Three novel species of coccoid green algae within the *Watanabea* clade (Trebouxiophyceae, Chlorophyta)

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Coccoid green algae are extremely diverse despite their simple coccoid phenotype, a phenotype that may be the result of convergent evolution. In this study, we used a polyphasic approach combining molecular phylogenetic analyses, morphology and ultrastructure to investigate isolated coccoid strains from China, and our results reveal three new lineages of Trebouxiophyceae: the novel genus and species *Mysteriochloris nanningensis* gen. et sp. nov., and the two novel species *Phyllosiphon coccidium* sp. nov. and *Desertella yichangensis* sp. nov. (Trebouxiophyceae, Chlorophyta). We provide a detailed characterization of the novel microalgae which they are autosporic coccoid unicells and have parietal chloroplasts. In phylogenies based on 18S rDNA sequences and the chloroplast ribulose-bisphosphate carboxylase gene (*rbc*L), these three algae are nested within the *Watanabea* clade and are different from any known algae. *M. nanningensis* FACHB-1787 is not really close to any known algae within the *Watanabea clade*. *Phyllosiphon coccidium* FACHB-2212 is within the *Phyllosiphon* lineages. *D. yichangensis* FACHB-1793 is closely related to *Desertella californica* and described as a representative of a novel species of the genus *Desertella*.

INTRODUCTION

Coccoid green algae are very common in all types of aquatic and terrestrial habitats. While classification for the group has been challenging for traditional algae taxonomists using morphology-based approaches, molecular phylogenetic methods have provided new genotypic characteristics that have led to the discovery of new diversity. Unicellular coccoid species which reproduce exclusively by autospores have been documented in the Chlorophyceae and Trebouxiophyceae within the division Chlorophyta (Lewin et al., 2000; Krienitz et al., 2003; Aslam et al., 2007; Němcová et al., 2011; Leliaert et al., 2012). Within the Trebouxiophyceae, many new lineages of coccoid green algae have been described in the past decade, mainly based on molecular markers (e.g. nuclear-encoded 18S rDNA and ITS, and plastid-encoded 16S rDNA and *rbc*L), and it has become clear that coccoid morphotypes independently evolved several times, e.g. in the *Watanabea* clade (Zhang et al., 2008; Ma et al., 2013; Neustupa et al., 2009; Song et al., 2015), the Chlorellales (Luo et al., 2010; Bock et al., 2011; Krienitz et al., 2015), the Xylochloris clade (Neustupa et al., 2011), and the Leptochlorella clade (Neustupa et al., 2013a).

As defined by Karsten et al. (2005), the *Watanabea* clade is a monophyletic lineage of the Trebouxiophyceae, and the majority of its presently known members are coccoid unicells that have spherical or ellipsoidal cells, and parietal chloroplasts; pyrenoids or pyrenoglobuli may be present or absent, reproduction is exclusively by autospores, and they occur mostly in subaerial habitats. The genus *Phyllosiphon* Kühn is an exception to this general pattern: it was originally described as branched siphonal filaments parasitic on...
leaves (Kühn, 1878; Abola & Werner, 2011), and in addition to the type species *Phyllosiphon arisari*, four additional species of the genus *Phyllosiphon* have been described from the leaves of tropical aracaean plants, but they have not been reported since their original taxonomic descriptions. Procházková et al. (2015) demonstrated that members of the *Phyllosiphon* clade might also occur as free-living microalgae in subaerial microhabitats such as tree bark, and furthermore, some environmental sequences derived from sandstone substrates may also represent members of the genus *Phyllosiphon* (Hallmann et al., 2013). The genus *Desertella* Fučíková, Lewis and Lewis was also a member of the *Watanabea* clade, as was established by Fučíková et al. (2014b). There is only one species of this genus, *Desertella californica*, and it is found in desert habitats in the USA.

This study presents a survey of six coccoid green algae isolates from China. We use a polyphasic approach with small subunit rDNA phylogeny, *rbcL* phylogeny, light microscopy and electron microscopy to characterize the algae. Our results indicate that three of the isolates are genetically identical to previously investigated trebouxiophycean strains, and the other three isolates represent three previously unknown trebouxiophycean taxa within the *Watanabea* clade (Trebouxiophyceae, Chlorophyta). We describe these three novel algae as (1) a new genus and species, *Mysteriochloris nanningensis* gen. et sp. nov., (2) a novel species of the genus *Phyllosiphon* Kühn, *Phyllosiphon coccidium* sp. nov., and (3) a novel species of the genus *Desertella* Fučíková et al., *Desertella yichangensis* sp. nov.

