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# Morphological diversity and phylogeny of the palmelloid chrysophyte genera *Chrysotilos* and *Globulochrysis*, gen. nov.

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**ABSTRACT**

Chrysophytes are a diverse group of stramenopile protists comprising a variety of taxa with different cell organizations. Here, we morphologically and genetically characterize a novel chrysophyte genus isolated from three freshwater localities in the Czech Republic, described as *Globulochrysis compacta* gen. nov. sp. nov. The genus is well characterized by the frequent formation of closely packed cells, developing into large, spherical sporangia. This alga also forms solitary cells, mucilaginous colonies, amoebae, and teardrop-shaped zoids. We also investigated the morphologically similar genus *Chrysotilos*, isolated from its type locality in the Austrian Alps. In culture, *Chrysotilos* showed characteristic neustonic growth, which was, however, not observed in *Globulochrysis*. Phylogenetic analyses based on 18S rRNA and *rbc*L genes showed that these two algae belong to two distinct lineages. Whereas *Chrysotilos* was inferred within the clade Hibberdiales, *Globulochrysis* formed a completely new lineage within the order Hydrurales.

**HIGHLIGHTS**

- *Globulochrysis* is described as a new genus in Hydrurales, Chrysophyceae.
- The genus is characterized by closely packed cells.
- The phylogenetic position of neustonic *Chrysotilos* is determined.

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**KEYWORDS** Chrysophytes; freshwater algae; neustonic growth; phylogeny; sporangia; taxonomy

## Introduction

Chrysophytes, or golden algae, are a diverse group of protists belonging to the stramenopiles. Nine orders can be identified within the chrysophytes, according to the phylogenetic reconstruction of morphologically well-characterized taxa (Kristiansen & Škaloud, 2017). Chrysophytes appear to have had a very complex evolutionary history, with several independent origins of morphologically similar species (Kristiansen & Škaloud, 2017). The morphology spans from unicellular to colonial forms, with different cell organizations such as flagellates, amoebae, coccoids, and capsal forms. Members of this group are characterized by heterokont flagella, yellow accessory pigments, and silicified endogenous resting stages called stomatocysts (Kristiansen, 2009). In addition to having a wide range of ultrastructural characteristics, they also adopt a variety of lifestyles, including phototrophic, mixotrophic, and heterotrophic ones, and are found all over the planet in freshwater, brackish, and marine settings. This protist group was chosen as a model for the investigation of algal life cycles (Čertnerová *et al.*, 2022).

Molecular studies published during the last decade point to the enormous cryptic diversity within the chrysophytes. Traditional, morphologically defined genera have often proven to be polyphyletic and rich in new, undescribed genera. Such genera include *Spumella* Cienkowsky (Findenig *et al.*, 2010; Grossmann *et al.*, 2016), *Paraphysomonas* De Saedeleer (Scoble & Cavalier-Smith, 2014), *Ochromonas* Vysotskii (Andersen *et al.*, 2017) and *Uroglena* Ehrenberg (Pusztai & Škaloud, 2019, 2022). In addition, sequencing of environmental samples revealed the existence of several order-level lineages within the chrysophytes, with unknown morphology and ecological roles (Lepère *et al.*, 2009; Del Campo & Massana, 2011; Bock *et al.*, 2022). On the other hand, molecular genetic data are still lacking for many morphologically well-defined genera. More than half of the genera described so far, which undoubtedly belong to the class Chrysophyceae, lack genetic characterization. These include the morphologically distinct and occasionally reported genera *Bitrichia* Woloszynska, *Chrysococcus* Klebs, *Chrysolykos* B.Mack, *Chrysopyxis* F.Stein, *Chrysostephanosphaera* Scherffel, *Chrysotilos* Pascher, *Polylepidomonas* H.R.Preisig & D.J. Hibberd, *Stipitochrysis* Korshikov, and many others

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(Kristiansen, 2001). Such a knowledge gap makes both taxonomic studies and the identification of environmental sequences very difficult. Accordingly, great efforts should be made to cultivate and genetically characterize newly isolated strains of chrysophytes, including re-isolations of species of genetically yet uncharacterized genera from their type localities.

During our recent investigations of freshwater algae in the Czech Republic, we repeatedly observed and isolated into culture two morphologically distinct chrysophytes, one producing spherical packets of 'closely packed' cells, the second forming zoids with a peculiar teardrop-like morphology with hyaline posterior regions. In one locality, both morphotypes were detected together. In cultured strains, both morphotypes are produced by the same organism, which also produces palmelloid colonies and amoeboid cells. This organism resembles in some features the freshwater genus *Chrysotilos*, described from a small alpine pond in the vicinity of Lunz am See, Austria (Pascher, 1931). This genus currently includes two species, *C. ferrea* Pascher and *C. tatrica* Czosnowski, neither of which have genetic data available. *Chrysotilos ferrea*, the type species, is characterized by the formation of bivalved thick iron-encrusted walls and palmelloid colonies composed of pairs or tetrads of cells coated with their own mucilage (Pascher, 1931). The second species, *C. tatrica*, distinguished by the formation of cell packets, resembled the morphs we observed. However, both *Chrysotilos* species are neustonic, forming gelatinous flakes or cuticles floating on the water surface. In addition, the flagellates of both species are morphologically very different from the zooids we observed.

The goal of this study was to morphologically and genetically characterize, as well as to taxonomically evaluate, a newly recognized organism, representing a potentially new genus of chrysophytes. To evaluate the possibility that this organism is related to the genus *Chrysotilos*, we further attempted to isolate and genetically characterize the type species of this genus, *C. ferrea*, from its type locality.

