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Morphology, fine structure, life cycle and phylogenetic analysis of *Phyllosiphon arisari*, a siphonous parasitic green alga

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Phyllosiphon arisari Kühn (Phyllosiphonaceae, Chlorophyta) commonly occurs in *Arisarum* leaves in coastal Mediterranean areas of the Iberian Peninsula and Balearic islands. The genus *Phyllosiphon* was first considered to be a member of the Xanthophyceae but was later transferred to the chlorophytes. However, there are few data about its morphology, ultrastructure, ecology or phylogenetic affinities. In this paper we describe the morphology of *Phyllosiphon*, as studied in field material and in culture; the fine structure, analysed by transmission electron microscopy; and phylogenetic relationships, inferred from DNA sequences. The siphonous filaments were seen to divide and penetrate leaf tissues. The cytoplasm divided into spherical or subspherical sporocysts producing autospores inside. Cytoplasmic remains could be observed between autospores or on their cell walls. Phylogenetic analysis of 18S rDNA and 16S rDNA sequences showed that the closest relatives of *Phyllosiphon* are subaerial strains of *Heterochlorella*, *Heveochlorella* and *Kalinella*, demonstrating that *Phyllosiphon* should be transferred to Trebouxiophyceae. An evolution from unicells to a siphonous thallus, and from aerophytic to endophytic and parasitic habits, is proposed for Trebouxiophyceae.

Key words: 16S rDNA, 18S rDNA, Chlorophyta, life cycle, morphology, *Phyllosiphon*, phylogeny, taxonomy, Trebouxiophyceae, ultrastructure

Introduction

The genus *Phyllosiphon* is widely distributed in tropical areas, where it is found penetrating the leaves of several species of Araceae (Chapman & Waters, 1992), although it has also been reported from temperate areas (Bourrelly, 1966). In Europe it has been reported in France, Italy and Hungary (Bourrelly, 1966) and it seems to be common in some areas of North America (Wehr & Sheath, 2003), Australia (Day *et al*., 1995; Phillips, 2002) and Africa (French, 2006). In Spain it is common along the Mediterranean coast of the Iberian Peninsula and the Balearic Islands (Aboal, 1995) and may infect *Arisarum vulgare* and *A. simorrhinum*. It usually penetrates the intercellular spaces of the leaves of *Arisarum* and provokes necrosis due to the proliferation of its siphonous filaments. The production of ellipsoidal aplanospores was reported by Bourrelly (1981). The absence of chlorophyll in the coenocytic filaments but the presence of chloroplasts in aplanospores has been noted by several authors (Mangenot, 1948; Joubert & Rijkenberg, 1971; Round, 1985).

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Phyllosiphon was first included in the Xanthophyceae because of the absence of starch (Mangenot, 1948; Bourrelly, 1966; Round, 1971) but Leclerc & Couté (1976) reported the presence of chlorophyll *b* and *Phyllosiphon* was transferred to Chlorophyceae (Bourrelly, 1981, 1990). However, some confusion still remains with regard to its taxonomic position: Parra & Bicudo (1995), Christensen (1980), Systema Naturae 2000, the Catalogue of Life, Algaebase and the GBIF portals all consider it a xanthophyte, whereas Bold & Wynne (1985), and Wehr & Sheath (2003) follow and include it among the chlorophytes.

The siphonaceous habit is infrequent in freshwater or subaerial aerophytic algae but is very common in marine ecosystems, especially in green algae. Endophytic or parasitic algae are also scarce (Ueno *et al*., 2005; Aslam *et al*., 2007) and are poorly known even though they may cause problems in agricultural production in tropical countries (Strange, 2003; Agrios, 2005; French, 2006) or human illnesses.

The aim of this work was to study the morphology, fine structure, life cycle and phylogenetic position of *Phyllosiphon arisari* Kühn.

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Materials and methods

Field sampling

Field samples were collected in Pego, SE Spain (N 38° 51.16′ W 0° 03.41′), where *Arisarum* is common from autumn to spring. Part of the material was pressed and deposited in the University of Murcia Herbarium (MUB-ALGAE 1923, 1924, 3371, 3372, 3373).

Cultures

Leaves were surface sterilized with 7.5% (mass/volume) calcium hypochlorite solution by vigorously shaking for 5–10 min and rinsing three times with sterilized water. Fragments of decorticated leaves were put in Petri dishes with agarized (1.5%) Bold's Basal Medium (Andersen *et al*., 2005, p. 437) containing 25 or 50% of an extract of *Arisarum* leaves obtained by grinding and sonicating fresh leaves in distilled water. Subsequently, modified Provasoli's medium (ES: Andersen *et al*., 2005, p. 501) and the saltwater medium SWES (SAG Culture collection, www.epsag.uni-goettingen.de) were used, with the water obtained from a source close to the locality of collection. The cultures were maintained at 20°C and 79 µmol photons $m^{-2}s^{-1}$ under a 16 : 8 h light : dark cycle.

Light microscopy

Cuticle and epidermis of leaves were separated under the stereomicroscope to observe siphonous filaments. Lugol's iodine was added to reveal the possible presence of starch. Observations were made with an OLYMPUS BX50 microscope equipped with a digital camera. The presence of chlorophyll in the yellowish filaments and spores of *Phyllosiphon* was observed by fluorescence microscopy, under a MWBV2 filter.

Electron microscopy

In fresh material necrotic areas were cut from fresh leaves and preserved in 0.1 M cacodylate buffer with 2.5% glutaraldehyde at 20°C for 2 hours and then rinsed in 0.1 M cacodylate buffer with 8% sucrose for one night at the same temperature. For SEM, specimens were then dehydrated in an alcoholic series, finishing with pure acetone and before critical point treatment and coating with gold–palladium. The observations were made with a JEOL 6100 scanning microscope equipped with a digital camera.

For TEM preparations, material fixed as above was postfixed in 8% osmic acid for 2.5 h at 4°C, rinsed overnight, and dehydrated in increasing concentrations of ethanol (30%, 50%, 70%, 90% and 100%, 10 min in each) at room temperature. The samples were then treated with propylene oxide (two rinses of 20 min each). The material was embedded in 1 : 2 epon : propylene oxide, followed by 1 : 1 and finally 2 : 1 proportions of the same (1 h for each step). Finally, the samples were left in pure epon overnight. They were stained at the same temperature with uranyl acetate for 2 h, before rinsing in distilled water for 5 min. Using a Reichert–Jung Ultracut, the epon blocks were cut into ultrathin sections, which were contrasted with uranyl acetate and lead citrate and observed with a PHILIPS TECNAI 12 microscope.

