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Hidden diversity of *Chlorococcum* (Chlorophyta) in a shallow temporary freshwater lake: description of *Chlorococcum szentendrense* sp. nov.

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Running title: *Chlorococcum szentendrense* from a shallow lake

Abstract

Chlorococcum is a coccoid green algal genus, which contains almost 50 species. The genus is polyphyletic, but in a traditional sense, the cells are coccoid and non-motile with a cup-shape chloroplast in the vegetative stage, while the spores have chlamydomonad appearance. They are distributed worldwide mainly in terrestrial habitats, and the biotechnological potential of several strains has also been reported. In this study, three new green algal strains from a shallow temporary freshwater lake in Hungary are characterized using microscopic (light and transmission electron microscopy) and DNA–based methods (phylogenetic analysis of the ribosomal ITS region, the 18S ribosomal RNA and *rbcL* genes, and ITS secondary structure comparison). Based on the obtained results, one of the three new isolates is considered to represent a new species, which is described here as *Chlorococcum szentendrense* sp. nov.

Keywords

Introduction

Microalgae represent a great and yet unraveled diversity of the biosphere and have important roles in the carbon and nitrogen cycles, in the marine and freshwater food webs as well as in the global production of oxygen and in CO_2 fixation (Bhola *et al.*, 2014; Castro & Huber; 2019; Felföldi, 2020; Somogyi *et al.*, 2022). In addition, they also have great potential in many kinds of industrial applications including biofuel production (Mahapatra & Ramachandra, 2013; Selvarajan *et al.*, 2015; Khan *et al.*, 2018; Nagy *et al.*, 2018), application as biostimulant and biofertilizer in the agriculture (Abdel-Raouf *et al.*, 2012; County & County, 2015), and especially in the health, the cosmetic and the food industries (reviewed by e.g. Mimouni *et al.*, 2012; Heydarizadeh *et al.*, 2013; Gateau *et al.*, 2017). In spite of their obvious global importance, out of the 700,000 microalgal species that are expected to exist only approximately ten thousand have been described so far (Guiry, 2012). This clearly outlines the importance of the identification and description of novel species.

Chlorococcum (Chlamydomonadales, Chlorophyceae) was first described by Meneghini in 1842 (Guiry & Guiry, 2020), and an amended description of this genus was provided recently by Watanabe and Lewis (2017) and by Nakada *et al.* (2019). The 47 species accepted taxonomically to date (Guiry & Guiry, 2020) represent a polyphyletic group, the type species of the genus [*Chlorococcum infusionum* (Schrank) Meneghini] with others belong to the Moewusinia macroclade, while several other species to the Stephanosphaerinia macroclade (Nakada *et al.*, 2008; Watanabe & Lewis, 2017). Members of the genus *Chlorococcum* are mainly edaphic (i.e. terrestrial) algae, but they were also reported from diverse aquatic habitats and subaerial surfaces (e.g.

Klochkova *et al.*, 2006; Wehr *et al.*, 2015; Temraleeva & Moslalenko, 2019), and even symbiotic forms have been described within the genus (e.g. Feng *et al.*, 2016). Traditional members of this genus, similarly to several other green algal species, exist in two morphological forms. The longer period of the lifespan is spent as a spherical, non-motile form as round cells with a rigid, thick cell wall. These vegetative cells are constantly growing and, under given conditions, are divided into a number of oval, biflagellate zoospores that are morphologically very similar to the vegetative cells of certain *Chlamydomonas* Ehrenberg species (Miller, 1978).

In this work, our aim was to investigate the hidden diversity of microalgae in Hungarian temporary freshwater habitats. As a result, three new *Chlorococcum* strains were isolated from a temporary freshwater lake,   and analysis of molecular phylogenies and internal transcribed spacer (ITS) structures, as well as light and electron microscopy have been applied to determine their taxonomic status. Based on morphological and molecular data, the examined strains represented two species, and one of them is described here as *Chlorococcum szentendrense* sp. nov.

Materials and methods

Site description, strain isolation and maintenance

Three algal strains (K2/5, K2/9 and K2/11) were isolated from the water of Kőhegyi Lake, a temporary freshwater lake in Hungary (near Szentendre; 47°40'32.94″ N, 19°01'6.93″ E; Central Europe) in July 2016. Isolation was carried out with the spread-plate technique using PHM–1 medium (slightly modified from Borowitzka &

Borowitzka, 1988) which contained 1 g KNO_3, 0.2 g $MgSO_4$, 0.2 g K_2HPO_4, 0.1 g

sodium acetate, 20 ml saturated $CaSO_4$ solution, 10 ml micronutrient solution [61 mg

H_3BO_3, 38 mg $(NH_4)_6Mo_7O_{24} \times 4\ H_2O$, 6 mg $CuSO_4 \times 5\ H_2O$, 6 mg $Co(NO_3)_2 \times 6\ H_2O$,

2 mg $ZnSO_4 \times 7\ H_2O$ and 4 mg $MnCl_2 \times 4\ H_2O$, in 1000 ml distilled water] and 10 ml

iron solution (3.8 g Fe(III)NaEDTA $\times$ 3 H_2O in 1000 ml distilled water) in distilled

water in a final volume of 1000 ml. The medium was solidified with 15 g l^{-1} agar. All

chemicals were purchased from Molar Chemicals (Halásztelek, Hungary), except

Fe(III)NaEDTA $\times$ 3 H_2O (Acros Organics, Geel, Belgium).

For maintenance, cultures were grown on PHM–1 agar slants at room temperature

(~24°C) on a 16 : 8 h light : dark cycle and illuminated from above with a light intensity

of 180 µmol photons $m^{-2}\ s^{-1}$ irradiance.

For cryopreservation at –80°C, 0.1 ml methanol was added to 0.9 ml liquid

culture.

Light microscopy

Light microscopy was performed using a Nikon Eclipse 80i microscope (Tokyo, Japan)

and an Image–ProPlus 6.0 software package (Media Cybernetics, Rockville, MD,

USA).

In order to visualize the lipid droplets in algal cells, 2-week-old cultures were

investigated by nile red (9-diethylamino-5H-benzo[alpha]phenoxazine-5-one; Sigma,

St. Louis, MO, USA) staining following the protocol of Alemán-Nava *et al.* (2016).

