INTRODUCTION

The so-called Watanabea clade was defined by Karsten et al. (2005) as a monophyletic lineage of the Trebouxiophyceae that includes the type strain SAG 211-9b of Watanabea reniformis N. Hanagata, Karube, Chihara, & P.C. Silva, and a group of Chlorella-like strains that were formerly included by Darienko et al. (2010) in the genus Chloroidium Nadson. Additional studies on molecular phylogeny of trebouxiophytes showed that the Watanabea clade includes other microalgal genera, such as Viridella P. Albertano, Pollio, & Taddei, Heterochlorella Neustupa, Němcová, Eliáš, & Škaloud, Heveochlorella J. Zhang, V.A.R. Huss, X. Sun, K. Chang, & D. Pang, Kalinella Neustupa, Němcová, Eliáš, & Škaloud and Phyllophorton Kühn (Huss et al. 2002; Zhang et al. 2008; Neustupa et al. 2009, 2013; Ma et al. 2013). Most of these taxa are Chlorella-like coccoid autosporine microalgae that thrive in various subaerial and freshwater habitats, but the genus Phyllophorton, which is characterized by a siphonous habit, is known as a parasite of vascular plants (Aboal & Werner 2011). However, sequences corresponding to this lineage were recently also reported from epilithic biofilms (Hallmann et al. 2013). Because of their relatively uniform morphology, most of the coccoid taxa were only recognized by molecular methods. Recent molecular data from studies that used environmental sequencing and the sequencing of various strains deposited in culture collections revealed that several taxa of this clade, such as Heterochlorella luteoviridis and species within the genus Chloroidium, are probably relatively widely distributed, and frequently occur in subaerial and freshwater phytobenthic microbial biofilms (Darienko et al. 2010; Hallmann et al. 2011; Lee & Hur 2012). Conversely, four species of the genera Kalinella and Heveochlorella J. Zhang, V.A.R. Huss, X. Sun, K. Chang, & D. Pang have so far only been reported from the type localities in corticolous subaerial microhabitats of subtropical and tropical ecosystems (Zhang et al. 2008; Neustupa et al. 2009, 2013; Ma et al. 2013). In addition, several taxonomically undetermined 18S ribosomal (r)DNA sequences that probably represent undescribed taxa of the Watanabea clade were deposited in GenBank. These include the sequence AM260450 that originated from photobiont cells of the lichen Psoroglaena epiphylla Lücking (Nyati et al. 2007) and the sequences AB058305 and AB006045 from unidentified Chlorella-like strains. These records indicate that the taxonomic diversity of the Watanabea clade could be considerably higher than previously believed, and that this phylogenetic lineage may actually include several Chlorella-like microalgae that frequently occur in subaerial biofilms. In this study, we present a taxonomic description of Parachloroidium, a new genus of the Watanabea clade, according to the International Code of Nomenclature for Algae, Fungi and Plants. A combination of morphological observations and molecular phylogenetic analyses was used to characterize this previously unknown lineage of trebouxiophyccean chlorellloid microalgae, which was discovered in phototrophic corticolous biofilms in Mediterranean Europe.

Besides various phenotypic and phylogenetic data the compensatory base changes (CBCs) in the secondary structure

Parachloroidium gen. nov. (Trebouxiophyceae, Chlorophyta), a novel genus of coccoid green algae from subaerial corticolous biofilms

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The Watanabea clade of the Trebouxiophyceae included mostly unicellular coccoid microalgae that thrived in various terrestrial microhabitats. The diversity of these morphologically uniform microalgae was little known, and several new genus-level lineages had recently been described on the basis of molecular data. In this study, we provided a taxonomic description of a new trebouxiophyccean genus, Parachloroidium, found in the Mediterranean in corticolous phototrophic biofilms. Their simple chlorellloid morphology did not unambiguously distinguish the Parachloroidium strains from other similar green algae. However, ultrastructural characteristics and molecular phylogenetic analyses based on the 18S ribosomal (r)DNA, internal transcribed spacer region (ITS) and the chloroplast ribulose-bisphosphate carboxylase gene sequences provided a basis for the discrimination of Parachloroidium from related genera of the Watanabea clade. The four strains investigated formed two species, P. laureanum and P. lobatum, which differed in plastid morphology and in ITS and 18S rDNA sequences. All four strains were characterized by globular or ellipsoidal cell shapes, single parietal plastids and asexual reproduction by autospores. Their plastids lacked typical pyrenoids; however, plastids included peculiar thylakoid-free regions of irregular shape. On the basis of accumulating molecular data, we concluded that the Watanabea clade was a diverse phylogenetic lineage within the subaerial chlorellloid green algae.

