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Phyllosiphon duini sp. nov. (Trebouxiophyceae, Chlorophyta), a species isolated from a corticolous phototrophic biofilm

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Abstract – The *Watanabea* clade of the Trebouxiophyceae includes unicellular coccoid green microalgae that mostly thrive in subaerial microhabitats. Recently, a number of new genera and species were described on the basis of the DNA sequence data and morphological observations. The peculiar genus *Phyllosiphon,* which forms a monophyletic clade within the *Watanabea* clade, is characterized by forming siphonous parasitic stages thriving in the leaves of the Araceae. In addition, several previous studies demonstrated that members of the genus *Phyllosiphon* also include free-living chlorelloid individuals that occur on various subaerial substrates. A number of *Phyllosiphon* members sampled from European subaerial microhabitats have so far not been taxonomically described, because they were not available in cultures. In this study, we provide a taxonomic description of a new species of the genus *Phyllosiphon*, *P. duini*, isolated from a corticolous biofilm growing on *Quercus pubescens* in a sub-Mediterranean forest stand. The simple chlorelloid morphology of this strain did not unambiguously distinguish *Phyllosiphon duini* from other closely related members of the *Watanabea* clade. However, phylogenetic analyses based on the 18S rDNA sequences showed that this species clustered in a sister position to a *Phyllosiphon* species previously described from eastern Asia. Similar phylogenetic pattern was also supported by the plastid-encoded *rbc*L gene sequences of members of the *Watanabea* clade. Our data demonstrate that the genus *Phyllosiphon* represents a diverse phylogenetic lineage within the subaerial chlorelloid green microalgae of the *Watanabea* clade.

18S rDNA / green algae / *Phyllosiphon* / *rbc*L / subaerial algae / Trebouxiophyceae / *Watanabea* clade

INTRODUCTION

Phototrophic corticolous biofilms are typically composed of green microalgae belonging to the division Chlorophyta (Štifterová & Neustupa, 2015). The diversity of these microalgae initially appears low due to their simple, unicellular coccoid morphology. However, examination of their DNA sequence data showed that corticolous green microalgae are considerably phylogenetically diversified (Freystein & Reisser, 2010; Rindi *et al.,* 2010; Kulichová *et al.,* 2014). Most notably, the species and genera belonging to the phylogenetically defined *Watanabea* clade in the class Trebouxiophyceae are especially abundant in corticolous and other subaerial microhabitats, such as rock or soil surfaces (Kulichová *et al.,* 2014; Song *et al.,* 2016). Members of the genus *Chloroidium* have often been frequently encountered in corticolous and epilithic microhabitats (Darienko *et al.,* 2010).

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Chloroidium's sister genus *Parachloroidium* includes two recently described species that are only known from European corticolous biofilms (Neustupa *et al.,* 2013b; Kulichová *et al.,* 2014). The genera *Heterochlorella*, *Heveochlorella*, *Kalinella* and *Mysteriochloris* were described from tropical South-East Asian corticolous microhabitats (Zhang *et al.,* 2008; Neustupa *et al.,* 2009; Ma *et al.,* 2013; Song *et al.,* 2016). However, both species of the genus *Kalinella* were also encountered in sub-Mediterranean corticolous microhabitats in Europe (Kulichová *et al.,* 2014). In addition, genotypes belonging to the genus *Heveochlorella* were also reported as frequently occurring folicolous lichen photobionts in tropical habitats of Florida, USA (Sanders *et al.,* 2016). The genera *Watanabea* and *Viridiella*, two deep lineages of the *Watanabea* clade, as well as the genus *Polulichloris*, and one species of the genus *Desertella*, have been found in biofilms growing on soil (Albertano *et al.,* 1991; Fučíková *et al.,* 2014; Song *et al.,* 2015). These chlorelloid microalgae all exhibit the relatively small dimensions of cells that reproduce exclusively asexually by unequally sized autospores. Apart from the above mentioned epiphytic, epilithic and edaphic taxa, the *Watanabea* clade also contains a peculiar parasitic genus, *Phyllosiphon*, which is characterized by the presence of parasitic stages that exhibit a siphonous habit (Aboal & Werner, 2011). Formation of these parasitic stages does not seem to be related to sexual process. Parasitic populations of *Phyllosiphon* form branched filaments in the intracellular matrix of the leaf parenchyma of species in the Araceae family (Procházková *et al.,* 2015; 2016). Previously, the only known DNA sequences from the parasitic members of the *Phyllosiphon* were acquired from infected leaves of subtropical and temperate *Arisarum vulgare* and *Arum italicum* (Aboal & Werner, 2011; Procházková *et al.,* 2015; 2016). Interestingly, the life cycle of these parasitic species of the genus *Phyllosiphon* also includes chlorelloid individuals thriving in corticolous biofilms as free-living algae (Procházková *et al.,* 2016). Recently, an additional *Phyllosiphon* species, *P. coccidium*, was described from a tree bark biofilm in China (Song *et al.,* 2016). Moreover, several additional *Phyllosiphon* genotypes that are closely related to the parasitic and free-living species of *Phyllosiphon* were also reported from various European corticolous and epilithic biofilms (Cutler *et al.,* 2013; Hallmann *et al.,* 2013; Procházková *et al.,* 2015). These genotypes may belong to additional undescribed *Phyllosiphon* species that form their parasitic stages in other species or genera of Araceae. However, they cannot be formally described until the strains are isolated into cultures.