Cells were suspended in 1 ml of 4.5 g l^{-1} Na_2EDTA (Molar Chemicals, Halásztelek,

Hungary) solution, and then 5 µl of 0.25 mg ml^{-1} nile red (dissolved in acetone) was

added. After this, the samples were incubated for 10 minutes in the dark. Fluorescence

microscopic investigations were carried out with the same instrument using 510–550 nm excitation light and 575–640 nm emission filter.

Electron microscopy

Transmission electron microscopic (TEM) sample preparation was carried out as in Somogyi *et al.* (2011, 2013). For this purpose, 2.5% glutaraldehyde (Sigma; dissolved in 70 mM K-Na phosphate buffer, pH 7.2) was pipetted to the algal colonies grown on the agar plate. Cells were suspended in the fixative, fixed for 3 h at room temperature, and then centrifuged at 20°C with 5000 × g for 5 min. The pellet was resuspended into solidifying agar (2%), and the solid agar plates were processed further. In another set of experiments solid plaques of algal culture were immersed in the 2.5% glutaraldehyde fixative for 3 h. After fixation both the agar plates and the algal plaques were treated similarly. The samples were rinsed with the phosphate buffer described above three times for 15 min, post-fixed in 1% OsO_4 (Sigma; dissolved in the same buffer) for 2 h, and then rinsed three times for 15 min with the same buffer. After dehydration in mounting ethanol series (25%, 50%, 70% and 90% ethanol, 15 min each; then 2×20 min in 96% and finally 20 min in absolute ethanol), the samples were embedded in Durcupan ACM epoxy resin (Sigma). Ultrathin (70 nm) sections were cut with a Ultracut E microtome (Reichert–Jung, Vienna, Austria) using a Diatome diamond knife (Nidau, Switzerland). The sections were stained with 5% uranyl acetate dissolved in methanol for 5 minutes and by Reynold's lead citrate for 5 minutes. Samples were investigated using a Hitachi 7100 TEM at 75 kV accelerating voltage and a JEM 1011 (Jeol, Tokyo, Japan) at 80 kV accelerating voltage. Images were taken using Morada

digital camera (Olympus, Tokyo, Japan) and were analyzed using ImageJ software (Schneider *et al.*, 2012) when necessary.

DNA-based studies

Total genomic DNA was extracted from the strains using the DNeasy Plant Mini kit (Qiagen, Hilden, Germany) according to the instructions given by the manufacturer. PCR amplification of the whole 18S rRNA gene was completed with two separate Chlorophyta-specific reactions using the primer pairs Euk328f–Chlo02R (Moon-van der Staay *et al.*, 2000; Zhu *et al.*, 2005) and ChloroF–Euk329r (Moro *et al.*, 2009; Moon-van der Staay *et al.*, 2000) as described in detail by Somogyi *et al.* (2013). The ribosomal ITS region (containing ITS-1, the 5.8S rRNA gene, ITS-2 and a short region from the 28S rRNA gene) was amplified with primers ITS_f and ITS_r (Liu *et al.*, 2014), while the *rbcL* gene was amplified with primers rbcL–F1 and rbcL–R1 (Fawley *et al.*, 2005). Purification of PCR products and Sanger sequencing were carried out by the Biomi Ltd. (Gödöllő, Hungary). The obtained sequences were deposited in the GenBank database under the accession codes MG784550–MG784555 and MG977017–MG977019.

In the case of the 18S rRNA gene, sequences were aligned with the SINA Alignment Service (Pruesse *et al.*, 2012), while in the case of other regions the ClustalW incorporated in the MEGA 7 software (Kumar *et al.*, 2016) was used. For concatenation, the SequenceMatrix v1.8 software (Vaidya *et al.*, 2011) was used. Maximum likelihood analysis (including the search for the best-fit model) was conducted using the MEGA 7 software. Bayesian analysis for phylogenetic

reconstruction was performed with the Markov Chain Monte Carlo algorithm in two simultaneous, completely independent analyses running for 1,000,000 generations (sampled every 100 generations) using MrBayes version 3.1 (Huelsenbeck & Ronquist, 2001). The first 25% of the calculated trees were discarded and posterior probabilities were calculated after the two independent runs had reached convergence.

The prediction of ITS-2 secondary structures was performed with the RNAstructure web server (Bellaousov *et al.*, 2013), while compensatory base changes (CBCs) were detected with 4SALE 1.7.1 (Seibel *et al.*, 2008).

Results

Taxonomic results

Chlorococcum szentendrense Greipel, Kutasi, Solymosi & Felföldi, sp. nov.
(Figs 1–4, 11–17)

DIAGNOSIS: Vegetative cells single, spherical, diameter 5.9–13.5 µm when young and up to 30 µm in old cultures. Zoospores 9.0 µm long and 4.0 µm wide on average. Cell wall smooth, bilayered and thin in zoospores and in young vegetative cells, but thicker in older vegetative cells. Single nucleus, one cup-shaped chloroplast with in general one large pyrenoid and small plastoglobuli and few starch grains in young cells. In older vegetative cells, diffuse or lobed chloroplasts with two pyrenoids and more starch grains. Pyrenoid matrix surrounded by a continuous, smooth starch sheath, traversed by

few intrapyrenoidal channels lined with double membranes, and penetrating the pyrenoid matrix as intramatricial channels. Thylakoids grouped into lamellae (5–10 thylakoids). Zoospores oval with thin bilayered cell wall, parietal chloroplast, one pyrenoid similar to that in vegetative cells, and intraplastidial eyespot. Stigma elliptical. No sexual reproduction observed. Unequivocal distinction from related *Chlorococcum* species based on molecular taxonomic methods (18S rRNA, *rbcL* and ITS sequence analysis).

HOLOTYPE: Lyophilized material from strain K2/9 has been deposited at the Algological Collection of the Hungarian Natural History Museum (Budapest, Hungary) under HNHM-ALG-H7965.

TYPE LOCALITY: Kőhegyi Lake, near Szentendre, Hungary.

ETYMOLOGY: The specific epithet 'szentendrense' (neut. adj.) means of belonging to Szentendre and refers to the town which is close to the isolation source of the new species.

