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Polulichloris henanensis gen. *et* sp. nov. (Trebouxiophyceae, Chlorophyta), a novel subaerial coccoid green alga

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Abstract

Coccoid green algae are abundant in subaerial habitats, but they are largely unexplored because of their morphological uniformity. Several new genus-level lineages have recently been described on the basis of molecular data. In this study, a coccoid green alga was isolated from surface soil in Zhoukou, Henan Province, China, and the cultured cells were described using light and electron microscopy. The ellipsoidal cell had smooth cell wall and parietal chloroplast with a pyrenoid surrounded by a starch envelope. Reproduction occurred by formation of 2–16 autospores. Molecular phylogenetic analyses based on the nuclear 18S rDNA gene and the chloroplast ribulose-bisphosphate carboxylase gene (*rbc*L) indicated that this coccoid green alga represents a new lineage of the *Watanabea* clade (Trebouxiophyceae, Chlorophyta). Here, we describe this organism as a new genus and species, *Polulichloris henanensis, gen. et sp. nov.*

Key words: Phylogeny, Taxonomy, Subaerial alage, Trebouxiophyceae, *Watanabea* clade

Introduction

Coccoid green algae have traditionally been classified as a subgroup of the green algae, the order Chlorococcales (Komárek & Fott 1983). Introduction of molecular phylogenetic methods into the taxonomy of green algae led to a fundamental revision of these algae, and most of the taxa have been transferred to other orders. Autosporic coccoid green algae are currently known in the Chlorophyceae, Trebouxiophyceae, and Prasinophyceae within the division Chlorophyta (Melkonian *et al*. 1990, Fawley *et al*. 2000, Luo *et al*. 2003, Krienitz *et al*. 2003, Leliaert *et al*. 2012). In the Trebouxiophyceae, autosporic coccoid green algae occur in three major clades: the *Watanabea* clade, the Chlorellales, and the Trebouxiales. Several other clades that include coccoid green algae are the *Elliptochloris* clade (Eliáš *et al*. 2008), the *Xylochloris* clade (Neustupa *et al*. 2011), the *Leptochlorella* clade (Neustupa *et al*. 2013a), the *Chloropyrula* clade (Gaysina *et al*. 2013) and the *Eremochloris* clade (Fučíková *et al*. 2014).

Most members of the *Watanabea* clade are subaerial coccoid autosporic microalgae. This clade was defined by Karsten *et al*. (2005) as a monophyletic lineage of the Trebouxiophyceae. Molecular phylogenetic studies of the Trebouxiophyceae showed that the *Watanabea* clade includes *Viridiella* Albertano, Pollio & Taddei (1991: 347); *Heterochlorella* Neustupa, Němcová, Eliáš & Škaloud (2009: 167); *Heveochlorella* Zhang, Huss, Sun, Chang & Pang, *Kalinella* Neustupa, Němcová, Eliáš & Škaloud (2009: 167); *Chloroidium* Nadson (1906: 189); *Phyllosiphon* Kühn (1878: 25); *Watanabea* Hanagata, Karube, Chihara & Silva (1998: 226); *Desertella* Fučíková, Lewis & Lewis (2014:303); and *Parachloroidium* Neustupa & Škaloud (2013: 413) (Albertano *et al*. 1991, Hanagata *et al*. 1998, Zhang *et al*. 2008, Neustupa *et al*. 2009, 2013b, Darienko *et al*. 2010, Aboal & Werner 2011, Ma *et al*. 2013, Fučíková *et al*. 2014).

Most coccoid autosporic microalgae are not distinguishable using traditional methods because of their reduced, uniform morphology; molecular methods are the primary means of recognizing their taxonomic position. Broad sampling of the 18S rDNA gene among members of the Trebouxiophyceae has made this marker extremely useful for identifying previously unknown lineages. The chloroplast large subunit of the ribulose bisphosphate carboxylase/

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oxygenase (*rbc*L) gene has recently been used in taxonomic and diversity analyses (Neustupa *et al*. 2013b, Fučíková *et al*. 2014). In this study, we chose 18S rDNA and *rbc*L as molecular markers to learn more about this alga. We used a polyphasic approach with small subunit rDNA, *rbc*L, and internal transcribed spacer (ITS) rDNA phylogeny, light microscopy, and electron microscopy to characterize the subaerial coccoid green alga. This novel alga is morphologically similar to *Chlorella* species, but phylogenetic analysis indicated that the isolate represents a distinct species within the *Watanabea* clade (Trebouxiophyceae, Chlorophyta). Therefore, we propose this alga as a new genus and species, *Polulichloris henanensis* Song, Zhang & Liu *gen. et sp. nov.*

Material and methods

Algal isolation and culture:—The strain FACHB-1765 was isolated from a soil sample collected by huiyin Song from Zhoukou, Henan Province, China (33°48' 40.02" N, 114° 28' 20.80" E, elevation 56 m a.s.l.) in February 2013. Unialgal cultures were established by serial streaking on 1.5% BG11 agar and by single colony isolates. FACHB-1765 was cultivated on 1.5% agar plates maintained at 21 °C under 30 μmol $m^{-2}s^{-1}$ of cool-white fluorescent light on a 14 h light: 10 h dark cycle.