In this study, we investigated a single *Phyllosiphon* strain that was isolated from a corticolous biofilm in a single sub-Mediterranean forest habitat. We characterized the morphology and ultrastructure using light and transmission electron microscopy. We also obtained 18S rDNA and *rbc*L sequences to characterize the phylogenetic position of the strain. The results indicate that it represents a previously unknown lineage of the genus *Phyllosiphon*, which we are describing as a new species, *Phyllosiphon duini*.

MATERIALS AND METHODS

Locality, sampling and cultivation

The novel strain was isolated from the phototrophic corticolous biofilm of *Quercus pubescens* growing in a natural forest close to Duino, Italy (GPS coordinates:

45°46′42″ N, 13°35′37″ E; altitude: 25 m a.s.l.) in April 2016. The sample was isolated from approximately 1 cm^2 of tree bark surface taken from northern side of the trunk at a height of 150 cm. The sample was put into a sterile bag and processed carefully to prevent any cross-contamination. The biofilm was scraped using a sterile dissecting needle into 1.5 ml Eppendorf tube and vortex-mixed for 10 seconds with 1.0 ml of sterile liquid Bold's Basal Medium (BBM) and 0.75 mm diameter sterile glass beads. Then, 40 µl of suspension from each Eppendorf tube was placed onto agar-solidified BBM in Petri dishes. After 6 weeks, algal microcolonies with chlorelloid morphology were isolated onto agar-solidified BBM in test tubes.

Light and electron microscopy

Microphotographs of strains were taken under an Olympus BX51 light microscope with a Canon EOS 700D (Canon, Tokyo, Japan). Transmission electron microscopy was performed following the method described by Procházková *et al.* (2015).

DNA extraction, PCR, and sequencing

The genomic DNA of single algal cultures growing in test tubes was isolated following the protocol described by Procházková *et al.* (2015), or by using the Invisorb Spin Plant Mini Kit (Invitek, Hayward, CA, USA) according to manufacturer's protocol. The extracted solution was diluted to 5-10 ng µl-1 and used for PCR. The plastid *rbc*L gene and the nuclear 18S rDNA marker were PCR-amplified from the genomic DNA. Consequently, the *rbc*L gene was amplified from the strain belonging to the genus *Phyllosiphon* acquired from the corticolous biofilm using the primers phyllrbcLF (5′-TTCCGTATGACTCCACAACAAGG-3′, Procházková *et al.,* 2015) or PRASF1 (5′-ATGGTTCCACAAACAGAAAC-3′, Sherwood *et al.,* 2000) and ellaR2 (5′-TCACGACCTTCATTACGAGCTTG-3′, Neustupa *et al.,* 2013a). The 18S rDNA sequences were obtained using these primer combinations: (a) phyllos-F1 (5′-CGGAGAGAAGGCTTGAGAATCGGCCTT-3′) and 1263R (5′-GAACGGCCATGCACCACC-3′, Pichrtová *et al.,* 2013); (b) NS1 (5′-GTAGTCATATGCTTGTCT-3′, Hamby *et al.,* 1988) and phyllos-R (5′-GGCAGCAAGGCGGGCCGCG-3′); (c) phyllos-F3 (5′-GACTAGGGATCGGC GGGCGTTTCTCGAA-3′) and 1636-57R (5′-GGTAGGAGCGACGGGCGGTGTG-3′, Katana *et al.,* 2001) or 18L (5′-CACCTACGGAAACCTTGTTACGACTT-3′, Hamby *et al.,* 1988). The PCR mix was performed using the methods described by Procházková *et al.* (2015). The PCR amplification was carried out under the following conditions: initial denaturation at 94°C for 5/4 minutes (*rbc*L/18S rDNA); 40/35 cycles of denaturation at 95/94°C for 1 minute/45 seconds, annealing at 50/47/54/52/52°C (for primer combination rbcL-203F and rbcL-991R/phyllrbcLF and ellaR2/PRASF1 and ellaR2/NS1 and phyllos-R/phyllos-F1 and 1263R) for 1/1.5 minutes, and elongation at 72°C for 2/2.5 minutes; final extension at 72°C for 10 minutes. The final segment of the 18S rDNA, which was impossible to obtain using the AmpliTaq Gold 360 DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), was amplified using the PrimeSTAR Max DNA Polymerase (Takara, Kusatsu, Shiga Prefecture, Japan). The PCR reactions were performed in a total volume of 15 µl containing 7.5 µl of PrimeSTAR Max DNA Polymerase (0.1 U), 5.9 µl of sterile Milli-Q water, and 0.3 µl of each primer (phyllos-F3 and 1636-57R or 18L; 25 pmol). In this case the PCR protocol consisted of 10 seconds at 98°C,

