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Freshwater 'microcroissants' shed light on a novel higher-level clade within the Trebouxiophyceae and reveal the genus *Chlorolobion* to be a trebouxiophyte

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ABSTRACT

The Trebouxiophyceae is a widespread and species-rich green algal class encompassing mostly coccoid algae with a simple spherical, ovoid or ellipsoidal outline. However, some poorly sampled lineages have evolved more elaborate shapes or even complex thalli, adding to the morphological diversity of the class. By investigating new and previously established strains, this study expands the range of morphologies exhibited by the class members by uncovering a clade of croissant-like trebouxiophytes. Phylogenetic analyses inferred from nuclear 18S rDNA and chloroplast *rbc*L sequences confirmed the monophyly of the 'microcroissant' clade, which we propose to be classified as a new family, Ragelichloridaceae. This family includes two novel genera, *Ragelichloris* and *Navichloris*, and the previously described *Thorsmoerkia*. The position of Ragelichloridaceae within Trebouxiophyceae stayed unresolved but chloroplast phylogenomics showed that the family belongs to the broader incertae sedis group that also includes *Xylochloris* and *Leptosira*. In addition, our study showed that the similar morphotype-bearing genus *Chlorolobion*, previously classified within Chlorophyceae, is a genuine trebouxiophyte, potentially related to Ragelichloridaceae.

HIGHLIGHTS

- A new family-level clade uncovered within the Trebouxiophyceae.
- Two new genera, *Ragelichloris* and *Navichloris*, are described.
- The genus *Chlorolobion* is shown to be a trebouxiophyte.

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Introduction

The Trebouxiophyceae is one of the major groups of green algae and encompasses organisms with a broad range of lifestyles, from free-living photoautotrophs and endosymbiotic partners to heterotrophic parasites. They also exhibit a great diversity in morphological features, though the most widespread morphotype is a simple coccoid form, with cells typically exhibiting a spherical, ovoid or ellipsoidal outline. This morphology appears to represent the plesiomorphic state, as it occurs across the trebouxiophyte phylogenetic tree, intermingled with lineages exhibiting different, more intricate morphotypes (Friedl & Rybalka, 2012). A great example of contrasting morphologies is found in the order Microthamniales, which encompasses both the multicellular branched filamentous *Microthamnion* and the unicellular coccoid *Coleochlamys* which molecular phylogenetics have revealed as sister taxa (Lemieux *et al.*, 2014; Barcytė *et al.*, 2021). Another similar example is the *Prasiola* clade accommodating organisms with a wide range of thallus morphologies and organization, spanning from unicellular through filamentous to pseudoparenchymatous (Pröschold & Darienko, 2020). Therefore, morphological traits alone are highly unreliable in inferring phylogenetic relationships or defining higher-order taxa in Trebouxiophyceae.

Despite the growing knowledge of trebouxiophyte diversity, with new and previously overlooked taxa (species and genera) being described at a steady pace (e.g. Barcytė *et al.*, 2017; Li *et al.*, 2020; Malavasi *et al.*, 2022; Kato *et al.*, 2023), the higher-level phylogenetic relationships within Trebouxiophyceae, and formal delimitation of taxa at the suprageneric level, remain highly unsettled. Identifying monophyletic clades with full statistical support is primarily hampered by unstable phylogenetic relationships caused largely by poorly sampled lineages 'jumping' across the Trebouxiophyceae phylogenies depending on the taxa sampled, molecular marker used or tree inference method (Li *et al.*, 2020; Barcytė *et al.*, 2021). Phylogenomics could overcome this problem and generate a reliable backbone phylogeny as well as potentially reshape our understanding of some of the major groupings (Lemieux *et al.*, 2014; Kato *et al.*, 2023). However, for many understudied lineages, multigene data are so far missing and nuclear 18S rDNA trees (sometimes combined with phylogenies derived from

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the chloroplast *rbc*L gene or the nuclear ITS2 sequences) remain the primary means to infer the relationships within the group (Barcytė *et al*., 2017; Li *et al*., 2020; Pröschold & Darienko, 2020; Malavasi *et al*., 2022).

Since 'green balls' represent most of the Trebouxiophyceae diversity, organisms with conspicuous morphology attract attention in terms of their phylogenetic placement within the class. In some cases, such organisms form deeply branching isolated lineages. A notable example is the recently described semi-aerophytic, crescent-shaped *Thorsmoerkia curvula*, which was not assigned to any of the existing orders or clades and allegedly lacked sequenced close relatives (Nicoletti *et al*., 2021). Another example of an enigmatic and phylogenetically unresolved lineage is represented by a ship-shaped coccoid alga (strain SAG 2477) isolated from soil in Germany, provisionally considered a candidate new species in a new genus (Hodač, 2015). This alga is phylogenetically close to the un-named organism under the strain code BCP-BC4VF9, isolated and sequenced from the Central Desert in Baja California, Mexico (Fučíková *et al*., 2014). Unfortunately, the BCP-BC4VF9 strain did not survive and its fine morphological features were undocumented. Finding sister taxa of such orphan trebouxiophyte lineages could not only help to resolve their position among other subgroups but also to assist in stabilizing the phylogenetic backbone of the class, a prerequisite for a better understanding of Trebouxiophyceae evolution.

We recently isolated two *Sphagnum*-associated croissant-shaped algal strains that we aimed to place in the green algal phylogeny and uncover their closest relatives. Utilizing 18S rDNA and *rbc*L as phylogenetic markers, these new isolates were found to belong in the Trebouxiophyceae, specifically to a novel trebouxiophyte clade which included the enigmatic lineages mentioned above. Microscopic observations confirmed a similar morphology of the three distinct genus-level lineages (*Thorsmoerkia, Ragelichloris* gen. nov. and *Navichloris* gen. nov.) that constituted the novel clade, suggesting that the croissant-shaped trebouxiophytes should be recognized as a novel higher-level taxon within the class. This notion was further supported by the multigene phylogenomic analysis based on the chloroplast genome data, demonstrating the deeply branching nature of the croissant lineage within Trebouxiophyceae, forming sister relationships with other incertae sedis lineages of the class. Finally, sequence analysis of the similar morphotype-bearing genus *Chlorolobion* (including the type species *C. obtusum*) provided the first evidence of its affiliation to the Trebouxiophyceae and elucidated its polyphyletic nature, with two of the species belonging to the novel 'microcroissant' clade.

Materials and methods

Strains and microscopy

Strain 'PLY' was isolated from a *Sphagnum* moss sample taken in October 2021 from the raised bog Plynoja (55°19′23″N, 22°8′18″E) situated in the Pagramantis Regional Park, Tauragė, Lithuania (Supplementary fig. S1). Strain 'VMJ' was isolated from a *Sphagnum* sample taken in May 2022 from the shore of the Great Moss Lake (Velké mechové jezírko; 50°13′10.92″N, 17°17′12.84″E) situated in the National Natural Reserve Rejvíz, Czech Republic (Supplementary fig. S2). The two strains have been deposited at the SAG (Sammlung von Algenkulturen Göttingen) collection under the strain designations SAG 2660 and SAG 2659, respectively, and are further referred to with these names. We additionally obtained and studied strain SAG 2477, deposited to the collection as an 'unidentified Trebouxiophyte', and nine ACOI (Coimbra Collection of Algae) strains identified by the ACOI curator as five different species of the genus *Chlorolobion* (Table 1). All strains were cultivated in liquid Bold's Basal Medium (BBM; Bischoff & Bold, 1963) in 25 cm^2 cell culture flasks with filter caps (SPL Life Sciences), as well as on

Table 1. SAG and ACOI strains evaluated for their connection to the 'microcroissant' clade.

Strain	Original identification	Habitat, collection site	Updated species name	Classification
SAG 2477	unidentified Trebouxiophyte	soil in spruce forest, Swabian Alb, Germany	*Navichloris terrestris*	Ragelichloridaceae, Trebouxiophyceae
ACOI 732	*Chlorolobion obtusum*	Serra da Estrela, Covão do Boeiro, Portugal		Trebouxiophyceae, *incertae sedis*
ACOI 231	*Chlorolobion lunulatum*	pond near the river, São João do Campo, Portugal		Selenastraceae, Chlorophyceae
ACOI 811	*Chlorolobion lunulatum*	puddle, Serra do Gerês, Leonte, Portugal		Selenastraceae, Chlorophyceae
ACOI 2681	*Chlorolobion lunulatum*	puddle, Serra do Gerês, Portugal	*Thorsmoerkia lunulata*	Ragelichloridaceae, Trebouxiophyceae
ACOI 3192	*Chlorolobion lunulatum*	puddle, Serra do Gerês, Leonte, Portugal	*Thorsmoerkia lunulata*	Ragelichloridaceae, Trebouxiophyceae
ACOI 208	*Chlorolobion saxatile*	pond near Arazede, Amieiro, Portugal		Selenastraceae, Chlorophyceae
ACOI 3295	*Chlorolobion guanense*	unknown		Selenastraceae, Chlorophyceae
ACOI 820	*Chlorolobion braunii*	Leiria, Portugal		Selenastraceae, Chlorophyceae
ACOI 2567	*Chlorolobion braunii*	pond, Castelo Branco, Penha Garcia, Portugal		Selenastraceae, Chlorophyceae

agarized BBM medium (1.5% agar) in Petri dishes. Light was provided continuously by a tri-band fluorescent tube (LT 36 W T8/865 NARVA) with an irradiance of 40–50 µmol photons m^{-2} s^{-1}, and the cultivation temperature was maintained at 20°C.

