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Study program: Botany

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Fylogenetická diverzita rodu *Phyllosiphon* (Trebouxiophyceae, Chlorophyta) v Evropě

Phylogenetic diversity of the genus *Phyllosiphon* (Trebouxiophyceae, Chlorophyta) in Europe

Ph.D. thesis

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Prague, 2020

Declaration

I declare that I have written this thesis independently, using the listed references; or in cooperation with other paper co-authors. I have not submitted this thesis, or any of its parts, to acquire any other academic degree.

Prague, 21.6. 2020 …….

Kateřina Procházková

Acknowledgements

Firstly, I would like to thank my supervisor, Jiří Neustupa, for leadering of this work, patience, and especially for the idea and opportunity for studying such a charming and interesting alga such as the *Phyllosiphon*. I am aware that without Jiří, who bring me infected leaves of *Arisarum* and *Arum*, this thesis would not be written. I also thank for the freedom in the research I have taken, it has given me a lot. Jiří also helped me every time I needed to.

I would like thank to Jana Kulichová for supervising me during first two years of my Ph.D. study and for inspiring ideas, helpful advices and comments to this thesis. I also thank to a co-author Yvonne Němcová for her encouragement and support.

Many thanks go to Lenka Flašková, a laboratory technician, for her care and very helpful advices.

I am also grateful my friends and colleagues from the Phycology Research Group for kind and friendly atmosphere and for a lot of out-of-school experiences.

I would also like to thank my dear colleagues from my current job for encouraging me to finish my study.

Finally, I would like to thank especially my family, Dan and his family, for supporting me and encouraging me during the study and writing of this thesis.

ORIGINAL PAPERS

This thesis is based on the following three papers:

I. Procházková, K., Němcová, Y., Kulichová, J. & Neustupa, J. (2015) Morphology and phylogeny of parasitic and free-living members of the genus *Phyllosiphon* (Trebouxiophyceae, Chlorophyta). *Nova Hedwigia* 101: 501–518.

II. Procházková, K., Němcová, Y. & Neustupa, J. (2016) *Phyllosiphon ari* sp. nov. (*Watanabea* clade, Trebouxiophyceae), a new parasitic species isolated from leaves of *Arum italicum* (Araceae). *Phytotaxa* 283: 143–154.

III. Procházková, K., Němcová, Y. & Neustupa, J. (2018) *Phyllosiphon duini* sp. nov. (Trebouxiophyceae, Chlorophyta), a species isolated from a corticolous phototrophic biofilm. *Cryptogamie, Algologie* 39: 23–34.

Author´s contribution

Paper I. Jiří Neustupa planned the study. Jiří and Jana Kulichová helped me with the sampling. I isolated and cultivated the samples, photographed the infected leaves, branched filaments and cultures, and performed the molecular reactions. Yvonne Němcová performed the transmission electron microscopy. I prepared picture tables. J. Neustupa hepled me with phylogenetical analyses. I deposited sequences into the GenBank database. J. Neustupa wrote a part concerning on a single codon instertion of the *rbc*L gene. I wrote the remaining part of the manuscript, and J. Neustupa helped me to improve the text.

Paper II. Jiří Neustupa and I planned the study. J. Neustupa and I perfored sampling. I isolated and cultivated the samples, photographed the infected leaves, branched filaments, endospores and monoclonal cultures, performed the molecular reactions and phylogenetic analyses. I also deposited sequences into the GenBank database. Y. Němcová performed the transmission electron microscopy. I prepared picture tables. I wrote the paper, and J. Neustupa helped me with the final improvements on the manuscript.

Paper III. Jiří Neustupa and I planned the study. I performed the sampling. I isolated and cultivated the samples, photographed monoclonal cultures, performed the molecular reactions and phylogenetic analyses. I also deposited sequences into the GenBank database. Y. Němcová performed the transmission electron microscopy. I prepared picture tables. I wrote the paper, and J. Neustupa helped me with the final improvements on the manuscript.

On behalf of all the co-authors, I declare the keynote participation (as first author) of Kateřina Procházková in completing the research and writing the papers, as described above.

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Jiří Neustupa

Abstact

The trebouxiophycean genus *Phyllosiphon* includes unique and little known phototrophic green algae that form endophytic siphonous stages within the members of Araceae. In total, six parasitic species of the genus *Phyllosiphon* were originally described from the leaves of Araceae, but the only known DNA sequences were acquired from *P. arisari*, a species that infects leaves of *Arisarum vulgare* growing in the Mediterranean region. Surprisingly, the environmental sequences closely related to those obtained from *P. arisari* were recently detected in samples of phototrophic biofilms thriving on sandstone substrates in Germany and Northern Ireland. However, these studies did not provide any information about morphology of these microalgae.

In this thesis, I was looking for *Phyllosiphon* parasites in the leaves of terrestrial members of the Araceae growing in the Mediterranean Europe in order to find out whether parasitic algae of the *Phyllosiphon* isolated from different taxa of the Araceae are phylogenetically differentiated, or they form a single lineage within the *Watanabea* clade of Trebouxiophyceae. In addition, corticolous biofilms from various localities in Europe were sampled in order to detect any presence of *Phyllosiphon* cells outside their hosts. Both the parasitic thalli and chlorelloid unicells of *Phyllosiphon* isolated from corticolous biofilms were cultivated to understand their life cycle. The unialgal cultures were established for subsequent morphological and ultrastructural observations and for sequencing of the 18S rDNA region and the *rbc*L gene to clarify their phylogenetic position.

Infected leaves of *Arisarum* were found in several regions of the Mediterranean. In addition, *Phyllosiphon*-infected leaves of the genus *Arum* were also found at a single locality in the sub-Mediterranean. It was shown that the parasitic members of the genus *Phyllosiphon* thriving in the leaves of the Araceae are phylogenetically non-homogenous. While the parasitic populations of *P. arisari* isolated from infected leaves of *Arisarum vulgare* represent a single homogenous lineage, the investigation of parasitic specimens isolated from leaves of *Arum italicum* showed that these parasitic microalgae formed a lineage separated from *P. arisari*, which suggest concerted host-pathogen co-evolution driving diversification within the genus. Subsequently, the parasitic populations from *Arum italicum* were described as a new species, *P. ari*. In subsequent analyses it was shown that the free-living populations with identical DNA sequences as both parasitic *Phyllosiphon* species occur in microalgal corticolous biofilms close to the infected plants. In addition, several additional genotypes related to the *P. arisari* and *P. ari* were also identified in corticolous biofilm communities. One of these microalgae was described as a new species *P. duini*. These free-living *Phyllosiphon* microalgae asexually reproduce by autospores. Likewise, parasitic populations of *P. arisari* and *P. ari* also form *in vitro* chlorelloid stages, which suggests that they are capable of autonomous reproduction outside their hosts.

Abstrakt

Rod *Phyllosiphon* (Trebouxiophceae) zahrnuje unikátní a málo známé fototrofní želené řasy, které vytvářejí endofytní sifonální stádia uvnitř zástupců čeledi Araceae. Dosud bylo popsáno šest parazitických druhů rodu *Phyllosiphon* z listů áronovitých rostlin, avšak jediné známé DNA sekvence byly získány z *P. arisari*, druhu, který napadá listy *Arisarum vulgare* rostoucí ve Středomoří. Environmentální sekvence blízce příbuzné s těmi získanými z *P. arisari* byly překvapivě nedávno objeveny ve vzorcích fototrofních biofilmů rostoucích na pískovcových substrátech v Německu a Severním Irsku. Tyto studie však neposkytly žádné informace o morfologii těchto řas.

V této práci jsem hledala parazity rodu *Phyllosiphon* v listech suchozemských zástupců Araceae rostoucí v mediteránní Evropě za účelem zjištění, zda jsou parazitické řasy rodu *Phyllosiphon* izolované z různých taxonů čeledi Araceae fylogeneticky odlišené, anebo vytvářejí jednu linii uvnitř *Watanabea* kladu z třídy Trebouxiophyceae. Kromě toho byly odebrány vzorky kortikoidních biofilmů z různých lokalit v Evropě, s cílem zaznamenat přítomnost buněk rodu *Phyllosiphon* mimo jejich hostitele. Parazitické stélky a chlorelloidní stádia rodu *Phyllosiphon* izolované z kortikolních biofilmů byly kultivovány za úřelem pochopení jejich životních cyklů. Jednořasové kultury byly ustanoveny pro následné morfologické a ultrastrukturální pozorování a pro sekvenování jejich 18S rDNA oblasti a *rbc*L genu pro objasnění jejich fylogenetické pozice.

Napadené listy rodu *Arisarum* byly nalezeny v několika oblastech Mediteránu. Kromě toho, listy rodu Arum napadené řasou *Phyllosiphon*, byly také nalezeny na jedné lokalitě v sub-Mediteránu. Ukázalo se, že paraziticti zástupci rodu *Phyllosiphon* žijící v listech Araceae jsou fylogeneticky různorodé. Zatímco parazitické populace *P. arisari* isolované z napadených listů rostliny *Arisarum vulgare* představují jednu homogenní linii, zkoumání parazitických řas z listů *Arum italicum* ukázalo, že tyto parazitické mikrořasy vytvářejí linii oddělenou od *P. arisari*. Tato zjištění naznačují koevoluci patogenu a hostitele řídící genetické rozrůznění v rámci tohoto rodu. Parazitické populace druhu *Arum italicum* byly následně popsány jako nový druh, *P. ari*. V následujících analýzách bylo ukázáno, že volně žijící populace s DNA sekvencemi identickými s oběma parazitickými druhy rodu *Phyllosiphon* se vyskytují v kortikolních biofilmech v blízkosti napadených rostlin. Několik dalších genotypů příbuzných *P. arisari* a *P. ari*, bylo navíc zaznamenáno ve společenstvech kortikolních biofilmů. Jedna z těchto mikrořas byla popsána jako nový druh *P. duini*. Tyto volně žijící mikrořasy rodu *Phyllosiphon* se asexuálně rozmnožují pomocí autospor. Stejně tak, parazitické populace *P. arisari* a *P. ari* vytvářejí chlorelloidní stádia *in vitro*, což naznačuje, že jsou schopny autonomní reprodukce mimo jejich hostitele.

CONTENT

1 INTRODUCTION

1.1. Delimitation of algal species

For more than two centuries, scientists attempt to describe the diversity of all the species inhabiting our planet. The importance of describing and naming new taxa (alpha taxonomy) transcends the mere cataloguing of species. This discipline is essential to all other biological disciplines, which use scientific names of species as one of basic comparative units (de Queiroz 2005). Therefore, the appropriate species delimitation is essential for a correct understanding of their ecology, biogeography or phylogeny. However, using of the species names is much more important for political conventions as currency in framing their conservation efforts (De Clerk 2013). Although most taxonomists would probably agree that species are real and important, their delimitation is sometimes extremely difficult, because the absence of a general agreement regarding both the definition of the species and methods for inferring of species boundaries and number of species (de Queiroz 2007). The problem of the species delimitation is complicated by the fact that species are not stable entities and they tend to evolve over time. Therefore, we are surrounded by a plethora of species that vary based on their evolutionary ages, which can make it extremely difficult to perform species delimitation and identification. To date, more than twenty species concepts have been proposed (Mayden 1999). These species concepts reflect different biological properties of species and different approaches to their study. Many of these concepts are thus mutually incompatible, as they can lead to different conclusions concerning the boundaries and number of species (de Queiroz 2007).

1.1.1. Species concept in algal systematics

Until recently, three species concepts (morphological, biological and phylogenetic species concept) have been most commonly used in algal systematics. Traditionally, species have been delimited using morphological species concept, which is based on discontinuities in morphological variation of the comparable species observable by the bare eye or with the assistance of light or electron microscope (Mann 1999, Pröschold & Leliaert 2007). This concept has long dominated in algal systematics. Although discontinuities in morphological

variation will in many instances correspond to species boundaries based on variation in DNA sequence data, there are many phenomena, such as convergent morphological evolution, morphological stasis, phenotypic plasticity or polymorphism, which underestimate or overestimate the conclusions on species diversity strictly based on morphological criteria (Verbruggen 2014). Consequently, the morphological characters have lost credibility as the primary species delimitation criteria for many algal groups (De Clerck et al. 2013). In some cases, though, morphological criteria still prevail in assessing of species boundaries in some algal groups, such as silica-scaled chrysophytes (Kristiansen 2007), diatoms (Van de Vijver & Houk 2019) or desmids (Šťastný & Kouwets 2012).

To overcome difficulties associated with morphological data, other species concepts have been applied to assess species boundaries in algae. The biological species concept has been applied in some algal groups that frequently reproduce sexually (Mann 1999). However, this concept is not applicable for most of algae, because the direct observation of sexual processes is lacking for majority of protist species, including algae (Dunthorn & Katz 2010). The invention and routine use of DNA sequencing techniques during the past two decades has resulted in an explosion of additional data that have been used for species discovering and delimitation (De Clerk et al. 2013). These data have been incorporated into the phylogenetic species concept that defines species as the smallest biological entities that are diagnosable and/or reciprocally monophyletic (Mayden 1997). The genetic data have helped to identify species complexes that consist of two or more species hidden under one traditionally defined species practically within all algal groups (Huss et al. 1999, Amato et al. 2007, Pawlowski et al. 2008, Nemjová et al. 2010, Belton et al. 2014). These findings suggest that algal diversity is far from being completely described. Presence of such cryptic diversity is related to "low morphological complexity problem", i.e. with the absence of sufficient morphological characters that could be used for a clear distinguishing of one species from another (Verbruggen 2014). This problem is especially significant in morphologically simple taxa that have a few discriminating features (Verbruggen et al. 2009), such as subaerial green algae with coccoid morphology discussed in chapter 1.2.1.

1.1.2. The genetic markers used for identification and delimitation of green algae

Among the green algae, nuclear-encoded 18S rDNA sequences have been the primary phylogetic markers for inferring phylogenetic relationships (Pröschold & Leliaert 2007). The 18S rDNA is especially very useful for the recognition of individual genus-level taxa

(Němcová et al. 2011, Hodač et al. 2012, Neustupa et al. 2013b). However, phylogenetic analyses based on the 18S rDNA sequences showed that this marker alone can provide neither sufficient phylogenetic signal to resolve all relationships among genus-level lineages nor enough variability to distinguish closely related species (Darienko et al. 2018, Li et al. 2020). A broad sampling of the 18S rDNA among various species and genera of green algae makes this marker a very useful tool for identification of new, previously unknown lineages (Fučíková et al. 2014, Song et al. 2016). The *rbc*L gene, ITS rDNA (i.e. ITS1-5.8S-ITS2 region) or ITS2 rDNA sequences has gradually become the second markers that has been together with the 18S rDNA (separated or concatenated) used in most of recent taxonomic studies (Luo et al. 2010, Fučíková et al. 2014, Song et al. 2016, Li et al. 2020). All these markers were recommended for species delimitation of green algae (Leliaert et al. 2012). Phylogeny based on the ITS rDNA or *rbc*L sequences generally coincides with the 18S rDNA-based tree topologies, even though they are more variable than the 18S rDNA sequences. However, using of the ITS rDNA sequences brings several problem, such as slow coalescence and intragenomic variation (Leliart et al. 2014). The *rbc*L gene contrary offers a number of practical aspects, such as faster coalescence within species lineages and relatively easy amplification, compared with nuclear loci.

Recently, several additonal methods were used for the species delimitation of green algae. The mapping of compensatory base changes (CBCs) in the secondary structure of the ITS-2 rRNA is probably the most used of them (Bock et al. 2011). Previous studies showed that occurrence of the CBCs in the conserved regions of helices II and III of the ITS-2 rDNA correlate with reproductive isolation of the biological species (Coleman 2000, Müller et al. 2007). However, later studies illustrated that the absence of CBCs should not be interpreted as proof that the specimens belong to a single species (Caisová et al. 2011). Therefore, the CBCs are not always suitable for the species differentiation.

1.1.3. The unified species concept and polyphasic approach

Nowadays, most algal taxonomists often prefer the multidisciplinary (also called polyphasic) approach for species delimitation. This approach combines several modern methods, such as light microscopy, transmission electron microscopy, mating experiments or sequencing techniques. However, as was mentioned above, application of these methods,

which are related to different species concepts, may generate the data that can lead to contradictory conclusions regarding species boundaries. The recently proposed unified species concept offers the solution for elimination of conflicts between rival species concepts (de Queiroz 2005, 2007). According to the unified species concept, species are considered as separately evolving metapopulation lineages; the species definition criteria, such as morphological differentiation, reproductive isolation, reciprocal monophyly or distinct occupation of ecological niches, are considered as secondary properties that serve as important biological evidences relevant to assessing separation of lineages (Fig. 1). These properties commonly arise at different times or in irregular order during the process of lineage divergence, causing discrepancies between different species concepts. Some properties may not even appear during the process of speciation. The presence of any one of the properties provides evidence for lineage separation. However, a highly corroborated hypothesis of existence of separate species requires multiple lines of evidence (de Queiroz 2007).

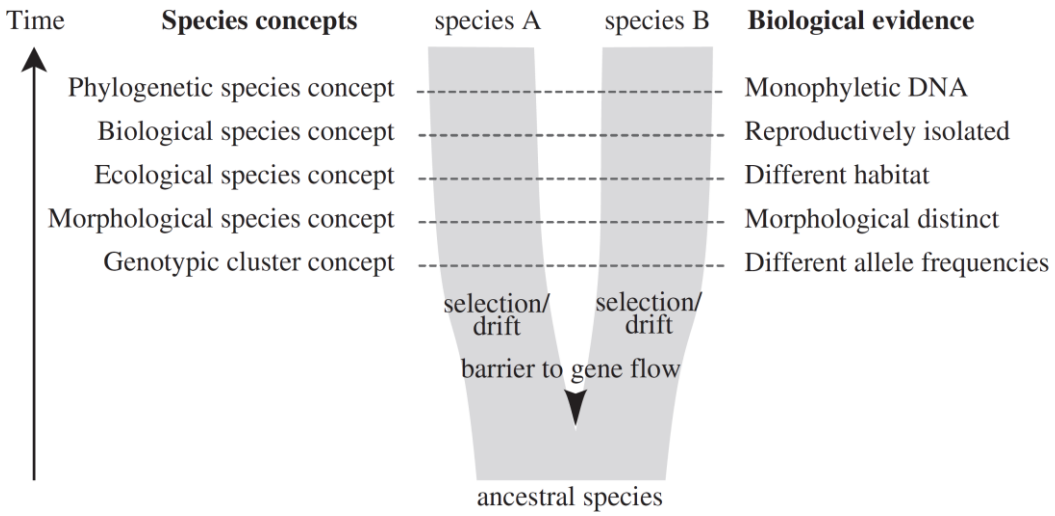

Fig. 1. A highly simplified diagram representing the speciation process. Populations of ancestral species are separated by interrupted gene flow at the beginning of speciation. The selection and drift will result in divergence of two daughter lineages. During the speciation process, these daughter lineages will acquire different properties, which have traditionally served as biological evidence for species delimitation, corresponding to different species concepts. These properties do not necessarily arise at the same time or in regular order. Some properties may not even appear. After Leliaert et al. (2014).

1.2. Aero-terrestrial environment and its algal inhabitants

Aero-terrestrial algae are widespread across many non-aquatic habitats. They include, according to the definition of Ettl & Gärtner (1995), both terrestrial algae and aerophytic algae. Terrestrial algae include euterrestrial (occuring on soil surface), hypolithic/endolithic (inside the rocks) and hydroterrestrial (on permanently wet soil) taxa. Aerophytic algae are further divided by substate type to include epiphytic (occuring on bark and leaves), xylophytic (on wood), lithophytic (on natural rocks and artificial substrates) algae, as well as lichen photobionts. Aerophytic algae may also form parasitic stages within the plant tissues (see chapters 1.3 and 1.4, **papers I, II**).

Aero-terrestrial habitats represent extreme environments characterized by fluctuating dry conditions, overall low nutrient availability, extreme temperature fluctuations and usually also intense light irradiation, including the UV part of the spectrum. Microalgae occurring in these microhabitats are faced with frequent extreme levels of these environmental conditions, which is in contrast with generally more stable aquatic habitats (Hoffmann 1989). To overcome these stress situations, aero-terrestrial microalgae have developed morphological and physiological adaptations, e.g. the thallus simplification with low surface-to-volume ratios, production of mucilaginous envelopes retaining moisture, production of resting stages, the capability to utilize water in the form of vapour or production of pigments acting as protection against the UV irradiation (Hoffmann 1989, Lopez-Bautista et al. 2007). Tolerance of individual algae to stress factors, such as drought, UV radiation or air pollution, ultimately affects their occurrence in aero-terrestrial habitats (Freystein et al. 2008, Lüttge & Büddel 2010).

Although phycologists have been describing the terrestrial microalgae since the beginning of the 19th century (Agardh 1824, Kützing 1849), these organisms have generally received far less attention compared to marine and freshwater microalgae (Johansen & Shubert 2001, Lopez-Bautista et al. 2007). However, knowledge of their diversity and ecology has recently rapidly increased due to application of molecular genetic methods in microalgal research. Primarily, it has been proved that a great deal of phylogenetic diversity is often hidden behind identical or highly similar morphologies (Lopez-Bautista et al. 2007). Although this phenomenon is common in all groups of organisms, including plants, animals and other microorganisms, it is much more

pronounced in aero-terrestrial microalgae that have the limited number of phenotypic characters useful for their reliable morphological identification (de Quieroz 2007). The morphological structure of their cells converged, as a result of stress connected with frequent desiccation, towards a relatively limited number of types (coccoid, sarcinoid and filamentous morphology) in multiple different and often unrelated lineages. Due to this phenomenon microscopic identification of these algae is usually difficult, and is often only possible to higher taxonomic units, such as genera or species complexes (Neustupa & Štifterová 2013). Given the impossibility of their reliable morphological identification, it is necessary to use the DNA sequencing to identify species and to describe new ones using the polyphasic approach (Neustupa et al. 2013a, b). Such detailed examination of taxonomic status of different populations requires previous cultivation of acquired samples on agar plates, morphology-based identification of algal colonies and subsequent establishment of unialgal clonal cultures. This approach based on cultivation also makes it possible to observe and describe the life history of aero-terrestrial algae in detail. However, on the other hand, culture-dependant approach itself affects the obtained pattern of community composition. In fact, culturing selects the genotypes, which culture conditions meet (Lakeman et al. 2009). The different growth rates also may affect abundance and composition of microalgal assemblages (Campo et al. 2013). For example, the fast-growing species are favored in culture conditions, while they may have been present in low abundance in the original natural sample.

