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# Revisiting *Chrysococcus* (Chrysophyceae): new phylogenetic evidence and evolutionary implications

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**Abstract:** Loricate golden algae (Chrysophyceae) are photosynthetic microorganisms characterized by a lorica, a rigid or semi–rigid protective casing made of organic material, sometimes reinforced with silica or iron. The lorica's diverse shapes and intricate ornamentation serve as both adaptive strategies and taxonomic markers. Here, we identified, for the first time, the molecular phylogenetic position of a loricate genus *Chrysococcus*, based on genetic investigations of two freshwater populations in Poland. The genus was resolved to form a well–supported clade with *Chrysosaccus* within the order Chrysosaccales. Accordingly, this order represents one of the morphologically most diverse lineages of Chrysophyceae, including naked flagellates, coccoid organisms, amoebae and flagellates dwelling in loricae, and mucilage–secreting cells. The phylogenetic resolution of *Chrysococcus* provides key evidence for understanding the evolutionary transitions within Chrysophyceae, highlighting the complex relationships between loricate and non–loricate taxa.

**Keywords:** Chrysophyceae, Chrysosaccales, evolutionary relationships, freshwater plankton, loricate golden algae

## Introduction

Loricate golden algae, belonging to the class Chrysophyceae, are a fascinating group of photosynthetic algae characterized by the presence of a protective structure known as a lorica. The lorica is rigid or semi–rigid casing that surrounds the cell, offering protection and often aiding in buoyancy. It is typically composed of organic materials, sometimes reinforced with silica or iron (Starmach 1985; Malavasi et al. 2024). It can vary greatly in shape and have intricate ornamentation such as pores, ridges, or spines. For instance, in *Dinobryon*, it is vase– or beaker–shaped; in *Lagynion*, it is flask–shaped; and in *Chrysococcus*, it is globular with pores (Kristiansen & Škaloud 2017). The diversity in lorica morphology not only reflects the adaptive strategies of different species but also provides valuable taxonomic markers for identifying and classifying these algae.

Members of Chrysophyceae thrive primarily in freshwater environments, although some species are also found in brackish or marine habitats (e.g. *Dinobryon balticum* (Schütt) Lemmermann; Throndsen 1996). They play a significant role in contributing to primary production and forming a part of the planktonic community (Andersen 2004; Kristiansen & Škaloud 2017). Their ability to form colonies and the structural complexity of their loricae also make them important subjects of study in evolutionary biology, ecology, and paleontology (Piątek et al. 2020; Jeong et al. 2023). The intricate interplay between their morphology, ecology, and evolutionary history continues to intrigue researchers, making loricate golden algae a key group for understanding the diversity and adaptability of protists in aquatic environments.

During the investigation of small, eutrophic ponds at two localities – Duchnice and Jelonki (Poland), we discovered in the samples an organism with a globular lorica bearing a pore that initially resembled a member of the euglenid genus *Trachelomonas*, characterized by a

rusty lorica. However, after breaking the lorica, we observed golden chloroplasts, making it clear the organisms represent the genus *Chrysococcus* Klebs. Subsequently, two gatherings of cells ('Jelonki' and 'Duchnice') were established, allowing us to characterize these specific strains in detail using both genetic and morphological approaches without the requirement of obtaining a growing culture.

*Chrysococcus* was described by Georg Albrecht Klebs, a German botanist, in 1892, based on the species *Chrysococcus rufescens*. This genus, as other chrysomonads, is characterized by including unicellular planktonic, golden–brown algae surrounded by globular lorica encrusted by deposits of manganese or iron (Preisig 1986; Heinrich et al. 1986; Dunlap et al. 1987; Nicholls & Wujek 2015). The genus is characterized by the presence of both a long and a short flagellum, with the long flagellum extending through a pore in the lorica. This feature differentiates it from *Kephyrion*, which has a significantly larger opening.

A total of 31 *Chrysococcus* species are currently recognized as taxonomically accepted (Guiry & Guiry 2024). Key characteristics for distinguishing taxa include lorica shape and size, ornamentation, number of pores, and the presence or absence of stigma and pyrenoids. All species are planktonic and commonly found in freshwater habitats, such as ponds, lakes, and rivers, but also in some brackish environments (Starmach 1985; Nicholls & Wujek 2015). *Chrysococcus* primarily reproduces asexually, usually through cytokinesis. In some species, stomatocysts (statospores) develop inside the lorica or after the cell has escaped from it, often remaining attached (Pascher 1914). Sexual reproduction has not been observed so far.