## Materials and methods

### *Isolation and cultivation*

The samples were collected in the Czech Republic either by harvesting macroscopically visible algal growth at the bottom of a pond (strain CZ 61A) or using a plankton net with 20 µm mesh (other strains). Abiotic factors including water pH, temperature and specific conductivity were measured using a combined pH/conductometer (WTW 340i; WTW GmbH, Weilheim, Germany). Single cell packets and zoids were isolated by micropipetting and cultivated in either Hepes- or MES-buffered DY IV liquid medium (Andersen *et al.*, 1997), at 15°C, under constant illumination of 20–40 µmol photons $m^{-2}$ $s^{-1}$. Strain CZ 54B (a flagellate) was isolated from the Doubravník pond (49.6497497°N, 15.8513172°E) in May 2020 (17.1°C, pH 7.2, conductivity 102 µS $cm^{-1}$). Strain CZ 61A (a cell packet) was isolated from a small pond near Skalka monastery (49.8781336°N, 14.2533894°E) in April 2020 (abiotic factors not measured). Strains CZ 170A (a cell packet) and CZ 170B (a flagellate) were isolated from the Rájecký pond (50.8066750°N, 14.0084372°E) in April 2021 (5.3°C, pH 5.4, conductivity 92 µS $cm^{-1}$). Unialgal non-axenic cultures of these strains were established.

To obtain the strain of *Chrysotilos ferrea* from its type locality in the Alps, we had first to determine the exact location of the original site, described by Pascher (1931) as 'in the alpine pond at the Pauschenalm and Herrnalm, Lunz in Lower Austria'. Considering the local names and the altitude specified in the original paper (1200–1700 m a.s.l.), we selected a small pond named Kaltluftsee Grünloch (47.8202678°N, 15.0451428°E) as the most likely location corresponding to the type locality. The sampling was conducted in June 2022. Since no neustonic layer was observed at the time of sampling, the sample was obtained by both squeezing submerged vascular plants and using a plankton net with 20 µm mesh. In the laboratory, the individual chrysophyte cells embedded in mucilaginous colonies were isolated by micropipetting and cultivated as described above. A unialgal non-axenic culture of the chrysophyte was established as strain AT 26A.

### *Morphological observations*

Field samples were observed under an Olympus CH light microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) with LED light source. In the laboratory, the strains were thoroughly checked under an Olympus CKX41 light microscope. Colonies and single cells were measured, drawn, and photographed with a Canon EOS 40D system (Canon, Tokyo, Japan). Cultures of the newly established strain AT 26A were cultivated in a 24-well polypropylene plate in a combined culture medium consisting of MES-buffered DY IV liquid medium (Andersen *et al.*, 1997) enriched with excess $FeCl_3$ $6H_2O$ or with Fe-oxidizing bacteria collected directly from natural iron-rich microbial mats from the Čertoryje Brook (50.5150831°N, 15.1837456°E) or with both. Cultivation was carried out at 15°C under constant illumination of 20–40 µmol photons $m^{-2}$ $s^{-1}$. Each week, the cultures were checked for the formation of pseudocysts, shell-like structures strongly impregnated with iron (Pascher, 1931; Czosnowski, 1948). We also tried to induce cyst formation by various manipulations including cultivation in two different

media (WC TES and WC MES) (Guillard & Lorenzen, 1972), imitating *in situ* conditions at the type locality during the summer dry period: temperature (24°C) and photon irradiation (40–80 µmol photons $m^{-2}$ $s^{-1}$), and using different cultivation media in various Erlenmeyer flasks but no stomatocysts were observed in any of these experiments.

### *DNA extraction, PCR amplification, and sequencing*

After centrifugation, algal cells were mechanically disrupted by shaking in the presence of glass beads (0.5 mm diameter, Sigma). Genomic DNA was extracted using the Invisorb® Spin Plant Mini Kit (Invitek, Berlin, Germany) following the instructions given by the manufacturer. Two molecular loci were amplified by PCR: nuclear SSU rDNA and chloroplast *rbc*L. The SSU rDNA sequences were obtained by PCR amplification using the primers 18S-F (5'-AAC CTG GTT GAT CCT GCC AGT-3') and 18S-R (F (5'-TGA TCC TTC TGC AGG TTC ACC TAC G-3'; Katana *et al.*, 2001). PCR amplification was performed using a thermal cycler Eppendorf Mastercycler ep S with the following protocol: denaturation at 94°C for 4 min; 35 cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min and elongation at 72°C for 1 min; final elongation at 72°C for 10 min. PCR amplification of the *rbc*L locus was performed according to Jo *et al.* (2011), using the primers rbcL-Chrys-F2 (5'-TTA TTA ACW GCT TGT GAT-3') and rbcL-Chrys-R (5'-TCC ATR TCR AAG AAA ATW CC-3'; Škaloudová & Škaloud, 2013). PCR amplification was performed as described above, with the following protocol: denaturation at 94°C for 4 min; 40 cycles of denaturation at 94°C for 1 min, primer annealing at 40°C for 1 min and elongation at 72°C for 1 min; final elongation at 72°C for 10 mins. The quality and yield of the PCR products were checked under UV light using 1% agarose gel containing ethidium bromide. PCR products were purified and sequenced at Macrogen (Amsterdam, Netherlands). Sequences were deposited in GenBank under the accession numbers OR622487; OR622488; OR622489; OR622490; OR622491 (18S rDNA) and OR663905; OR663906; OR663907 (*rbc*L).

