Taxonomy and phylogeny of the family Fuscideaceae (Umbilicariales, Ascomycota) with special emphasis on *Fuscidea*

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Preface

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I would like to express my deepest gratitude to my principal supervisor, Professor Tor Tønsberg, for his tireless patience in discussing both taxonomic and personal issues, and for his constructive comments on the manuscripts. His passion for collecting lichens, including *Fuscidea*, provided me with hundreds of samples from Norway and Alaska. Thank you very much Tor.

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My sincere thanks to the curators of the following herbaria for loans of specimens: BM, H-Ach, HO, LD, MSC, S, and UPS, and to VER for accepting me as visitor. Professor Mark R.D. Seaward provided important specimens of *Fuscidea* for the phylogenetic analyses. Alan M. Fryday, Gintaras Kantvilas, John Inge Johnsen, Jiří Malíček, Ondřej Peksa, Josef Halda, Reidar Haugan, Jan Vondrák, and Jana Kocourková generously provided fresh material from Australia, Tasmania, British Isles, Central Europe, North America, and Norway.

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Contents

Abstract

Introduction: For several decades, the taxonomic position of the lichen family Fuscideaceae and its associated genera, including the type genus *Fuscidea* V. Wirth & Vězda, has been debated. Amongst the species of *Fuscidea*, there are many questions about species limitation. The identity of the photobiont in *Fuscidea* is poorly known and has not been studied by molecular methods. To fill these gaps, the objectives of this thesis were the following:
1) Investigate the placement of Fuscideaceae within Ascomycota and to designate the genera to be included in this family (Paper I);
2) Examine the phylogenetic relationships within the genus *Fuscidea* (Paper I);
3) Elucidate the taxonomy of the *F. lightfootii-F. pusilla* species complex (Paper II);
4) Assess the infraspecific taxonomy of *F. cyathoides* (Ach.) V. Wirth & Vězda and the status of *F. fagicola* (Zschacke) Hafellner & Türk and *F. stiriaca* (A. Massal.) Hafellner (Paper III);
5) Identify the photobiont in *Fuscidea* and clarify its systematic placement (Paper IV).

Methods: The concatenated data sets comprising the mitochondrial SSU and nuclear LSU, ITS rDNA regions of the fungal sequences (Papers I–III) as well as the individual 18S rDNA and partitioned ITS data sets of algal sequences (Paper IV) were analysed by Bayesian inference, maximum parsimony and maximum likelihood (ML) analysis. Principal Components Analysis (PCA) was used to assess selected morphological characters among varieties of *F. cyathoides* (Paper III). The ITS data set of algal sequences was run in the program Gblocks under relaxed and stringent masking to minimize the ambiguous positions in the alignment. The program SATé-II was used to analyse the non-aligned data matrix of ITS. The topologies and support values of the Bayesian and the ML trees recovered from the resulting aligned data sets were compared and their topologies with the ML tree calculated by SATé-II. The secondary structures of the ITS2 region were folded in order to identify Compensatory Base Changes (CBCs) and hemi-CBCs of the retrieved ITS groups (Paper IV).

Results: Fuscideaceae included four genera and was located in Umbilicariales. The new genus *Printzeniella* Palice, Tønsberg & Zahradn. ined. was found to be closely related to *Fuscidea*. *Ropalospora* A. Massal. appeared as the first diverging lineage within Fuscideaceae and *Maronea* A. Massal. was nested within the *Fuscidea*-clade. The lichenicolous *Lettauia* D. Hawksw. & R. Sant. and *Cryptodiscus* Corda were nested in Stictidaceae within Ostropales.

Loxospora A. Massal. was grouped with *Sarrameana* Vězda & P. James in Sarrameanaceae, Sarrameanales, and closely related to Ostropales. *Orphniospora* Körb. may be related to Lecideaceae *s. str.* within Lecideales (Paper I). *Fuscidea lightfootii* (Sm.) Coppins & P. James and *F. pusilla* Tønsberg were not conspecific, but phylogenetically well distinct. Fertile specimens of *F. pusilla* were recorded for the first time (Paper II). Genetic, chemical or morphological differences were not significant among the current varieties of *F. cyathoides*. The variation in apothecia and the presence of tuberculate apothecia were not significant for *F. fagicola* and *F. stiriaca* (Paper III). The photobiont in *Fuscidea* was identified as *Apatococcus* F. Brand, but its taxonomic position remained unresolved within Trebouxiophyceae due to poor supports in the deep phylogeny. *Apatococcus fuscideae* A. Beck & Zahradn. ined. differs from *A. lobatus* (Chodat) J.P. Petersen by the presence of typical reticulate chloroplasts in the mature cells and by three CBCs and five hemi-CBCs on the conserved part of helix III. The photobiont of *F. lightfootii* differs from *A. fuscideae* by having four CBCs and three hemi-CBCs on the conserved part of helix III. Six ITS groups, including both lichenized and free-living species, were retrieved and supported by different CBCs and hemi-CBCs found on ITS2. (Paper IV).