Light microscopy was carried out with an Olympus BX53 light microscope (Tokyo, Japan). Micrographs were taken with an Olympus DP73 digital camera (Tokyo, Japan). The Olympus cellSens Imaging Software v1.6 was used to process images and obtain morphometric measurements of the cells. In addition, strain SAG 2660 was further investigated using transmission electron microscopy (TEM). For TEM, a flat specimen carrier of 3 mm diameter (Leica Microsystems, Vienna, Austria) was first filled with a drop of 20% BSA (Bovine Serum Albumin) cryoprotectant, followed by a drop of the centrifuged cell suspension. The specimen carriers were then rapidly frozen (within 30 ms at a pressure of 2100 Pa) using a Leica EM ICE High Pressure Freezer (Leica Microsystems). Following freezing, the flat specimen carriers were transferred to screw-capped containers filled with a pre-cooled (−90°C) substitution medium (2% osmium tetroxide in 100% acetone). The containers were placed into a freeze substitution device Leica AFS (Leica Microsystems) pre-cooled to −90°C. The samples were substituted for a week started at −90°C for 96 h, followed by warming at 5°C h^{-1} to −20°C. After 24 h at −20°C, warming was continued at 3°C h^{-1} to 3°C. This was followed by 8 h at 3°C and 18 h at 4°C. After a week, the specimen carriers were rinsed three times with 100% acetone at room temperature. During the rinsing procedure round pieces of cell suspensions were released from the specimen carriers and those pieces of cells were infiltrated into the resin. Finally, ultrathin sections of the resin samples were cut on a Reichert-Jung Ultra-cut E ultramicrotome (Wien, Austria) and stained using uranyl acetate and lead citrate. Sections were examined using a JEOL JEM-1011 electron microscope (Tokyo, Japan).

DNA extraction, PCR and sequencing

Total genomic DNA from each strain was extracted using a Geneaid Plant Genomic DNA Mini Kit (New Taipei City, Taiwan). Nuclear 18S rDNA sequences were PCR-amplified with the primer pairs 18SF plus 18SR (Katana *et al.*, 2001) or 20F (Thüs *et al.*, 2011) plus CH1750R (Hallmann *et al.*, 2013). A segment of the chloroplast RuBisCO large subunit gene (*rbc*L) was amplified with primers M35F plus M1338R (McManus & Lewis, 2011) and rbcL1F plus rbcL23R (Hoham *et al.*, 2002). The internal transcribed spacer 2 (ITS2) rDNA region was amplified using the primers AL1500af (Helms *et al.*, 2001) plus LR3 (Vilgalys & Hester, 1990). All PCR reaction mixtures were prepared using either 2× PCRBIO Taq Mix Red (PCR Biosystems, London, UK) or Qiagen Multiplex PCR Plus Kit (Hilden, Germany). The PCR amplification thermal profile for each primer pair is listed in Supplementary table S1. The PCR products were purified with QIAquick PCR & Gel Cleanup Kit (Qiagen, Hilden, Germany). All amplicons were directly Sanger-sequenced at Eurofins Genomics (Ebersberg, Germany). The primer pair mci01_F (5′ ACAAATCACAAGCAGAAACGGG 3′) plus mci01_R (5′ AATGTCCCTTAACCTCCAAATAAGG 3′) were additionally designed for sequencing the *rbc*L intron. The obtained reads were assembled using SeqAssem ver. 07/2008 (Hepperle, 2004). Sequences were deposited in GenBank (www.ncbi.nlm.nih.gov/genbank/) and are available under accession numbers PP668153–PP668156 (18S rDNA), PP668157–PP668161 (ITS2 rDNA) and PP680714–PP680718 (*rbc*L).

Phylogenetic analyses

In addition to newly acquired sequences, we compiled 18S rDNA and *rbc*L sequences from GenBank to represent all major known lineages of the class Trebouxiophyceae, with Chlorophyceae representatives serving as outgroup taxa. The 18S rDNA dataset was aligned with the online MAFFT version 7 (https://mafft.cbrc.jp/alignment/server/) using the L-INS-i method (Katoh & Standley, 2013). The resulting alignment was trimmed with trimAl v1.2rev59 using the -automated1 mode (Capella-Gutiérrez *et al.*, 2009), yielding the final dataset of 83 sequences with 1686 aligned positions. Maximum likelihood (ML) analysis was performed with IQ-TREE multicore version 2.2.5 (Nguyen *et al.*, 2015) using the TIM2e + I + R4 model and a non-parametric bootstrapping with 100 replicates. The evolutionary model was automatically selected by ModelFinder (Kalyaanamoorthy *et al.*, 2017) implemented in IQ-TREE. Bayesian inference (BI) was carried out with MrBayes v3.2.7 (Ronquist *et al.*, 2012) using the GTR + I + G model and two independent runs with one cold and three heated chains each ran for 3 000 000 generations and sampled every 100th generation. The first 25% sampled datapoints were discarded as burn-in. Parameter stability and run convergence were assessed using Tracer v1.7.2 (Rambaut *et al.*, 2018). The *rbc*L dataset of 73 sequences was aligned using the same method, resulting in 1431 aligned positions. For the ML analysis, this dataset was partitioned to the 1st, 2nd, and 3rd codon positions, and respective models GTR + F + R4, SYM + I + R2 and TIM3 + F + I + R4 were applied to each partition. Non-parametric bootstrapping technique with 100 replicates was used to assess node support. For BI analysis, GTR + I + G model was set to each codon position, followed by the steps described above.

For phylogenomic analysis, we extracted plastome-derived transcript sequences from our unpublished transcriptome assembly generated from strain SAG 2660. Briefly, the total RNA was isolated using TRI Reagent® (TR 118) (Molecular Research Center, Inc., Cincinnati, USA), following standard procedures. Transcriptome sequencing was performed by the Institute of Applied Biotechnologies a.s. (Olomouc, Czech Republic) with the Illumina NovaSeq 6000 platform and the pair-end sequencing strategy. The obtained sequence data were quality trimmed with Trimmomatic v0.39 (Bolger *et al.*, 2014). *De novo* transcriptome assembly was performed using Trinity v2.1.1 (Grabherr *et al.*, 2011). The deduced plastome-encoded protein sequences were included into 79 chloroplast genome-encoded protein datasets assembled previously by Turmel *et al.* (2016) (see also Supplementary table S2). The same datasets were further updated by including sequences from *Kalinella pachyderma, Massjukichlorella minus* and *Medakamo hakoo* (Liu *et al.*, 2023; Takusagawa *et al.*, 2023). Single-gene datasets were prepared by aligning amino acid sequences with MAFFT (E-INS-i method was used) and trimming them with trimAl (-automated1 option was used). The alignments were concatenated into a single supermatrix (16700 aligned positions in total) using FASconCAT-G_v1.05 (Kück & Longo, 2014). ML analysis was carried out with IQ-TREE employing the automatically selected Q.yeast + I + G4 model and a non-parametric bootstrapping with 100 pseudoreplicates. All sequence alignments employed in this study are available from the Figshare repository (10.6084/m9.figshare.25625283).

ITS2 rDNA secondary structures

The ITS2 region was annotated using the ITS2 database (Merget *et al.*, 2012). The secondary structure of each sequence was generated using the UNAFold web server (Zuker, 2003), and the minimum energy structure was exported as a Vienna-formatted text file. The structures were inspected and edited in 4SALE v1.7.1 (Seibel *et al.*, 2006) to correct misfolded regions. Sequences with their secondary structures were aligned using the same program and the ClustalW algorithm. The resulting alignment was used to compute a matrix of compensatory base changes between all pairs of sequences and structures (in 4SALE), and to calculate pairwise phylogenetic distances in the program ProfDist v0.9.9. (Wolf *et al.*, 2008). The retrieved phylogenetic distances between sequences + structures were graphically visualized as a UPGMA cladogram without constraints (cophenetic correlation = 0.53) in the program PAST 4.14 (Hammer *et al.*, 2001). Folded ITS2 structures were visualized using the online tool Pseudoviewer (http://pseudoviewer.inha.ac.kr) with default settings. Graphical elements were adapted in Inkscape 1.1.

Results

Morphology

Vegetative cells of strain SAG 2660 were crescent-shaped, with narrowly rounded ends (Figs. 1, 2). In mature cells, the ends were also slightly bent. Some cells exhibited a tapered shape, being wider at one end than the other (Figs 2, 3). The cell wall was smooth and robust. The cells were 12–20 µm long and 3–8 µm wide. They contained a single chloroplast without an obvious pyrenoid. In young cells the chloroplast was trough-shaped and occupied a half of the cell volume. With age, the chloroplast became lobed into two or more parts (Figs 1–3). Small starch grains were seen squeezed between thylakoids under TEM (Fig. 2). The nucleus was on the opposite side to the chloroplast, in the central part of the cell. Oil droplets were also observed in some cells (Fig. 3). When cultivated on agar plates, the majority of cells became drop-like with one end prominently broader than the other (Fig. 4). Reproduction took place by autosporulation, forming two, four or eight daughter cells within a single mother cell. The serially arranged daughter cells were released by a rupture of the mother cell wall (Fig. 5).

Vegetative cells of strain SAG 2659 were also crescent-shaped, with narrowly rounded ends (Figs 6, 7). One cell end was often slightly slenderer than the other. Cell size ranged from 6–24 µm in length and 3–8 µm in width. A trough-shaped chloroplast occupied most of the cell volume. The pyrenoid was often obscure in liquid-grown culture of the strain (Fig. 6). However, single pyrenoids became clearly discernible in cells grown on agar plates (Fig. 7; arrowheads). Cells were frequently vacuolated, with either two prominent vacuoles or a chain of smaller ones (Fig. 7). A single nucleus was present within the cell. Reproduction occurred by producing 4–8 autospores per autosporangium. In mother cells, the autospores were arranged serially.

