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Two Freshwater Cryptomonads New to Korea: *Cryptomonas marssonii* and *C. pyrenoidifera*

Jee-Hwan Kim, Sung Min Boo and Woongghi Shin*

Department of Biology, Chungnam National University, Daejeon 305-764, Korea

We described two brownish freshwater *Cryptomonas* species, *C. marssonii* Skuja and *C. pyrenoidifera* Geitler as first records in Korea. The identification was based on light microscopy, scanning electron microscopy, and nuclear SSU rDNA sequences analysis. *Cryptomonas marssonii* is characterized by its sigmoid shape with a sharply pointed and dorsally curved antapex, dorso-ventrally flattened cell, two lateral plastids without pyrenoid, and its dimension of 18-25 μm in length and 8-13 μm in width. *Cryptomonas pyrenoidifera* is characterized by ovoid to elliptical shape with a partially twisted or rounded antapex, dorso-ventrally biconvex cell, lateral plastids with two pyrenoids, and the dimensions of 15-22 μm in length and 10-14 μm in width. Nuclear SSU rDNA sequences between *C. marssonii* WCK01 from Korea and CCAC0086 from Gernmay, and between *C. pyrenoidifera* WCK02 from Korea and CCMP152 from Australia were identical, respectively.

Key Words: *Cryptomonas marssonii, C. pyrenoidifera*, Cryptophyceae, morphology, nuclear SSU rDNA sequences

INTRODUCTION

Cryptomonads (Class Cryptophyceae) are biflagellate, unicellular organisms with approximately two hundred species in many kinds of aquatic habitats (Cerino and Zingone 2006). They make nuisance blooms both in marine and freshwater ecosystems (Nishijima 1990; Novarino 2003). They are easy to recognize due to their peculiar behavior of swaying swimming and unique plastid complex derived from eondosymbiosis with a red alga (Douglas and Penny 1999). Cryptomonads contain only phycoerythrin of the red algal phycobilisomes and a highly reduced endosymbiont nucleus known as nucleomorph. The presence of a secondary nucleus and the distinctive pigment reflects the complex evolutionary history of cryptomonads (Douglas *et al.* 2001; Archibald 2007).

Cryptomonas (Ehrenberg 1831) is a unicellular freshwater genus that often forms mucilaginous palmelloid colonies. The genus is characterized by having the furrow-gullet complex, two chloroplasts and purple phycoerythrin 566 as an accessory pigment (Butcher 1967; Clay *et al.* 1999; Hoef-Emden and Melkonian 2003). In addition, *Cryptomonas* exhibits two different cell types within a clonal culture: one cryptomorph type posses an inner periplast component (IPC) consisting of polygonal to oval-shaped plates, and the other campylomorph type posses a sheet-like inner periplast component (Hill 1991; Hoef-Emden and Melkonian 2003; Hoef-Emden 2007). Classification system of the genus was based mainly on morphological criteria such as cell size, cell shape, furrow/gullet system, periplast structure, and internal anatomy (Novarino and Lucas 1993; Clay *et al.* 1999; Deane *et al.* 2002). However, it is difficult to identify cryptomonad species because of their small size, brittleness, and less maintenance of type materials. Recently, DNA sequence data have been used to test hypotheses on cryptomonad diversity (Novarino and Lucas 1993; Clay *et al.* 1999) and to describe five species of the genus (Hoef-Emden 2007).

To date, two species of freshwater *Cryptomonas* has been recorded in Korea by floristic study (Chang 1981). However, morphology and relative importance of the genus in Korea are scarcely known. We reported two brownish freshwater cryptomonads, *Cryptomonas marssonii* and *C. pyrenoidifera* as a first record in Korea. They were examined by light microscopy, scanning electron microscopy, and nuclear small subunit ribosomal DNA (SSU rDNA) sequences.

*Corresponding author (shinw@cnu.ac.kr)

MATERIALS AND METHODS

Algal cultures and microscopy

Specimens were collected from various freshwater habitats in Korea during October 2006 to March 2007. Live cells were isolated by micropipette technique and the isolates were grown in f/2 medium (Guillard 1975). The clonal cultures were maintained at 22 ± 1°C on a 14:10h LD photoperiod using cool-white fluorescence lamps with illuminations of 30-μmol m^{-2} sec^{-1}. The morphology was examined with a light microscope (Nikon Eclipse 80i, Nikon) and scanning electron microscope (JSM-7000F, Jeol). For SEM, cells were fixed in 2.5% glutaraldehyde in culture medium for 1 h, collected on 2.5 μm pore size filters, and rinsed in distilled water. Then, the cells were post fixed in 1% osmium tetroxide for 1 h, dehydrated through an alcohol series (50%, 60%, 70%, 80%, 90%, and 100% ethanol), and critical point dried. Dried cells were mounted on stubs, coated with gold and observed under a SEM (Cerino and Zingone 2006). Species identification was confirmed based on nuclear SSU rDNA sequence.