Taxon sampling for molecular systematics

Since at the start of the project the taxonomic position of *Phyllosiphon* was not clear, taxon sampling was decided after an initial BLAST search that suggested a close relationship with several trebouxiophycean algae. Special care was taken to include several parasitic taxa. The prasinophycean genera *Nephroselmis*, *Tetraselmis* and *Scherffelia* were used as outgroup. Two Ulvophyceae, 16 Chlorophyceae and 44 Trebouxiophyceae 18S rDNA sequences were added. Due to the more limited availability of 16S rDNA sequence data, only *Nephroselmis olivacea* was used as outgroup. Two *Chlamydomonas* accessions and 12 trebouxiophycean sequences were added to the alignment. Taxa, strains and GenBank accession numbers are given in Table 1.

DNA isolation and amplification of the nuclear 18S rDNA and chloroplast 16S rDNA

For DNA extraction, *P. arisari* specimens were used from new collections, herbarium sheets and cultures (filaments and spores). In the case of dry herbarium material, infected spots of dark green colour surrounded by apparently dead leaf tissue were excised. Unialgal cultures were centrifuged and the pellets used for extraction. Total DNA was extracted using the NaOH extraction method as explained in Werner *et al*. (2002). The 18S rRNA gene was amplified using the primers WACCTGGTTGATCCTGCCAGT and GATCCTTCYGCAGGTTCACCTAC (Huss *et al*., 1999) at a final concentration of 400 µM. Stock DNA (4 µl) was added as template, followed by 200 µM of each dNTP, 2 mM $MgCl_2$, 2 units Taq polymerase (Oncor Appligene), 1 µl BLOTTO (10% skimmed milk powder and 0.2% NaN_3 in water) and the buffer provided by the enzyme supplier. BLOTTO attenuates PCR inhibition caused by plant compounds (De Boer *et al*., 1995). The amplification conditions were: 3 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at 50°C and 2 min at 72°C, and a final 7 min extension step at 72°C. Amplification products were controlled on 1% agarose gels and successful reactions were cleaned with the help of the GenElute PCR Clean-Up Kit (Sigma–Aldrich). Cycle sequencing was performed using a standard protocol at Secugen (Madrid). In addition to the sequencing primers, the internal primers CGGTAATTCCAGCTCC and GGGCATCACAGACCTG (Gunderson *et al.,* 1986) were added to the different sequencing reactions. The 5′-part of the16S rRNA gene was amplified with the help of the primer pair PSf (GGGATTAGATACCCCWGTAGTCCT) and 16S-UR (ACGGYTACCTTGTTACGACTT) (Stiller & McClanahan, 2005). Reaction conditions were set as indicated for the 18S rDNA sequence. We could sequence four 18S rDNA probes from cultivated

Table 1. Accessions examined for nuclear and chloroplast SSU rDNA sequence variation.

Taxon	Strain	GenBank acc. no. 16S rRNA	GenBank acc. no. 18S rRNA
Ankistrodesmus stipitatus (Chodat) Legnerová	SAG 202-5		X56100
Auxenochlorella protothecoides (W. Krüger) Kalina & Punčochárová	SAG 211-7a		X56101
Bracteacoccus aerius H.W. Bischoff & H.C. Bold	UTEX 1250		U63101
Bulbochaete hiloensis (Nordstedt) Tiffany	unknown		U83132
Chaetopeltis orbicularis Berthold	unknown		U83125
Characium hindakii K.W. Lee & H.C. Bold	UTEX2098		M63000
Characium perforatum K.W. Lee & H.C. Bold	unknown		M62999
Characium vacuolatum K.W. Lee & H.C. Bold	unknown		M63001
Chlamydomonas debaryana Goroschankin	SAG 26.72		AF008240
Chlamydomonas fimbriata H. Ettl	SAG 17.72		U70784
Chlamydomonas moewusii Gerloff	CGC CC-419		U41174
Chlamydomonas moewusii Gerloff	unknown	X15850	
Chlamydomonas reinhardtii P.A. Dangeard 2	unknown	J01395	
Chlamydomonas reinhardtii P.A. Dangeard 1	unknown		M32703
'*Chlorella angustoellipsoidea*' N. Hanagata & M. Chihara	MES A7-4		AB006047
Chlorella lobophora Andreyeva	Andreyeva 750_I		X63504
'*Chlorella mirabilis*' Andreyeva	Andreyeva 748-I	X65100	X74000
Chlorella sorokiniana Shihira & Krauss	SAG 211-8k	X65689	X62441
Chlorella vulgaris Beijerinck	SAG 211-11b		X13688
Chlorella vulgaris Beijerinck	SAG 211-1e	D11347	
'*Chlorella*' sp.	UTEX 318		EF159951
Chlorococcum oleofaciens Trainor & H.C. Bold	UTEX 105		U41176
Chloroidium ellipsoideum (Krüger) Darienko, Gustavs, Mudimu, Menendez, Schumann, Karsten, Friedl & Pröschold	MES-A1-2		AB006048
Chloroidium saccharophilum (Krüger) Darienko, Gustavs, Mudimu, Menedez, Schumann, Karsten, Friedl & Pröschold 1	SSAG 211-9a		X63505
Chloroidium saccharophilum (Krüger) Darienko, Gustavs, Mudimu, Menedez, Schumann, Karsten, Friedl & Pröschold 2	MBIC10037		AB183575
Chloroidium saccharophilum (Krüger) Darienko, Gustavs, Mudimu, Menedez, Schumann, Karsten, Friedl & Pröschold 3	3-80	D11348	
Chloroidium saccharophilum (Krüger) Darienko, Gustavs, Mudimu, Menedez, Schumann, Karsten, Friedl & Pröschold 4	SAG 211-1d	D11349	
Choricystis minor (Skuja) Fott	SAG 251-1		X89012
Closteriopsis acicularis (Chodat) Belcher & Swale	SAG 11.86	Y17632	
Coccomyxa sp.	BC98		AJ302940
Coenocystis inconstans Hanagata & Chihara	unknown		AB017435
Dictyochloropsis reticulata (Tschermak-Woess) Tschermak-Woess	CCHU 5616		Z47207
Dunaliella salina (Dunal) Teodoresco	unknown		M84320
Elliptochloris bilobata Tschermak-Woess	SAG 245.80		AM422984
Elliptochloris subsphaerica (H. Reisigl) H. Ettl & G. Gärtner	SAG2202		FJ648518
Elliptochloris sp. 1	SAG2201		FJ648516
Elliptochloris sp. 2	ZC102		FJ217366
Elliptochloris sp. 3	SAG2117		FJ648515
Helicosporidium sp.	unknown	AF538865	AF317893
Heterochlorella luteoviridis Chodat 1	MES A5-4		AB006045
Heterochlorella luteoviridis Chodat 2	SAG 211-2a		X73997
Heveochlorella hainangensis Zhang, Huss, Sun, Chang & Pang	FGG01	EF595525	EF595524
Kalinella bambusicola	CAUP H7901		EU346910
Koliella longiseta (Vischer) Hindák	UTEX 339		U18520
Koliella sempervirens (Chodat) Hindák	Hindák 1961/11		AF278743
Koliella spiculiformis (Vischer) Hindák	Vischer 1940/208		AF278744
Leptosira obovata Vischer	SAG 445-1		Z68695
Microthamnion kuetzingianum Nägeli	UTEX 1914		Z28934
Muriella sp.	AS2-4		AY195969

(*continued*)

Table 1. Continued.