Despite significant advancements in the field, molecular genetic data are still lacking for many morphologically well–defined Chrysophyte genera, hindering comprehensive phylogenetic analyses and a deeper understanding of their evolutionary relationships (Stancheva et al. 2019; Pusztai et al. 2023; Remias et al. 2020; Pusztai & Škaloud 2019, 2022; Malavasi et al. 2024). To our knowledge, no molecular phylogenetic analysis of *Chrysococcus* including its type species, *C. rufescens*, has been performed to date. This study aims to elucidate the phylogenetic position of the genus *Chrysococcus* for the first time, contributing to a more comprehensive understanding of its evolutionary history and classification.

## Materials and Methods

**Sampling and cell isolation.** Environmental samples were collected in June and August 2022 from two locations in Poland, district of Warsaw: Schnajdar's Pond in Jelonki (52°13'04.0" N, 20°54'40.6" E) and a pond in Duchnice village (52°11'43.0" N, 20°48'03.0" E). Samples were collected using a plankton net with mesh size 10 µm and screened for diversity. About 100–120 morphologically identical *Chrysococcus* cells were isolated from each sample using a micromanipulator (MM–89 Narishige) with a micropipette attached and mounted on a Nikon Ni–U microscope (Nikon, Tokyo, Japan). The obtained isolates were then transferred through several drops of sterile modified f/2 medium without seawater (Guillard & Ryther 1962; Guillard 1975) for sample purification and frozen at –80 °C until DNA isolation.

**Documentation and observation.** Light microscope observations, photographs and video clips were made using a NIKON Eclipse E–600 microscope with differential interference contrast, equipped with the software NIS Elements BR v.3.1 (Nikon) and NIKON DX–1200 digital camera for image recording and processing. The ultrastructure of lorica was examined with FE–SEM ZEISS Ultra Plus (ZEISS Oberkochen, Germany) scanning electron microscope (SEM). Samples from both sites were dropped onto a small piece of aluminium foil using the micromanipulator (see above), dried, rinsed with five drops of distilled water and dried again. The samples were then attached to the aluminium stubs using double–sided carbon tape and cleaned with RF plasma (Evactron) for 10 min before SEM analysis. SEM images were acquired at an accelerating voltage of 5 kV at low probe current (about 15 pA) using an InLens secondary electron detector with SmartSEM software.

**DNA extraction, PCR amplification and sequencing.** DNA extraction was performed using the Chelex 100 (Bio–Rad, Hercules, California, USA.) chelating resin according to the protocol in Fells et al. (2023). The nuclear–encoded SSU rDNA was amplified using the primers 18S–F (5'–AAC CTG GTT GAT CCT GCC AGT–3') and 18S–R (5'–TGA TCC TTC TGC AGG TTC ACC TAC G–3') (Katana et al. 2001). The PCR amplification was carried out under the following conditions: initial denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 2 min; final extension at 72 °C for 10 min.

The chloroplast–encoded rbcL was amplified using the primers Chrys–F2 (5'–TTA TTA ACW GCT TGT GAT– 3') and Chrys–R (5'–TCC ATR TCR AAG AAA ATW CC–3') (Škaloudová & Škaloud 2013). The PCR amplification was carried out under the following conditions: initial denaturation at 94 °C for 4 min; 40 cycles of denaturation at 94 °C for 1 min, annealing at 40 °C for 1 min, and elongation at 72 °C for 1:30 min; final extension at 72 °C for 10 min. The quality and yield of the PCR products were checked under UV light using 1% agarose gel containing ethidium bromide. Amplified PCR products were purified using Exosap–IT (Life Technologies Corporation, Carlsbad, CA, USA). PCR products were sequenced at Macrogen (Amsterdam, Netherlands). Sequences were deposited in GenBank under the accession numbers PQ635348, PQ635349, and PQ635350.