LIVING CULTURE: Strain K2/9 is available at the Sammlung von Algenkulturen der Universität Göttingen (Culture Collection of Algae at Göttingen University, Göttingen, Germany) under the code SAG 2595.

DNA SEQUENCES: MG784551 (18S rRNA gene), MG784555 (ITS region) and MG977018 (*rbcL* gene).

Description

The vegetative cells are green, spherical (Figs 1–2, 11–12) with a diameter of 5.9–13.5 µm when young and up to 30 µm in old cultures. Cells have single nucleus with nucleolus (Fig. 12), one cup-shaped chloroplast (Figs 7, 11) that becomes diffuse or

lobed in older cells (Fig. 12). Regular mitochondria with cristae, normal Golgi apparatus, and some vacuoles sometimes accumulating electron-dense deposit are observed (Figs 11–12). The chloroplast of young cells has in general one large pyrenoid grain surrounded by a continuous starch sheath (Fig. 13), in older cells two pyrenoids are also sometimes observed within the same chloroplast (Fig. 15). Pyrenoids are surrounded by a continuous starch sheath that is traversed by intrapyrenoidal channels lined with double membranes which penetrate the pyrenoid matrix as intramatricial channels (Figs 13, 15). Individual smaller starch grains are also observed in the plastids (Figs 12, 13, 15), as well as small electron-dense plastoglobuli (Figs 11–13). Thylakoids are grouped into bands (lamellae) consisting in general of 5–10 thylakoids (Figs 13–15). The cell wall is 150–250 nm thick, with thinner cell walls typical in younger cells and the cell wall thickness slightly increasing with cell age (up to 650 nm).

No sexual reproduction has been observed. Zoosporangia (Fig. 1) produce zoospores with oval cell shape, 9.0 µm long and 4.0 µm wide (Figs 1, 2, 16–17) and thin (40–50 nm) bilayered cell wall (Figs 16–17). The cytoplasm of zoospores has basically similar ultrastructure to those in vegetative cells, but contains eyespots located inside the chloroplast lamellae (Fig. 17), and fewer starch grains in the chloroplast (Figs 16–17).

Other strains studied: *Chlorococcum* sp. K2/5 and K2/11

LYOPHILIZED MATERIAL: Lyophilized material of strain K2/5 has been deposited at the Algological Collection of the Hungarian Natural History Museum (Budapest, Hungary) under HNHM-ALG-H7966.

LIVING CULTURE: Strain K2/5 is available at the Sammlung von Algenkulturen der
Universität Göttingen (Culture Collection of Algae at Göttingen University, Göttingen,
Germany) under the code SAG 2594.

DNA SEQUENCES: for strain K2/5: MG784550 (18S rRNA gene), MG784553 (ITS
region) and MG977017 (*rbcL* gene); and for strain K2/11: MG784552 (18S rRNA
gene), MG784554 (ITS region) and MG977019 (*rbcL* gene).

Characterization

Vegetative cells are green, single, spherical or ellipsoidal, and have a diameter of 6.5–
14.5 µm (Figs 5–10, 18–19). Lipid droplets are observable (Figs 7–8).Their cell wall is
thin (130–160 nm) (Figs 18–19). Cells contain a single nucleus, one cup-shaped
chloroplast located parietally, several mitochondria with cristae, a large multilamellar
Golgi apparatus, and one to few vacuoles sometimes filled with electron-dense material
(Fig. 18). The chloroplast contains several smaller starch grains embedded in the matrix
(Fig. 18), one large pyrenoid surrounded by a continuous starch sheath with few
intrapyrenoidal channels lined with double membranes which penetrate the pyrenoid
matrix as intramatricial channels (Fig. 19). In addition, small electron-dense
plastoglobuli were observed in the plastids, and the thylakoids were grouped into bands
(lamellae) consisting in general of 2–4 thylakoids (Figs 18–19).

        Aggregates (Figs 9 and 10) or 'microcolonies', i.e. an irregular group of
individual vegetative cells (some of them at least seemingly empty) are sometimes
formed (Figs 6, 9–10). In some cells of these 'microcolonies' zoospores are surrounded
by a common sheath, their mother cell wall (Fig. 20). Zoospores are 7.5 µm long and
3.5 µm wide on average, have thin but peculiarly striated cell wall (26–42 nm) (Figs 20,

21), intraplastidial eyespot (Fig. 22) and two anterior flagella (Fig. 23). Ultrastructural features of the zoospores are in other respect very similar to that of vegetative cells (compare Figs 18–19 and 20–24). No sexual reproduction has been observed. Unequivocal distinction from other *Chlorococcum* species based on molecular taxonomic methods (18S rRNA, *rbcL* and ITS sequence analysis).

Results of DNA-based analyses

The 18S rRNA gene sequence of strains K2/5 and K2/11 was identical based on 1750 nucleotide positions and shared high similarity values (99.8–99.9%, only 1–2 nucleotide difference) with other *Chlorococcum* isolates (RK261 from an oligotrophic lake in Japan and GRK7WB5 from karstwater stream biofilm in Germany; Fujii *et al.*, 2010; Hodač *et al.*, 2015) and with '*Chlamydomonas debaryana*' CCAP 11/1 (which was isolated from a freshwater pool in the Czech Republic). These strains were only distantly related (98.4–98.6% similarities) to *Chlorococcum aquaticum* P.A.Archibald UTEX 2222 and *C. minutum* R.C.Starr SAG 213-7. Strain K2/9 showed 98.7–98.9% similarities to the authentic strains of *Chlorococcum vacuolatum* R.C.Starr, *C. rugosum* P.A.Archibald & Bold and *C. isabeliense* P.A.Archibald & Bold. The new strains formed distinct lineages on the phylogenetic trees (Fig. 25, Supplementary Figs S1–S3) from the authentic strains of *Chlorococcum* species based on all three studied DNA region (18S rRNA and *rbcL* genes, ITS region), and their position was supported with high bootstrap and posterior probability values.