KEY WORDS: Green algae, Subaerial algae, Taxonomy, Trebouxiophyceae, Watanabea clade

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model of the second part of the internal transcribed spacer (ITS2) were also used for species delimitation (Müller et al. 2007; Bock et al. 2011). Previous studies illustrated that occurrence of CBCs in the conserved regions of helices II and III of the ITS2 models of two closely related taxa are highly correlated with their sexual incompatibility (Müller et al. 2007; Coleman 2009). Consequently, CBCs in the above-mentioned parts of the ITS2 secondary structure model may be used as an auxiliary feature for species discrimination even in tentatively asexual organisms, such as many subaerial coccolid green algae.

MATERIAL AND METHODS

The algal strains were isolated from samples of corticolous microbial biofilms taken from three trees growing in different European locations. Strain CAUP H8501 was obtained from the bark of Laurus nobilis Linnaeus growing near Ankaran, Slovenia [45°35’11.93”N, 13°42’35.15”E, altitude 5 m above sea level (asl)]. Two strains, CAUP H8502 and H6e, were isolated from the bark of Fraxinus ornus Linnaeus growing near Pacug, Slovenia [45°31’33.55”N, 13°35’40.95” E, altitude 55 m asl]. Finally, strain CAUP H8503 was isolated from the corticolous film of Ficus carica var. caprifolius (Risso) Tschirch & Ravasini in Polícoro, Italy [40°10’35.75”N, 16°41’49.71”E, altitude 10 m asl]. Each of the four strains was isolated from a sample of approximately 1 cm² of bark surface that was taken from the shaded north face of the tree trunk, at 120–150 cm above the soil surface. The strains are available from the Culture Collection of Algae of Charles University in Prague (CAUP; http://botany.natur.cuni.cz/algo/caup. html). For this study, the strains were cultivated on agar-solidified Bold’s basal medium (Andersen et al. 2007; Bock et al. 2011). Previous studies illustrated that CBCs in the above-mentioned parts of the ITS2 secondary structure model may be used as an auxiliary feature for species discrimination even in tentatively asexual organisms, such as many subaerial coccolid green algae.

Total genomic DNA was isolated using the InstaGene matrix (Bio-Rad, Hercules, California, USA) as described by Škaloud et al. (2012). Sequences of the 18S rDNA, the ITS region, and the chloroplast ribulose-bisphosphate carboxylase gene (rbcL) were obtained by polymerase chain reaction (PCR) amplification using an XP thermal cycler (Boer, Tokyo, Japan). Each 20-µl PCR reaction contained 13.1 µl of sterile Milli-Q water (Millipore Corp., Billerica, Massachusetts, USA), 2 µl of AmpliTaq Gold® 360 buffer 10× (Life Technologies, Carlsbad, California, USA), 2.2 µl of MgCl₂ (25 mM), 0.4 µl of deoxyribonucleotide triphosphate mix (10 mM), 0.25 µl of each primer (25 nM), 0.6 µl of 360 GC Enhancer (Life Technologies), 0.2 µl of AmpliTaq Gold® 360 DNA polymerase, and 1 µl of DNA (10 ng µl⁻¹). The subunit- (SSU) rDNA was amplified using the primers 18S-F (5’-AAC CTG GTT GAT CCT GCC AGT-3’) and 18S-R (5’-TGA TCC TTC TGC AGG TCC ACC TAC G-3’; Katana et al. 2001), and the ITS rDNA region was amplified using the primers ITS1 (5’-TCC GTA GGT GAA CTT GCG G-3’) and ITS4 (5’-TCC TTC TCC TAT TGA TAT GC-3’; White et al. 1990). To amplify the rbcL gene, we used the primers PRASF1 (5’-ATG CCT GCA CAA ACA GAA AC-3’; Sherwood et al. 2000), rblL-203F (5’-GAA TCW TCW ACW GGW ACT TGG ACW AC-3’; Nelsen et al. 2011), and ellaR2 (5’-TCA CGA CCT TCA TTG CGA GGT GC-3’; Neustupa et al. 2013). Amplification of the SSU rDNA/ITS/rbcL markers started with an initial denaturation at 94°C for 4/4/5 min, followed by 35/35/40 cycles of denaturing at 94/94/95°C for 1/1/0.75 min, annealing at 52/50/50°C for 1/1/1.5 min, and elongation at 72°C for 2.5/1.5/2 min, with a final extension at 72°C for 10 min, respectively. The PCR products were stained with bromophenol blue loading dye, quantified on a 1% agarose gel, stained with ethidium bromide, and cleaned with the JETQUICK PCR Purification Kit (Genomed, Löhne, Germany). The purified amplification products were sequenced using an Applied Biosystems (Foster City, California, USA) automated sequencer (ABI 3730xl) by Macrogen, Seoul, Korea. The sequences are available in the EMBL Nucleotide Sequence Database under accession numbers HF586459-HF586467 and HF674884-HF674885. Summing up, two new rbcL sequences for the type strains of Chloroidium ellipsoideum (Gerneck) T. Darienko et al. (CAUP H1904) and C. saccharophilum (Krüger) T. Darienko et al. (H1912) and, in addition, three 18S rDNA, rblL and ITS sequences of the strains H8501, H8502 and H8503 were published.