Light and electron microscopy:—Microphotographs were taken with an Olympus BX53 light microscope (Olympus Corp., Tokyo, Japan) and an Olympus BX53 camera using differential interference contrast. Photographs were taken under an oil immersion objective lens. For transmission electron microscopy (TEM), cells undergoing exponential growth were collected. Algal samples were fixed for 2 h at 5 °C in 2% glutaraldehyde in 0.05 M phosphate buffer and postfixed for 2 h at 5 °C in 1% osmium tetroxide in 0.05 M phosphate buffer and overnight at 5 °C in 1% uranyl acetate in methanol. After dehydration through an ethanol series, the samples were embedded in Spurr medium via propylenoxide. Ultrathin sections, cut on an Leica UC7, were poststained with uranyl acetate and bismuth oxynitrate and examined with a Hitachi HT-7700 TEM at 120kV.

DNA extraction, PCR amplification, and sequencing:—DNA was extracted using the Universal DNA Isolation Kit (AxyPrep, Shuzhou, China). PCR amplification was performed using 3 μL template DNA, 0.4 μmol/L each primer, and 25 μL 2× Tap Master Mix (ExTaq; Takara, Dalian, China) in a 50 μL reaction volume. Nuclear-encoded small subunit ribosomal DNA (SSU rDNA) was amplified using primers 5'-TGGTTGATCCTGCCAGT-3' and 5'-TGATCCTTCTGCAGGTTCACC-3' (Medlin *et al*. 1988). The amplification conditions were as follows: 5 min at 94 °C, 32 cycles of 50 s at 94 °C, 50 s at 55 °C, 90 s at 72 °C, and a final 10 min extension step at 72 °C. The ITS region, including ITS1, 5.8S rDNA, and ITS2, was amplified using primers 5'-CAAGGTTTCCGTAGGTGA-3' and 5'-GGCATCCTGGTTAGTTTCT-3'. The amplification conditions were as follows: 5 min at 94 °C, 32 cycles of 50 s at 94 °C, 50 s at 55 °C, 1 min at 72 °C, and a final 10 min extension step at 72 °C. The *rbc*L gene sequence was amplified using primers 5'-ATGTCACCACAAACAGAAACTAAAGCA-3' and 5'-GATCTCCTTCCATACTTCACAAGC-3' (Zechman 2003). The amplification conditions were as follows: 5 min at 94 °C, 32 cycles of 50 s at 94 °C, 50 s at 55 °C, 70 s at 72 °C, and a final 10 min extension step at 72 °C. The amplification products were separated along with a sample of the control and a 5000 bp DNA marker (Cebio, Beijing, China) in 1.0% (w/v) agarose gels cast in TAE buffer. The gels were electrophoresed at 100 V for 35 min and viewed under ultraviolet light. The purified amplification products were sequenced by TSINGKE Biotechnologies (China). Sequences were deposited in GenBank under the accession numbers KM085344–KM085346.

Phylogenetic analyses:—The SSU rDNA, *rbc*L, and ITS sequences, selected based on a BLAST search or representation of reference species in the relevant taxonomic class, were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/). Sequences were aligned using ClustalX v 2.0 (Larkin *et al*. 2007) and were further manually edited and adjusted by eye. Positions of SSU rDNA and SSU rDNA + *rbc*L sequences that could not be aligned with confidence were removed prior to the analysis. Sequence alignments were exported as nexus files from ClustalX and were analyzed using maximum likelihood (ML) and Bayesian inference (BI) as implemented in PAUP 4.0* 4.0b10 (Swofford 2002) and MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001). In ML analyses, appropriate substitution models and parameters were determined for each alignment by running likelihood ratio tests in PAUP*4.0 (Swofford 2002) and using ModelTest (Posada & Crandall 1998). The evolutionary models used in ML for the SSU and SSU + *rbc*L phylogenies were TrNef + I + G and GTR + I + G, respectively; a heuristic search option with random addition of sequences (100 replicates) and the nearest-neighbor interchange branch-swapping algorithm (NNI) were used for tree searching. Phylogenetic Bayesian analyses (Huelsenbeck & Ronquist 2001) were performed using the GTR model (Rodriguez *et al*. 1990), and Markov Chain Monte Carlo (MCMC) analyses were run with four Markov chains (three

heated, one cold) for $3 \cdot 10^6$ generations, with trees sampled every 1000 generations. Every time the diagnostics were calculated, a fixed number of samples (burnin = 250) were discarded from the beginning of the chain. Parameter stability and run convergence were inspected using Tracer v1.6 (Rambaut & Drummond 2003). We obtained posterior probability (PP) values for the branching patterns in BI trees and bootstrap (BP) values in ML trees.