Comparative analysis (including strains *C. isabeliense* SAG 65.80, *C. rugosum* UTEX 1785, *C. vacuolatum* SAG 213-8, *C. aquaticum* UTEX 2222, *C. minutum* SAG 213-7, '*Chlamydomonas debaryana*' CCAP 11/1, and strains K2/5 and K2/9) of ITS-2 secondary structures (Fig. 26) revealed relatively low number of CBCs. No CBCs were found comparing K2/9 with the strains of the closest-related species (*C. isabeliense*, *C. rugosum* and *C. vacuolatum*). We should mention here that if there is no CBC in the ITS-2, there is still a probability of ~0.24 to that the two strains studied belong to different species, since no causal relationship exist between the presence of CBC and speciation (Wolf *et al.*, 2013). Nevertheless, 4 CBCs were detected between K2/5 and *C. aquaticum* UTEX 2222.

Discussion

Molecular studies have revealed that *Chlorococcum* is a polyphyletic genus (Nakada *et al.*, 2008; Krienitz & Bock, 2012; Kawasaki *et al.* 2015; Watanabe & Lewis, 2017; Temraleeva & Moslalenko, 2019). Our new strains belonged to the Stephanosphaerinia macroclade but outside the Oleo clade which was proposed by Kawasaki *et al.* (2015) (Fig. 25). General morphological features (spherical green vegetative cells, single cells or forming aggregates, an average cellular diameter around 10–20 µm, single parietal chloroplast and Chlamydomonad-like zoospores with two equal flagella) and ultrastructure [smooth cell wall and presence of pyrenoid(s) with pyrenoid channels] of the novel species were similar to that described for unicellular green microalgae traditionally belonging to the *Chlorococcum* genus (Starr, 1955;

Archibald & Bold 1970; Miller, 1978; Ettl & Gärtner, 1988, 2014; Péterfi *et al.*, 1988; Klochkova *et al.*, 2006; Feng *et al.*, 2016; Watanabe & Lewis, 2017). Although we studied cultures of different age, we could not observe and identify sexual reproduction in any of the three studied strains.One of our new isolates showed remarkable morphological and phylogenetic differences with other strains isolated previously. The cell wall of K2/9 is thinner (150–250 nm) than that of the closest-related species, *C. rugosum* and *C. vacuolatum* (0,5–1 µm or thicker; Ettl & Gärtner, 2014; Kawasaki *et al.* 2015), which clearly distinguishes the new strain from them. The cell wall of the zoospores is smooth in case of K2/9 and vegetative cells sometimes have two pyrenoids which clearly distinguishes them ultrastructurally from *C. minutum* (Péterfi *et al.*, 1988) and *C. oleofaciens* (Miller, 1978). The continuous starch sheath surrounding the pyrenoids distinguishes the new strain from species belonging to *Chlorococcum sensu* Watanabe & Lewis in which the starch sheath is discontinuously covered with starch segments or grains (Watanabe & Lewis, 2017). Archibald & Bold (1970) described that several *Chlorococcum* species (e.g. *C. isabeliense*) usually have a single pyrenoid. Therefore, it may be possible that the cells containing two pyrenoids were old cells. Some of the pyrenoids observed in our work have atypical shape with shrunk matrices. It should be noted, that pyrenoid structure is also influenced by sampling time (daytime vs. night time), culture conditions and the age of the cells (Meyer *et al.*, 2017).

In addition to these morphological differences, distinction of the new strain was confirmed by DNA sequence analysis of the 18S rRNA gene, the ribosomal ITS region and the *rbcL* gene, and they showed 1.1–1.6% pairwise dissimilarity values to the closest related authentic strains based on the 18S rRNA gene. According to Temraleeva & Moslalenko (2019) the average dissimilarity values among closely related

Chlorococcum species within the Stephanosphaerinia macroclade is even lower (0–1.1%), which supported that our strain represents a new species to science. Since K2/9 was positioned to a different clade than the *Chlorococcum* species *sensu* Watanabe & Lewis (including the type species of the genus, *C. infusionum*), therefore we concluded that the new strain is a member of new genus also. For strain K2/9, the establishment of a new genus and species, *Chlorococcum szentendrense* sp. nov., is proposed.

The other two new isolates, K2/5 and K2/11, had vegetative cells with similar cell size and ultrastructural features than those of K2/9, but the oval zoospores were slightly larger in these two strains. Some ultrastructural differences were also observed: the number of thylakoid lamellae was 5–10 on average in case of K2/9, while in K2/5 and K2/11 thylakoids were grouped into only 2–4-layered assemblies; and the thickness of the bilayered cell wall varied with the age of the cells in case of the K2/9 strain (150–250 nm, in some cases extraordinarily thick, ~ 450–650 nm), and had a relatively constant thickness (130–160 nm) in case of the K2/5 and K2/11 strains. These strains belonged to a different phylogenetic group as K2/9. It seems that strain CCAP 11/1, closely related to K2/5 and K2/11, labeled as '*Chlamydomonas debaryana*' Goroschankin [reclassified recently as *Edaphochlamys debaryana* (Goroschankin) Pröschold & Darienko (Pröschold *et al.* 2018)] is currently misclassified. This could be due to that motile cell (spores) in the genus *Chlorococcum* are chlamydomonad in appearance (biflagellar, cup-shaped chloroplast, persistent eyespot, etc.; Wehr *et al.*, 2015), although micrographs available at the homepage of Culture Collection of Algae and Protozoa (www.ccap.ac.uk; downloaded: 15 November 2020) show cellular morphology similar to *Chlorococcum* species (non-flagellar spherical gregarious cells with a cellular diameter around 5–15 µm). To clarify the taxonomic status of strain

CCAP 11/1 with direct comparison was not possible, since the strain is not available any more from the culture collection (i.e. it was archived). Morphological features (e.g. the size of vegetative cells and zoospores, the uninterrupted starch shell characteristic for pyrenoids) made the new isolates (K2/5 and K2/11) similar to the closely related species, *C. aquaticum* and *C. minutum* (Ettl & Gärtner, 1988; Kawasaki *et al.*, 2015; Temraleeva & Moslalenko, 2019), and unequivocal distinction from them is possible only with molecular taxonomic methods. Since morphological results did not support the description of another new species, we did not propose that for strains K2/5 and K2/11. Additional formal *Chlorococcum* species descriptions for strains distinguishable solely based on DNA sequence data would probably create an even more complicated taxonomic situation for this polyphyletic genus.