### *Phylogenetic analyses*

The newly determined SSU rDNA and *rbc*L sequences were aligned with other sequences of Chrysophyceae from GenBank selected according to Pusztai & Škaloud (2019) to encompass all chrysophycean lineages. This selection was further extended to include environmental clades according to Wilken *et al.* (2020), as well as all sequences closely related to the newly determined sequences according to BLAST searches. A concatenated 2,606 bp long SSU rDNA and *rbc*L alignment was produced, including sequences from a total of 106 chrysophytes plus two outgroup taxa – *Synchroma* R. Schnetter and *Nannochloropsis* D.J.Hibberd (Supplementary table S1). The SSU rDNA sequences were aligned using MAFFT v. 6 software (Katoh *et al.*, 2002) and poorly aligned positions were removed. *rbc*L 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 *rbc*L dataset according to Škaloud *et al.* (2013). For each of the alignment partitions, the most appropriate substitution model was estimated using the Bayesian information criterion (BIC) as implemented in jModelTest 2.1.4 (Darriba *et al.*, 2012). This procedure selected the following models: (1) GTR +I + Γ for SSU rDNA and the second codon position of the *rbc*L gene; and (2) GTR+Γ for the first and third codon position of the *rbc*L gene. The phylogenetic tree was inferred by Bayesian inference (BI) using MrBayes version 3.2.6 (Ronquist *et al.*, 2012). The analysis was carried out on partitioned datasets using the substitution models best matching those selected by jModelTest 2.1.4. All parameters were unlinked among partitions. Two parallel MCMC runs were carried out for 8 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). The SDSF value was 0.0063. Finally, the burn-in value was determined using the 'sump' command. The maximum likelihood (ML) analysis was performed using RAxML 8.2.12 (Stamatakis, 2014) on the concatenated dataset partitioned to individual loci and codon partitions, applying the hybrid parallelization on 4 threads. The evolutionary model used was the default GTR+Γ. 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 nine chrysophycean orders and three environmental clades (Fig. 1). Based on these genes, *Chrysotilos ferrea* strain AT 26A was included in the clade Hibberdiales, encompassing generally colonial chrysophytes having the palmelloid level of organization. *Chrysotilos* was genetically most closely related to *Dermatochrysis reticulata* (K.I.Meyer) Entwisle & R.A.Andersen, *Naegeliella flagellifera*

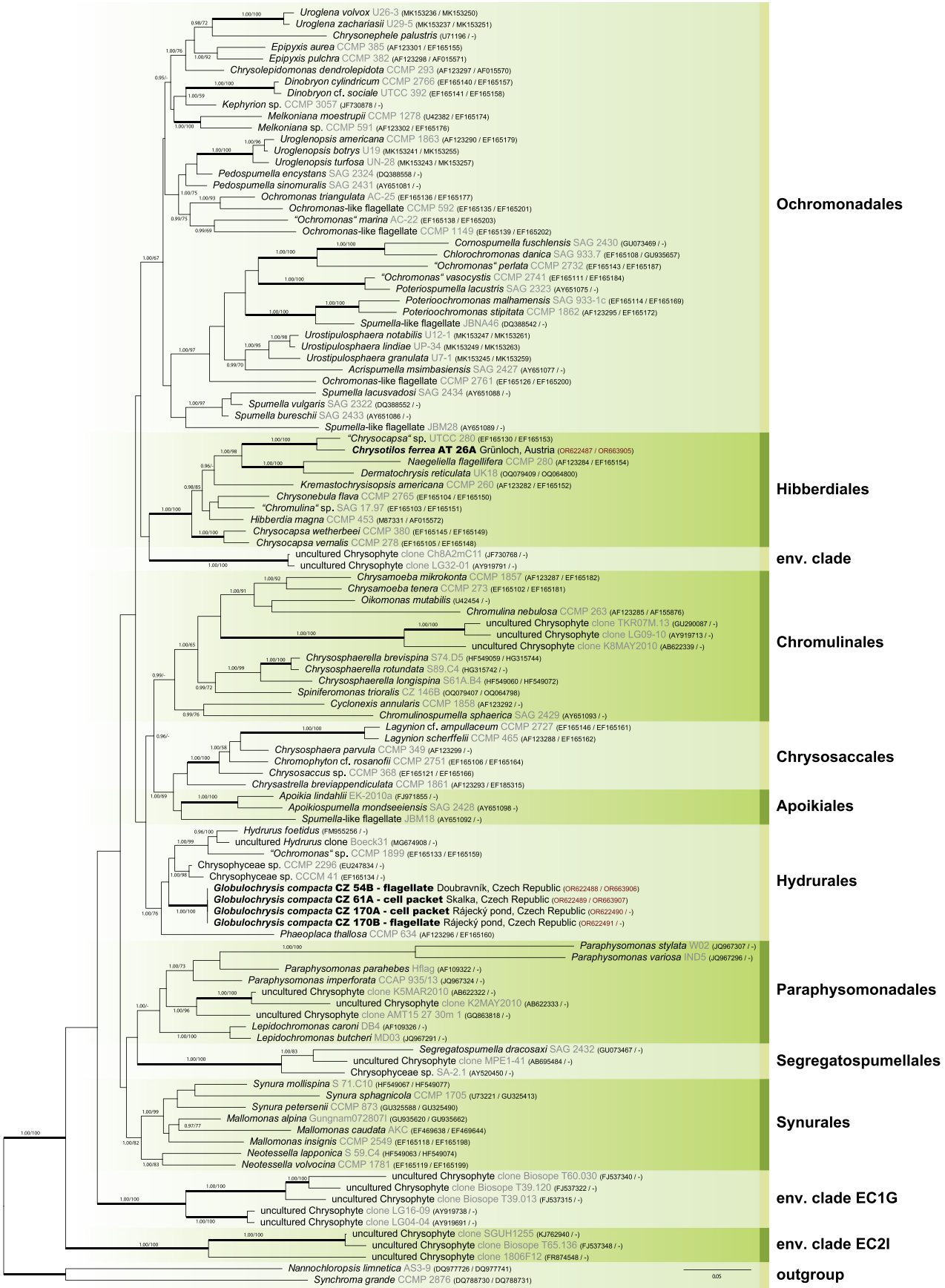



Fig. 1. Phylogeny of the Chrysophyceae obtained by Bayesian inference of the concatenated and partitioned SSU rDNA and rbcL dataset. For each sequence, GenBank accession numbers, taxonomic designations, and, if known, strain information are provided. Values at the nodes indicate statistical support estimated 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 *Chrysotilos ferrea* and *Globulochrysis compacta* gen. et sp. nov. sequences are given in bold. Scale bar shows the estimated number of substitutions per site.