Subaerial (a synonym for aerophytic) biofilms represent one of the specific microhabitats in the aero-terrestrial environment. They represent miniature worlds that are located at the interface of atmosphere and lithosphere (Gorbushina & Broughton 2009). Subaerial biofilms occur practically on all types of solid aero-terrestrial substrates, such as external parts of soil, surfaces of rocks, tree barks, perennial leaves of vascular plants, and even artificial substrates (Thompson & Wujek 1997, Gorbushina & Broughton 2009). These biofilms are formed by heterogeneous matrix of diverse microorganisms held together and tightly bound to substrate surface (Rosenberg 1989). They comprise heterotrophic and phototrophic microorganisms, such as bacteria, cyanobacteria, algae and fungi, in diverse proportions (Gorbushina & Broughton 2009). Consequently, this differences in composition are reflected in varying macroscopic colouring (green, orange, brown or black) of subaerial biofilms.

For historical reasons, most studies of the diversity, taxonomy and ecology of subaerial algae have been carried out in Europe (Lopez-Bautista et al. 2007). Therefore, almost any detailed, modern investigation of subaerial microhabitats from other continents have led to the discovery of new species or even genera, as evidenced by multiple recent taxonomic studies performed outside Europe that lead to description of new diatom (Van de Vijver et al. 2002) and green algal flora (Li et al. 2020). In other words, the lack of exploration of subaerial habitats is considerable. A number of taxa have never been recorded after their description; so, we are missing the information about their biogeography. However, increasing interest of phycologists in subaerial microhabitats have gradually been improving this situation. Typically, members closely related to species recently described from tropics, were later recorded during the studies performed elsewhere (Hodač et al. 2012). Kulichová et al. (2014) even identified multiple genotypes identical to the described coccoid green microalgal species that have been so far known only from their type localities. In addition, a few recent studies based on the 18S rDNA sequencing of environmental samples of various subaerial communities suggested that a lot of genera are phylogenetically diversified and widely distributed in subaerial microhabitats (Hallmann et al. 2013, Johnston et al. 2018, Kirchhoff et al. 2018, Zhu et al. 2018). Some of these molecular OTUs (operational taxonomic units) may represent true species. If this prediction is right, or these OTUs are only the sequencing artefacts, can only be tested after isolation of these specimens into clonal cultures and their subsequent detailed examination by polyphasic approach.

Microbial communities are permanently disturbed by wind, which cause that small fragments of substrate along with biofilms are picked up from the surface and transported over long distances (Gorbushina et al. 2007). In contrast to freshwater algae, subaerial algae are well adapted to fast changing and adverse environmental conditions. Their resting stages are resistant to desiccation and high UV radiation, which are crucial abilities for successful survive transportation over long distances in the air (Karsten et al. 2005, Gustavs et al. 2010, Souffreau et al. 2013). These facts suggest that the subaerial algae should be uniformly and randomly distributed compared to freshwater microalgae, for which water bodies represent isles in the mainland and dispersion between them is often difficult. However, biogeography of subaerial microalgae is still far from explored due to insufficient data.

During the last decades, two contrasting views regarding diversity and biogeography of protist taxa have emerged. On the one hand, the ubiquity model suggests that most protist have cosmopolitan distribution. According to this model, small size of protists and ability to create dormant stages allow the large-scale dispersal of these microorganisms, which is not limited by geographical barriers (Finlay 2002, Fenchel & Finlay 2004). This dispersal is, due to the huge size of their populations, so frequent that allopatric speciation or extinction are very rare. Consequently, individual protist species occur worldwide, wherever they have suitable conditions. Thus, this model supposes that the global protist diversity is low (Fenchel & Finlay 2003). Symbiotic or parasitic host-specific protists limited by the distributrion of their host organism are, of course, excluded (Fenchel 2005). According to the contrasting moderate endemicity model, the global protist diversity is expected to be extraordinaly high and represented by a wide range of distribution patterns, i.e. some species have cosmopolitan distribution, whereas other (probably rare) species have restricted distribution facilitated by their limited spatial dispersal due to lack of any suitable resting stages (Foissner 2006). Recent molecular studies support the second model; they showed that at least some members of subaerial microalgae probably have cosmopolitan distribution areas (Kulichová et al. 2014, Ryšánek et al. 2015, 2016, Pinseel et al. 2019). However, it should be mentioned that the ubiquitous genera apparently include species with limited geographical distribution (Souffreau et al. 2013, Ryšánek et al. 2015, Pinseel et al. 2019). Nevertheless, it is also possible that the presumably restricted distribution of some subaerial species may be given by their insufficient sampling. Some members may appear endemic only as long as no other close relatives to them are known and their status may easily be lost with the availability of new sequences from different regions and ecosystems.

1.2.1. Corticolous biofilms and their algal inhabitants

Corticolous biofilms that occur on the tree bark represent an important part of epiphytic microhabitats. This ecological group includes biofilms on perennial parts of vascular plants, i.e. on tree barks, perennial leaves and needles. They are relatively common and well visible in various ecosystems; they may be green or orange in color, which is caused by the different groups of microalgae that dominate in these biofilms (Fig. 2). Corticolous biofilms generally represent microhabitats that are more arid than the soil, because most of the rainfall that falls on the tree trunk is not maintained and falls to the

ground or evaporates (Hoffmann 1989). However, fissures in tree bark create a special microclimate that retains moisture for a longer time, as they are protected against the desiccation. Consequently, these fissures may have slightly higher amounts of nutrients compared to the surrounding tree bark surface that microalgae need for their survival and reproduction (Štifterová & Neustupa 2017).

Fig. 2. Phototrophic corticolous biofilms. A. Tree bark of beech (*Fagus sylvatica*) covered by green biofilm. B. A detail of a green biofilm on a beech tree (*Fagus sylvatica*). C. Cells of the green algal genera *Desmococcus* (Chlorophyceae) and/or *Apatococcus* (Chlorellales, Trebouxiophyceae). D. Tree bark of an apple tree (*Malus* sp.) covered by red biofilm. E. A detail of a red biofilm on an apple tree. E. Filaments of *Trentepohlia umbrina* (Trentepohliales, Ulvophyceae). After Lütge & Büdel (2010).

Despite physiognomic uniformity of corticolous biofilms, these assemblages are non-randomly structured by combination of various small to large scale processes, affecting species distribution and abundance. Several recent studies have clarified some general patterns of their spatial distribution. On larger scale, such as among climatically different regions, the geographic distance, correlated with the differences in temperature and humidity, seems to be the most significant factor for the distribution of major microalgal taxa in corticolous and other subaerial biofilms or soil (Rindi & Guiry 2004, Bates et al. 2013, Neustupa & Štifterová 2013). On a smaller (mesoscale) level, i.e., tens of kilometers within a single region, or tens of meters within a single locality, the physicochemical factors related to specific characters of tree bark substrata, such as pH, nutrients or water retention potential of individual tree species, seem to determine community structure of corticolous biofilms in the sense of their species composition and abundance (Neustupa & Štifterová 2013, Kulichová et al. 2014, Štifterová & Neustupa 2015). These factors probably act as environmental filters in local species sorting. In addition, some other factors, such as light conditions or substrate age, also determine the occurrence of individual microalgae in these biofilms (Hedenås et al. 2007, Neustupa & Škaloud 2010, Neustupa & Štifterová 2013). On an even smaller scale, i.e. on a single host tree, microstructural heterogeneity of the substrate (such as rough or smooth tree bark), as well as neutral processes, such as local extinction, high rates of dispersal, and colonization of the substate by newly arrived taxa, have significant effect on structuring of corticolous communities rather than environmental or spatial factors (Štifterová & Neustupa 2017).

The corticolous biofilms are mainly composed of green algae (Streptophyta and Chlorophyta) and cyanobacteria, while other microalgal groups with taxa occuring in terrestrial ecosystems, such as diatoms, xantophyceans and eustigmatophyceans, are considerably less abundant (Rindi et al. 2010, Štifterová & Neustupa 2015). The green algal

phylum Streptophyta is the evolutionary lineage that gave rise to land plants and within this lineage the corticolous microalgae are found in the classes Zygnematophyceae, Klebsormidiophyceae and Chlorokybophyceae (Fig. 3). The majority of corticolous green microalgae, nevertheless, belong to three lineages of the phylum Chlorophyta, the classes Trebouxiophyceae, Chlorophyceae and Ulvophyceae. Out of these three classes, the Trebouxiophyceae comprise most corticolous microalgae, and many of them are very common and widespread genera (e.g. *Coccomyxa*, *Stichococcus* and *Asterochloris*) (Kulichová et al. 2014, Darienko et al. 2015, Škaloud et al. 2015). Compared to that, the members of the classes Chlorophyceae and Ulvophyceae are especially abundant in freshwater and coastal marine habitats, respectively, though they comprise several aero-terrestrial genera (e.g. *Bracteacoccus* and *Chlorococcum* within the Chlorophyceae; and all the representatives of the order Trentepohliales within Ulvophyceae) (Fig. 3; Škaloud 2009, Leliaert et al. 2012).

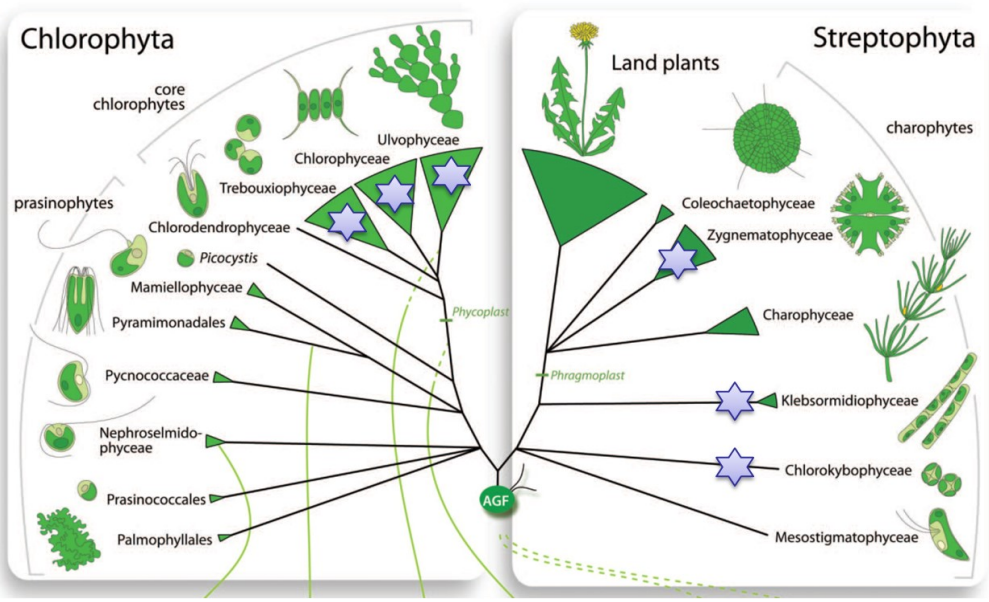

Fig. 3. Diversity of corticolous green algae (blue star symbols) mapped along tree of life of the Viridiplantae. After Leliaert et al. (2012).

As I mentioned in the previous paragraph, the majority microalgae occuring in corticolous microhabitats belong to the chlorophyte class Trebouxiophyceae. Their morphology is mostly coccoid, sometimes filamentous or sarcinoid. The coccoid life forms are probably best adapted to the significant stress connected with frequent desiccation

typically encountered in terrestrial habitats as they include the highest number of representatives in corticolous biofilms. In addition, recent investigations have proved that traditional taxa are composed of a number of phylogenetic lineages that have identical or almost identical morphologies. The cryptic species diversity is especially conspicuous in the "*Chlorella*" morphotypes (e.i. unicellular green algal species with globular to oval cells reproducing entirely by asexual autospores), which was found to be polyphyletic, comprising several unrelated phylogenetic lineages dispersed over two classes of Chlorophyta, the Trebouxiophyceae and the Chlorophyceae (Huss et al. 1999). Although a number of chlorelloid species have been isolated from various freshwater habitats (Krienitz & Bock 2012) and (more rarely) from marine habitats (Aslam et al. 2007), these microalgae are probably most abundant and diversified in subaerial biofilms and in soil (Ettl & Gärtner 1995). Most of these subaerial chlorelloid microalgae probably belong to the *Watanabea* clade of the class Trebouxiophyceae (Neustupa et al. 2009, Kulichová et al. 2014, Darienko & Pröschold 2019a).

1.2.2. The Watanabea *clade of Trebouxiophyceae*

According to the recent study of phylogeny of green algae published by Friedl & Rybalka (2011), the *Watanabea* clade represents one of five well-supported clades of the Trebouxiophyceae. A number of recent taxonomic studies illustrated that the *Watanabea* clade is phylogenetically diversified and represents one of the trebouxiophycean lineages that includes taxa mostly occurring in aero-terrestrial habitats (Fig. 4).

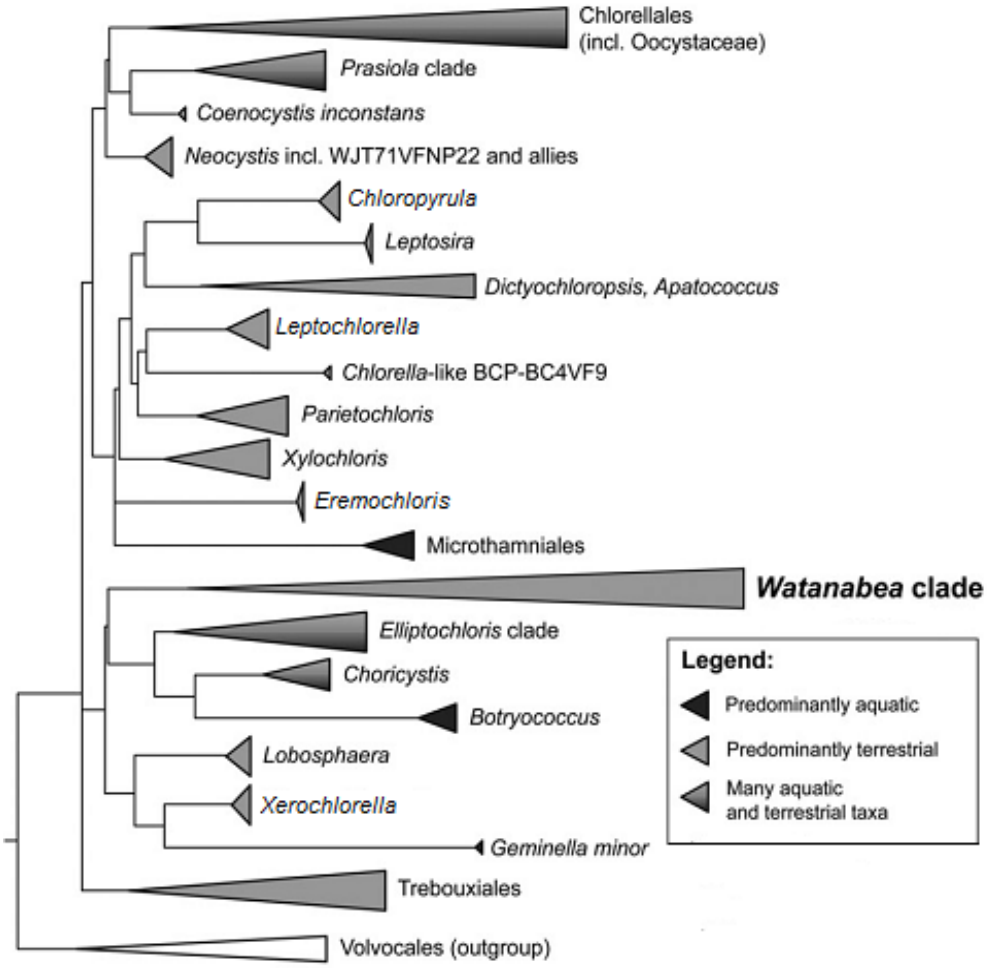

Fig. 4. A schematic phylogenetic tree of the Trebouxiophyceae based on analyses of the 18S rDNA dataset illustrating the habitat distribution of major lineages. Triangles represented by collapsed major clades of trebouxiophyceans are filled with colors corresponding to the predominant habitat preference found in the clade (gray color represents terrestrial taxa, black color the aquatic taxa, mixed gray illustrates aquatic and terrestrial taxa). After Fučíková et al. (2014).

The *Watanabea* clade includes ten genera. Practically all of them form well-supported lineages within the the 18S rDNA phylogenetic tree (Fig. 5). Several genera are represented by only one or two species that are often only known from their type locality. However, the genera *Chloroidium* Nadson, *Watanabea* Hanagata, Karube, Chihara & Silva and *Jaagichlorella* Reisigl are widely distributed across different habitats and regions. The genus *Chloroidium* currently includes nine species, four of them are known only from various aero-terrestrial habitats, the remaining five species were found in marine, freshwater

and aero-terrestrial environment (Darienko et al. 2018). These nine species also include two species of the sister genus *Parachloroidium* Neustupa & Škaloud that are only known from European corticolous biofilms (Neustupa et al. 2013b, Kulichová et al. 2014), which, however, was recently merged with the genus *Chloroidium* (Darienko et al. 2018). The genus *Watanabea* includes five species, three of them were found in European epilithic biofilms or in soil; two species were described from freshwater habitats (Darienko & Pröschold 2018). The recently described genus *Massjukichlorella* Darienko & Pröschold was described from a corticolous biofilm in temperate Europe (Darienko & Pröschold 2019a). Two other species of the *Massjukichlorella* were isolated from epiphytic biofilms in tropical China (Li et al. 2020). The recently re-established woldwide genus *Jaagichlorella* includes six species (Darienko & Pröschold 2019b). Two species of the genus *Heveochlorella* Zhang, Huss, Sun, Chang & Pang were transferred to this genus together with one species of the *Heterochlorella* Neustupa, Němcová, Eliáš & Škaloud that were originally described from tropical South-East Asian tree bark biofilms. Later, additional specimens of these species were isolated from subaerial microhabitats outside the tropics (Darienko & Pröschold 2019b). In addition, genotypes belonging to *Jaagichlorella* were reported as frequently occurring foliicolous lichen photobionts in tropical habitats of Florida, USA (Sanders et al. 2016). The genus *Kalinella* Neustupa, Němcová, Eliáš & Škaloud and *Mysteriochloris* Song, Hu, Zhu, Wang, Liu & Hu 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, members of the genus-level lineages are probably much more widely distributed. Both species of the genus *Kalinella* were also encountered in the sub-Mediterranean corticolous microhabitats in Europe (Kulichová et al. 2014). The genus *Viridiella* Albertano, Pollio & Taddei, a deep lineage of the *Watanabea* clade, as well as the type species of the genus *Polulichloris* Song, Zhang, Liu & Hu, and one species of the genus *Desertella* Fučíková, Lewis & Lewis, have been found in biofilms growing on various soil surfaces (Albertano et al. 1991, Fučikova et al. 2014, Song et al. 2015). Interestingly, another species of the *Desertella* was described from river phytoplankton in China (Song et al. 2015). Two species of the *Polulichloris* were recently decribed from epiphytic biofilms from tropical China (Li et al. 2020). All these chlorelloid microalgae are typical by relatively small dimensions of cells that reproduce exclusively by asexual, unequally sized autospores. Consequently, these relatively widely differentiated species and genera cannot be clearly distinguished by morphology alone. Their clear identification requires DNA sequencing methods that help to determine exact phylogenetic affiliation at

species or genus level. These methods were also recently used in study by Aboal & Werner (2011) that suggested that, in addition to the above mentioned predominantly subaerial taxa, the *Watanabea* clade also contains a parasitic genus *Phyllosiphon* Kühn, which is characterized by the presence of parasitic stages that exhibit a siphonous habit dissimilar to other members of the *Watanabea* clade. This genus is the subject of this thesis.

