

**One step closer to eliminating the nomenclatural problems of minute coccoid green algae: *Pseudochloris wilhelmii*, gen. et sp. nov. (Trebouxiophyceae, Chlorophyta)**

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## Abstract

'*Chlorella*' and '*Nannochloris*' were traditional genera of minute coccoid green algae with numerous species described in the past century, including isolates used as experimental test organisms. In the last few years, the introduction of DNA-based phylogenetic analyses resulted in a large number of taxonomic revisions. We investigated and reclassified a taxonomically problematic group within the Trebouxiophyceae (comprising '*Nannochloris eucaryotum*' UTEX 2502, '*N. eucaryotum*' SAG 55.87 and '*Chlorella minutissima*' SAG 1.80), distantly related to the recently described *Chloroparva* isolates (97.5–97.9 % 18S rRNA gene pairwise similarity). Cryopreserved material of SAG 55.87 was selected as holotype for a novel species – *Pseudochloris wilhelmii* Somogyi, Felföldi & Vörös – whose phylogenetic position confirmed the proposal of a new genus. *Pseudochloris wilhelmii* had spherical to oval cells with an average diameter of  $2.6 \times 2.8 \mu\text{m}$  and a simple ultrastructure characteristic of small green algae. Vegetative cells sometimes contained several lipid droplets occupying a large portion of the cells. The cell wall consisted of an outer trilaminar layer and an inner microfibrillar sheet. Cells divided by autosporulation, forming two or four daughter cells per autosporangium. The pigment composition was typical of green algae, with chlorophylls *a* and *b*, and lutein as the dominant carotenoid.

**Key words:** 18S rRNA gene analysis, brackish, *Chlorella*, coccoid chlorophytes, green algae, marine, *Nannochloris*, picoeukaryote, *Pseudochloris wilhelmii*, taxonomic revision

## Introduction

The taxonomic and nomenclatural problems of coccoid green algae lacking distinctive morphological characters go back decades. Starting with uninformative species descriptions and continuing with re-classification of the taxa based on more detailed morphological (e.g. cell wall structure) and chemotaxonomical characterizations, nowadays the combination of morphological, ecophysiological and molecular phylogenetic methods offers the best approach to creating a new, more natural classification system (Bock *et al.*, 2010; Darienko *et al.*, 2010; Krienitz & Bock, 2012). Incorrect strain identifications, however, have sometimes led to contradictory morphological and/or chemotaxonomic descriptions and there are problematic groups whose members cannot be assigned to any existing genus (Henley *et al.*, 2004). An example is the group studied in the present paper, comprising three misinterpreted algal strains: ‘*Chlorella minutissima*’ C 1.1.9, ‘*C. minutissima*’ SAG 1.80 (in the Sammlung von Algenkulturen der Universität Göttingen, Germany) and ‘*Nannochloris eucaryotum*’ UTEX 2502 (in the Culture Collection of Algae at the University of Texas at Austin, U.S.A.). These strains have nearly identical 18S rRNA gene sequences but are distantly related to the ‘true’ *Chlorella* or *Nannochloris* species (Huss *et al.*, 1999; Krienitz *et al.*, 1999; Henley *et al.*, 2004). The closest known relative of this group was recently described as *Chloroparva pannonica* (Somogyi *et al.*, 2011).

Kalina & Puncochárová (1987) were the first to suggest that ‘*C. minutissima*’ C 1.1.9 is distinct from the nominal *C. minutissima* isolate (Lefevre ALCP no. 87; Fig. 1). Their conclusion was based on ultrastructural differences and the chemotaxonomical results of Kessler (1982). Later, Huss *et al.* (1999) confirmed this separation based on 18S rRNA gene sequences, while Krienitz *et al.* (1999) drew attention to the similarly uncertain phylogenetic position of ‘*C. minutissima*’ SAG 1.80 (Fig. 1). Nevertheless, Huss *et al.* (1999) proposed that, provisionally, the name ‘*C. minutissima*’ should be kept for C 1.1.9. Unfortunately, the C 1.1.9 strain is not maintained in any current culture collection (Henley *et al.*, 2004).

‘*Nannochloris eucaryotum*’ UTEX 2502 was studied by Yamamoto *et al.* (2003), who claimed that this strain was closely related to ‘*C. minutissima*’ C 1.1.9 (Fig. 1). One year later, Henley *et al.* (2004) renamed *N. eucaryotum* as *Picochlorum*

*eucaryotum*, based on the 18S rRNA gene sequence of the authentic strain, Mainz 1. At the same time, they pointed out, that '*N. eucaryotum*' UTEX 2502 was distinct from the authentic strain (Fig. 1).

According to the UTEX catalogue, '*N. eucaryotum*' UTEX 2502 was acquired from the SAG culture collection, where the original strain (SAG 55.87) has been continuously maintained. Both collections claim that the two isolates are identical. Nevertheless, the status of SAG 55.87 is not so clear, since Huss *et al.* (1999) and Yamamoto *et al.* (2003) attributed the 18S rRNA gene sequence X06425 to this strain, which was originally published as that of *P. eucaryotum* Mainz 1 (Sargent *et al.*, 1988). Hence, the identity of this isolate is unclear, and sequencing it is necessary as suggested by Henley *et al.* (2004).

The aim of the present study was therefore to clarify the taxonomic status of the above-mentioned problematic group ('*C. minutissima*' SAG 1.80, '*N. eucaryotum*' UTEX 2502 and '*N. eucaryotum*' SAG 55.87) by morphological and molecular phylogenetic methods.

## **Materials and methods**

### *Algal cultures*

Cultures were obtained from SAG and UTEX culture collections. '*Chlorella minutissima*' SAG 1.80 was isolated from a mangrove swamp by R.A. Lewin and deposited in SAG by E. Kessler in 1977. According to the SAG catalogue, this strain might be identical with '*C. minutissima*' C 1.1.9. '*Nannochloris eucaryotum*' SAG 55.87 was isolated from a seawater aquarium (which contained marine animals from Adria, Rovinj) by C. Wilhelm in 1981, who deposited the strain in SAG in 1986. In 1988, SAG sent this strain to UTEX, who gave it the number 2502. Two *Chloroparva* strains (ACT 0602 and ACT 0622), which were isolated from the water of Zab-szék and Böddi-szék soda pans, respectively, in December 2005 (the isolation was carried out on a modified brackish water medium as previously described in Somogyi *et al.*, 2009), were also involved in the phylogenetic analysis.