Taxon	Strain	GenBank acc. no. 16S rRNA	GenBank acc. no. 18S rRNA
Mychonastes homosphaera (Skuja) Kalina & Punčochárová	unknown		AB025423
Myrmecia astigmatica Vinatzer	IB T76		Z47208
Myrmecia biatorellae J.B. Petersen	UTEX 907		Z28971
Myrmecia bisecta H. Reisigl	IB T74		Z47209
Myrmecia incisa H. Reisigl	SAG 2007		AY762602
Myrmecia israeliensis (Chantanachat & H.C. Bold) Friedl	unknown		M82995
Nannochloris sp.	JL 4-6		AY195983
Nephroselmis olivacea F. Stein	SAG 40.89		X74754
Nephroselmis olivacea F. Stein	NIES 484	AF137379	
Parachlorella kessleri (Fott & Nováková) Krienitz, E. Hegewald, Hepperle, V. Huss, T. Rohr & M. Wolf	SAG 211-11g		X56105
Parachlorella kessleri (Fott & Nováková) Krienitz, E. Hegewald, Hepperle, V. Huss, T. Rohr & M. Wolf	SAG 211-11h	D11346	
Parietochloris pseudoalveolaris (T.R. Deason & H.C. Bold) S. Watanabe & G.L. Floyd	unknown		M63002
***Phyllosiphon arisari* J.G. Kühn**	**MUB-ALGAE 3373**	**FJ829885**	**FJ829884**
***Phyllosiphon arisari* J.G. Kühn**	**PY4a1**	**JF304472-JF304473**	**JF304468**
***Phyllosiphon arisari* J.G. Kühn**	**PY6a2**	**JF304474-JF304477**	**JF304469**
***Phyllosiphon arisari* J.G. Kühn**	**PY7a1**	**JF304478-JF304481**	**JF304470**
***Phyllosiphon arisari* J.G. Kühn**	**PY9a1**	**JF304482-JF304483**	**JF304471**
Picochlorum eucaryotum (C. Wilhelm, Eisenbais, Wild & Zahn) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley	Mainz 1	X76084	
Picochlorum eucaryotum (C. Wilhelm, Eisenbais, Wild & Zahn) Henley, Hironaka, Guillou, M. Buchheim, J. Buchheim, M. Fawley & K. Fawley	unknown		X06425
Protosiphon botryoides (Kützing) Klebs	UTEX 99		U41177
Prototheca wickerhamii Tubaki & Soneda	Pore 1283		X56099
Prototheca wickerhamii Tubaki & Soneda	263-11	X74309	
Pseudochlorella pringsheimii (Shihira & Krauss) Darienko, Gustavs, Mudimu, Menendez, Schumann, Karsten, Friedl & Proschöld	IAM C-87	X12742	D13324
Pseudochlorella sp.	CCAP 264-2		AB006049
Scenedesmus costato-granulatus Skuja	SAG 18.81		X91265
Scherffelia dubia (Perty) Pascher	unknown		X68484
Stichococcus bacillaris Nägeli	UTEX 314		U18524
Stigeoclonium helveticum Vischer	unknown		U83131
Tetraselmis convolutae (Parke & Manton) R.E. Norris, Hori & Chihara	#208 from the North East Pacific Culture Collection		U05039
Tetraselmis striata Butcher	Ply 443		X70802
Trebouxia asymmetrica Friedl & Gärtner	SAG 48.88		Z21553
Trebouxia impressa Ahmadjian	UTEX 892		Z21551
Trebouxia magna P.A. Archibald	UTEX 902		Z21552
Ulothrix zonata (Weber & Mohr) Kützing	SAG 38.86		Z47999
Ulva rigida C. Agardh	EL0102		AJ005414
Viridiella fridericiana Albertano, Pollio & Taddei	237		AJ439401
Watanabea reniformis Hanagata, Karube, Chihara & P.C. Silva	SAG 211-9b		X73991

samples directly. All the other samples (one 18S rDNA from a herbarium specimen and all 16S rDNA sequences) were obtained after cloning the fragments with the help of the CloneJET™ PCR Cloning Kit (Fermentas, Lithuania) following the manufacturer's instructions. Positive clones were used to reamplify the gene fragments by transferring a small proportion of the positive colonies with a sterile toothpick to the PCR mix. Successful amplifications were sequenced with the help of the amplification primers.

Data analysis

The raw sequence data were edited with Bioedit 5.0.9 (Hall, 1999). As indicated, the sequences were obtained

aligned from the ARB-SILVA database, which provides fully aligned and up-to-date small (16S/18S, SSU) and large (23S/28S, LSU) subunit ribosomal RNA sequences. This was also true for some of our own sequence data, as they had been already submitted to GenBank when the final alignments were made. Some minor adjustments were introduced when checking the alignment using Bioedit 5.0.9 (Hall, 1999). The alignments are available online as supplementary material (via the Supplementary Content tab of the article's online page at http://dx.doi.org/10.1080/09670262.2011.590902).

P-distances between the sequences of the alignment were calculated with the help of MEGA 4 (Tamura *et al.,* 2007). Ambiguous regions were excluded from the following steps. The aligned sequences were analysed using Maximum Parsimony (MP; Fitch, 1971). The MP analysis, run with PAUP*4b10 (Swofford, 2002), used the following settings: RANDOM additions (100 replicates), TBR branch-swapping, MULTREES = yes, steepest descent = no, COLLAPSE = yes. The number of maxtrees (1000) was not reached. All characters were equally weighted. A bootstrap analysis (Felsenstein, 1985) with 1000 replicates was performed with the settings as mentioned. Neighbour joining (NJ) analyses were run using MEGA 4 (Tamura *et al*., 2007) with uncorrected pairwise distances. Branching confidence for MP and NJ was assessed using 1000 bootstrap replicates. Additionally, the data were analysed by Bayesian inference as implemented with MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The best models for nucleotide substitution were determined for each region with Modeltest (Posada & Crandall, 1998). Three runs were conducted with 2 000 000 generations. Trees were sampled every 100th generation and the first 10 000 trees were discarded (burn-in) in order to exclude the trees before the chain reached the stationary phase. Trees were edited with the help of TreeGraph2 (Stöver & Müller, 2010).

Results

Morphology and life cycle

In the first stages of infection, light green areas could be observed in the *Arisarum* leaves (Fig. 1), with some yellowish filaments inside, but later green branched radial filaments were evident in the necrotic areas of leaves (Figs 2, 6, 7, 11, 12).