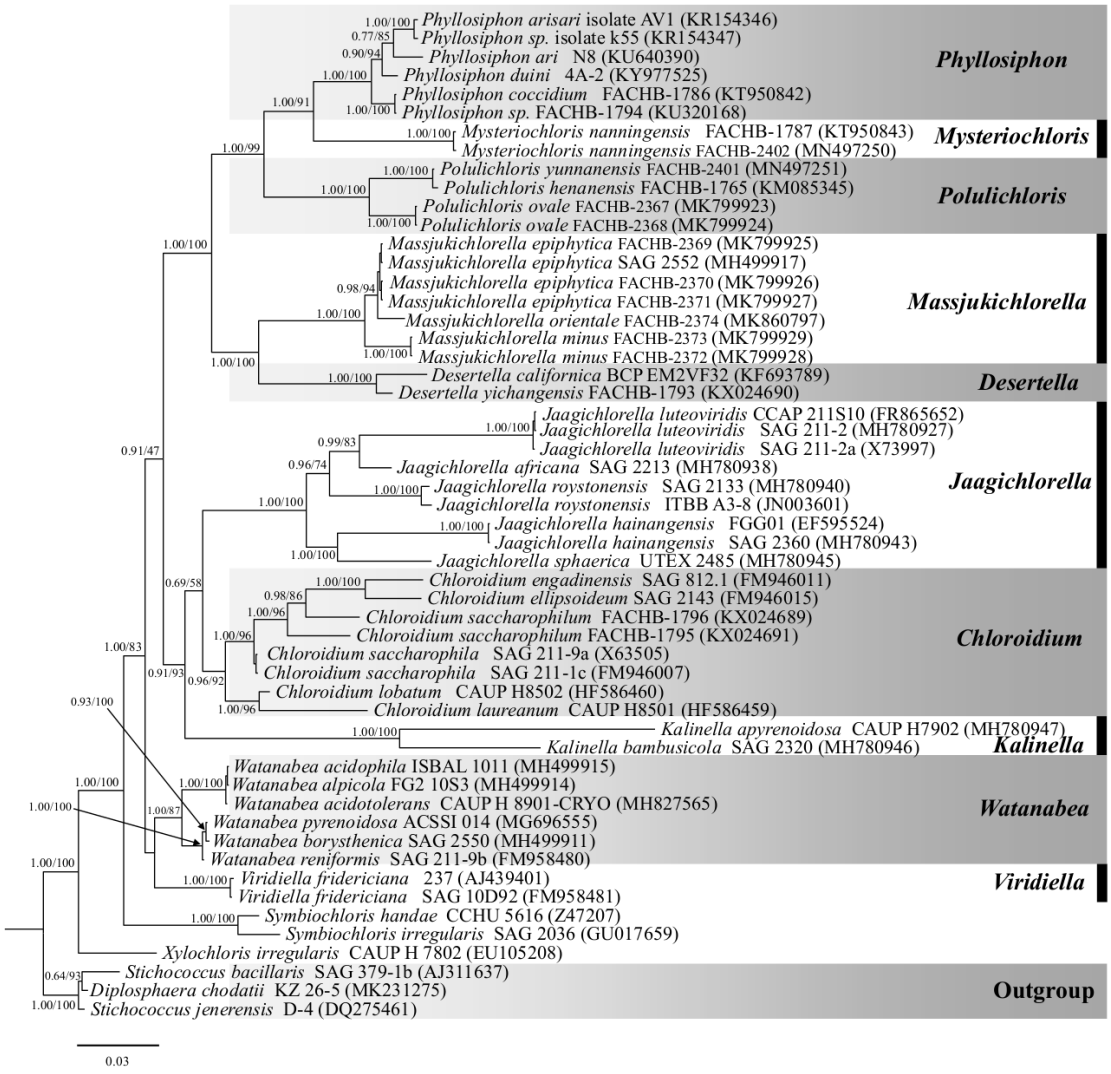

Fig. 5. Phylogenetic tree of the *Watanabea* clade of Trebouxiophyceae based on 18S rDNA sequences of ten described genera. After Li et al. (2020).

1.3. The peculiar genus *Phyllosiphon*

The genus *Phyllosiphon* traditionally included unique green microalgae that thrive as endophytic leaf parasites in various genera of the plant family Araceae. The type species, *P. arisari* Kühn, was described from leaves of *Arisarum vulgare* O. Targ. Tozz., which he found at the beginning of April in South-Eastern France and Western Italy (Kühn 1878). Later, *P. arisari* was recorded in the leaves of *A. vulgare* in other areas of the Mediterranean region, such as Southern Europe (Maire 1908, Mangenot 1948b, Aboal 1995, **papers I, II**) and Northern Africa (Maire 1908), where the genus *Arisarum* Mill. is naturally distributed. Since the description of the type species, five additional *Phyllosiphon* species were described from the leaves of different genera of the Araceae growing in tropical South America, and Western and Eastern Africa (Lagerheim 1892, Tobler 1917, Mangenot 1948b). *Phyllosiphon* infection was also reported in association with other taxa of the Araceae growing in temperate localities of Europe and North America, such as *Arum maculatum* L. (Maire 1908), *Arum italicum* Mill. (Nicolas 1912, **paper II**) and *Arisaema triphyllum* (L.) Schott. (Collins 1909, Smith 1933). *Phyllosiphon* was also recorded on unspecified taxa of the Araceae during a floristic survey at several areas in eastern Australia (Bostock & Holland 2010).

The parasitic *Phyllosiphon* thalli are formed by branched filaments containing unicellular endospores, which penetrate the intercellular matrix of the leaf parenchyma leading to leaf necrosis (Figs 6.3-7; Kühn 1878, Maire 1908, **papers I, II**). This infection is, in general, macroscopically similar to infection caused by other leaf non-algal parasites, such as fungi or viruses, which can also cause circular spots on the leaves. However, *Phyllosiphon* infection is characterized by macroscopic yellow-green spots on the leaves (Fig. 6.1, **papers I, II**). The *Phyllosiphon* spots remain unchanged as the leaves become dry, and thus appear as notably greener areas against the brown-colored dry leaf tissue (Fig. 6.2).

The genus *Phyllosiphon* was originally considered a member of the Xanthophyceae because of the absence of visible starch grains in chloroplasts and siphonous habit of parasitic thalli (Mangenot 1948a). Later, when the spectrophotometric analyses of photosynthetic pigments showed that plastids of the *Phyllosiphon* filaments contains chlorophyll *b* that is typical for green algae, the *Phyllosiphon* was transferred to the Chlorophyceae (Leclerc & Couté 1976). The precise phylogenetic position of the

Phyllosiphon was only recently elucidated by molecular phylogenetic methods. Aboal & Werner (2011) re-discovered *Phyllosiphon arisari* in the leaves of *Arisarum vulgare* at a single locality in South-Eastern Spain. Their phylogenetic analyses of its nuclear 18S rDNA and plastid-encoded 16S rDNA sequences showed that *Phyllosiphon* forms a separate lineage of *Watanabea* clade within Trebouxiophyceae. These results were surprising, because neither siphonous thalli nor parasitic lifestyle has yet been recorded in other representatives of the *Watanabea* clade. As I mentioned in Chapter 1.2.2, all other known representatives of this clade are chlorelloid unicells that mostly occur in various subaerial microhabitats as free-living algae (e.g. Zhang et al. 2008, Neustupa et al. 2009, Darienko et al. 2010).

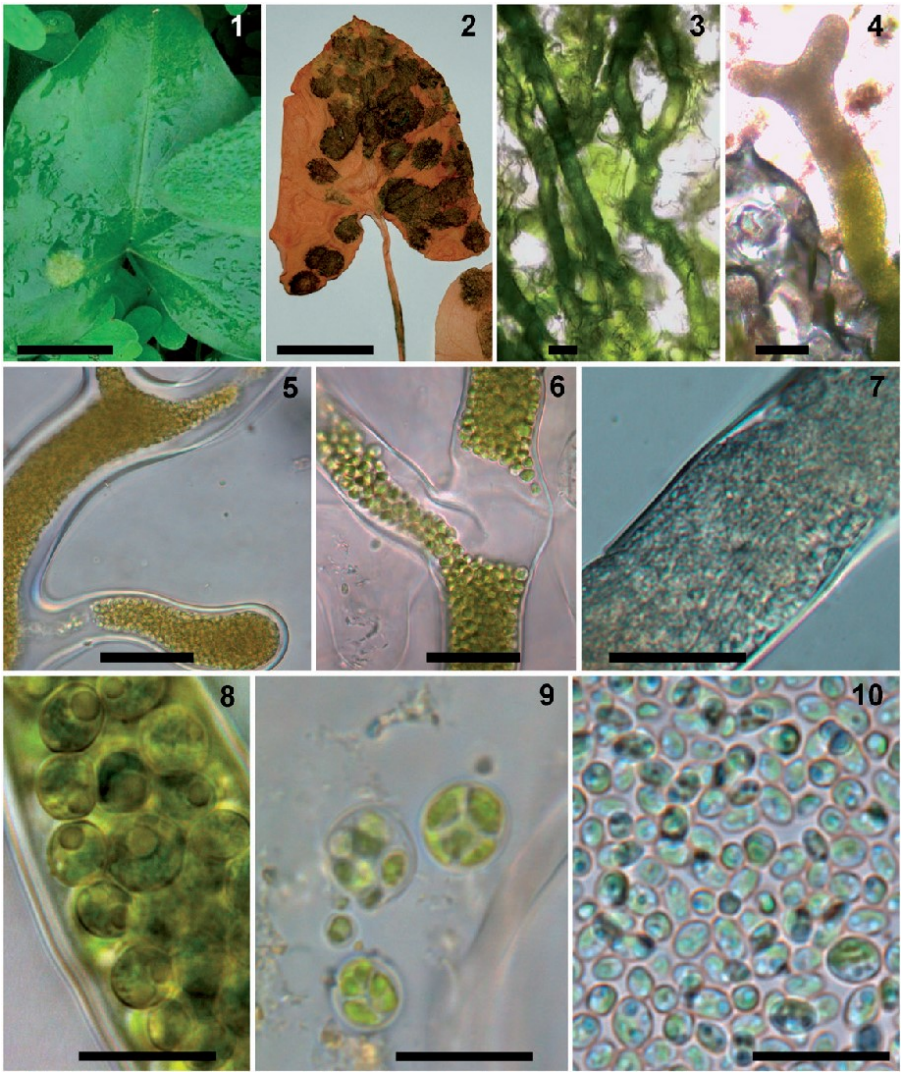

Fig. 6. Morphology of the *Phyllosiphon* (*P. arisari*). 1, 2. *Arisarum* leaf at different stages of infection. 3, 4. Leaf parenchyma containing branched filaments. 5, 6. Branching of filaments. 7. Yellowish filament at higher magnification and without conspicuous organelles. 8. Detail of endospores full of oil droplets. 9. Autospore formation. 10. Released autospores. Scale bars: 1 cm (Figs 1– 2), 50 mm (Figs 3–7) and 10 mm (Figs 8–10). After Aboal & Werner (2011).

Aboal & Werner (2011) also cultivated the parasitic filaments of *Phyllosiphon* to elucidate its life cycle. Interestingly, during the cultivation of parasitic filaments on agar-solidified medium, autosporangia were formed (Fig. 6.9), and autospores were released from them after the addition of water (Fig. 6.10). This observation led these authors to a conclusion that these autosporangia may represent resting stages that are able to survive periods of adverse conditions and, after their overcoming, the autospores contained in autosporangia can infect other leaves of the host plant species. Thus, Aboal & Werner (2011) considered the *Phyllosiphon* to be an obligate parasite that is not able to thrive and reproduce outside of its host plant. However, environmental 18S rDNA sequences closely related to *Phyllosiphon arisari*, were later detected in epilithic biofilms in Germany and Belfast, i.e. in regions located far from the Mediterranean distribution area of the genus *Arisarum* (Cutler et al. 2013, Hallmann et al. 2013). Likewise, the *rbc*L sequences of the *Phyllosiphon* were also recorded in samples of corticolous biofilms acquired from the sub-Mediterranean forests (Kulichová et al. 2014). However, in the later study, these sequences were listed without any taxonomic name, because it was not clear what taxa it belonged to (whether it represents a previously undescribed taxon or a taxon with no *rbc*L sequences published, as yet). The fact that these *rbc*L sequences actually belonged to the *Phyllosiphon* clade was confirmed in the **paper I**, in which we characterized these corticolous strains using the 18S rDNA sequences and compared them to the published 18S rDNA sequences of *Watanabea* clade members. In addition, in the **papers I, II**, we sampled additional corticolous assemblages from the several (sub-)Mediterranean localities to find out if members of the *Phyllosiphon* clade are really common and autochthonous members of subaerial microhabitats. Finally, in the **paper III**, we described a free-living *Phyllosiphon* species, *Phyllosiphon duini* Prochazkova, Němcova & Neustupa, which we obtained from a corticolous biofilm growing at a single sub-Mediterranean locality, from which Kulichová et al. (2014) originally obtained their *Phyllosiphon* sequences.

1.4. Algal parasites within the green algae (Viridiplantae)

Parasitism is defined as an interspecific interaction, where one species (the parasite) spends the whole or part of its life on or inside cells or tissues of another living organism (the host), from whom it derives most of its food (Salomon & Mai 2006). However, parasitism is not necessarily associated with a disease. Parasites constitute numerically and ecologically very important components of ecosystems and they play a key role in regulating host populations (Skovgaard 2014).

Parasitism is extremely common and widespread consumer strategy across the eukaryotic phylogenetic tree (Bass et al. 2018). It has even been estimated that about half of species on the Earth are actually parasites (Windsor 1998). Nevertheless, the parasitic life strategy is relatively uncommon in phototrophic microorganisms compared to parasitic members within heterotrophic protists. The known parasitic algae belong to the Viridiplantae, Rhodoplantae and Stramenopiles; they infect wide range of hosts including another algae, animals or plants (Bavestrello et al. 2000, Kim et al. 2008, Preuss et al. 2017).

Here, I will briefly describe the parasitic taxa classified within the phylum Chlorophyta. In this phylum, there are several parasitic algal lineages belonging to the classes Ulvophyceae, Chlorophyceae and Trebouxiophyceae. The class Trebouxiophyceae contains three parasitic genera that mostly infect terrestrial hosts. Two of them, *Prototheca* Krüger and *Helicosporidium* Keilin, are the only known true non-photosynthetic genera of the Chlorophyta; however, they are believed to possess colourless degenerated plastids because of the presence of plastid genomes (Suzuki et al. 2018). Ultrastructural studies even showed that *Prototheca* cells have a plastid-like structure surrounded by two membranes and filled by starch granules (Nadakavukaren & McCracken 1977). Both genera are closely related to the free-living photosynthetic alga *Auxenochlorella* Kalina & Punčochářová with mixotrophic nutrition (Ueno et al. 2005). The genus *Prototheca* consists of free-living heterotrophic species with simple chlorelloid morphology, which thrive in the various terrestrial and aquatic habitats worldwide (Nelson et al. 1987). They may cause infection of skin and internal organs, known as protothecosis, in animals, including humans (Lass-Flörl & Mayr 2007). The closely related genus *Helicosporidium* is a highly adapted parasite infecting a wide range of arthropods (Bläske-Lietze & Boucias 2005, Pombert et al. 2014). Morphologically, the genus *Helicosporidium* is characterized by discoid cysts each

containing a single filamentous and three ovoid cells. The infectious cysts are ingested by the host, they rupture in the digestive tract, and release both the short-lived ovoid cells and the barbed filamentous cells that penetrate into the hemocoel. In the hemocoel, these cells transit to vegetative cells that massively reproduce by autosporulation and differentiate into mature cysts. These cysts are obviously directly transmitted by insect predators that feed on infected prey or cannibalized insects (Yamar & Radek 2007). However, unlike most entomopathogenic protists *Helicosporidium* is capable of *in vitro* reproduction in simple nutritional media, while maintaining infectivity, which suggest that *Helicosporidium* may have a so far undescribed free-living stages until cysts are ingested by a host organism (Boucias et al. 2001). The infection of the host by the species of *Helicosporidium* is manifested by variable symptoms, such as change of hemolymph color or decreased host weight. However, the infection can also lead to the death of the host (Tartar 2013).

The class Trebouxiophyceae also includes the genus *Coccomyxa* Schmidle, which contains several members that are known as pathogens of marine bivalve molluscs (Vázquez et al. 2010). However, the genus *Coccomyxa* also includes both free-living terrestrial and freshwater species, photobiont of lichens and intracellular endosymbionts of *Ginkgo biloba* L. (Trémouillaux-Guiller & Huss 2007). The species of the genus *Coccomyxa* are typically the unicellular microalgae with elliptical cell shape that reproduce by autosporulation. Cells of the parasitic *Coccomyxa* species initially infiltrate into hemolymph and subsequently settle in the organs most exposed to light, such as connective tissues of the mantle edge, siphon or gonads (Vázquez et al. 2010, Sokolnikova et al. 2016). The infested tissues gradually degenerate and this process ultimately leads to organ dysfunctions, which may negatively affect reproduction of these molluscs (Gray et al. 1997). This parasitic relationship is considered as facultative, as the *Coccomyxa* is also capable of reproduction in cultures on an inorganic medium (Sokolnikova et al. 2016).

The ulvophycean order Trentepohliales contains genera *Cephaleuros* Kunze ex Fries and *Stomatochroon* Palm that thrive as parasites and endophytes in the leaves of various land plants (Zhu et al. 2017). These genera are widespread in regions with humid climate and may cause considerable damage to plants. Both genera have similar morphology and both have alternation of heteromorphic generations (Thompson & Wujek 1997). The genus *Cephaleuros* consists of branched filaments that are macroscopically visible as orange to dark brown irregular spots with velvety appearance on the leaves or fruits (Nelson 2008). These spots are formed, when fragments of thallus with sporangia or sporangia themselves

are deposited on susceptible host leaves or fruits. The infection occurs during the humid conditions and begins by releasing of motile zoospores from the sporangia. The zoospores penetrate the host cuticle and generate disc-like thalli, which mostly grow under the cuticle or sometimes penetrate under epidermis of the host plant. The increased infection of the leaves occurs during the monsoon season (Muthukumar et al. 2014). There is a variation in the frequency of lesions and their shape among host plants, which is attributed to the fact that infections can be caused by different *Cephaleuros* species. The growth of *Cephaleuros* on plants causes tissue necrosis, reduces photosynthetic area of the leaves, causes leaf falling and reduces market value of the infected leaves or fruits of important tropical crops, such as tea, coffee, vanilla, avocado, mango, guava, cacao or some citrus cultivars (Nelson 2008, Chapman & Good 2011).

The genus *Stomatochroon* is another parasitic taxon within the ulvophycean order Trentepohliales. It consists of few-celled thalli that grow in the substomatal chambers of plants leaves and in the intercellular spaces between mesophyll cells (Chapman & Good 2011). The reproduction takes place through the sporangia protruding from the stomata. *Stomatochroon* is sometimes referred as an obligate endophyte or parasite. It should be noted that it is sometimes difficult to clearly distinguish parasites from epiphytes and endophytes, as the relationship between the host and alga may change over time (Joubert & Rijkenberg 1971). The endophytes are also known from the class Chlorophyceae. The genus *Chlorochytrium* Cohn occupies intercellular spaces of various freshwater plants (Lewin 1984). Thus, it cannot be ruled out that the relationship between other microalgae and their hosts cannot at some point turn to parasitism.

1.5. The plant family Araceae

The Araceae is one of the oldest and the third largest family of monocotyledonous flowering plants, after orchids and grasses (Moodley et al. 2016). It was estimated that Araceae diverged from the remaining Alismatales at the beginning of the Early Cretaceous (130-146 million of years ago; Nauheimer et al. 2012). In total, the family comprises about 3800 described species in 120 genera (Cusimano et al. 2011). More than two-thirds of aracean species occur in the Neotropics. The remaining one-third of species occur in other

parts of world (mainly in subtropical and temperate regions) except the polar and high alpine regions and the dry deserts (Bown 2000, Li et al. 2010).

A unique feature of the Araceae is the spathe-and-spadix inflorescence, a floral structure consisting of a petal-like leaf (the spathe) and flowers-bearing protuberance (the spadix) (Bown 2000). Members of the Araceae family are highly diverse in life forms, leaf morphology, and inflorescence characteristics. Their life forms range from submerged and free-floating aquatic forms to terrestrial and epiphytic forms. Most aroids tend to grow in places with sufficient moisture and shade. The aroids growing in temperate and subtropical region, i.e. in areas that are drier than tropics, tend to be tuberous and seasonally dormant, escaping the cold or hot weather, which is associated with drought by losing all overground parts and completely disappearance for several months of year. In cold climates, dormancy takes place in the winter and renewed growth in spring. In warmer regions, dormancy take place in the summer or dry season, and new growth begins when cooler, rainy weather returns (Bown 2000).

The parasitic stages of *Phyllosiphon* were only found in several genera of the Araceae. Interestingly, these genera are included in both the *Stylochaeton* clade and Aroideae clade within phylogenetic tree of the Araceae (Fig. 7). These genera occur in tropical, subtropical or temperate regions. *Phyllosiphon asteriforme* Tobler was described from the leaves of *Zamioculcas* (Lodd.) Engl. from tropical East Africa (Tobler 1917), *P. deformans* Mangenot was described from the leaves of *Anchomanes* Schott from tropical West Africa (Mangenot 1948b), *P. philodendri* Lagerheim was described from the leaves of *Philodendron* Schott from tropical South America (Lagerheim 1892), *P. alocasia* Lagerheim was described from the leaves of *Alocasia* (Schott) Don from tropical South America (Lagerheim 1892), *P. arisari* was described from the leaves of subtropical *Arisarum*. (Kühn 1878). The parasitic stages of *Phyllosiphon* were also reported from leaves of *Arum* L. spp. in temperate Europe (Maire 1908, Nicolas 1912, Sowter 1949) and *Arisaema* Mart in North America (Collins 1909, Smith 1933).

In this thesis, I studied the algal parasites in the leaves of terrestrial members of the Araceae growing in the Mediterranean Europe, where there is relatively high diversity of the Araceae (Bown 2000). However, there are only a few terrestrial genera of Araceae in comparison to the tropics: *Arisarum*, *Arum*, *Biarum* Schott, *Eminium* (Blume) Schott, *Helicodiceros* Schott, *Dracunculus* Mill. and *Ambrosina* Bassi. The genus *Arum* is the only

one of them, which also occurs in temperate Europe; the remaining genera are restricted to the subtropical Mediterranean region. During my study of algal parasites of the Araceae, I mostly focused on the genera *Arisarum* and *Arum*, because *Phyllosiphon* infections were repeatedly observed on their leaves.