Some *Chlorococcum* species have been reported to accumulate abundant amounts of lipids (as it was observed in the case of strain K2/5) and are thus considered as potential algae for biofuel production (Mahapatra & Ramachandra, 2013; Prabakaran *et al.*, 2019). *Chlorococcum minutum* produces biological hydrogen (Paramesh *et al.*, 2018), while other strains within this genus could be potential natural source of B-vitamin (Fujii *et al.*, 2010). Furthermore, with the use of a carotenoid biosynthesis inhibitor, relatively high amounts of phytoene could be accumulated by *Chlorococcum* strains (Laje *et al.*, 2019). Phytoene is a colorless highly added value compound with potential UV-protective, anti-aging and photo-protective cosmetic applications. In addition, some of these species are proliferating in municipal wastewater (Mahapatra & Ramachandra, 2013) and can be effectively used for the treatment of wastewater effluents (Aravantinou *et al.*, 2016). Therefore, the description, cultivation and

characterization of novel microalgal species has not only taxonomic importance, but new strains may represent direct links to various kinds of industrial applications.

Supplementary information

This article contains three supplementary figures.

Author contributions

E. Greipel: isolation and cultivation of the algae, light microscopy, and drafting of the manuscript. T. Felföldi: supervision of the DNA-based studies, phylogenetic analyses, drafting and editing the manuscript. K. Solymosi: electron microscopy, drafting and editing of the manuscript. H. Nagy: DNA isolation and PCR. J. Kutasi: supervision of cultivation. Each author has participated sufficiently in the work to take public responsibility for appropriate portions of the content. All authors read and approved the final manuscript.

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Figure legends
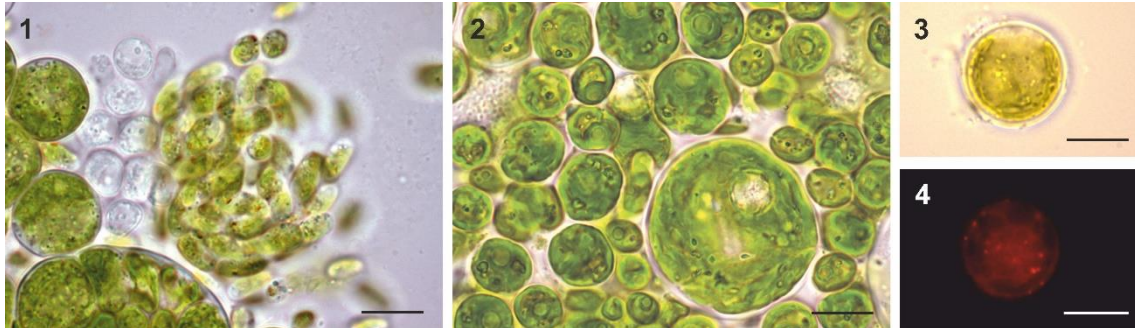
Figs 1–4. Light micrographs of *Chlorococcum szentendrense* K2/9One-day-old (Fig. 1) and two-week-old cultures (Figs 2–4; Fig. 3, epifluorescence microscopy picture after nile red staining). Scale bars = 10 µm.
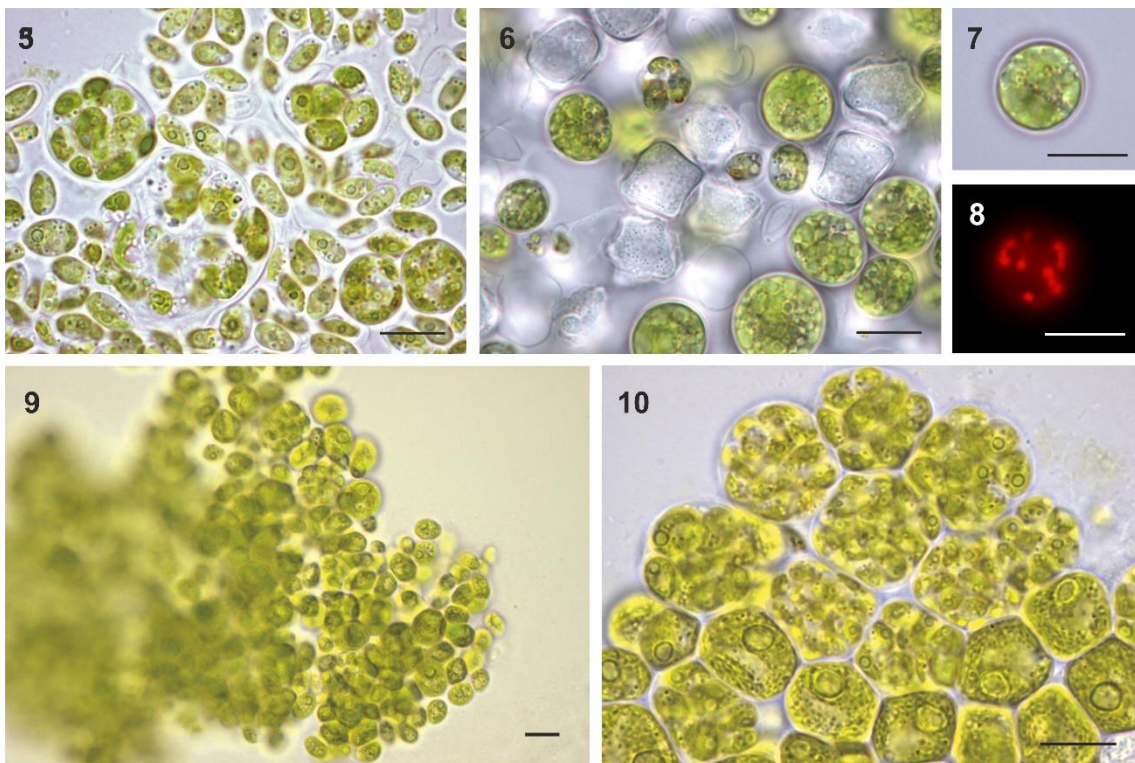
Figs 5–10. Light micrographs of *Chlorococcum* strains K2/5 and K2/10. **Figs 5–8.** *Chlorococcum* sp. K2/5. **Figs 9–10.** *Chlorococcum* sp. K2/10. One-day-old (Fig. 5) and