In contrast, the cells of strain SAG 2477 were ellipsoidal to broadly ellipsoidal in shape with broadly rounded ends (Figs 8, 9). Some cells were also slightly bent, but the degree of inclination was not as significant as in the other studied strains. They were 7–16 µm long and 2.5–6.5 µm wide. When cultivated on agar plates, the cell width reached up to 9 µm, with occasional variations in cell shape, some of which appeared triangular (Fig. 9). The chloroplast was fragmented, divided into 2–4 main parts, without a pyrenoid. Older cells contained two or more vacuoles. Reproduction via serially arranged 4–6 autospores per autosporangium was observed.

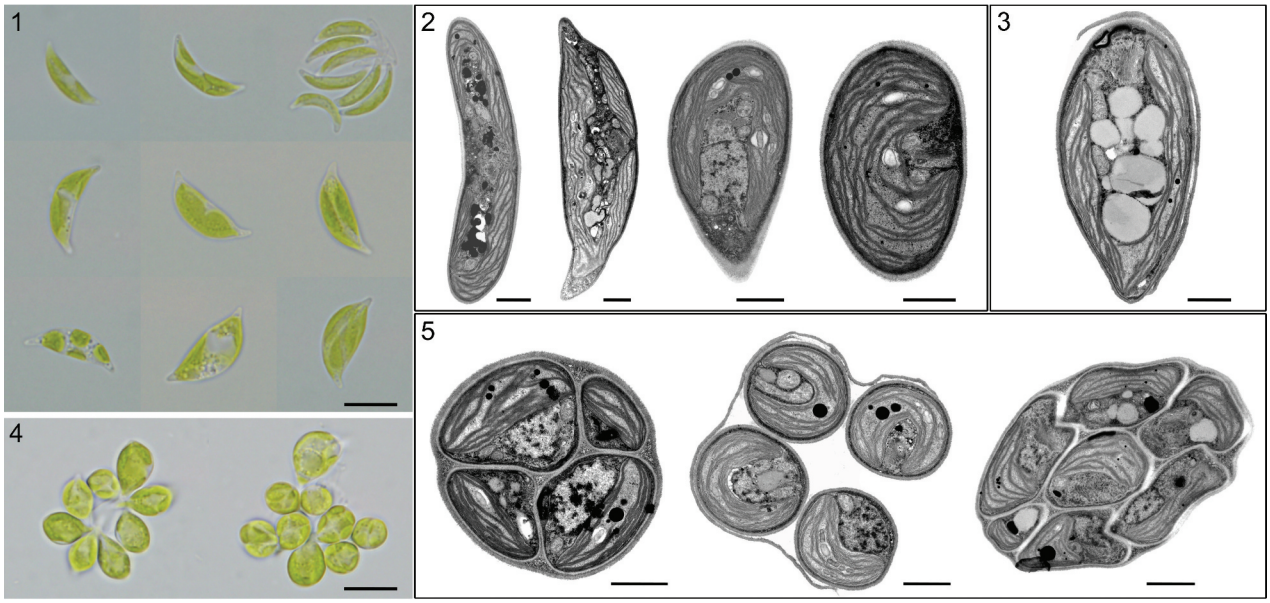


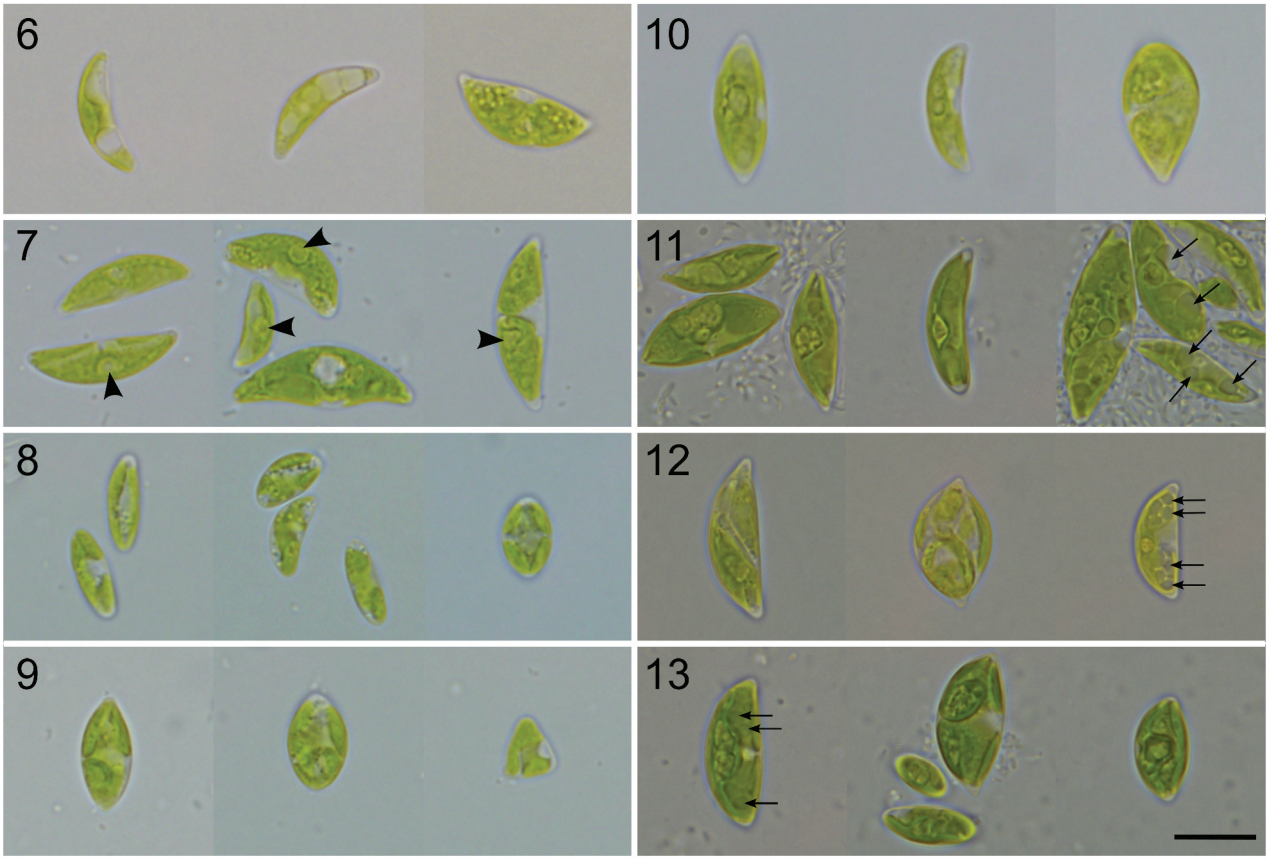
Fig. 1–5. Morphology and ultrastructure of the newly isolated strain SAG 2660 (*Ragelichloris palustris* gen. et sp. nov.). **Fig. 1**. Light micrographs of the strain cultivated in liquid medium. **Fig. 2**. Ultrastructure of vegetative cells. **Fig. 3**. Vegetative cell full of lipid droplets. **Fig. 4**. Cells grown on agar plates. **Fig. 5**. Autosporangia with four and eight daughter cells. Scale bars = 10 μm in Figs 1, 4 and 1 μm in Figs 2, 3, 5.

Considering morphological similarity to species of *Chlorolobion* of some of the previously mentioned strains, we also studied a series of strains assigned to this genus obtained from the ACOI collection (Table 1). Two of them, ACOI 2681 and ACOI 3192, both isolated from Serra do Gerês in Portugal and previously identified as *C. lunulatum*, demonstrated similar cell morphologies to our new isolate SAG 2659 (Figs 10–13). More specifically, they both had a crescent-like outline with both ends narrowly rounded or slightly tapered, with the cell length ranging from 7–25 μm and the width from 3–9 μm. The chloroplast was parietal and trough-shaped and contained a single prominent pyrenoid. Rows of numerous vacuoles were also present within the cells (Figs 11–13; arrows). Reproduction by four or eight serially arranged autospores per autosporangium was observed. Another ACOI strain investigated in detail, *C. obtusum* ACOI 732, was found to neatly match the original description of the species by Korshikov (1953) (Figs 14, 15). The six remaining *Chlorolobion* strains departed morphologically from these two species by having needle-like shapes (Supplementary fig. S3).