followed by 35 cycles of 15 seconds at 65°C, 30 seconds at 72°C, and a final extension of 5 minutes at 72°C. All PCR products were analysed by electrophoresis on 1% agarose gel, and stained with ethidium bromide. Correctly amplified products were cleaned using the GenElute PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol. The purified PCR products were sequenced with the amplification primers at Macrogen in Amsterdam, Netherlands, or at Biocev in Vestec, Czech Republic. Sequencing reads were assembled and edited using SeqAssem 09/2004 (Hepperle, 2004). The sequences of the newly described species *Phyllosiphon duini* are available in the GenBank database under the accession numbers KY977525 and KY977526.

Phylogenetic analyses

The newly determined 18S rDNA and *rbc*L sequences were added to alignments published by Procházková *et al.* (2016), and manually aligned with additional newly published sequences from the GenBank database using MEGA 6 (Tamura *et al.,* 2013). In parallel, we performed the automatically generated 18S rDNA alignment using ClustalW with default settings, integrated in MEGA 6, and subsequently manually edited there. The final 18S rDNA alignments consisted of 1773 and 1778 nucleotides, respectively. The *rbc*L gene alignment was done manually only, because it did not contain any gaps. The final *rbc*L alignment consisted of 1211 nucleotides. All these alignments are available at https://botany.natur.cuni.cz/neustupa/phyllosiphon_duini.html. The appropriate evolutionary models were determined using the Bayesian information criterion (BIC) in MEGA. The BIC selected the GTR+G+I model for the entire 18S rDNA dataset and the 3rd codon position of *rbc*L, the GTR+G model for the 1st codon position of *rbc*L, and the JC+G+I model for the 2nd codon position of *rbc*L. Phylogenetic trees were inferred with Bayesian inference using MrBayes 3.2.2. (Ronquist *et al.,* 2012). Two parallel Markov chain Monte Carlo (MCMC) runs were carried out for 2 million generations, each with one cold and three heated chains. Analyses of the *rbc*L dataset were carried out using a partitioned dataset to assign distinct substitution models to the codon positions. Parameters and trees were sampled every 100th generation for a total of 20 000 trees. After visual inspection of log-likelihood values of sampled trees, the initial 5001 trees of each run were discarded, and posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining 30 000 (15 000 × 2) trees. The maximum likelihood (ML) and weighted maximum parsimony (wMP) analyses for bootstrap supports of individual phylogenetic lineages were calculated using Garli 2.01 (Zwickl, 2006) and PAUP 4.0b10 (Swofford, 2002), respectively. The maximum likelihood (ML) analyses consisted of 100 replicates, using default settings with automatic termination set at 100 000 generations, under the unpartitioned 18S rDNA (GTR + G + I model) and partitioned *rbc*L (GTR + G, JC + G + I, GTR + G + I model for the first, second and third position, respectively) 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), with gap characters treated as a fifth character state. 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. The phylogenetic trees were graphically adjusted in FigTree 1.3.1 (Rambaut, 2009) and Adobe Illustrator CS3.

RESULTS

Phylogenetic analyses

The determined 18S rDNA sequence from the species described below as *Phyllosiphon duini* comprised 2140 bp, including one intron (424 bp), which was identified as the intron group I based on literature data, was not shared with any known member of the *Phyllosiphon* clade. The entire intron was excluded from the alignment. Phylogenetic analyses based on the manually edited alignment of 18S rDNA sequences of the major trebouxiophycean clades showed that our strain clustered within the monophyletic *Phyllosiphon* clade of the *Watanabea* clade in Trebouxiophyceae (Fig. 14) with high statistical support (1.00 BPP /95 ML bootstrap support/100 wMP bootstrap support). *Phyllosiphon duini* formed a moderately supported lineage (0.94/65/53) together with *Phyllosiphon coccidium* (KT950842) and "*Watanabea*" sp. (KU320168). *Phyllosiphon duini* differed from these taxa by 27 and 29 changes, respectively, out of 1773 nucleotide positions of the 18S rDNA alignment. The similar results were given by phylogenic analyses of automatically generated alignment of 18S rDNA sequences. However, a sister position of our strain to a clade comprising *Phyllosiphon coccidium* (KT950842) and "*Watanabea*" sp. (KU320168) was inferred with Bayesian phylogenetic inference only with a low statistical support (0.56 BPP). *Phyllosiphon duini* differed from these taxa by 27 and 28 changes, respectively, out of 1778 nucleotide positions of the automated 18S rDNA alignment.