The genus *Arisarum* comprises four species, which thrive only in the Mediterranean and in Macaronesia (Govaerts 2018). The most widespread species of the genus *Arisarum*, *A. vulgare*, occurs in Mediterranean region of southern Europe and northern Africa, Canary Islands, Madeira and Azores. *A. vulgare* is a tuberous geophyte growing in groups, with leaves looking like a heard, hooded spathe friar's cowl with maroon stripes, and slightly curved spathe termination (Govaerts 2018). Additional species *A. simorrhinum* Durieu is morphologically similar to *A. vulgare*, but it only occurs in Portugal, Spain, Balearic Islands, Morocco and Algeria. The hybrid *Arisarum* × *aspergillum* Dunal, originated by hybridization of *A. vulgare* and *A. simmorhinum*, occurs in Spain, Morocco and Algeria. Another species *A. proboscideum* (L.) Savi is a rhizomatous geophyte growing in groups, with white spathe, which is stretched and ended at the end, resembling mouse's tail. It occurs in Spain and Italy (Bown 2000, Govaerts 2018). It is interesting that the *Phyllosiphon* infection was so far only reported from leaves of *A. vulgare* (Aboal & Werner 2011, **papers I, II**).

The members of the genus *Arum* look very similar to *Arisarum*, and it is sometimes difficult to distinguish them, especially when they are not flowering. The species of the genus *Arum* are tuberous geophytes with sagittate (arrowhead-shaped) leaves, frequently coloured spathe and straith spadix, on which orange or red berries develop (Bown 2000). The genus *Arum* comprises 29 species that are mainly distributed in the Mediterranean, but they also grow in Central and Northern Europe, the Middle East and Central Asia (Boyce 2006, Linz et al. 2010, Govaerts 2018). Nevertheless, *Phyllosiphon* infection was reported only from leaves of two *Arum* species: *A. maculatum*, which is naturally distributed in Europe and its distribution area ranges from northern Turkey to western Caucasus; and *A. italicum*, which is distributed in Macaronesia, and area from western Europe to Iraq (Linz et al. 2010, Govaerts 2018). In the **paper II**, we found the parasitic stages of the *Phyllosiphon* growing in the leaves of *Arum italicum* at a single sub-Mediterranean locality. Since these parasites were genetically different (based on the 18S rDNA and *rbc*L sequences) from those previously obtained from *Arisarum* leaves, we decided to describe these parasites as a new *Phyllosiphon* species, *Phyllosiphon ari* Procházková, Němcová & Neustupa.

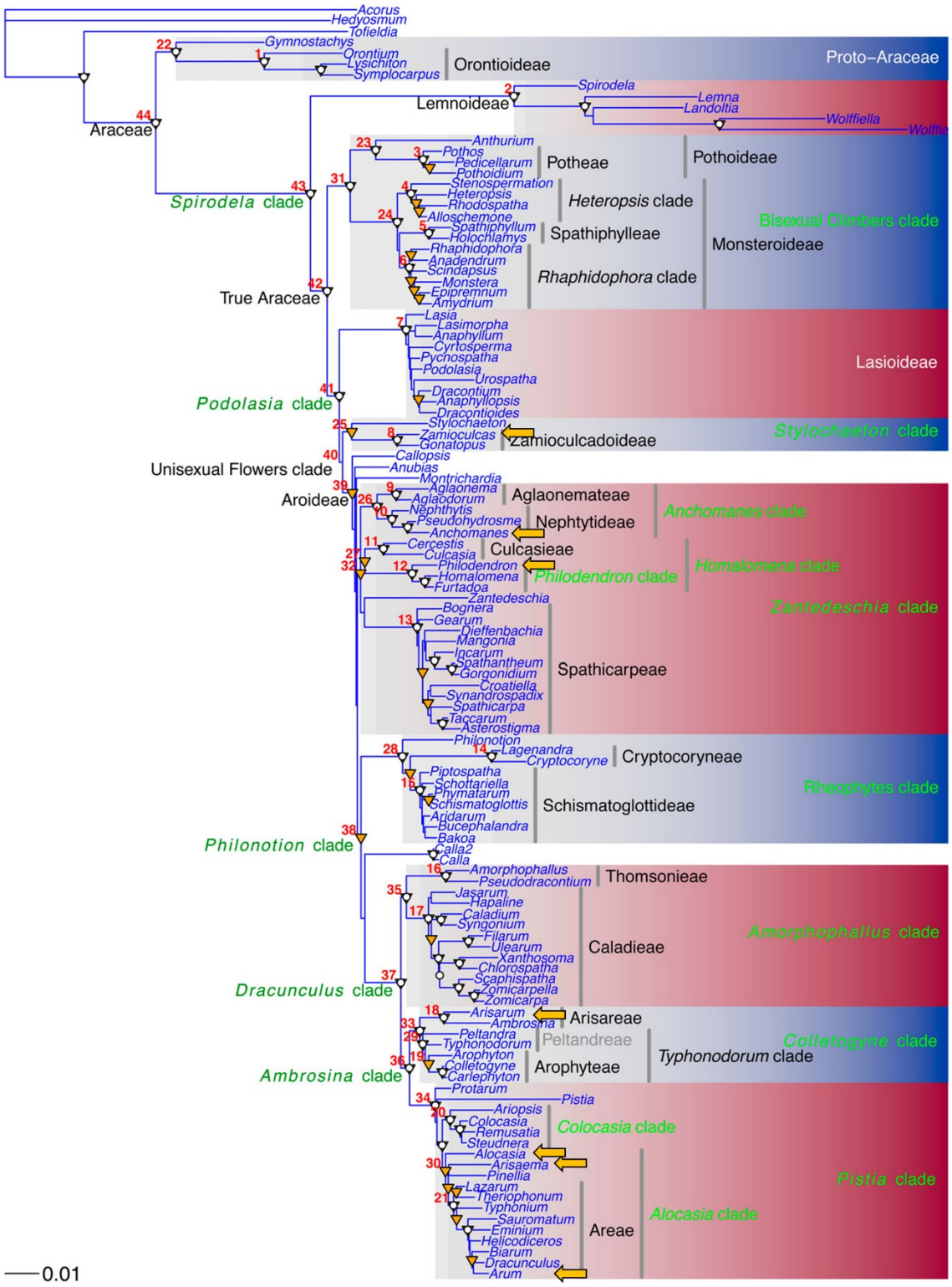
Acorus
Hedyosmum
Tofieldia
Gymnostachys
Orontium
Lysichiton
Symplocarpus
Orontioideae
Proto–Araceae
Spirodela
Lemna
Landoltia
Wolffiella
Wolffia
Lemnoideae
Araceae
Anthurium
Pothos
Pedicellarum
Pothoidium
Potheae
Pothoideae
Stenospermation
Heteropsis
Rhodospatha
Heteropsis clade
Alloschemone
Spathiphyllum
Holochlamys
Spathiphylleae
Bisexual Climbers clade
Monsteroideae
Rhaphidophora
Anadendrum
Scindapsus
Monstera
Epipremnum
Amydrium
Rhaphidophora clade
Spirodela clade
True Araceae
Lasia
Lasimorpha
Anaphyllum
Cyrtosperma
Pycnospatha
Podolasia
Urospatha
Lasioideae
Podolasia clade
Dracontium
Anaphyllopsis
Dracontioides
Stylochaeton
Zamioculcas
Gonatopus
Zamioculcadoideae
Stylochaeton clade
Callopsis
Anubias
Unisexual Flowers clade
Montrichardia
Aglaonema
Aglaodorum
Aroideae
Aglaonemateae
Anchomanes clade
Nephthytis
Pseudohydrosme
Nephtytideae
Anchomanes
Cercestis
Culcasia
Culcasieae
Philodendron
Philodendron clade
Homalomena clade
Homalomena
Furtadoa
Zantedeschia
Zantedeschia clade
Bognera
Gearum
Dieffenbachia
Mangonia
Incarum
Spathantheum
Gorgonidium
Spathicarpeae
Croatiella
Synandrospadix
Spathicarpa
Taccarum
Asterostigma
Philonotion
Lagenandra
Cryptocoryne
Cryptocoryneae
Piptospatha
Schottariella
Phymatarum
Schismatoglottis
Schismatoglottideae
Rheophytes clade
Aridarum
Bucephalandra
Bakoa
Philonotion clade
Calla2
Calla
Amorphophallus
Pseudodracontium
Thomsonieae
Jasarum
Hapaline
Caladium
Syngonium
Filarum
Ulearum
Caladieae
Amorphophallus clade
Xanthosoma
Chlorospatha
Scaphispatha
Zomicarpella
Zomicarpa
Dracunculus clade
Arisarum
Arisareae
Ambrosina
Peltandra
Typhonodorum
Peltandreae
Colletogyne clade
Typhonodorum clade
Arophyton
Colletogyne
Carlephyton
Arophyteae
Ambrosina clade
Protarum
Pistia
Ariopsis
Colocasia
Remusatia
Steudnera
Colocasia clade
Alocasia
Arisaema
Pinellia
Lazarum
Theriophonum
Typhonium
Sauromatum
Eminium
Helicodiceros
Biarum
Dracunculus
Arum
Areae
Pistia clade
Alocasia clade
0.01

Fig. 7. Diversity of the parasitic members of the *Phyllosiphon* (yellow arrows) mapped along the phylogenetic tree of the Araceae. The type species of the *Phyllosiphon*, *P. arisari*, was described from the leaves of subtropical *Arisarum*. Additional species of the *Phyllosiphon* were traditionally described from the leaves of the following aracean plants: *P. asteriforme* from *Zamioculcas*, *P. deformans* from *Anchomanes*, *P. philodendri* from the *Philodendron*, *P. alocasia* from *Alocasia*. *Phyllosiphon* infections were also reported from the leaves of *Arisaema* and *Arum*. The existence of *P. arisari* and *P. ari* species has recently been supported by DNA sequencing data. Modified after Cusimano et. al. (2011).

1.6. Research aims and methods

The main aim of this thesis was to examine the life forms of the genus *Phyllosiphon* using the cultivation and DNA sequencing of clonal isolates acquired from subaerial corticolous biofilms and infected leaves of the two aracean genera collected at several (sub-)Mediterranean localities. We were also interested in phylogenetic relationships among the isolates obtained from different samples and localities. Furthermore, we wanted to find out whether the particular *Phyllosiphon* lineages can be distinguished morphologically or ultrastructurally.

The particular questions we asked were:

Do the genotypes of parasitic *Phyllosiphon* populations previously obtained from the leaves of *Arisarum vulgare* in Spain also occur in other locations across the Mediterranean region?

Parasitic populations of *Phyllosiphon arisari* from the *Arisarum vulgare* leaves were collected in Cyprus and Sardinia (papers I, II). The isolated specimens were cultivated on an agar-solidified medium on Petri dishes. The 18S rDNA sequences were obtained and compared with those previously obtained from infected leaves of *Arisarum vulgare* in Spain. We also examined their morphology and ultrastructure using light (LM) and transmission electron microscopy (TEM).

Are the parasitic species of the genus *Phyllosiphon* isolated from different taxa of the Araceae phylogenetically differentiated?

Several parasitic *Phyllosiphon* specimens from the *Arum* leaves at a single sub-Mediterranean locality were collected (paper II). The 18S rDNA and *rbc*L gene sequences were obtained to characterize their phylogenetical positions and to compare them with those previously acquired from *Arisarum* leaves. In addition, morphology and ultrastructure of these specimens were investigated using LM and TEM.

Are there any *Phyllosiphon* populations in corticolous biofilms? If yes, do their morphological and molecular features correspond to the parasitic populations?

The corticolous biofilms were collected from a number of sites (papers I, II, III). Chlorelloid unicells with morphology typical for the members of the *Watanabea* clade were

isolated. The 18S rDNA and *rbc*L gene sequences were obtained to characterize the phylogenetical positions of these isolates. We also compared morphology and ultrastructure of free-living and parasitic members using LM and TEM.

Do the genus *Phyllosiphon* includes some lineages, which occurs solely as free-living algae and do not form parasitic stages?

The 18S rDNA and *rbc*L sequences of the free-living chlorelloid stages of the *Phyllosiphon* isolated from corticolous biofilms (papers I, II, III) were compared with those acquired from parasitic specimens isolated from the leaves of *Arum* and *Arisarum* (papers I, II) to find out which *Phyllosiphon* lineages form parasitic or free-living stages, or both. In addition, we were looking for parasitic stages inside the leaves of any aracean taxa at the same localities, where we sampled corticolous biofilms, in order to determine whether the same lineages occur in corticolous biofilms and infected leaves, or not (papers II, III).

Can the parasitic specimens of the *Phyllosiphon* clade thrive independently of their hosts on a long-term basis?

Parasitic specimens of the *Phyllosiphon* were isolated from the leaves of *Arum* and *Arisarum* and cultivated them *in vitro* on an agar-solified inorganic medium to find out whether these specimens are able to capable of autonomous reproduction and long-term survival outside their host vascular plant, or whether they cannot be grown and require host plant for growing (papers I, II).

2.1. Paper I.

Morphology and phylogeny of parasitic and free-living members of the genus *Phyllosiphon* (Trebouxiophyceae, Chlorophyta)

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Abstract

The trebouxiophycean genus *Phyllosiphon* occurs as an endophytic leaf parasite of the Araceae. However, sequences similar to those acquired from siphonous parasitic thalli were also recently identified in various subaerial biofilms. In this study, we confirmed that free-living *Phyllosiphon* populations occur in corticolous biofilms as chlorelloid unicells with morphological features typical of the *Watanabea* clade. These free-living unicellular *Phyllosiphon* populations asexually reproduce by autospores and are culturable outside of the host plants. While the majority of the 18S rDNA sequences from the parasitic thalli were the same as those in previously published research, the free-living populations from the biofilms probably represented several different species of the *Phyllosiphon* clade. Phylogenetic analyses using the *rbc*L gene sequences confirmed the position of the genus *Phyllosiphon* within the *Watanabea* clade, but distinct from the clade of the genera *Chloroidium* and *Parachloroidium* typified by a single codon insertion at position 286 of the gene.

Key words: 18S rDNA, chlorelloid microalgae, *Phyllosiphon*, *rbc*L, subaerial algae, Trebouxiophyceae, *Watanabea* clade

Introduction

The genus *Phyllosiphon* contains unique green algae that are leaf parasites of various members of the Araceae. The type species, *P. arisari* J.G. Kühn, was described from leaves of *Arisarum vulgare* growing in the Mediterranean region (Kühn 1878). Several additional studies reported occurence of *P. arisari* in the leaves of *A. vulgare* in various regions of

Southern Europe (Maire 1908, Mangenot 1948a, Aboal 1995) and North Africa (Maire 1908). *P. arisari* was also reported in association with other members of the Araceae, such as *Arum maculatum* (Maire 1908), *Arum italicum* (Nicolas 1912) and *Arisaema triphyllum* (Collins 1909, Smith 1933). Five additional *Phyllosiphon* species were also described from the leaves of tropical aracean plants, but they have not been reported since their original taxonomic descriptions.

Parasitic *Phyllosiphon* thalli consist of branched siphonal filaments growing in the intercellular matrix of the leaf parenchyma without cell penetration. Macroscopically, *Phyllosiphon* infection is visible as yellow-green spots on the leaves. The *Phyllosiphon* spots remain unchanged as the leaves become dry, and thus appear as notably greener areas against the brown-colored dry leaf tissue. Asexual reproduction proceeds by segmentation of the cytoplasm in siphonal filaments and production of unicellular endospores (Kühn 1878, Maire 1908, Aboal & Werner 2011).

The genus *Phyllosiphon* was originally considered a member of the Xanthophyceae (Mangenot 1948a). Later, it was transferred to the Chlorophyceae (Leclerc & Couté 1976), but its precise phylogenetic position was only recently elucidated by molecular phylogenetic analysis (Aboal & Werner 2011). Surprisingly, these analyses found that *Phyllosiphon arisari* formed a separate lineage of the trebouxiophycean *Watanabea* clade, which otherwise included solely coccoid unicellular algae (Darienko et al. 2010, Neustupa et al. 2009, 2013a, b). The relationship of *P. arisari* to the Trebouxiophyceae was also supported by morphological and ultrastructural signatures of the autospores (Aboal & Werner 2011). These authors showed that *Phyllosiphon* reproduced by ellipsoidal cells that contained numerous oil droplets and had a parietal chloroplast without a pyrenoid. They isolated and cultivated siphonal filaments and found that endospores were produced within the filaments. The endospores later developed into autosporangia containing four ellipsoidal autospores. Endospores were released from the siphonal filaments after the addition of water, leading Aboal & Werner (2011) to assume that autosporangia were resting structures that resisted adverse environmental conditions, and, after re-wetting, the released autospores would then be able to infect other leaves of the host plant.

Siphonal morphology in the Trebouxiophyceae had not been known prior to the above mentioned study. However, several other studies showed that unicellular chlorelloid taxa may evolve relatively rapidly into more complex thalli, probably as a consequence of the selective pressure for colonial or spine-bearing morphologies (Luo et al. 2006, Krienitz et al. 2010, Bock et al. 2011). Therefore, it can be assumed that the *Watanabea* clade, which

to date only contains coccoid unicellular species, may also include a lineage with a profoundly different morphology that evolved as a consequence of switching to an endophytic and parasitic life strategy.

Recently, environmental 18S rDNA sequences were detected in epilithic phototrophic biofilms in Germany that were closely related to those acquired from the parasitic population of *Phyllosiphon arisari* (Hallmann et al. 2013). The biofilms in question frequently occurred in sun-exposed microhabitats on sandstone substrates. Hallmann et al. (2013) noted that the *Phyllosiphon* clones corresponded to the morphologically defined *Lobosphaeropsis pyrenoidosa*, an alga with a morphology distinct from that of chlorelloid unicells as illustrated by Aboal & Werner (2011). However, Hallmann et al. (2013) did not provide additional data or figures illustrating their *Phyllosiphon*-like findings. Sequences closely related to *P. arisari* were also found in samples of phototrophic biofilm communities growing on sandstone substrates in Belfast, UK (Cutler et al. 2013). Both these *Phyllosiphon*-like sequences were acquired in regions located far from the predominantly Mediterranean distribution area of the genus *Arisarum*. This raises questions regarding the relationship of *Phyllosiphon* to its host species. First, could the free-living coccoid stage of *P. arisari* thrive independently of their hosts on a long-term basis? Second, could the *Phyllosiphon* clade include distinct parasitic and free-living taxa? Finally, could free-living *Phyllosiphon* populations, occurring in areas outside the *Arisarum* distribution, form parasitic siphonal stages in the leaves of other aracean plants?

The purpose of the present study was to determine the distribution of the genus *Phyllosiphon* in various European microhabitats using molecular and morphological methods. We tested whether samples from infected leaves of the Mediterranean *Arisarum* population corresponded to the molecular and morphological data published by Aboal & Werner (2011). We wished to determine whether free-living *Phyllosiphon* populations were also found in corticolous subaerial biofilms and whether the morphological and molecular features of such populations corresponded to the parasitic populations. We therefore sampled tree bark and infected *Arisarum vulgare* leaves in various European locations. Morphology and ultrastructure of the isolates was investigated with an emphasis on their possible life strategies. Nuclear 18S rDNA and plastid *rbc*L gene loci were sequenced to determine the phylogenetic position of isolated clones. With the exception of several members of the *Watanabea* clade, which have a single codon insertion at position 286 of the gene sequence (Neustupa et al. 2013b), the *rbc*L gene is invariably 1428 nucleotides long in the Trebouxiophyceae. Thus, sequence data from additional *rbc*L gene sequences of

lineages belonging to the *Watanabea* clade could clarify evolution of this rare insertion in one of the most widely used phylogenetic markers among the Viridiplantae. Therefore, we also sequenced the *rbc*L gene from eight additional strains of the *Watanabea* clade acquired from public microalgal culture collections.

Materials and methods

Isolation and cultivation of strains

Phyllosiphon strains were isolated from samples of subaerical corticolous biofilms and from infected leaves at six locations in South and North Italy and Cyprus (Table 1). The strains AG13 and AV1 isolated from corticolous biofilm has been deposited in the Culture Collection of Algae of Charles University in Prague (CAUP) (http://botany.natur.cuni.cz/algo/caup.html). The biofilm samples were isolated from ~1 cm^2 of tree bark surface taken from shaded northern parts of tree trunks at a height of approximately 150 cm above the soil surface. The phototrophic biofilm from each sample was scraped using a sterile dissecting needle into the 1.5 ml Eppendorf tubes 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 (10 cm in diameter). After 6 weeks, 2 to 4 algal microcolonies with chlorelloid morphology were isolated onto agar-solidified BBM medium in test tubes.

Clones from leaves were isolated from ~3 mm^2 of infected spots on *Arisarum vulgare* leaves under a Olympus SZ61 stereomicroscope (Olympus, Japan) (Fig. 1). These 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 cell colony from each Petri dish were used for DNA isolation. Another piece of the same cell colony was isolated onto agar-solidified BBM medium in test tubes after 1 week.

In addition, to reconstruct the evolution of the *rbc*L gene, additional members of the *Watanabea* clade from various culture collections were sequenced (Table 1). Three strains of unknown taxa previously used by Kulichová et al. (2014) as the OTUs cort08 and cort21, which originated from North Italy, were also included in this study to identify these taxa by additional molecular marker (Table 1). Strains were cultivated on agar-solidified BBM

medium (Andersen et al. 2005) at 23°C, with illumination of 40 mmol m^{-2} s^{-1} provided by 18 W cool fluorescent tubes (Philips TLD 18W/33).

Light and electron microscopy

Microphotographs of strains and infected leaves were taken under an Olympus BX51 light microscope with an Olympus Z5060 camera (Olympus, Japan) and an Sony Cyber-shot DSC-HX20V camera (Sony, Japan), respectively. Cells for transmission electron microscopy (TEM) were fixed for 2 h at 5°C in 2% glutaraldehyde in 0.05 M phosphate buffer, postfixed for 2 h in 1% osmium tetroxide (OsO_4) in 0.05 M phosphate buffer, and finally incubated for 12 h at 5°C in 1% uranyl acetate solution. The samples were then dehydrated through an ethanol series and embedded in Spurr medium via propylenoxide. Ultrathin sections were cut with a diamond knife on an Ultracut E (Reichert-Jung), post-stained with lead citrate, and examined using a JEOL 1011 transmission electron microscope (JEOL Ltd, Japan).