Discussion: Fuscideaceae accommodates genera with a brownish hypothallus (sometimes inconspicuous in *Maronea* or invisible in *Printzeniella*), a green coccoid alga, a distinct pigmentation of the apothecium, slightly tapered or cylindrical-clavate asci of the *Fuscidea*-type and short bacilliform conidia. The genus *Fuscidea* is tentatively split into three groups, possibly defined by the shape of the ascospores and the secondary chemistry. Some *Fuscidea* species remained unresolved. *Fuscidea* is paraphyletic as *Maronea* is nested inside *Fuscidea*. To make *Fuscidea* monophyletic there are three possibilities: to lump all *Fuscidea* species in *Maronea*, to transfer the *Fuscidea* species in the *F. pusilla*-clade (the sister to *Maronea*) to *Maronea*, or to introduce a new genus for the *F. pusilla* clade. As the backbone of the *Fuscidea*-clade is poorly resolved, at this point no nomenclatural changes at the generic level have been proposed (Paper I). *Fuscidea lightfootii* and *F. pusilla* are chemically identical, anatomically and morphologically similar but molecularly different. The two species are difficult to identify without molecular methods. The records of non-sequenced material need revision (Paper II). The diagnostic characters for *F. cyathoides* are the sessile apothecia with persistent margin, the bean-shaped ascospores becoming brown when mature and the presence of fumarprotocetraric acid (Paper III). The photobiont in *F. lightfootii* differed

from *A. fuscideae* and may represent another new *Apatococcus* species. SATé-II provides a phylogeny similar to those from the aligned ITS matrices (Paper IV).

Conclusion: Fuscideaceae belongs to Umbilicariales and is comprised of *Fuscidea*, *Maronea*, *Ropalospora* and *Printzeniella* gen. nov. *Hueidea* is treated as a tentative member of the family. The four genera *Lettauia*, *Loxospora*, *Orphniospora* and *Sarrameana* are not closely related to Fuscideaceae (Paper I). Although some morphotypes of *F. lightfootii* and *F. pusilla* appear to be distinguishable based on morphology, DNA sequencing is recommended for their definitive identification (Paper II). The varieties of *F. cyathoides* are synonymized with the typical saxicolous form and *F. fagicola* and *F. stiriaca* synonymous with *F. cyathoides* (Paper III). *Apatococcus fuscideae* is the photobiont in most of the studied *Fuscidea* species and *Apatococcus* is treated as a genus with uncertain position within Trebouxiophyceae (Paper IV).

List of publications

Paper I Zahradníková, M., Palice. Z., Tønsberg, T. & Andersen, H.L. **Phylogeny and taxonomy of the lichen family Fuscideaceae (Ascomycota: Umbilicariales).** Manuscript.

Paper II Zahradníková, M., Andersen, H.L. & Tønsberg, T. ***Fuscidea lightfootii* and *F. pusilla* (Fuscideaceae, Umbilicariomycetidae, Ascomycota), two similar, but genetically distinct species.** Manuscript submitted for *The Lichenologist*.

Paper III Zahradníková, M., Tønsberg, T. & Andersen, H.L. **The taxonomy of the lichen *Fuscidea cyathoides* (Fuscideaceae, Umbilicariomycetidae, Ascomycota) in Europe.** *The Lichenologist*, in print.

Paper IV Zahradníková, M., Andersen, H.L., Tønsberg, T. & Beck, A. **Molecular evidence of *Apatococcus*, including *A. fuscideae* sp. nov., as photobiont in the genus *Fuscidea*.** Manuscript submitted to *Protist*, reviewed and resubmitted.

Introduction

Lichens are symbiotic organisms comprised of at least two partners; a heterotrophic mycobiont and an autotrophic photobiont (Schwendener, 1867; Hawksworth, 1988; Honegger, 2000). The mycobiont, typically a member of Ascomycetes, provides nutrients and moisture to the photobionts and shelters them from the harsh environment. The photobiont is usually a green alga (e.g. *Asterochloris* Tschermak-Woess, *Coccomyxa* Schmidle, *Trebouxia* Puymaly) or a cyanobacterium (e.g. *Nostoc* Vauch., *Stigonema* C. Agardh ex Bornet & Flahault), or both as is common in Peltigerales (e.g. Miadlikowska & al., 2006; Friedl & Büdel, 2008). In some cases, other fungi may take part such as the so-called lichenicolous fungi (e.g. Rambold & Triebel, 1992; Werth & al., 2013). Recently, a basidiomycete yeast was found as an obligate symbiont in cortex of Parmeliaceae (Spribille & al., 2016). Its teleomorph state had previously been described as lichenicolous (Millanes & al., 2016).