For maintenance, all cultures were transferred to modified liquid BG11 medium in which only one tenth of the recommended micronutrient solution was used (Rippka *et al.*, 1979) and grown at 21°C and a photon flux density of 40  $\mu\text{mol m}^{-2} \text{s}^{-1}$  from F33 cool-white fluorescent lights (Tungsram, Budapest, Hungary) on a 14 : 10 h light: dark cycle.

### *Microscopy and pigment analysis*

Live cells from young cultures were examined under an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with an Olympus UPLFLN 100 $\times$  objective and differential interference contrast optics. Digital images were made with an Olympus DP71 digital camera. For cell size calculations, approximately 400 cells were measured manually using Olympus Cell<sup>D</sup> software v3.1. Transmission electron microscopy (TEM) analysis of '*N. eucaryotum*' SAG 55.87 was performed according to Somogyi *et al.* (2011). The samples were fixed in 2.5% glutaraldehyde for 3 h at room temperature. After fixation, the cells were centrifuged (4000  $\times g$  for 5 min at 20°C) and the pellet was resuspended and mixed into solidifying agar (2%). The agar plates were then rinsed three times for 15 min with 70 mM Na-K phosphate buffer (pH 7.2), post-fixed for 2 h in 1% OsO<sub>4</sub>, and rinsed again three times for 15 min in the phosphate buffer. The same buffer was used to dilute the fixatives. Following dehydration in an alcohol series (20-20 min in 25%, 50%, 70% and 90% ethanol, then 2  $\times$  20 min in 96% and finally 20 min in absolute ethanol), the samples were embedded in Durcupan ACM epoxy resin (Sigma-Aldrich, St. Louis, MO, U.S.A.). Ultrathin (60 nm) sections were cut with a Reichert–Jung Ultracut E microtome. The sections were stained with 5% uranyl acetate dissolved in methanol for 5 min and treated with Reynolds' lead citrate solution for 5 min. Cells were investigated using a Hitachi 7100 TEM at 75 kV accelerating voltage.

Pigment analysis of '*N. eucaryotum*' SAG 55.87 was performed in three replicates according to Frommolt *et al.* (2001). Pigments were extracted from a lyophilized algal suspension in a solvent consisting of 90% methanol : 0.2 M ammonium acetate (90 : 10, v/v) and 10% ethyl acetate. The suspension was then centrifuged twice for 1 min at 20 800  $\times g$  (Centrifuge 5417C, Eppendorf, Hamburg, Germany), and injected into a Nucleosil 100-5 C18 HPLC column (12.5  $\times$  4 mm,

Macherey & Nagel, Düren, Germany). Reversed phase HPLC was made on Ultimate 3000 HPLC system (Dionex, Sunnyvale, USA). The detection wavelength for integration was 440 nm. Peaks were identified by comparing retention times ( $t_r$ ) and on-line spectra of pigment fractions with those of authentic standards.

### *Phylogenetic analysis*

Genomic DNA was extracted according to the procedure described by Somogyi *et al.* (2009). To avoid the formation of non-chlorophyte amplicons, PCR amplification of the whole 18S rRNA gene was completed with two separate Chlorophyta-specific reactions, as some of the strains were not axenic and therefore might contain other eukaryotic taxa, e.g. UTEX 2502 was heterogeneous according to Yamamoto *et al.* (2003). PCRs performed with primer pairs Euk328f–Chlo02R and ChloroF–Euk329r (Table 1) generated two overlapping fragments that were merged after sequencing. Reactions were carried out in a final volume of 50  $\mu$ l using approximately 2  $\mu$ l genomic DNA, 0.2 mM of each deoxynucleotide, 1 U LC *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania), 1 $\times$  PCR buffer (Fermentas), 2 mM MgCl<sub>2</sub>, 0.325 mM of each primer and 20  $\mu$ g of BSA (Fermentas). The thermal profile consisted of a first denaturing step at 98°C for 3 min, followed by 32 amplification cycles and a final extension step at 72°C for 10 min. In the case of the primer pair Euk328f–Chlo02R, an amplification cycle consisted of 94°C for 45 s, 55°C for 1 min and 72°C for 1.5 min, while in the case of the primer pair ChloroF–Euk329r the cycle was 94°C for 1 min, 59°C for 1 min and 72°C for 1 min. Amplicons were purified with the PCR-M<sup>TM</sup> Clean Up System (Viogene, Sijhih, Taiwan), and sequencing was done with the BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, U.S.A.) using the primers listed in Table 1. Chromas software v1.45 (Technelysium, South Brisbane, Queensland, Australia) was used for the manual correction of automatic base calling on chromatograms and for the removal of primer sequences. The 18S rRNA gene sequences generated were compared to the GenBank nucleotide database using Blast (Altschul *et al.*, 1997). Sequence alignment of various trebouxiophycean sequences (excluding introns) was performed with SINA v1.2.9. (Pruesse *et al.*, 2012) and

ambiguous regions were subsequently removed using MEGA5 software (Tamura *et al.*, 2011). The alignment is available as a Supplementary file.

Maximum likelihood and maximum parsimony analyses were carried out with GARLI 2.0 web service (Bazinnet & Cummings, 2011) and MEGA5, respectively. Bayesian analysis for phylogenetic reconstruction was conducted with the Markov Chain Monte Carlo algorithm in two simultaneous, completely independent analyses running for 2,000,000 generations (sampled every 100 generations) using MrBayes version 3.1 (Huelsenbeck & Ronquist, 2001). The first 10% of the calculated trees were discarded and posterior probabilities were calculated after the two independent runs had reached convergence. Phylogenetic analyses were performed on the basis of the likelihood settings calculated with Modeltest version 3.7 (Posada & Crandall, 1998). The 18S rRNA gene sequences obtained are available in GenBank under the accession numbers: JX235961 (UTEX 2502), JX235962 (SAG 55.87), JX235963 (ACT 0602) and FJ013258 (ACT 0622). Since the 18S rRNA gene sequence of SAG 1.80 determined in this study was identical to the currently available GenBank record (AB006046), no sequence was deposited in this case.