DNA isolation, polymerase chain reaction (PCR) and DNA sequencing

Single algal colonies were placed into a tube containing 80 µl InstaGene matrix (Bio-Rad laboratories). The cells were mechanically disrupted by shaking for 10 min in a Mixer Mill MM 400 (Retsch) in the presence of glass beads (1.5 mm diameter; Sigma-Aldrich). The mixture was subsequently centrifuged at 700 rpm and incubated at 56°C for 30 min. The mixture was then vortex-mixed for 10 s, and incubated at 99°C for 8 min. After vortexing for a second time, the tubes were centrifuged at 12000 rpm for 2 min and the supernatant was removed. The solution was diluted to 5–10 ng/µl and used for amplification by PCR.

Two molecular markers were amplified by PCR: the nuclear 18S rRNA and the plastid large subunit of the ribulose-1,5-bisphosphate carboxylase oxygenase (*rbc*L) gene. The 18S rDNA was amplified by forward (18S-F) and reverse (18S-R) primers as described in Katana et al. (2001) or using a combination of newly designed primers as follows: phyllF1 (5′-CAYGTGTAAGTATGAACCGCTC-3′), phy-F2 (5′-ACTGCGAATGGCTCA TTAAATC-3′), and 18S-R (5′-TGATCCTTCTGCAGGTTCACCTACG-3′) or 1636-57-R (5′- GGTAGGAGCGACGGGCGGTGTG-3′). All new primers were designed to specifically amplify *Phyllosiphon* members. The *rbc*L gene was amplified using the forward primer PRASF1 (5′-ATGGTTCCACAAACAGAAAC-3′, Sherwood et al. 2000), rbcL203F (5′-GAATCWTCWACWGGWACTTGGACWAC-3′, Nelsen et al. 2011) or the

newly designed forward primer phyllrbcLF (5′-TTCCGTATGACTCCACAACAAGG-3′) with reverse primers ellaR2 (5′-TCACGACCTTCATTACGAGCTTG-3′, Neustupa et al. 2013a), PRASR1 (5′-TTGTCAATAGTATCAAATTC-3′, Sherwood et al. 2000), or rbcL-991R (5′-CCTTCTARTTTACCWACAAC-3′, Nelsen et al. 2011). All PCR reactions were performed in a total volume of 20 µl containing 14.2 µl of sterile Milli-Q water, 2 µl of 10 × AmpliTaq Gold® 360 buffer, 1.1 µl of $MgCl_2$ (50 mM), 0.4 µl of dNTP mix (10 mM), 0.25 µl of each primer (25 pmol), 0.6 µl of 360 GC enhancer, 0.2 µl of AmpliTaq Gold® 360 DNA polymerase (1 U), and 1 µl of DNA (5–10 ng). Amplifications were performed in a XP thermal cycler (Bioer, Japan) or a Touchgene gradient cycler (Techne, UK). Amplification of the 18S rDNA/*rbc*L regions was as follows: initial denaturation at 94/94°C for 4 min/5 min; 35/40 cycles of denaturation at 94/95 °C for 1 min/45 s, annealing at 54/52 °C for 1/1.5 min, and elongation at 72 °C for 2.5/2 min; final extension at 72 °C for 10 min. PCR products were quantified using 1% agarose gel electrophoresis with ethidium bromide staining and purified using a GenElute PCR Clean-Up Kit (Sigma-Aldrich) according to the manufacturer's protocol. The purified amplification products were sequenced with the amplification primers using an automated sequencer (ABI 3730xl, Applied Biosystems) at Macrogen, Korea. Sequencing reads were assembled and manually edited using SeqAssem (version 09/2004; Hepperle 2004).

Phylogenetic analyses

Newly determined 18S rDNA and *rbc*L sequences were manually aligned with the partial *rbc*L sequences used in Kulichová et al. (2014) and existing sequences from the GenBank database using MEGA version 6 (Tamura et al. 2013). Alignment of the 18S rDNA sequences was guided by the 18S rDNA secondary structure model of *Chlamydomonas reinhardtii* (Wuyts et al. 2000). The final alignments consisted of 1769 nucleotides in the case of the 18S rDNA and 1206 nucleotides for the *rbc*L gene. Both alignments are available at http://botany.natur.cuni.cz/phyllos. 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 gene dataset and the GTR+G, K2+G+I, and GTR+G+I models for the 1st, 2nd, and 3rd codon positions of *rbc*L, respectively. Phylogenetic trees were inferred with Bayesian inference using MrBayes version 3.2.2. (Ronquist et al. 2012). Two parallel Markov chain Monte Carlo (MCMC) runs were carried out for 3 million generations, each with one cold and three heated chains. Analysis of the *rbc*L dataset was 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 30 000 trees. After visual inspection of log-likelihood values of sampled trees, the initial 7501 trees of each run were discarded and posterior probabilities of tree bipartitions were calculated on the basis of the consensus of the remaining 45000 (22500 × 2) trees. The maximum likelihood (ML) and weighted maximum parsimony (MP) analyses for bootstrap supports of individual phylogenetic lineages were calculated using the *phangorn* package version 1.7-4 (Schliep 2011) in R version 2.15.2 (R Development Core Team 2012). The GTR+G+I substitution model for the ML analysis of both markers was selected using the Akaike information criterion (AIC) by the *modeltest* function (Posada & Crandall 1998) implemented in the *phangorn* package. The function *bootstrap.pml* was then used for non-parametric bootstrap analysis (with 1000 replicates) of the individual tree nodes recovered by the ML analysis. Two parallel strategies for finding trees with the lowest parsimony score were used in the weighted maximum parsimony (wMP) analysis. The *optim.parsimony* function was used for the nearest-neighbor tree rearrangements and *pratchet* was used for the parsimony ratchet searches (Nixon 1999). Sankoff's algorithm was used for weighting the parsimony function (Felsenstein 2004). Bootstrap analysis of the nodes of the optimal wMP tree was based on 1000 replicates. The phylogenetic trees were graphically adjusted in FigTree version 1.3.1 (Rambaut 2009) and Adobe Illustrator version CS3.

Results

Morphology, ultrastructure and inoculation experiments

Phyllosiphon strains were taken from samples of corticolous biofilms and infected leaves of *Arisarum vulgare* (Table 1). Free-living populations from subaerial biofilms were isolated from seven independent samples and formed a majority of investigated strains. The infected leaves of *Arisarum vulgare* contained characteristic green-colored spots 2–17 mm in size (Fig. 1). Branched siphonous filaments grew in the intercellular matrix of the leaf parenchyma (Figs 2–3). Typically, the inner space of the filaments was filled with 3.0-6.0 μm diameter ellipsoidal endospores. Endospores were released from the filaments after re-wetting of thalli with cultivation medium. When transferred to agar-solidified medium in Petri dishes, the endospores developed into spherical coccoid cells (diameter 5.3-9.2 μm) that subsequently propagated by autosporulation (Fig. 4). The autosporangia were typically 7.3-9.0 μm in diameter. The cultured isolates of *Phyllosiphon* from the free-living

corticolous populations had coccoid spherical vegetative cells 5.5–11.8 µm in diameter (Figs 5–6, 8–9). These strains reproduced by formation of autospores. In most cells, a single large autospore was produced in addition to one, three, five, or seven smaller autospores produced within a single autosporangium. These autosporangia were typically 6.5–10.4 µm in diameter, but those with diameters of more than 11.5 µm were encountered as well (Figs 5–9). The cells contained a single parietal lobed chloroplast. Neither pyrenoids nor starch grains were observed within the chloroplast. However, the chloroplast included electron-dense globular plastoglobuli scattered within the chloroplast matrix. The vegetative cells often contained substantial numbers of extraplastidial oil droplets and carbohydrates (Figs 10–11).

Phylogenetic analyses

Sequences of the *rbc*L gene were obtained from 18 *Phyllosiphon* strains (Table 1). Sequences of 18S rDNA were obtained from 17 strains (the 18S rDNA from isolate k115 could not be amplified despite repeated attempts). Analyses of the 18S rDNA sequences indicated that the *Phyllosiphon* strains formed a moderately supported monophyletic lineage (0.98 Bayesian posterior probability/100 ML bootstrap support/98 wMP bootstrap support) within the *Watanabea* clade of the Trebouxiophyceae (Fig. 12). The 18S rDNA sequences of the strains isolated in this study were identical to the *Phyllosiphon arisari* sequence JF304470 acquired by Aboal & Werner (2011) with the exception of strain *k55*, which differed in the deletion of one nucleotide and the presence of two ambiguous nucleotides. The *Phyllosiphon* sp. (JX127175) strain, acquired by Hallmann et al. (2013) from epilithic biofilms in Germany, differed by five substitution changes and the deletion of 18 nucleotides from the sequence JF304470 acquired by Aboal & Werner (2011).

The *Phyllosiphon* lineage clustered in a sister position with the uncultured "*Chlorella*" strain (AM260450) with high statistical support (1.00/100/91). This lineage clustered in a sister position with *Desertella californica* (KF693789) with high support (1.00/99/83). This lineage, which includes *Phyllosiphon* spp., uncultured "*Chlorella*" strain and *Desertella californica,* was placed in an unsupported sister position to a firmly supported clade comprising the genera *Chloroidium* and *Parachloroidium*. *Lobosphaeropsis pyrenoidosa* (KM020128), together with the18S rDNA sequence from an organism labelled as "*Stichococcus bacilaris*" (KM020054), and *Watanabea* sp. (EU090195) formed highly supported monophyletic clade (1.00/100/100), which clustered in a sister position with higly supported clade (1.00/100/100) comprising *Watanabea* sp. (FM958480) and *Watanabea* sp.

(KF693804). The *Watanabea* clade as a whole represented a moderately supported lineage of the Trebouxiophyceae (0.98/95/0) and also included the genera *Kalinella*, *Heterochlorella*, *Heveochlorella*, *Watanabea*, and *Viridiella*.

The topology of the phylogenetic tree derived from analysis of the *rbc*L sequences (Fig. 13) also illustrated that the *Phyllosiphon* lineage formed a highly supported monophyletic clade (1.00/100/100) with an unresolved position within the *Watanabea* clade (1.00/95/65). The *rbc*L gene sequences of most of the *Phyllosiphon* isolates were identical. The *rbc*L sequence in strain *k55* contained two ambiguous nucleotides, and the sequences from strains *k115* and *k17* differed by 3 and 51 nucleotide substitutions, respectively. The authentic strain of *Heveochlorella hainangensis* Zhang, Huss, Sun, Chang and Pang (SAG 2360) Zhang clustered with *Heterochlorella luteoviridis* (Chodat) Neustupa et al. (CAUP H1906). The *Watanabea reniformis* strain SAG 211-9b formed a highly supported monophyletic clade (1.00/100/100) with *Watanabea* sp. strain CCAP 6091/1 and *Watanabea* sp.(KF693823). The monophyletic genera *Chloroidium* and *Parachloroidium* formed a joint lineage that was moderately supported by the statistical analyses (1.00/92/69).

All of the sequences belonging to the genera *Chloroidium* and *Parachloroidim*, including the three newly acquired *Chloroidium* sequences, had a single codon insertion at position 286 of the *rbc*L gene sequence. This insertion (AAA in *Chloroidium* and AAG in *Parachloroidim*) resulted in the insertion of a lysine residue in the encoded protein. Interestingly, none of the sequences from other members of the *Watanabea* clade, including the newly acquired *rbc*L sequences from the genera *Viridiella*, *Watanabea*, *Phyllosiphon* and *Heveochlorella hainangensis*, possessed either of these insertions. Therefore, the unique insertion of lysine in the coding region of the *rbc*L gene was clearly a synapomorphic character of the *Chloroidium* / *Parachloroidium* lineage.

Discussion

This study demonstrated that members of the trebouxiophycean *Phyllosiphon* clade exist not only as leaf parasites of the aracean species (Aboal &Werner 2011), but might also occur as free-living microalgae in subaerial microhabitats such as tree bark. This corresponded with previous research by Hallmann et al. (2013) and Cutler et al. (2013), who also detected *Phyllosiphon*-related sequences in samples of phototrophic biofilms thriving on sandstone substrates in Germany and the UK, respectively. The previous studies

were based only on molecular data; in this study we investigated molecular and morphological characteristics of parasitic and free-living *Phyllosiphon* lineages. The sequence data acquired by Hallmann et al. (2013) and Cutler et al. (2013) illustrated that the free-living taxa belonging to the *Phyllosiphon* lineage might occur not only in subaerial biofilms thriving near to natural populations of *Arisarum vulgare*, the primary host species of the parasitic stages, but also in temperate regions distant to the predominantly Mediterranean distribution area of the plant species.

We also discovered *Phyllosiphon* isolates in corticolous biofilms; this supported the previous data and further indicated that this genus might be a frequently occurring and autochthonous member of these microhabitats. Alternatively, the endospores, produced by the parasitic thalli, could be readily dispersed to relatively long distances so that they can frequently reach places with not adequate host. Interestingly, the free-living corticolous isolates *cort21* and *cort08*, characterized by unique *rbc*L sequences in a recent study of Kulichová et al. (2014), also proved to be representatives of the *Phyllosiphon* lineage. The lack of earlier records of *Phyllosiphon* from subaerial biofilms may be explained by its simple chlorelloid morphology that may have resulted in misidentification in previous morphology-based studies dealing with the diversity of phototrophic subaerial biofilms. However, it should be noted that we did not find any clear-cut evidence that the free-living stages actually reproduced within the biofilms. Therefore, we cannot reject the hypothesis that the free-living *Phyllosiphon* cells, recovered from the subaerial biofilms, might have been the resistance stages originating directly from the parasitic thalli.

Our molecular phylogenetic analyses, based on 18S rDNA and *rbc*L gene sequences, confirmed the monophyly of the available *Phyllosiphon* specimens. The phylogenetic analysis of the 18S rDNA sequences illustrated a sister relationship of the *Phyllosiphon* lineage with an unidentified photobiont alga isolated from the lichen *Psoroglaena epiphylla* (Nyati et al. 2007). This undescribed trebouxiophycean taxon may thus possibly be considered a sister genus to *Phyllosiphon*. However, as isolate L-1016 acquired by Nyati et al. (2007) is presently unavailable, the eventual taxonomic description of this taxon rests on its eventual re-isolation from natural habitats.

Our isolates, which were acquired from infected leaves of *Arisarum vulgare* in Cyprus and from corticolous biofilms in Puglia and Trieste in Italy, represented a lineage (with one exception) with homogenous18S rDNA sequences that were identical to a previously published *P. arisari* 18S rDNA sequence from *Arisarum vulgare* leaves from Southern Spain (Aboal & Werner 2011). However, sequence JX127175, which originated

from epilithic biofilms in Germany (Hallmann et al. 2013), differed by 23 nucleotide positions and clustered in a closely related sister position to the above mentioned clade. Given the genetic differences in this conservative marker, as well as the present molecular taxonomic conception of species and genera within the *Watanabea* clade (Ma et al. 2013, Neustupa et al. 2013b), we presume that the sequences studied by Hallmann et al. (2013) were possibly acquired from a separate, and so far undescribed, taxon of the *Phyllosiphon* lineage. However, whether this taxon solely occurs as a free-living member of subaerial biofilms or might also thrive as a parasite of the Araceae remains to be ascertained by future studies. The precise phylogenetic position of the *Phyllosiphon* sequences recovered in the epilithic biofilms from Northern Ireland (Cutler et al. 2013) could not be assessed as the authors did not submit sequences to public databases. However, as their 18S rDNA sequences were approximately 96.5% similar to those of Aboal & Werner (2011), they may probably also have represented an additional *Phyllosiphon* taxon.

Based on the data presented in this study, we can rule out the possibility that cocoid stages of the *Phyllosiphon* taxa could be identical with morphologically defined *Lobosphaeropsis pyrenoidosa*, described originally from an alpine soil sample by Reisigl (1969). First, the *Phyllosiphon* cells consistently lack any pyrenoids, whose presence has been considered typical for *L. pyrenoidosa*. In addition, the coccoid stages cells of the *Phyllosiphon* taxa are typically about 25-50% larger than in *L. pyrenoidosa*. However, it should be noted that one of the well supported lineages of the *Watanabea* clade also includes an unpublished sequence KM020128 that originated from a strain morphologically identified as *L. pyrenoidosa*. This lineage also includes two additional sequences from strains that probably did not unambiguously correspond to any of the previously described taxa (one of them was apparently mislabelled as *Stichococcus bacillaris*). Therefore, it may possibly accommodate the traditional generic name *Lobosphaeropsis*. However, such taxonomic change could possibly only be conducted on the basis of combined phenotypic and molecular investigation of the strains belonging to this lineage. In contrast with the almost homogenous 18S rDNA sequences, phylogenetic analyses of the *rbc*L gene sequences in this study revelaed at least two closely related *Phyllosiphon* lineages. The first lineage comprised 15 isolates from various locations and formed a cluster with distinct sequences from strains *k55* and *k115*, which were isolated from corticolous biofilm sample from North Italy. The second consisted solely of sequence *k17*, which was also found in the corticolous biofilm sample from North Italy (Kulichová et al. 2014). As the *k17* genotype was recovered from only a single sample, we could not determine whether it might

constitute a separate species with a free-living life strategy, or whether it might also be capable of a parasitic life style in leaves of the Araceae.

Our extended sampling of the *rbc*L gene within the members of *Watanabea* clade also aimed to clarify the peculiar evolution of a single codon insertion at position 286 of the gene sequence (encoding a lysine residue) that was previously noted in the genera *Chloroidium* and *Parachloroidium* by Neustupa et al. (2013b). Such short insertions in the *rbc*L gene of actively phototrophic organisms are extremely rare as their acquisition must not lead to a shift in the reading frame of the DNA strand. The only other documented case of such an insertion in the *rbc*L gene was described by Daugbjerg & Guillou (2001), who illustrated that diatoms and bolidophytes share a two-codon insertion at position 1336 that forms a unique synapomorphy of their joint phylogenetic clade. As only a limited *rbc*L dataset was available from the *Watanabea* clade, it was not clear what evolutionary scenario was most probable for the 286-Lys insertion. However, our additional *rbc*L sequence data acquired from the members of the genera *Phyllosiphon*, *Chloroidium*, *Viridiella*, *Watanabea* and *Heveochlorella* clearly illustrated that the 286-Lys insertion was not shared by any other known members of the *Watanabea* clade. Thus, this insertion may be considered a synapomorphy of the *Chloroidium*/*Parachloroidium* clade that probably evolved uniquely in their common ancestor.

Given the relatively frequent occurrence of *Phyllosiphon* populations in subaerial biofilms, we also wished to evaluate the possibility that previous records of these sequences from the leaves of *Arisarum vulgare* (Aboal & Werner 2011) might be caused by contamination of the leaves by free-living cells from adjacent habitats. However, we believe that this eventuality may now be excluded. We obtained endospores directly from parasitic siphonous growth in the infected leaves of *Arisarum vulgare.* The endospores were isolated onto agar-solidified BBM medium, where they rapidly developed into autosporangia that produced chlorelloid cells with morphologies typical of the *Watanabea* clade members (Neustupa et al. 2009, 2013a). The sequences acquired from the cultured populations were identical to those of the parasitic stages, as well as to the sequences reported by Aboal & Werner (2011). This suggests that, after release from siphonous parasitic thalli, endospores are dispersed relatively easily by wind or rain and can travel extended distances due to their small size. In a suitable new locality, endospores could then reproduce via sphaerical autospores and eventually infect other populations of *Arisarum*. We were able to cultivate *Phyllosiphon* isolates acquired from both parasitic and free-living populations *in vitro* on a standard culture medium designed for green microalgae (Andersen et al. 2005). We

therefore conclude that this alga does not have any specific nutritional requirements and can easily survive outside its host plant. Consequently, we consider *Phyllosiphon* to be a facultative parasite that possesses free-living stages in its life cycle and is capable of autonomous reproduction and long-term survival outside the host plant. This life strategy is somewhat similar to the other trebouxiophycean parasitic genera *Helicosporidium* and *Prototheca* (Boucias et al. 2001) that, unlike most pathogenic protists, can also be easily cultured *in vitro*.

However, it is not certain that all *Phyllosiphon* species are the facultative parasites. It is possible that solely some species of the *Phyllosiphon* lineage retained the parasitic life strategy, while the other species might have secondarily lost this capacity. Existing results are based on a relatively small amount of samples, which were phylogenetically characterized using the conservative molecular markers. However, ecological strategies of particular species within *Phyllosiphon* lineage will be examined in the future, in a study based on additional samples of parasitic and free-living populations characterized by the highly variable molecular markers.

Despite recent advances, little is known about regarding the diversity of trebouxiophycean chlorelloid green microalgae. Numerous novel taxa, such as *Chloroidium*, *Heveochlorella*, *Kalinella*, *Leptochlorella,* and *Parachloroidium*, were recently described from various subaerial microhabitats (Darienko et al. 2010, Ma et al. 2013, Neustupa et al. 2013a, b). Notably, the genera and species belonging to the *Watanabea* clade tend to be highly abundant in subaerial biofilms. Therefore, reports of free-living *Phyllosiphon* populations from these microbial assemblages (Cutler et al. 2013, Hallmann et al. 2013, this study) supplement a steadily growing body of knowledge regarding morphologically homogenous chlorelloid members of the *Watanabea* clade. The genus *Phyllosiphon* is currently the only member of the *Watanabea* clade known to form morphological stages other than unicellular coccoid thalli. However, data on the life cycles of trebouxiophycean microalgae remain extremely limited. Most chlorelloid microalgae have been taxonomically described solely on the basis of an examination of cultivated clonal strains isolated from biofilms growing on substrates such as tree bark, stone surfaces, or soils (Ettl & Gärtner 1995, Darienko et al. 2010, Ma et al. 2013, Neustupa et al. 2013a, b). Moreover, these taxa are often only known from their type localities. Thus, it is not yet clear whether these chlorelloid microalgae form only the simple coccoid stages typical of cultured clonal populations or whether other life stages also exist but have not yet been observed.