**METHODS**

**Algal isolation and culture.** The strains described herein were isolated by the authors from field material and deposited in the Freshwater Algae Culture Collection at the Institute of Hydrobiology (FACHB-collection), Wuhan, Hubei Province, China. Localities of origin of the isolates are listed in Table 1. Strain FACHB-1787 was isolated from tree bark samples collected in Nanning, Guangxi Province, China (22° 51’ 23.79” N 108° 22’ 14.04” E, elevation 91 m a.s.l.) in March 2013. Strain FACHB-2212 was isolated from tree bark samples collected from Zhoukou, Henan Province, China (33° 48’ 40.02” N 114° 28’ 20.80” E, elevation 56 m a.s.l.) in February 2013. Strain FACHB-1793 was isolated from Gaolan River in Yichang, Hubei Province, China (31° 7’ 32.60” N 110° 49’ 6.32” E, elevation 163 m a.s.l.) in March 2015. Strains FACHB-1794 and FACHB-1795 were isolated from rock in Tibetan Autonomous Prefecture of Garze, Sichuan Province, China (29° 55’ 46.57” N 101° 97’ 81.82” E) in May 2015. Strain FACHB-1796 was isolated from a tree stump in Wuhan, Hubei Province, China (30° 55’ 21.58” N 114° 36’ 65.51” E) in March 2013. Unialgal cultures were established by serial streaking on 1.5 % BG11 agar and by single colony isolates. The strains were cultivated in BG11 medium maintained at 23 °C under 30 µmol m⁻² s⁻¹ of cool-white fluorescent light on a 12 h light:12 h dark cycle.

**Light and electron microscopy.** Microphotographs were taken with a BX53 light microscope (Olympus), an oil immersion lens and an Olympus BX53 camera using differential interference contrast. For transmission electron microscopy, 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 then 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 propyleneoxide. Ultrathin sections, cut on a Leica UC7, were poststained with uranyl acetate and bismuth oxyunate and examined with a Hitachi HT-7700 transmission electron microscope at 120 kV.

**DNA extraction, PCR amplification, and sequencing.** DNA was extracted using a Universal DNA Isolation Kit (AxyPrep). PCR amplification was performed using 3 µl template DNA, 0.1 µM each primer, and 25 µl 2× Tap Master Mix (ExTaq; Takara) in a 50 µl reaction volume. Nuclear-encoded small subunit ribosomal DNA (SSU rDNA) was amplified using the primers 18F (5'-TGTTGTAGCTCTGCTCAAGT-3') and 18R (5'-TGCCTGCTGTAGGTTACCC-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, and 90 s at 72 °C, and a final 10 min extension step at 72 °C. The *rbcL* gene sequence was amplified using the primers *rbcL* A (5'-ATGTTCAACAACACAGGACTAAGC-3') and *rbcL* B (5'-GATCTCCTTCACATCTCAGACC-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, and 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) 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).

**Phylogenetic analyses.** GenBank accession numbers for sequences obtained in the present study are listed in Table 1. SSU rDNA and *rbcL* sequences, selected based on a BLAST search or as representative of reference species in the relevant taxonomic class, were downloaded from the

