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Phyllosiphon ari sp. nov. (*Watanabea* clade, Trebouxiophyceae), a new parasitic species isolated from leaves of *Arum italicum* (Araceae)

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Abstract

The trebouxiophycean genus *Phyllosiphon* contains unique green algae that thrive as endophytic parasites in the leaves of various members of the Araceae. The DNA sequences of the parasitic populations were originally acquired from infected leaves of subtropical *Arisarum vulgare*. However, several previous studies showed that the members of the *Phyllosiphon* clade also occur as free-living algae on various subaerial substrates across Europe. *Phyllosiphon* infection was also observed on the leaves of the temperate genus *Arum*, but no molecular data was available for these parasites. We recently found these parasites at a single sub-Mediterranean locality. These algae were genetically different from those previously obtained from *Arisarum* leaves on the basis of their 18S rDNA and *rbc*L gene sequences. In the present study, we describe this organism as a new species, *Phyllosiphon ari*. Phylogenetic differentiation of *Phyllosiphon* taxa, correlated with host specificity to different genera of the Araceae, suggests concerted host-pathogen co-evolution driving species diversification within this peculiar green algal lineage.

Keywords: 18S rDNA, Arum, parasitic algae, Phyllosiphon, rbcL, subaerial microalgae, Trebouxiophyceae, Watanabea clade

Introduction

Parasitic microorganisms form a considerable part of eukaryotic diversity. They have evolved from free-living ancestors multiple times during eukaryotic evolution. However, some parasitic taxa transitioned back to a free-living lifestyle from parasitism (Poulin 2007). Knowledge about the strength of a relationship between the host taxa and its parasite helps us to understand the mechanisms that generate and maintain species diversity in parasitic taxa. Parasites are often specialized to a single or a few host species (Poulin 2007).

The parasitic life strategy is relatively uncommon in phototrophic microorganisms. However, a few parasitic algae have adapted to infecting not only other algae or animals, but also vascular plants. Interestingly, microalgae that infect vascular plants belong exclusively to the division Chlorophyta (Smith 1933, Joubert & Rijkenberg 1971).

The chlorophyte order Trentepohliales represents futher green algal lineage thriving on vascular plants in subaerial habitats, both epiphytically, endophytically and parasitically. The most known genera *Trentepohlia* and *Phycopeltis* grows on tree bark or leaf surface of various plants (Guiry & Guiry 2016). This group also includes the genus *Stomatochroon* that occupy the substomatal chambers of plants leaves in tropics, and the genus *Cephaleuros* thriving in intercellular spaces of various plants in tropics and subtropics and cause leaf necrosis (Joubert & Rijkenberg 1971). The division Chlorophyta also encompasses the parasitic genera *Chlorochytrium* and *Rhorochytrium* thriving in intercellular spaces of various freshwater and terrestrial plants, respectively (Joubert & Rijkenberg 1971).

In this study, we focused on the genus *Phyllosiphon*, a little-known member of the trebouxiophycean *Watanabea* clade with a parasitic lifestyle, which infect the leaves of various plants of the Araceae. The type species, *P. arisari* J.G. Kühn, was described from leaves of *Arisarum vulgare* O. Targ. Tozz., growing in the Mediterranean region (Kühn 1878). Five additional *Phyllosiphon* species were described from the leaves of five different aracean genera growing in the tropics, such as *Alocasia* sp., *Philodendron* sp. (Lagerheim 1892), *Zamioculcas zamiifolia* (Tobler 1917), and *Anchomanes difformis* (Mangenot 1948). However, *Phyllosiphon* infection was also morphologically observed on leaves of other host taxa of the Araceae growing in temperate regions, such as *Arisaema triphyllum* (L.) Scott. in North America (Collins 1909, Smith 1933) and *Arum italicum* Mill. (Nicolas 1912) and *Arum maculatum* L. in Eastern France and Scotland (Maire 1908, Sowter 1949). The *Phyllosiphon* thalli are formed by branched filaments containing

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unicellular endospores, which penetrate the intercellular matrix of the leaf parenchyma and thus cause leaf necrosis. This infection is macroscopically manifested by the formation of yellow-green spots on the leaves (Kühn 1878, Maire 1908).

The only known DNA sequences of the parasitic members of the *Phyllosiphon* were acquired from infected leaves of subtropical *Arisarum vulgare* (Aboal & Werner 2011, Procházková *et al.* 2015a). Interestingly, several recent studies demonstrated that populations closely related to those obtained from parasitic populations thriving within the leaves of *A. vulgare* also occur in various subaerial microhabitats throughout Europe as free-living microalgae (Cutler *et al.* 2013, Hallmann *et al.* 2013, Procházková *et al.* 2015a). They thrive there as chlorelloid unicells with morphology typical of the trebouxiophycean *Watanabea* clade (Procházková *et al.* 2015a).

The species and genera belonging to the phylogenetically defined *Watanabea* clade of Trebouxiophyceae occur especially in various subaerial microhabitats. These unicellular chlorelloid microalgae typically reproduce by autospores of unequal size. Three of four species of the genus *Chloroidium* have beeen frequently found in subaerial biofilms, while a single species occurs in freshwater habitats (Darienko *et al.* 2010). The genera *Kalinella* and *Heveochlorella* were described from corticolous biofilms in tropical South-East Asia (Zhang *et al.* 2008, Neustupa *et al.* 2009, Ma *et al.* 2013). The genus *Parachloroidium* was recently described from corticolous samples in sub-Mediterranean habitats (Neustupa *et al.* 2013b). *Desertella*, another recently described genus of the *Watanabea* clade, was found in desert soil in Western USA (Fučíková *et al.* 2015). Futher genus *Polulichloris* was described from soil biofilms in Eastern China (Song *et al.* 2015). *Watanabea* and *Viridiella*, two deep lineages of the *Watanabea* clade, occur in freshwater and terrestrial, ofter very extreme, habitatats (Albertano *et al.* 1991, Hanagata *et al.* 1998, Fučíková *et al.* 2015). However, this clade also containes several taxonomically undetermined DNA sequences that probably represent undescribed taxa (Nyati *et al.* 2007, Kulichová *et al.* 2014).