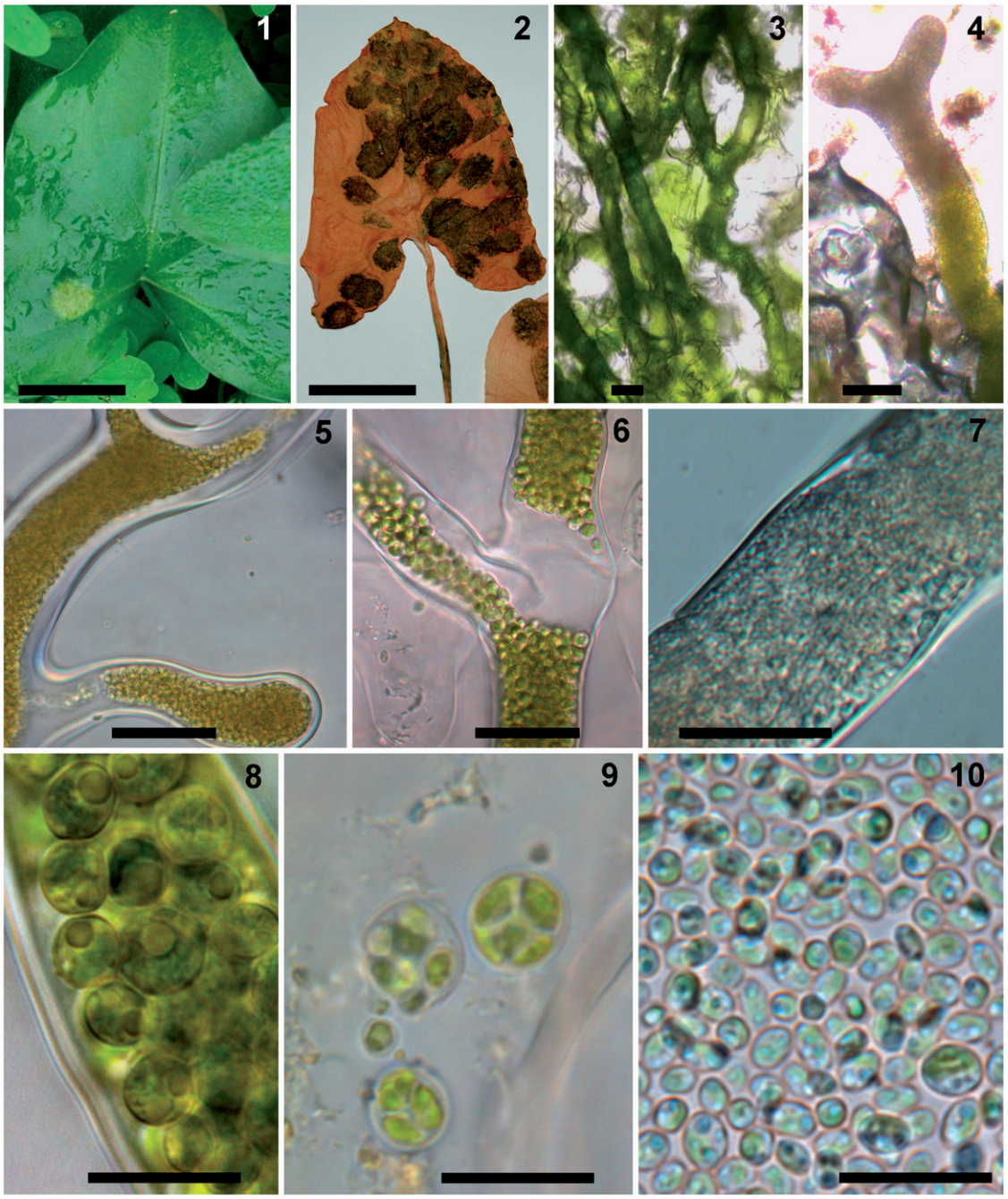
The filaments (25–45 μm in diameter) grew in the intercellular spaces of the leaf parenchyma and could be branched (Figs 5, 6). Fluorescence observations showed that the yellowish filaments formed initially did not contain chlorophyll. Staining with Lugol's iodine indicated the absence of starch granules but the presence of star-like brown-staining granules. A great number of chloroplasts, mitochondria, nuclei and lipid droplets could be observed in the cytoplasm of siphonous filaments. Spores were produced by a progressive division of the cytoplasm, with small vesicles fusing to separate portions of cytoplasm that became sporocysts (Figs 16, 17); the latter were spherical and 6–8 μm in diameter (Figs 8, 9, 18). The filaments were sometimes so full of sporocysts that their cell wall became undulate (Fig. 13). The sporocysts were dark-green and divided to produce more or less ellipsoidal pale green or yellow-green autospores (4–6 × 2.5–4 μm) (Figs 10, 14, 15). Undivided cytoplasm and cytoplasmic remains could be observed between the spores or on their cell walls (Figs 14, 15). Autospores were quickly released when water was added to dry material (Fig. 10). Groupings of three or more thylakoids were clearly visible in the chloroplasts of siphonous filaments and autospores, as were numerous lipid droplets (Figs 19–21). No evidence of pyrenoids was found.

In culture, the siphonous filaments grew for a period and then produced autospores. When released, the autospores were ellipsoidal and bluish in colour but later transformed into dark green sporocysts producing tetrads of ellipsoidal autospores.

A hypothetical life cycle can be summarized as follows (Fig. 22): after infection, *Phyllosiphon* produces yellow spots in host leaves. At this stage the siphonous filaments are yellowish and do not contain chlorophyll. Then the filaments become progressively greener and completely full of sporocysts. At the same time the host leaf tissues die and become brownish. The sporocysts are probably a resting phase. The germination of the sporocysts and the formation and release of autospores is probably related with the presence of water since, when infected dead leaves are collected and studied under the microscope, the addition of water immediately promotes liberation of autospores.