two-week-old cultures (Figs 6–10; Fig. 8, epifluorescence microscopy picture after nile red staining). Scale bars = 10 μm.
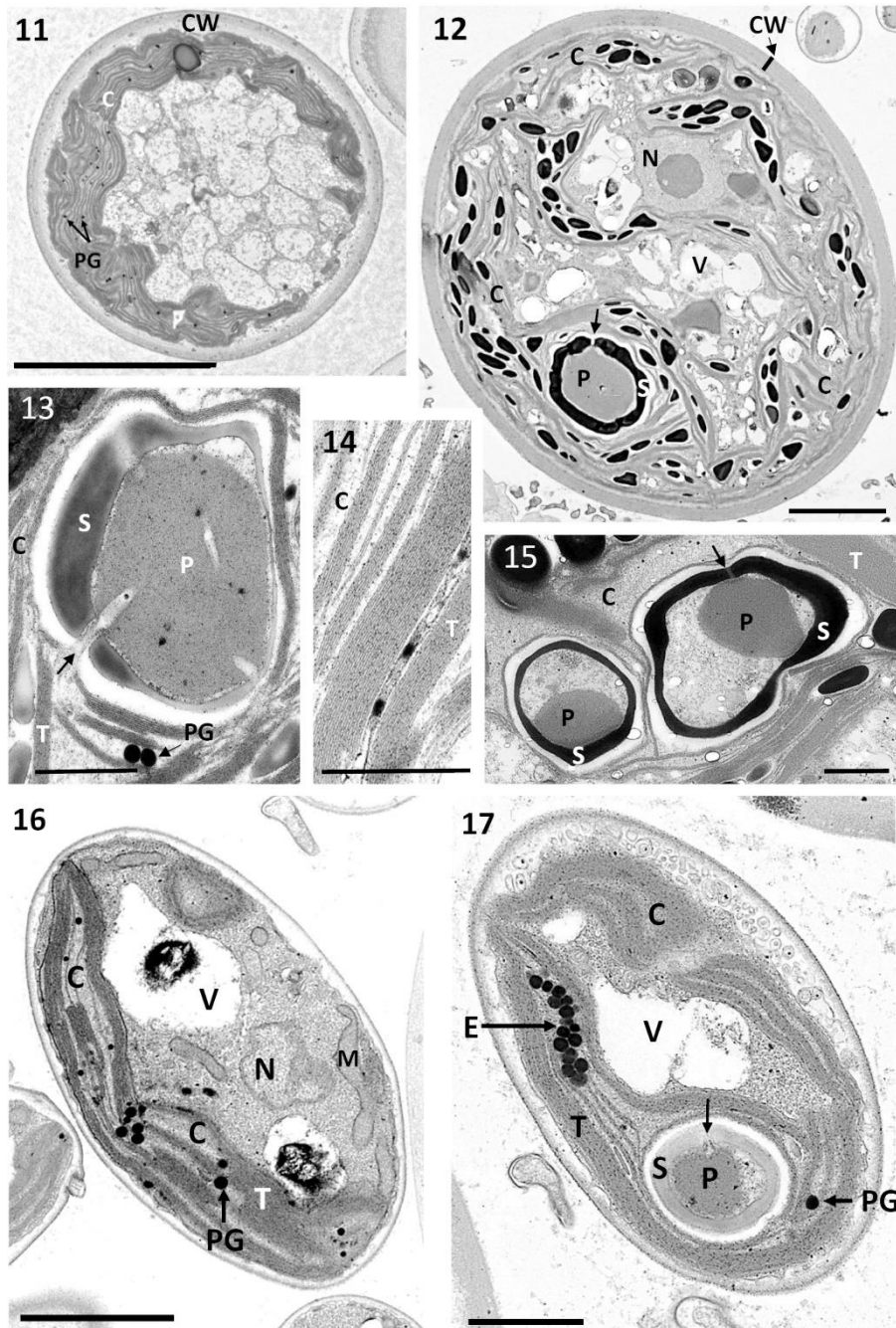
Figs 11–17. Transmission electron micrographs of *Chlorococcum szentendrense* K2/9.

Figs 11–17. Vegetative cells of two-week old cultures. **Figs 16–17.** Zoospores of one-