Analyses of nuclear SSU rDNA sequence

Live cells were harvested by centrifugation and total genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen Gmbh, Hilden, Germany), according to the manufacturers' protocol. Nuclear SSU rDNA region was amplified using CrN1F and modified BR primers and sequenced using CrN1F, 826F, 956R, and modified BR primers of Hoef-Emden *et al.* (2002). PCR was carried out under the following conditions: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturing at 95°C for 1 min, annealing at 47°C for 1 min, and elongation at 72°C for 2 min, followed by a final extension at 72°C for 6 min. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocols. The bi-directional sequences were determined for all specimens using an ABI PRISM^{TM} 3730 xl DNA Sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). GenBank accession numbers for nuclear SSU rDNA analyzed in this study are EU163586 for *Cryptomonas marssonii* and EU163587 for *C. pyrenoidifera*.

The alignment of 19 nuclear SSU rDNA sequences, two new sequences determined here and 17 previous known sequences from GenBank, were refined by eye in the Genetic Data Environment (GDE 2.2) program (Smith *et al.* 1994). The entire conserved areas of the SSU rDNA genes were readily alignable across taxa, and were used for phylogenetic analyses.

Phylogenetic trees were constructed using maximum parsimony (MP), maximum likelihood (ML), and Bayesian analyses. MP analysis was conducted with PAUP* (Swofford 2002) using a heuristic search algorithm with the following settings: 100 random sequence-addition replicates, tree bisection-reconnection (TBR) branch swapping, MulTrees, all characters unordered and unweighted, and branches with a maximum length of zero collapsed to yield polytomies. The bootstrap values (BS) for the resulting nodes were assessed using bootstrapping with 1000 replicates (Swofford 2002).

For ML and Bayesian analyses, evolutionary best-fit model (TrN+I+G) was selected by ModelTest 3.7 (Posada and Crandall 1998) under the Hierarchical Likelihod Ratio Tests (hLRTs) and Akaike Information Criterion (AIC). ML analysis was performed with PAUP* (Swofford 2002) by heuristic search with 100 random sequence-addition replicates, TBR branch swapping, and MulTrees options. Bootstrapping (BP) analysis was preformed with 1,000 replicates.

Bayesian analysis was performed with MrBayes 3.2 (Huelsenbeck and Ronquist 2001). Each analysis was initiated from a random starting tree, and the program was set to run four chains of Markov chain Monte Carlo iterations simultaneously for 2,000,000 generations. Tree and parameters were sampled every 100 generations and the first 800 trees were burned to ensure that they had stabilized. A majority rule consensus tree was calculated from the remaining trees to examine the posterior probabilities of each clade.

RESULTS AND DISCUSSION

Cryptomonas marssonii Skuja 1948

Specimens examined: Ojeong pond, Naju, Korea (Nov. 04, 2006; WCK01); Daepyeong swamp, Haman, Korea (Mar. 02, 2007); Gahang swamp, Haman, Korea (Mar. 02, 2007); Jillal swamp, Haman Korea (Mar. 02, 2007).

Light microscopy: Cells are 18-25 μm in length and 8-13 μm in width, and are ovoid to asymmetrical in ventral or dorsal view. The cells have two lateral plastids without a pyrenoid and contain a contractile vacuole seen in ventral view (Fig. 1A). In dorsal view, the anterior end of the cell is a slightly protuberant shape (Fig. 1B).