The newly determined SSU rDNA and rbcL sequences were aligned with other sequences, obtained from the DDBJ/EMBL/GenBank database, using MAFFT software (v. 6, Q-INS-i strategy; Katoh et al. 2005). Four newly determined ITS rDNA sequences were highly divergent from all other sequences stored in the database. Their alignment was produced manually by using MEGA5 (Tamura et al. 2011). To evaluate the effect of different outgroup taxon and alignment strategies on the phylogenetic reconstructions, four different SSU rDNA alignments were produced for the phylogenetic analyses. Initially, 82 sequences were selected to encompass all known lineages in Trebouxiophyceae (Friedl & Rybalka 2011; Leliaert et al. 2012), and were manually aligned with two different outgroup taxa. The alignment followed the secondary structure model of Chlamydomonas reinhardtii Dangeard vSSU rDNA (Wuyts et al. 2000). First, the sequences were aligned with the chlorophycean taxa C. bilatus Ettl, Chloromonas rosae Ettl, and Pleurostrum insigne Chodat. As an alternative, the outgroup was composed of prasinophytes Nephrolepis olivacea Stein, N. pyriformis (N. Carter) Ettl, and Pseudooursfeldia marina (Thronsden) Manton. Next, we eliminated ambiguously aligned regions in both SSU rDNA alignments by using Gblocks software (v.
RESULTS

Parachloroidium Neustupa & Škaloud gen. nov.

DESCRIPTION: Vegetative cells solitary and uninucleate, with globular outlines and thin, smooth cell walls. Chloroplast parietal, without a pyrenoid surrounded by starch grains, but with a central cluster composed of numerous pyrenoglobuli. Asexual reproduction only by autospores. Sexual reproduction not observed. Secondary carotenoids not produced. The type species of this genus differs from other members of Trebouxiophyceae in the 18S rDNA, ITS, and rbcL sequences.

ETYMOLOGY: Generic name chosen to emphasize the apparent sister phylogenetic position to the genus Chloroidium [the Greek prefix πυρην- (pyren-) means ‘next to’ or ‘near’].

TYPE SPECIES: Parachloroidium laureanum Neustupa & Škaloud sp. nov.

Parachloroidium laureanum Neustupa & Škaloud sp. nov.

Figs 1–8, 17–20

DESCRIPTION: Vegetative cells solitary, uninucleate. Mature cells spherical, (2.5–)3.0–7.5(–9.8) μm in diameter. Single parietal, cup-shaped chloroplast, sometimes divided into several lobes. Pyrenoid absent, but chloroplasts typically include a central cluster of pyrenoglobuli and starch grains. Asexual reproduction via two to eight elliptical or egg-shaped autospores, 2.5–3.5 × 3.5–5.5 μm in diameter. Sexual reproduction not observed.

HOLOTYPE: Strain CAUP C-H8501 permanently cryopreserved in CAUP Culture Collection (http://botany.natur.cuni.cz/algo/caup.html). Also available from CAUP as a perpetually transferred culture, strain H8501, from which the holotype was derived.

HABITAT: Subaerophytic on the bark of trees.

SPECIES LOCALITY: Ankaran, Slovenia (45°35′11.93″N, 13°42′35.15″E), on the bark of Laurus nobilis.