day old cultures. Black arrow, pyrenoid channel; C, chloroplast; CW, cell wall; E, eyespot; M, mitochondrion; N, nucleus; P, pyrenoid; PG, plastoglobuli; S, starch; T, thylakoid; V, vacuole. Scale bars = 10 µm (Fig. 11), 5 µm (Fig. 12), 2 µm (Figs 16 and 17), 1 µm (Figs 13 and 15), 0.5 µm (Fig. 14).
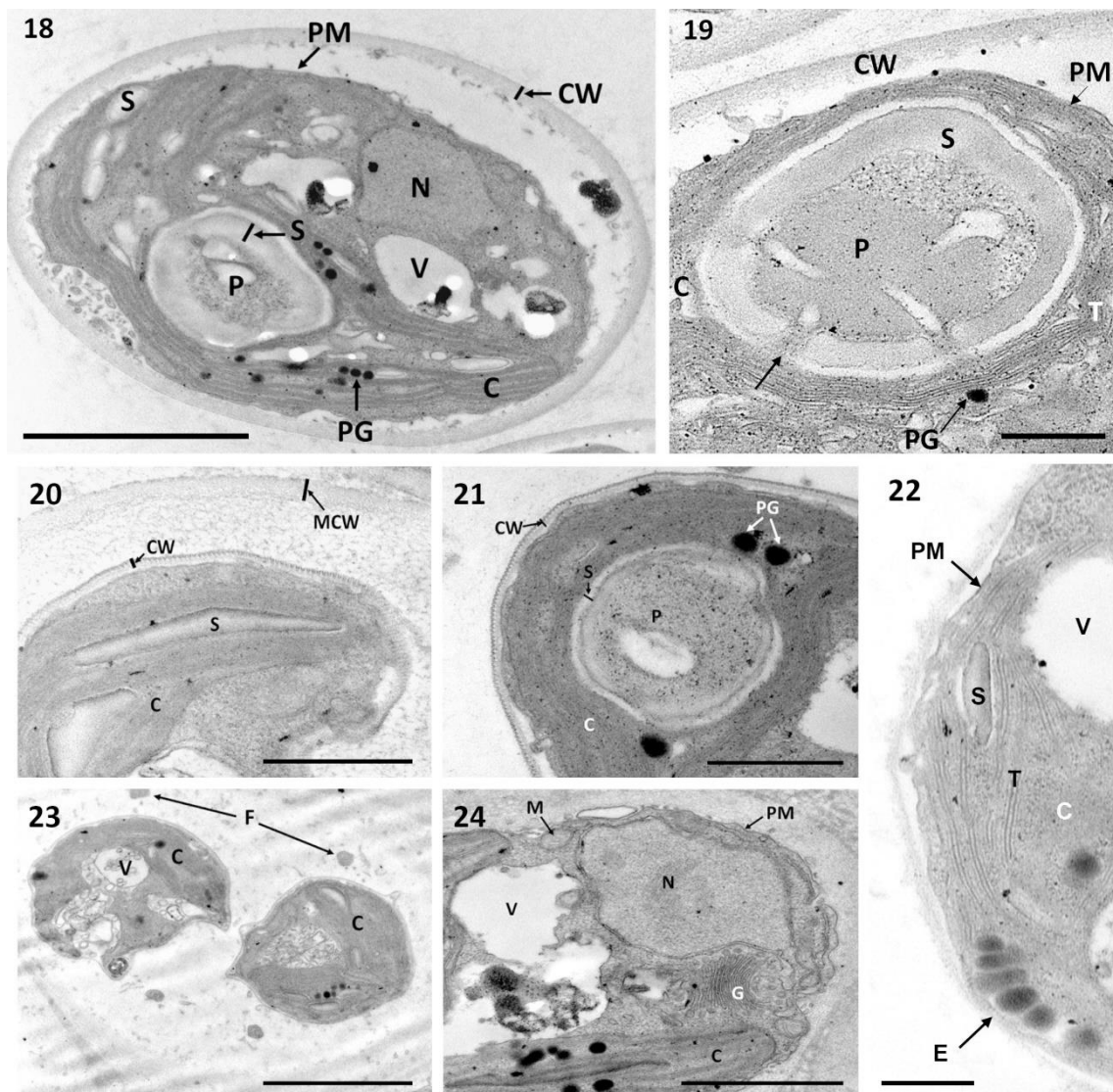
Figs 18–24. Transmission electron micrographs of *Chlorococcum* strains K2/5 and K2/11 vegetative cells and zoospores. **Figs 18–19.** Vegetative cells of two-week old cultures (Fig. 18, K2/5 and Fig. 19, K2/11). **Figs 20–25.** Zoospores of one-day old

cultures (K2/5). Black arrow, pyrenoid channel; C, chloroplast; CW, cell wall; E, eyespot; F, flagella; G, Golgi apparatus; MCW, mother cell wall; N, nucleus; P, pyrenoid; PG, plastoglobule; PM, plasma membrane; S, starch; T, thylakoid; V, vacuole. Scale bars = 2 µm (Figs 18 and 23), 1 µm (Figs 21 and 24), 0.5 µm (Figs 19, 20 and 22).
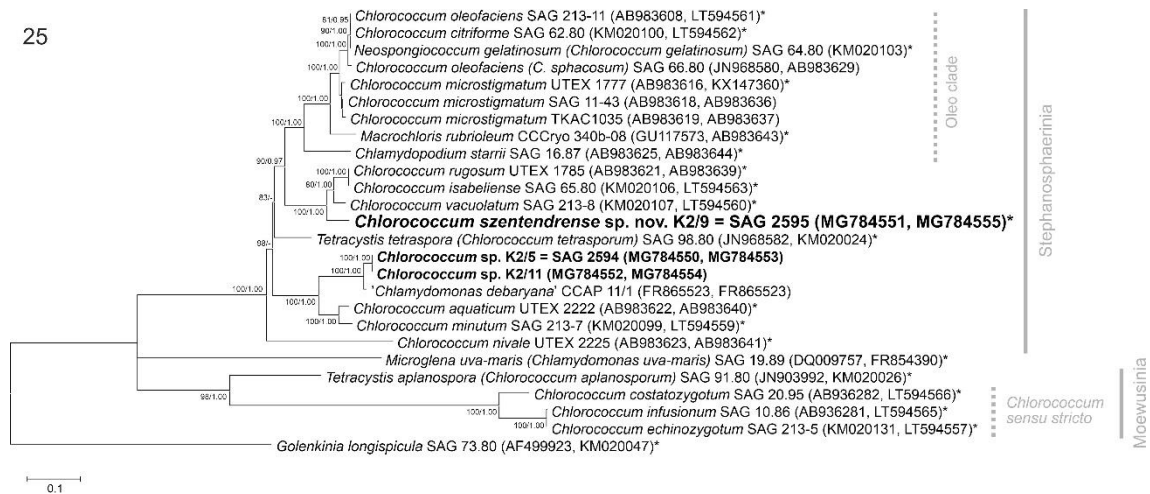