During our recent investigations of algal leaf parasites thriving in leaves of the Araceae in Mediterranean Europe, we discovered that *Phyllosiphon* populations that occur in the leaves of *Arum italicum*, as well as in corticolous biofilms close to infected plants, form a new, previously unknown phylogenetic lineage within the genus. The main purpose of this study is to describe this lineage as a new species of the genus *Phyllosiphon*, *Phyllosiphon ari*.

Materials and methods

Origin and cultivation of investigated strains

In total, 17 *Phyllosiphon* strains were isolated from infected leaves of *Arisarum vulgare.* At the same time, 10 strains were isolated from infected leaves of *Arum italicum* and subaerial biofilms on tree bark growing in the vicinity of an infected *Arum* population (Table 1). The strain CAUP H8803 isolated from the leaf of *Arum italicum* has been deposited in the Culture Collection of Algae of Charles University Prague (CAUP) (http://botany.natur.cuni.cz/algo/caup.html). Isolation and culturing techniques were performed as described by Procházková *et al.* (2015a). Clones from leaves were isolated from approximatelly 3 mm^2 of infected spots on *Arisarum* and *Arum* leaves under a stereomicroscope Olympus SZ61 (Japan). The leaf sections containing the siphonal filaments with endospores inside were trasferred onto agar-solidified BBM medium in Petri dishes. The endospores were released from these filaments after the addition of liquid BBM medium. After one-week cultivations, these endospores were transformed into autosporangia. In this stage, a piece of clonal colony from each Petri dish was used for DNA isolation. Another piece of the same cell colony was isolated onto agar-solidified BBM medium in test tubes after 1 week.

The biofilm samples were isolated from approximatelly 1 cm^2 of tree bark surface taken from northern parts of tree trunks at a heigh of approximatelly 150 cm above the soil surface. The phototrophic biofilm from each sample was scraped using a sterille dissecting needle into 1.5 ml Eppendorf tube and vortex-mixed for 10s with 1.0 ml of sterile liquid BBM medium and sterile glass beads (0.75 mm in diameter). Then, 40 µl of suspension from each Eppendorf tube was placed onto agar-solidified BBM medium in Petri dishes. After 6 weeks, 2 to 4 algal microcolonies with chlorelloid morphology were isolated onto agar-solidified BBM medium in test tubes.

Light and electron microscopy

Microphotographs of strains and infected leaves of *Arum* were taken under an Olympus BX51 light microscope with a Canon EOS 700D (Canon, Japan) and a Sony Cyber-shot DSC-HX20V camera (Sony, Japan), respectively. For transmission electron microscopy (TEM) preparation, leaf sections with endospore-containing filaments were used 10 months after their collection, together with cells from a culture (strain N8). TEM was performed as described by Procházková *et al.* (2015b).

TABLE 1. List of the *Phyllosiphon* strains used in this study, including collection data, and accession numbers of 18S rDNA and *rbc*L sequences.

Strain no.	Species	Origin	Geographic coordinates	Isolation date	18S rDNA GenBank accession no.	*rbc*L GenBank accession no.
S3-1	*Phyllosiphon arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°18'00.4"N 8°30'28.4"E	02/2014	-	identical to KR154334
S3-2	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°18'00.4"N 8°30'28.4"E	02/2014	-	identical to KR154334
S4-1	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°49'0.5"N 8°28'20.7"E	02/2014	-	identical to KR154334
S4-2	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°49'0.5"N 8°28'20.7"E	02/2014	-	identical to KR154334
S4-3	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°48'59.7"N 8°27'51.9"E	02/2014	-	identical to KR154334
S5-1	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°35'44.1"N 8°16'54.9"E	02/2014	-	identical to KR154334
S5-3	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°35'44.0"N 8°16'53.1"E	02/2014	-	identical to KR154334
S6-1	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°32'45.1"N 8°19'44.6"E	02/2014	-	identical to KR154334
S6-2	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°32'45.1"N 8°19'44.6"E	02/2014	-	identical to KR154334
S6-3	*P. arisari*	*Arisarum vulgare* leaf, Sardinia, Italy	40°32'45.1"N 8°19'44.6"E	02/2014	-	identical to KR154334
LOS-2	*P. arisari*	*Arisarum vulgare* leaf, Lošinj, Croatia	44°30'03.8"N 14°30'12.9"E	03/2015	-	identical to KR154334
LOS-5	*P. arisari*	*Arisarum vulgare* leaf, Lošinj, Croatia	44°29'57.0"N 14°30'16.0"E	03/2015	-	identical to KR154334
ILO-3	*P. arisari*	*Arisarum vulgare* leaf, Ilovik, Croatia	44°27'48.8"N 14°32'40.3"E	03/2015	-	identical to KR154334
ILO-4	*P. arisari*	*Arisarum vulgare* leaf, Ilovik, Croatia	44°27'40.0"N 14°32'36.2"E	03/2015	-	identical to KR154334
ILO-5	*P. arisari*	*Arisarum vulgare* leaf, Ilovik, Croatia	44°27'24.1"N 14°33'07.4"E	03/2015	-	identical to KR154334
ILO-7	*P. arisari*	*Arisarum vulgare* leaf, Ilovik, Croatia	44°27'19.2"N 14°33'12.4"E	03/2015	-	identical to KR154334
ILO-PL	*P. arisari*	*Pistacia lentiscus* bark, Ilovik, Croatia	44°27'24.1"N 14°33'07.4"E	03/2015	-	identical to KR154334
N-A	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°09'58.0"N 14°33'53.4"E	04/2014	-	identical to KU640391
N-B	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°09'58.0"N 14°33'53.4"E	04/2014	-	identical to KU640391
N1	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°09'56.1"N 14°33'08.5"E	04/2015	-	identical to KU640391
N2	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°09'56.1"N 14°33'08.5"E	04/2015	-	identical to KU640391
N3	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°09'56.1"N 14°33'08.5"E	04/2015	-	identical to KU640391
N5	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°9'55.73"N 14°33'43.25"E	04/2015	identical to KU640390	identical to KU640391
N8	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°09'55.6"N 14°33'46.4"E	04/2015	KU640390	KU640391
N10	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°10'01.3"N 14°33'59.9"E	04/2015	-	identical to KU640392
N11	*P. ari*	*Arum italicum* leaf, Krk, Croatia	45°10'01.3"N 14°33'59.9"E	04/2015	-	KU640392
KRK-12	*P. ari*	*Ulmus minor* bark, Krk, Croatia	45°10'1.71"N 14°34'3.6"E	04/2015	identical to KU640390	identical to KU640392