<table>
<thead>
<tr>
<th>Strain</th>
<th>Taxa</th>
<th>Locality of origin</th>
<th>GenBank accession number for sequence of:</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACHB-2212</td>
<td><em>Phyllosiphon coccidium</em> sp. nov.</td>
<td>Bark sample, Henan Province, China</td>
<td>KT950842, KT950844</td>
</tr>
<tr>
<td>FACHB-1787</td>
<td><em>Mysteriochloris nanningensis</em> gen. et sp. nov.</td>
<td>Bark sample, Guangxi Province, China</td>
<td>KT950843, KT950845</td>
</tr>
<tr>
<td>FACHB-1793</td>
<td><em>Desertella yichangensis</em> sp. nov.</td>
<td>Gaolan River, Hubei Province, China</td>
<td>KX024690, KX024694</td>
</tr>
<tr>
<td>FACHB-1794</td>
<td><em>Watanabea</em> sp.</td>
<td>Rock, Sichuan Province, China</td>
<td>KU320168, KU077899</td>
</tr>
<tr>
<td>FACHB-1795</td>
<td><em>Chloroidium saccharophilum</em></td>
<td>Rock, Sichuan Province, China</td>
<td>KX024691, KX024692</td>
</tr>
<tr>
<td>FACHB-1796</td>
<td><em>Chloroidium saccharophilum</em></td>
<td>Tree stump, Hubei Province, China</td>
<td>KX024689, KX024693</td>
</tr>
</tbody>
</table>
GenBank database (http://www.ncbi.nlm.nih.gov/). The sequences were aligned using MAFFT software (v. 6, QINS-i strategy; Katoh et al., 2005), and alignments were further manually edited and adjusted by eye using MEGA6 software (Tamura et al., 2013). SSU rDNA and rbcL sequence positions that could not be aligned with confidence were removed prior to the analysis. Sequence alignments were exported as NEXUS files from MEGA6 and were analysed using Bayesian inference (BI), maximum-likelihood (ML) approaches with MrBayes 3.1.2 (Haugen et al., 1998) and PAUP*4.0 4.0b10 (Swofford, 2003), respectively. Bayesian analyses were performed using MrBayes version 3.1.2 (Haugen et al., 1998; Ronquist & Huelsenbeck, 2003) with the models suggested by MrModeltest 2.2 (Nylander, 2004), and Markov Chain Monte Carlo (MCMC) analyses were run with four Markov chains (three heated, one cold) for 5 × 10^6 generations, with trees sampled every 1000 generations. Every time the diagnostics were calculated, a fixed number of samples (burn-in=250) were discarded from the beginning of the chain. In PAUP ML analyses, appropriate substitution models and parameters were determined for each alignment by running likelihood ratio tests in PAUP*4.0 (Swofford, 2003) and using ModelTest (Posada & Crandall, 1998). The evolutionary models used in BI and ML for the SSU and rbcL phylogenies were TrNef + I + G and GTR + I + G, respectively; a heuristic search option with random addition of sequences (100 replicates) and the nearest-neighbour interchange branch-swapping algorithm (NNI) were used for tree searching.

RESULTS


Mysteriochloris (Mys.te.ri.o.chlo’ris. Gr. n. mysterion mystery; Gr. adj. chloros green; N.L. masc. n. Mysteriochloris mysterious green organism).

Vegetative cells solitary, uninucleate, with smooth cell walls. Mature cells elongated–ellipsoidal or ellipsoidal. Chloroplast parietal, with a pyrenoid surrounded by a starch envelope. Pyrenoid penetrated more or less by thylakoids; pyrenoglobuli surrounding the pyrenoid in the pyrenoid matrix. Asexual reproduction by autospores. Sexual reproduction not observed.

Type species. Mysteriochloris nanningensis Song, Hu, Zhu, Wang, Liu & Hu sp. nov.

Description of Mysteriochloris nanningensis Song, Hu, Zhu, Wang, Liu & Hu sp. nov. (Figs 1 and 2)

Mysteriochloris nanningensis (nan.ning.en’sis. N.L. masc. adj. nanningensis pertaining to Nanning, the locality of the type strain).

With the characters of the genus. Vegetative cells solitary, uninucleate. Young cells elongated–ellipsoidal, mature cells elongated–ellipsoidal or ellipsoidal. Dimensions of the vegetative cells are 2.4–7.2 × 4.6–11.1 μm. Cells with parietal, cup-shaped chloroplast with a pyrenoid surrounded by a starch envelope; pyrenoid transacted by one or several bands of thylakoids dividing the starch envelope into several parts; pyrenoglobuli around the thylakoids in the pyrenoid matrix. Asexual reproduction via 2 to 8 elliptical or elongated–elliptical autospores. Sexual reproduction not observed.

Holotype. Glutaraldehyde-fixed specimen number FACHB-f1787 deposited in the Freshwater Algae Specimen Station, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China.

Habitat. Subaerophytic on the bark of trees.

Species locality. Nanning Province, China.

Authentic culture. Culture strain FACHB-1787 deposited in the Freshwater Algae Specimen Station, Institute of Hydrobiology, Chinese Academy of Sciences (http://algae.ihb.ac.cn/).

Description of Phyllosiphon coccidium Song, Hu, Zhu, Wang, Liu & Hu sp. nov. (Figs 1 and 3)

Phyllosiphon coccidium (coc.ci’di.um. N.L. neut. adj. coccidium pertaining to the morphology of the type strain).

Vegetative cells solitary, uninucleate. Young cells ellipsoidal or oval, 2.5–3.6 × 4.0–5.2 μm; mature cells spherical or sub-spherical, 4.1–15.5 μm. Cell with parietal, cup-shaped chloroplasts without pyrenoids, but with some starch grains. Asexual reproduction via 2 to 32 ellipsoidal or irregularly egg-shaped autospores. Sexual reproduction not observed.

Holotype. Glutaraldehyde-fixed specimen number FACHB-f2212 deposited in the Freshwater Algae Specimen Station, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China.

Habitat. Subaerophytic on the bark of trees.

Species locality. Henan Province, China.