## **Results and Discussion**

### *Morphology, ultrastructure and reproduction*

All of the strains had green, spherical or oval cells, which propagated by autosporulation. Light microscopy revealed two, three or four autospores per sporangium. The cells of SAG 55.87 were 1.6–3.9  $\mu\text{m}$  wide and 1.8–4.0  $\mu\text{m}$  long, with an average size of  $2.6 \times 2.8 \mu\text{m}$ . Similar results were found in case of UTEX 2502 (with an average size of  $2.6 \times 2.9 \mu\text{m}$ ) and SAG 1.80 (with an average size of  $2.7 \times 2.9 \mu\text{m}$ ).

TEM investigation of SAG 55.87 revealed that the cell organization was very simple: usually a single nucleus, chloroplast and mitochondrion occupied the major portion of the cells (Figs 2-4). The chloroplast was parietal and cup-shaped, usually containing electron-dense plastoglobuli and starch grains (Figs 2, 4). Thylakoids were grouped into bands (lamellae) of three to six thylakoids. No grana were observed. A single elongated mitochondrion with cristae was found in the central cytoplasm, closely

associated with the concavity of the chloroplast and also with the nucleus (Fig. 2). Vacuoles and a peroxisome were also observed, surrounded by the cytoplasm, which contained ribosomes (Fig. 2). A Golgi body was also found (Fig. S1 as Supplementary material). There were no flagella and no basal bodies were detectable by electron microscopy. In some cases, vegetative cells contained several lipid droplets (Fig. 3).

The cell wall had electron-dense and electron-transparent layers and an average thickness of 50–90 nm. It consisted of an outer layer with a trilaminar structure (an inner electron-dense, a middle electron-transparent and an outer electron-dense layer) and an inner, dense and relatively thick layer (inner microfibrillar sheet) covering the plasma membrane (Fig. 5). No mucilaginous envelope was observed on the outer surface of the cells (Figs 2–4).

Autosporulation was also confirmed by electron microscopy: each mother cell formed two or four autospores (Figs 6–11). The layers of the mother and the daughter cell walls were clearly visible during autosporulation (Figs 9, 10). Sporangia having three or four autospores were also observed as a result of two separated processes of autosporulation. Daughter cells were released by the rupture of the mother cell wall (Figs 8, 11). Sometimes ghost-like, curling, empty walls were observed after the release of the daughter cells.

#### *Pigment composition*

SAG 55.87 contained typical chlorophyte pigments, including chlorophylls *a* and *b*, and lutein as the dominant carotenoid (Table 2, Fig. 12). The chlorophyll *a* content was  $13.4 \pm 1.037$  mmol g<sup>-1</sup> dry weight. The chlorophyll *b* ( $t_r$ : 23.55 min) and *a* ( $t_r$ : 24.6 min) ratio of SAG 55.87 was 0.25 by weight, and 0.29 by molarity (Table 2). The main carotenoids were lutein, violaxanthin and neoxanthin (Table 2, Fig. 12).

#### *18S rRNA gene analysis*

Almost complete 18S rRNA gene sequence analyses were performed with the three strains (SAG 1.80, SAG 55.87 and UTEX 2502), which resulted in completely identical 1754 nucleotide long regions. These sequences showed 99.9% pairwise similarity to C-



1.1.9 (a single C nucleotide insertion was observed between the positions 1704 and 1705 of X56102). Re-sequencing of SAG 1.80 resulted in a completely identical sequence to the one currently available (AB006046), while in the case of UTEX 2502 an additional single nucleotide insertion/deletion was observed, which could be the result of PCR amplification and sequencing bias (a T at position 490 of AB080304, which occurred within a TTTT motif; our sequence had TTT instead).

Sequencing the 18S rRNA gene of SAG 55.87 confirmed its identity (100 % pairwise similarity) with UTEX 2502. Thus, our finding suggests that Huss *et al.* (1999) and Yamamoto *et al.* (2003) used incorrect sequence data (strain Mainz 1, GenBank X06425) for SAG 55.87 in their phylogenetic analysis. The cause of this mistake was probably that both Mainz 1 and SAG 55.87 strains were isolated together from the same place (a seawater aquarium in Mainz) by Wilhelm, as stated by the SAG catalogue and Wilhelm *et al.* (1982). Unfortunately the Mainz 1 strain does not seem to be available in any culture collection.

Based on the phylogenetic analysis, the three strains we studied formed a distinct, well-separated group close to the recently described *Chloroparva pannonica* (Fig. 13). Excluding the regions containing introns, the *Chloroparva* strains showed 99.6–100% similarity to each other but only 97.5–97.9% pairwise nucleotide similarity values with the three strains (Fig. 13). The relatively low pairwise similarities between the three strains studied here and *Chloroparva*, and the high bootstrap values (100/100/1.00 for *Chloroparva* and -/98/0.93 for the studied group; Fig. 13) support the designation of a novel genus separate from *Chloroparva*.

#### *Morphological and ultrastructural comparisons*

On the basis of the morphological analysis, SAG 55.87 differed significantly from strain Mainz 1 (*Picochlorum eukaryotum*), which confirmed that the two isolates are non-identical even though they have the same origin. According to the original description, the cell size of *P. eukaryotum* ranges from 0.8 to 1.5  $\mu\text{m}$  in width and 1.1 to 2.2  $\mu\text{m}$  in length (Wilhelm *et al.*, 1982). SAG 55.87 had significantly larger cells with an average diameter of  $2.6 \times 2.8 \mu\text{m}$ . The cellular organization and the cell wall structure were very similar in the two isolates (Wilhelm *et al.*, 1982; Menzel & Wild, 1989), but this is not

uncommon among coccoid green algae (Krienitz *et al.*, 1996; Krienitz *et al.*, 1999; Krienitz *et al.*, 2011). On the other hand, in the case of strain Mainz 1, only two autospores were formed per mother cell (Wilhelm *et al.*, 1982), in contrast to the two, three or four in SAG 55.87 strain. As stated by Krienitz *et al.* (1996), however, the number of autospores may depend on culture conditions.

