *cox*1 (CO1-5P) using the primer pair GWSLF2 and GWSRx (Saunders and Moore, 2013), and the nuclear 18S rRNA region using alternative combinations of the forward primers 18SFn2 or 18SFn42 with the reverse primers 18SRn1760 and 18SRn1772. PCR products were purified and either directly sequenced or cloned (CloneJET PCR Cloning Kit, Fermentas) prior to sequencing by standard dye-terminator methods. Sequences of all primers used are provided in supplemental Table S2. Finally, complete sequences of the plastid gene encoding the ribulose-1,5-bisphosphate carboxylase: oxygenase large subunit (*rbc*L) and the mitochondrial gene *cox*1 of particular *Cyanophora* species (supplemental Table S3), obtained from parallel DNA sequencing by synthesis (Illumina) projects in our lab, were used for additional pairwise distance estimations (Section 2.4). GenBank accession numbers of DNA sequences generated are provided in supplemental Table S1.

2.3. Phylogenetic analysis

DNA sequences were aligned with MAFFT v7 (Katoh and Standley, 2013) and manually refined using Se-Al v2.0a11 (http://tree.bio.ed.ac.uk/software/seal/). Regions with missing data on both ends of the multiple DNA alignments were excluded from further analyses. All multiple alignments are available upon request. The best-fit nucleotide substitution model for each multiple alignment was selected with JModelTest 2.1.4 (Darriba et al., 2012). Maximum likelihood (ML) trees were estimated using the corresponding best-fit substitution model (AIC criterion) and using the nearest neighbor interchange (NNI) topology improvement implemented in PhyML 3.0 (Guindon et al., 2009). Branch support was assessed with 500 non-parametric bootstrap replicates. Bayesian posterior probabilities were calculated with MrBayes 3.2.1 (Ronquist et al., 2012) using the GTR + I + Γ substitution model running a Metropolis-coupled Markov Chain Monte Carlo (MC3) for 2 million generations. Two independent MC3 runs were performed simultaneously starting from different random trees. Chain convergence was evaluated every 150,000 generations, until the average standard deviation of split frequencies dropped below 0.01. Trees were sampled every 100th generation. Final posterior probabilities were estimated after discarding trees from the first 500,000 generations. Trees were visualized and graphic versions prepared with Archaeopteryx beta v0.997 (Han and Zmasek, 2009).

2.4. Pairwise genetic distance estimation

In order to quantify the amount of genetic divergence in glaucophyte species and strains, we generated matrices of uncorrected