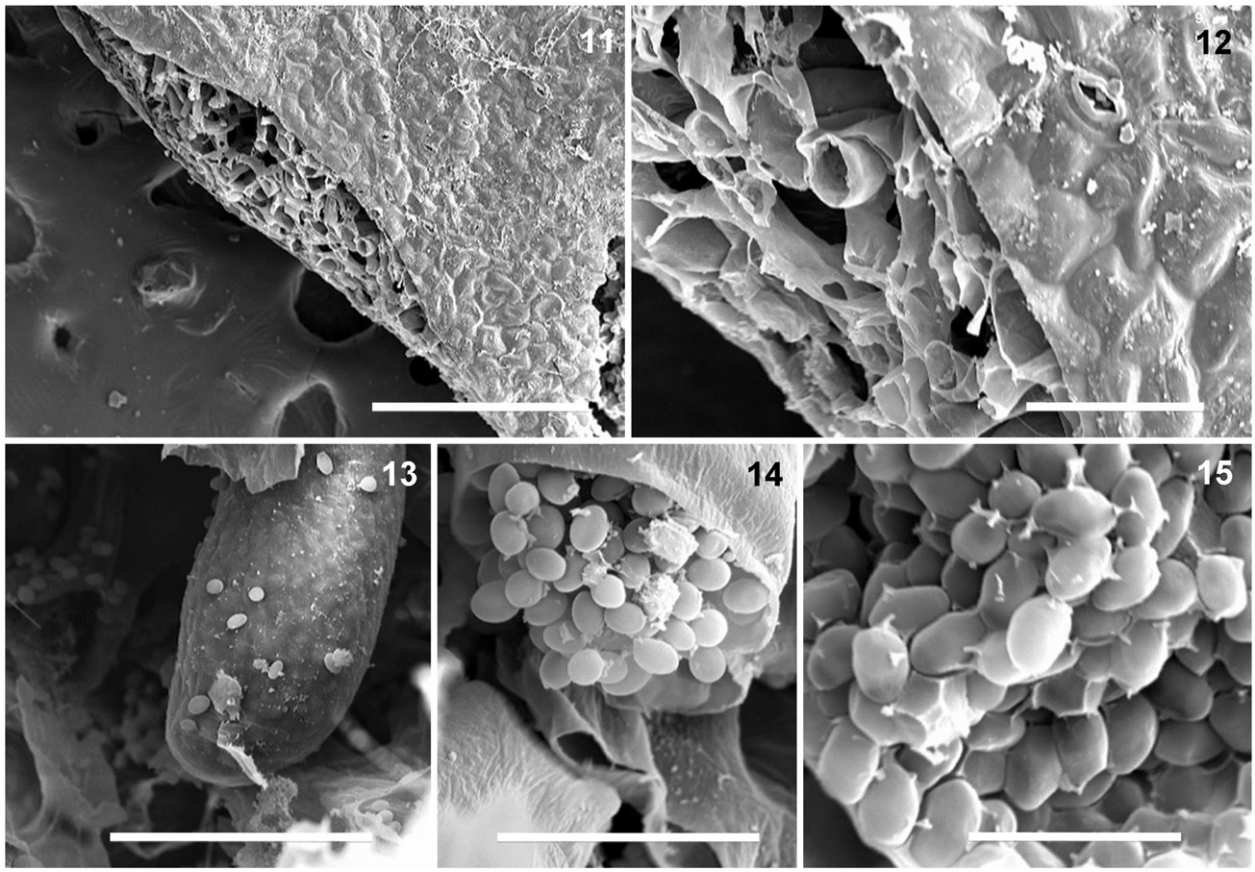
Phylogenetic analysis

The partial sequence of the 18S rDNA gene used had a length of 1722 bp. No intron sequences were present. The initial BLAST search suggested that some '*Chlorella*' and related taxa were the closest relatives of *Phyllosiphon arisari*. '*Chlorella*' sp. UTEX 318, '*Chlorella*' *angustoellipsoidea*, *Chloroidium saccharophilum* strains MBIC10037 and SAG 211-9a, and *Pseudochlorella* CCAP 264-2 were the best hits with 93% identity. These preliminary results served to narrow down taxa sampling and to concentrate especially on Trebouxiophyceae. The initial alignment was used to calculate *p*-distances between the sequences. *Pseudochlorella* CCAP 264-2 and '*Chlorella*' sp. UTEX 318 had the lowest distance values (0.064 and 0.063, respectively) confirming the results of the BLAST search.



Figs 1–10. *Phyllosiphon*, light microscopy. **1–2**. *Arisarum* leaf at different stages of infection. **3, 4**. Yellowish and green filaments inside leaves. **5, 6**. Branching of filaments. **7**. Yellowish filament at higher magnification and without conspicuous organelles. **8**. Detail of sporocysts full of oil droplets. **9**. Autospore formation. **10**. Released autospores. Scale bars: 1 cm (Figs 1, 2), 50 μm (Figs 3–7) and 10 μm (Figs 8–10).

All introns and ambiguously aligned positions were excluded for the phylogenetic analyses. The final alignment had a length of 1794 bp. Of these positions, 1060 were constant, 328 were variable but parsimony-uninformative, and 406 parsimony-informative. The general time reversible model of nucleotide substitution with invariant sites and gamma distribution (GTR + I + G) was selected for the Bayesian analysis under the hierarchical likelihood-ratio tests and the Akaike information criterion. All three methods used to reconstruct the phylogenetic relationships (MP, NJ and Bayesian) resulted in highly similar trees (Fig. 20) and confirmed the results of the BLAST search. *Phyllosiphon arisari* was placed with good support (1.00 Bayes, 83% MP, 94% NJ) in a clade with '*Chlorella*' sp. UTEX 318, '*Chlorella angustoellipsoidea*', *Heterochlorella luteoviridis* strains MES A5-4 and SAG 211-2a, *Chloroidium saccharophilum* strains MBIC10037 and SAG 211-9a, *Chloroidium ellipsoideum* MES A1-2, *Heveochlorella hainangensis* FGG01, *Kalinella*



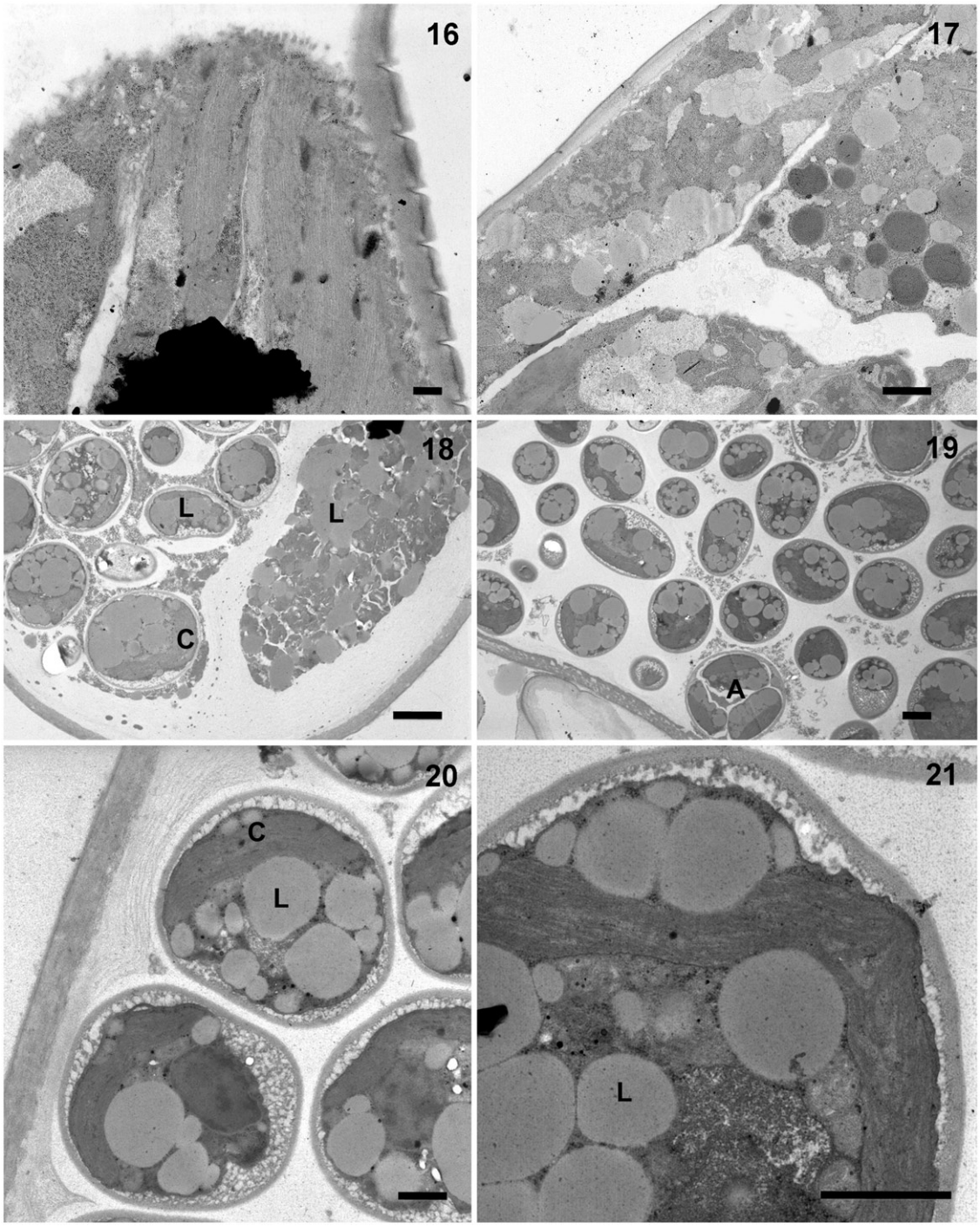
Figs 11–15. *Phyllosiphon*, scanning electron microscopy. **11, 12**. Section of an *Arisarum* leaf with *Phyllosiphon* filaments inside. **13**. Filament apex with autospores inside. **14, 15**. Autospores inside filaments with cytoplasmic remnants. Scale bars: 500 μm (Fig. 11), 100 μm (Fig. 12), 25 μm (Fig. 13), 20 μm (Fig. 14) and 5 μm (Fig. 15).