Correns and '*Chrysocapsa*' sp. UTCC 280, while *Chrysotilos* and '*Chrysocapsa*' sp. UTCC 280 are sister lineages.

Strains CZ54B, CZ61A, CZ170A, and CZ170B were genetically identical, forming a completely new lineage within the order Hydrurales. According to the genetic data available, this order comprises the freshwater genus *Hydrurus* C.Agardh forming macroscopic thalli usually growing in cold water, pseudoparenchymatous marine chrysophyte *Phaeoplaca* Chodat, and several unspecified *Ochromonas*-like flagellates. Since our newly recognized lineage is not attributable to any so far known chrysophyte genus, we refer to it here as *Globulochrysis compacta*, gen. et sp. nov.

## *Morphological investigations*

The detailed overview of the morphological variability of investigated strains both in the field and in the culture is summarized in Figs 2–9 and in Figs 10–27.

### *Microscopic observations of Chrysotilos ferrea*

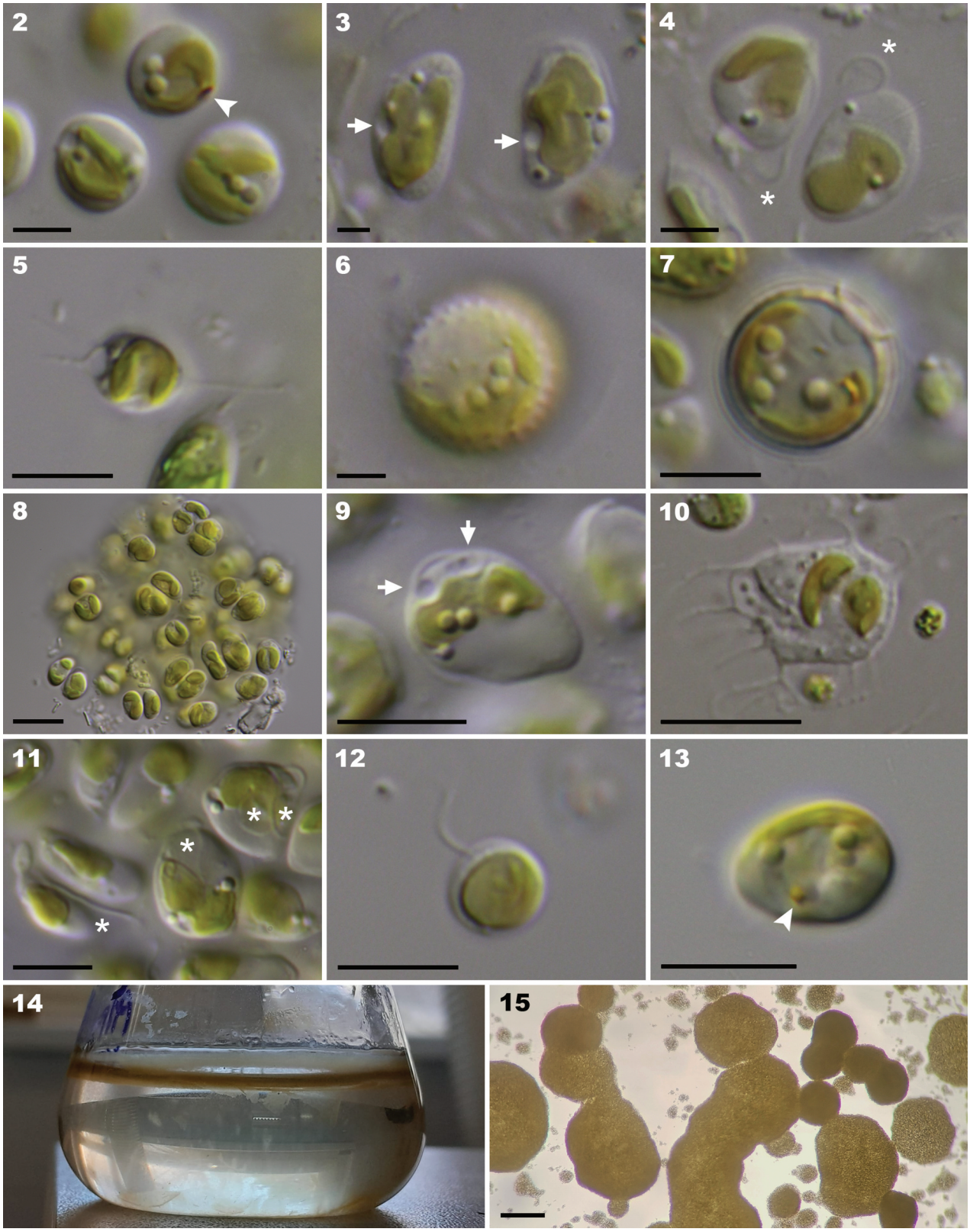
In the field material, this alga formed free-living mucilaginous colonies composed of either spherical (Fig. 2) or elongated to irregular non-motile cells (Fig. 3). The cells possessed a single lobed parietal chloroplast, up to two contractile vacuoles, and rarely a small red or orange stigma (Figs 2, 3). Occasionally, cells in mucilaginous colonies had fully formed flagella (Fig. 4). We also observed the presence of amoeboid cells (Fig. 5) and the formation of cysts with a warty surface and a bowl-shaped collar (Figs 6, 7). Cysts were almost spherical to slightly oblate, 16–17 µm wide and 15 µm in length with a warty surface.equally embellished with numerous regular granules smaller than 1 µm in diameter and clearly visible in LM. The pore (c.2–3 µm in diameter) was surrounded by a 7–7.5 µm wide, obconical to bowl-shaped, simple collar.