The analyses of the *rbc*L gene sequence (Fig. 15) suggested that *Phyllosiphon duini* clustered in a sister position with *Phyllosiphon* sp. *k17* (KR154336) with high statistical support (1.00/100/100). This lineage clustered in a sister position with *Phyllosiphon coccidium* (KT950844) with moderate support (0.78/-/66). *Phyllosiphon duini* differed from *Phyllosiphon* sp. *k17* and *Phyllosiphon coccidium* by 2 and 39 substitutions out of 1211 nucleotide positions of the *rbc*L alignment. This lineage was part of the highly supported (1.00/100/100) *Phyllosiphon* clade of the *Watanabea* clade in Trebouxiophyceae, which also included the *Phyllosiphon ari* lineage (KU640391, KU640392), and a lineage comprising *Phyllosiphon arisari* (KR154334) and *Phyllosiphon* sp. (KR154335).

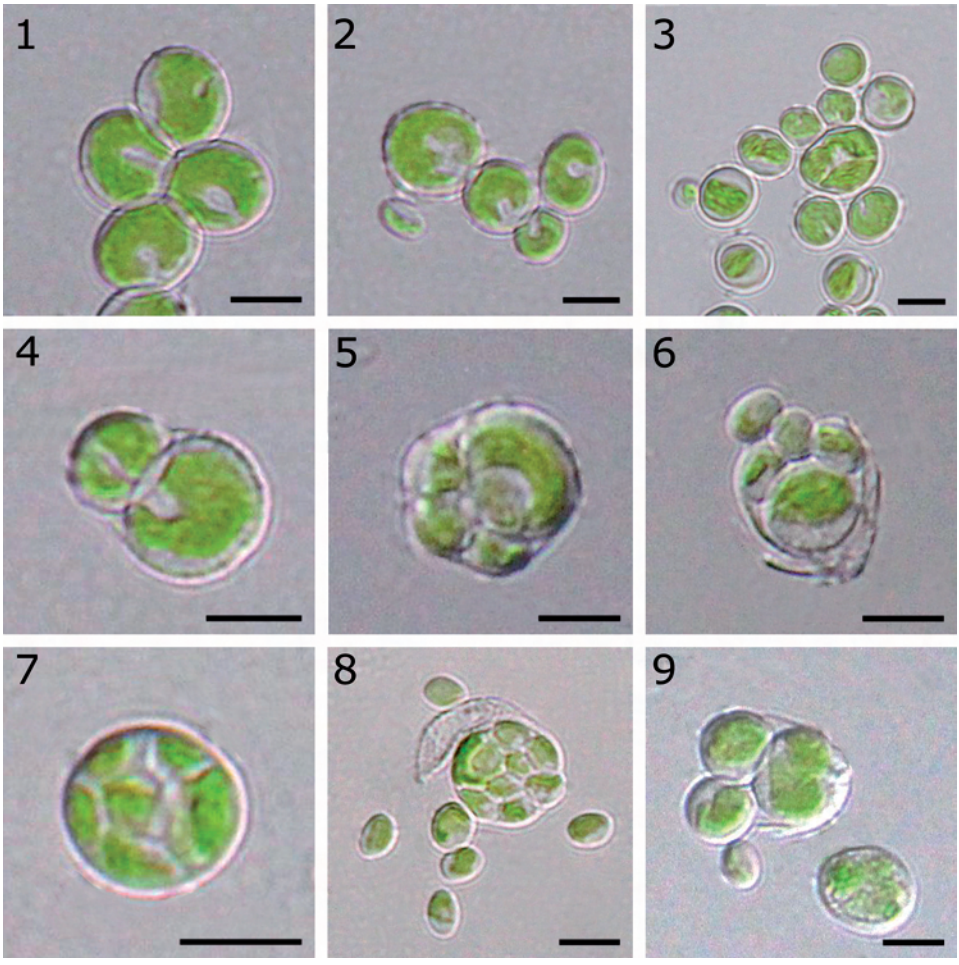
Morphology and ultrastructure

Phyllosiphon duini strain CAUP H8804 formed elliptical cells, 5.5-9.5 μm in diameter. The cells reproduced by 2 to 8 autospores (Figs 4-9). Mostly, there was a single large autospore and 1, 3, 5 or 7 smaller autospores produced within a single sporangium (Figs 4-9). Autosporangia were typically 6.9-12 μm in diameter, however, sporangia with diameter larger than 16 μm were also rarely, but occasionally, observed. The cells contained a single parietal chloroplast (Figs 10, 12-13), and occasionally divided into two or three lobes both in mature vegetative cells and autospores (Figs 1-4). The chloroplast contained electron-dense plastoglobuli (Fig. 1) and small elliptical