ETYMOLOGY: Specific epithet reflects the host species (Laurus nobilis) of the holotype.

Parachloroidium lohatum Neustupa & Škaloud sp. nov.

Figs 9–16, 21–22

DESCRIPTION: Vegetative cells solitary, uninucleate. Mature cells spherical, (3.5–)4.0–10.5(–13.5) μm in diameter. Single parietal, cup-shaped chloroplast, often with two lobes. True pyrenoid absent, but chloroplasts typically include a thylakoid-free space with starch grains. Sexual reproduction via two to eight elliptical or spherical autospores, 3.0–6.5 μm in diameter. Sexual reproduction not observed. This species differs from the type species of the genus Parachloroidium in the 18S rDNA, ITS, and rbcL sequences.

HOLOTYPE: Strain CAUP C-H8502 permanently cryopreserved in CAUP Culture Collection (http://botany.natur.cuni.cz/algo/caup.html). Also available from CAUP as a perpetually transferred culture, strain H8502, from which the holotype was derived.

HABITAT: Subaerophytic on the bark of trees.

TYPE LOCALITY: Pacug, Slovenia (45°31′33.55″N, 13°35′40.95″E), on the bark of Fraxinus ornus.

ETYMOLOGY: Specific epithet reflects the characteristic lobate shape of the chloroplasts.

All four strains investigated in this study shared general morphological characteristics common to the Chlorella-like green microalgae. They were unicellular, with spherical or elliptical cells, and with single parietal plastids (Figs 1–4, 9–13). They reproduced by two to eight asexual autospores
Figs 1–16. Morphology of *Parachloroidium laureanum* sp. nov. and *P. lobatum* sp. nov.

Figs 1–3. Vegetative cells and autosporangia of *P. laureanum*, strain CAUP H8501. Scale bar = 5 μm.

Fig. 4. Detail of mature vegetative cell of *P. laureanum*, strain CAUP H8501. Scale bar = 1 μm.

Fig. 5. Vegetative cells and the two-celled autosporangium of *P. laureanum*, strain CAUP H8501. Scale bar = 5 μm.

Fig. 6. Young vegetative cells of *P. laureanum*, strain CAUP H8501. Scale bar = 5 μm.

Figs 7, 8. Two-celled autosporangia of *P. laureanum*, strain CAUP H8501. Scale bar = 1 μm.

Figs 9–11. Vegetative cells and autosporangia of *P. lobatum*, strain CAUP H8502. Scale bar = 5 μm (Figs 9, 11); scale bar = 1 μm (Fig. 10).

Fig. 12. Vegetative cells and autosporangia of *P. lobatum*, strain CAUP H8502. Scale bar = 5 μm.

Figs 13, 14. Detail of mature vegetative cell with two distinct chloroplast lobes and the two-celled autosporangium of *P. lobatum*, strain CAUP H8502. Scale bar = 1 μm.

Figs 15, 16. Vegetative cells and autosporangia of *P. lobatum*, strain CAUP H8503. Scale bar = 5 μm.
(Figs 5–8, 14–16). In most cells, a single relatively large autospore and one, three, or seven considerably smaller autospores were produced within a single sporangium (Figs 7–8, 12, 14–15). The autospores of Parachloroidium laurenum, strain CAUP H8501, were typically elliptical or irregularly egg shaped (Fig. 6). Conversely, the autospores of P. lobatum, strain CAUP H8502, were more or less spherical in shape (Fig. 12). The mature cells and autosporangia of all the strains assigned here into the genus Parachloroidium were regularly spherical. The plastids of P. laurenum were often slightly detached from the cytoplasmic membrane at its parietal side. Therefore, in a side view, the plastid often appeared as a flat plate separating two cytoplasmic regions of the cell (Figs 8, 17, 18). Conversely, the plastids of P. lobatum were more or less cup shaped, i.e. they typically had conspicuous lobes and a central incision (Figs 10–13). True pyrenoids were absent in both species, but plastids of P. laurenum cells included numerous pyrenoglobuli arranged in a central thylakoid-free region that sometimes also included oval to spherical starch grains (Figs 17–20). The chloroplasts of P. lobatum also typically included thylakoid-free regions with irregularly arranged strach grains, but pyrenoglobuli were absent (Figs 21, 22). The cell walls of Parachloroidium strains were unsulptured. Autosporangial cell wall remnants of P. laurenum rapidly dissolved in the medium. Conversely, cell wall remnants of P. lobatum were significantly more resistant and they were frequently observed surrounding the former autospores (Fig. 21).

