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Leptochlorella corticola gen. et sp. nov. and *Kalinella apyrenoidosa* sp. nov.: two novel *Chlorella*-like green microalgae (Trebouxiophyceae, Chlorophyta) from subaerial habitats

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The diversity of green microalgae in subaerial habitats remains largely unexplored and a number of new genus- and species-level lineages have been discovered recently. The traditional green algal genus, *Chlorella*, which accommodated coccoid unicellular green algal species with globular to oval cells, reproducing entirely by autospores, has been found to be polyphyletic. In this study, we provide a detailed characterization of two strains of microalgae isolated from tree bark in the Mediterranean. These algae share the general *Chlorella*-like morphology and their 18S rRNA and *rbcL* gene sequences place them in the Trebouxiophyceae. Strain CAUP H8401 forms an independent trebouxiophycean lineage, together with three previously published 18S rRNA gene environmental sequences of undescribed microalgae, which were retrieved from profoundly different habitats. In contrast, strain CAUP H7902 is related to *Kalinella bambusicola* in the *Watanabea* clade of the Trebouxiophyceae on the basis of its 18S rRNA gene sequence. This relationship is also supported by the *rbcL* gene sequence, acquired from the type strain of *K. bambusicola*. The investigated strains are described as representatives of a novel species in a new genus, *Leptochlorella corticola* gen. et sp. nov., and a novel species, *Kalinella apyrenoidosa* sp. nov., according to the International Code of Nomenclature for Algae, Fungi and Plants.

INTRODUCTION

The morphologically defined genus *Chlorella* accommodated coccoid unicellular green algae with globular to oval cells that reproduce entirely by autospores, which were defined as asexual, non-motile reproductive cells developing simultaneously within maternal sporangium (Fott & Nováková, 1969; Ettl & Gärtner, 1995). The members of this genus were also characterized by smooth cell walls, the absence of extracellular mucilage and single chloroplasts in cells. However, despite detailed light microscopic, ultrastructural and biochemical studies of these organisms (Kalina & Punčochářová, 1987; Kessler & Huss, 1992), taxonomy of the *Chlorella*-like microalgae has been hampered by the lack of suitable phenotypic characteristics for the delimitation of species. For this reason, molecular phylogenetic data, based primarily on 18S rRNA gene sequences, provided the first real insights into the phylogenetic structure of these microalgae (Friedl, 1995; Huss *et al.*, 1999). Most notably, they were recognized as members of two separate classes: the Chlorophyceae and Trebouxiophyceae.

In the Trebouxiophyceae, the *Chlorella*-like microalgae form several independent lineages. The Chlorellales include several genera, i.e. *Chlorella*, *Marinichlorella*, *Meyerella*, *Parachlorella* and *Picochlorum*, which typically inhabit various freshwater and, to a lesser extent, marine habitats, either as free-living organisms or as endosymbionts of protists and metazoans (Henley *et al.*, 2004; Krienitz *et al.*, 2004; Fawley *et al.*, 2005; Aslam *et al.*, 2007; Bock *et al.*, 2011; Pröschold *et al.*, 2011). Additional *Chlorella*-like lineages were recently classified as separate trebouxiophycean genera *Elliptochloris*, *Pseudochlorella* and *Xylochloris* (Rindi *et al.*, 2007; Letsch *et al.*, 2009; Darienko *et al.*, 2010; Neustupa *et al.*, 2011).

Several other traditional *Chlorella*-like taxa have been recognized as separate genus-level trebouxiophycean lineages

Abbreviations: ASL, above sea-level; BIC, Bayesian information criterion; ML, maximum-likelihood; BS, bootstrap support; BPP, Bayesian posterior probability; TEM, transmission electron microscopy; wMP, weighted maximum-parsimony.

The GenBank/EMBL/DDBJ accession numbers for the 18S rRNA gene sequences of strains CAUP H7902 and CAUP H8401 are HE984578 and HE984579, respectively. The GenBank/EMBL/DDBJ accession numbers for the *rbcL* gene sequences of strains CAUP H1906, CAUP H7901, CAUP H7902, CAUP H8401 and CAUP 7801 are HE984580–HE984584, respectively.

Supplementary material is available with the online version of this paper.

in the so-called *Watanabea* clade. The frequently occurring members of the genus *Chloroidium* have often been encountered in subaerial microhabitats, such as tree bark or rock surfaces (Hoffmann & Darienko, 2005; Darienko *et al.*, 2010; Neustupa & Škaloud, 2010). They may also be found less frequently as lichen photobionts (Thüs *et al.*, 2011) and a single species of this genus, *Chloroidium saccharophilum*, occurs in freshwater localities (Darienko *et al.*, 2010). Further *Chlorella*-like taxa in the *Watanabea* clade include the genera *Heterochlorella*, *Heveochlorella* and *Kalinella*, typically encountered in corticolous subtropical to tropical habitats (Neustupa *et al.*, 2009; Zhang *et al.*, 2008). Interestingly, the profoundly morphologically different, epiphyllous and parasitic genus *Phyllosiphon*, which is characterized by a siphonous habit, was recently recovered in a sister position to the three corticolous genera mentioned above (Aboal & Werner, 2011). *Watanabea reniformis* and *Viridiella fridericiana*, two additional taxa that form deep, independent lineages of the *Watanabea* clade are known to occur in freshwater and terrestrial environments (Albertano *et al.*, 1991; Hanagata *et al.*, 1998).