In culture, we observed the frequent formation of mucilaginous colonies, usually composed of 1–4 celled sub-colonies, 80 µm in size (Fig. 8). Cells were spherical to elongate, 7–9 µm, with a single parietal chloroplast and two anterior contractile vacuoles (Fig. 9). Sometimes, we observed the formation of amoeboid cells with multiple filopodia extending in all directions (Fig. 10). Asexual reproduction took place by frequent formation of zoospores, 7–8.5 µm, with two flagella oriented in opposite directions, typical for the Hibberdiales (Remias *et al.*, 2020). Zoospores developed within mucilaginous colonies (Fig. 11) and were subsequently released as spherical or elongated swimmers (Figs 12, 13). The stigma was minute, red or orange, and clearly visible only in a few cells (Fig. 13). Zoospores tended to spin in small circles (Supplementary data S1). After a few days of cultivation, this alga started to form neustonic flat colonies (Fig. 14). The cells formed yellow-brown to black-brown flakes (up to 1 mm) on the water surface (Fig. 15), but it is unclear if these were epineustonic or hyponeustonic layers.

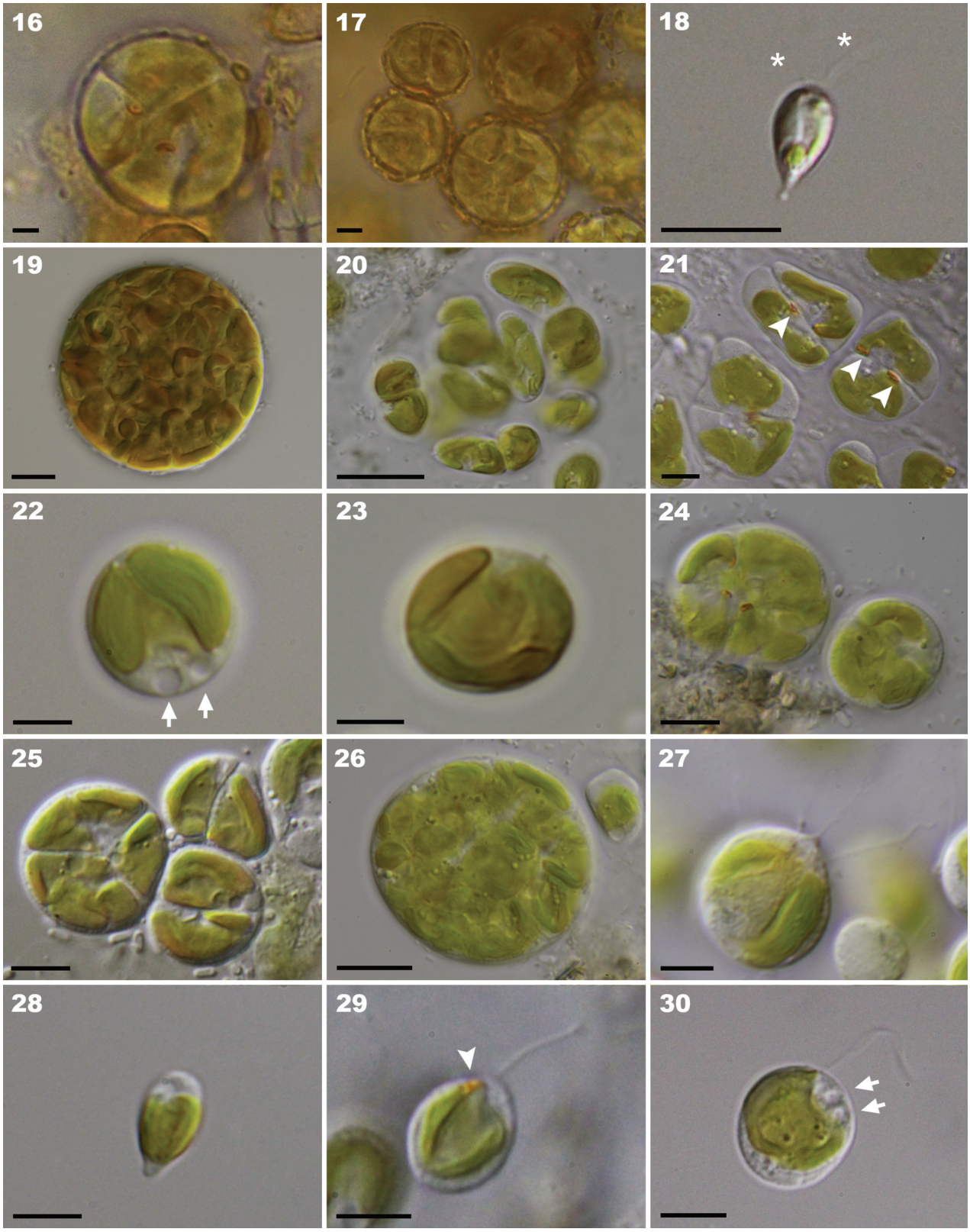
### *Microscopic observations of Globulochrysis compacta*

In field material, this alga formed solitary cells or, much more frequently, mucilaginous colonies composed of cell packets of compacted cells that varied in number from 10 to 30 (Fig. 16). The colonies were frequently covered by iron deposits and the cells possessed one lobed chloroplast and a small, orange stigma (Fig. 17). In Rájecký pond, we also observed zoospores with a peculiar teardrop-like morphology with hyaline lateral or posterior regions (Fig. 18).