Results

Chlorophyta
Trebouxiophyceae, the *Watanabea* clade

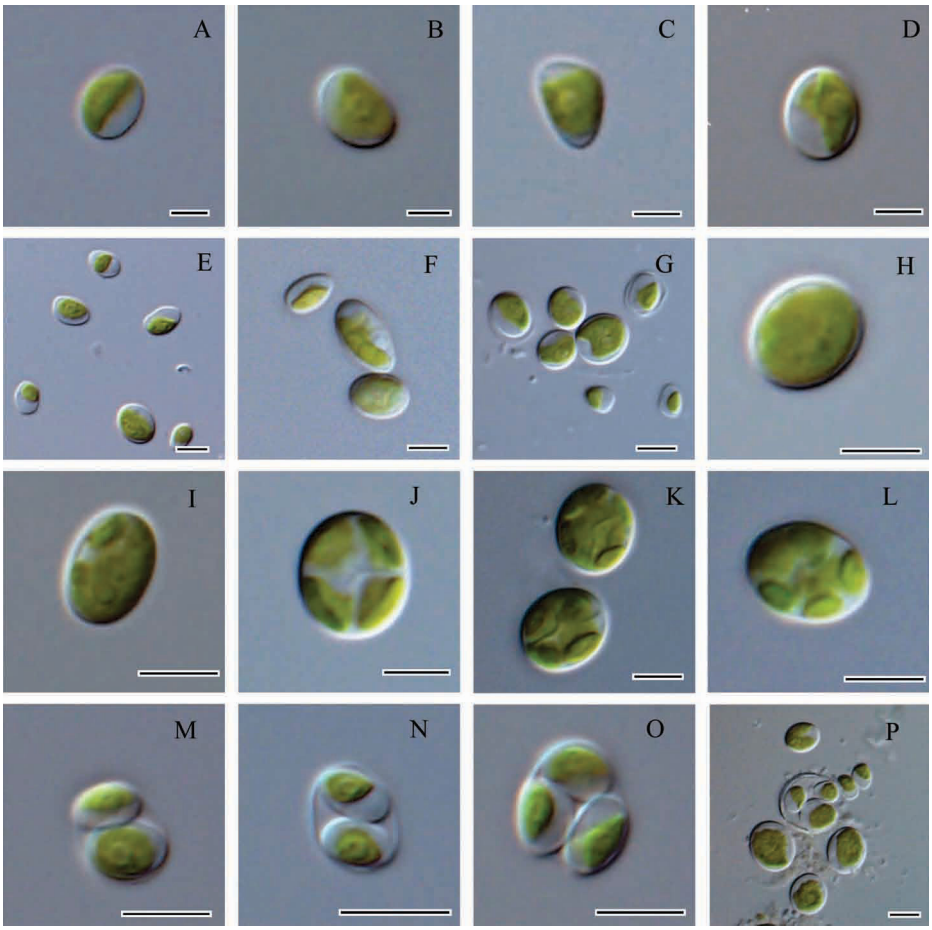

FIGURE 1. Morphology of *Polulichloris henanensis* strain FACHB-1765. A–G: young vegetative cells. H, I: mature vegetative cell. J–O: autosporangium. P: vegetative cells and liberation of autospores. Scale bars: A–D = 2 µm, E–P = 5 µm.

Polulichloris H.Y. Song, Q. Zhang, G.X. Liu & Z.Y. Hu, *gen. nov.* (Figs. 1, 2)

Vegetative cells solitary, uninucleate, and ellipsoidal. Chloroplast single, parietal, with a pyrenoid surrounded by starch envelope. Cell walls smooth and double-layered. Asexual reproduction via 2–4–8 autospores; sexual reproduction not observed. Secondary carotenoids not produced. The genus differs from other members of the *Watanabea* clade (Trebouxiophyceae) by the 18S rDNA, ITS and *rbc*L sequences.

Type species:—*Polulichloris henanensis* H.Y. Song, Q. Zhang, G.X. Liu & Z.Y. Hu (see below)

Etymology:—The genus name *Polulichloris* consists of "*Polul*" (the Latin prefix "poluius" means "small volume") and "chloris" (the Greek suffix "χλωρος" (chloros) means "green").

Polulichloris henanensis **HY. Song, Q. Zhang, GX. Liu & ZY. Hu , *sp. nov.* (Figs. 1, 2)**

Vegetative cells solitary, uninucleate. Young cells irregularly egg-shaped or ellipsoidal, 2.98–3.70 × 3.95–5.67 µm; mature cells ellipsoidal, 4.86–5.84 × 6.08–8.20 µm. Cell have parietal, cup-shaped chloroplast with a pyrenoid surrounded by starch envelope. Asexual reproduction via 2 to 8 elliptical or irregularly egg-shaped autospores, 5.71–8.70 × 6.90–9.28 µm. Sexual reproduction not observed.