To the best of our knowledge, TEM investigations of the isolates have not been carried out previously, but two strains (SAG 55.87 and UTEX 2502) were studied by light microscopy (Tschermak-Woess, 1999; Yamamoto *et al.*, 2001; 2003). As described by Tschermak-Woess (1999), SAG 55.87 had ellipsoidal cells somewhat less than  $2 \times 2 \mu\text{m}$ , and the size of mother cells reached  $3.5 \mu\text{m}$ . The other strain, UTEX 2502 was first characterized as having spherical cells  $2.5 \mu\text{m}$  in diameter (Yamamoto *et al.*, 2001), but later said to have larger cells, the autospores being  $3\text{--}4 \mu\text{m}$  in diameter and the mother cells  $5\text{--}6 \mu\text{m}$  (Yamamoto *et al.*, 2003). According to our morphological analysis, the vegetative cells of SAG 55.87; UTEX 2502 and SAG 1.80 were larger than those stated by Tschermak-Woess (1999) but smaller than those described by Yamamoto *et al.* (2003). The results of Yamamoto *et al.* (2001), however, are in good agreement with our findings. Autosporulation (with two, three or rarely four autospores per mother cell) was also described earlier, as well as the presence of oil droplets and starch grains in vegetative cells (Tschermak-Woess, 1999; Yamamoto *et al.*, 2001; 2003).

*Chloroparva pannonica*, the sister group of the novel species (Fig. 13; Somogyi *et al.*, 2011), is characterized by spherical to ovoid cells of  $1.4 \times 1.7 \mu\text{m}$ , a simple cell organization, typical chlorophyte pigment composition (chlorophylls *a* and *b* and lutein as the main carotenoid), and a trilaminate cell wall with an inner microfibrillar and an innermost electron-transparent layer (Somogyi *et al.*, 2011). In comparison, SAG 55.87 has larger cells, but a similar internal cell organization and pigment composition. Starch grains, which are usually present in SAG 55.87, were only rarely observed in *C. pannonica* chloroplasts under the same culture conditions. The cell wall structure also differs: the innermost electron-transparent layer is absent in SAG 55.87. Lipid droplets, sometimes found in SAG 55.87, were never found in the vegetative cells of *C. pannonica* (Somogyi *et al.*, 2011).

Fatty acid analysis has been carried out for both genera. In the case of *C. pannonica*, oleic acid (18:1 n-9) was found to be the main component, constituting approximately 90% of the total extracted fatty acid content (Somogyi *et al.*, 2011). Lang *et al.* (2011) described the fatty acid profiles of SAG 55.87 and SAG 1.80, which were not identical. The main fatty acid of SAG 55.87 was oleic acid, as in *C. pannonica*, but it constituted only 20% of the total extracted fatty acid content. Palmitic acid (16:0) and alpha-linoleic acid (18:3 n-3) were also found in large amounts (17% and 18%, respectively). On the other hand, the main fatty acids of SAG 1.80 were linoleic acid (18%) and alpha-linoleic acid (16%) and this strain contained only 6% of oleic acid. A relatively high content (14%) of 9-octadecanamide was found instead (Lang *et al.*, 2011). These different fatty acid profiles, however, may be the result of different culture conditions.

#### *Taxonomic considerations*

The results of both the morphological investigation and the phylogenetic analysis clearly support the re-classification of the studied isolates into a newly designated genus, clarifying the taxonomic position of a ‘problematic group’ within the Trebouxiophyceae. As the original names (‘*Nannochloris eucaryotum*’ and ‘*Chlorella minutissima*’) were incorrect and the group is only distantly related to the ‘true’ *Chlorella*, *Nannochloris*, *Mychonastes* or *Picochlorum* isolates (Figs 1, 13), a new genus name – *Pseudochloris* – is proposed. The species is named in honour of Christian Wilhelm (who isolated strains SAG 55.87 and Mainz 1 and described *N. eucaryotum*). Strain SAG 55.87 is selected as holotype for the new species, because UTEX 2502 was indicated to be heterogeneous by Yamamoto *et al.* (2003) and SAG 1.80 is not authentic according to the SAG catalogue. *Pseudochloris wilhelmii* is associated with marine or brackish environments, as SAG 55.87 was isolated from a seawater aquarium and SAG 1.80 from a mangrove swamp.

***Pseudochloris wilhelmii* Somogyi, Felföldi & Vörös, *gen. et sp. nov.***

DESCRIPTION: Cells green, spherical to oval, measuring  $2.6 \times 2.8 \mu\text{m}$ , growing in marine/brackish waters. Each cell with one nucleus, one mitochondrion and one lateral chloroplast without a pyrenoid; starch grains usually present. Chloroplast pigments comprise chlorophylls *a*, *b*, lutein, violaxanthin and neoxanthin. Flagella absent. Lipid droplets sometimes present. Cell wall trilaminate, with an inner microfibrillar layer. Reproduction by autospore formation into two or four daughter cells. Sexual reproduction unknown. Analysis of 18S rRNA gene sequence (JX235962) shows differences from sequences of other trebouxiophycean species.

HOLOTYPE: Material of strain SAG 55.87, cryopreserved at the Sammlung von Algenkulturen der Universität Göttingen, Germany.

ICONOTYPE: Fig. 2.

TYPE LOCALITY: marine/brackish environment; presumed source Rovinj, Croatia (the SAG database states “isolated from a seawater aquarium at University of Mainz (Germany) that contained marine animals from Adria (Rovinj, former Yugoslavia”): <http://sagdb.uni-goettingen.de/>).

ETYMOLOGY: The specific epithet refers to the name of the isolator (C. Wilhelm, in 1981).

#### *General discussion*

Pico-sized ( $< 3 \mu\text{m}$ ) coccoid chlorophytes have evidently evolved by convergent evolution and are often unidentifiable by microscopy, including EM (Krienitz & Bock, 2012 and references therein). Their uniform morphology conceals an extremely high phylogenetic and physiologic diversity (Krienitz & Bock, 2012) and within the Trebouxiophyceae a number of lineages comprise picoplankton species, including the marine or saline *Picochlorum*, *Chloroparva* and *Pseudochloris*, as well as the

freshwater *Choricystis* clade, *Meyerella* and *Marvania* species (Table S1). All of these genera have members with indistinguishable morphology: they are spherical to oval cells with a diameter of 2  $\mu\text{m}$  (classical ‘green balls’, Table S1), simple ultrastructure, and similar pigment composition and cell wall structure. Nowadays, the identification of these species is mainly based on 18S rRNA gene analysis, however in some cases, species delineation can require multigene analysis (using ITS regions, *rbcL*, etc.) and/or detailed taxon sampling (Coleman, 2003; Fawley *et al.*, 2005; Krienitz & Bock, 2012). For example, considerable genetic variation (mainly based on *rbcL*) suggests the presence of several cryptic species within the *Choricystis* clade, but additional sequences will be necessary to clarify phylogenetic relationships (Hepperle & Krienitz, 2001; Hepperle & Schlegel, 2002; Fawley *et al.*, 2005). In many cases, the greatest challenge comes from conflicts between the old, morphology-based classification system and the new, more natural phylogenetic system (Krienitz & Bock, 2012). The types of older species descriptions were usually figures or rarely specimens, but not strains, and sometimes authentic strains are missing, such as in the case of *Choricystis* species. This can lead to nomenclatural difficulties: sometimes it is very hard to link the previously described morphospecies with the observed genetic variations, as can be seen in the *Choricystis* clade (Krienitz *et al.*, 1996; Fawley *et al.*, 2005). Difficulties can also arise from incorrect strain identifications, as we have shown here. The present study brings us one step closer to eliminating the nomenclatural problems related to minute coccoid green algae by describing *Pseudochloris wilhelmii* as a new genus and species.