Interestingly, several lineages of the Trebouxiophyceae, such as the genera *Prototheca*, *Helicosporidium*, *Auxenochlorella*, some members of the Oocystaceae, and members of the *Watanabea* clade, have relatively divergent 18S rDNA sequences. This is illustrated by their extremely long branches in phylogenetic reconstructions and suggests an elevated rate of molecular evolution at this molecular locus (Krienitz et al. 2004, Darienko et al. 2010, Aboal & Werner 2011, Krienitz & Bock 2012, Hallmann et al. 2013, Neustupa et al. 2013a). The rate of molecular evolution is generally believed to be faster in parasitic taxa than in their non-parasitic relatives (Bromham et al. 2013). Thus, it may be possible that members of additional genera within the *Watanabea* clade that are represented by extremely long branches in phylogenetic trees, such as *Heterochlorella*, *Heveochlorella*, or *Kalinella*, might also have complex life cycles that include stages with dramatically different morphologies and ecological strategies.The present study, alongside previous research by Aboal & Werner (2011), demostrated that *Phyllosiphon arisari* thrives in the leaves of *Arisarum vulgare* across the Mediterranean region (i.e., from Spain to Cyprus) and also occurs as a free-living alga in subaerial biofilms in the Mediterranean regions and in localities distant to the distribution area of the host plant genus *Arisarum*. Close relatives of the parasitic genotypes also occur in subaerial biofilms throughout Europe. Although the genus *Phyllosiphon* is presently the only known trebouxiophycean alga infecting vascular plants, it is possible that future surveys of microorganisms occuring in vascular plants may reveal additional unknown microalgal taxa with parasitic or endophytic life strategies.

Acknowledgements

We thank Jana Steinová for her assistance with DNA isolation, Pavel Škaloud for his assistance with design of primers. This work was supported by a grant from the Czech Science Foundation P506/12/0955 and by the research project no. 394315 of the Charles University Grant Agency. The authors also thank BioEdit proofreading service for the English language and style corrections.

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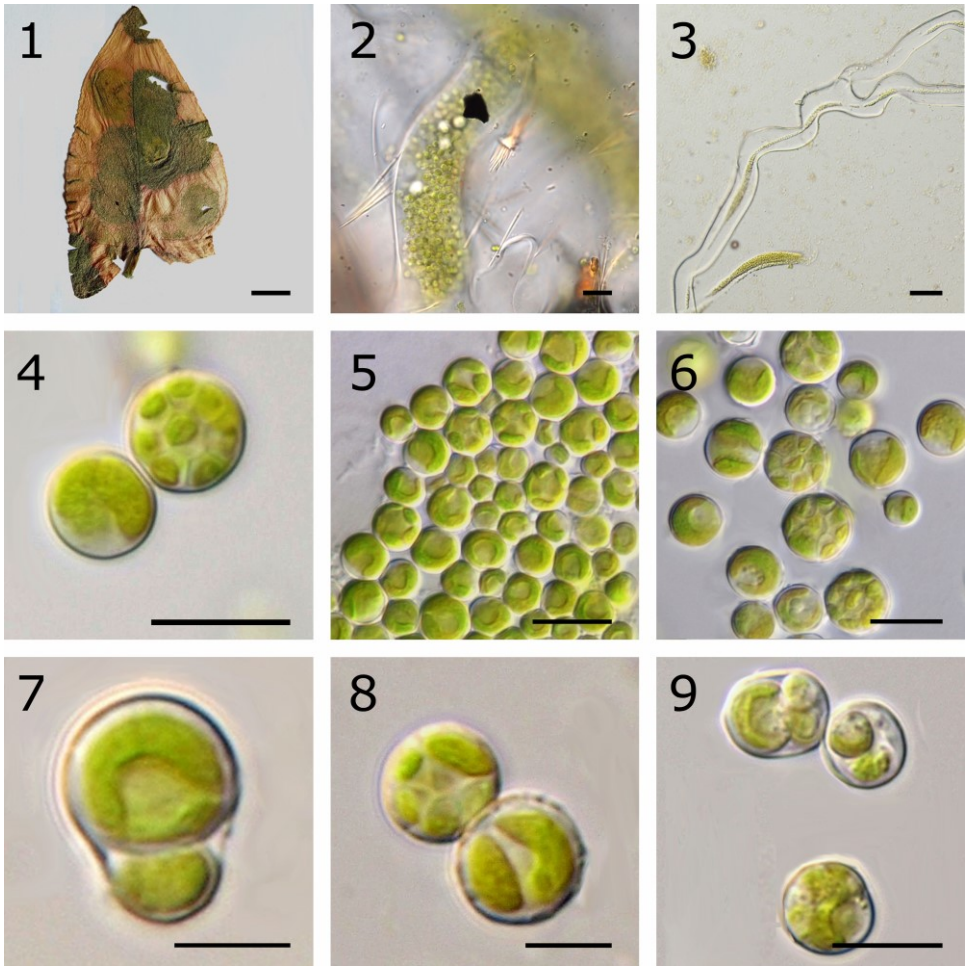
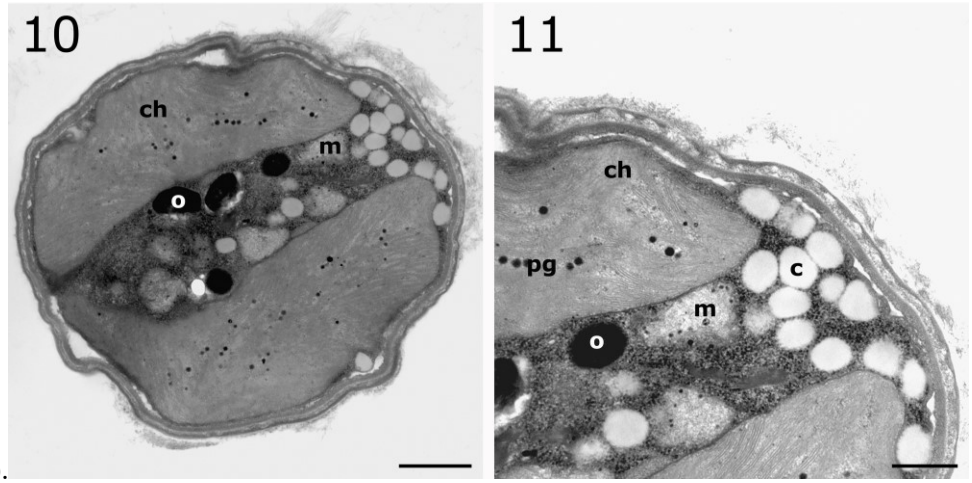


Figs 1–9. Light microscopic morphology of *Phyllosiphon*. Fig. 1. *Arisarum vulgare* leaf infected with *Phyllosiphon arisari*. Figs 2–3. Leaf parenchyma containing branching siphonal filaments, with endospores. Fig. 4. Vegetative cell and autosporangium, strain AV1. Fig. 5. Vegetative cells and autosporangia, strain B3e. Fig. 6. Vegetative cells and autosporangia, strain B4a. Fig. 7. Detail of two-celled autosporangium, strain AG10. Fig. 8. Vegetative cell and autosporangium, strain AG10. Fig. 9. Vegetative cell and autosporangia, strain QI2-9. Scale bars: 1 cm (Fig. 1), 20 µm (Fig 3), 10 µm (Figs –6, 9), 5 µm (Figs 7–8).

8). 

Figs 10–11. Transmission electron micrographs of *Phyllosiphon arisari* from corticolous biofilm, strain B4a. Fig 10. Vegetative cells with plastids, mitochondria and electron-dense extraplastidial oil droplets. Fig. 11. Detail of cell with plastid containing pyrenoglobuli and numerous extraplastidial oil droplets. c = carbohydrates, ch = chloroplast, m = mitochondrion, o = oil droplets, pg = plastoglobuli. Scale bars: 1 μm.

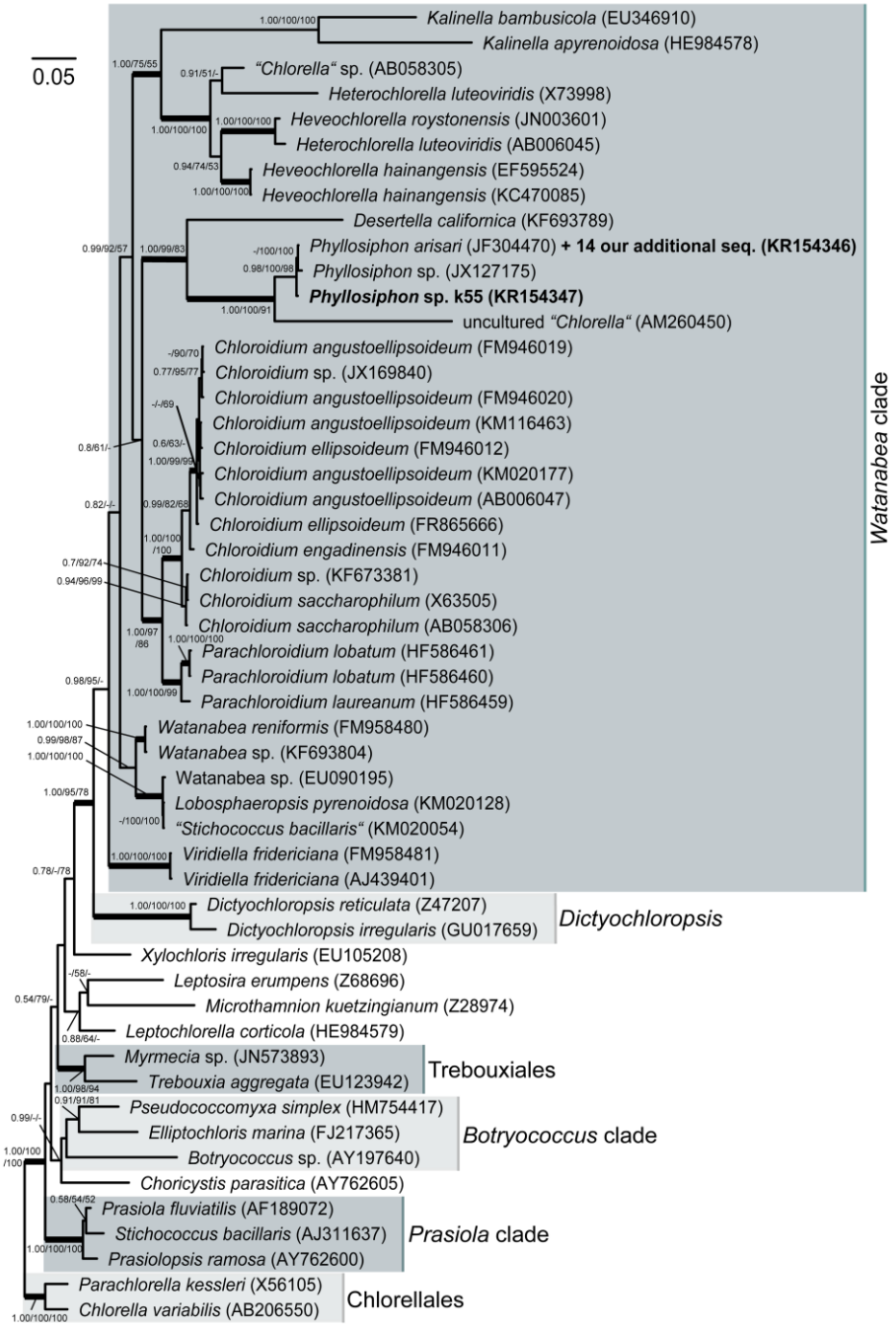


Fig. 12. Bayesian unrooted tree of Trebouxiophyceae based on 18S rDNA gene sequences. Numbers at nodes indicate statistical support (BPP > 0.95 / ML > 50 % / MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequences newly acquired in this study are shown in bold. The scale bar shows the estimated number of substitutions per site.

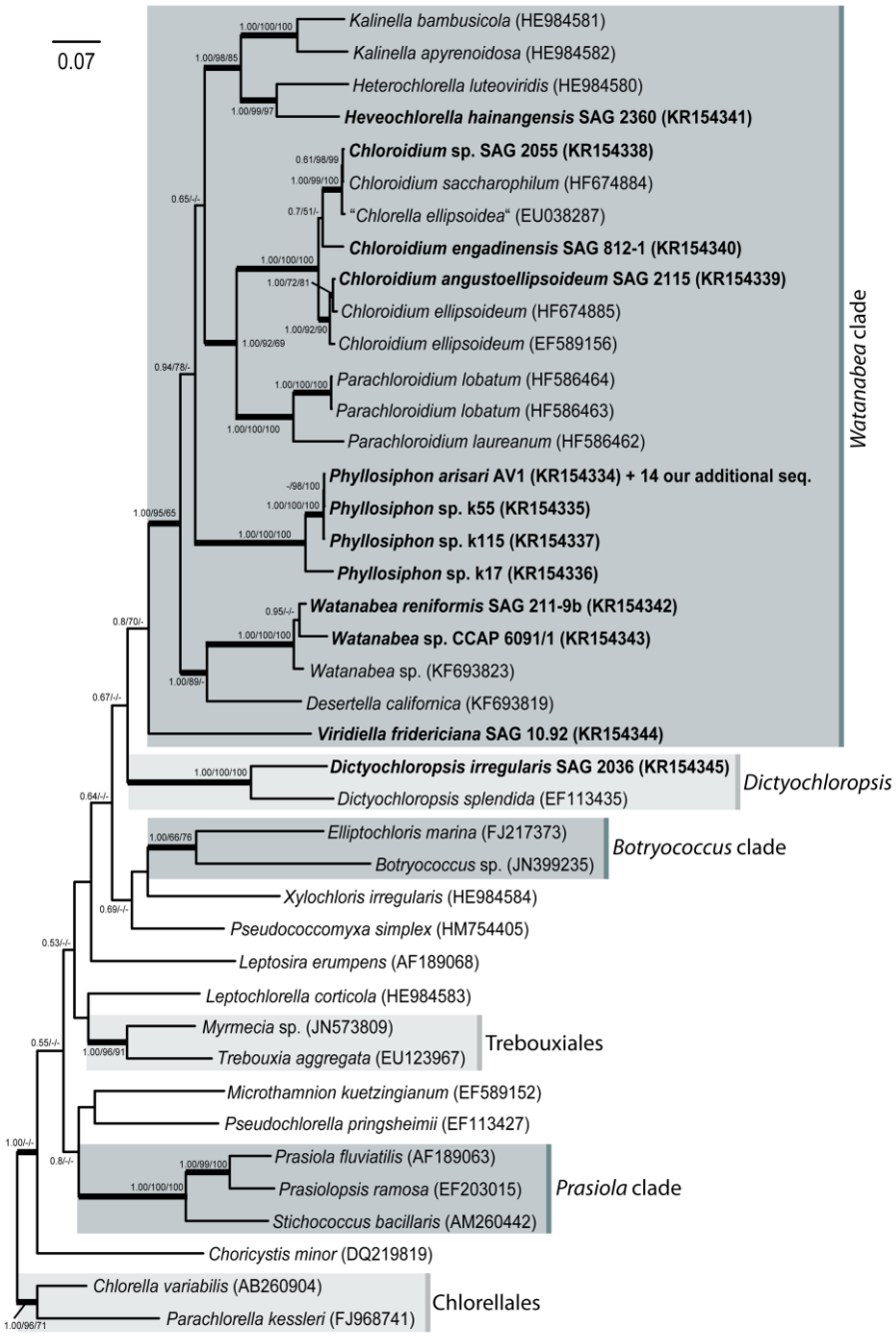


Fig. 13. Bayesian unrooted tree of Trebouxiophyceae based on *rbc*L sequences. Numbers at nodes indicate statistical support (BPP > 0.95 / ML > 50 % / MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequences newly acquired in this study are shown in bold. The scale bar shows the estimated number of substitutions per site.

Table 1. Characteristics of strains used in this study

Strain no.	Species	Origin	Geographic coordinates	Isolation date	18S rDNA GenBank accession no.	*rbc*L GenBank accession no.
AV1	*Phyllosiphon arisari*	*Arisarum vulgare* leaf, Cyprus	34°53'52.60" N 32°21'51.28" E	03/2013	KR154346	KR154334
AV2	*P. arisari*	*Arisarum vulgare* leaf, Cyprus	34°53'50.63" N 32°21'42.30" E	03/2013	identical to KR154346	identical to KR154334
AV3	*P. arisari*	*Arisarum vulgare* leaf, Cyprus	34°53'50.63" N 32°21'42.30" E	03/2013	identical to KR154346	identical to KR154334
B3e	*P. arisari*	*Pistacia lentiscus* bark, Basilicata, Italy	40°10'03.03" N 16°41'16.38" E	02/2012	identical to KR154346	identical to KR154334
B4a	*P. arisari*	*Ficus caprificus* var. *caprificus* bark, Basilicata, Italy	40°10'03.03" N 16°41'16.38" E	11/2012	identical to KR154346	identical to KR154334
B4b	*P. arisari*	*Ficus caprificus* var. *caprificus* bark, Basilicata, Italy	40°10'03.03" N 16°41'16.38" E	11/2012	identical to KR154346	identical to KR154334
AG6	*P. arisari*	*Alnus glutinosa* bark, Basilicata, Italy	40°10'04.44" N 16°41'15.03" E	11/2012	identical to KR154346	identical to KR154334
AG10	*P. arisari*	*Alnus glutinosa*	40°10'04.44"	11/2012	identical to	identical to

		bark, Basilicata, Italy	N 16°41'15.03" E		KR154346	KR154334
AG13	*P. arisari*	*Alnus glutinosa* bark, Basilicata, Italy	40°10'04.44" N 16°41'15.03" E	11/2012	identical to KR154346	identical to KR154334
AG16	*P. arisari*	*Alnus glutinosa* bark, Basilicata, Italy	40°10'04.44" N 16°41'15.03" E	11/2012	identical to KR154346	identical to KR154334
PH1	*P. arisari*	*Pinus halepensis* bark, Puglia, Italy	40°09'27.42" N 17°58'03.18" E	11/2012	identical to KR154346	identical to KR154334
QI1-1	*P. arisari*	*Quercus ilex* bark, Puglia, Italy	40°37'59.64" N 17°16'23.31" E	11/2012	identical to KR154346	identical to KR154334
QI2-6	*P. arisari*	*Quercus ilex* bark, Puglia, Italy	40°37'59.64" N 17°16'23.31" E	11/2012	identical to KR154346	identical to KR154334
QI2-8	*P. arisari*	*Quercus ilex* bark, Puglia, Italy	40°37'59.64" N 17°16'23.31" E	11/2012	identical to KR154346	identical to KR154334
QI2-9	*P. arisari*	*Quercus ilex* bark, Puglia, Italy	40°37'59.64" N 17°16'23.31" E	11/2012	identical to KR154346	identical to KR154334
k17	*Phyllosiphon* sp.	*Quercus ilex* bark, Duino, Italy	45°46'38.47" N 13°35'46.67"	04/2012	identical to KR154346	KR154336

			E			
k55	*Phyllosiphon sp.*	*Quercus ilex* bark, Duino, Italy	45°46'38.79" N 13°35'42.49" E	04/2012	KR154347	KR154335
k115	*Phyllosiphon sp.*	*Quercus ilex* bark, Duino, Italy	45°46'38.79" N 13°35'42.49" E	04/2012	-	R154337
SAG 2055	*Chloroidium* sp.	*Bacidina inundata* lichenized thallus, Davos, Switzerland	-	07/2003	-	KR154338
SAG 2115	*C. angustoellipsoideum*	Epilithic biofilm, Göttingen, Germany	51°32'19.06" N 09°56'16.07" E	2004	-	KR154339
SAG 812-1	*C. engadinensis*	Soil, Unterengadin, Switzerland	46°42'19.08" N 10°24'07.00" E	1940	-	KR154340
SAG 2360	*Heveochlorella hainangensis*	*Hevea brasiliensis* bark, Hainan, China	19°58'59.00" N 110°19'37.00 "E	05/2005	-	KR154341
SAG 211-9b	*Watanabea reniformis*	Garden basin, Dorking, United Kingdom	51°15'15.02" N 00°19'59.04" W	1939	-	KR154342
CCAP 6091/1	*Watanabea* sp.	Lake Caviahue, Patagonia, Argentina	-	2007	-	KR154343

SAG 10.92	*Viridiella fridericiana*	Soil of sulphurous vents, Campania, Italy	-	1985	-	KR154344
SAG 2036	*Dictyochloropsis irregularis*	*Picea abies* bark, Austria	47°03'00.01" N 14°43'01.02" E	09/1991	-	KR154345

2.2. Paper II.

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.

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.) Schott. 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 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).

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′-TCACGACCTTC ATTACGAGCTTG-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 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).

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*.

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.

Acknowledgement

This work was supported by a grant from the Czech Science Foundation P506/12/0955 and by the research project no. 394315 of the internal Charles University Grant Agency. We also thank the anonymous reviewers for their critique and recommendations that improved the manuscript and Editage for English language editing.