pyrenoids that were covered by the starch envelope (Figs 2b). Cells also contained extraplastidial oil droplets (Fig. 10). The cell wall was smooth and thin, composed of two layers. An outermost layer had fibrillar character (Figs 10-11).

Phyllosiphon duini K. Procházková, Y. Němcová & J. Neustupa **sp. nov. Figs 1-14**

Description: Vegetative cells solitary, uninucleate. Cells elliptical, 5.5-9.5 µm in diameter. Single parietal chloroplast containing both starch grains and pyrenoids, divided into two or three lobes. Asexual reproduction via 2-8 autospores, 6.9-12(-16) µm in diameter. Sexual reproduction not observed. Differs from all the other species of the *Watanabea*-clade of Trebouxiophyceae in "GCC" triplet codon of alanine at the position 163 to 165 of the single copy chloroplast-encoded *rbc*L gene sequence, and in a presence of the unique intron within 18S rDNA.

Holotype specimen: Strain CAUP C-H8804, based on strain 4A-2 obtained from the holotype, has been cryopreserved in the Culture Collection of Algae of Charles University Prague (CAUP) (http://botany.natur.cuni.cz/algo/caup.html). The strain has also been deposited in CAUP as an active culture, CAUP H8804.

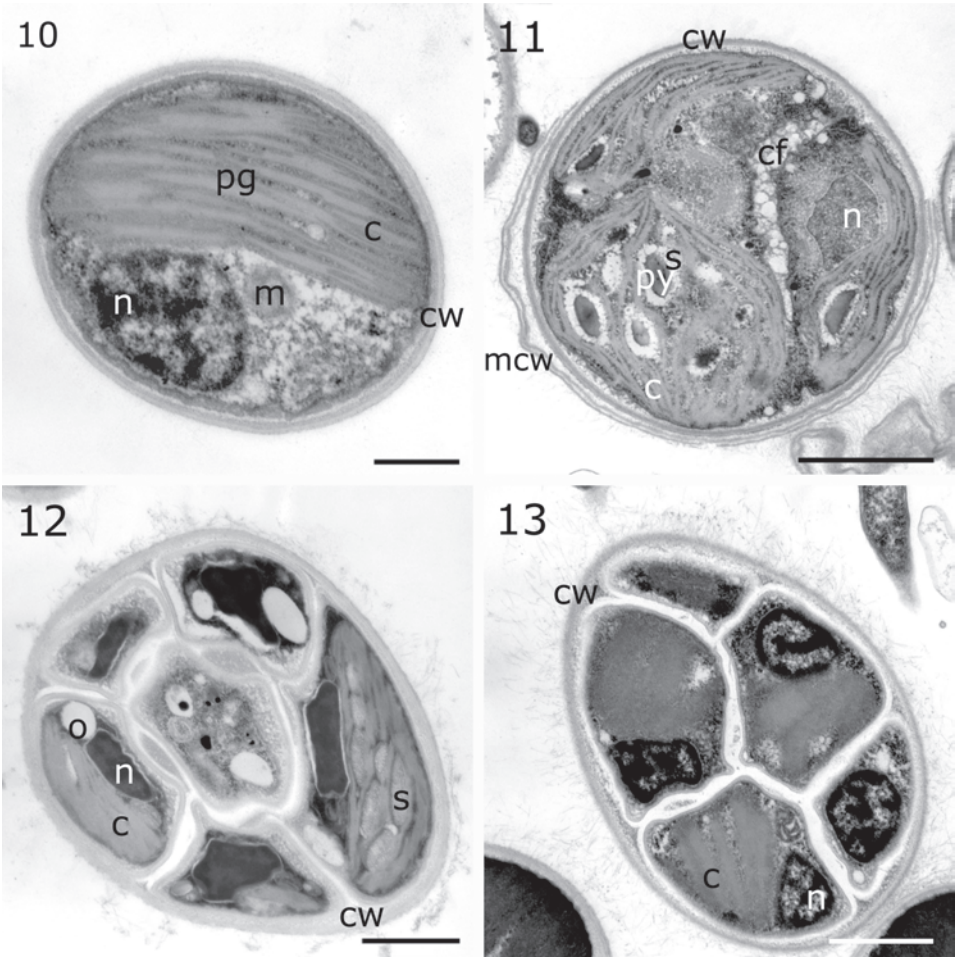


Figs 1-9. Morphology of *Phyllosiphon duini* sp. nov. **1-3.** Vegetative cells. **4.** Two-celled autosporangium. **5-6.** Four-celled autosporangium. **7.** Six-celled autosporangium. **8.** Eight-celled autosporangium and autospores. **9.** Autosporangium, autospore and vegetative cell. Scale bars: 5 µm.