DNA extraction, PCR, and sequencing

The genomic DNA of single algal colonies was isolated following the protocol described by Procházková *et al.* (2015a). The extracted solution was diluted to 5–10 ng/μL and used for PCR. Two molecular markers were PCR-amplified

from the genomic DNA: the plastid *rbc*L gene and the nuclear 18S rDNA. The *rbc*L gene was primarily amplified from the strains using the nonspecific primers rbcL-203F (5′-GAATCWTCWACWGGWACTTGGACWAC-3′) and rbcL-991R (5′-CCTTCTARTTTACCWACAAC-3′) as described in Nelsen *et al.* (2011). Consequently, the *rbc*L gene was amplified both from the strains belonging to the genus *Phyllosiphon* acquired from the corticolous biofilms and those isolated from samples of plant leaves using the primers phyllrbcLF (5′-TTCCGTATGACTCCACAACAAGG-3′, Procházková *et al.* 2015a) or PRASF1 (5′-ATGGTTCCACAAACAGAAAC-3′, Sherwood *et al.* 2000) and ellaR2 (5′-TCACGACCTTCATTACGAGCTTG-3′, Neustupa *et al.* 2013). The 18S rDNA sequences were obtained from *Phyllosiphon* strains with unique *rbc*L sequences using the primers 18S-F and18S-R as described in Katana *et al.* (2001), or using the primer combination phy-F2 (5′-ACTGCGAATGGCTCATTAAATC-3′, Procházková *et al.* 2015a) and 1636-57-R (5′-GGTAGGAGCGACGGGCGGTGTG-3′, Katana *et al.* 2001). The PCR mix was performed as described in Procházková *et al.* (2015a). The amplification conditions were as follows: initial denaturation at 94°C for 5/4 min (*rbc*L/18S rDNA); 40/35 cycles of denaturation at 95/94°C for 1 min /45 s, annealing at 50/47/54/52/54°C (primer combination rbcL-203F and rbcL-991R/phyllrbcLF and ellaR2/PRASF1 and ellaR2/phyF2 and 1636-57-R/18S-F and 18S-R) for 1/1.5min, and elongation at 72°C for 2/2.5 min; final extension at 72°C for 10 min. 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) according to the manufacturer's protocol. The purified PCR products were sequenced with the amplification primers at Macrogen Inc. in Seoul, Korea. Sequencing reads were assembled and edited using SeqAssem 09/2004 (Hepperle 2004). The unique 18S rDNA and *rbc*L sequences of the species, described below as *Phyllosiphon ari*, are available in the GenBank database under the accession numbers KU640390-KU640392 (Table 1).

Phylogenetic analyses

Newly determined *rbc*L and 18S rDNA sequences were added to alignments published by Procházková *et al.* (2015a) and manually aligned with newly published sequences from the GenBank database using MEGA 6 (Tamura *et al.* 2013). Two sequences of the Chlorellales were used as an outgroup. The final 18S rDNA alignment consisted of 1773 nucleotides and 1211 nucleotides for *rbc*L gene. Both alignments are available at http://botany.natur.cuni.cz/neustupa/phyllosiphon_ari.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 1st codon position of *rbc*L, and the JC+G+I model for 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. ML analyses consisted of 100 replicates, using default settings and the automatic termination set at 100 000 generations, under the unpartitioned 18S rDNA and partitioned *rbc*L 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

Phylogenetic analyses based on the 18S rDNA sequences of the major trebouxiophycean clades, together with the representatives of the Chlorellales, showed that our isolates clustered within a monophyletic *Phyllosiphon* lineage of the *Watanabea* clade in Trebouxiophyceae (Fig. 3) with moderate statistical support (0.88 BPP/86 ML bootstrap