Type:—CHINA. Henan Province: microbial biofilm on surface soil in Zhoukou, 33° 48' 40.02" N, 114° 28' 20.80" E, elevation: 56 m a.s.l., *H.Y. Song, February 2013*(holotype: FACHB!, fixed specimen *shy053* deposited in the Freshwater Algae Specimen Station, Institute of Hydrobiology, Chinese Academy of Sciences. Reference strain: living culture (ex-holotypus), accession no. FACHB-1765, deposited in FACHB: http://algae.ihb.ac.cn/).

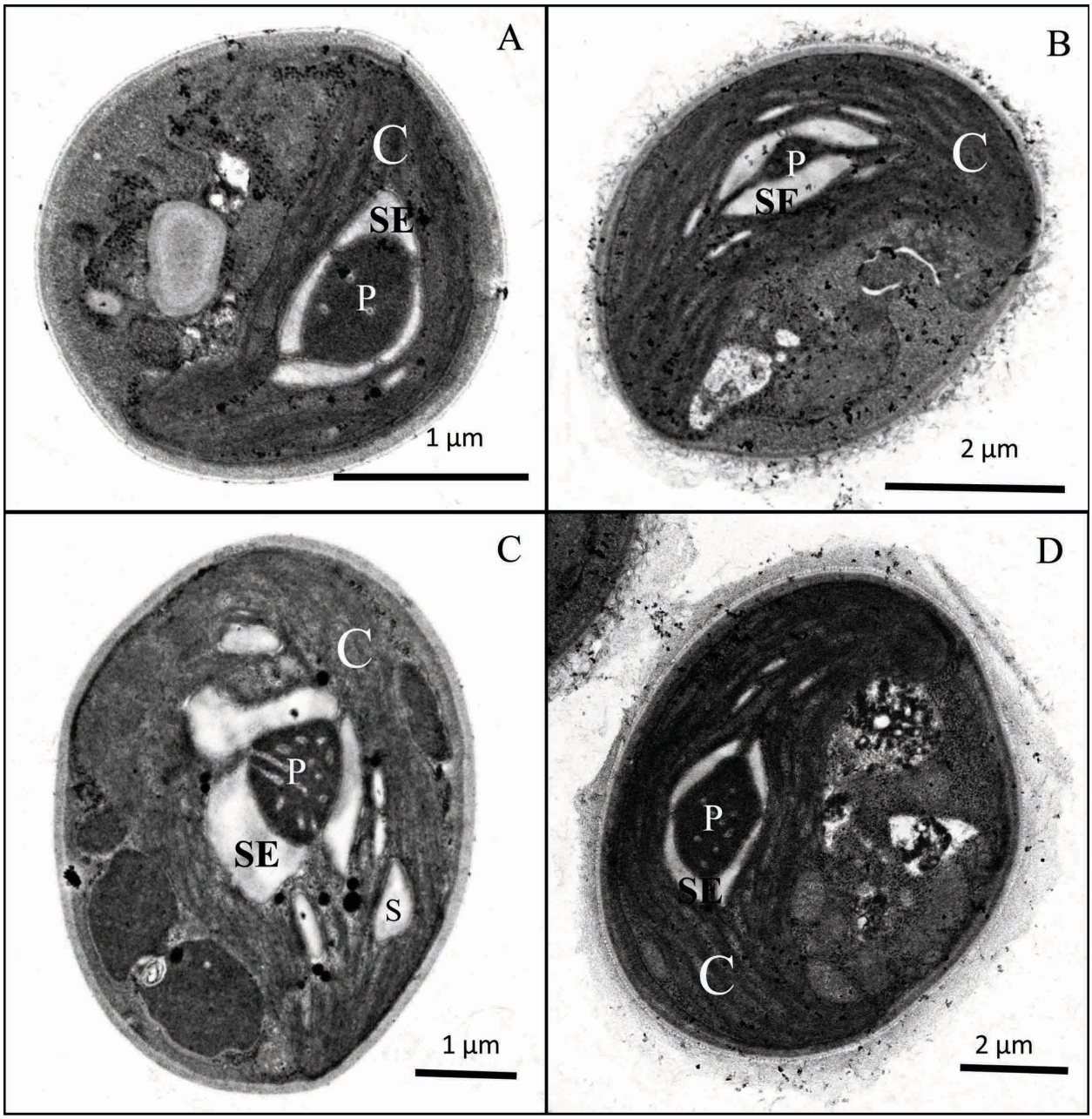

FIGURE 2. Ultrastructure of *Polulichloris henanensis*. C: chloroplast, P: pyrenoid, SE: starch envelope, S: starch grains. Cell of *P. henanensis* with a parietal and cup-shaped chloroplast, pyrenoid bisected by a few thylakoid bands, and starch envelope composed of 2–4 plates surrounding the pyrenoid. A. Young cell. B–D. Vegetative cell.