Lichen-forming fungi (estimated to a number between 17 500 and 20 000 species) represent more than 40% of the known Ascomycota (Kirk & al., 2008). The largest class of the lichenized Ascomycota is Lecanoromycetes (Kirk & al., 2008) accommodating five main subclasses (Acarosporomycetidae, Candelariomycetidae, Ostropomycetidae, Lecanoromycetidae and Umbilicariomycetidae) and 17 accepted orders (Lücking & al., 2016).

Although Miadlikowska & al. (2014) provided a comprehensive molecular study comprising 66 families across Lecanoromycetes, some of the groups remained unresolved. This is the case for Umbilicariales/Umbilicariomycetidae particularly when Fuscideaceae and Ropalosporaceae were included in the analyses. Without these two families, Umbilicariales was a well-supported group.

Magnusson's study of the *Rivulosa*-group of *Lecidea* Ach. (Magnusson, 1925) became a basis for the introduction of *Fuscidea* V. Wirth & Vězda by Wirth & Vězda (1972). *Fuscidea* species have an esorediate, sometimes sorediate thallus, a dark prothallus at the thallus edge, lecideine or aspicilioid apothecia with pseudothalline margins, simple or sparingly branched, sometimes anastomosing paraphyses with a swollen apical cell and brown cap, ellipsoid to bean-shaped or medianly constricted ascospores and the chemical constituents divaricatic (mostly), alectorialic, fumarprotocetraric or sekikaic (rarely) acids or are acid deficient. This cosmopolitan genus is comprised of saxicolous and corticolous taxa preferring acid substrates (Hertel, 1974, 1984; Inoue 1981a,b; Oberhollenzer & Wirth, 1984; Galloway, 1985; Brusse, 1989a; Tønsberg 1992; Kantvilas, 2001, 2004; Øvstedal & Smith, 2001; Fryday, 2008; Gilbert & al., 2009; van de Boom & al., 2014). Most previous taxonomic studies of *Fuscidea* were

based on morphology only and included only a limited number of species. The current taxonomy of *Fuscidea* is therefore in need of a thorough revision.

The phylogenetic position (Paper 1: Fig. 1, Table 1) and taxonomy (Paper I: Table 2) of the family Fuscideaceae have been matters of debate recently. As there is no generally accepted solution, a revision is needed. Based on the similarities in the ascus apex, the two genera, *Fuscidea* and *Maronea* A. Massal., comprised the family Fuscideaceae *sensu* Hafellner (1984). The two genera differ in the presence of a thalline margin in the apothecia (only in *Maronea*), the morphology of their brown paraphyses (with swollen apices as in *Fuscidea vs*. not swollen in *Maronea*), the number of ascospores in the ascus (8 in *Fuscidea vs*. many in *Maronea*) and in their ecology. *Fuscidea* species prefer cool and maritime climates, whereas *Maronea* prefers warmer, more temperate climates (Magnusson, 1936; Kantvilas, 2004). The inclusion of *Maronea* in Fuscideaceae has been disputed.

Based on the *Fuscidea*-type asci as the diagnostic character, Eriksson & al. (2006) included the genera *Fuscidea* (Fig. 2A) and *Hueidea* Kantvilas & P.M. McCarthy in Fuscideaceae. The genera *Maronea* (Fig. 2B), *Ropalospora* A. Massal. (Fig. 2C), *Lettauia* D. Hawksw. & R. Sant. (Fig. 2D), *Orphniospora* Körb. (Fig. 2E) and *Sarrameana* Vězda & P. James (Fig. 2F) were tentatively assigned to the family. Lumbsch & Huhndorf (2007) excluded *Sarrameana* and placed it in the family Sarrameanaceae with *Loxospora* A. Massal., but *Loxospora* was later transferred to Fuscideaceae by Tehler & Wedin (2008). According to Lücking & al. (2016) Fuscideaceae accommodates *Fuscidea*, *Hueidea*, *Maronea* and *Orphniospora*.