Authentic culture. Culture strain FACHB-2212 deposited in the Freshwater Algae Specimen Station, Institute of Hydrobiology, Chinese Academy of Sciences (http://algae.ihb.ac.cn/).

Description of Desertella yichangensis Song, Hu, Zhu, Wang, Liu & Hu sp. nov. (Figs 4 and 5)

Desertella yichangensis (yi.chang.en’sis. N.L. fem. adj. yichangensis pertaining to Yichang, the locality of the type strain).

Vegetative cells solitary, uninucleate, mucilage present or absent. Young cells elongated–ellipsoidal, reniform, or elongated–oval, ends rounded or slightly pointed, 1.9–3.3 × 3.3–7.7 μm; mature cells ellipsoidal, oval, or sub-spherical, 4.0–7.1 × 6.4–11.6 μm. Chloroplast often one, rarely two or four, parietal, plate-, band-, or cup-shaped. Pyrenoid often one, rarely two or a few, surrounded by starch envelope. Asexual reproduction via 2 to 32 ellipsoidal, sub-spherical, or irregularly egg-shaped autospores. Sexual reproduction not observed.
Holotype. Glutaraldehyde-fixed specimen number FACHB-f1793 deposited in the Freshwater Algae Specimen Station, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, China.

Habitat. Planktonic in the Gaolan River.

Species locality. Hubei Province, China.

Authentic culture. Culture strain FACHB-1793 deposited in the Freshwater Algae Specimen Station, Institute of Hydrobiology, Chinese Academy of Sciences (http://algae.ihb.ac.cn/).

Morphology and ultrastructure. The three strains described herein as the new taxa *Mysteriochloris*
Three novel species of coccoid green algae

Mysteriochloris nanningensis gen. et sp. nov., Phyllosiphon coccidium sp. nov. and Desertella yichangensis sp. nov., belong to the taxonomically challenging coccoid green microalgae. They are characterized as unicellular, with spherical, sub-spherical, elongated-ellipsoidal, or elliptical cells, and a single chloroplast, and they reproduce entirely by autospores.

Young cells of M. nanningensis FACHB-1787 are narrowly ellipsoidal, and the chloroplast is parietal, cup-shaped, and with a smooth edge (Fig. 1a). When mature, most cells are elongated-ellipsoidal or ellipsoidal, with the chloroplast edge smooth (Fig. 1b, d, e) or with a wavy margin (Fig. 1c), and with the chloroplasts parietal and cup-shaped, the pyrenoid barely visible under light microscope (Fig. 1b–e) but developed well in most cells, and surrounded by a starch envelope (Fig. 2). The pyrenoid is entered by several thylakoid bands that divide the starch envelope into several sections, and pyrenoglobuli surround the thylakoid in the pyrenoid matrix (Fig. 2a–d). The alga reproduces by means of 2, 4, or 8 asexual autospores (Figs 1e–g and 2e, f). The autosporangium is ellipsoidal (Fig. 1e–g), and the autospores are ellipsoidal or oval (Fig. 1g, h). Usually, a single relatively large autospore and several smaller autospores are produced within a single autosporangium (Fig. 1g, h). The autospores are discharged through an aperture (Fig. 1h). Sometimes the vegetative cell is within the mother cell’s wall remnants (Fig. 2a, d).

Young cells of Phyllosiphon coccidium FACHB-2212 are ellipsoidal, oval, rarely reniform or sub-spherical, 2.5–3.6×4.0–5.2 µm in size (Fig. 1i). When mature, most cells are spherical, a few are sub-spherical or ellipsoidal and 4.1–15.5 µm in diameter (Fig. 1j–l). Chloroplasts are parietal and cup-shaped, without a pyrenoid (Figs 1j–l and 3a, b), but with some starch grains (Fig. 3a–c). The alga reproduces by 2, 4, 8, 16, or 32 asexual autospores (Fig. 1m–p). The autosporangium is spherical or sub-spherical (Fig. 1m). Autospores are ellipsoidal or irregularly egg-shaped (Figs 1n–p and 3d). Usually, a single relatively large autospore and several smaller autospores are produced within a single sporangium (Fig. 1m, o). The autospores are discharged through an aperture (Fig. 1p).

Young cells of D. yichangensis are elongated-ellipsoidal, reniform, or elongated-oval (Fig. 4a), with ends rounded or

Fig. 2. Ultrastructure of Mysteriochloris nanningensis FACHB-1787. (a) Longitudinal section of vegetative cells, elongated ellipsoidal, within the mother cell wall remnants. (b) Longitudinal section of vegetative cells, elongated ellipsoidal. (c, d) Transverse section of vegetative cell. (e) Autosporangium contains four autospores. (f) Autosporangium contains two autospores. Bars, 2 µm. ch, chloroplast; MCW, mother cell wall; P, pyrenoid; Pi, pyrenoglobuli; s, starch envelope.