Light and electron microscopy:—*Polulichloris henanensis* shares general morphological characteristics with the *Chlorella*-like green microalgae: unicellular, elliptical, parietal plastid with a pyrenoid surrounded by a starch envelope. Young cells are ellipsoidal or irregularly egg shaped, 2.98–3.70 × 3.95–5.67 µm in size. When mature, the cells are

ellipsoidal or broadly ellipsoidal and 4.86–5.84 × 6.0–8.20 µm. Chloroplasts are parietal and cup-shaped, sometimes occupying most of the cell. The pyrenoid is barely visible by light microscope but is well developed in most cells and surrounded by a starch envelope composed of 2–4 plates; with a few thylakoid bands transecting the pyrenoid matrix (Fig. 2). The alga reproduces by 2, 4, or 8 asexual autospores. The autosporangium of *P. henanensis* strain FACHB-1765 was typically elliptical. Autospores were elliptical or irregularly egg-shaped. Sometimes, a single relatively large autospore and several smaller autospores were produced within a single sporangium; some autospores within a sporangium were almost equal in size. The autospores were discharged through an aperture.

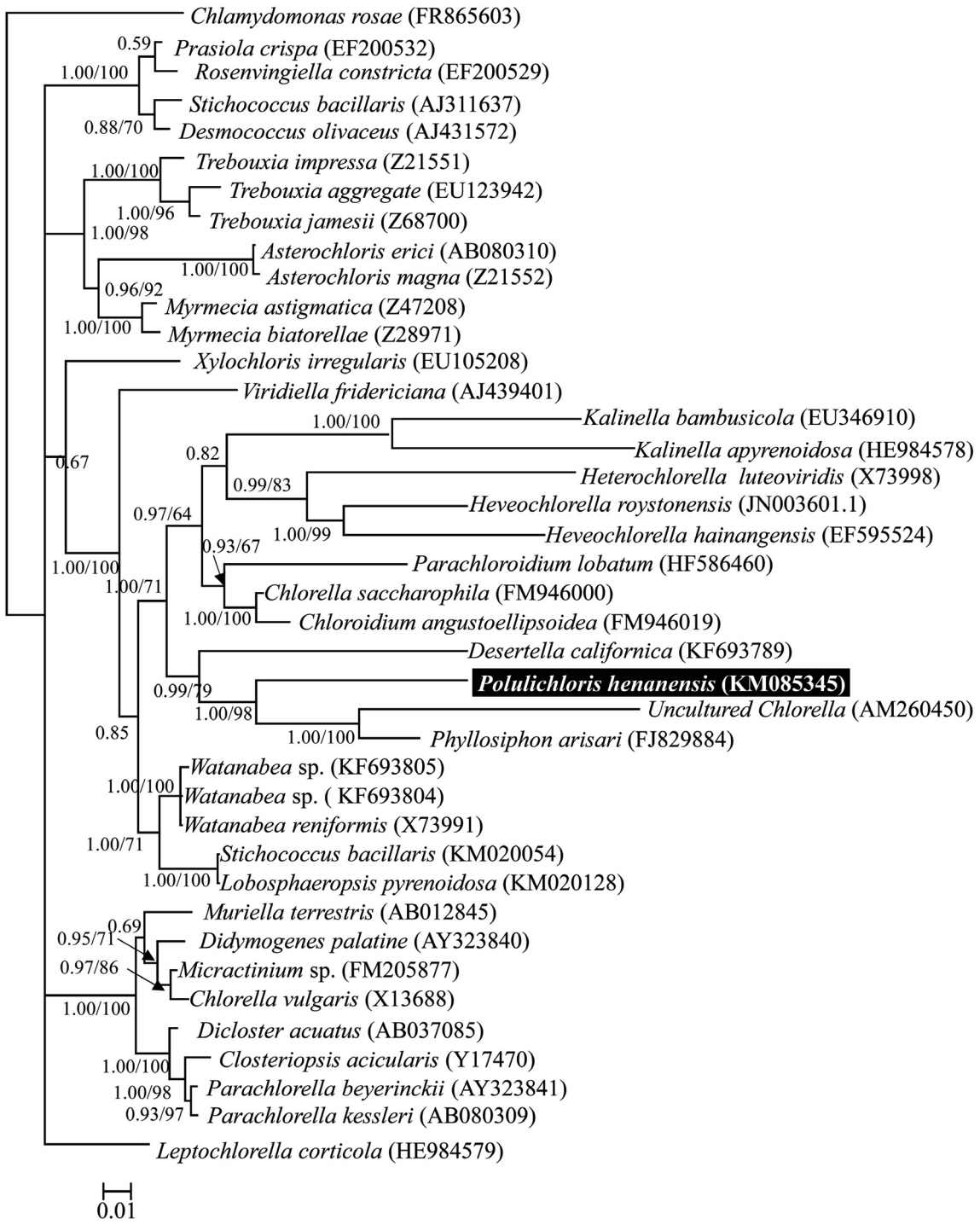

FIGURE 3. Phylogenetic position of *Polulichloris henanensis* within class Trebouxiophyceae (Chlorophyta), based on 18S rDNA sequences. The analysis was based on reduced alignment with an outgroup formed by the chlorophycean species *Chlamdomonas rosae*. The tree was inferred using PAUP*4.0 with the TrNef + I + G evolutionary model. Numbers at branches correspond to MrBayes posterior probabilities (BPP)/maximum likelihood (ML) bootstrap values. Values below 0.95 BPP and 50% ML bootstrap support are not shown. Scale bar shows estimated number of substitutions per site.

Molecular phylogeny:—The 18S rDNA and *rbc*L gene sequences were obtained from strain FACHB-1765, and the sequenced lengths were 1686 and 1248 bp, respectively. The phylogenetic position of *P. henanensis* was inferred by analyzing the 18S rDNA and *rbc*L DNA sequences. The 18S rDNA and the18S rDNA + *rbc*L alignments consisted of 1637 and 2721 characters, respectively. The corresponding ML and Bayesian topologies were consistent for these clades, and the best ML trees for 18S rDNA and 18S rDNA + *rbc*L are shown in Figures 3 and 4, respectively, with Bayesian posterior probability (BPP) and bootstrap support values (BP) indicating branch support. According to the phylogenetic tree based on 18S rDNA (Fig. 3), FACHB-1765 was positioned on a solitary branch nested within the *Watanabea* clade (Trebouxiophyceae, Chlorophyta), likely sister to a well-supported clade including *Phyllosiphon arisari* (FJ829884) and the uncultured strain (AM260450) (1.00 / 98). However, it differed from *P. arisari* (FJ829884) by 126 of 1692 positions of the 18SrDNA gene and Blast searches resulted in hits of 93% similarity to *P. arisari*. The topology of the phylogenetic tree derived from analysis of the concatenated 18S rDNA + *rbc*L sequences (Fig. 4) was consistent with the tree based on 18S rDNA (Fig. 3). We did not find *rbc*L sequence data for *Phyllosiphon* in the NCBI database; the phylogenetic tree based on 18S rDNA + *rbc*L does not include *Phyllosiphon*. Strain FACHB-1765 was in a supported sister position with the genus *Desertella* (0.99 / 71) in the *Watanabea* clade. The phylogenetic tree based on ITS (Fig. S1) is available online as supplementary material.