**Phylogenetic analyses.** The newly determined SSU rDNA and rbcL sequences were aligned to other sequences of Chrysophyceae from the GenBank database, including all closely related sequences according to BLAST searches. The SSU rDNA sequences were aligned using MAFFT v. 6 software (Katoh et al. 2002) and poorly aligned positions were removed. rbcL sequences were manually aligned using MEGA 6 (Tamura et al. 2013). The site–stripping method was used to remove over–saturated nucleotide positions from the rbcL dataset according to Škaloud et al. (2013). A concatenated 2,606 bp long SSU rDNA and rbcL alignment was produced, including

sequences from a total of 83 chrysophytes plus two outgroup taxa – *Synchroma grande* and *Nannochloropsis limnetica* (Supplementary Table S1). Prior to performing the concatenated phylogenetic analysis, maximum likelihood (ML) analyses were performed separately for each locus in RAxML 8.1.20 (Stamatakis 2014) to verify there were no obvious topological incongruencies among the loci. The final phylogenetic trees were inferred by ML in RAxML 8.1.20 and Bayesian inference (BI) using MrBayes version 3.2.6 (Ronquist et al. 2012). The analyses were carried out on partitioned datasets (SSU rDNA and three codon positions of the rbcL gene) using the GTR+I+Γ substitution models. In BI analysis, two parallel MCMC runs were carried out for 10 million generations, each with one cold and three heated chains. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was assessed during the run by calculating the average standard deviation of split frequencies (SDSF). Finally, the burn–in value was determined using the 'sump' command. In ML analysis, we applied the hybrid parallelization on four threads. Bootstrap analyses were performed with the rapid bootstrapping procedure using two independent runs and 1,000 pseudoreplicates.

## Results

### Phylogenetic analyses

The tree topologies inferred from the Bayesian and ML analyses of a concatenated SSU rDNA and rbcL dataset were generally congruent, resolving that *Chrysococcus* is a member of the well–supported order Chrysosaccales (PP = 1.00, MLBS = 99). The genus *Chrysococcus* forms a well–supported monophyletic lineage (PP = 1.00, MLBS = 92) in a sister position to the genus *Chrysosaccus* (Fig. 1). *Chrysococcus* populations from Jelonki and Duchnice were genetically identical in their 18S rDNA gene, while the rbcL gene exhibited a 3% difference.

### Morphology

According to the SEM and LM morphological investigations, both investigated populations correspond well to the description of *Chrysococcus triporus* B. Mack. The lorica of 'Duchnice' population was spherical, smooth, 5.6–6.9 µm in diameter and light rusty (Figs 2–6, 10, 12, 15–16), containing a monad with a single, parietal, gold, bilobed plastid with a stigma and locomotive flagellum (Figs 2–6, Video S1). The lorica of 'Jelonki' population was a little larger (6.6–7.7 µm in diameter) and dark rusty (Figs 7–8, 9, 11, 13–14), containing a monad with the same morphology as the Duchnice population (Figs 7–8). These slight differences in the size and color of the lorica were most likely due to ontogeny or environmental conditions. Both populations possessed a unique pore arrangement – two pores lying on opposite poles (Figs 4, 9–12) plus a third pore in close proximity to the apical pore (Figs 13–16). Moreover, there was a small apical wart surrounding one of apical pores (Figs 13–16). Based on SEM and LM pictures we observed that the number of pores depends on the age of the lorica what is well visible, especially in the case of the 'Duchnice' population (see Figs 5–6 LM imagine – lorica with 4 pores).

## Discussion

From a phylogenetic perspective, the order Chrysosaccales Bourrelly 1957, which newly includes the genus *Chrysococcus*, occupies a distinct and well–supported position within the Chrysophyceae (Kristiansen & Škaloud 2017). The order Chrysosaccales includes a morphologically very diverse group of taxa that have experimented with different types of cell envelopes (Starmach 1985; Kristiansen & Škaloud 2017). It is encompassing the *Ochromonas*– or *Chromulina*–like naked flagellates, the coccoid *Chrysosphaera*, the amoeboid loricate *Lagynion* or sister lineage of *Chrysococcus*, the genus *Chrysosaccus* with cells embedded in mucilage. Moreover, mucilage–secreting cells of *Chromophyton rosanoffii* have an unusual phenology and form well–known summer neustonic (palmelloid) layer. However, all the representatives of the genus *Chrysococcus* described so far produce lorica. *Chrysococcus triporus* is characterized by three pores with specific locations. However, the number of pores may not be a completely stable taxonomic character, as the populations we studied also exhibited four pores. In contrast, pore position appears to be a stable and taxonomically valuable trait. Mack (1951) in his description of *C. triporus* clearly states that in addition to the two pores lying on opposite poles, there is a third pore in close proximity to the apical pore, which he illustrates in the published drawings. There are several other later–described *Chrysococcus* taxa with three or four pores that differ in this very characteristic feature.