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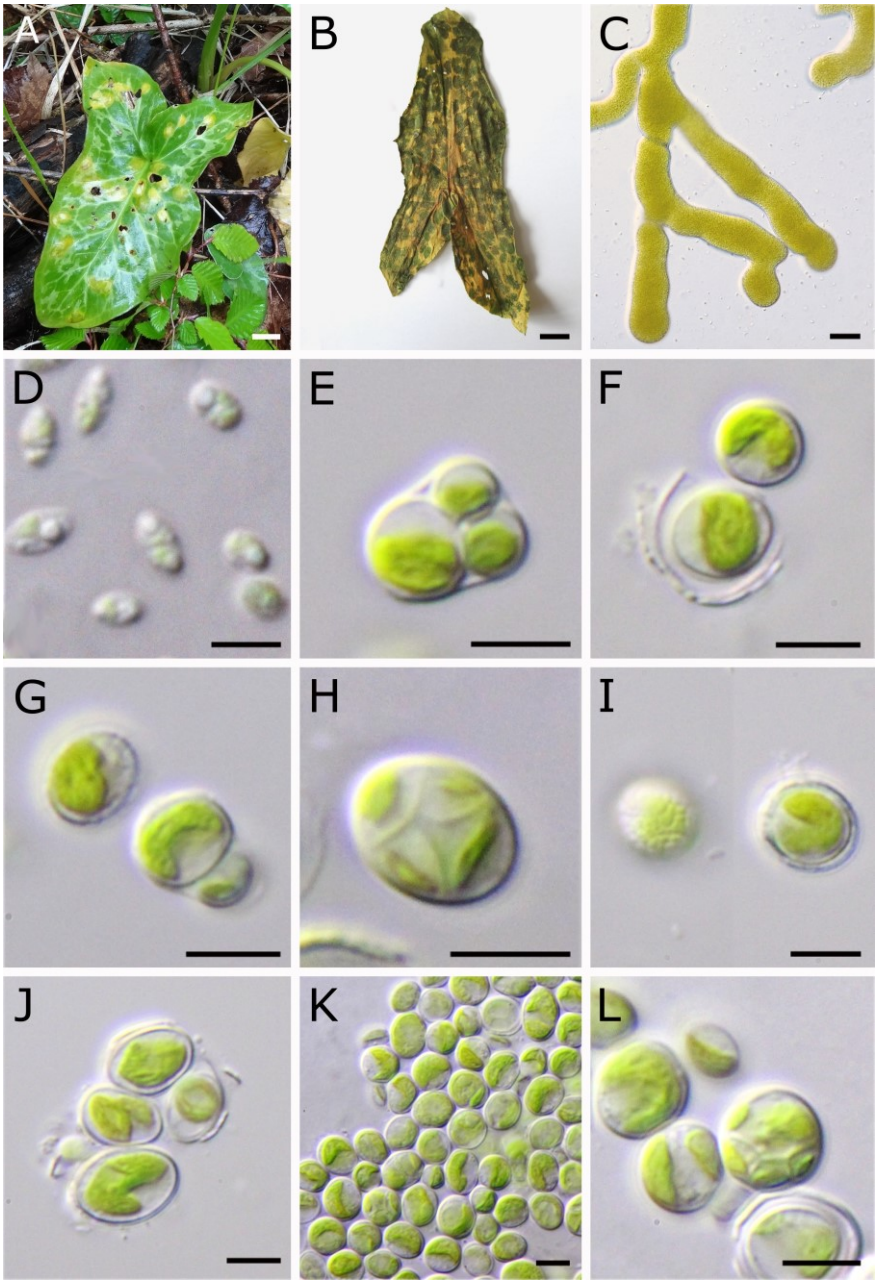

FIGURE 1. Morphology of *Phyllosiphon ari* sp. nov. A–B. A leaf of *Arum* at different stages of infection caused by *Phyllosiphon ari*. C. Branching filaments in the leaf parenchyma containing endospores. D. Detail of *P. ari* endospores released from filaments. E. Autosporangium of *P. ari*, strain 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).

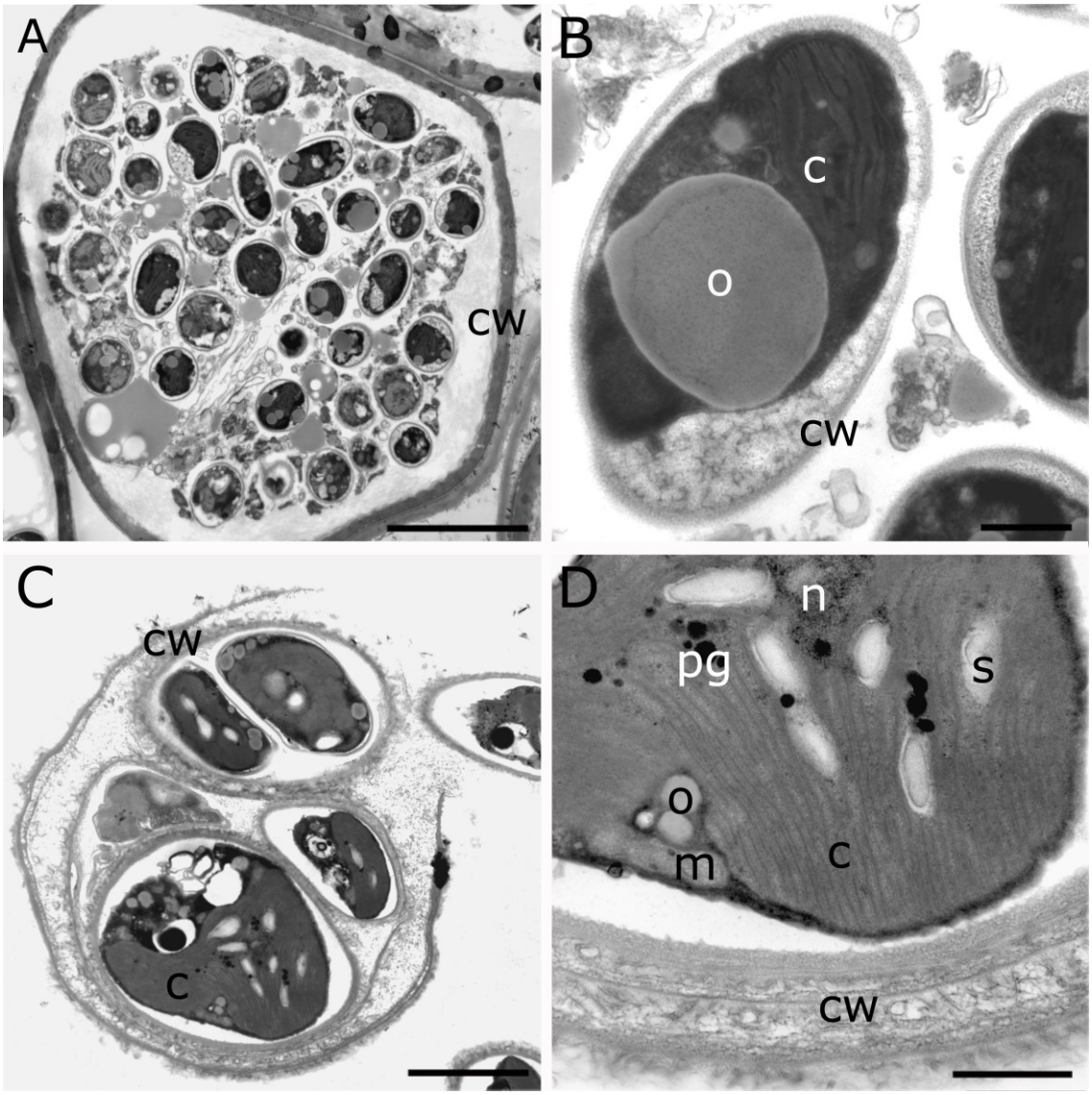

FIGURE 2. Ultrastructure of *Phyllosiphon ari* sp. nov. A. Cross section of parasitic filaments with thick cell wall containing endospores. B. Detail of an endospore with parietal chloroplast and large extraplastidial oil droplet. C. Autosporangium with autospores inside. Note the autospore released 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).

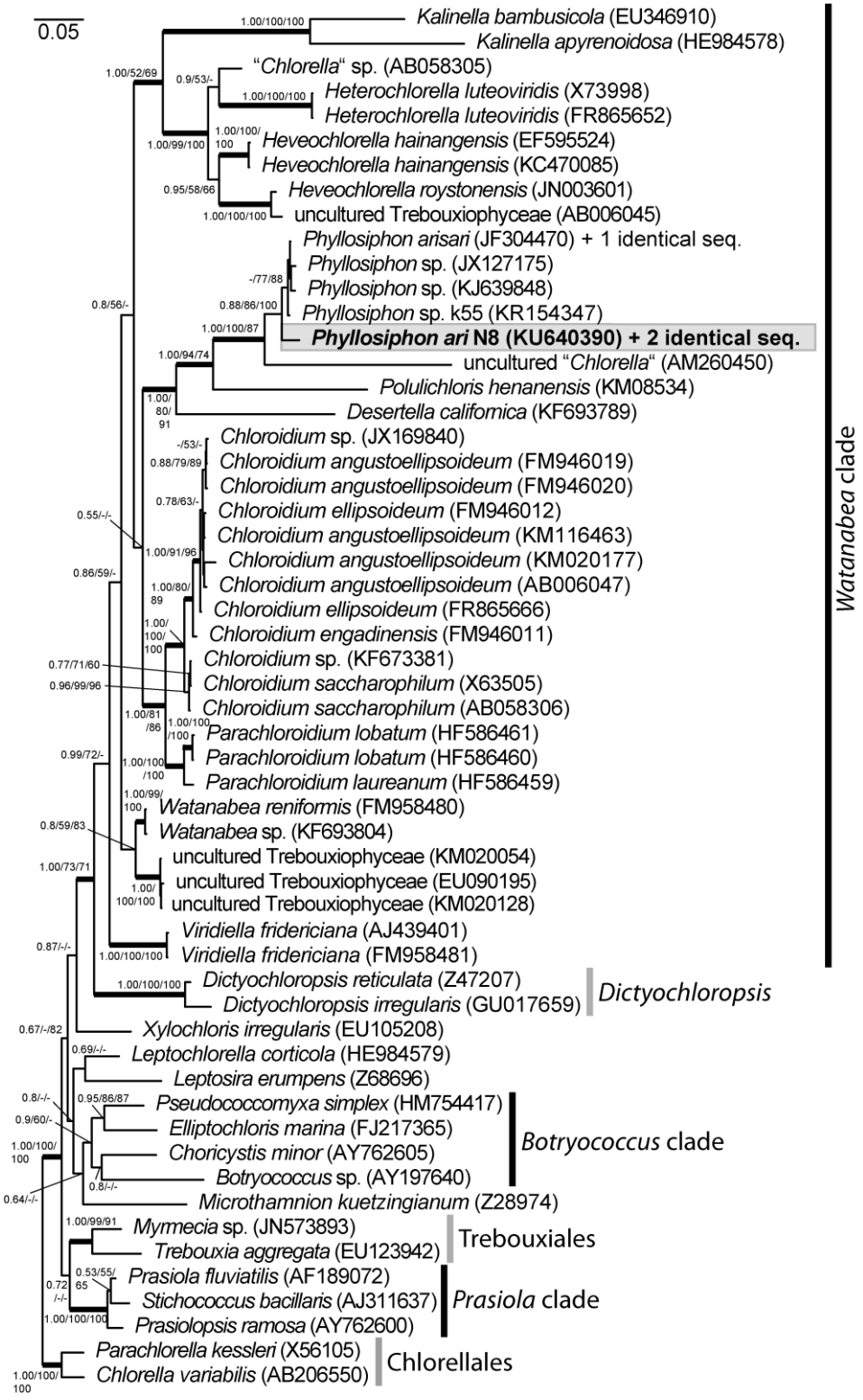

FIGURE 3. Bayesian analysis of Trebouxiophyceae based on the 18S rDNA dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ML > 50 %/MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequences newly acquired in this study are shown in bold. The scale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

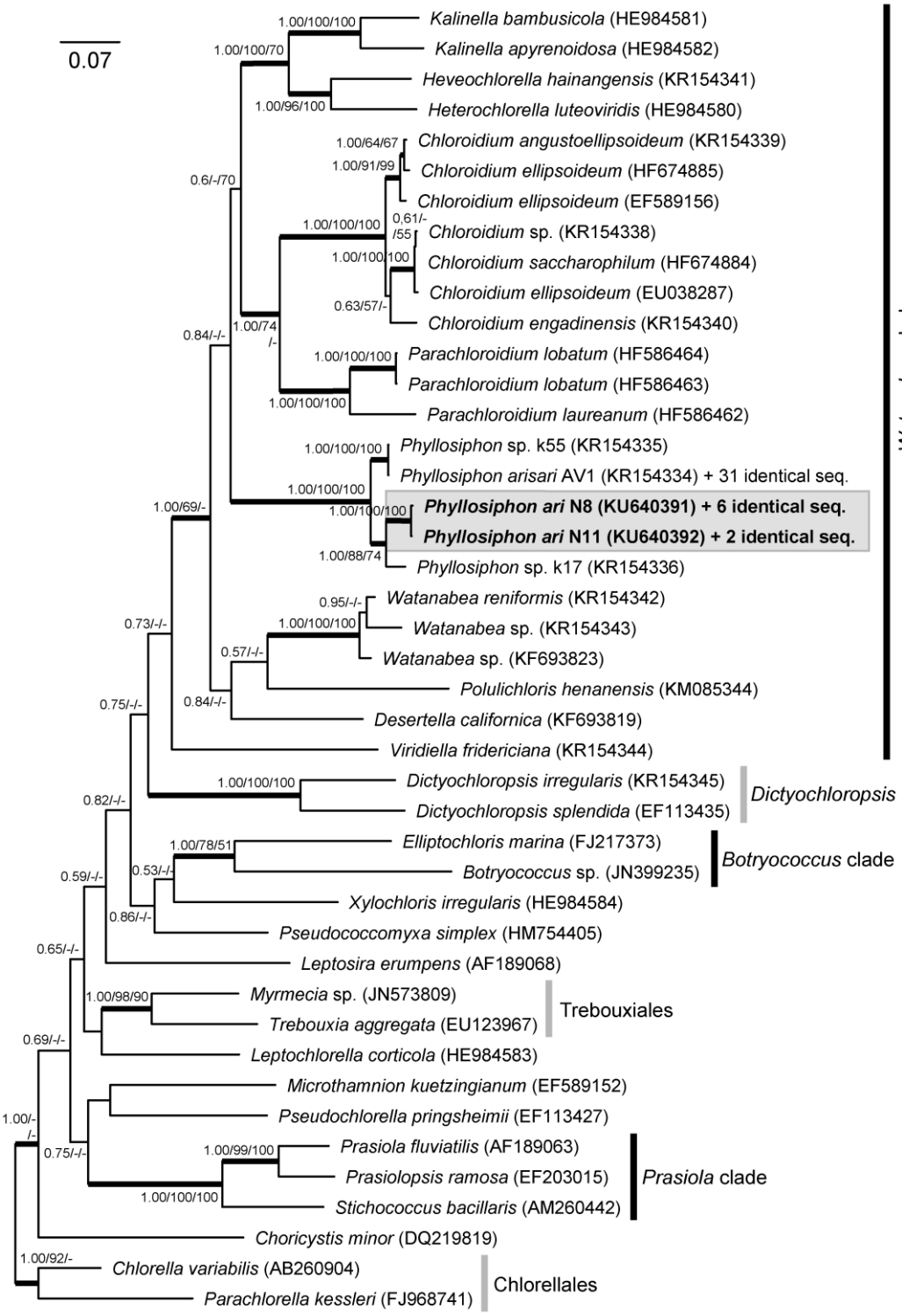

FIGURE 4. Bayesian analysis of Trebouxiophyceae based on the *rbc*L dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ML > 50 %/MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequences newly acquired in this study are shown in bold. The scale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

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,	44°27'19.2"N 14°33'12.4"E	03/2015	-	identical to KR154334

		Ilovik, Croatia				
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

2.3. Paper III.

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). *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′-CGGAGAGAAGGCTTGAGAATC GGCCTT-3′) and 1263R (5′-GAACGGCCATGCACCACC-3′, Pichrtová *et al.,* 2013); (b) NS1 (5′-GTAGTCATATGCTTGTCT-3′, Hamby *et al.,* 1988) and phyllos-R (5′-GGCAGCAAGGCG GGCCGCG-3′); (c) phyllos-F3 (5′-GACTAGGGATCGGCGGGCGTTTCTCGAA-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 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* sp. nov. K. Procházková, Y. Němcová & J. Neustupa**

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.

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.

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.

Acknowledgements. We would like to thank to our laboratory technician, Lenka Flašková, for her helpful advice and care. We also thank the anonymous reviewers for their critique and recommendations that improved the manuscript and Editage for English language editing. This work was supported by a grant from the Czech Science Foundation P506/12/0955 and by the research project no. 394315 from the internal Charles University Grant Agency.

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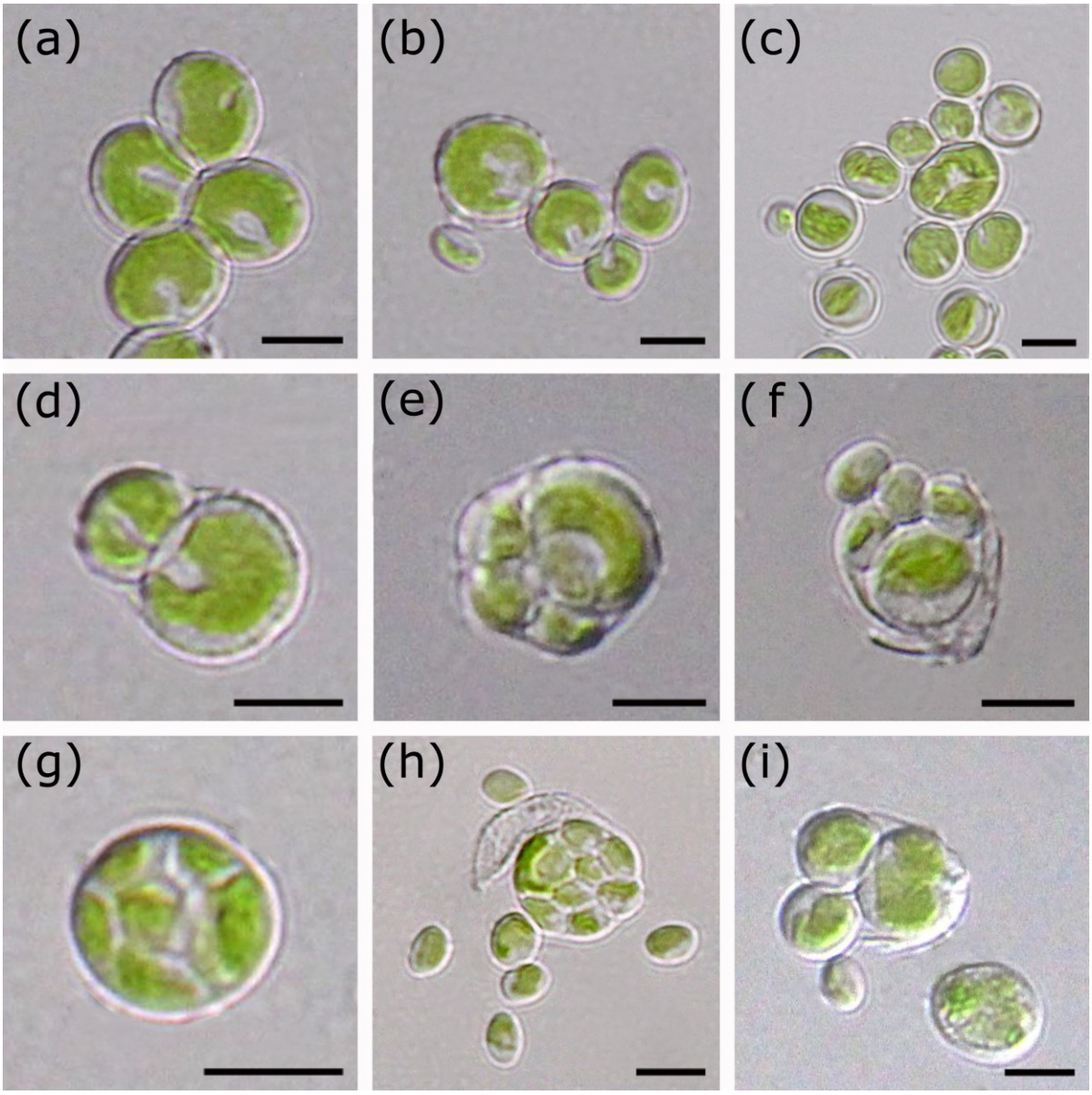
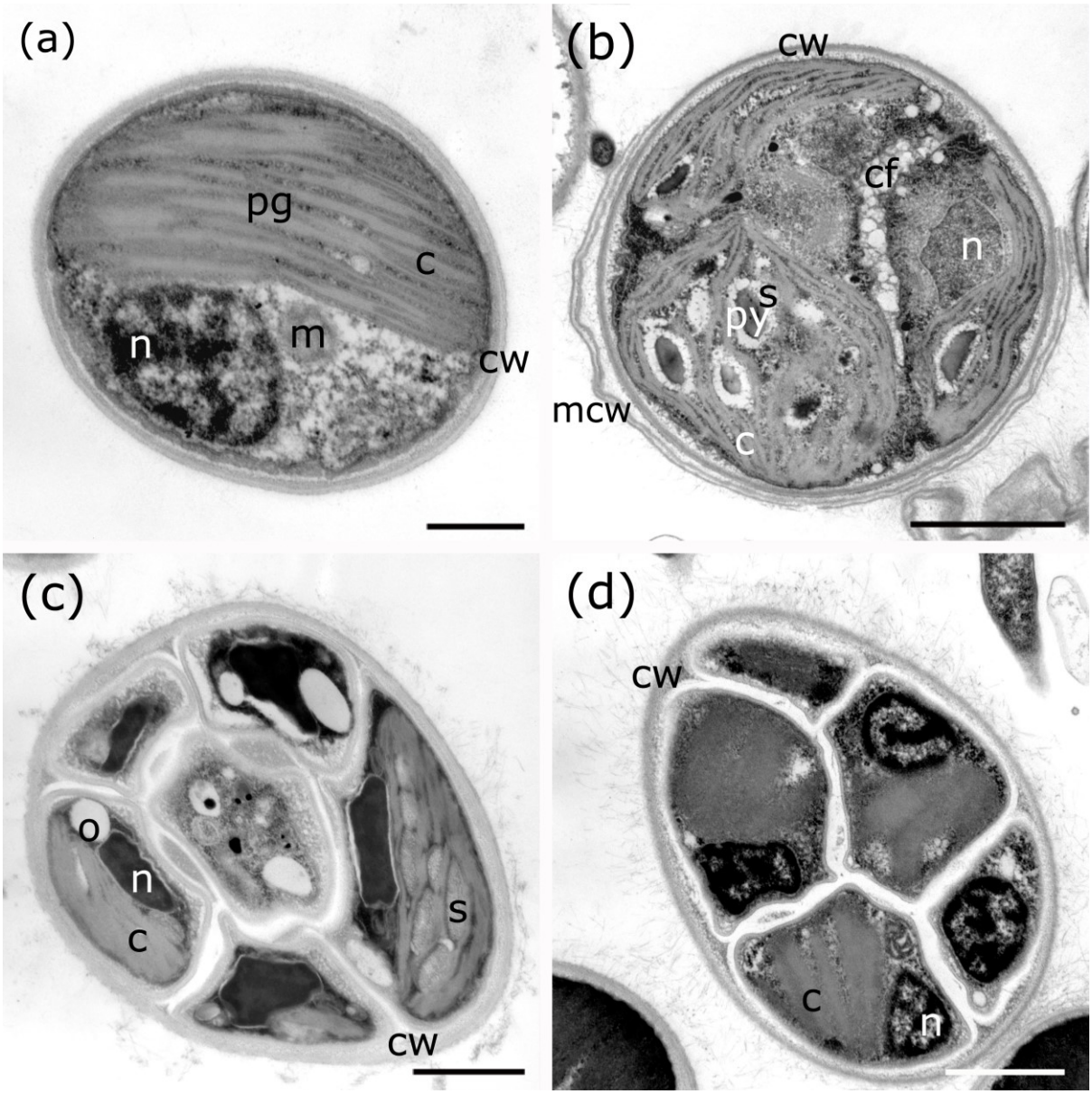


Figs 1–9. Morphology of Phyllosiphon duini sp. nov. 1–3. Vegetative cells. 4. Two-celled autosporangium. 5–6. Four-celled autosporangium. 7. Six-celled autosporangium. 8. Eight-celled autosporangium and autospores. 9. Autosporangium, autospore and vegetative cell. Scale bars: 5 μm.