bambusicola CAUP H7901 and *Pseudochlorella* CCAP 264-2. Within this clade, all three methods indicated a relatively isolated position for *Phyllosiphon arisari*. Interestingly, other parasitic algae included in the analyses, viz. *Helicosporidium*, *Elliptochloris* and *Coccomyxa*, were clearly separated from *P. arisari*.

In the case of the 16S rRNA gene, the total length of the partial sequence was 701 bp. There were minor differences (maximum two mutations compared with the consensus sequence) between some of the cloned fragments, which did not affect the tree topology. A BLAST search of the 16S rRNA gene gave *Chloroidium saccharophilum* (91% identity) and *Heveochlorella hainangensis* (92% identity) as the best hits, when short sequences and uncultured unidentified algae were excluded. Because the number of well identified, almost complete trebouxiophycean 16S rRNA gene sequences is quite limited, only 15 sequences, in addition to our own, were included in the phylogenetic analyses (Fig. 22). The *p*-distance between *P. arisari* and *Chloroidium saccharophilum* strain SAG 211-1d was 0.087 and between *P. arisari* and *Heveochlorella hainangensis*, 0.113. After the exclusion of all intron sequences and ambiguously aligned regions, the alignment had a length of 742 bp, 406 of which were constant, 131 variable but parsimony-uninformative and 205 parsimony-informative. For the Bayesian analysis the GTR + I + G model of nucleotide substitution was implemented as suggested by the Modeltest results. As in the case of the 18S rDNA, all three methods for the reconstruction of the phylogenetic relationships confirmed a relatively close relationship of *Phyllosiphon arisari* with *Chloroidium saccharophilum* (strain SAG 211-1d) and *Heveochlorella hainangensis* with a very solid support (Fig. 21; Bayes 1.00, MP 97%, NJ 100%). Surprisingly, the second sequence of *Chloroidium saccharophilum* (strain 3-80, GenBank D11348) was clearly separated from this clade, which points in the direction of possible problems concerning the identification or taxonomy.

Discussion

There are few siphonous algae among aerophytic or freshwater chlorophytes. *Protosiphon*, which lives in wet soils, may be the only one with a vesicular siphonous thallus and is the only one whose structure and cell division has been studied in detail with electron microscopy (Kouwets & Schaaf, 1992). Using light microscopy, Bold (1933) described the complex life cycle of



Figs 16–21. *Phyllosiphon*, transmission electron microscopy. **16, 17**. Detail of filaments with progressive cleavage of cytoplasm. **18**. Sporocyst and autospore formation. **19–21**. Details of autospores with parietal chloroplasts and lipid droplets. C = chloroplast, L = lipid droplets, A = autospores. Scale bars: 2 μm.

Protosiphon and the main mechanisms of cell division, which include the delimitation of aplanospores by progressive cleavage in a similar way to that observed in *Phyllosiphon*. This same process of division is common in Siphonocladales with siphonous thalli (Bold & Wynne, 1985).

Chlorella-like species have a multinucleate stage for a short period (Ettl, 1988). Probably *Phyllosiphon* maintains a coccal stage in vegetative–resting stages but becomes filamentous and siphonous only inside leaves, between the cells of the host tissues. The filaments are initially yellowish and have no chlorophyll but later the presence of parietal chloroplasts without pyrenoids can be confirmed with TEM, even when they are usually masked by lipid droplets. Large stellate granules, which stained purple with iodine, suggesting they were composed of polysaccharide, were observed by Mangenot (1948).

As no signs of flagella could be detected, the spores produced by *P. arisari* must be regarded as autospores, but Mangenot (1948) and