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## Supplementary information

The following supplementary material is available for this article, accessible via the Supplementary Content tab on the article's online page at

Table S1. Main properties (habitat, morphological and phylogenetic attributes) of pico-sized (< 3  $\mu\text{m}$ ) coccoid chlorophytes within the Trebouxiophyceae.

Fig. S1. Transmission electron micrograph showing the Golgi apparatus (white arrowhead) in a vegetative cell of SAG 55.87. C, chloroplast; CW, cell wall; N, nucleus; PM, plasma membrane; T, thylakoid. Black arrowhead indicates the outer trilaminar layer of the cell wall. Asterisk indicates a starch grain. Scale bar represents 0.2  $\mu\text{m}$ .

Supplementary file. Alignment of 18SrDNA sequences (fasta format).

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**Table 1.** Oligonucleotide primers used in this study.

Primer name	Position <sup>1</sup>	Sequence	Reference
Euk328f <sup>2</sup>	2–20	5'-ACCTGGTTGATCCTGCCAG-3'	Moon-van der Staay <i>et al.</i> , 2000
Chlo02r <sup>2,3</sup>	996–978	5'-CTTCGAGCCCCCAACTTTC-3'	Zhu <i>et al.</i> , 2005
ChloroF <sup>2,3</sup>	825–845	5'-TGGCCTATCTTGTTGGTCTGT-3'	Moro <i>et al.</i> , 2009
Euk329r <sup>2</sup>	1797–1778	5'-TGATCCTTCYGCAGGTTAC-3'	Moon-van der Staay <i>et al.</i> , 2000
18S rRNA 5'-PCR 1F <sup>3</sup>	2–21	5'-ACCTGGTTGATCCTGCCAGT-3'	Yamamoto <i>et al.</i> , 2003
18S rRNA 5'-PCR 1R <sup>3</sup>	786–767	5'-CGTAGGCYTGCTTTGAACAC-3'	Yamamoto <i>et al.</i> , 2003
18S rRNA 5'-PCR 2F <sup>3</sup>	544–563	5'-CMATTGGAGGGCAAGTCTGG-3'	Yamamoto <i>et al.</i> , 2003
18S-544R <sup>3</sup>	563–544	5'-CCAGACTTGCCCTCCAATTG-3'	Somogyi <i>et al.</i> , 2011
18S-1270F <sup>3</sup>	1270–1289	5'-GTGGTGCATGGCCGTTCTTA-3'	Somogyi <i>et al.</i> , 2011
18S rRNA 5'-PCR 2R <sup>3</sup>	1289–1270	5'-TAAGAACGGCCATGCACCAC-3'	Yamamoto <i>et al.</i> , 2003
18S rRNA 5'-PCR 3F <sup>3</sup>	980–999	5'-AAGTTRGGGGMTCGAAGACG-3'	Yamamoto <i>et al.</i> , 2003
18S rRNA 5'-PCR 3R <sup>3</sup>	1793–1774	5'-CCTTCYGCAGGTTACCTAC-3'	Yamamoto <i>et al.</i> , 2003

<sup>1</sup> annealing sites refer to nucleotide positions of the *Chlorella vulgaris* SAG 211-11b 18S rRNA sequence (X13688, Huss & Sogin, 1989)

<sup>2</sup> used for PCR amplification

<sup>3</sup> used for sequencing

**Table 2.** Isolated pigments of SAG 55.87 in relation to the concentration of chlorophyll *a*: measurements are mean  $\pm$  s.d. ( $N = 3$ ).

Pigment type	Content (mM M <sup>-1</sup> chlorophyll <i>a</i> )
Neoxanthin	67.83 $\pm$ 9.12
Violaxanthin	64.19 $\pm$ 5.42
Antheraxanthin	32.24 $\pm$ 6.83
Lutein	283.83 $\pm$ 0.86
Zeaxanthin	48.68 $\pm$ 3.36
Chlorophyll <i>b</i>	297.15 $\pm$ 10.03
$\alpha$ -carotene	2.55 $\pm$ 0.31
$\beta$ -carotene	45.07 $\pm$ 4.41

Figure captions:

**Fig. 1.** Summary of the nomenclatural problems in the case of some coccoid green algal isolates [*Chlorella minutissima* Lefevre ALCP no. 87, '*C. minutissima*' C 1.1.9 and SAG 1.80, *Nannochloris eucaryotum* Mainz 1 and '*N eucaryotum*' UTEX 2502 (=SAG 55.87)] according to Fott & Nováková (1969), Andreyeva (1975), Wilhelm *et al.* (1982), Kalina & Puncochárová (1987), Menzel & Wild (1989), Huss *et al.* (1999), Krienitz *et al.* (1999), Yamamoto *et al.* (2003) and Henley *et al.* (2004). Misinterpreted strains (C 1.1.9; SAG 1.80 and UTEX 2502) are framed with thin dashed lines and authentic strains with thick, continuous lines. Strains possessing nearly identical 18 rRNA gene sequences are grouped in a box with thick dashed lines and are the subjects of this study.

**Figs 2–5.** *Pseudochloris wilhelmii*, transmission electron micrographs of SAG 55.87. C, chloroplast; CW, cell wall; L, lipid droplet; M, mitochondrion; N, nucleus; P, peroxisome; PM, plasma membrane; V, vacuole. Black and white arrowheads indicate the outer trilaminar layer of the cell wall and plastoglobuli, respectively. Asterisks indicate starch grains. **2, 4.** Vegetative cell. **3.** Vegetative cell with lipid droplets. **5.** A close-up from Fig. 4. showing the cell wall structure: note the outer trilaminar layer and the granulo-fibrillar inner layer covering the plasma membrane. Scale bars = 0.5  $\mu\text{m}$  (Fig. 2), 1  $\mu\text{m}$  (Figs 3, 4), and 0.2  $\mu\text{m}$  (Fig. 5).