Fig. 3. Ultrastructure of Phyllosiphon coccidium FACHB-2212. (a) Vegetative cells, spherical. (b) Vegetative cells, ellipsoidal. (c) The cells before autospores formation. (d) Autosporangium. Bars, 2 µm. ch, chloroplast; n, nucleus; P, pyrenoid; s, starch grains.
Fig. 4. Morphology of *Desertella yichangensis* sp. nov. strain FACHB-1793: (a) young cells, (b–d) vegetative cells, (e–h) mature cells before formation of autosporangia, (i–j) autosporangia, (k–m) liberation of autospores (n–q) negative staining with India ink. Bars, 5 µm.
slightly pointed, 1.9–3.3×3.3–7.7 µm; mature cells are ellipsoidal, oval, or sub-spherical (Fig. 4b–e), 4.0–7.1×6.4–11.6 µm. The chloroplast in young cells is parietal, plate- or band-shaped (Fig. 4a); when mature, each cell usually has a single plate- or cup-shaped parietal chloroplast (Figs 4b–d and 5a), sometimes with an undulated edge surface (Fig. 4b, c), a few cells were seen with 2 or 4 chloroplasts (Fig. 4g, h), probably due to division of the chloroplast before cell division. Most often, only one pyrenoid is present, with two or a few observed only rarely (Fig. 4c, e, f), and surrounded by a starch envelope (Fig. 5); the pyrenoid matrix is penetrated by the thylakoid membranes (Fig. 5). Most of the cells are surrounded by thick or thin amorphous mucilage, visible by negative staining with India ink (Fig. 4o–q); a few cells have no mucilaginous envelope (Fig. 4n). Asexual reproduction is accomplished by 2, 4, 8, 16, or 32 ellipsoidal, sub-spherical, or irregularly egg-shaped autospores (Fig. 4i–m). Sexual reproduction was not observed.

The other three strains are morphologically consistent with the species in which the phylogeny places them. Morphological characteristics of Watanabea sp. FACHB-1794 are accord with genus Watanabea, which have two forms of cells, one (E-form) narrowly to broadly ellipsoidal, the other (S-form) ovoid to spheroidal, the chloroplast is parietal and without pyrenoid. Reproduction by autospores. Morphological characteristics of Chloroidium saccharophilum FACHB-1795 and FACHB-1796 are accord with species Chloroidium saccharophilum; the two strains have narrowly ellipsoidal, ellipsoidal to almost spherical shape, the chloroplast is parietal and with a pyrenoid, reproduction by autospores.

**Molecular phylogeny.** 18S rDNA gene sequences were obtained from the strains FACHB-2212, FACHB-1787 and FACHB-1793, and their sequenced lengths were 1306 bp, 1300 bp and 1292 bp, respectively. The BLAST search of 18S rDNA suggested that the closest relative of strain FACHB-2212 was Phyllosiphon arisari (GenBank accession no. FJ829884) with 98% identity, differing by six nucleotide indels and 32 substitutions out of 1695 bp. The BLAST search of rbcL suggested that the closest relative of strain FACHB-2212 was Phyllosiphon sp. K17 (KR154336) with 97% identity, differing only by two single-nucleotide indels and 34 substitutions out of 1039 bp. The BLAST search of 18S rDNA suggested that the closest relative of strain FACHB-1787 was Phyllosiphon arisari (FJ829884) with 94% identity, differing only by 20 nucleotide indels and 82 substitutions out of 1715 bp. The BLAST search of rbcL suggested that the closest relative of strain FACHB-1787 was Phyllosiphon arisari isolate AV1 (KR154334) with 88% identity, differing by 150 substitutions out of 1202 bp. The BLAST search of 18S rDNA suggested that the closest relative of strain FACHB-1793 was D. californica (KF693789) with 98% identity, differing only by two single-nucleotide indels and 34 substitutions out of 1100 bp. The BLAST search of rbcL suggested that the closest relative of strain FACHB-1793 was D. californica (KF693819) with 91% identity, differing only by two single-nucleotide indels and 96 substitutions out of 1100 bp.