Fig. 25. Concatenated maximum likelihood (ML) phylogenetic tree based on the 18S rRNA gene and the ITS region. Bootstrap values greater than 70 and Bayesian posterior probabilities (BP) greater than 0.70 are shown at the nodes (ML/BP). Trees were reconstructed using the following datasets and model parameters: 1766 nucleotide positions and K2P+G+I for the 18S rRNA gene, 781 nucleotide positions and GTR+G for the ITS region. Sequences determined in this study appear in bold. Asterisks mark authentic strains.
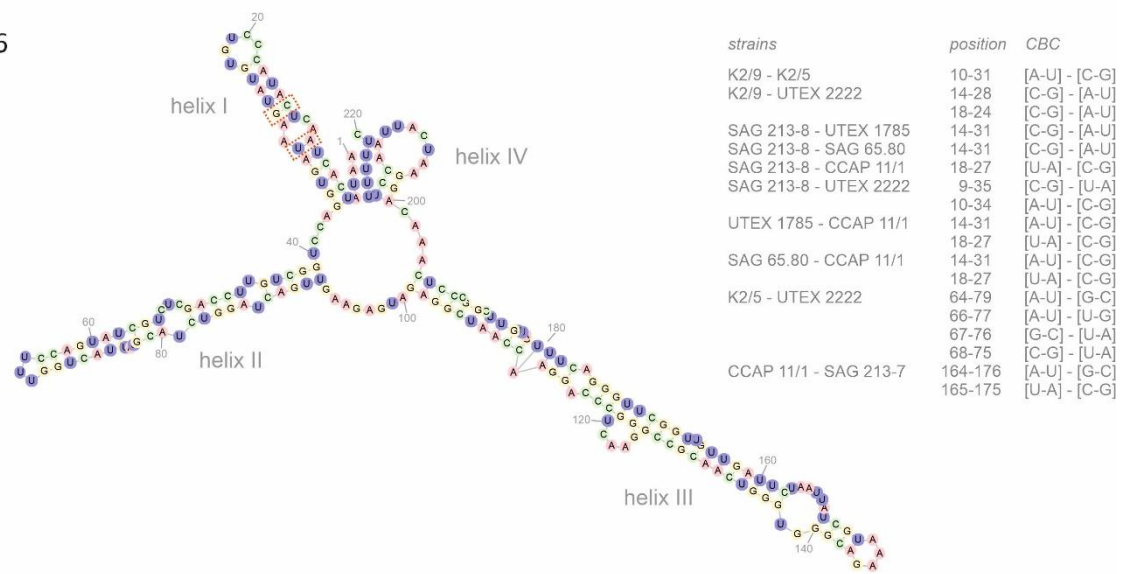
Fig. 26. Predicted ITS-2 secondary structure of *Chlorococcum szentendrense* K2/9 and the identified CBCs comparing the closest related strains of K2/9 and K2/5. Strains included in the analysis: *C. szentendrense* K2/9, *C. vacuolatum* SAG 213-8, *C. rugosum* UTEX 1785, *C. isabeliense* SAG 65.80, *C. aquaticum* UTEX 2222, *C. minutum* SAG 213-7, '*Chlamydomonas debaryana*' CCAP 11/1 and *C.* sp. K2/5 (for GenBank accession codes, see Fig. 25). CBCs of K2/9 are indicated with red boxes.

SUPPLEMENTARY MATERIAL

Hidden diversity of Chlorococcum-like algae (Chlorophyta) in a shallow temporary freshwater lake: description of *Chlorococcum szentendrense* sp. nov.

Greipel E, Kutasi J, Solymosi K, Nagy H, Felföldi T
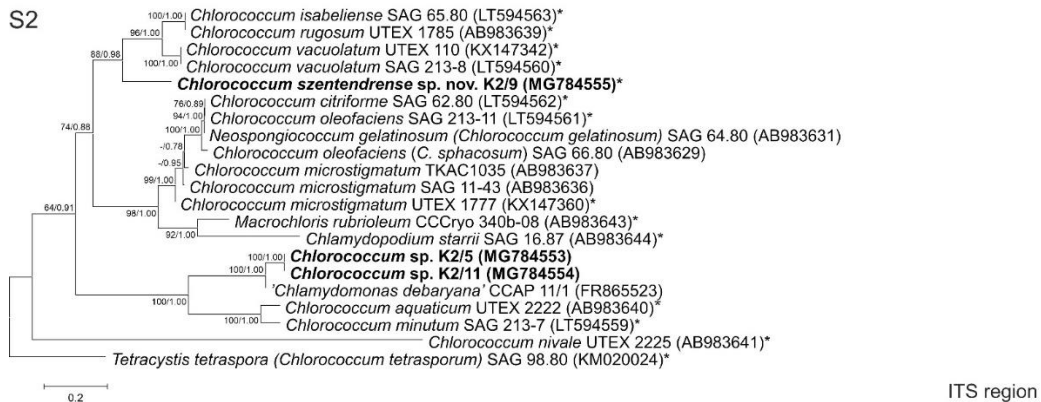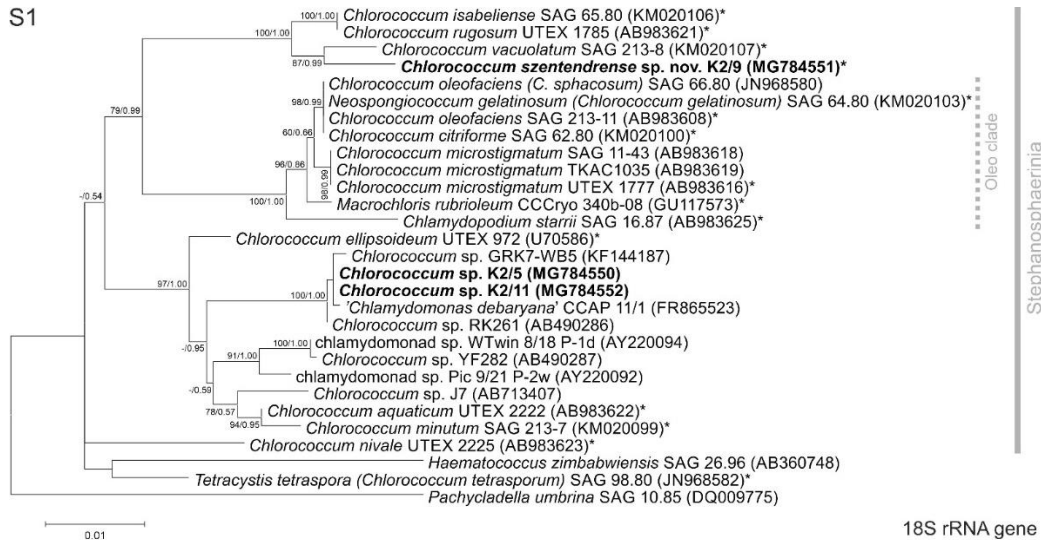
Supplementary Figs S1–S3. Phylogenetic position of the new strains within the Stephanosphaerinia macroclade. Maximum likelihood (ML) trees based on 18S rRNA gene (Fig. S1), ITS region (Fig. S2) and *rbcL* gene (Fig. S3) sequences. Trees were reconstructed using the following datasets and model parameters: 1699 nucleotide positions and K2P+G+I for the 18S rRNA gene, 727 nucleotide positions and K2+G for the ITS region and 424 nucleotide positions and GTR+G for the *rbcL* gene. Bootstrap values greater than 50 and Bayesian posterior probabilities (BP) greater than 0.50 are shown at the nodes (ML/BP). Sequences determined in this study appear in bold. Asterisks mark authentic strains.