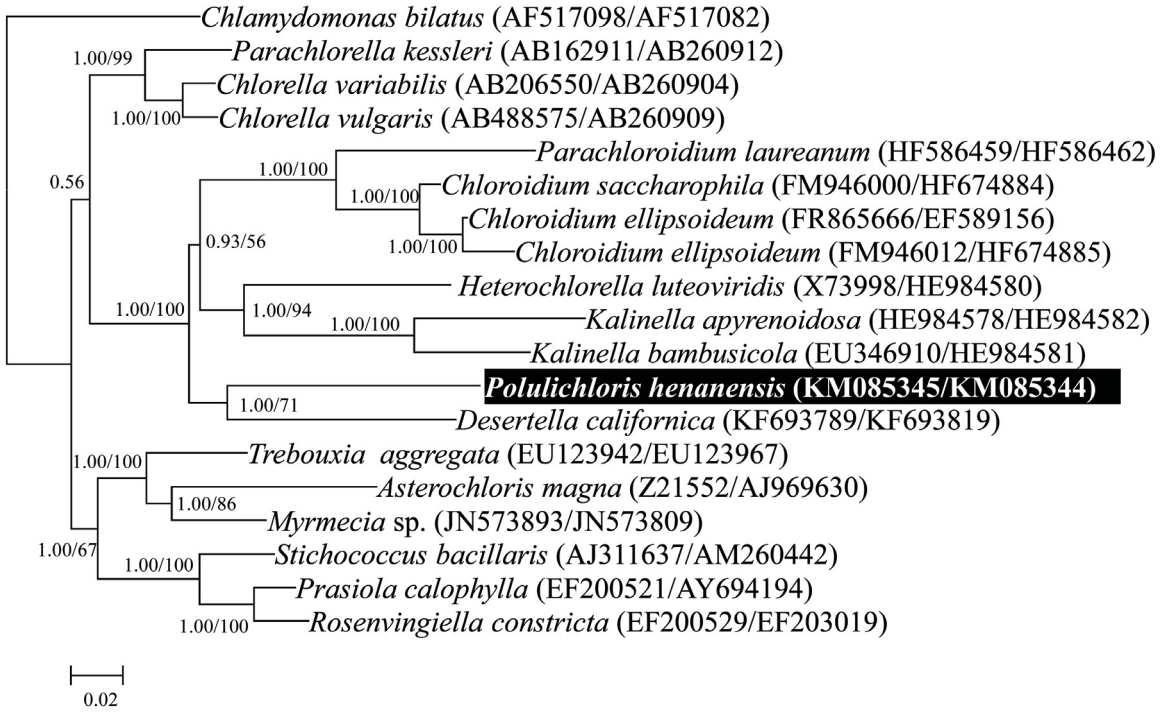

FIGURE 4. Phylogenetic position of *Polulichloris henanensis* within class Trebouxiophyceae (Chlorophyta), based on 18S rDNA + *rbc*L sequences. The analysis was based on reduced alignment with an outgroup formed by the chlorophycean *Chlamydomonas bilatus*. The tree was inferred using PAUP*4.0 with the GTR + I + G evolutionary model. Numbers at branches correspond to MrBayes posterior probabilities (BPP)/maximum likelihood (ML) bootstrap values. Values below 0.95 BPP and 50% ML bootstrap support are not shown. Scale bar shows estimated number of substitutions per site.

Discussion

Several recent studies based on molecular data have revealed unexpected phylogenetic diversity in the coccoid green algae. These microalgae are probably most abundant and diversified in subaerial biofilms. Numerous coccoid green taxa have been isolated from subaerial ecosystems (*e.g*., Krienitz *et al*. 2004, Darienko *et al*. 2010, Luo *et al*. 2010, Bock *et al*. 2011). In the Trebouxiophyceae, *Leptochlorella* Neustupa, Veselá, Němcová & Škaloud in Neustupa *et al.* (2013a: 379) was found on the bark of *Cupressus sempervirens*, *Xylochloris irregularis* Neustupa, Eliáš & Škaloud in Neustupa *et al*. (2011: 59) was found on bark and wood of tropical trees, *Chloropyrula uraliensis* Gaysina, Němcová, Škaloud , Ševčíková, & Eliáš in Gaysina *et al*. (2013: 476) was found on soil; and *Eremochloris* Fučíková, Lewis & Lewis in Fučíková *et al*. (2014: 304), *Xerochlorella* Fučíková, Lewis & Lewis in Fučíková *et al*. (2014: 304) and

Desertella Fučíková, Lewis & Lewis in Fučíková *et al*. (2014: 303) were found in the desert. In the Chlorophyceae, *Jenufa perforata* Němcová, Eliáš, Škaloud & Neustupa in Němcová *et al.* (2011: 930) and *Jenufa minuta* Němcová, Eliáš, Škaloud & Neustupa in Němcová *et al*. (2011: 930) were found on the bark of trees in tropical forest habitats, and the type strain of *Hylodesmus singaporensis* Eliáš, M., Němcová, Y., Škaloud, P., Neustupa, J., Kaufnerová, V., & Šejnohová, L. in Eliáš *et al.* (2010: 1224) was isolated from decaying bare wood in a tropical forest. The known members of the *Watanabea* clade mostly occur in subaerial habitats, but there are exceptions: siphonous *P. arisari* Kühn is parasitic on vascular plants, and the uncultured strain AM260450 originated from photobiont cells of the lichen *Psoroglaena epiphylla* (Nyati *et al*. 2007), possibly indicating habitat diversity in the *Watanabea* clade that has yet to be discovered.