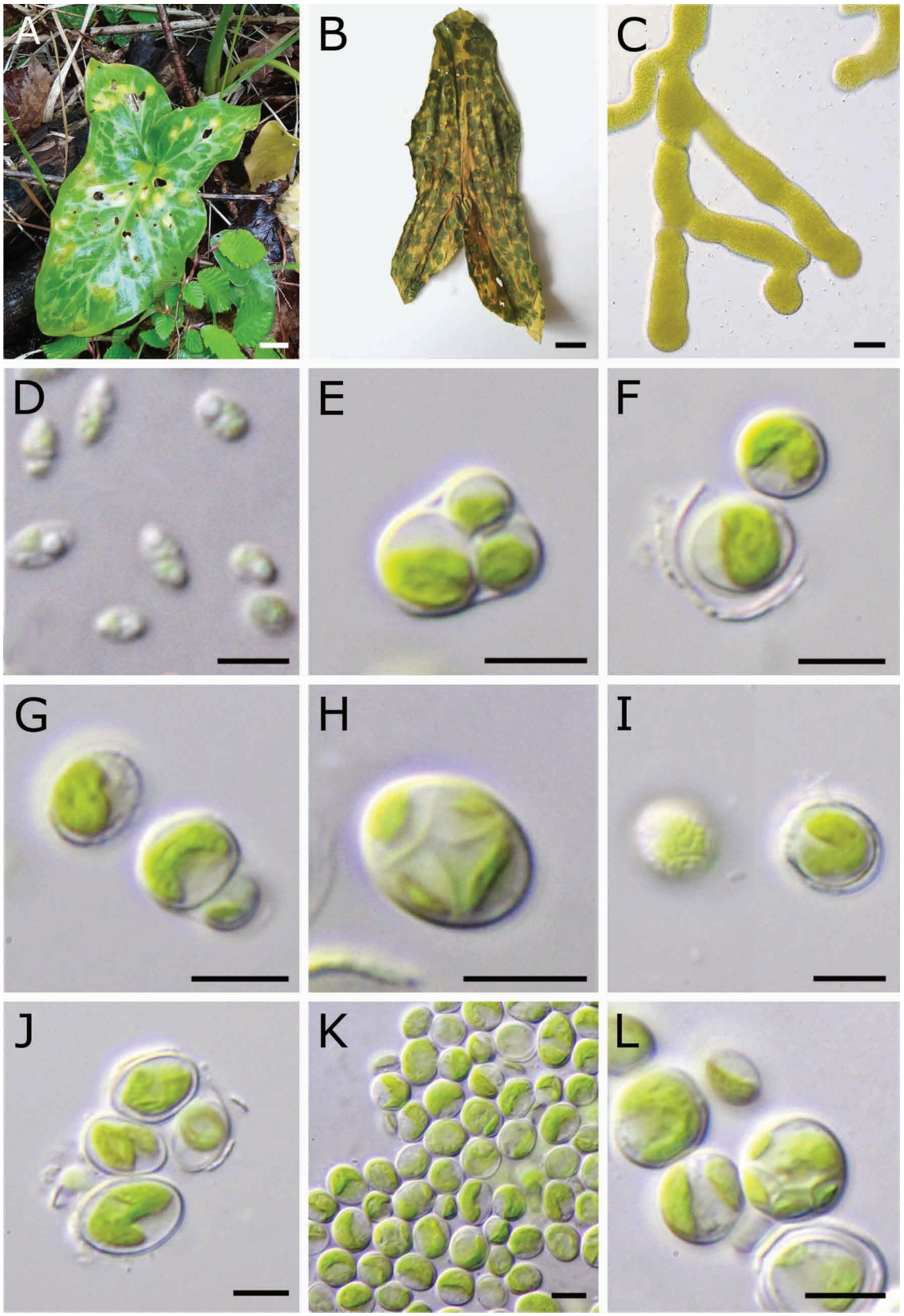

FIGURE 1. Morphology of *Phyllosiphon ari* sp. nov. A–B. A leaf of *Arum* at different stages of infection caused by *Phyllosiphon ari*. C. Branching filaments in the leaf parenchyma containing endospores. D. Detail of *P. ari* endospores released from filaments. E. Autosporangium of *P. ari*, strain N8. F. Vegetative cells with a remnant of the mother cell wall, strain N8. G. Vegetative cell and two-celled autosporangium, strain N8. H. Four-celled autosporangium, strain KRK-12. I. Vegetative cell with a detail of an ornamented cell wall, strain N5. J. Vegetative cells, strain N5. K. Vegetative cells, strain N6. L. Four-celled autosporangium, vegetative cells and autospore, strain N6. Scale bars: 1 cm (A–B), 20 µm (C), 5 µm (D–L).