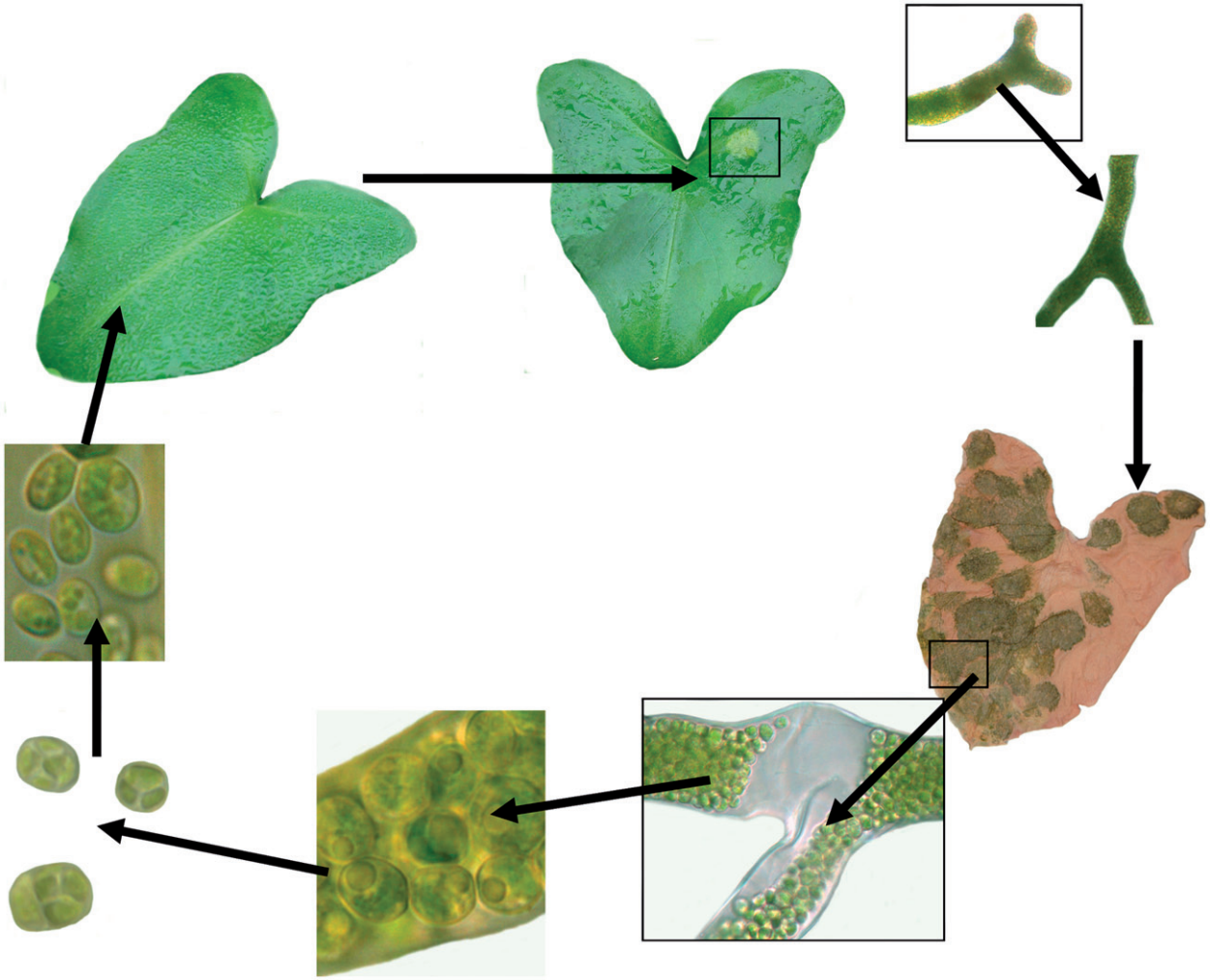


Fig. 22. Schematic representation of the hypothetical life cycle of *Phyllosiphon arisari*: yellow spots are the first sign of infection; yellow filaments become green while all the cytoplasm transforms into sporocysts and the leaves die; sporocysts divide to form autospores, which are released after rewetting of dead leaves.

Bourrelly (1990) considered them to be aplanospores. Joubert & Rijkenberg's (1971) report of aplanospores and microspores probably refers to what we call sporocysts and autospores.

The phylogenetic analysis points to a clear relationship between *Phyllosiphon* and some species of *Chlorella* and *Heveochlorella*, and indicates that *Phyllosiphon* belongs to the Trebouxiophyceae. This is surprising, because a siphonous habit and progressive cleavage have never previously been observed in Trebouxiophyceae, even in other parasitic algae belonging to this group. Analyses of both the nuclear 18S rDNA and the chloroplast 16S rDNA strongly suggest that the closest relatives of *Phyllosiphon arisari* (with available sequence information) are '*Chlorella*' sp. UTEX 318, '*Chlorella*' *angustoellipsoidea*, *Heterochlorella luteoviridis* strains MES A5-4 and SAG 211-2a, *Chloroidium saccharophilum* strains MBIC10037 and SAG 211-9a, *Ch. trebouxioides* MES A1-2, *Heveochlorella hainangensis* FGG01, *Kalinella bambusicola* CAUP H7901 and *Pseudochlorella* CCAP 264-2. All of them are coccoid algae and therefore morphologically clearly separated from *Phyllosiphon*. But recent advances in taxonomy and systematics, mostly based on DNA sequencing, have shown that morphologically similar algae can be polyphyletic and that morphologically very different taxa are sometimes closely related. For example, Huss *et al*. (1999) showed that coccoid algae with a *Chlorella*-like appearance are distributed over two classes (Trebouxiophyceae and Chlorophyceae), while Katana *et al*. (2001) and Krienitz *et al*. (2010) have shown that '*Chlorella*' and other trebouxiophycean genera are polyphyletic. At the same time, '*Chlorella*' *protothecoides* var. *acidicola* seems to be related to two *Nannochloris*-strains as well as another '*Chlorella*' sp. and *Koliella spiculiformis* (Huss *et al*., 2002).

Although *Phyllosiphon arisari* is placed with very good support in a clade with the above-mentioned species, it occupies an isolated position in this clade. This is confirmed by the tree topology (Figs 23, 24) and the *p*-distances with the closest