The majority of members of the *Watanabea* clade were *Chlorella*-like coccoid autosporic microalgae, with the exception of *Phyllosiphon*. *Phyllosiphon henanensis* was morphologically distinguished from a relatively close genus in the *Watanabea* clade (Table 1). The cell of *P. henanensis* were ellipsoidal, parietal plastid with a pyrenoid surrounded by a starch envelope. Chloroplast in *Desertella californica* was plate- or cup-shaped and single in young cells and 2–3 in older cells. *Parachloroidium* had regular spherical chloroplasts; those of *Chloroidium* species were parietal, plate-like or band-shaped, lobed or unlobed (but not cup-shaped). *Chloroidium ellipsoideum* (Gerneck 1907: 250) Darienko *et al*. (2010: 92) and *P. henanensis* had similar pyrenoid. *Parachloroidium laureanum*, *Parachloroidium lobatum*, and *Chloroidium saccharophilum* lacked pyrenoid.

Phylogenetic trees have shown that taxa with large morphological differences are sometimes closely related. The type stain of *P. henanensis* was positioned on a solitary branch nested within the *Watanabea* clade (Trebouxiophyceae, Chlorophyta), likely sister to a well-supported clade including *P. arisari* and the uncultured strain AM260450. It is clear that the apparent relatedness between *P. henanensis* and *P. arisari* is due to the fact that the closest relatives of these algae (particularly *P. henanensis*) have not yet been discovered or sequenced. *Phyllosiphon arisari* is most likely the closest relative to *P. henanensis* among the currently cultured algae, but the morphology and ecology of the two species are markedly different: the siphonous *P. arisari* is parasitic on vascular plants, while the *Chlorella*-like coccoid *P. henanensis* was discovered from surface soil. The taxonomic diversity of the *Watanabea* clade could be considerably higher than is currently known.