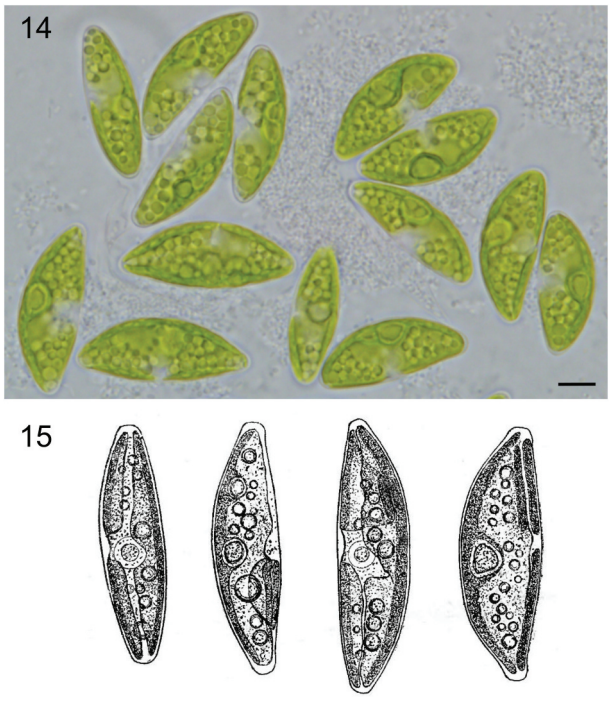
Phylogenetic analyses

To examine the phylogenetic position of the two new isolates (SAG 2660 and SAG 2659) and the ACOI strains 2681, 3192 and 732, we obtained sequences of their 18S rRNA genes and used them in a phylogenetic reconstruction with ML and Bayesian methods, sampling widely across Trebouxiophyceae to represent all major lineages of the class. The new isolates were part of a strongly supported clade (ML/BI: 99/1.00) containing several previously studied yet phylogenetically unresolved trebouxiophytes (Fig. 16). Strain SAG 2660 was sister to the unidentified desert organism BCP-BC4VF9 sequenced by Fučíková *et al.* (2014) but not studied in further detail, while SAG 2659 branched with ACOI strains 2681 and 3192 (*C. lunulatum*). Sequences of ACOI 2681 and 3192 were identical for all three molecular markers sequenced and thus we treat these two strains as conspecific. The SAG 2659 + ACOI 2681/3192 cluster was then sister to the Icelandic *Thorsmoerkia curvula* described by Nicoletti *et al.* (2021) (Fig. 16). Noteworthy, SAG 2659 contained an intron in its 18S rRNA gene, but its insertion site differed from the position of the intron found in the *T. curvula* 18S rRNA gene (Nicoletti *et al.*, 2021). All the previously mentioned organisms constituted a clade further united with strain SAG 2477 as a more deeply diverged sister lineage. This newly emerged broader clade, which we termed 'microcroissants', received high statistical support in both 18S rDNA analyses (ML/BI: 95/1.00). Meanwhile, *C. obtusum* ACOI 732 also belonged to Trebouxiophyceae based on the newly sequenced 18S rRNA gene. However, instead of clustering with 'microcroissants' in the 18S rDNA phylogeny it branched with *Leptosira* and *Chloropyrula*, although statistical support for this position was obtained only in the BI analysis (Fig. 16).

To further test the grouping of all 'microcroissants', we computed a *rbc*L-based phylogenetic tree



Figs 6–13. Morphology of the 'microcroissant' clade. **Fig. 6**. SAG 2659 strain in liquid medium. **Fig. 7**. SAG 2659 strain on agar plates. **Fig. 8**. SAG 2477 in liquid medium. **Fig. 9**. SAG 2477 on agar plates. **Fig. 10**. ACOI 2681 in liquid medium. **Fig. 11**. ACOI 2681 on agar plates. **Fig. 12**. ACOI 3192 in liquid medium. **Fig. 13**. ACOI 3192 on agar plates. Arrowheads indicate the pyrenoids, while arrows mark the vacuoles. The scale bar is consistent across all figures, representing 10 μm.



Figs 14, 15. *Chlorolobion obtusum* Korshikov. **Fig. 14**. The matching morphology of ACOI 732. Scale bar = 10 μm. **Fig. 15**. Original drawings of the species from Korshikov (1953).

(Fig. 17). The analysis recovered the same 'microcroissant' clade, though only with moderate statistical support in the ML analysis and virtually no support in BI (ML/BI: 70/0.84), consistent with a generally lower resolution of the *rbc*L tree compared with the 18S rDNA tree. Crucially, the internal topology of the 'microcroissant' clade in the *rbc*L tree was the same as indicated by 18S rDNA sequences. However, in contrast to the 18S rDNA analyses, *C. obtusum* ACOI 732 this time branched off as a sister lineage of the 'microcroissants', with support for this topology to be particularly high (0.99) in the BI analysis (Fig. 17).

Notably, the *rbc*L gene of each SAG 2659 and ACOI 2681/3192 contains a group I intron with an intronic ORF encoding a LAGLIDADG family homing endonuclease. No other case of introns interrupting the *rbc*L gene in any Trebouxiophyceae member was identified by our literature search and inspection of *rbc*L gene sequences available in the GenBank database. (We note that the chloroplast genome sequence ON645925.1, which is assigned in the database to '*Symbiochloris* sp. SG-2018', i.e. a trebouxiophyte genus, and whose *rbc*L gene is interrupted by

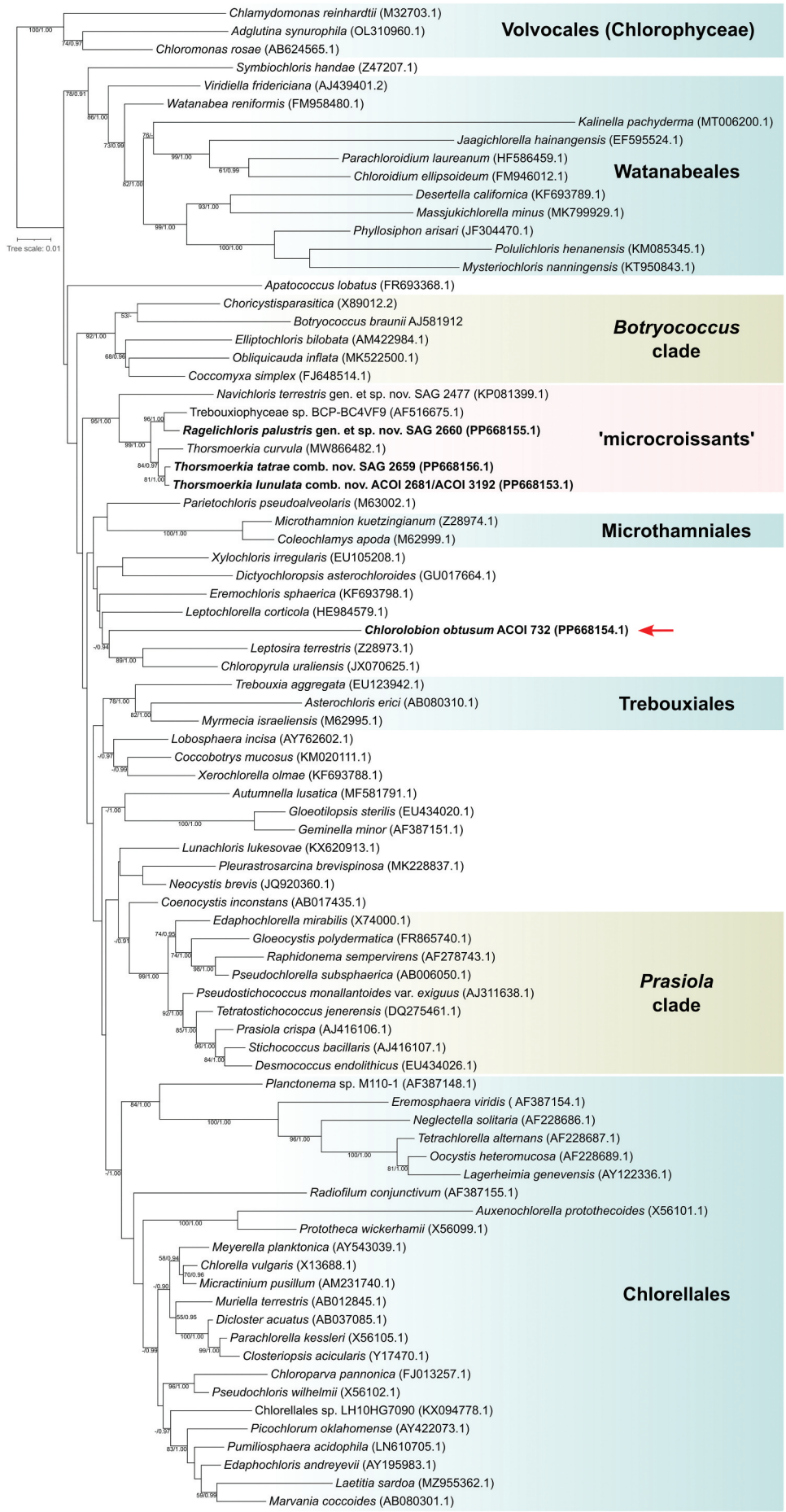


Fig. 16. Maximum-likelihood (ML) phylogenetic tree of the class Trebouxiophyceae based on 18S rDNA. Numbers next to branches indicate statistical support values: ML bootstraps/BI posterior probabilities (shown when ML > 50 and BI ≥ 0.90). New sequences are in bold. Red arrow points to the position of the genus *Chlorolobion*. Tree scale indicates substitutions/site.