Electron microscopy: Two subequal flagella emerge

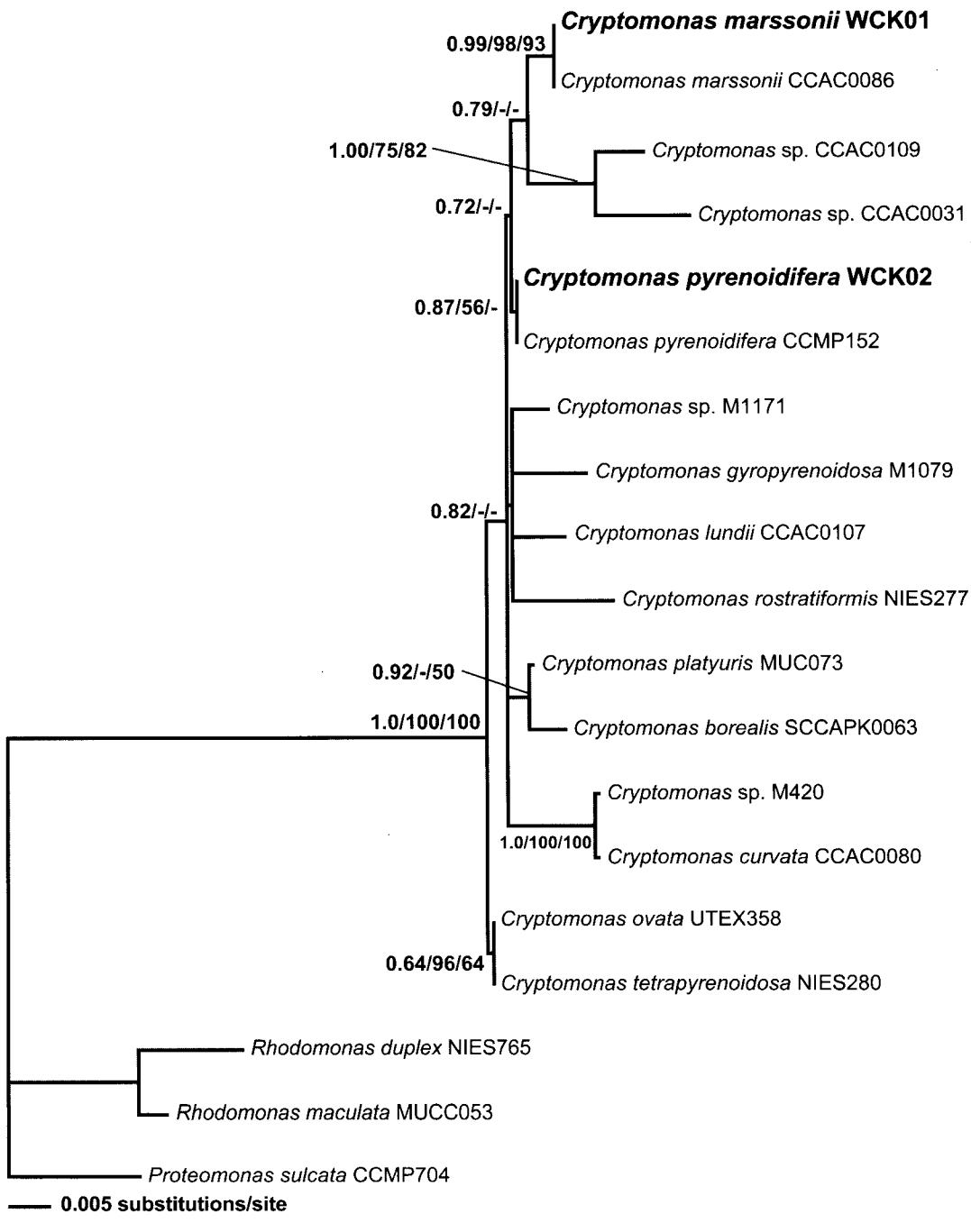

Fig. 3. Rooted Bayesian tree for *Cryptomonas* inferred from nuclear SSU rDNA sequences data (TrN + I + G model). -InL = 3848.4033, proportion of invariable sites (I) = 0.7226, gamma distribution substitution rates (G) = 0.6745. Species designations in bold face label newly determined here. Bootstrap values in order form left to right: Bayesian posterior probabilities/maximum parsimony/ML. Dashed indicate < 50% bootstrap or < 0.5 Bayesian posterior probabilities.

Korea was 0.6%. Sequence divergence between *Cryptomonas* species from Korea and other countries ranged from 0.4% (between *C. pyrenoidifera* WCK02 and *C. ovata* UTEX358) to 2.1% (between *C. marssonii* WCK 01 and *Cryptomonas* sp. CCAC0031).

The phylogenetic tree constructed by MP, ML and Bayesian analyses resulted in similar topologies as their composition (Fig. 3). All taxa within *Cryptomonas* were monophyletic (1.0/100/100 for BA/MP/ML), as in a previous study (Hoef-Emden 2007). However, *C. pyrenoidifera* and *C. marssonii* were not grouped together (Hoef-Emden 2007).

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