*Chrysococcus matvienkoae* Kapustin, *C. quadriporus* Hortobágyi and *C. minutus* var. *multipora* Wawrik do not have any of the pores located near another or in pairs. As mentioned in the introduction, the apparent close similarity of some *Chrysococcus* species with darker lorica not allowing direct observation of the protoplast to the genus *Trachelomonas* has puzzled many researchers. It is therefore not surprising that the latter species *C. minutus* was originally described as *Trachelomonas volvocina* f. *minuta* Fritsch (see p. 603 and fig. 43F in Fritsch 1918). The two pores located at opposite poles are clearly visible in his drawing, and he discusses the potential role of the second pore. Fritsch observed very dense populations of this taxon in numerous African water bodies, making the discovery of similarly dense populations in Poland particularly noteworthy.

In 2019, Kapustin clarified the nomenclature of triporous *Chrysococcus* taxa in the form of a short note. Although he does not provide further details in the text, this revision is entirely consistent with our observations presented and discussed here. Accordingly, *C. triporus* Mack should be recognized as a separate species with unique lorica ultrastructure. Both synonymous taxa *C. rufescens* f. *triporus* J.W.G. Lund and *C. lundianus* Lilitskaya et Klochenko show similar size (7–9 µm) and identical pore arrangement with a small apical wart surrounding one of pores. Kapustin also proposed replacing the name *C. triporus* Matvienko by