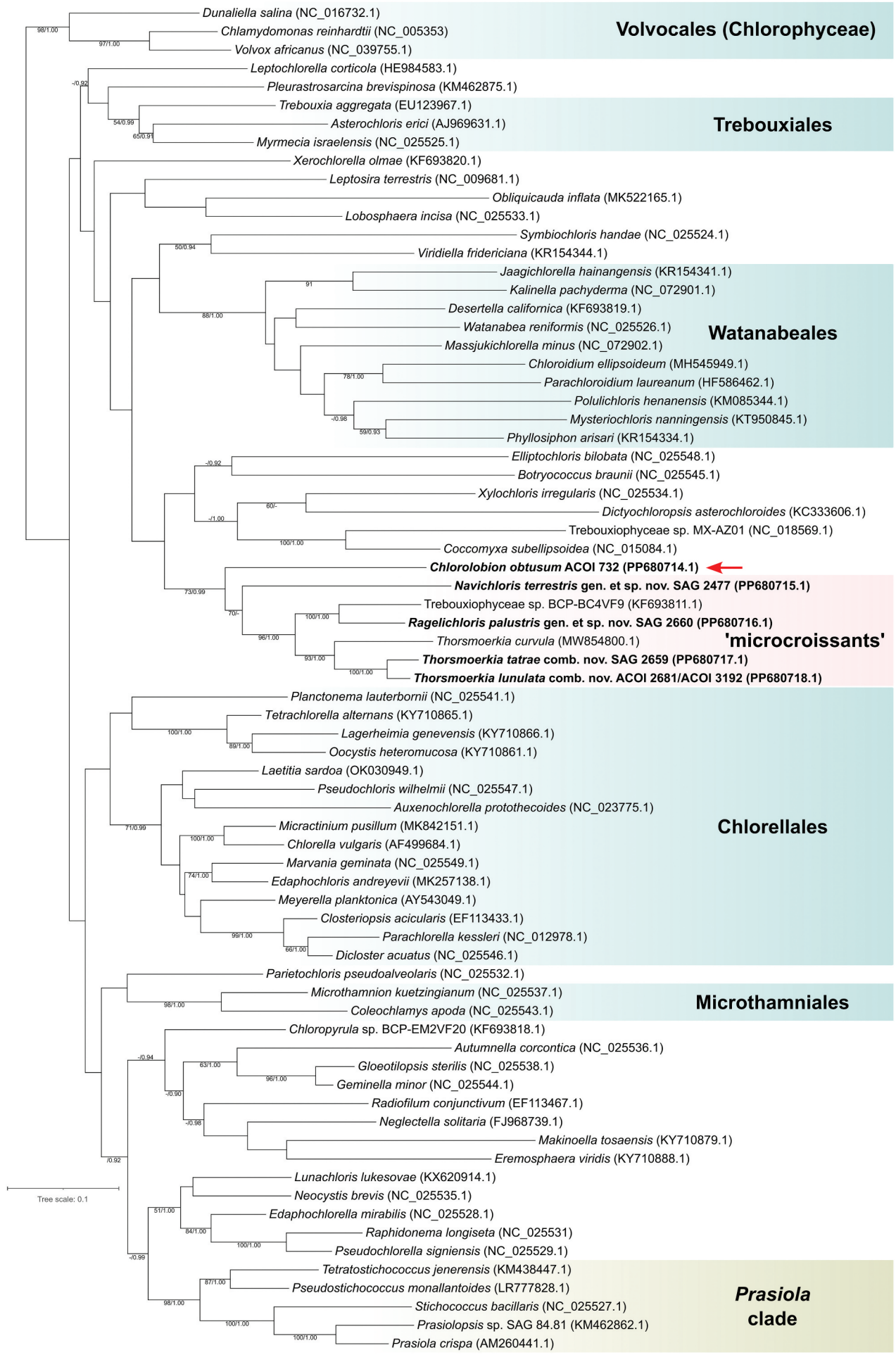


Fig. 17. Maximum-likelihood (ML) phylogenetic tree of the class Trebouxiophyceae based on the *rbc*L gene. The display conventions are the same as for Fig. 16.

two introns, in fact corresponds to the chloroplast genome of the ulvophyte *Symbiochlorum hainanensis*.) The *rbc*L introns of SAG 2659 and ACOI 2681/3192, as well as the proteins encoded by the respective intronic ORFs, are mutually highly similar, consistent with a single acquisition of the intron in the common ancestor of these two microcroissant lineages. Intronic ORFs closest to those of SAG 2659 and ACOI 2681/3192 come from chloroplast genomes of various non-trebouxiophyte green algae and are contained in introns occupying the same position in the *rbc*L gene, but the donor lineage from which the intron was transferred into the microcroissant subclade cannot be discerned.

In order to determine the position more accurately of 'microcroissants' in the Trebouxiophyceae tree, we used plastome-encoded protein sequences of strain SAG 2660 for phylogenomic analysis. The inferred phylogenetic tree placed SAG 2660 as a sister lineage to the coccoid subaerial alga *Xylochloris irregularis*, with the two having the filamentous soil alga *Leptosira terrestris* as their immediate relative. The close phylogenetic relationship among these three algae received full statistical support. Moreover, the clade comprising *Leptosira, Xylochloris* and strain SAG 2660 emerged with robust support (ML: 93%) as a group sister to Microthamniales (Fig. 18).

ITS2 rDNA secondary structures

To gain additional insights into the genetic diversity and relationships of 'microcroissants', we sequenced the ITS2 regions of the respective algae and predicted their secondary structures. Secondary structure analysis allows comparison of the base-pair interactions and their compensatory mutations (= compensatory base changes; CBCs) or base substitutions on only one side of the stem structures (hemi-CBCs) between close relatives. As illustrated in Fig. 19, the most striking difference among the examined strains was the absence of a typical helix IV motif in SAG 2659, ACOI 2681/3192 and *Thorsmoerkia curvula* (MW866482.1), i.e. all representatives of a particular subclade of 'microcroissants' phylogenetic analyses (Figs 16, 17). In addition, ACOI 2681/3192 lacked a short side loop in helix III, a feature that was present in the two closest relatives, making the ITS2 secondary structure of this strain pair the most distinctive one within the 'microcroissant' clade. A detailed comparison of SAG 2659 and ACOI 2681/3192 revealed the presence of a single CBC in helix I and two hemi-CBCs in helix III between the two organisms, while comparison with *T. curvula* (MW866482.1) displayed a total of three CBCs distributed across the helices I and II as well as in the 5.8S and 28S stem in both strains. The comparison of two *Sphagnum*-associated strains, SAG 2660 and SAG 2659, showed five CBCs (one in the helix I, three in the helix III and one in the stem) and four hemi-CBCs. Interestingly, the ITS2 secondary structure of strain SAG 2660 exhibited a closer resemblance to that of the more distantly related SAG 2477 compared with the closer relatives (Fig. 19; the ITS2 sequence from BCP-BC4VF9, the closest known relative of SAG 2660, is not available). They exhibited six CBCs and three hemi-CBCs between themselves. The highest number of CBCs (eight) were found when comparing strain SAG 2660 with ACOI 2681/3192 and *T. curvula*. As expected, the ITS2 secondary structure of ACOI 732 was the most different one (Fig. 19). An UPGMA cladogram, based on genetic distances within the sequence + structure alignment of the 'microcroissant' clade (with ACOI 732 as an outgroup), exhibited topology agreeing with strain similarities evident from the graphical visualization of the ITS2 structural models (Fig. 19).

Discussion

Identification of the new strains

Morphologically, the two newly isolated strains (SAG 2660 and SAG 2659) resemble certain members of the family Selenastraceae (Chlorophyceae) (Krienitz *et al.*, 2001). Examples of highly similar selenastracean algae are different species of the genus *Monoraphidium* Komárková-Legnerová, including *M. terrestre* (Bristol) Krienitz & Klein and *M. dybowskii* (Wołoszyńska) Hindák & Komárková-Legnerová. However, some differences do exist. For example, *M. dybowskii* and *M. terrestre* have a pyrenoid, with and without a starch envelope, respectively, and the latter species is also characterized by a prominent secretion of mucilage (Krienitz *et al.*, 2001). Strain SAG 2659 also contains a starch-covered pyrenoid as evidenced by the light microscopy (Fig. 7), but it does not produce cylindrical cells as *M. dybowskii*, and the maximum cell length measured for SAG 2659 considerably exceeds that of *M. dybowskii*. In contrast, strain SAG 2660 did not show the presence of the pyrenoid matrix even under TEM (Figs 2, 3, 5), and we did not detect any mucilage secretion in either of the two newly isolated strains. Most crucially, the position of the two discussed *Monoraphidium* species in Selenastraceae has been confirmed by molecular means (Krienitz *et al.*, 2001).

Of the so far unsequenced species historically placed in the genus *Monoraphidium*, strain SAG 2659 most closely resembles *M. tatrae* (Hindák) Hindák, originally described as *Chlorolobion tatrae* Hindák and later recombined by the same author as *Choricystis tatrae* (Hindák, 1988). As our results dismiss both new combinations (see below), we refer to this species using its basionym in the subsequent