**Figs 6–11.** *Pseudochloris wilhelmii*, transmission electron micrographs of SAG 55.87 during autosporulation. C, chloroplast; DCW, daughter cell wall; M, mitochondrion; MCW, mother cell wall; PM, plasma membrane; T, thylakoid; V, vacuole. Single and double black arrowheads indicate the outer trilaminar layer of the cell wall of the daughter cell and of the mother cell, respectively. Asterisks indicate starch grains, white arrowheads show plastoglobuli. C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub> and C<sub>4</sub> correspond to the chloroplasts of the different daughter cells within the sporangium. **6.** Mother cell with two autospores. **7.** Mother cell with four autospores. **8.** A daughter cell at a late phase of autosporulation. **9.** A close-up from Fig. 6, showing the structure of the mother cell wall and the

developing daughter cell walls. The daughter cell wall has a fine trilaminar layer and a firm granulo-fibrillar inner layer. **10.** A close-up from Fig. 7, showing the structure of the mother cell wall and the developing daughter cell walls. At this stage, the daughter cell wall has only a fine trilaminar layer. **11.** A close-up from Fig. 8, showing the rupture of the mother cell wall. Scale bars = 1  $\mu\text{m}$  (Figs 6–8) and 0.2  $\mu\text{m}$  (Figs 9–11).

**Fig. 12.** HPLC chromatogram of pigments extracted from SAG 55.87 at 440 nm. Retention time (min) on the *x*-axis and absorbance (mAU) on the *y*-axis. Peak identities: (1) neoxanthin, (2) violaxanthin, (3) antheraxanthin, (4) lutein, (5) zeaxanthin, (6) chlorophyll *b*, (6a) isomer of chlorophyll *b*, (7) chlorophyll *a*, (7a) isomer of chlorophyll *a*, (8)  $\alpha$ -carotene and (9)  $\beta$ -carotene.

**Fig. 13.** Maximum likelihood (ML) tree of 18S rRNA gene sequences retrieved from the studied isolates (SAG 55.87, SAG 1.80 and UTEX 2502) and their closest relatives (Trebouxiophyceae, Chlorophyta) based on 1691 nucleotide positions (best tree likelihood score,  $\ln L = -4575.832$ ). Bootstrap values greater than 70 [based on 100 and 1000 replicates for ML and maximum parsimony (MP), respectively] and Bayesian (B) posterior probabilities higher than 0.70 are shown (ML/MP/B). Sequences determined in this study appear in bold. The number of introns is shown in square brackets, if present. Asterisks mark authentic strains according to Henley *et al.* (2004), Luo *et al.* (2010), Krienitz *et al.* (2011), Somogyi *et al.* (2011) and this study.

FIGURES:

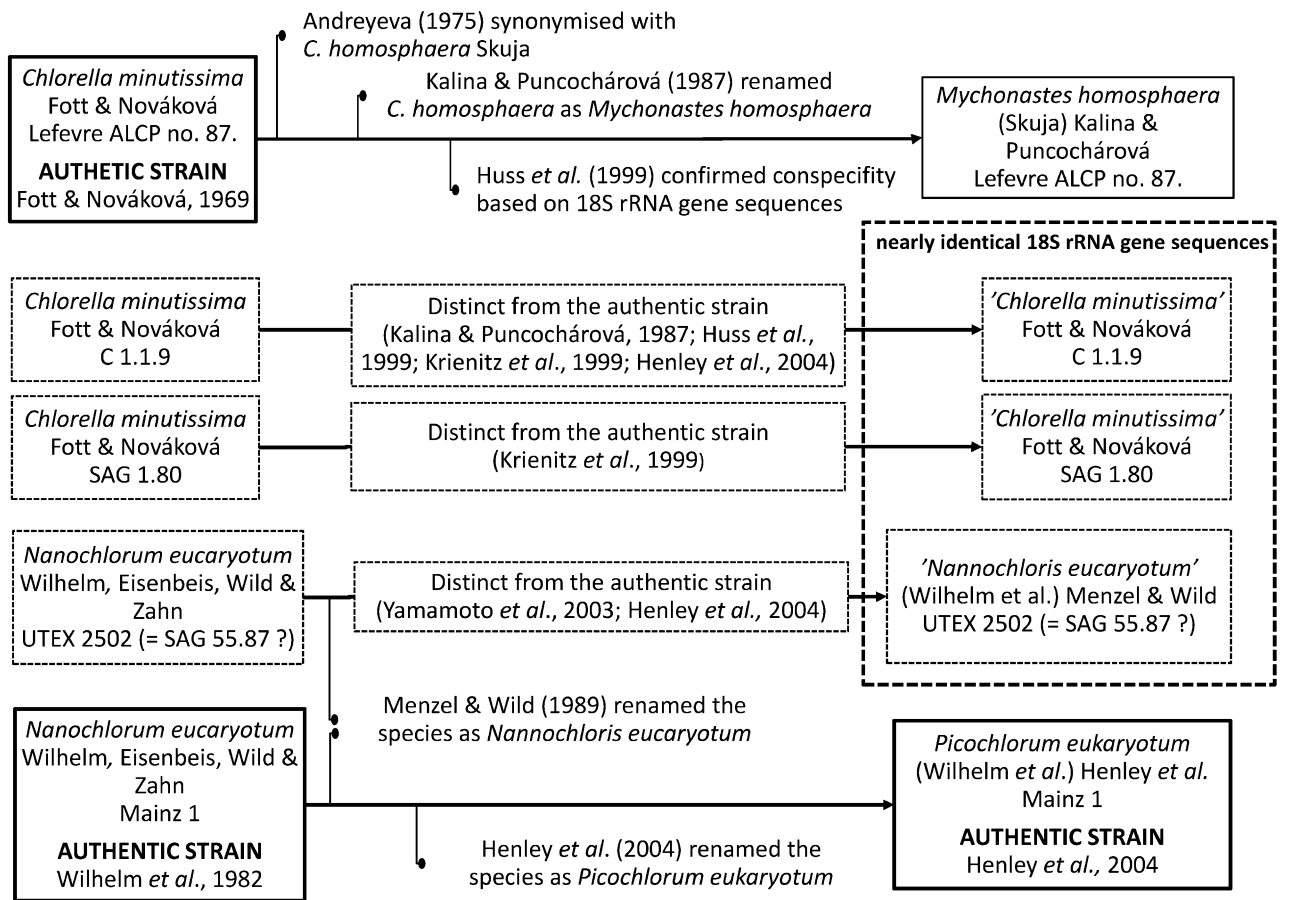
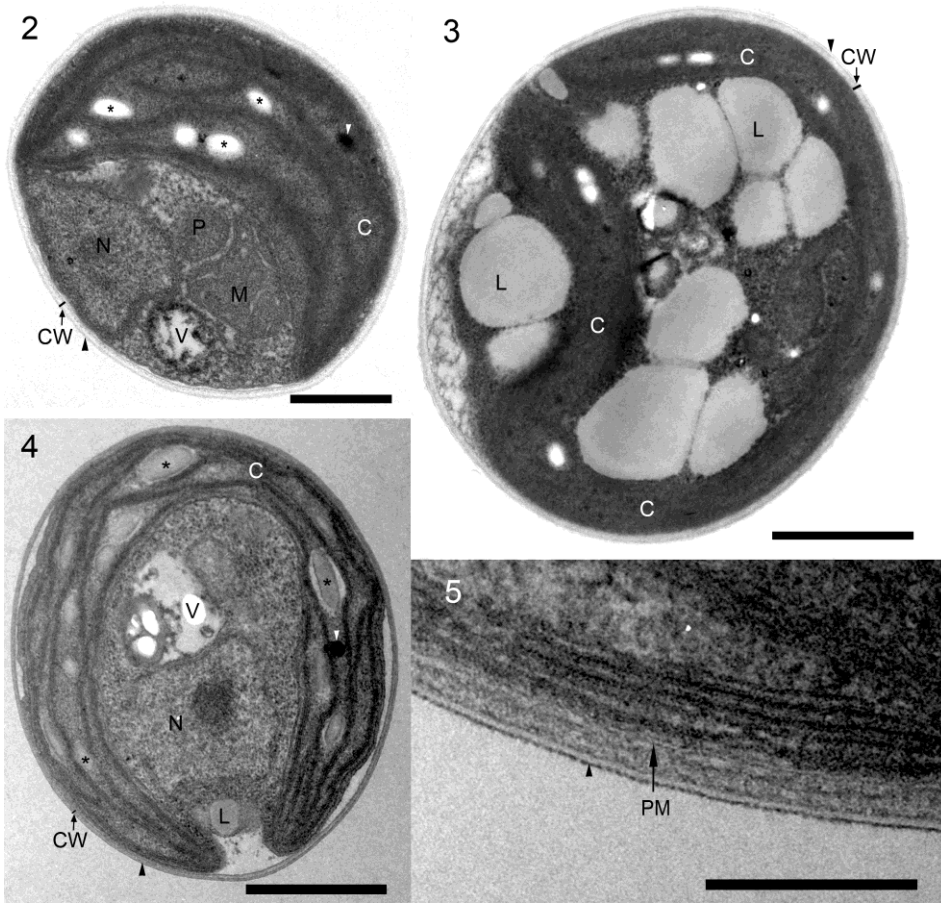
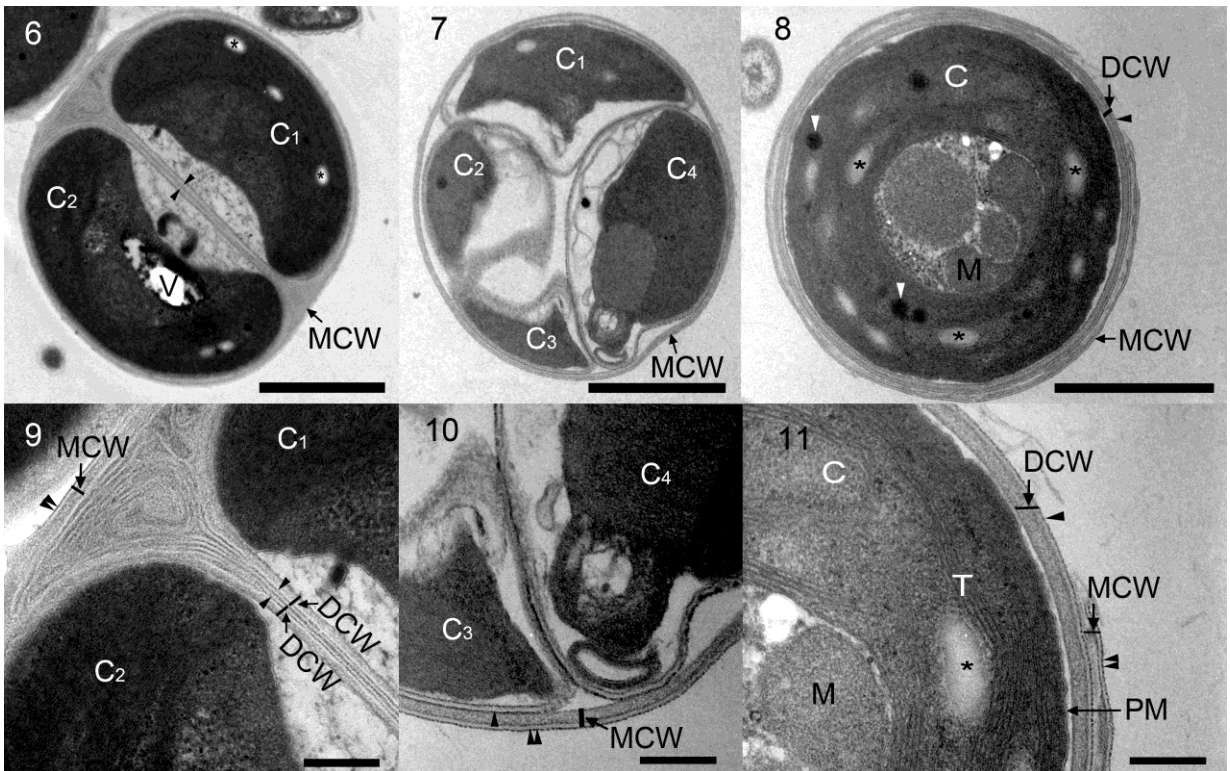


Fig.1.



Figs 2-5.