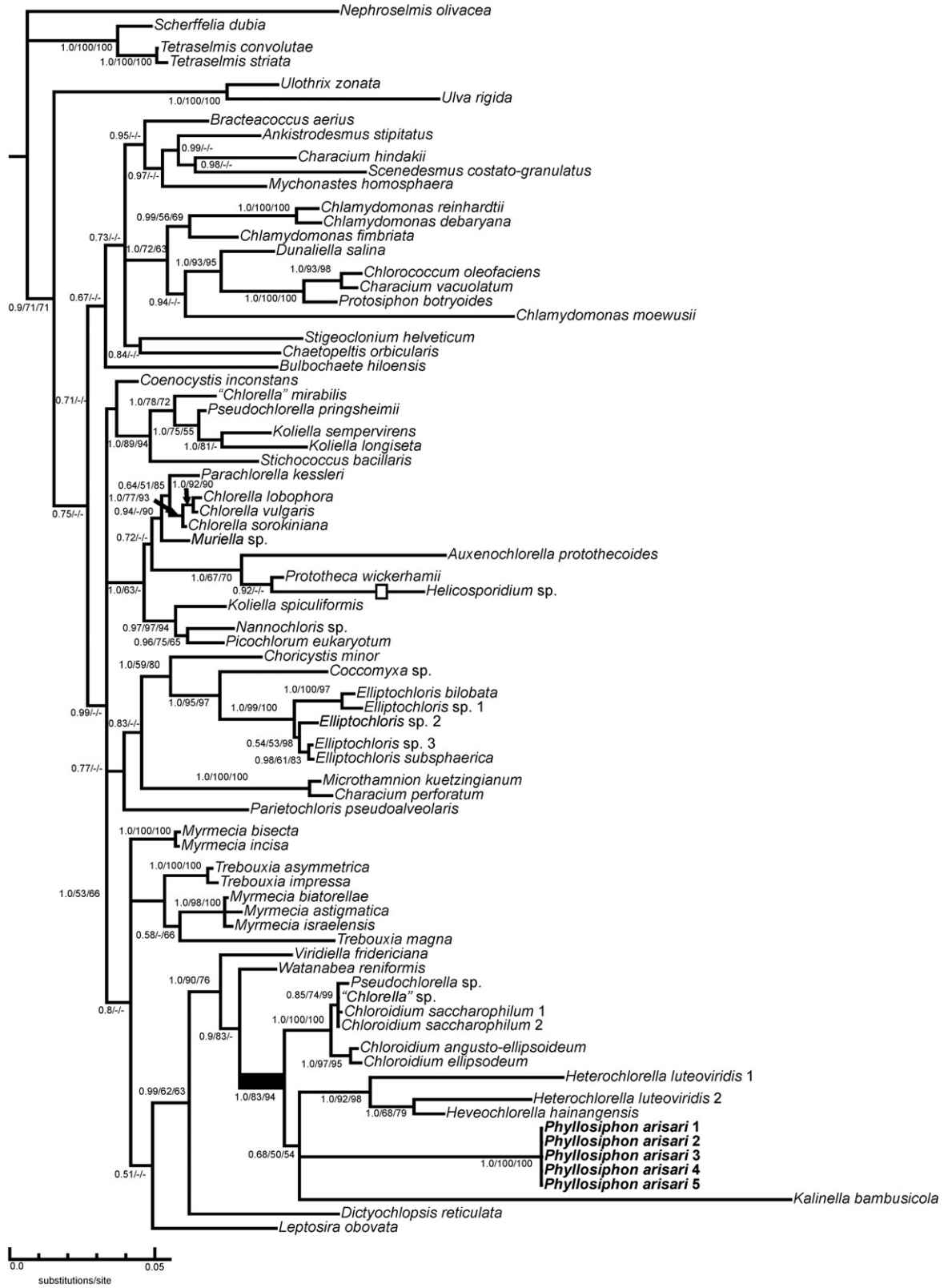


Fig. 23. Phylogram indicating the phylogenetic relationships of *Phyllosiphon arisari* based on Bayesian analysis of the 18S rDNA gene. Posterior probabilities and bootstrap support values obtained with MP and NJ are indicated (PP/MP/NJ). *Phyllosiphon* is deeply nested within the Trebouxiophyceae. Note the clear separation of *Phyllosiphon* from the chlorophycean siphonous *Protosiphon botryoides*. The clade of *Helicosporidium* was shortened by a factor of 10.

relatives in the range of 0.06 to 0.11. Interestingly, some of the closest relatives of *Phyllosiphon arisari* are known to live on trees and show coccoid morphology: *Heveochlorella hainangensis* was isolated from rubber trees (Zhang *et al*., 2008), and *Chloroidium saccharophilum* occurs on bare rocks and the bark of trees (Huss *et al*., 2002). *Kalinella bambusicola* is known to grow as an epiphyte on

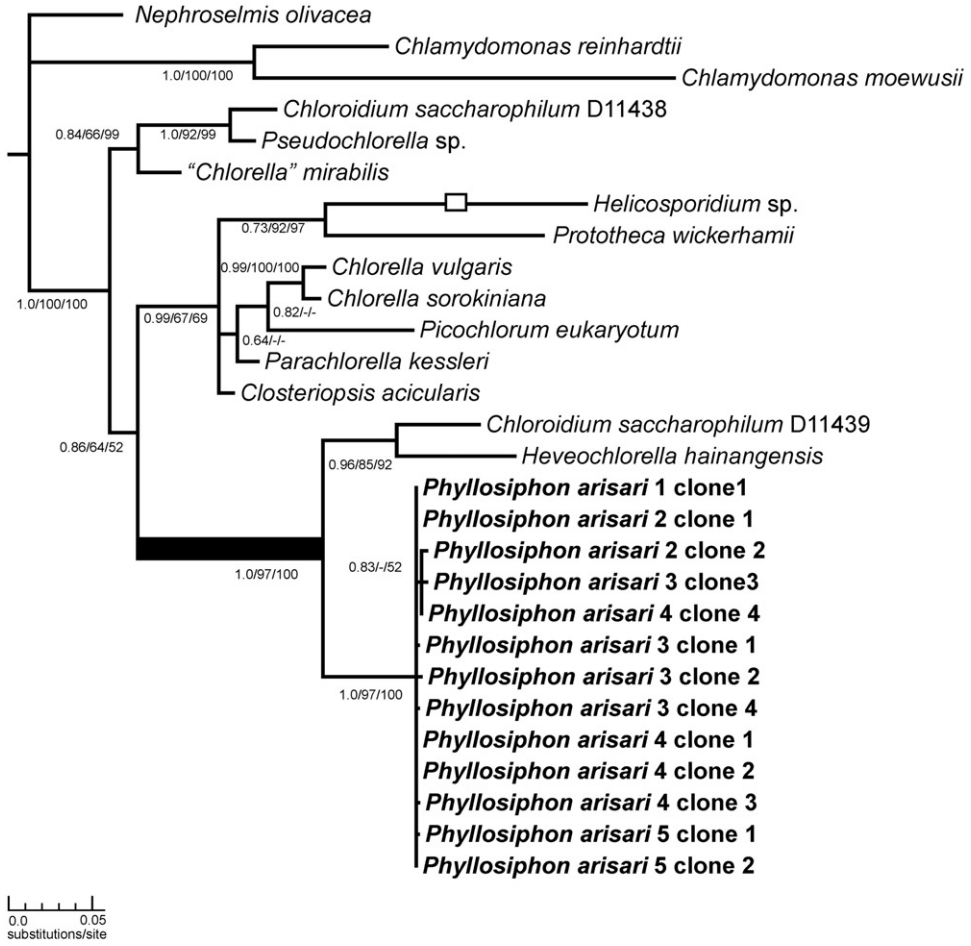


Fig. 24. Phylogram indicating the phylogenetic relationships of *Phyllosiphon arisari*, based on Bayesian analysis of 16S rDNA gene sequences. Posterior probabilities and bootstrap support values obtained with NJ and MP are indicated (PP/MP/NJ). The trebouxiophycean *Heveochlorella hainangensis* and *Chloroidium saccharophilum* are the closest relatives of *Phyllosiphon*. The clade of *Helicosporidium* was shortened by a factor of 5.

bamboo stalks (Neustupa *et al*., 2009). It is therefore tempting to speculate that the evolutionary sequence that led to *Phyllosiphon* started with forms like *Chloroidium saccharophilum* living on the plant surface; these later adapted to grow in the interior layers of plant tissues and finally became parasitic and, in order to better penetrate the surrounding tissues, changed to a siphonous habit.

Siphonous algae are common in marine habitats, where some of them may penetrate rocks or even penetrate in other algae, but all of these siphonous marine forms belong to the Ulvophyceae (Brodie *et al*., 2007). This is the first reported siphonaceous Trebouxiophyceae parasitic in flowering plants.

Supplementary material

The following supplementary material is available for this article, accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2011.590902:
Phyllosiphon 16 s nexus.nex
Phyllosiphon 18 s nexus.nex

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