This overview of studies describing different, often widely unrelated taxa of the morphologically rather uniform *Chlorella*-like microalgae illustrates that the real taxonomic diversity of these organisms is probably only starting to emerge as result of the extended sampling and molecular phylogenetic investigation of various phototrophic microbial communities. Indeed, several studies on the molecular diversity of microbial eukaryotes in various extreme microhabitats, such as desert soil crusts (Lewis & Lewis, 2005), Antarctic lake epipelon (De Wever *et al.*, 2009), endolithic (Horath & Bachofen, 2009) or hypolithic biofilms (Wong *et al.*, 2010), have revealed unique environmental sequences of additional, so far undescribed, lineages within the Chlorophyceae and Trebouxiophyceae. One of these lineages, which was originally known only from environmental sequencing of an endolithic microbial community thriving in alpine dolomite rocks in Switzerland (Horath & Bachofen, 2009), was recently morphologically characterized and taxonomically described as a new chlorophycean genus, *Jenufa*, typified by *Chlorella*-like morphology (Němcová *et al.*, 2011). Given the morphological uniformity of the 'little green balls' that occur in terrestrial microhabitats, and which are often difficult to identify by microscopic methods, it can be presumed that some of these lineages, known only as environmental sequences, may in fact represent further *Chlorella*-like taxa that have so far evaded taxonomic description.

In this study, we report the results of an investigation of two morphologically similar microalgal strains that were recently isolated from two tree bark samples taken in similar sub-Mediterranean habitats of south-west Slovenia, Central Europe. Our results indicate that these isolates represent previously unknown trebouxiophycean taxa, described herein as a new genus, *Leptochlorella*, and a novel species of the genus *Kalinella*, *Kalinella apyrenoidosa*, according to the International Code of Nomenclature for Algae, Fungi and Plants (Knapp *et al.*, 2012). Interestingly, phylogenetic analyses of previously published environmental 18S rRNA gene sequences reveal that at least two additional undescribed species of the newly described genus *Leptochlorella* inhabit profoundly different habitats. These results indicate that *Leptochlorella* may in fact be a widely distributed alga that has so far been overlooked because of its minute dimensions and simple *Chlorella*-like morphology.

METHODS

Isolation and cultivation. The two novel algal strains were obtained from samples of subaerial corticolous biofilms. Strain CAUP H8401 was isolated from the bark of a *Cupressus sempervirens* growing near Portorož, Slovenia [45° 31′ 28.23″ N 13° 34′ 43.75″ E, altitude 10 m above sea-level (ASL)]. Strain CAUP H7902 was isolated from the bark of a *Laurus nobilis* near Ankaran, Slovenia (45° 35′ 11.93″ N 13° 42′ 35.15″ E, altitude 5 m ASL). The samples were isolated from approximately 1 cm^2 of bark surface taken from a shaded north part of the tree's trunk, at a height of about 120 cm above the soil surface. Strains CAUP H8401 and CAUP H7902 are available from the Culture Collection of Algae of Charles University in Prague (CAUP) (http://botany.natur.cuni.cz/algo/caup.html). The strains were cultivated on agar-solidified Bold's basal medium (BBM) (Andersen *et al.*, 2005) at 23 °C with illumination of 40 μmol m^{-2} s^{-1}, provided by 18 W cool fluorescent tubes (Philips TLD 18W/33).

Light and electron microscopy. Microphotographs of cells were taken with an Olympus BX51 light microscope and Olympus Z5060 camera using differential interference contrast. For transmission electron microscopy (TEM), samples of each strain were fixed for 2 h at 5 °C in 2 % glutaraldehyde in 0.05 M phosphate buffer and post-fixed for 2 h in 1 % osmium tetroxide (OsO_4) in 0.05 M phosphate buffer and, subsequently, for 12 h at 5 °C in 1 % uranyl acetate solution. Then, the samples were dehydrated through an ethanol series and embedded in Spurr medium via propylenoxide. Ultrathin sections, cut with a diamond knife on an Ultracut E (Reichert-Jung), were post-stained with lead citrate and examined using a JEOL 1011 TEM.

DNA isolation, PCR and DNA sequencing. After centrifugation of algal cells, 100 ml InstaGene matrix (Bio-Rad Laboratories) was added to the pellet. The cells were then mechanically disrupted by shaking for 5 min in the presence of glass beads (3 mm diameter; Sigma-Aldrich) in a Mixer Mill MM 400 (Retsch). Subsequently, the solution was incubated at 56 °C for 30 min, vortex mixed for 10 s, and heated to 99 °C for 8 min. After vortex mixing for a second time, the tubes were centrifuged at 12 000 r.p.m. for 2 min, and the supernatant was used directly as a PCR template. Two molecular markers were amplified by PCR: the nuclear 18S rRNA genes and the chloroplast-encoded large subunit of the ribulose-1,5-bisphosphate carboxylase oxygenase (*rbcL*) genes. The PCR mix contained 13.1 μl sterile MilliQ water, 2 μl AmpliTaq Gold 360 buffer 10×, 2.2 μl $MgCl_2$ (25 mM), 0.4 μl dNTP mix (10 mM), 0.25 μl of each primer (25 nM), 0.6 μl 360 GC enhancer, 2 μl AmpliTaq Gold 360 DNA polymerase, and 1 μl DNA (10 ng μl^{-1}) in a total volume of 20 μl. The 18S rRNA gene was amplified using 18S-F/18S-R primers published by Katana *et al.* (2001) and the *rbcL* gene was amplified using PRASF1/PRASR1 and PRASF2/PRASR2 primers designed by Sherwood *et al.* (2000), or by PRASF1/ellaR2 newly designed primer (5′-TCACGACCTTCATTACGAGCTTG-3′). The 18S rRNA/*rbcL* genes were amplified in a Touchgene gradient thermal cycler (Krackeler Scientific), starting with initial denaturation at 94 °C for

4/5 min (18S rRNA/*rbcL*), followed by 35/40 cycles of denaturing at 94/95 °C for 1 min/45 s, annealing at 54/50 °C for 1/1.5 min, and elongation at 72 °C for 2.5/2 min, with a final extension at 72 °C for 10 min. 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). The purified amplification products were sequenced using an Applied Biosystems automated sequencer (ABI 3730xl) at Macrogen in Seoul, Korea.