Figs 6-11.

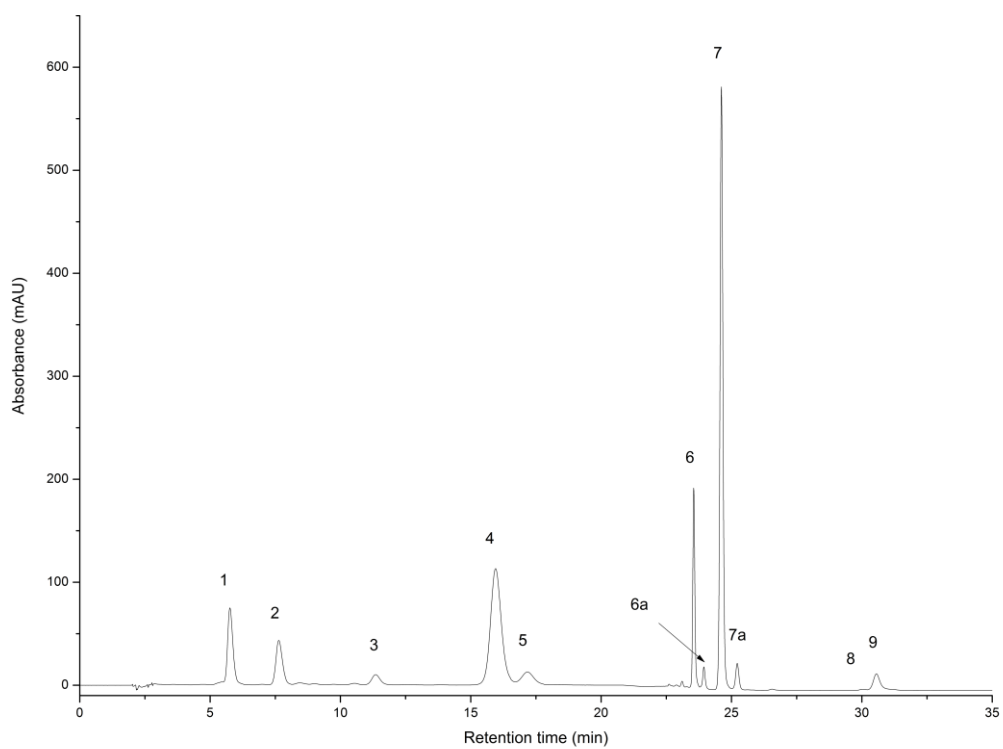


Fig. 12.

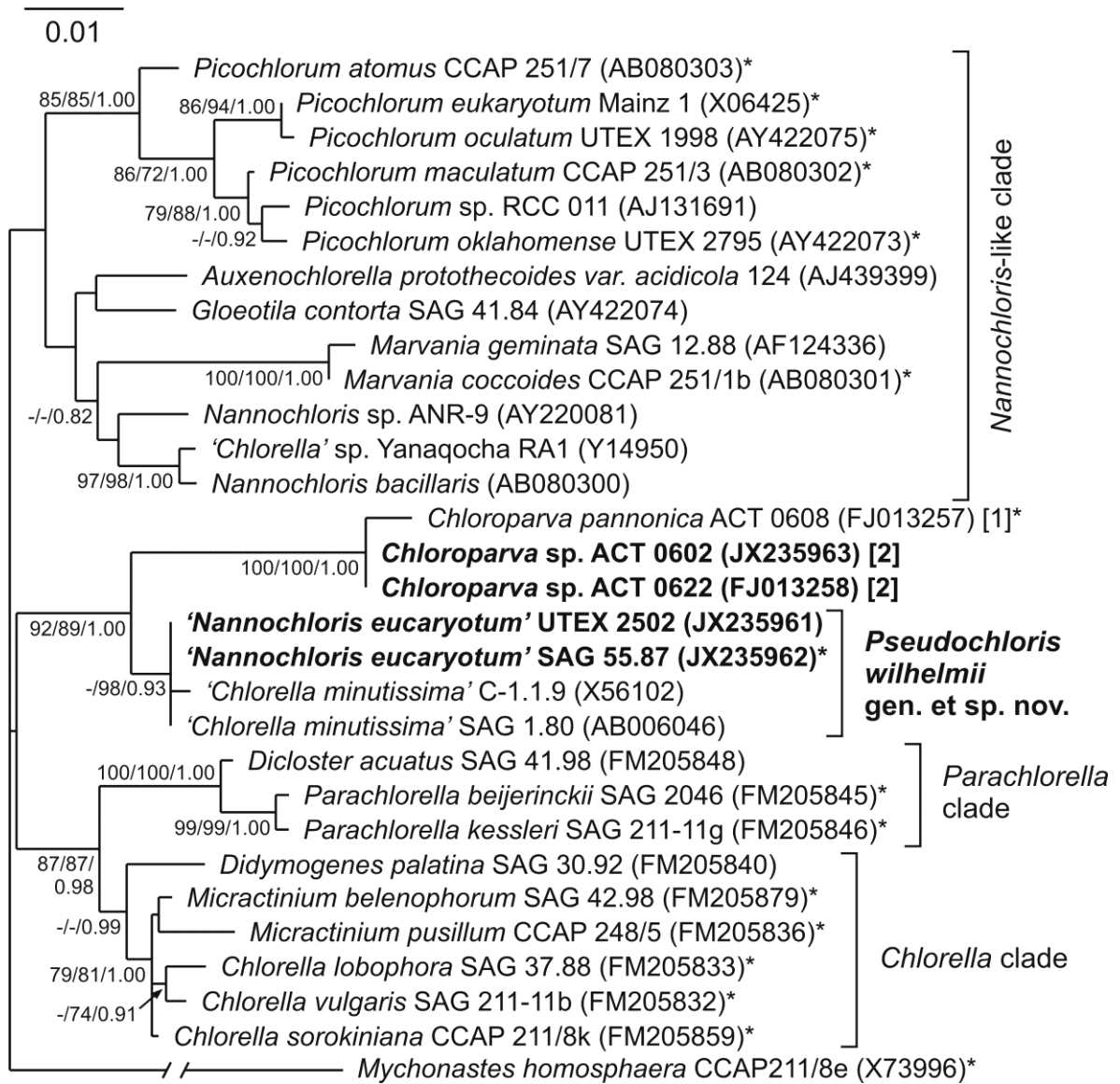


Fig. 13.