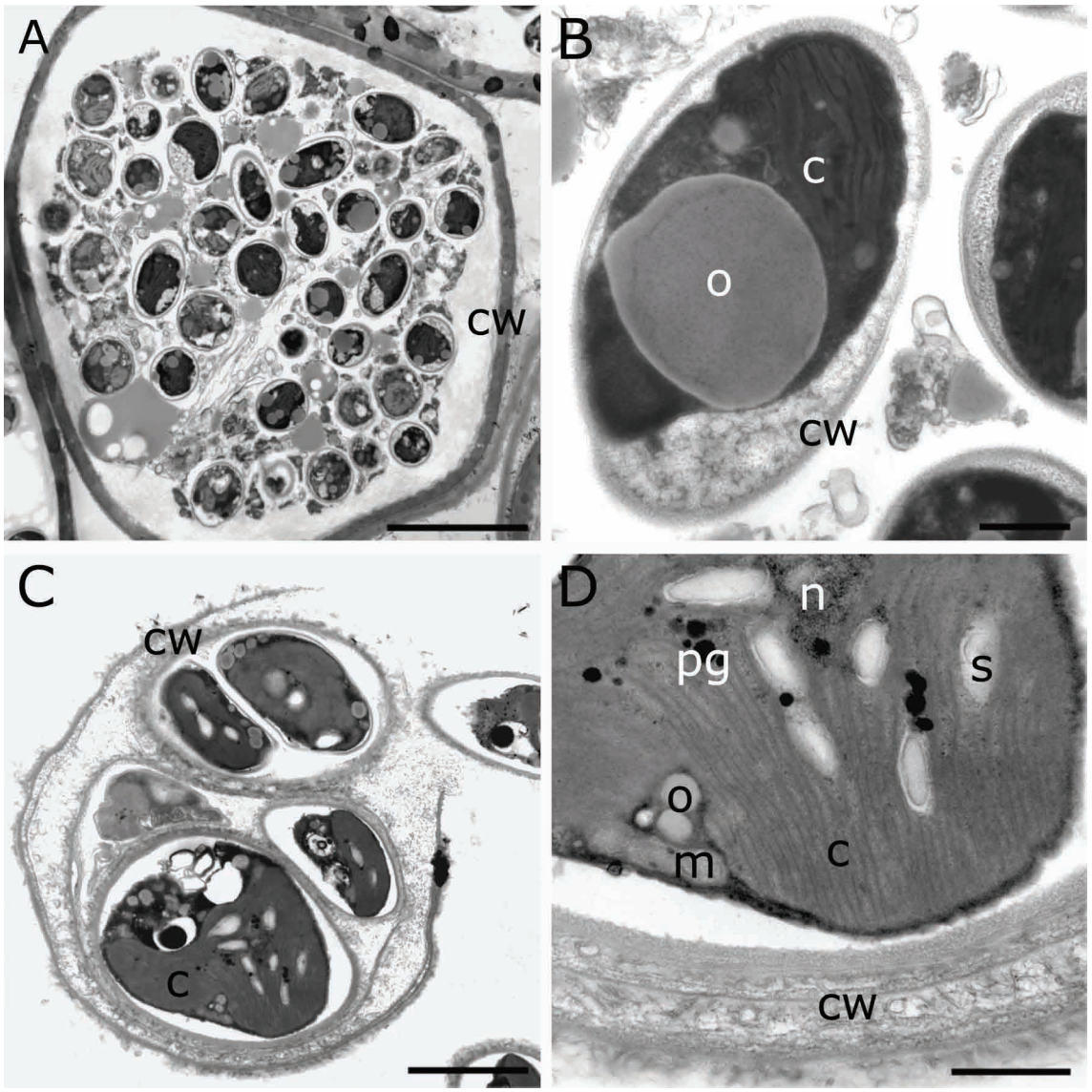

FIGURE 2. Ultrastructure of *Phyllosiphon ari* sp. nov. A. Cross section of parasitic filaments with thick cell wall containing endospores. B. Detail of an endospore with parietal chloroplast and large extraplastidial oil droplet. C. Autosporangium with autospores inside. Note the autospore released by rupture of autosporangial cell wall, strain CAUP H8803. D. Detail of chlorelloid cell with mitochondria, plastid containing plastoglobuli and starch grains, and extraplastidial oil droplet, strain CAUP H8803. Abbreviations: c, chloroplast; cw, cell wall; m, mitochondria; n, nucleus; o, oil droplet; s, starch grain. Scale bars: 0.5 μm (A–B, D), 2 μm (C).

support/100 MP bootstrap support). Three strains of the species, described below as *P. ari*, had identical 18S rDNA sequences, which differed from the *Phyllosiphon arisari* sequence (JF304470) by 26 out of 1773 nucleotide positions of the final 18S rDNA alignment. These 26 nucleotide changes were distributed across whole 18S rDNA, including the variable region V4, which contained six changes, and the V9 region of the 18S rDNA with 6 changes.

Sequences of *rbc*L gene were established for 27 new *Phyllosiphon* strains isolated from samples of corticolous biofilms and leaves of *Arisarum* and *Arum* (Table 1). Analyses of the *rbc*L gene sequences of these strains also illustrated that our isolates clustered within a monophyletic *Phyllosiphon* lineage of the *Watanabea* clade in Trebouxiophyceae (Fig. 4) with high statistical support (1.00/100/100). Seventeen *Phyllosiphon* isolates acquired from samples of *Arisarum* leaves had the *rbc*L sequences identical with the *Phyllosiphon arisari* sequence (KR154334). The remaining ten isolates of the taxon, described below as *Phyllosiphon ari*, had almost identical *rbc*L gene sequences to each other (three *rbc*L gene sequences differed by 1 out of 1773 nucleotide positions of the *rbc*L alignment). These isolates formed a highly supported clade (1.00/100/100), which clustered in a sister position with the *Phyllosiphon* sp. sequence *k17* (KR154336) with high support (1.00/88/74). This clade clustered in a sister position with a highly supported clade (1.00/100/100) comprising *Phyllosiphon arisari* AV1 (KR154334) and *Phyllosiphon* sp. *k55* (KR154335).