Phylogenetic analyses. Two different alignments were constructed for the phylogenetic analyses, based on the 18S rRNA and *rbcL* gene sequences (Table S1, available in IJSEM Online). Newly determined sequences and those selected from the GenBank database were edited manually using MEGA5 (Tamura *et al.*, 2011). The alignment of 18S rRNA gene sequences was guided by the secondary structure model of *Chlamydomonas reinhardtii* 18S rRNA (Wuyts *et al.*, 2000). Suitable substitution models were selected using the Bayesian information criterion (BIC) in MEGA5. The BIC selected the GTR + G + I model for the entire 18S rRNA gene dataset and the 1st and 3rd codon position of *rbcL*, and the JC + G + I model for the 2nd codon position of *rbcL*. Two members of the Nephoselmidophyceae, a class that is among the closest relatives of the UTC-clade (including Trebouxiophyceae), but safely positioned outside of this group, were selected as the outgroup in our analyses.

The phylogenetic trees were inferred with Bayesian inference using MrBayes version 3.1 (Ronquist & Huelsenbeck, 2003). Two parallel Markov chain Monte Carlo (MCMC) runs were carried out for 10 million generations, each with one cold and three heated chains. Analysis of the *rbcL* dataset was carried out on a partitioned dataset, to assign distinct substitution models to the codon positions. Trees and parameters were sampled every 100 generations. Convergence of the two cold chains was checked and 'burnin' was determined by use of the 'sump' command. A 50 % majority rule consensus of the sampled trees was constructed to calculate the Bayesian posterior probabilities (BPP) of the tree nodes. Bootstrap analysis was performed by maximum-likelihood (ML) and weighted maximum-parsimony (wMP) criteria using GARLI v. 2.0 and PAUP v. 4.0b10 (Swofford, 2002), respectively. ML analyses consisted of 100 replicates, using default settings and the automatic termination set at 100 000 generations, under the unpartitioned 18S rRNA gene and partitioned *rbcL* datasets. The wMP bootstrapping (1000 replicates) was performed using heuristic searches, with 1000 random sequence addition replicates, tree bisection, and reconnection (TBR) swapping, and random addition of sequences (the number was limited to 10 000 for each replicate). The rescaled consistency index was used to assign weight to the characters on a scale of 0–1000. New weights were based on the mean of the fit values for each character over all of the trees in the memory.

RESULTS

Leptochlorella Neustupa, Veselá, Němcová & Škaloud gen. nov.

Description

Vegetative cells solitary, spherical, uninucleate. Cell wall composed of two layers. Chloroplast parietal, without a pyrenoid. Asexual reproduction by autospores. Sexual reproduction not observed. Secondary carotenoids not produced. The genus differs from other members of the Trebouxiophyceae by the 18S rRNA gene (HE984579) and *rbcL* sequences (HE984583).

Type species

Leptochlorella corticola Neustupa, Veselá, Němcová & Škaloud

Etymology

The generic name was chosen to emphasize the minute dimensions of the *Chlorella*-like cells of the genus.

Leptochlorella corticola Neustupa, Veselá, Němcová & Škaloud sp. nov.

Description

Vegetative cells solitary, uninucleate. Cells spherical, (2.5–) 3.0–9.5(–12.5) μm in diameter. Single parietal chloroplast without a pyrenoid, typically divided into two or three lobes. Asexual reproduction via 2–8 spherical to oval autospores, 2.5–4.0 μm in diameter.

Holotype

Strain CAUP C-H8401, which has been permanently cryopreserved in the CAUP (http://botany.natur.cuni.cz/algo/caup.html). The strain has also been deposited in CAUP as an active culture, CAUP H8401, from which the holotype was derived. The strain has also been preserved in the form of a permanent slide (CAUP P-H8401).

Type locality

Microbial biofilm growing on the bark of a *Cupressus sempervirens* near Portorož, Slovenia (45° 31′ 28.23″ N 13° 34′ 43.75″ E, altitude 10 m ASL).

Etymology

The species name was chosen to emphasize the original habitat of the type strain.

Morphology and ultrastructure

L. corticola had mostly globular cells, (2.5–)3.0–9.5 (–12.5) μm in diameter. The mature cells were consistently globular (Fig. 1a–d), but the freshly released autospores sometimes had slightly oval shapes (Fig. 1e–h). The alga reproduced solely by autospores and these were predominantly produced in tetrads of four identical cells (Fig. 1e). However, autosporangia containing 2 or 8 autospores were also rarely observed. The autospores were released by irregular fracturing of the maternal cell wall. The freshly released daughter cells were often briefly connected by a short mucilaginous stalk, which dissolved later during ontogenesis (Fig. 2a). The mature cells typically transformed