The phylogenetic position of the strains was inferred by analysing the DNA sequences of 18S rDNA and rbcL. The 18S rDNA alignment comprised 1682 characters, and parsim-informative sites are 468 characters; the rbcL alignment consisted of 1038 characters, and parsim-informative sites are 411 characters. The ML and Bayesian topologies were basically consistent, and the best ML trees for 18S rDNA and rbcL are shown in Figs 6 and 7, respectively, with Bayesian posterior probability (BPP) and bootstrap support (BS) values indicating branch support. M. nanningensis (strain FACHB-1787), Phyllosiphon coccidium (strain FACHB-2212), and D. californica (strain FACHB-1793) are nested within the Watanabea clade.
(Trebouxiophyceae, Chlorophyta). According to the phylogenetic tree based on 18S rDNA sequences (Fig. 6), *M. nanningensis* FACHB-1787 is likely sister to an uncultured strain (GenBank accession no. AM260450), but this clade
was supported by low values in all analyses (0.76/49). *Phyllosiphon coccidium* FACHB-2212 is found in a strongly supported cluster (1.00/96) together with one of the strongly supported *Phyllosiphon* lineages (1.00/98) including
**Table 2.** Morphological comparison between the novel species and similar species

<table>
<thead>
<tr>
<th>Species</th>
<th>Shape of cell</th>
<th>Shape of Chloroplast</th>
<th>Pyrenoid</th>
<th>Size of cell (µm)</th>
<th>Number of autospores</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mysteriochloris nanningensis</em></td>
<td>Elliptoidal to elliptoidal</td>
<td>One, surrounded by starch envelope</td>
<td>2.4–7.2×4.6–11.1</td>
<td>2–8</td>
<td>This research</td>
<td></td>
</tr>
<tr>
<td>FACHB-1787</td>
<td>Elliptoidal, oval or sub-spherical</td>
<td>One, rarely two or three, surrounded by starch envelope</td>
<td>1.9–3.3×6.4–11.6</td>
<td>2–32</td>
<td>This research</td>
<td></td>
</tr>
<tr>
<td><em>Desertella yichangensis</em></td>
<td>Elliptoidal</td>
<td>Band or cup</td>
<td>Surrounding by starch envelope</td>
<td>3.2–5×12</td>
<td>2–4</td>
<td>Fučíková et al. (2014b)</td>
</tr>
<tr>
<td>FACHB-1793</td>
<td>Elliptoidal, oval or sub-spherical</td>
<td>Ellipsoidal, plate, cup</td>
<td>Surrounding by starch envelope</td>
<td>2.7–4.4×6.0–10.4</td>
<td>2–4</td>
<td>Darienko et al. (2010)</td>
</tr>
<tr>
<td><em>Chloroidium ellipsosideum</em></td>
<td>Elliptoidal, sometimes almost spherical</td>
<td>Band</td>
<td>Surrounding by many starch grains</td>
<td>3.0–4.0×10.1–20.8</td>
<td>2–4 (16)</td>
<td>Darienko et al. (2010)</td>
</tr>
<tr>
<td>SAG 3.95</td>
<td>Elliptoidal</td>
<td>Band, smooth margin</td>
<td>Absent</td>
<td>2.7–4.2×7.6–10.6</td>
<td>2–4</td>
<td>Darienko et al. (2010)</td>
</tr>
<tr>
<td><em>Chloroidium engadinensis</em></td>
<td>Elliptoidal</td>
<td>Deeply, lobed</td>
<td>Surrounding by many starch grains</td>
<td>3.6–3.9×13.6–16.2</td>
<td>2–4–8–16</td>
<td>Darienko et al. (2010)</td>
</tr>
<tr>
<td>SAG 812-1</td>
<td>Elliptoidal, sometimes ovoid or spherical</td>
<td>Parietal, band-shaped to slightly lobed</td>
<td>Indistinct, naked</td>
<td>6.9–5.3×13.6–9.4</td>
<td>2–4–8–16</td>
<td>Darienko et al. (2010)</td>
</tr>
<tr>
<td><em>Polulichloris henanensis</em></td>
<td>Elliptoidal</td>
<td>Parietal, cup</td>
<td>Surrounding by starch envelope</td>
<td>2.5–3.6×4.0–5.2</td>
<td>2–4–8–16</td>
<td>Song et al. (2015)</td>
</tr>
<tr>
<td>FACHB-1765</td>
<td>Elliptoidal</td>
<td>Parietal, band-shaped to slightly lobed</td>
<td>Indistinct, naked</td>
<td>6.9–5.3×13.6–9.4</td>
<td>2–4–8–16</td>
<td>Darienko et al. (2010)</td>
</tr>
<tr>
<td><em>Phyllosiphon coccidium</em></td>
<td>Elliptoidal</td>
<td>Parietal, cup</td>
<td>Absent</td>
<td>2.5–3.6×4.0–5.2</td>
<td>2–4–8–16</td>
<td>This research</td>
</tr>
<tr>
<td>FACHB-2212</td>
<td>Elliptoidal</td>
<td>Parietal, cup</td>
<td>Absent</td>
<td>2.5–3.6×4.0–5.2</td>
<td>2–4–8–16</td>
<td>This research</td>
</tr>
<tr>
<td><em>Kalinaella aipyrenoidosa</em></td>
<td>Elliptoidal</td>
<td>Parietal, cup</td>
<td>Absent</td>
<td>2.5–3.6×4.0–5.2</td>
<td>2–4–8–16</td>
<td>This research</td>
</tr>
<tr>
<td>CaUP C-H7902</td>
<td>Elliptoidal</td>
<td>Parietal, cup</td>
<td>Absent</td>
<td>2.5–3.6×4.0–5.2</td>
<td>2–4–8–16</td>
<td>This research</td>
</tr>
<tr>
<td><em>Viridiella fridericiana</em></td>
<td>Elliptoidal</td>
<td>Paraphyletic, cup</td>
<td>Absent</td>
<td>2.5–3.6×4.0–5.2</td>
<td>2–4–8–16</td>
<td>This research</td>
</tr>
</tbody>
</table>