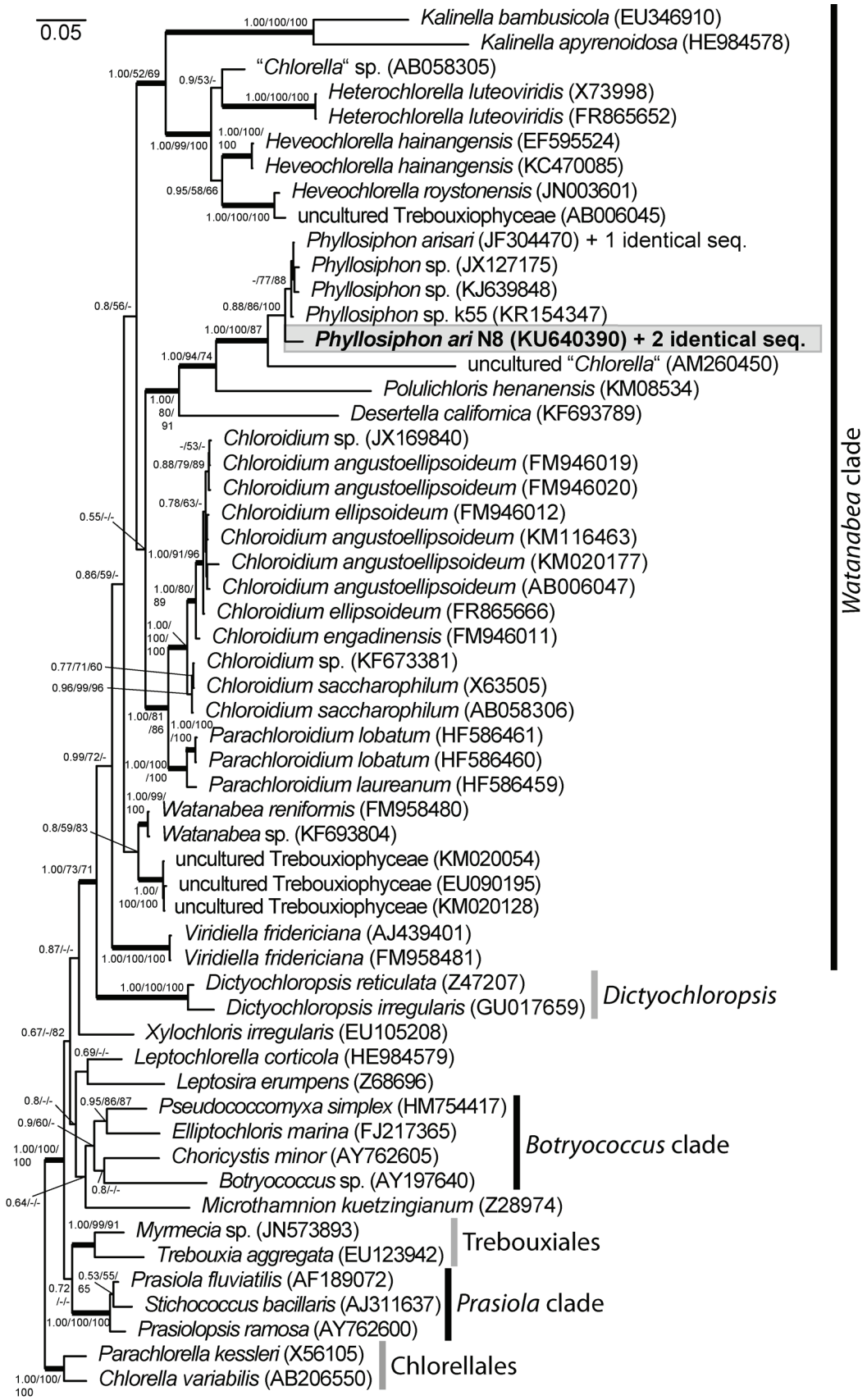

FIGURE 3. Bayesian analysis of Trebouxiophyceae based on the 18S rDNA dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ML > 50 %/MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequences newly acquired in this study are shown in bold. The scale bar shows the estimated number of substitutions per site.

Morphology and ultrastructure

Phyllosiphon ari formed branched filaments in the leaf parenchyma of *Arum italicum*, which were visible as yellowish spots on the fresh leaves (Fig. 1A). Later, as the leaves became dry, they appeared as considerably greener areas against the yellow-brown dry leaf tissue (Fig. 1B). These spots had spherical to ellipsoidal shape, 3–8 mm in diameter (Figs 1A–B). The filaments were 20–45 µm in diameter and grew in the intercellular matrix of the leaf parenchyma (Fig. 1C). The filaments had thick cell walls (Fig. 2A), and their inner space was filled with ellipsoidal endospores 1.5–5.4 µm in diameter (Fig. 1D, 2A–B). Endospores were released from the filaments after re-wetting of the thalli with liquid medium. In culture with agar-solidified medium, the endospores developed into spherical to ellipsoidal coccoid cells 3.7–6.9 µm in diameter. These cells reproduced by two to six asexual autospores with ellipsoidal shape (Figs 1J–L). In most cells, there was a single large autospore, together with one or three smaller autospores produced within a single sporangium (Figs 1E, G, K, L). These autosporangia were typically 5.1–9.6 µm in diameter. Cultured *Phyllosiphon* isolates, acquired from corticolous biofilms, also had coccoid spherical to ellipsoidal vegetative cells 3.7–8.6 µm in diameter, which reproduced by formation of autospores. The autosporangia of these isolates had spherical to ellipsoidal shape 5.4–8.8 µm in diameter (Fig. 1H). The cells contained a single parietal lobed chloroplast, and no pyrenoid was observed within the chloroplast matrix. However, the chloroplast was filled with starch grains (Fig. 2C–D) and electron-dense plastoglobuli (Fig. 2C–D). Cells contained extraplastidial oil droplets (Fig. 2C–D). The cell walls of the vegetative cells and autosporangia were ornamented with numerous small papilae (Fig. 1I).