Type locality: Subaerial biofilm on the bark of a *Quercus pubescens* growing in Duino, Italy (GPS coordinates: 45°46′42″N, 13°35′37″E; altitude: 25 m a.s.l.) in April 2016.

Distribution: North-east Italy; species was only found at the type locality.

Etymology: The species name is derived from Duino, a small town close to the type locality.



Figs 10-13. Ultrastructure of *Phyllosiphon duini* sp. nov. **10.** Autospore with a single nucleus, mitochondria and chloroplast. **11.** Ongoing autosporogenesis; note a single autospore that is already separated by cleavage furrow (the right side of the cell); the pyrenoids are covered by the starch envelope; the autosporangium is partly wrapped in a mother cell wall. **12.** Autosporangium with mature autospores inside; autospores have a single nucleus, plastid containing starch grains, and extraplastidial oil droplets. **13**. Autosporangium with five visible autospores. Note the fibrillar nature of the outer layer of the cell wall. Abbreviations: c, chloroplast; cf, cleavage furrow; cw, cell wall; m, mitochondria; mcw, mother cell wall; n, nucleus; o, oil droplet; py, pyrenoid; s, starch grain. Scale bars: 0.5 μm (10), 2 μm (11), 1 μm (12, 13).

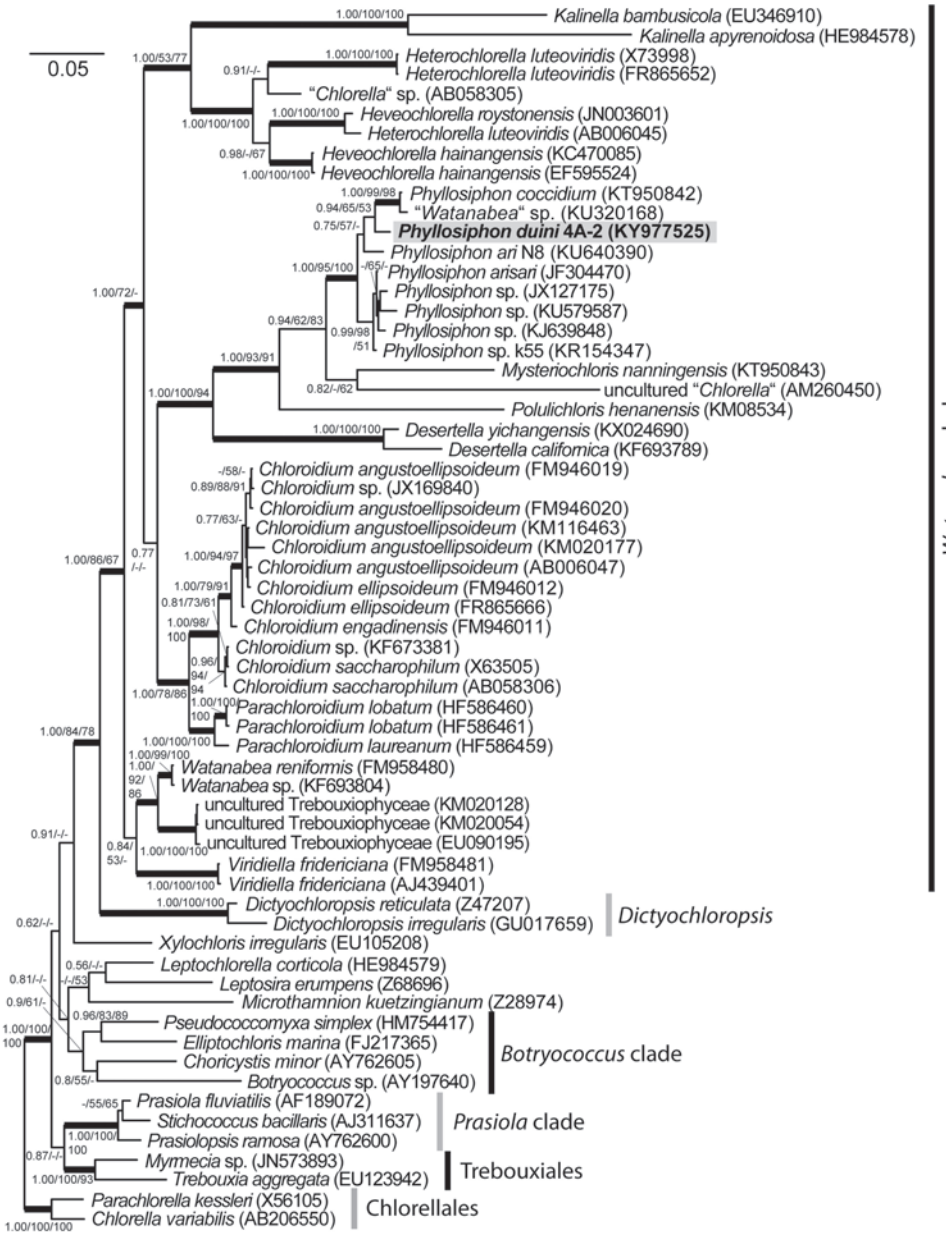


Fig. 14. Bayesian tree of Trebouxiophyceae based on the 18S rDNA dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ML > 50%/MP > 50%). Thick branches represent nodes receiving the highest BPP support (1.00). The sequence newly acquired in this study are shown in bold. The scale bar shows the estimated number of substitutions per site.