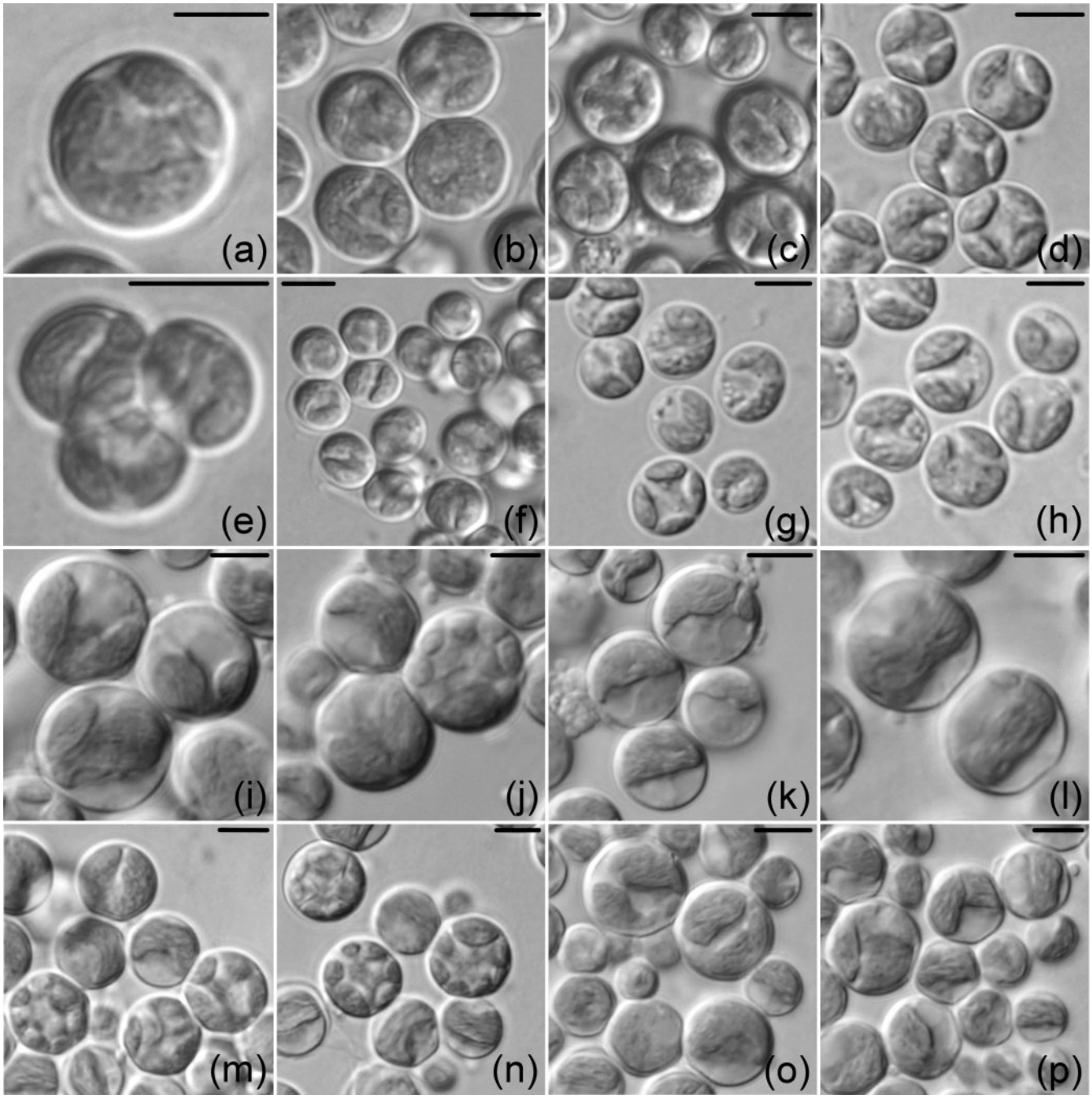

Fig. 1. Morphology of *L. corticola* gen. et sp. nov., strain CAUP H8401, and *K. apyrenoidosa* sp. nov., strain CAUP H7902. (a–h) *L. corticola*. (a–d) Vegetative cells. (e, f) Autospores (note the arrangement of autospores in groups of four identical cells). (g, h) Young vegetative cells. (i–p) *K. apyrenoidosa*: vegetative cells and autosporangia (note the unequal size of autospores within autosporangia). Bars, 5 μm.

into autosporangia with diameters of about 8.0–9.5 μm, but vegetative cells with diameters of more than 12.0 μm were encountered as well, albeit less frequently. The cells possessed single parietal chloroplasts that often filled most of the volume of the cell. The plastids of mature cells were typically divided into two or three lobes, separated by narrow incisions (Figs 1a and 2b–c). Two serial plastid divisions resulted in four daughter plastids that could be observed in the mature cells prior to asexual reproduction. No pyrenoids were observed but there were numerous elliptical starch grains scattered within the chloroplast matrix (Fig. 2b–c). The cytoplasmic, electron-dense globular