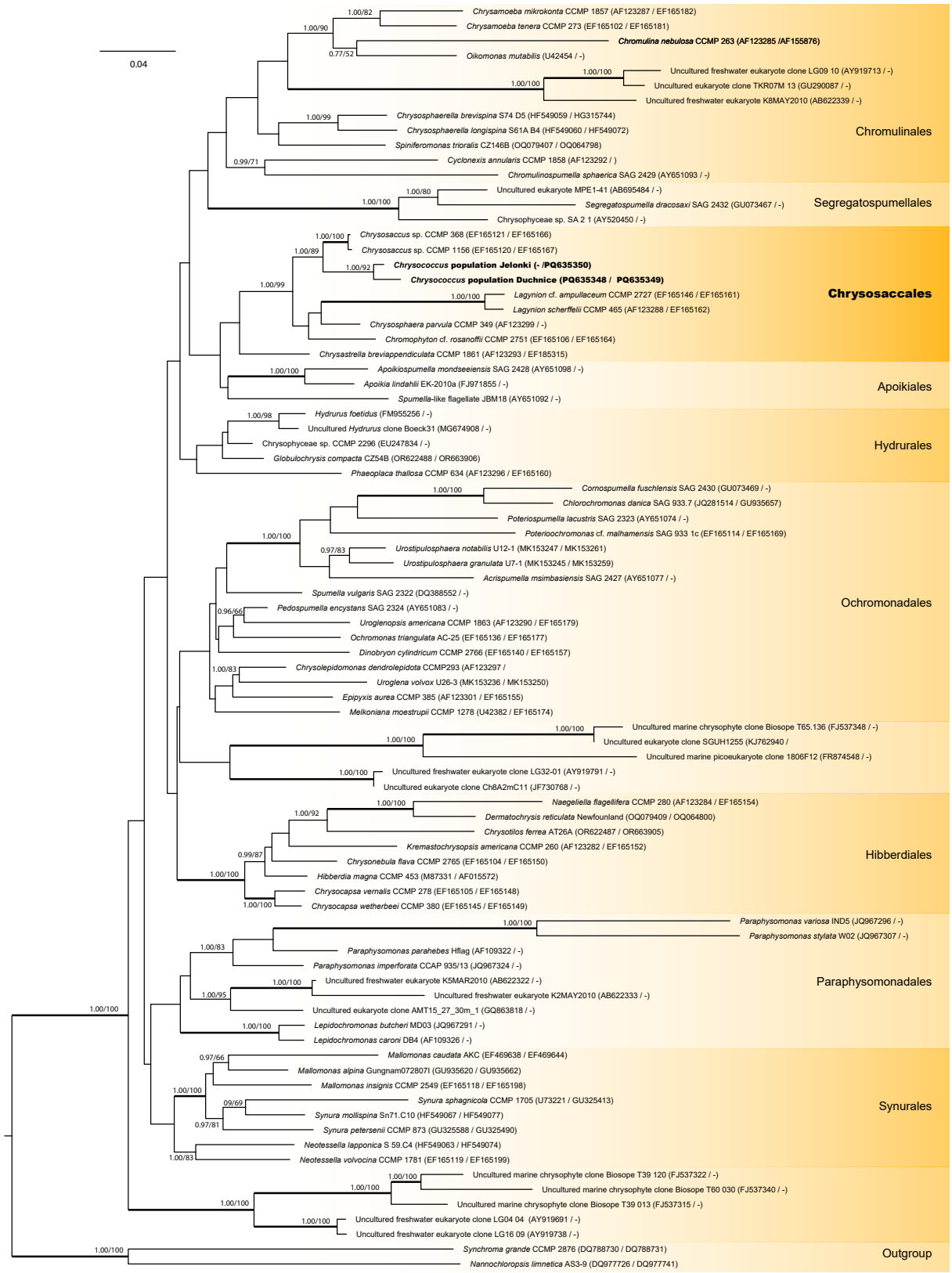
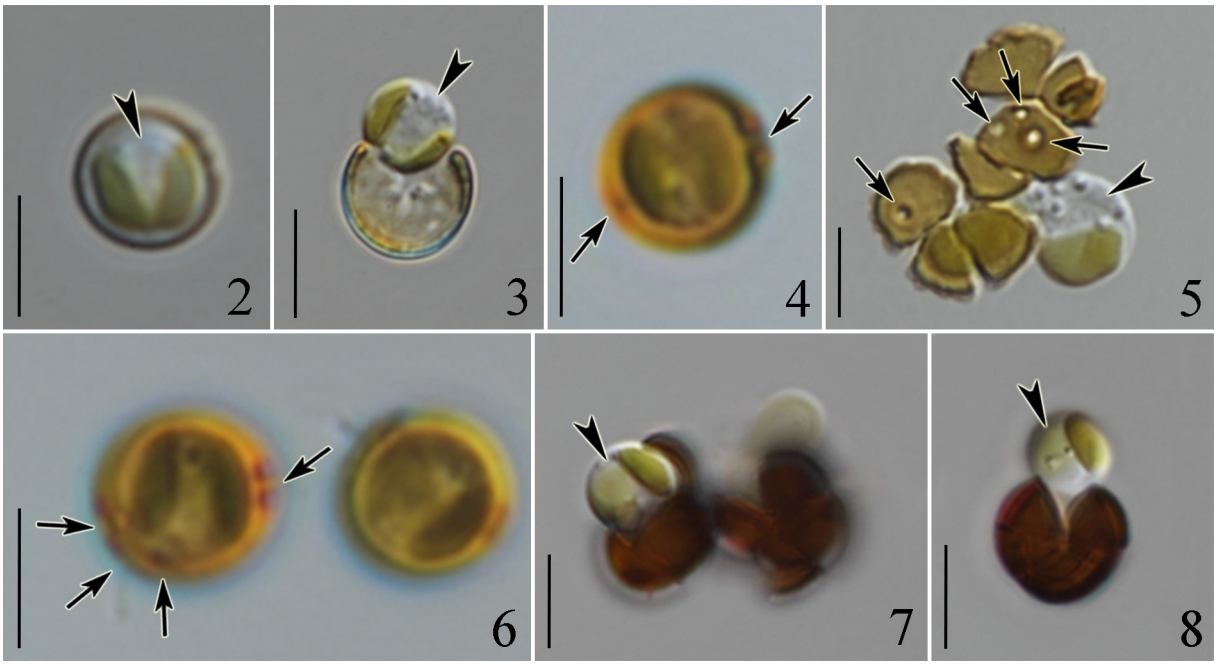
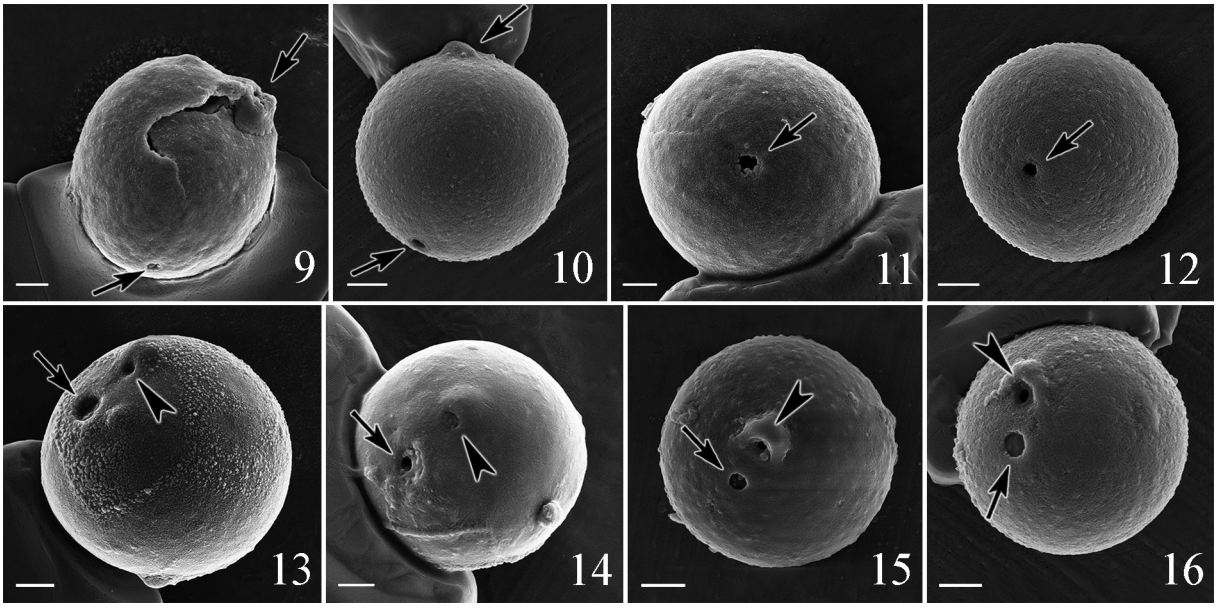