**Molecular phylogeny**

ITS rDNA, SSU rDNA, and rbcL DNA sequences were obtained from all four Parachloroidium strains. In addition, the plastid-encoded rbcL gene sequences were determined for the authentic strains of two Chloroidium species, C. ellipsoidium (Gerneck) Darienko *et al.* (CAUP H1904) and C. saccharophilum (W. Krüger) Darienko *et al.* (CAUP H1912). The phylogenetic position of Parachloroidium strains within Trebouxiophyceae was inferred by analysing the DNA sequences of the slowly evolving 18S rDNA and rbcL genes. To test the robustness of the phylogeny, four different 18S rDNA gene analyses were conducted using variations in outgroup taxa and alignment strategies. The results of these four analyses were in agreement: the major clades were resolved with very similar statistical support (Table 1). All Parachloroidium strains formed a firmly supported monophyletic lineage (1.00 Bayesian posterior probability/100 ML bootstrap support/100 wMP bootstrap support) within the Watanabea clade of Trebouxiophyceae (Fig. 23). Three P. lobatum strains (CAUP H8502, CAUP H8503, I6e) were closely related (0.98/84/89). The 18S rDNA sequences of the strains CAUP H8502 and I6e were identical, but strain CAUP H8503 differed by five substitution changes and one deletion of three nucleotides from two previous strains of this species. The entire genus Parachloroidium was consistently placed in a highly supported sister position to the genus Chloroidium (1.00/93/90). The Watanabea clade also comprised the genera Heterochlorella, Heveochlorella, Kalimella, Phyllosiphon, Viridiella, and Watanabea. Sequences of three taxonomically unidentified trebouxiophycean strains (“Chlorella” sp. MBIC 10057, uncultured “Chlorella” L-1016 and “Chlorella luteoviridis” MES A5-4) were also firmly nested within this clade.

The topology of the phylogenetic tree derived from the analysis of the concatenated 18S rDNA + rbcL sequences (Fig. 24) illustrated that the monophyletic genus Parachloroidium (1.00/100/100) was recovered in a firmly supported sister position with the genus Chloroidium (1.00/100/100). The Parachloroidium strains and two authentic strains of the Chloroidium species shared a single amino acid insertion (Lys), coded as ‘AAG’ in Parachloroidium and ‘AAA’ in Chloroidium, at position 286 in the rbcL gene sequence. The genera Chloroidium and Parachloroidium formed a part of the Watanabea clade, together with the sequences of the authentic strains of Heterochlorella luteoviridis (Chodat) Neustupa *et al.*, Kalimella bambuscicola Neustupa *et al.*, and K. aperyrenoidosa Neustupa *et al.* (1.00/100/100). Three strains of P. lobatum (CAUP H8502, CAUP H8503, I6e) had identical rbcL sequences.

ITS rDNA sequences were obtained for all four Parachloroidium strains. Intragenomic variation in four nucleotide sites was found in the ITS1 region of P. laurenum (CAUP H8501). The basic local alignment search tool (BLAST) searches of 5.8S rDNA sequences showed that the best matches were to GenBank sequence no. FJ792803 [assigned as Dictyochloropsis reticulata (Tschermak-Woess) Tschermak-Woess] and to various Chloroidium species. However, the BLAST searches of ITS1 and ITS2 regions did not suggest any similar GenBank sequences, with the exception of the P. laurenum sequence, receiving 88% identity with the FJ792803 sequence. No Chloroidium sequences were returned by the BLAST searches used to evaluate the homologies of the Parachloroidium ITS rDNA sequences. The ITS regions of P. lobatum strains CAUP H8502 and I6e were identical to one another; they differed from strain CAUP H8503 by four substitution changes in ITS1 and one deletion in ITS2. The single deletion was located in the terminal loop of helix II (Fig. 25). Consequently, no compensatory base changes were detected among the strains of P. lobatum. Conversely, the ITS rDNA sequence of P. laurenum CAUP H8501 was highly diverse. The overall divergence between the ITS2 rDNA sequences of P. laurenum and P. lobatum was approximately 27%. Most of the differences were in helices III and IV and a total of seven compensatory base changes were identified (Fig. 25).