Phyllosiphon sp. K55 (KR154347), Phyllosiphonarisari (FJ829884), uncultured Trebouxiophyceae (JX127175), and Phyllosiphonarisari AV1 (KR154346). The Phyllosiphon lineages are sister to the clade containing *M. nanningensis* and the uncultured strain (AM260450) with moderately supported values in all analyses (1.00/75). *D. yichangensis* FACHB-1793 clustered in a sister position with *D. californica* (KF693819) with high support (1.00/99).

The topology of the phylogenetic tree derived from the analysis of the rbcL sequences (Fig. 7) was basically consistent with phylogenetic tree based on 18S rDNA (Fig. 6). *M. nanningensis* FACHB-1787 clustered in a sister position with *Polulichloris henanensis* with low support values (0.69) in MrBayes analyses and occupied a solitary branch in PAUP analyses. However, *M. nanningensis* FACHB-1787 was relatively close to *Polulichloris henanensis* and the Phyllosiphon lineages. *Phyllosiphon coccidium* FACHB-2212 was nested within the Phyllosiphon lineages, clustering together with a clade including *Phyllosiphon* sp. K17 (KR154336) and environmental sequence Trebouxiophyceae sp. cort08 (HG793065) (Kulichová et al., 2014) with high support (0.98/54). *D. yichangensis* FACHB-1793 clustered in a sister position with *D. californica* (KF693819) with high support (1.00/99).

**DISCUSSION**

Current research on the Watanabea clade suggests that it contains mainly coccoid green algae from subaerial habitats. The known members include the subaerial or aquatic genera *Chloroidium* Nadson (1906: 189) and *Heterochlorella* Neustupa et al. (2009: 167), the epiphytic *Kalinaella* Neustupa et al. (2009: 167), the epiphytic and endophytic *Heterochlorella* Zhang et al. (2008: 186), the extremely
Three novel species of coccoid green algae

acidophilic Viridiella Albertano et al., (1991: 347), the parasitic and free-living Phyllospihon Kühn (1878: 25), the edaphic or aquatic Watanabea Hanagata et al. (1998: 226) and the edaphic Polulichloris Song et al. (2015: 139), the desert Desertella Fučíková et al. (2014a: 303), and the corticous Parachloroidium Neustupa et al. (2013b: 413). In addition to the corticous FACHB-1787 and FACHB-2212 strains, we also isolated the epilithic Watanabea sp. FACHB-1794 and C. saccharophilum FACHB-1795, the epiphytic C. saccharophilum FACHB-1796, and the aquatic D. yichangensis FACHB-1793. This is the first report of members of genera Watanabea and Desertella from China, and the first description of a member of the genus Desertella from aquatic habitats. The uncultured strain (GenBank accession no. AM260450) represents an undescribed taxon that originated from photobiont cells of the lichen Psorograena epiphylla Lücking (Nyiay et al., 2007). This may indicate the cryptic diversity of habitats within the Watanabea clade, the members of which are probably relatively widely distributed in various habitats. As additional members of this clade are discovered, the full extent of the clade's habitat diversity will become clearer.

The three novel coccoid green algae were not only distinguished from other known taxa of the Watanabea clade by differences in their phylogenetic positions, but also morphological characteristics, and the morphological comparison with other known taxa of the Watanabea clade is shown in Table 2.