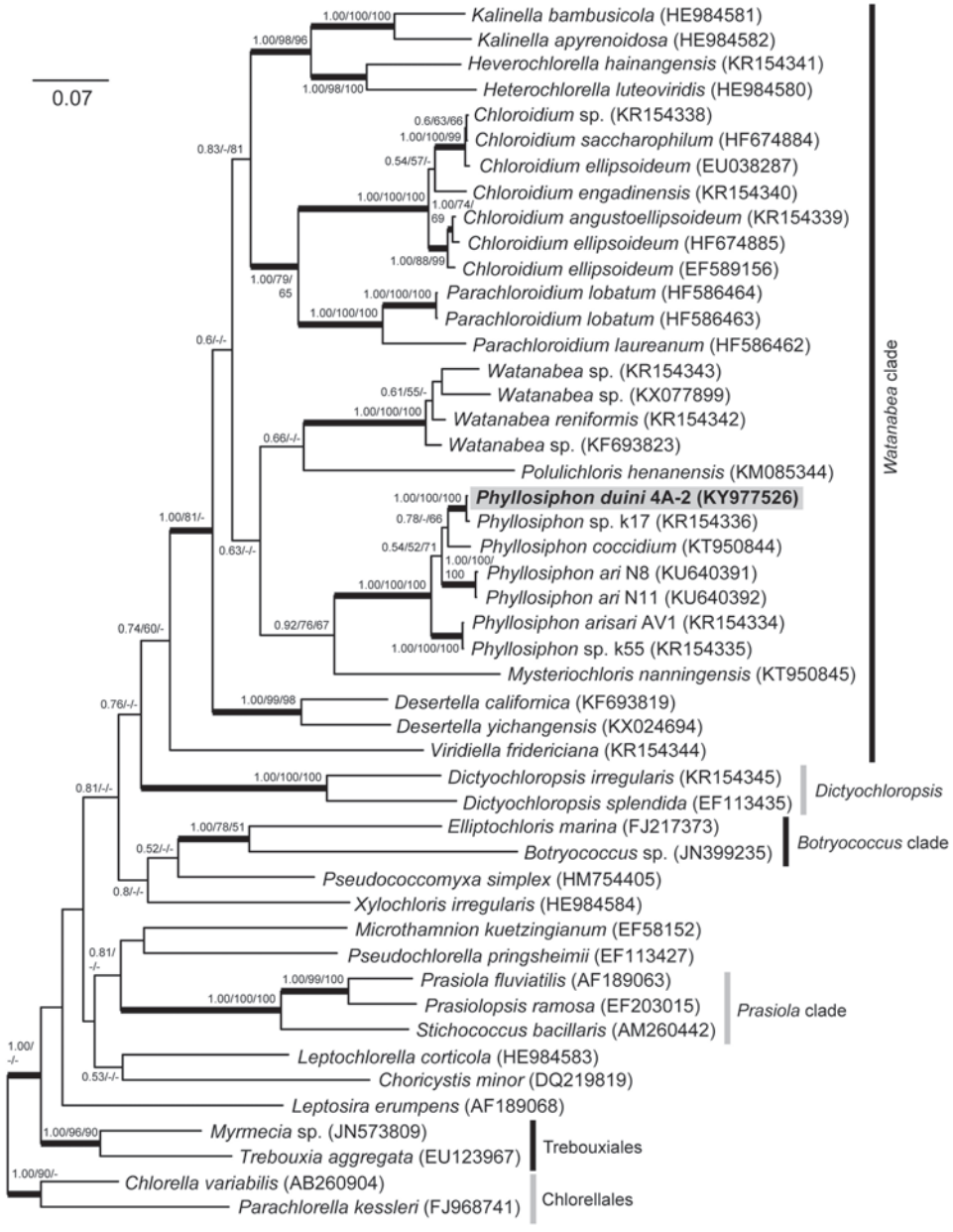


Fig. 15. Bayesian tree of Trebouxiophyceae based on the *rbc*L dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ML > 50%/MP > 50%). Thick branches represent nodes receiving the highest BPP support (1.00). The sequence newly acquired in this study are shown in bold. The scale bar shows the estimated number of substitutions per site.

DISCUSSION

The genus *Phyllosiphon* is a little known member of the trebouxiophycean *Watanabea* clade with a parasitic lifestyle that infects the leaves of various genera of the Araceae (Aboal & Werner, 2011). However, several recent phylogenetically based studies have demonstrated that the members of the *Phyllosiphon* genus also occur as free-living algae on various subaerial substrates throughout Europe and Asia (Cutler *et al.,* 2013; Hallmann *et al.,* 2013; Procházková *et al.,* 2015; 2016, Song *et al.,* 2016). In this paper, by describing a new *Phyllosiphon* species, *Phyllosiphon duini*, which was found in a corticolous biofilm, we are adding a new piece to the puzzle of understanding the diversity of this clade.

The phylogenetic analyses of the 18S rDNA sequences indicated a sister relationship of the newly described species *P. duini* with a lineage containing *P. coccidium*, a coccoid free-living microalga recently described from a corticolous biofilm in China, and "*Watanabea*" sp. isolated from a rock surface in China (Song *et al.,* 2016). The *rbc*L analysis showed that *P. duini* formed a well-supported lineage with *Phyllosiphon* sp. *k17* (Procházková *et al.,* 2015), which clustered in a sister position to *P. coccidium* (Song *et al.,* 2016). Two isolates of *P. duini 4A-2* and *k17* were acquired from the same location in Duino, but on different occasions (*P. duini* was obtained four years later), and are considered as representatives of the same species. Thus, our newly described species *P. duini* is, according to the 18S rDNA and *rbc*L analyses, closely related to *Phyllosiphon* species that also thrive as free-living algae in various subaerial microhabitats. Conversely, both known parasitic *Phyllosiphon* species, *P. ari* and *P. arisari*, are more distantly related to *P. duini* and form their own lineages within the genus. Thus, despite a low number of independent observations of *Phyllosiphon* taxa in natural habitats, it is possible that the genus may be phylogenetically differentiated into lineages with free-living chlorelloid species and lineages containing taxa with alternating parasitic and free-living stages in their life cycle.