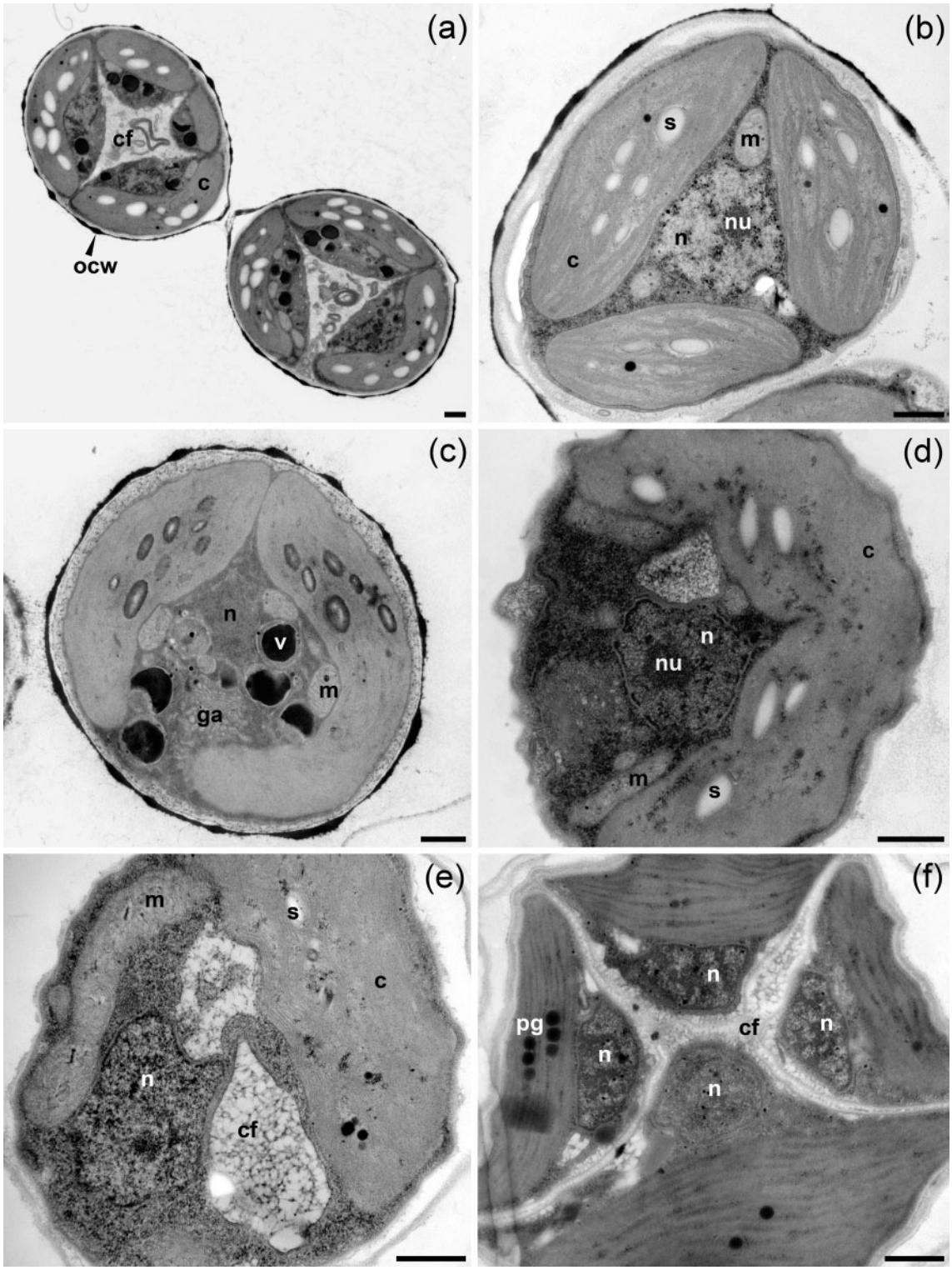

Fig. 2. Ultrastructure of *L. corticola* gen. et sp. nov. and *K. apyrenoidosa* sp. nov. (a–c) *L. corticola*: (a) two mature vegetative cells during division (note the connection by a short mucilaginous stalk); (b) autospore within a mother cell wall; (c) vegetative cell. (d–f) *K. apyrenoidosa*: (d) autospore; (e) vegetative cell during division; (f) autosporogenesis (note one considerably larger autospore). c, Chloroplast; cf, cleavage furrow; ga, Golgi apparatus; m, mitochondrion; n, nucleus; nu, nucleolus; ocw, outer cell wall; pg, plastoglobuli; s, starch grain; v, cytoplasmic electron-dense vacuoles. Bars, 500 nm.

vacuoles were usually also present in the vegetative cells (Fig. 2c). The cell wall was smooth and thin; no thickenings were observed. A conspicuous outermost layer was characteristically formed by an electron-dense material (Fig. 2a–c).

Kalinella apyrenoidosa Neustupa, Veselá, Němcová & Škaloud sp. nov.

Description

Vegetative cells solitary, uninucleate. Cells spherical, (3.5–)6.0–9.5(–11.0) μm in diameter. Single parietal chloroplast without a pyrenoid, occasionally divided into two or three lobes. Asexual reproduction via 2–16 spherical autospores, 3.5–6.0 μm in diameter. The species differs from the type species of the genus *Kalinella* by the absence of a pyrenoid and by 18S rRNA gene and *rbcL* sequences.

Holotype

Strain CAUP C-H7902, which has been permanently cryopreserved in the CAUP (http://botany.natur.cuni.cz/algo/caup.html). The strain has also been deposited in CAUP as an active culture, CAUP H7902, from which the holotype was derived. The strain has also been preserved in the form of a permanent slide (CAUP P-H7902).

Type locality

Microbial biofilm on the bark of a *Laurus nobilis* growing approximately 10 m from the Adriatic sea coastline near Ankaran, Slovenia (45° 35′ 11.93″ N 13° 42′ 35.15″ E, altitude 5 m ASL).

Etymology

The species name was chosen to emphasize the lack of a pyrenoid in the plastid compared to *K. bambusicola*, the type species of the genus.

Morphology and ultrastructure

K. apyrenoidosa had globular cells, (3.5–)6.0–9.5(–11.0) μm in diameter (Fig. 1i–l). The cells reproduced by 2 to 16 autospores. In most cells there was a single large autospore and 1, 3, 7 or 15 smaller autospores produced within a single sporangium (Figs 1j, m–n and 2f). The autospores were released by irregular fracturing of the maternal cell wall. The globular autosporangia were typically 8.0–9.5 μm in diameter. The cells possessed single parietal band-like chloroplasts that were occasionally divided into two or several lobes in mature cells (Fig. 1o–p). In many cells, the plastid was slightly detached from the cytoplasmic membrane at its parietal side (Fig. 1l–m). No pyrenoids were observed. The older cells were often filled up with extraplastidial globular vacuoles. The chloroplasts often included small elliptical starch grains, which were scattered within the chloroplast matrix, and several electron-dense globular plastoglobuli (Fig. 2d–f). The cell wall was smooth and thin, without any visible thickenings.

Molecular phylogenetic analysis

The sequences of the 18S rRNA genes of *L. corticola* strain CAUP H8401 and *K. apyrenoidosa* CAUP H7902 comprised 1775 and 1740 bp, respectively. BLAST searches against the nucleotide database at NCBI (http://www.ncbi.nlm.nih.gov/BLAST) placed both strains firmly into the Trebouxiophyceae, Chlorophyta. Phylogenetic analyses focusing on representatives of all trebouxiophycean lineages confirmed that both strains belonged to this algal class (Fig. 3). Strain CAUP H8401, described here as *L. corticola*, was placed into a well-supported clade (1.00 BPP/100 ML bootstrap support/100 wMP bootstrap support) with three unidentified trebouxiophycean environmental sequences (FJ946881, FJ790649, FJ790655). The sequence FJ946881 differed from that of CAUP H8401 by 17 out of 1746 positions of the final 18S rRNA gene alignment. The environmental sequences FJ790649 and FJ790655 differed by 8 positions from each other, and by 25 and 23 positions from the sequence of CAUP H8401, respectively. This lineage clustered together with a clade including *Microthamnion kuetzingianum* (Z28974), *Coleochlamys perforata* (M62999), *Parietochloris alveolaris* (EU878373) and *Parietochloris pseudoalveolaris* (M63002), but this position was not supported by the statistical analyses. The position of this entire lineage within the Trebouxiophyceae was also not resolved in our analyses.