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).

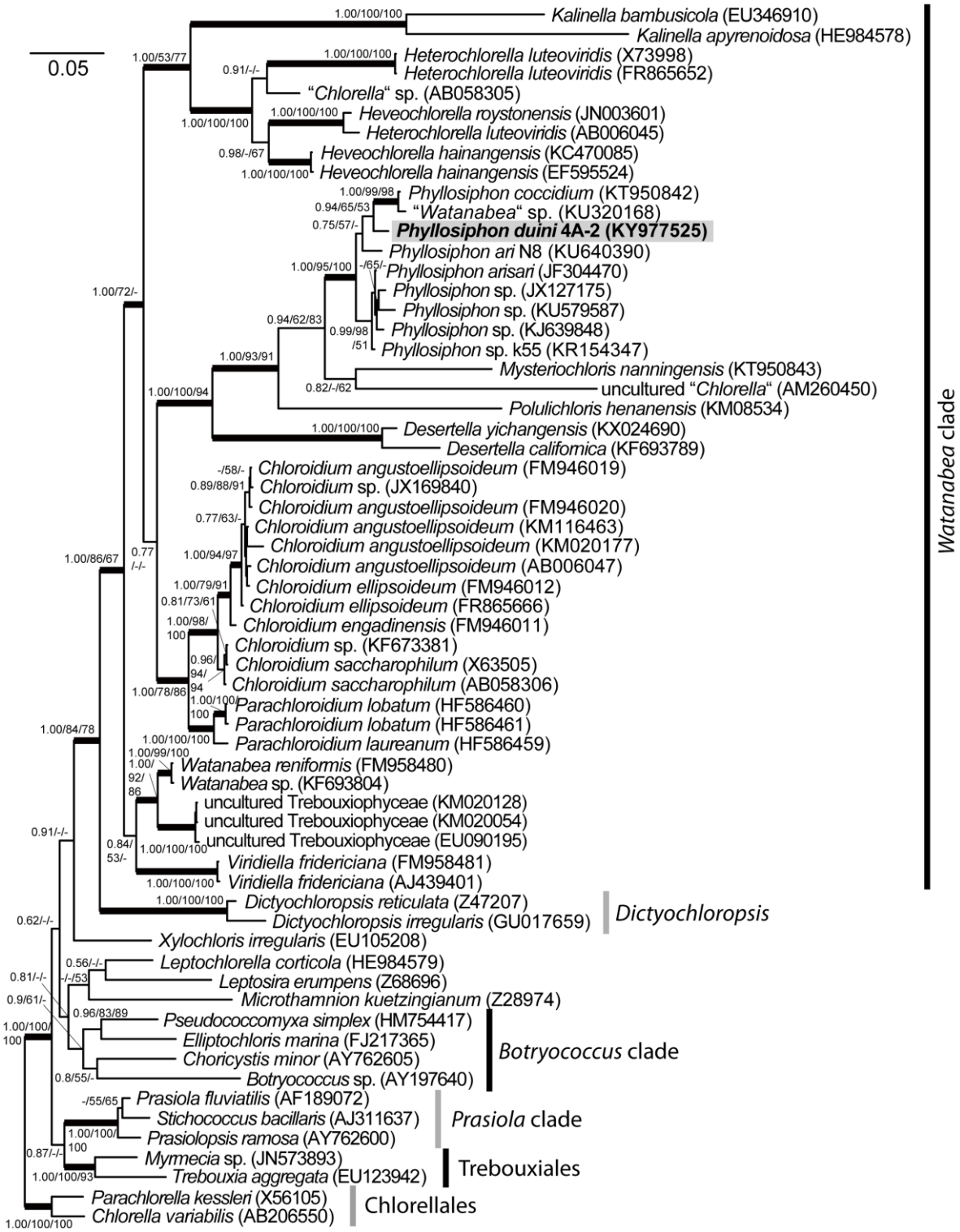


Fig. 14. Bayesian tree of Trebouxiophyceae based on the 18S rDNA dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ML > 50 %/MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequence newly acquired in this study are shown in bold. The scale bar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

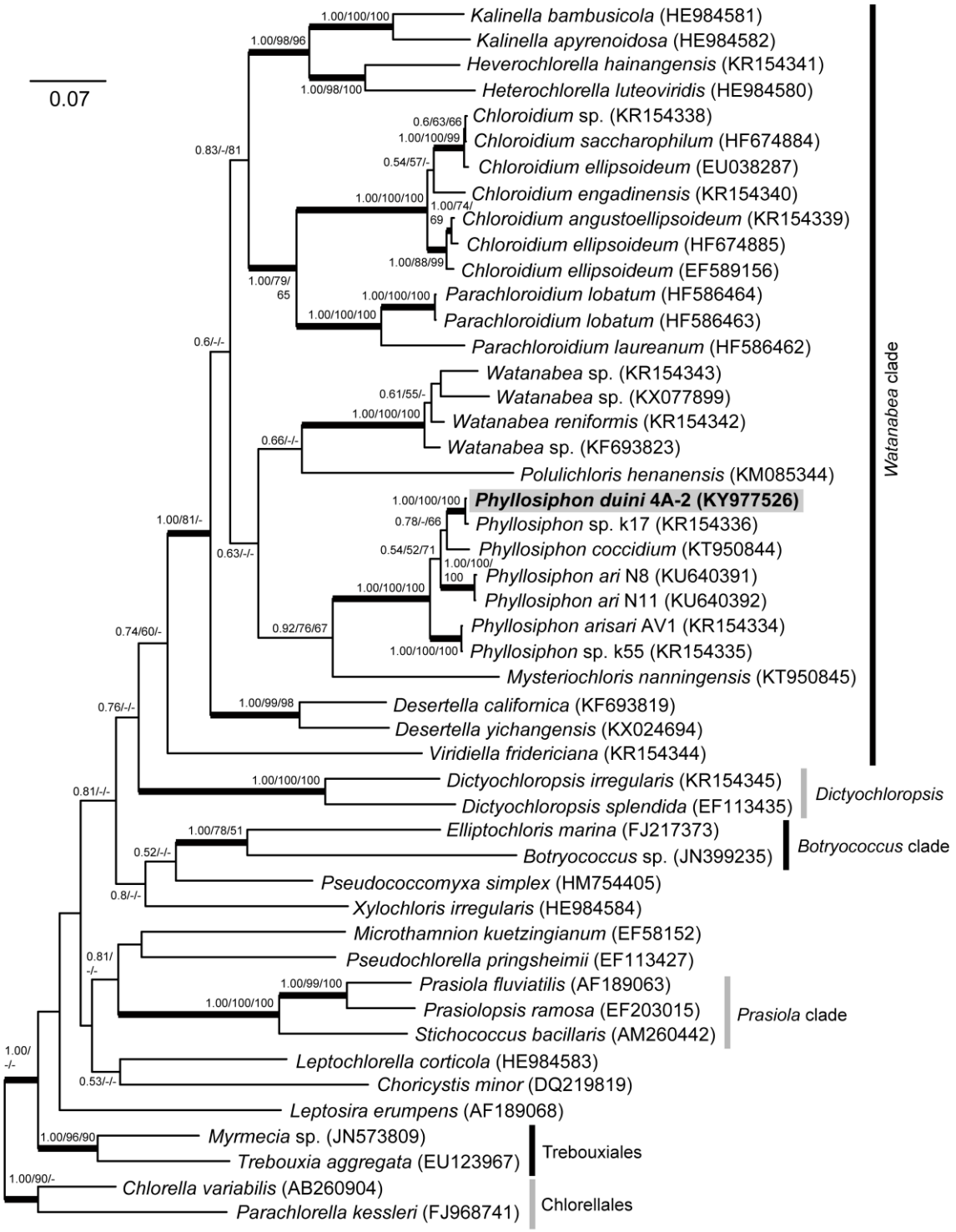


Fig. 15. Bayesian tree of Trebouxiophyceae based on the *rbc*L dataset. Numbers at nodes indicate statistical support (BPP > 0.95/ML > 50 %/MP > 50 %). Thick branches represent nodes receiving the highest BPP support (1.00). The sequence newly acquired in this study are shown in bold. The scale bar shows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

3 SUMMARY AND CONCLUSIONS

Results of presented studies provided several new pieces to the puzzle of genetic diversity of the genus *Phyllosiphon*. The thesis also provided several new findings about morphology, life cycle and ecology of the *Phyllosiphon* taxa.

Phylogenetic diversity and geographic distribution of parasitic Phyllosiphon *species*

It was found out that the parasitic members of the genus *Phyllosiphon* thriving in the leaves of the Araceae are phylogenetically non-homogenous. Our isolates of parasitic *Phyllosiphon* populations, which were acquired from infected leaves of *Arisarum vulgare* in Cyprus, Sardinia and Croatia (**papers I, II**), represented a lineage with homogenous 18S rDNA sequences that were identical to a previously published *P. arisari* 18S rDNA sequences from *Arisarum vulgare* growing in Southern Spain (Aboal & Werner 2011). Moreover, these isolates also formed a single homogenous lineage in the *rbc*L phylogenetic trees. Thus, these results, alongside the previous study of Aboal & Werner (2011), demonstated that *P. arisari* is widely distributed in the leaves of *Arisarum vulgare* across the Mediterranean region (i.e. from Spain to Cyprus).

The investigation of several parasitic specimens isolated from infected leaves of *Arum italicum* at single locality in the Krk island (Croatia) showed that these parasitic microalgae formed a phylogenetic lineage separated from *P. arisari* (**paper II**). These microalgae also differed from *P. arisari* by elipsoidal shapes of their autospores and autosporangia and by ornamented cell walls. Therefore, they were described as a new species of the genus *Phyllosiphon*, *P. ari*, which specifically infects leaves of the plant genus *Arum*. While 18S rDNA sequences of these specimens were identical to each other, the *rbc*L sequences from *P. ari* specimen differed by one substitution change from the remaining seven *rbc*L sequences, which we considered to be an intraspecific variability within *P. ari*. Currently, this species is only known from the type locality based on a single, spatially limited population of *Arum italicum*. However, this species may have broader distribution. It is possible that it may be the same as those *Phyllosiphon* populations reported more than 100 years ago from the leaves of *Arum italicum* (Nicolas 1912) or *Arum maculatum* in temperate Eastern France (Maire 1908). However, besides the type locality of *P. ari*, no other infected populations of the genus *Arum* were found, despite repeated field effort in different regions of both temperate and sub-mediterranean Europe.

Morphology and phylogeny of parasitic and free-living Phyllosiphon *members*

The chlorelloid cells with identical DNA sequences as *P. arisari* and *P. ari* were repeatedly found in corticolous biofilms at several Mediterranean localities (**papers I, II**). In the spring, both species were even found in corticolous biofilms near to the infected plants (**paper II**). Therefore, we confirmed the earlier findings of several recent studies focused on environmental sequencing of subaerial phototrophic biofilms and reporting that members of the *Phyllosiphon* clade also occur as free-living algae on various subaerial substrates throughout Europe (Cutler et al. 2013, Hallmann et al. 2013, Kirchhoff et al. 2018). In addition, the fact that members of the *Phyllosiphon* clade occur in subaerial biofilms as chlorelloid unicells was recently confirmed by Song et al. (2016), who described another free-living *Phyllosiphon* species, *P. coccidium*, from a corticolous biofilm in temperate China.

The morphological observation of cultivated parasitic stages of both *P. arisari* and *P. ari* showed that endospores produced within the siphonous filamens transform into autosporangia producing minute chlorelloid autospores (**papers I, II**). I was able to cultivate parasitic populations of both *Phyllosiphon* species *in vitro* for a long time on an agar-solidified inorganic medium, which is routinely used for the cultivation of aero-terrestrial microalgae. In these cultures, however, *Phyllosiphon* populations never formed any siphonous filaments, but behaved as typical chlorelloid microalgae reproducing solely by autospores with characteristic morphology typical for the members of the trebouxiophycean *Watanabea* clade.

It was also demonstrated that some additional *Phyllosiphon* genotypes, closely related to the *P. arisari* and *P. ari* species, also occur in corticolous biofilms (**papers I, III**). In the **paper I**, it was shown that isolates of unknown taxa, originally isolated in the study of Kulichová et al. (2014) from corticolous biofilms in the sub-Mediterranean, and proven to belong to the *Watanabea* clade based on their *rbc*L gene sequences, represent additional new genotypes of the *Phyllosiphon* clade according to my newly obtained 18S rDNA sequences. In the **paper III**, a single *Phyllosiphon* genotype was isolated from a corticolous biofilm at the same locality from where Kulichová et al. (2014) originally obtained their *Phyllosiphon* sequences. Since this strain formed a separate lineage in both the 18S rDNA and *rbc*L phylogenetic trees, the strain was described as a new species of the genus *Phyllosiphon*, *P. duini*.

Life strategy of parasitic Phyllosiphon *members*

Based on the facts mentioned in a previous paragraphs, I suppose that both *P. arisari* and *P. ari* should be considered as facultative parasites, which possess the free-living stages in their life cycles and are capable of autonomous reproduction and a long-term survival outside of their host plants. This life strategy is somewhat similar to the other trebouxiophycean parasitic genera *Helicosporidium*, *Prototheca* and *Coccomyxa* (Nelson et al. 1987, Boucias et al. 2001, Sokolnikova et al. 2016) that, unlike most pathogenic protists, can also be easily cultured *in vitro*. I also suppose that the free-living chlorelloid stages of *Phyllosiphon* taxa, which were found in corticolous biofilms, might get there from the decomposing infected leaves, which contained endospores released from the parasitic siphonal filaments. These chlorelloid stages then asexually reproduce on the tree bark surface and in other subaerial biofilms. During the spring, they may form parasitic siphonous stages in the leaves of their host plants *Arisarum* and *Arum*. However, the reason why they infect the leaves of the host plants, as well as the infection mechanism remain unknown.

Ecological differentiation within the Phyllosiphon

Based on the results summed up in the previous paragraphs, it can be concluded that the *Phyllosiphon* clade may actually be phylogenetically differentiated into lineages with the free-living chlorelloid species and the lineages containing taxa with alternating parasitic and free-living stages in their life cycle. This hypothesis is further supported by the fact that, unlike parasitic species *P. 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 (**paper II**), *P. duini* discovered in the **paper III**, occurred at a locality with lack of any taxa of the Araceae, despite repeated field efforts to locate them. Therefore, it is possible that the free-living taxa, such as *P. duini* (**paper III**), *P. coccidium* (Song et al. 2016), or other so far undescribed species belonging to the genus *Phyllosiphon* (Kulichová et al. 2014, **paper I**, Zhu et al. 2018), may only form the simple chlorelloid stages typical of other genera belonging to the *Watanabea* clade. However, this hypothesis is actually based on a low number of infected leaves of Araceae and subaerial biofilms. Therefore, it cannot be excluded that the parasitic stages of *Phyllosiphon* taxa, for which only chlorelloid stages are known, are formed occasionally and under specific conditions, and/or in other vascular plant hosts.

The evolution of parasitism in the Phyllosiphon

The *Phyllosiphon* taxa were reported from the leaves of several taxa of the subfamily Aroidae and one taxon within *Stylochaeton* clade, as indicated by the *Phyllosiphon* members on the phylogenetic tree of Araceae. These subfamilies evolved at the end of late Cretauceous (~ 70 Ma; Nauheimer et al. 2012). At the same time, the data obtained from the investigation of the European populations in this thesis suggested that phylogenetic differentiation of the *Phyllosiphon* taxa may be correlated to the host genus-level identity. Thus, it is possible that the concerted host-pathogen co-evolution may be a prime factor driving species diversification within the *Phyllosiphon* clade. Consequently, the parasitic lifestyle of the *Phyllosiphon* could have evolved at the latest during the late Cretaceous. This parasitic life strategy, which requires the formation of the complex endophytic filaments, probably originated once in the *Phyllosiphon* evolution. It is possible that only some lineages of the *Phyllosiphon* clade retained the parasitic lifestyle, while the other lineages might have secondarily lost this capacity. Existing results are, however, based on a relatively small amount of samples and should be supplemented in the future by additional samples of parasitic populations, especially from the tropics. Since the tropical regions are central to the diversity of the Araceae (Bown 2010), they could be central to the diversity of the genus *Phyllosiphon*, as well. This was also suggested in the recent phylogenetic study, which showed that a large number of undiscovered *Phyllosiphon* genotypes occur in the epiphytic assemblages of tropical China (Zhu et al. 2018). These genotypes may belong to additional underscribed *Phyllosphon* species that form their parasitic stages in other species or genera of Araceae.

Conclusions

The phylogenetic analyses of 18S rDNA and *rbc*L gene sequences of the *Phyllosiphon* isolates and their microscopical examinations led to the description of two new species. In addition, the data suggest that the currently known diversity of this genus is just the tip of an iceberg. In view of the growing body of knowledge regarding the diversity of this peculiar trebouxiophycean genus, which occurs not only in the leaves of plants of Araceae, but also in subaerial microhabitats, I expect that additional taxa of the *Phyllosiphon* clade might be discovered in little-explored regions, such as those in poorly explored tropical ecosystems, or in additional areas with a humid subtropical climate. The high diversity of tropical *Phyllosiphon* taxa was supported by both environmental sequencing in tropical Asia and indirectly by morphologically distinct parasitic populations of this alga described from various tropical plants

of the Araceae. Future studies also could support our hypothesis about the co-evolution in the genus *Phyllosiphon*, or ecological strategies of the *Phyllosiphon* clade. Additional studies also should to ascertain the geographical range and habitat distribution of the already described species of this genus.

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5 CURRICULUM VITAE

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Study and practice

since September 2017: Librarian in Municipal library of Prague

2014–2016: Laborant at the GAČR project

2013–2014: Teacher of courses of Biology and Pharmaceutical Botany at VOŠZ and SZŠ Alšovo nábřeží 6, Prague 1

since 2012: Postgradual study, Department of Botany, Faculty of Science, Charles University

2010–2012: Master study in Botany, Department of Botany, Faculty of Science, Charles University; thesis topic: Diversity and species concept of the *Vischeria*/*Eustigmatos* complex (Eustigmatophyceae)

2007–2010: Bachelor study in Biology, Department of Botany, Faculty of Science, Charles University; thesis topic: Sexual reproduction of ochrophyte algae

Publications in SCI journals

Procházková, K., Němcová, Y., Kulichová, J. & Neustupa, J. (2015) Morphology and phylogeny of parasitic and free-living members of the genus *Phyllosiphon* (Trebouxiophyceae, Chlorophyta). *Nova Hedwigia* 101: 501–518.

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Posters and presentations

Procházková, K., Němcová, Y. & Neustupa, J.: *Phyllosiphon ari* sp. nov. (*Watanabea* clade, Trebouxiophyceae), a new parasitic species isolated from leaves of *Arum italicum* (Araceae). 56th Conference of the Czech Algological Society, Prague, Czech Republic, 18. – 21.9. 2016.

Procházková K, Němcová Y, Kulichová J, Neustupa J: Morphology and phylogeny of parasitic and free-living members of the genus *Phyllosiphon* (Trebouxiophyceae, Chlorophyta). 55th Conference of the Czech Algological Society, Rožmberk nad Vltavou, Czech Republic, 15. – 19.9. 2014.

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