This notion was further supported by the fact that, unlike parasitic species *Phyllosiphon arisari* and *P. ari* that usually form the free-living populations in the biofilms on trees occurring close to the vascular plants that host their parasitic stages (Procházková *et al.,* 2015; 2016), we did not find any taxa of the Araceae near to the type locality, nor in other places around Duino, despite our efforts in the field to locate them. Thus, it is possible that the free-living taxa, such as *P. duini*, *P. coccidium*, or other so far undescribed species belonging to the genus *Phyllosiphon,* may only form the simple coccoid stages similar to other members of the *Watanabea* clade (Neustupa *et al.,* 2013a;b; Fučíková *et al.,* 2014; Song *et al.,* 2016). However, it is also possible that parasitic stages of these *Phyllosiphon* taxa are formed occasionally.

In conclusion, our phylogenetic analyses and microscopic observations of the 18S rDNA and *rbc*L gene sequences indicated that *P. duini* represents a new species of the genus *Phyllosiphon*. Considering the growing body of knowledge regarding the diversity of this genus, which occurs not only in the leaves of aracean plants, but also in subaerial microhabitats, we expect that additional taxa of the *Phyllosiphon* lineage might be discovered in other little-known phototrophic microbial biofilms, such as those from other areas with a subtropical climate, or in poorly explored tropical ecosystems. The probable high diversity of tropical *Phyllosiphon* taxa is indirectly supported by observations of parasitic populations of this alga in various tropical aracean plants that form thalli that are morphologically

distinct from those known from the European genera *Arisarum* or *Arum* (Lagerheim, 1892; Tobler, 1917). Additional field surveys are also necessary in subtropical and temperate regions to ascertain the geographical range and habitat distribution of the already described species of this genus.

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