K. apyrenoidosa CAUP H7902 clustered in a sister position with *Kalinella bambusicola* (EU346910) with high support (1.00/100/100). This lineage was part of the *Heterochlorella/Chloroidium* clade, which includes members of the genera *Heterochlorella*, *Heveochlorella*, *Phyllosiphon*, *Chloroidium*, and two taxonomically unresolved sequences (AB058305 and AB006045). These generic lineages formed a part of the moderately supported *Watanabea* clade of the Trebouxiophyceae (1.00/84/58).

The plastid-encoded *rbcL* gene sequences were determined for strains of the newly described taxa, *L. corticola* (CAUP H8401) and *K. apyrenoidosa* (CAUP H7902). They were also determined for the type strain of *K. bambusicola* (CAUP H7901), which was recovered in a sister position to CAUP H7902 in 18S-rRNA-gene-based analyses, and for *Xylochloris irregularis* strain CAUP H7801. The sequences of this marker comprised 1173 bp and BLAST searches illustrated that various trebouxiophycean taxa were most similar to both CAUP H8401 and CAUP H7902. Subsequent phylogenetic analyses considered all available *rbcL* sequences of individual trebouxiophycean lineages. Interestingly, the Chlorellales did not include Oocystaceae on the basis of the *rbcL* gene sequences. *L. corticola* CAUP H8401 formed an independent lineage in the *rbcL*-based phylogenetic tree of the Trebouxiophyceae (Fig. 4). It was recovered in a larger clade that included members of the

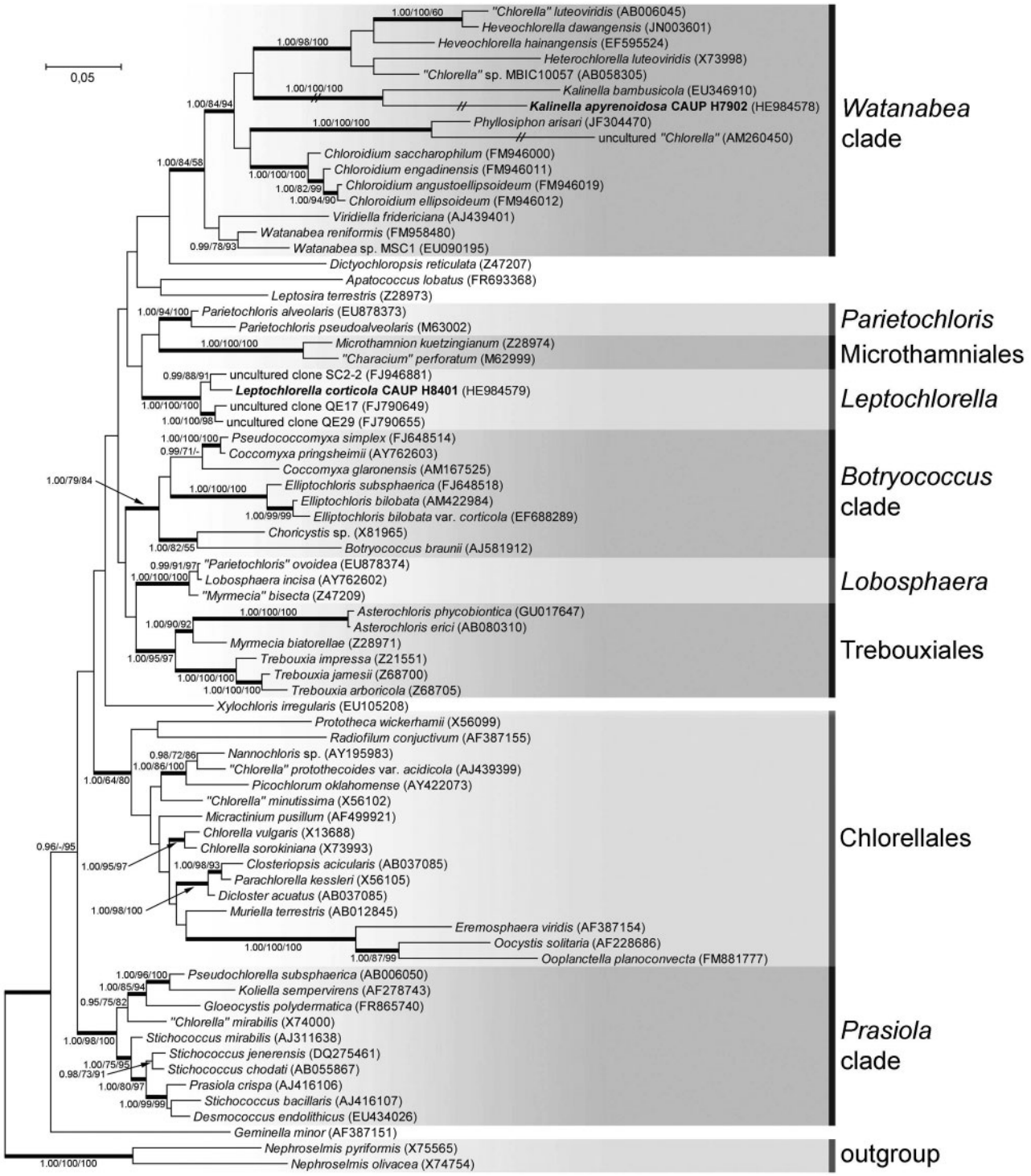

Fig. 3. Phylogenetic position of the investigated microalgae within the class Trebouxiophyceae (Chlorophyta) based on 18S rRNA gene sequences. The tree was inferred using MrBayes with the GTR+G+I evolutionary model. The tree was arbitrarily rooted with strains of *Nephroselmis pyriformis* (X75565) and *Nephroselmis olivacea* (X74754). Numbers at branches correspond to BPP/ML bootstrap values/wMP bootstrap values. Values below 0.95 BPP or 50 % ML and wMP bootstrap support are not shown. Thick branches represent nodes receiving the highest BPP support (1.00). Important, named clades are annotated on the right. Bar, estimated number of substitutions per site. The branches assigned with double slashes have been scaled down to 50 % of their original lengths.