**DISCUSSION**

Several recent studies based on molecular data have revealed rather unexpected phylogenetic diversity within the Chlorella-like green algae (Krienitz *et al.* 2004; Darienko *et al.* 2010; Luo *et al.* 2010; Bock *et al.* 2011). The taxa are now distributed over multiple genus-level lineages in the green algal classes Trebouxiophyceae and Chlorophyceae (Leliaert *et al.* 2012). Numerous Chlorella-like taxa have been isolated from various freshwater ecosystems (Krienitz & Bock 2012); they have also, more rarely, been observed in marine habitats (Aslam *et al.* 2007). These microalgae are probably most abundant and diversified in subaerial biofilms and in soil, where they are constituents of microbial assemblages (Ettl &
Gärtner 1995). In these habitats, microalgal populations are subject to aperiodic drying and rewetting cycles and, therefore, they must be able to cope with significant desiccation stress (Lütgge & Büdel 2010). The conditions they experience generally lead to selection for spherical cells with relatively low surface-to-volume ratios. Consequently, Chlorella-like morphotypes evolved several times independently in multiple trebouxiophycean and chlorophycean lineages in terrestrial habitats (Friedl & Rybalka 2011).

Correct phylogenetic classification of these microorganisms is a prerequisite for evaluating the diversity and structure of these little-known microbial phototrophic communities.

The unicellular members of the Watanabea clade, which mostly occur in terrestrial habitats, cannot usually be identified by traditional light and electron microscopy. Morphological criteria, such as cell size, shape of the individual ontogenetic stages (autospores, vegetative cells, autosporangia), or plastid structure, typically cannot be used to discriminate between individual members of this trebouxiophycean lineage. However, morphological characters may be very useful for a posteriori phenotypic definition of genus-level lineages that were previously identified by means of molecular data. The Parachloroidium lineage may be morphologically distinguished from its sister genus Chloroi-

Figs 17–20. Ultrastucture of Parachloroidium laureanum sp. nov., strain CAUP H8501; ch = chloroplast, n = nucleus, pg = pyrenoglobuli, s = starch grains. Scale bars = 0.5 μm.

Figs 17, 18. Vegetative cells of P. laureanum, strain CAUP H8501, with single nuclei and plastids containing numerous pyrenoglobuli and starch grains.

Fig. 19. Details of pyrenoglobuli and starch grains located in the chloroplast matrix of P. laureanum, strain CAUP H8501.

Fig. 20. Vegetative cells of P. laureanum, strain CAUP H8501, with numerous starch grains and pyrenoglobuli located in the chloroplast matrix.
mainly by its regular spherical cell shapes and by the peculiar structure of its plastids, which typically contain irregular thylakoid-free regions with clusters of pyrenoglobuli and starch grains. In contrast, the cells of *Chloroidium* species typically possess oval pyrenoids, composed of an amorphous matrix that may sometimes be penetrated by thylakoid bands and surrounded by a starch envelope (Ikeda & Takeda 1995). Similar pyrenoids have been observed in several other taxa belonging to the *Watanabea* clade, such as *Heterochlorella luteoviridis*, *Heveochlorella hainangensis*, and *K. bambusicola* (Zhang et al. 2008; Neustupa et al. 2009). Conversely, no pyrenoids were observed in other members of the clade, such as *Viridiella fridericiana* P. Albertano, A. Pollio & R. Taddei, *Phyllosiphon arisari* Kühn, and *Kalinella apyrenoidosa* (Huss et al. 2002; Aboal & Werner 2011; Neustupa et al. 2013). Plastids of *W. reniformis* also lack pyrenoids, but they do include thylakoid-free regions with pyrenoglobuli, i.e. structures vaguely similar to those observed in *Parachloroidium* strains (Hanagata et al. 1998). However, the relatively distant phylogenetic position of the genera *Watanabea* and *Parachloroidium* indicates that this ultrastructural character cannot be used to identify evolutionary relationships within the clade.

Two species were identified within the newly described genus *Parachloroidium*. They were primarily distinguished by differences in their molecular data, but slight morphological differences were also observed. There were 17 different nucleotide positions in the SSU rDNA sequence between the type strains of the two species; these differences were even more pronounced in the *rbcL* gene. In addition, the ITS2 sequences of the two strains were highly diverse, which supported the independent species status of both taxa.