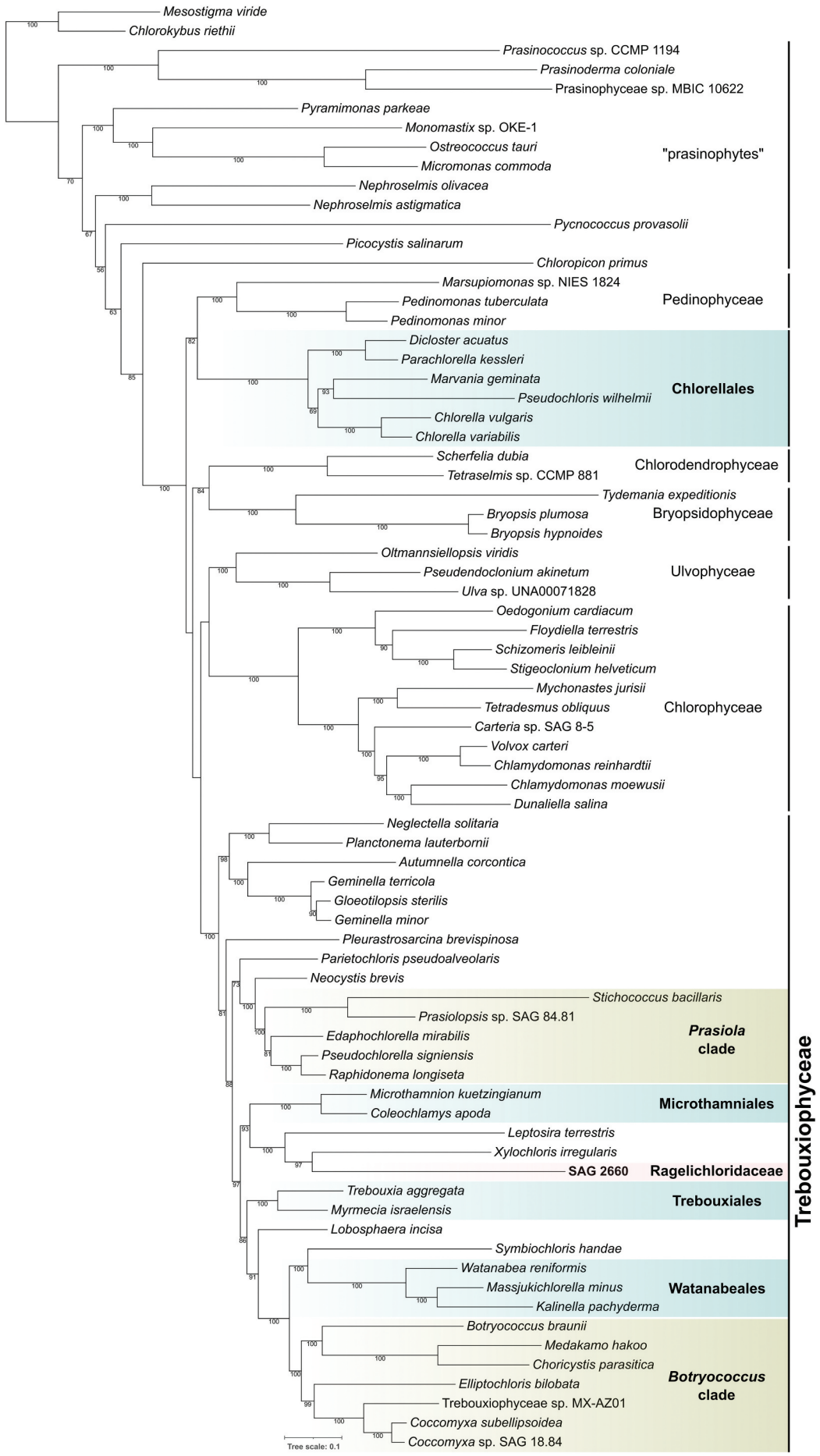


Fig. 18. Maximum-likelihood (ML) phylogenetic tree inferred from a concatenated data set of 79 plastome-encoded proteins from the green algae. The alignment used for the tree inference consisted of 16700 amino acid positions. Bootstrap support values shown when > 50. *Ragelichloris palustris* gen. et sp. nov. is represented by the strain SAG 2660. Tree scale indicates substitutions/site.

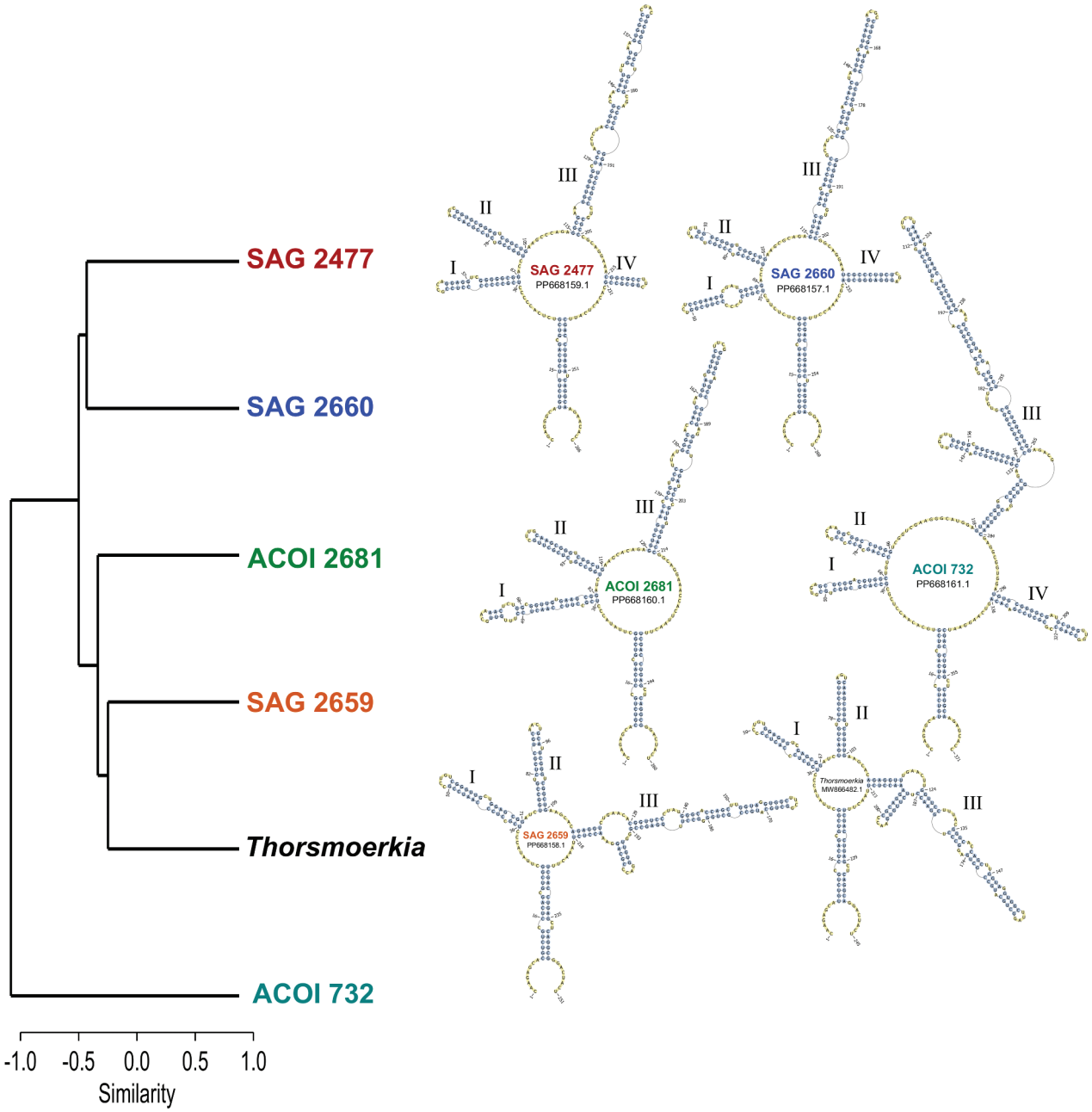


Fig. 19. The UPGMA cladogram based on genetic distances within the sequence + structure alignment of the 'micro-croissant' clade and ITS2 secondary structures of its members. Nucleotides that are paired are shown in blue, while nucleotides that are unpaired are shown in yellow. Helices are marked I to IV.

discussion. The similarity of SAG 2659 and *C. tatrae* concerns the size, cell and chloroplast shape, the presence of the two prominent vacuoles in some cells, as well as the presence of multiple smaller vacuoles in others. Thanks to the available detailed line drawings accompanying the original description of the species (Hindák, 1970), we could confirm all morphological matches of our strain to it. The only significant discrepancy between *C. tatrae* and strain SAG 2659 is the absence of the pyrenoid in the former. We could still link our isolate SAG 2659 to *C. tatrae* because the presence of the pyrenoid was not obvious in all cells when the strain was cultivated in liquid medium (Fig. 6). Noteworthy, Hindák's reclassification of the species as a member of *Monoraphidium* (Hindák, 1977) and eventually *Choricystis* (Hindák, 1988) was on the grounds of the apparent absence of the pyrenoid in *Chlorolobion tatrae*. However, many of the bona fide *Monoraphidium* species described as pyrenoid-less in the past have been shown to possess a pyrenoid after a thorough culture-based re-investigation (Krienitz *et al.*, 2001). Such discrepancies clearly suggest that the presence or absence of the pyrenoid in the original descriptions of green algae should be taken with caution, especially for the minute ones. *C. tatrae* was described from a field sample, so it is not at all surprising that the pyrenoid could have

been overlooked. In our case, the pyrenoid was consistently observed in strain SAG 2659 only when grown on agar plates (Fig. 7). Considering strain SAG 2659 as representing *C. tatrae* is also hinted to by the shared habitat of shallow waters in high elevations. While Hindák (1970) found *C. tatrae* in shallow pools in the mountain lakes area (Päť Spišských plies) in High Tatras, Slovakia, Lenarczyk & Tsarenko (2013) reported it on the Polish side of the same mountain range. Similarly, SAG 2659 was isolated from a *Sphagnum*-dominated shore of a lake (Velké mechové jezírko) in Jeseníky Mountains, Czech Republic.

Unlike strain SAG 2659, we were unable to confidently match our second isolate, SAG 2660, to any of the existing species' names. Apart from *Monoraphidium* spp., other carefully checked candidates included members of the genera *Podohedra* Düringer and *Keratococcus* Pascher (Komárek & Fott, 1983). However, *Podohedra* spp. have a long stalk, and *Keratococcus* spp. typically contain long spiny projections at both ends, features that are absent in strain SAG 2660. The possibility that our organism could have been found and described before cannot be completely ruled out but linking it to any of the existing names is extremely challenging considering the widespread uniformity (the lack of species-specific features) of the croissant-like morphotype, further complicated by phenotypic plasticity (Figs 1, 4). In order to avoid any confusion and misidentification, we propose to establish strain SAG 2660 as a novel taxon with a clearly defined phylogenetic position and reference sequences (see below for Formal taxonomy).