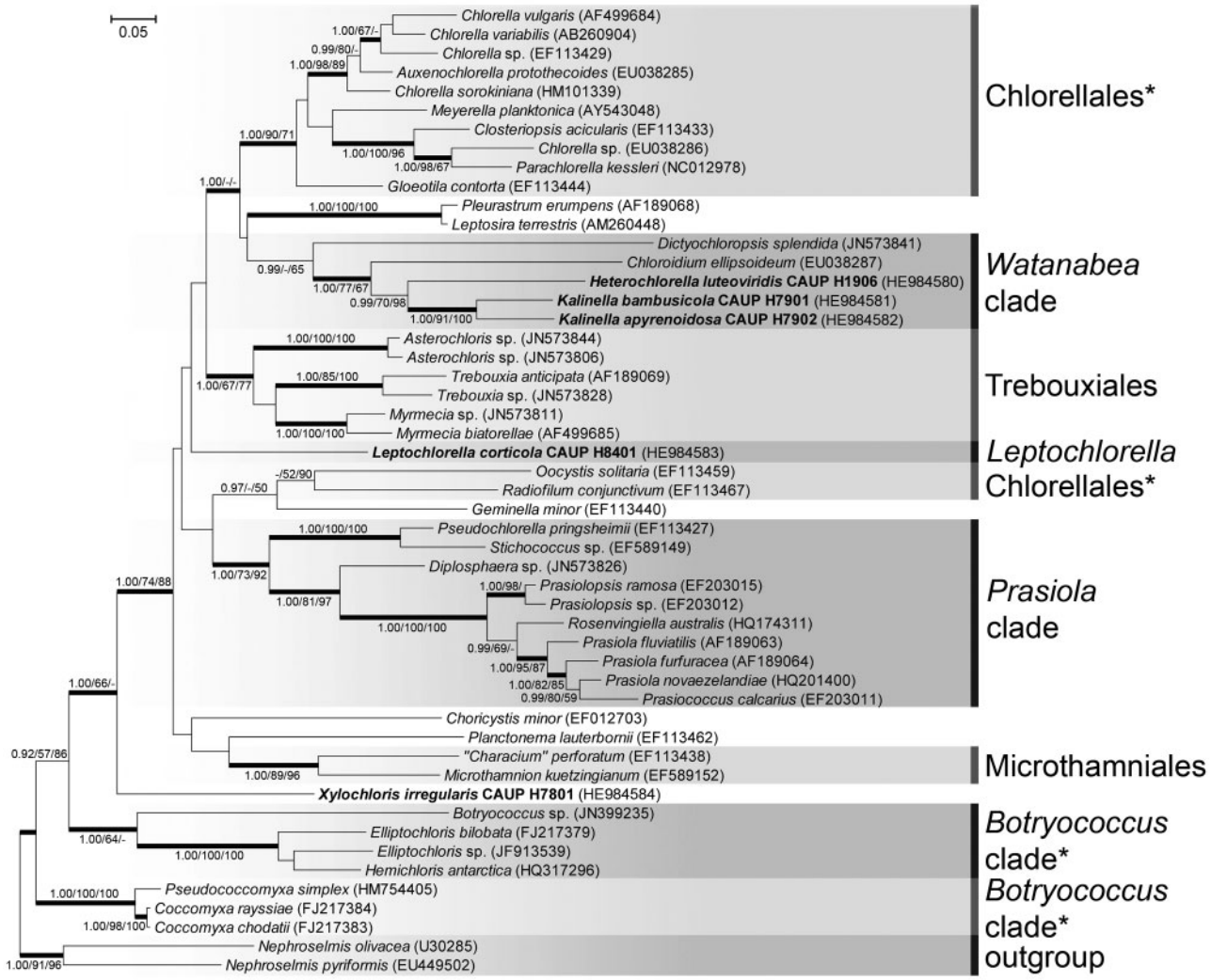

Fig. 4. Phylogenetic position of the investigated microalgae within the class Trebouxiophyceae (Chlorophyta) based on *rbcL* sequences. The tree was inferred using MrBayes. Three partitions were analysed separately using the GTR+G+I model for the 1st and 3rd codon positions, and the JC+G+I model for the 2nd codon position. The tree was arbitrarily rooted with strains of *Nephroselmis pyriformis* (EU449502) and *Nephroselmis olivacea* (U30285). Numbers at branches correspond to BPP/ML bootstrap values/wMP bootstrap values. The values below 0.95 BPP or 50 % ML and wMP bootstrap support are not shown. Thick branches represent nodes receiving the highest BPP support (1.00). The sequences newly acquired in this study are written in bold. Important named clades are annotated on the right. The asterisks mark the 18S rRNA groups that appeared polyphyletic on the basis of the *rbcL* data. Bar, estimated number of substitutions per site.

Chlorellales, the *Watanabea* clade, Trebouxiales, Oocystaceae, the *Prasiola* clade and Microthamniales (1.00/74/88). The exact position of *L. corticola* within this clade was not supported by the statistical analyses. Strains of the genus *Kalinella*, CAUP H7902 and CAUP H7901, formed a strongly supported clade (1.00/91/100), which was recovered in a sister position to the *rbcL* sequence of the type strain of *Heterochlorella luteoviridis* (0.99/70/98), within the *Watanabea* clade. This clade was monophyletic, but this monophyly was only moderately supported on the basis of the *rbcL* sequences (0.99/–/65).