Formal taxonomic description

Phyllosiphon ari Procházková, Němcová & Neustupa, ***sp. nov.***

Branched filaments 20–45 µm in diameter, macroscopically visible as yellow-green spots 3–8 mm in diameter on leaves of the genus *Arum*. Ellipsoidal endospores solitary, uninucleated, 1.5–5.4 µm in diameter. Vegetative cells solitary, uninucleate. Cells spherical to ellipsoidal, 3.7–6.9 µm in diameter. Single parietal chloroplast containing starch, but no pyrenoid. Asexual reproduction via 2–6 spherical to ellipsoidal autospores, 5.1–9.6 µm in diameter. The species differs from the type species of the genus *Phyllosiphon*, *Phyllosiphon arisari*, by different host species of the parasitic stages, by presence of autospores and autosporangia with a characteristic ellipsoidal shape, the ornamented cell wall, as well as by differences in 18S rDNA and *rbc*L gene sequences.

Type locality:—CROATIA. Krk: the leaf of *Arum italicum* growing along a route near Njivice, 45°09′55.6″N 14°33′46.4″E, 8 m a.s.l., leaf sample collected by K. Procházková, 28 April 2015.

Holotype:—A leaf of *Arum italicum* with the source population of *Phyllosiphon ari* was deposited in the Herbarium collection of the Charles University in Prague (PRC): holotype PRC3715 and isotype PRC3716. In addition, the strain CAUP C-H8803, based on strain N8 obtained from the holotype, has been cryopreserved in the CAUP Culture Collection (http://botany.natur.cuni.cz/algo/caup.html). The strain has also been deposited in CAUP as an active culture, CAUP H8803.

Etymology:—The specific epithet is derived from the host plant genus *Arum*.

Habitat:—Branched filaments thrive in the leaf parenchyma of *Arum*, while the chlorelloid unicells thrive in subaerial biofilms, such as those on a tree bark.

Distribution:—Croatia; species was only found at the type locality.

Discussion

The results presented here show that the parasitic members of the genus *Phyllosiphon* thriving in the leaves of the Araceae are phylogenetically non-homogenous. Our analyses, expanded by newly acquired 18S rDNA and *rbc*L gene sequences from parasitic populations of the *Phyllosiphon* from infected leaves of *Arisarum vulgare* in Sardinia and Croatia, show that these microalgae represent a single homogenous lineage that was previously identified as *P. arisari* (Aboal & Werner 2011, Procházková *et al.* 2015a). However, phylogenetic investigation of parasitic specimens isolated from infected leaves of *Arum italicum* showed that these parasitic microalgae formed a lineage separated from *P. arisari*. While the 18S rDNA sequences of strains belonging to the newly described species *P. ari* were identical to each other, the *rbc*L gene sequences from three *P. ari* samples differed by one substitution change at position 876 (from CTA to CTC; both codons encode the amino acid leucine) of the *rbc*L gene from the remaining seven *rbc*L gene

sequences. However, as all the samples of *P. ari* were acquired from a single population of *A. italicum* growing at a single locality, we did not consider this molecular variability as sufficient for any formal taxonomic conclusions. In addition, this single nucleotide substitution did not change the resulting *rbc*L protein sequence and, consequently, we assume that it represents intraspecific variability within *P. ari*.