<table>
<thead>
<tr>
<th>Table 1. Comparison of node resolutions [Bayesian inference (BI)/maximum likelihood (ML)/weighted maximum parsimony (wMP)] for the four different small-subunit (SSU) ribosomal (r)DNA phylogenetic analyses.</th>
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<tr>
<td><strong>Original alignment, outgroup:</strong> Chlorophyceae</td>
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<tr>
<td>Botryococcus clade</td>
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<td>Chlorellales</td>
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<td>Chloroidium–Kalinella clade</td>
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<td>Dictyochloropsis</td>
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<td>Leptochlorella</td>
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<td>Lobesphaera clade</td>
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<td>Microthamniales</td>
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<td>Parietochloris</td>
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<td>Xylochloris</td>
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<td>outgroup</td>
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*Figs 21, 22.* Vegetative cells and autospores of *Parachloroidium lobatum* sp. nov., strain CAUP H8502; ch = chloroplast, d = dictyosome, n = nucleus, s = starch grains. Scale bar = 0.5 μm.

*Fig. 21.* A vegetative cell of *P. lobatum*, strain CAUP H8502, showing the irregular thylakoid-free region located within the chloroplast.

*Fig. 22.* Two autospores of *P. lobatum*, strain CAUP H8502, with plastids containing scattered starch grains.
Fig. 23. Phylogenetic position of the genus *Parachloroidium* within the class Trebouxiophyceae (Chlorophyta), based on 18S rDNA sequences. The analysis was based on reduced alignment with an outgroup formed by the three chlorophycean species. The tree was inferred using MrBayes with the GTR + G + I evolutionary model. Numbers at the branches correspond to MrBayes posterior probabilities/maximum likelihood (ML) bootstrap values/weighted maximum parsimony (wMP) bootstrap values. Values below 0.95 Bayesian posterior probability (BPP) or 50% ML and wMP bootstrap support are not shown. Thick branches represent nodes receiving the highest BPP support (1.00). The scale bar shows the estimated number of substitutions per site.
Variation in sequence data was also observed between strains CAUP H8502 and H8503, which were assigned to *P. lobatum*. Five substitution changes and one deletion of three nucleotides in the 18S rDNA sequences were accompanied by a single deletion in the terminal loop of helix III of the ITS2 region. We did not interpret the observed variability in molecular data as sufficient for the formal taxonomic differentiation of these strains. This conclusion was also supported by the homogeneity in their morphological characteristics.

Friedl & Rybalka (2011) published an overview of Trebouxia lineages, and included the *Watanabea* clade as one of five well-recognized higher-level clades of this algal class. Following several recent taxonomic additions (Aboal & Werner 2011; Ma et al. 2013; Neustupa et al. 2013), the *Watanabea* clade may now be considered one of the Trebouxia lineages that mostly include taxa occurring in terrestrial microhabitats. The members of at least two genera of the clade—*Chloroidium* and *Heterochlorella*—were found in multiple molecular diversity studies, and several strains, identified by molecular methods, are available in culture collections (Hallmann et al. 2011; Thüs et al. 2011; Lee & Hur 2012). Conversely, the genus *Parachloroidium* has so far not been reported from comparable environmental sequence data and we may speculate that it is possibly a relatively rare taxon, or one with an ecologically restricted distribution. Interestingly, the ITS sequence no. FJ792803 was the most similar to *Parachloroidium* sequences. This sequence was isolated from the lichen *Lobaria pulmonaria* (Linnaeus) Hoffmann in Spain (Gasulla et al. 2010), and may represent an additional species of the *Chloroidium/Parachloroidium* lineage. However, detailed

Fig. 24. Phylogenetic position of *Parachloroidium* within the class Trebouxia (Chlorophyta), based on the combined 18S rDNA + rbcL data set. The tree was inferred using MrBayes with the GTR + G + I evolutionary model applied to the entire 18S rDNA data set and the first and third codon positions of rbcL, and the JC + G + I model applied to the second codon position of rbcL. Numbers at the branches correspond to MrBayes posterior probabilities/maximum likelihood (ML) bootstrap values/weighted maximum parsimony (wMP) bootstrap values. Values below 0.95 Bayesian posterior probability (BPP) or 50% ML and wMP bootstrap support are not shown. Thick branches represent nodes receiving the highest BPP support (1.00). The GenBank accession numbers for individual taxa refer to 18S rDNA/rbcL sequences. The scale bar shows the estimated number of substitutions per site.
distribution patterns of Parachloroidium and related genera in the Watanabea clade can only be established once more data on the molecular diversity of phototrophic corticolous biofilms and other subaerial microalgal assemblages have been collected.

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