In culture, both mucilaginous colonies and zoospores developed into large, spherical sporangia where the cells were angular, deformed by their close contact (Fig. 19). The sporangia often disintegrated into single cells embedded within the mucilage (Figs 20, 21), which frequently grew into new few-celled to multiple-celled colonies. The solitary cells were 4.5–8.5 µm wide and 6–12 µm long, variable in shape including obovate, spherical, or elliptic forms without scales or thecae (Figs 22, 23). The colonies were usually spherical or ellipsoidal, initially formed by 2–4 cells firmly attached to each other, the cell shape deformed by their close contact (Figs 24, 25), later developing into large sporangia 13–16 µm in diameter, containing 16–32 cells (Fig. 26). The cells possessed one parietal, often bi-lobed chloroplast without pyrenoids (Figs 22, 23), and two, rarely more anterior contractile vacuoles (Figs 22, 25, Supplementary data S2). A single prominent stigma with an oval, or usually triangular surface shape was commonly present in the cells of all growing types (Figs 21, 24), though in many cells the stigma was not developed (Figs 22, 26). Rarely, amoeboid cells were observed, of more or less spherical shape with several thin filopodia (Fig. 27, Supplementary data S2). Asexual reproduction took place either by above-mentioned disintegration of sporangia into non-motile, stigma- and contractile vacuoles-containing cells (so-called hemizoospores) or by the production of zoospores. Zoospores were of a teardrop-like shape (Fig. 28), quickly forming elongated or spherical shapes (Figs 29–30). They possessed one or two visible flagella emerging from the rounded apical side, two anterior contractile vacuoles, and anterior stigma (Fig. 29). The short, second flagellum was hardly visible in the light microscope. After settlement and flagellar retraction, the cells soon divided and formed cell packets.




Figs 2–15. Morphology of *Chrysotilos ferrea*, light microscopy. **Figure 2**. Free-living mucilaginous colonies containing spherical cells, one of them possessing a stigma (arrowhead). **Figure 3**. Elongated cells with contractile vacuoles (arrows). **Figure 4**. Cells with developed flagella (asterisks). **Figure 5**. Amoeboid cell. **Figure 6**. Cyst with warty surface. **Figure 7**. Cyst with bowl-shaped collar. **Figure 8**. Mucilaginous colony. Figure 9. Cell with two contractile vacuoles (arrows). **Figure 10**. Amoeboid cell. **Figure 11**. Cells with developed flagella (asterisks). **Figure 12**. Spherical zoospore. **Figure 13**. Zoospore with a small stigma (arrowhead). **Figure 14**. Culturing flask after a few days of cultivation with typical neustonic colonies floating on the water surface. **Figure 15**. Neustonic colonies on the water's surface. Scale bars: 5 μm (**Figs 2–7, 9–10, 12–13**) 10 μm (**Figs 9, 11**), 50 μm (**Fig. 15**).




Figs 16–30. Morphology of *Globulochrysis compacta* gen. et sp. nov., light microscopy. **Figure 16**. Mucilaginous colonies. **Figure 17**. Cell packets with stigmata and iron deposits. **Figure 18**. Zoospore of drop-like shape with two flagella (asterisks). **Figure 19**. Sporangium. **Figure 20**. Disintegration of sporangia into single cells (hemizoospores) by gelatinization of the mother cell wall. **Figure 21**. Disintegrated hemizoospores with stigmata (arrowheads). **Figure 22**. Solitary spherical cell with bi-lobed chloroplast and two contractile vacuoles (arrows). **Figure 23**. Elongated solitary cell with bi-lobed chloroplast. **Figure 24**. Young cell packets with well-developed stigmata. **Figure 25**. Young cell packets with visible contractile vacuoles. **Figure 26**. Sporangium. **Figure 27**. Amoeboid cell with filopodia. **Figure 28**. Drop-shaped zoospore. **Figure 29**. Ellipsoidal zoospore with a prominent triangular stigma. **Figure 30**. Rounded zoospore with well-visible contractile vacuoles (arrows). Scale bars: 2 μm (**Figs 18, 22**), 5 μm (**Figs 21, 23, 27, 29**), 10 μm (**Figs 16, 17, 19, 20, 24–26, 28, 30**).

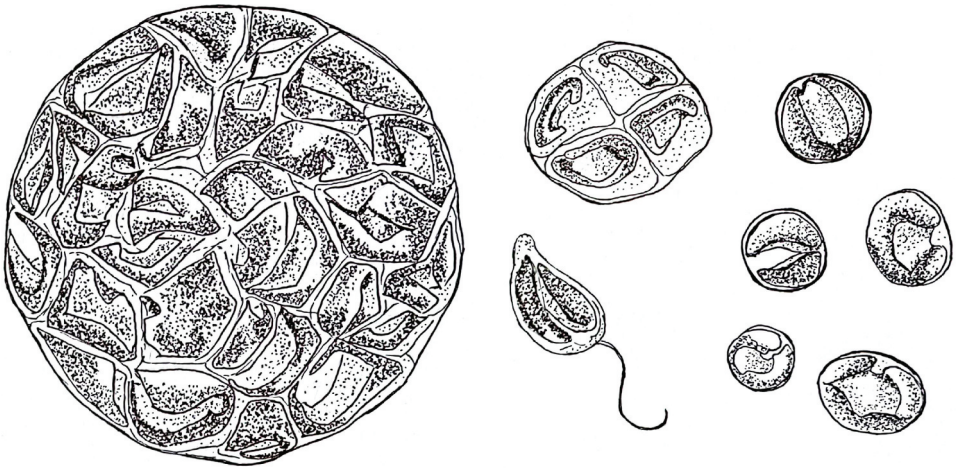


Fig. 31. Drawings of different developmental stages of *Globulochrysis compacta* gen. et sp. nov. Scale bar: 5 µm.