Fig. 1. Phylogeny of the Chrysophyceae obtained by Bayesian inference of the concatenated and partitioned SSU rDNA and rbcL dataset: for each sequence, GenBank accession numbers, taxonomic designations, and, if known, strain information are provided; values at the nodes indicate statistical support estimated by MrBayes posterior node probability (left) and maximum likelihood bootstrap (right); only statistical supports higher than 0.95/50 are shown; thick branches highlight nodes receiving the highest support (1.00/100); the newly obtained *Chrysococcus* sequences are given in bold. Scale bar shows the estimated number of substitutions per site.




Figs 2–8. (LM) Living cells of the studied 'population Duchnice' (2–6) and 'population Jelonki' (7–8) of *Chrysococcus triporus*: in each cell the monad with a single, gold, bilobed plastid and stigma (arrowheads) is surrounded by globular lorica with two pores lying on opposite poles (4); lorica with four pores and three of them in a close proximity to apical (4, 6) (arrows). Scale bars 5 μm.




Figs 9–16. (SEM) The ultrastructure of lorica of *Chrysococcus triporus*: (9–10) two pores lying on opposite poles (arrows) – 'population Jelonki' (9), 'population Duchnice' (10); (11–12) posterior pores (arrows) – 'population Jelonki' (11), 'population Duchnice' (12); (13–16) cell's anterior with two pores and the one is surrounded by a small wart (arrowheads) – 'population Jelonki' (13–14), 'population Duchnice' (15–16). Scale bars 1 μm.

*C. matvienkoae* D. Kapustin. However, the taxonomic status of the very similar species, *C. quadriporus* Hortobágyi and *C. minutus* var. *multipora* Wawrik, remains unresolved and will require a proper taxonomic revision of the entire genus. Our initial molecular genetic characterization of *C. triporus* presented here is thus a small piece in the puzzle and the first step in understanding its concept and taxonomy.

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Supplementary material

The following supplementary material is available for this article:

Table S1. List of sequences analyzed in this study. Classification, accession numbers are provided.

Video S1. *Chrysococcus* under standard culture conditions by LM.

This material is available as part of the online article (http://fottea.czechphycology.cz/contents)

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