Chlorolobion Korshikov belongs to the Trebouxiophyceae

Our identification of strain SAG 2659 as *C. tatrae* clarifies the specific phylogenetic position of the species in Trebouxiophyceae and thus has immediate implications towards its historically considered generic classification. The combination *Monoraphidium tatrae* (Hindák) Hindák is evidently unjustified, as the genus *Monoraphidium* has been firmly established as a member of Selenastraceae in the class Chlorophyceae, owing to the molecular evidence obtained from a strain identified as the generitype *M. griffithi* (Fawley *et al.*, 2005). *Choricystis* (Skuja) Fott is typified by *C. minor* (Skuja) Fott, a junior synonym of *C. parasitica* (K.Brandt) Pröschold & Darienko (Pröschold *et al.*, 2011), which belongs to the Trebouxiophyceae but is not directly related to 'microcroissants' and is a part of *Botryococcus* clade instead (Figs 16, 18). Hence, the combination *C. tatrae* (Hindák) Hindák must likewise be rejected. May then the most appropriate generic classification of *C. tatrae* be the original one?

Answering this question prompted further investigation into the genus *Chlorolobion* and where it falls in the green algal phylogeny. The genus was first described by Korshikov (1953) with the type species *C. obtusum* and further expanded by Hindák (1970) with his description of two species, *C. tatrae* and *C. lunulatum*. In the course of time, five other species (*C. braunii* (Nägeli) Komárek, *C. glareosum* (Hindák) Komárek, *C. saxatile* (Komárková-Legnerová) Komárek, *C. guanense* Comas, and *C. tianjinensis* Wang & Feng) were assigned to the genus (Komárek & Fott, 1983; Guiry & Guiry, 2023). Among the species described, only *Chlorolobion braunii* has been subjected to a molecular taxonomic investigation and phylogenetic analysis revealed its placement within Selenastraceae (Garcia da Silva *et al.*, 2017). However, the needle-like morphology of *C. braunii* and several other species currently classified as *Chlorolobion* differs substantially from the morphology of the type species *C. obtusum* (Komárek & Fott, 1983), raising the possibility *Chlorolobion* as currently circumscribed is a polyphyletic unit.

Thus, finding the general morphological similarity of our isolates to the 'typical' *Chlorolobion* species, we found it crucial to study additional strains identified as members of *Chlorolobion* and available from the ACOI culture collection (Table 1) in an attempt to clarify their possible relationship to the 'microcroissant' clade. Of the nine such strains investigated, six exhibited needle-like shapes (Supplementary fig. S3) and were found to represent members of the class Chlorophyceae, specifically the family Selenastraceae, based on complete or partial 18S rDNA sequences (data not shown). The remaining three, ACOI 732, 2681 and 3192, however, turned out to be trebouxiophytes, representing two independent lineages (Figs 16, 17). A crucial addition to our study was the strain ACOI 732 isolated and identified by the ACOI curator M. F. Santos as representative of *C. obtusum* – the type species of the genus (Fig. 14). We note that no authentic strain for Korshikov's *C. obtusum* exists, and no type locality was specified by the author, hindering future attempts to re-collect the species. The interpretation of the species thus relies solely on the author's drawings (Fig. 15). Upon examining the morphology of ACOI 732, we endorse the validity of its current identification (Figs 14, 15) and recommend this strain as a suitable candidate for future epitypification of *C. obtusum*.

By demonstrating the position of *C. obtusum* as an independent lineage in Trebouxiophyceae we validate the separate generic status of *Chlorolobion* and clarify its assignment into a particular green algal class. The generic assignment of other nominal *Chlorolobion* species requires further scrutiny. Considering our

results, some species may need to be reassigned to a different genus or genera in Selenastraceae, but a separate careful reassessment of the respective strains, ideally as part of a broader critical revision of the whole family Selenastraceae, is needed. Our results, however, provide a strong case for a taxonomic revision of *C. tatrae* and *C. lunulatum*, and this is presented below.

Expanding the genus Thorsmoerkia and introducing Ragelichloris gen. nov.

As revealed by our molecular phylogenetic analyses, the ACOI strains 2681 and 3192 are closely related to our SAG 2659 isolate. It is thus highly significant that both ACOI strains were identified as *C. lunulatum*, supporting our independent identification of SAG 2659 strain as a closely related species, *C. tatrae*. Indeed, all the morphological features exhibited by ACOI 2681 and 3192 strains are consistent with those of *C. lunulatum* (Figs 10–13). The other ACOI strains assigned by the culture collection to *C. lunulatum* (231, 811) represent selenastracean algae (Table 1) and were obviously misidentified, because their morphology does not match the description of the species (Supplementary fig. S3). Even though Komárek & Fott (1983) suggested possible conspecificity of *C. tatrae* and *C. lunulatum*, we do not endorse it. Both SAG 2659 and the two ACOI strains are morphologically very similar, but phylogenetic divergence (especially evident in the *rbc*L phylogeny) and substantial differences (the lack of a side loop in helix III in ACOI 2681/3192 and the presence of CBCs between SAG 2659 and ACOI strains) in their ITS2 rDNA secondary structures (Fig. 19) support their classification as distinct species.

Nevertheless, *C. tatrae* and *C. lunulatum* cannot stay in the genus *Chlorolobion* as they are not specifically related to *C. obtusum*. All molecular markers employed in this study instead consistently place *C. tatrae* and *C. lunulatum* as relatives of *Thorsmoerkia curvula*, the type and presently the only species of the recently established genus *Thorsmoerkia* Remias & Procházková (Figs 16, 17). It thus seems reasonable to resolve the untenable generic classification of the two nominal *Chlorolobion* species by expanding the circumscription of the genus *Thorsmoerkia* to embrace these two species as new combinations *Thorsmoerkia tatrae* and *Thorsmoerkia lunulata* (see Formal taxonomy).

It is then a matter of an arbitrary decision whether strain SAG 2660 should also be a part of *Thorsmoerkia* or not. The former treatment would be compatible with the general morphological similarity of strain SAG 2660 and *Thorsmoerkia* spp., but the phylogenetic distance between strain SAG 2660 and *Thorsmoerkia* is comparable to that observed between different genera within the Chlorellales or the *Prasiola*-clade (Figs 16, 17), making a case for classification of strain SAG 2660 as a separate genus. In addition, compared with the three *Thorsmoerkia* species, whose ITS2 secondary structure lacks helix IV, strain SAG 2660 has retained the plesiomorphic state with helix IV, making its ITS2 structure more similar to that of SAG 2477, which is even more distantly related to *Thorsmoerkia* than strain SAG 2660 (Fig. 19). In addition, strain SAG 2660 exhibits more tapered cell ends and lacks rows of vacuoles, the latter feature so characteristic for *Thorsmoerkia* species (Figs 1, 2, 6, 7, 10–13). We thus find it reasonable to place strain SAG 2660 into a genus separate from *Thorsmoerkia*, and as there seems to be no other previously described genus that could be considered as a taxonomic home of strain SAG 2660, we describe it as a new species in a new genus, *Ragelichloris palustris* (see Formal taxonomy). Based on our phylogenetic analyses, the now-lost strain BCP-BC4VF9 found in a Mexican desert (Fučíková *et al.*, 2014) is related to *R. palustris* and could be naturally classified in the same genus, yet clearly as a different species.

Navichloris terrestris: *a new genus and species of Trebouxiophyceae*

Touching on strain SAG 2477, there are similarities with other terrestrial coccoid algae, including certain species in the genus *Coccomyxa* Schmidle and *Chlorocloster* Pascher. The former is confidently a trebouxiophyte, yet unrelated to SAG 2477, whereas the latter was described as a member of Pascher's 'Heterokonten' and without evidence *contra* it is by default presently classified in a descendant of the traditionally circumscribed heterokont algae, i.e. in the ochrophyte class Xanthophyceae. However, numerous former members of this grouping have proved to belong to different major lineages of eukaryotes, including green algae (e.g. Eliáš *et al.*, 2013). Indeed, one of the nominal *Chlorocloster* species, *C. engadinensis* Vischer, has been formally transferred to the trebouxiophyte genus *Chloroidium* upon scrutiny with modern methods (Darienko *et al.*, 2010). We were, therefore, compelled to critically compare SAG 2477 with *Chlorocloster*, typified by *C. terrestris*, which was described as a common terrestrial alga occurring in meadow and forest soil (Pascher, 1925). Indeed, both organisms share the same habitat, cell size, and possess tripartite chloroplasts lacking pyrenoids. However, SAG 2477 has a single chloroplast that becomes deeply lobed, forming three apparent parts, while *C. terrestris* possesses three or more individual plastids. Furthermore, a key difference between them is that the cells of *C. terrestris* have clearly narrowed ends and also exhibit an S-shape,

while the cell termini of SAG 2477 are rounded and the alga is not noticeably S-shaped. Another similar species is *Chlorocloster simplex* Pascher also matching the general appearance of the 'microcroissant' but typically having one cell end narrower than the other (Pascher, 1938).

Finally, the ellipsoidal to oval cells and highly fragmented chloroplast of strain SAG 2477 also bear a resemblance to another putative xanthophyte genus *Ellipsoidion* Pascher, including the species *E. simplex, E. stichococcoides, E. acuminatum* and *E. pulchum*. Because of the morphological variability exhibited by strain SAG 2477 (Figs 8, 9), it is challenging to assign it to a specific *Ellipsoidion* species. It is though worth noting that one of the former *Ellipsoidion* species, *E. parvum*, has been reclassified as a conspecific member of the trebouxiophyte alga *Neocystis brevis* (Eliáš *et al.*, 2013). Therefore, a close relationship between strain SAG 2477 and certain *Ellipsoidion* species would not be unexpected. However, as for now, considering the deeply branching nature of the SAG 2477 from the other studied strains (*Ragelichloris* and *Thorsmoerkia*) along with its unmatched morphology with the previously described species, we place the SAG 2477 into a new genus and species, *Navichloris terrestris*, expanding thus the taxonomic diversity of trebouxiophyte algae (see Formal taxonomy).