### *Taxonomy*

Phylum OCHROPHYTA Cavalier-Smith
Class CHRYSOPHYCEAE Pascher
Order HYDRURALES Pascher

#### *Globulochrysis* Malavasi, Pusztai, Jadrná, Škvorová & Škaloud, gen. nov.

DIAGNOSIS: Cells either solitary or tightly united into cell packets, often covered by iron deposits. Chloroplast parietal, bi-lobed, without pyrenoids. Cells usually with two contractile vacuoles. Asexual reproduction by hemizoospores and zoospores. Sporangia spherical, with the angular cells deformed by their close contact, possessing stigmata. The genus differs from other genera by the frequent formation of large cell packets and by the 18S rDNA and *rbc*L sequences.
ETYMOLOGY: Epithet derived from the Latin noun *globulo*, globule, referring to the formation of the cell packets.
TYPE SPECIES: *Globulochrysis compacta*

#### *Globulochrysis compacta* Malavasi, Pusztai, Jadrná, Škvorová & Škaloud, sp. nov. (Figs 10–28)

DIAGNOSIS: Cells with characters of the genus. Young cells 4.5–8.5 µm in wide and 6–12 µm in length; older cells 6–10 µm in diameter. Asexual reproduction by zoospores and hemizoospores formed in sporangia developed from cell packets. Sporangia up to 13–16 µm in diameter. Zoospores 2–12 per sporangium, teardrop-shaped, with two flagella, anterior stigma, and lateral plastid. Hemizoospores are liberated by gelatinization of the mother cell wall. The species is distinctive in the presence of zoospores of a teardrop-shape with typical hyaline posterior region.
ETYMOLOGY: L. fem. adj. compacta, compact/firm, referring to the morphology of the cells.
TYPE LOCALITY: Doubravník pond (49.6497497, 15.8513172), Czech Republic.
HOLOTYPE: Strain CZ54B permanently cryopreserved in a metabolically inactive state (cryopreservation in liquid nitrogen) in the Culture Collection of Algae of the Charles University in Prague (http://botany.natur.cuni.cz/algo/caup.html) as the specimen CAUP-CRYO-B901. Drawings of the holotype are provided in Fig. 31.

## Discussion

In this study, we re-collected *Chrysotilos ferrea* from the type locality more than 90 years after its original description (Pascher, 1931). This procedure is appropriate when no cultures or other type material are preserved (Han *et al.*, 2018) and has been recently used for other chrysophytes such as *Ochromonas, Uroglena* and *Uroglenopsis* (Klaveness *et al.*, 2011; Andersen *et al.*, 2017; Pusztai & Škaloud, 2019). *Chrysotilos ferrea* has not been observed since its original description from the Austrian Alps. It is important to note that despite our effort to locate the exact type locality, it is possible that the original site may have been another pond nearby or one that no longer exists. Nevertheless, our morphological observations have convinced us that we have indeed recovered an isolate of *Chrysotilos ferrea*. The same applies to the second species of the genus, *Chrysotilos tatrica*, described from the Tatra Mountains (Czosnowski, 1948). Other authors who have written about the type species (e.g., Remias *et al.*, 2020) mention only Pascher's report without adding any new locations or additional information regarding its morphology or ecology. Therefore, the phylogenetic position of this genus, including its type species *C. ferrea*, remained unknown until today. *Chrysotilos*, like the other representatives of the order Hibberdiales, is characterized by a palmelloid level of organization with a substantial production of

mucilage (Kristiansen & Škaloud, 2017). Hibberdiales are known from standing or flowing waters. Some members of this order, such as *Kremastochrysopsis austriaca* or '*Chromulina*' sp. SAG 17.97, have recently been collected in other areas of the Austrian Alps, including melting summer snow (Remias *et al*., 2020).

The Alpine strain we acquired conformed with the description of the *Chrysotilos* type species by Pascher (1931). Indeed, our LM observations on *C. ferrea* from the type locality agreed with many of the observations reported by Pascher including the structures illustrated by the author in Fig. 14e, f., the cell morphology, cell division, and palmelloid stage. We observed all other features of this taxon, including the formation of two- and four-celled colonies enclosed in a common mucilage, and the production of pseudopodia. During our sampling, we were not able to observe the characteristic neustonic layer. It is likely, as suggested by Lund (1942), that 'when there is a stable surface film the dominant vegetative phase is non-motile'. It was constantly raining on the day of sampling, so this factor may have influenced the absence of neustonic formation. However, in cultured, the *C. ferrea* strain forms distinct neustonic flat colonies resembling yellow-brown to black-brown flakes (up to 1 mm) on the water surface (Fig. 1h, 1i), which is fully consistent with Pascher's (1931) observations: 'an der Wasseroberfläche treibende Flocken bildend'. In addition, based on the inferred phylogeny, *C. ferrea* differs significantly from other chrysophytes that are associated with the air–water interface, such as the hyponeustonic *Kremastochrysopsis* (Pascher, 1942) and the epineustonic, very common genus *Chromophyton* (Woronin, 1880).