DISCUSSION

The wide phylogenetic diversity of *Chlorella*-like microalgae has become apparent with the advent of molecular methods. Previously, detailed morphological studies of individual clonal strains defined characteristic morphological features, such as chloroplast form, presence or absence of a pyrenoid, and the number and arrangement of autospores, which were traditionally used for species identification (Fott & Nováková, 1969; Ettl & Gärtner, 1995; Andreyeva, 1998). Using such features, two closely similar *Chlorella*-like strains that were investigated in this

study would probably not be identifiable as any of the traditional species. Strain CAUP H7902 would resemble species of *Heterochlorella*, *Heveochlorella*, *Kalinella* or *Chloroidium*. Members of these genera, which belong to the *Watanabea* clade, typically have autospores of unequal size. Conversely, the Chlorellales, including *Chlorella sensu stricto*, are characterized by identical autospores that develop within a single sporangium (Fott & Nováková, 1969). However, despite these morphological hints towards genus-level assignment, reliable identification of the phylogenetic position of the investigated strains would be impossible without molecular data.

The 18S rRNA gene has been broadly sampled among the Trebouxiophyceae and has been established as the primary phylogenetic marker for this group (Leliaert *et al.*, 2012). Although the 18S rRNA gene alone can provide neither sufficient support for definition of phylogenetic relationships among deeper lineages of the Trebouxiophyceae nor enough variability to distinguish between closely related species, it is still very useful for the recognition of individual genus-level taxa (e.g. Aboal & Werner, 2011; Neustupa *et al.*, 2011). Furthermore, broad sampling of the 18S rRNA gene among various members of the class makes this marker extremely useful for identification of new, previously unknown lineages (De Wever *et al.*, 2009; Horath & Bachofen, 2009). The *rbcL* gene has gradually become the standard second choice among various molecular markers in the Trebouxiophyceae and it has recently been used in several taxonomic and diversity studies (Novis *et al.*, 2008; Thüs *et al.*, 2011; Novis & Visnovsky, 2012), providing relatively broad sampling of individual lineages within the class. The *rbcL*-gene-based trebouxiophycean phylogeny generally concurred with the 18S rRNA-based tree topologies. However, there was a notable difference in the position of Oocystaceae, which did not cluster within Chlorellales on the basis of the *rbcL* gene data (Thüs *et al.*, 2011; Novis & Visnovsky, 2012; this study). Strain CAUP H8401 consistently formed an independent trebouxiophycean clade on the basis of both the 18S rRNA gene and *rbcL* gene sequence data. The isolated phylogenetic position of this *Chlorella*-like microorganism is the main reason why it is being described in this study as a member of a new genus, *Leptochlorella*.

Interestingly, the 18S rRNA gene sequence of *L. corticola* was similar to three environmental sequences originating from two molecular diversity studies of profoundly different habitats. De Wever *et al.* (2009) reported the FJ946881 sequence from the summer phytobenthos of a lake in the Schirmacher Oasis, Antarctica. This 18S rRNA gene sequence was detected three times in a single locality, a possible indication that this alga frequently occurred in the phytobenthos of the lake. Wong *et al.* (2010) found another two similar environmental sequences (FJ790649 and FJ790655) in a hypolithic microbial community of quartz pavement in central Tibet. The sequence divergence and apparent differences in autecology of these organisms and *L. corticola* suggest that they almost certainly belong to different species of the genus *Leptochlorella*. However, they cannot be formally described until the strains are isolated from nature. Repeated findings of *Leptochlorella* sequences in profoundly different localities indicate that this trebouxiophycean genus may actually be widely distributed in various terrestrial and freshwater habitats worldwide, but it has been overlooked because its simple morphology has not allowed unequivocal genus-level identification.

Strain CAUP H7902 formed a monophyletic lineage with *K. bambusicola*, both on the basis of the 18S rRNA and *rbcL* gene sequences. Members of the *Heterochlorella* lineage, including the genus *Kalinella*, are typified by unusually divergent 18S rRNA gene sequences (Krienitz *et al.*, 2004; Zhang *et al.*, 2008; Neustupa *et al.*, 2009; Darienko *et al.*, 2010; Aboal & Werner, 2011). The taxa within this clade formed long branches in phylogenetic reconstructions based on this marker, indicating an increased rate of evolution of the 18S rRNA gene in this lineage. Consequently, the genetic divergence between *K. bambusicola* and *K. apyrenoidosa*, based on the 18S rRNA gene sequences, is relatively large. However, several monophyletic genera with similar degree of the 18S rRNA gene divergence have been taxonomically defined within Trebouxiophyceae, such as *Chloroidium*, *Pseudochlorella* (Darienko *et al.*, 2010), or *Parietochloris* (Neustupa *et al.*, 2011).

Thus, given the well-supported phylogenetic sister positions of CAUP H7901 and CAUP H7902, their similar morphologies and similar habitats, we treat them as two separate species of a single genus. As the species name suggests, *K. apyrenoidosa* lacks a pyrenoid, which, by contrast, has been regularly found in plastids of *K. bambusicola* (Neustupa *et al.*, 2009). This difference was traditionally considered important for microalgal taxonomy, but even the traditional microalgal monographs based on morphological data used this character for discrimination between species, rather than between genera (Fott & Nováková, 1969; Ettl & Gärtner, 1995). The two presently known species of the genus *Kalinella* occur in subaerial corticolous biofilms, in relatively warm habitats. *K. bambusicola* was isolated from a tropical ecosystem and *K. apyrenoidosa* was found in a part of the Mediterranean with a humid subtropical climate, according to the Köppen–Geiger climate classification system (Peel *et al.*, 2007). In addition, the phylogenetically closely related genera *Heveochlorella* and *Phyllosiphon* were also found in various subtropical or tropical subaerial localities (Zhang *et al.*, 2008; Aboal & Werner, 2011). Therefore, we expect that additional taxa of the *Heterochlorella* lineage might be discovered in the little-known phototrophic microbial biofilms of these habitats.

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