Three colonies of *P. henanensis* were found from one medium, and because the phylogenetic data and morphological characteristics of these colonies were identical, we think they represent one stain. Intraspecific divergence could not be estimated because only a single representative was found (Leliaert *et al*. 2014). Incomplete lineage sorting, trans-species polymorphism, hybridization, and introgression may cause inaccuracies in molecular approaches; because of this, we used different molecular markers (18S rDNA, 18S rDNA + *rbc*L and ITS) to explore the phylogenetic position. The marker results consistently showed that *P. henanensis* was positioned on a solitary branch nested within the *Watanabea* clade and was morphologically distinct from closely related genera under light and electron microscopy. The isolated phylogenetic position and distinct morphological and ultrastructural characteristics of this strain were the main reasons for our describing it as a new genus. We will next attempt to collect additional specimens to generate species boundaries; new genetic information and taxa of allied groups that resemble *P. henanensis* would help to clarify the relationships among these algae.

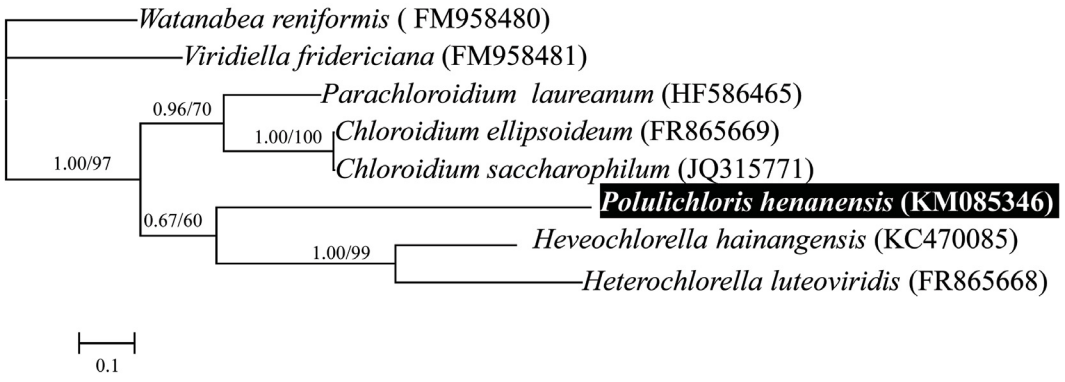

FIGURE S1. Phylogenetic position of *Polulichloris henanensis* within class Trebouxiophyceae (Chlorophyta), based on ITS1, 5.8S, and ITS2 sequences. The tree was inferred using PAUP*4.0 with the HKY + I + G evolutionary model. Numbers at branches correspond to MrBayes posterior probabilities (BPP)/maximum likelihood (ML) bootstrap values. Values below 0.50 BPP and 50%ML bootstrap support are not shown. Scale bar shows estimated number of substitutions per site.

TABLE 1. Morphological comparison between *Polulichloris henanensis* and several related species.

	P. henanensis FACHB-1765	*P. arisari* MUB-ALGAE 3373	*D. californica* BCPEM2VF32	*P. laureanum* CAUP H8501	*P. lobatum* CAUPH8502	*C. saccharophilum* SAG 211-9a
Habitat	subaerial	parasitic	subaerial	subaerial	subaerial	freshwater/subaerial
Cell shape	ellipsoidal	siphonous thallus	oval or ellipsoidal	spherical	spherical	ellipsoidal, spherical,
Cell size (μm)	2.98–3.7 × 6.08–8.2	*	3.2–5 × 12	2.5–9.8	3.5–13.5	6.9 – 5.3 × 13.6 – 9.4
Shape of chloroplasts	parietal, cup- shaped	*	Plate, cup-shaped	parietal, cup- shaped	parietal, cup- shaped	parietal, band-shaped to slightly lobed
Number of chloroplasts	1	*	1–3	1	1	1
Pyrenoid	surrounded by starch envelope	absent	surrounded by starch grains	absent	absent	indistinct, naked
Shape of autospores	elliptical or irregularly egg-shaped	ellipsoidal	*	elliptical or egg-shaped	elliptical or spherical	*
Size of autospores (μm)	5.71-8.70 × 6.90-9.28	4–6 × 2.5–4	*	2.5–3.5 × 3.5–5.5	3.0–6.5	*
Number of autospores	2–4–8	*	2–4	2–4–8	2–4–8	2–4–8–16
References	this research	Aboal *et al.* (2011)	Fučíková *et al.* (2014)	Neustupa *et al.* (2013b)	Neustupa *et al.* (2013b)	Darienko *et al.* (2010)

"*": no data.

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