The only feature that we did not observe in both natural material and cultures were the so-called pseudocysts. In both *C. ferrea* and *C. tatrica* they are described as bipartite shell-like (Pascher, 1931) or cap-like (Czosnowski, 1948) external structures encrusted with iron. As already noted by other authors it is usually hard to force these algae to produce such structures. We could speculate that their formation could be induced by feeding on ferric bacteria. The nutritional strategies of mixotrophic phytoflagellates, including chrysophytes, exhibit great variety, with a spectrum of mixotrophic nutrition ranging from nearly pure phototrophy to nearly pure heterotrophy (Jones, 1997, 2000; Rottberger, 2013). For example, some algae may ingest prey to obtain macronutrients (e.g., nitrogen or phosphorus) or essential trace growth factors (e.g. iron or vitamins), but largely use phototrophy to produce organic carbon for growth (Lie *et al*., 2018). We therefore carried out experimental cultivation of the *C. ferrea* strain under two specific conditions, i.e., with an excess Fe and in the presence of ferric bacteria. The culture produced outer thick-walled envelope structures encrusted with iron due to their yellow-rusting colour. However, the structures we observed differed from the described two-part structures observed by the authors of the original descriptions. Nevertheless, we got clear evidence that the isolated strain of *C. ferrea* is somehow able to use iron in the formation of the outer envelope structures (or iron is simply being chelated by organic compounds in the outer envelope), whatever we call these structures. It is likely that the production of bipartite structures is conditioned by the presence of a specific type of bacteria or takes place only under certain conditions. Interestingly, the production of various iron incrustations is relatively common among chrysophytes observed in nature. For example, Gisklhorn (1922) and Lund (1942) had already observed in *Chromulina smaragdina* and *Chromulina ferrea* ferric encrustation in a mucilage, similar to that described in *Chrysotilos ferrea* Pascher (1931). The production of ferric encrustation is also known in several loricate chrysophytes (Starmach, 1985) and in other algal lineages (e.g., *Trachelomonas* and *Strombomonas* in the Euglenophyceae). Moreover, in Starmach's 1985 book, there is no comparable organism to *Chrysotilos tatrica* and there is a possibility that, upon sequencing, it may be reclassified under *Globulochrysis* in the future. However, many developmental stages exhibit similarities across genera, such as the production of mucilage, the presence of flagellates, the existence of palmelloid colonies, and the utilization of Fe (e.g., *Chalkopyxis* bivalve shells). Potential future molecular analyses may assign other '*Ochromonas*-like' or '*Chomulina*-like' lineages into the newly established genus. It is important to note that only very young stages of *Naegeliella* or *Phaeoplaca* have angular cells in their globular 'mother plasma membrane', but differ when older. In our opinion, *Naegeliella* is related to *Chrysotilos* s.s., *Phaeoplaca* being associated with *Globulochrysis*. In addition, iron-encrusted two-part shell-like pseudocysts have been described in *Chalkopyxis tetrasporoides* (Pascher, 1931). Finally, newly described *Globulochrysis* produced multicelled globular colonies or sporangia-like stages regularly covered by yellow-rusty coloured pieces of some outer protective layer in the field. The colouration is very likely caused by ferric encrustation.

In our field morphological observations of the new genus *Globulochrysis*, the multicelled stages indeed resembled the morphology of *Chrysotilos tatrica*, as both taxa produce 'spheres with squeezed cells' and ferric encrustations. However, *Globulochrysis* is distinguished from *Chrysotilos* not only because it never forms neustonic layers but also by the morphology of wild flagellate cells. Interestingly, the specific teardrop-

shaped zoospores are morphologically similar to those of *Chrysonebula holmesii* (Lund, 1953), a species which also belongs to the order Hibberdiales.

Since we have a well-known neustonic chrysophyte genus *Chromophyton* in our personal culture collection, we were able to compare its growing characteristics with both *Chrysotilos* and *Globulochrysis*. While cultures of *Chrysotilos* and *Chromophyton* have maintained a consistent neustonic character even after multiple inoculations and long-term cultivation, this has not been the case for *Globulochrysis* in either the natural sample or any of the cultures of different ages and conditions. *Globulochrysis* produces teardrop-shaped flagellate cells in nature, which are characterized by a distinct hyaline basal region and the formation of conspicuous hyaline areas in cells, which are not known in *Chrysotilos*, but these teardrop-shaped flagellate cells were not observed in our *Globulochrysis* cultures. We can only speculate how crucial the composition of the bacterial flora is for the manifestations of golden algal life (Bock *et al.*, 2020), both for the formation of specific iron-encrusted structures and for the morphological diversity of their flagellate stages. 'To make the conditions of existence the same, it is not sufficient to attend merely to the basic fluid; the bacteria must also be the same' (Jennings, 1908).

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No potential conflict of interest was reported by the author(s).

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## Supplementary material

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at https://doi.org/10.1080/09670262.2024.2340026.

**Supplementary table S1**. List of sequences analyzed in this study, with classification, accession numbers, and affiliation to different alignment datasets.

**Supplementary data S1**. Video of *Chrysotilos ferrea* by LM. This video shows the zoospores tended to spin in small circles.

**Supplementary data S2**. Video of *Globulochrysis compacta* by LM. This video shows filopodia.

## Author contributions

V. Malavasi: obtaining the morphological data, sequencing, assembling figure panels, drafting and editing manuscript; M. Pusztai: culture experiments, drafting and editing manuscript; I. Jadrná: isolation of algal strains, sequencing; Z. Škvorová: isolation of algal strains, sequencing; P. Škaloud: conceiving the study, phylogenetic analyses, drafting and editing manuscript and funding acquisition.

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