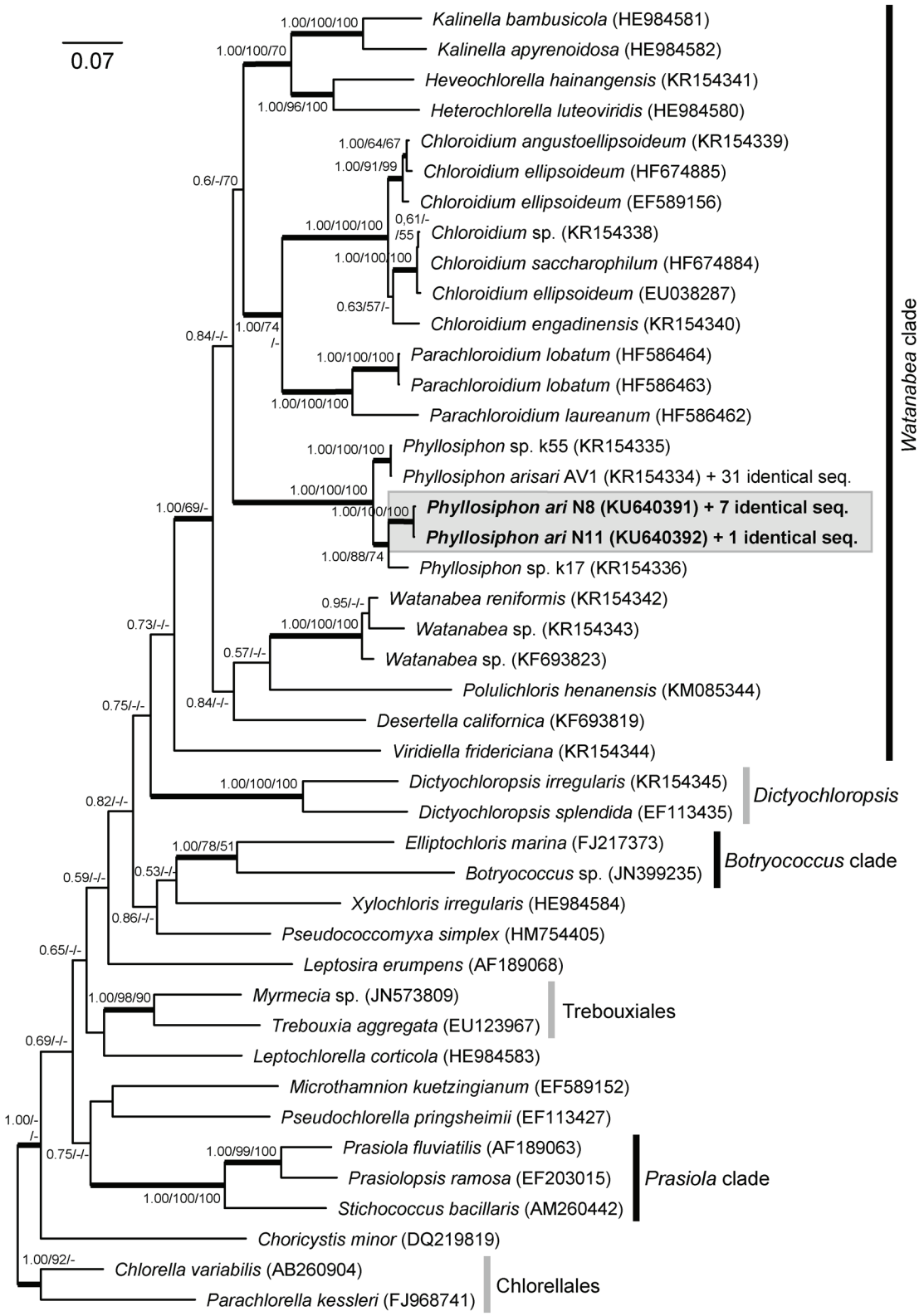

FIGURE 4. Bayesian analysis of Trebouxiophyceae based on the *rbc*L dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ ML > 50 %/MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequences newly acquired in this study are shown in bold. The scale bar shows the estimated number of substitutions per site.

Newly detected parasitic *Phyllosiphon* taxa thriving in the leaves of *Arisarum* and *Arum* supplement the recently recognized diversity of the free-living *Phyllosiphon* populations (Cutler *et al.* 2013, Hallmann *et al.* 2013, Procházková *et al.* 2015a). In the corticolous biofilms, we found the free-living cells with identical sequences as both parasitic *Phyllosiphon* species. However, several additional *Phyllosiphon* genotypes were also reported from the biofilm samples (Cutler *et al.* 2013, Hallmann *et al.* 2013, Kulichová *et al.* 2014). Given the generally scarce knowledge on diversity of microbial pathogens of non-cultivated vascular plants, these lineages may in fact represent additional undescribed *Phyllosiphon* taxa that form their parasitic stages in other species or genera of the Arales. Thus, future studies should characterize these free-living *Phyllosiphon* populations and, possibly, try to detect their parasitic stages in the field. It should be noted that free-living cells of both *Phyllosiphon arisari* and *P. ari* in corticolous biofilms were always found in proximity to the infected *Arisarum* and *Arum* plants. Thus, we suppose that populations of these *Phyllosiphon* species, which we have found in subaerial biofilms, might get there from the decomposing infected leaves, which contained endospores released from the parasitic siphonal filaments. Morphological observations of *Phyllosiphon* cultures suggest that these endospores transform into autosporangia producing minute chlorelloid autospores. Therefore, we presume that free-living *Phyllosiphon* populations may be capable of autonomous reproduction and long-term survival outside their host vascular plant. This hypothesis is indirectly supported by the fact that we were repeatedly able to cultivate parasitic populations of both *Phyllosiphon* species *in vitro* on an agar-solidified inorganic medium. In these cultures, however, *Phyllosiphon* populations never formed any siphonal filaments, but behaved as a typical chlorelloid microalga reproducing solely by autospores with characteristic morphology of the *Watanabea* clade (Ma *et al.* 2013, Neustupa *et al.* 2009, 2013). The *Watanabea* clade includes morphologically and ecologically similar taxa, such as *Parachloroidium*, *Kalinella*, *Heveochlorella* or *Polulichloris*, that have typical chlorelloid morfology and occupying subaerial microbial biofilms (Ma *et al.* 2013, Neustupa *et al.* 2013a,b, Song *et al.* 2015).

In both plant genera—*Arisarum* and *Arum*—known to be infected by *Phyllosiphon* algae in the European habitats, the actual infection mechanism is unknown (Aboal & Werner 2011, Procházková *et al.* 2015a). Tobler (1917) assumed that leaves of the host plants may be infected by *Phyllosiphon* cells during their germination when they penetrate the soil surface. In fact, we repeatedly observed macroscopically visible *Phyllosiphon* infection on young leaves of *Arisarum vulgare* developed during winter season in European Mediterranean habitats. However, whether the germinating leaves really are the critical life cycle stage that becomes infected by the alga remains to tested in future studies.

The fact that we recently discovered a new parasitic lineage of the *Phyllosiphon* might suggest that the currently known *Phyllosiphon* diversity is just the tip of the iceberg. Future studies should identify the diversity of the tropical *Phyllosiphon* members, including these, which were traditionally described based on specimens from the leaves of several different plants of the Araceae (Lagerheim 1892, Tobler 1917, Mangenot 1948). Given that the tropical regions are central to the diversity of the Araceae (Bown 2010), we expect that they could also be central to the diversity of the genus *Phyllosiphon*. Moreover, our data obtained from investigation of the European populations show that phylogenetic differentiation of the *Phyllosiphon* taxa is correlated with the host genus-level identity. This suggests that there is probably concerted host-pathogen co-evolution that may be a prime factor driving species diversification within this peculiar green algal lineage.

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