M. nanningensis FACHB-1787 appears likely to be sister to the uncultured strain (GenBank accession no. AM260450) according to 18S rDNA sequences, and likely to be sister to the Polulichloris lineages according to rbcL sequences; in fact, the large genetic differences involved indicate that strain FACHB-1787 is not really close to any known sequenced algae, and potentially closer relatives may not have been sequenced or even described yet. Furthermore, the morphological characteristics of M. nanningensis FACHB-1787 are clearly different from those of the related genera Phyllospihon and Polulichloris. M. nanningensis FACHB-1787 has an elongated-ellipsoidal or ellipsoidal cell outline, the chloroplasts are parietal and cup-shaped and have a pyrenoid surrounded by a starch envelope, while Phyllospihon arisari exists as siphonomous branched filaments or a spherical unicell, and its chloroplasts are without a pyrenoid. In the pyrenoid matrix of M. nanningensis FACHB-1787, the thylakoids are surrounded by some pyrenoglobuli, while pyrenoglobuli is absent in pyrenoid of Polulichloris henanensis.

According to both the 18S rDNA-based and the rbcL-based phylogenetic trees, Phyllospihon coccidium FACHB-2212 appears to belong to the Phyllospihon lineages, and it is independent of Phyllospihon arisari. The morphological features of strain FACHB-2212 are similar to those of free-living Phyllospihon populations (Procházková et al., 2015), both exhibiting spherical coccoid cells propagated by autospores, with a single relatively large autospore and several smaller autospores produced within a single autosporangia. In addition, both cells contain a single parietal chloroplast without a pyrenoid. However, there are some obvious differences: strain FACHB-2212 differs from free-living Phyllospihon arisari primarily in its chloroplast, which is cup-shaped and has no lobes, while the known free-living Phyllospihon arisari contain a single parietal lobed chloroplast. On the other hand, starch grains were observed within the chloroplasts of the FACHB-2212 strain, but not in those of free-living Phyllospihon populations.

D. yichangensis FACHB-1793 clustered together with D. californica and formed a highly supported monophyletic lineage in both the 18S rDNA-based and the rbcL-based phylogenetic trees. The morphological characteristics of strain FACHB-1793 resemble those of D. californica, which has oval or ellipsoidal cells, a plate- or cup-shaped chloroplast, and asexual reproduction via autospores. But their differences are also significant: firstly, cells of the FACHB-1793 strain often have only one chloroplast, even in older cells, and only rarely two or 4 chloroplasts, probably due to division of the chloroplast before cell division, whereas D. californica has 2–3 chloroplasts in older cells. Secondly, the pyrenoid of D. californica is always small and starch-covered, whereas the pyrenoid of D. yichangensis FACHB-1793 is relatively large and clearly visible under light microscope, rarely two or a few, and is penetrated by the thylakoid membranes. Thirdly, the autosporangia of D. yichangensis form 2, 4, 8, 16 or 32 autospores, while those of D. californica form 2–4 autospores. Lastly, the known habitat of D. yichangensis is aquatic (riverine), while the known habitat of D. californica is terrestrial in the desert, thus indicating the degree of habitat diversity within the genus Desertella.

In addition, we observed that D. yichangensis has a mucilaginous envelope surrounding the cells, but the function of this mucilage is ambiguous; we found Amoeba sp. accompanying the cultivation of algae in BG11 medium, so this mucilage may play a role in grazing protection (Reynolds, 2007).

It is worth mentioning that known members of the Watanabea clade are characterized by unusually divergent 18S rDNA and rbcL gene sequences, with the genetic divergence among the genera being relatively large. Furthermore, several monophyletic genera with high degrees of 18S rDNA and rbcL gene divergence have been taxonomically defined within the Watanabea clade, such as Chlororidiun, Kalinella and Heveochlorella. How can the divergent 18S rDNA and rbcL gene sequences within the Watanabea clade be explained? We believe that many members of the clade have not yet been described or sequenced, and limited sampling and the small amount of available genetic data have led to the appearance of unusual genetic divergence. Furthermore, the taxonomic diversity of the Watanabea clade could be considerably higher than we presently know, given that the actual species richness within the clade is difficult to determine with limited sampling and limited genetic data. There are other problems in the taxonomy of the Watanabea clade, including its uncertain higher-level classification,
with many genera having no family-level affiliation and no clear relationships. An approach combining dense and even taxon sampling (representing all major lineages) with sequence data from multiple loci will be necessary to solve these problems (Fučíková et al., 2014b; Štifterová & Neustupa, 2015).

**ACKNOWLEDGEMENTS**

We thank the Special Foundment of Science and Technology Basic Work of China (grant no. 2012FY112900), the National Natural Science Foundation of China (grant no. 31670202), and State Development and Investment Corporation (SDIC) of China for funding this research. We thank the reviewers for their helpful comments.

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