'Microcroissants' constitute a higher-level clade of Trebouxiophyceae

By employing the conservative molecular markers 18S rDNA and *rbc*L we obtained consistent support for the monophyly of a group encompassing the three genera, *Navichloris, Ragelichloris* and *Thorsmoerkia* (Figs 16, 17). The phylogenetic divergence of the 'microcroissants' (i.e. *Navichloris* + *Ragelichloris* + *Thorsmoerkia*) clade from other trebouxiophytes is comparable even to that of different formally delineated orders within the class. In addition, the similar croissant-like morphology of the three genera also suggests their assignment to the same higher-level taxonomic rank. As for now, we interpret the 'microcroissants' clade as a new family-level taxon, which we describe as Ragelichloridaceae (see Formal taxonomy).

Chloroplast phylogenomics, which surpasses the better-sampled single-gene phylogenies in terms of resolution of deeper branches of the trebouxiophyte phylogeny, uncovered Ragelichloridaceae, represented by *Ragelichloris palustris* (strain SAG 2660), as part of a broader clade additionally including the genera *Xylochloris* and *Leptosira* (Fig. 18). The latter two genera were found to be related to each other in a previous less-well sampled chloroplast phylogenomic tree and together denoted as the '*Xylochloris* clade' (Lemieux *et al.*, 2014). Our results thus expand this emerging grouping by adding Ragelichloridaceae. Considering the results of the 18S rDNA phylogeny (Fig. 16), the genera *Dictyochloropsis* (potentially related to *Xylochloris*; Figs 16, 17) and *Chloropyrula* (specifically related to *Leptosira*; see also Gaysina *et al.*, 2013) are also candidate members of the *Xylochloris* clade, which likely holds also for *Chlorolobion* based on the *rbc*L tree (Fig. 17). The *Xylochloris* clade thus seems to represent a candidate new order in Trebouxiophyceae.

We refrain from a formal definition of the new order in this study and advocate for further phylogenomic and phylotranscriptomic studies to test the aforementioned inferences on the taxonomic composition of the putative order. Nevertheless, we point to a potential synapomorphy of this grouping, namely the absence of the *ycf12* gene previously noticed to be shared by the chloroplast genomes of *Xylochloris* and *Leptosira* (Turmel *et al.*, 2015). The gene encodes a component (also known as Psb30) of the photosystem II complex (Inoue-Kashino *et al.*, 2011) and is virtually omnipresent in chloroplasts of green algae and plants, with additional exceptions found only among seed plants (all angiosperms and a subset of gymnosperms) (Turmel & Lemieux, 2018; Kwon *et al.*, 2020). In most trebouxiophytes, including taxa in the phylogenetic vicinity of the *Xylochloris* clade (Microthamniales, Trebouxiales), the gene is part of a gene block *psbK-ycf12-psaM*. Our *R. palustris* transcriptome assembly contains a transcript that includes the *psaM* gene directly downstream of *psbK*, with no *ycf12* in between them (Supplementary fig. S4), paralleling thus the situation in the fully sequenced chloroplast genomes of *Xylochloris irregularis* (NC_025534.1) and *Letosira terrestris* (NC_009681.1). It is thus likely that *R. palustris* shares the *ycf12* loss with the two previously investigated members of the *Xylochloris* clade. Indeed, no *ycf12* homologue could be found in the whole transcriptome assembly *R. palustris*, and it is missing also from the transcriptome assembly available for *Leptosira terrestris*. This indicates that the *ycf12* loss from the chloroplast genome in the *Xylochloris* clade has not been compensated for by the transfer of the gene to the nuclear genome, and that the photosystem II of these algae differs from that of other green algae by the absence of the *ycf12*-encoded subunit.

Formal taxonomy

***Ragelichloridaceae* Barcytė, fam. nov.**

Description

Unicellular croissant-shaped or broadly ellipsoidal cells with rounded ends. Solitary or in loose groups. Uninucleate. Chloroplasts are one per cell and lobed in older cells, with or without a pyrenoid. With or

without vacuoles. Asexual reproduction by autospores. Found in freshwater and terrestrial habitats.
TYPE GENUS: *Ragelichloris* Barcytė.
REMARKS: The family as delimited here presently includes *Ragelichloris* gen. nov., *Navichloris* gen. nov. and *Thorsmoerkia* Remias & Procházková.

Ragelichloris Barcytė, gen. nov.

Description
Unicellular, solitary or in loose groups, morphologically plastic algae demonstrating croissant-like to drop-like shapes with narrowly rounded ends. Uninucleate. Chloroplast single, parietal, trough-shaped or multilobed. Asexual reproduction by autospores. The genus differs from other genera in nuclear 18S and ITS2 rDNA and chloroplast *rbc*L sequences.
TYPE SPECIES: *Ragelichloris palustris* Barcytė, sp. nov.
ETYMOLOGY: The genus name *Ragelichloris* is derived from the Lithuanian word 'ragelis' meaning croissant and Greek word *khloros/χλωρός* meaning 'pale green'.

Ragelichloris palustris Barcytė, sp. nov. (Figs 1–5)

Description
In liquid cultures cells are croissant-shaped, 12–20 µm long and 3–8 µm wide; on agar plates cells are drop-like. Chloroplast parietal and trough-shaped, divided in several parts in older cells, without a pyrenoid. Reproduction by 2-4-8 autospores, liberated by rupture of sporangial cell wall. Sexual reproduction not observed.
HOLOTYPE: Metabolically inert (cryopreserved) culture SAG 2660 at the Culture Collection of Algae at the University of Göttingen, Germany (SAG). SAG CryoBank Number: Z000695311.
TYPE LOCALITY: Raised bog Plynoja, Pagramantis Regional Park, Tauragė, Lithuania (55°19′23″N, 22°8′18″E).
ETYMOLOGY: The Latin word '*palustris*' means 'of the marsh or swamp' or 'marshy, swampy'. It points to the fact that the species was isolated from the peat bog.

Navichloris Hodač & Barcytė, gen. nov.

Description
Vegetative cells solitary, ellipsoidal to broadly ellipsoidal. Uninucleate. The single chloroplast is parietal, fragmented. Asexual reproduction via autospores. The genus differs from other genera in nuclear 18S and ITS2 rDNA and chloroplast *rbc*L sequences.
TYPE SPECIES: *Navichloris terrestris* Hodač & Barcytė.
ETYMOLOGY: The genus name comes from a Latin word *navis* meaning 'ship', emphasizing ship-shaped cells and Greek word *khloros/χλωρός* meaning 'pale green'.

Navichloris terrestris Hodač & Barcytė, sp. nov. (Figs 8, 9)

Description
In liquid cultures cells are solitary, ellipsoidal to broadly ellipsoidal with broadly rounded ends, 7–16 µm long and 2.5–6.5 µm wide; on agar plates irregular shapes may appear. Can be slightly bent. Chloroplast single, parietal, and divided into two to four main parts. Pyrenoid absent. Vacuoles may be present. Asexual reproduction via 4–6 autospores. Sexual reproduction not observed.
HOLOTYPE: Metabolically inert (cryopreserved) culture SAG 2477 at the Culture Collection of Algae at the University of Göttingen, Germany (SAG). SAG CryoBank Number: Z000695331.
TYPE LOCALITY: Soil in spruce forest, Swabian Alb, Germany (48°24′44.145″N, 9°21′20.127″E).
ETYMOLOGY: The species name '*terrestris*' comes from the Latin word '*terra*' meaning 'earth' or 'soil', emphasizing that this species is isolated from soil.

Thorsmoerkia tatrae (Hindák) Barcytė, comb. nov. (Figs 6, 7)

BASIONYM: *Chlorolobion tatrae* Hindák, 1970, Algol. Stud. 1: 13, Fig. 4.
SYNONYMS: *Monoraphidium tatrae* (Hindák) Hindák, 1977: 109. *Choricystis tatrae* (Hindák) Hindák, 1988: 186.
EPITYPE: Metabolically inert (cryopreserved) culture SAG 2659 at the Culture Collection of Algae at the University of Göttingen, Germany (SAG). SAG CryoBank Number: Z000695321. Figs 6, 7 show epitype morphology.
TYPE LOCALITY: Päť Spišských plies, Tatra Mountains, Slovakia.

Thorsmoerkia lunulata (Hindák) Barcytė, comb. nov. (Figs 10, 11)

BASIONYM: *Chlorolobion lunulatum* Hindák, 1970, Algol. Stud. 1: 11, Figs 2, 3.

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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/10.1080/09670262.2024.2383626

Supplementary table S1. Thermal profiles of PCR reactions.

Supplementary table S2. GenBank accession numbers of chloroplast genomes used for phylogenomic analysis.

Supplementary fig. S1. Bog Plynoja where strain SAG 2660 was isolated from.

Supplementary fig. S2. The Great Moss Lake (Velké mechové jezírko) where strain SAG 2659 was isolated from.

Supplementary fig. S3. Light micrographs of the studied ACOI strains that do not belong to the Trebouxiophyceae.

Supplementary fig. S4. *Ragelichloris palustris* SAG 2660 polycistronic transcript containing coding sequences of the *psbK* and *psaM* genes but lacking the *ycf12* coding sequence in between them.

Author contributions

D. Barcytė: original concept, microscopy, molecular lab work, phylogenetic analyses, drafting and editing manuscript; **L. Hodač:** analysis of molecular data, drafting and editing manuscript; **M. Eliáš:** funding acquisition, analysis of molecular data, drafting and editing manuscript.

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