The identity of the photobiont in *Fuscidea* is poorly known. It has been identified as a protococcoid alga (Inoue, 1981a; Oberhollenzer & Wirth, 1984), *Trebouxia* Puymaly (Galloway, 1985), *Apatococcus lobatus* (Chodat) J.B. Petersen (Watanabe & al., 1997), achlorococcoid alga, probably *Chlorella* Beyerinck [Beijerinck] (Gilbert & al., 2009) and a coccoid green alga (Miadlikowska & al., 2014). Fryday (2008) and Gilbert & al. (2009) characterized the *Fuscidea* photobiont as a green alga with cells that duplicate by binary fission creating typical clusters of 2, 4 or 8 daughter cells that are often flattened on one side. This description agrees with *Apatococcus* F. Brand. Ettl & Gärtner (2014) considered the record of *A. lobatus* by Watanabe & al. (1997) uncertain, since this alga was reported as the photobiont in the lichen genus *Caloplaca*, known to associate with *Trebouxia* only (Castillo & Beck, 2012).

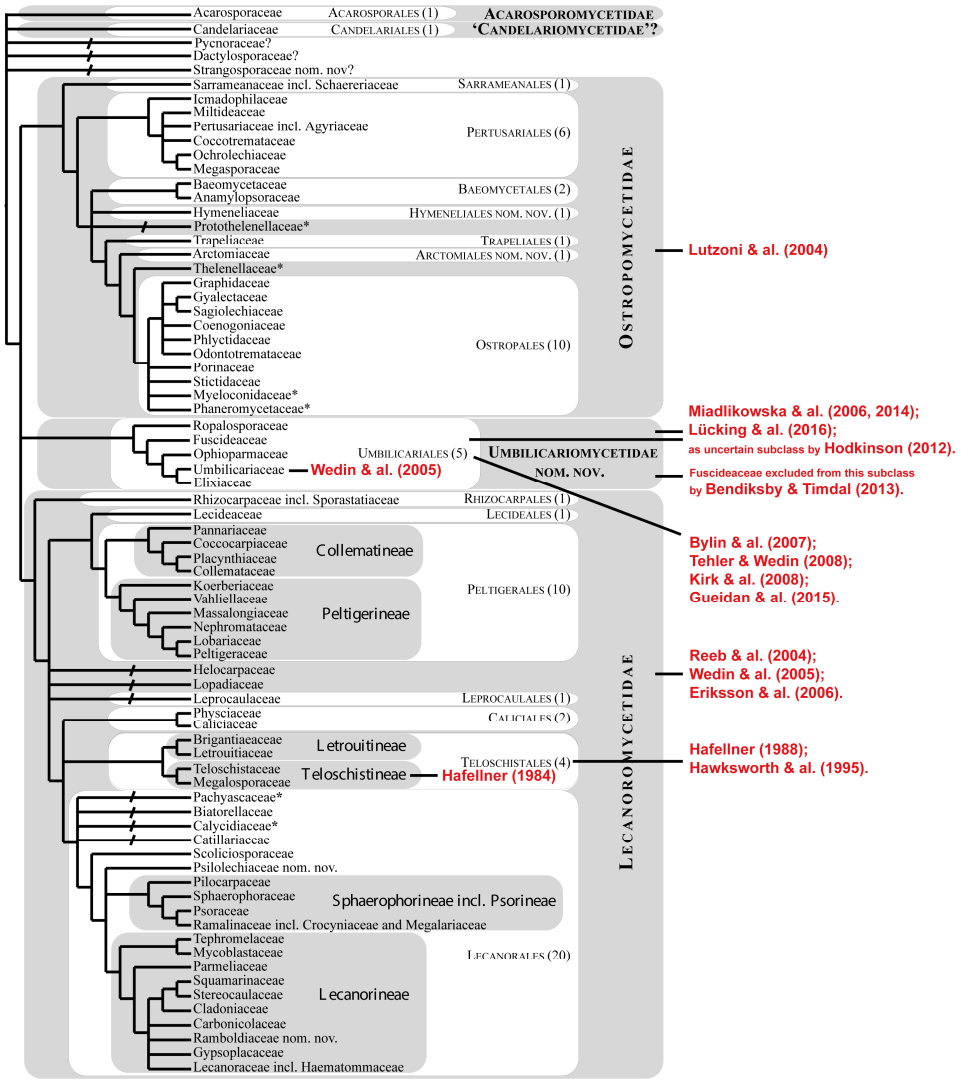

Figure 1. Taxonomic positions of the family Fuscideaceae according to the different studies depicted on the schematic presentation of phylogeny and classification of the class Lecanoromycetes made by Miadlikowska & al. (2014).

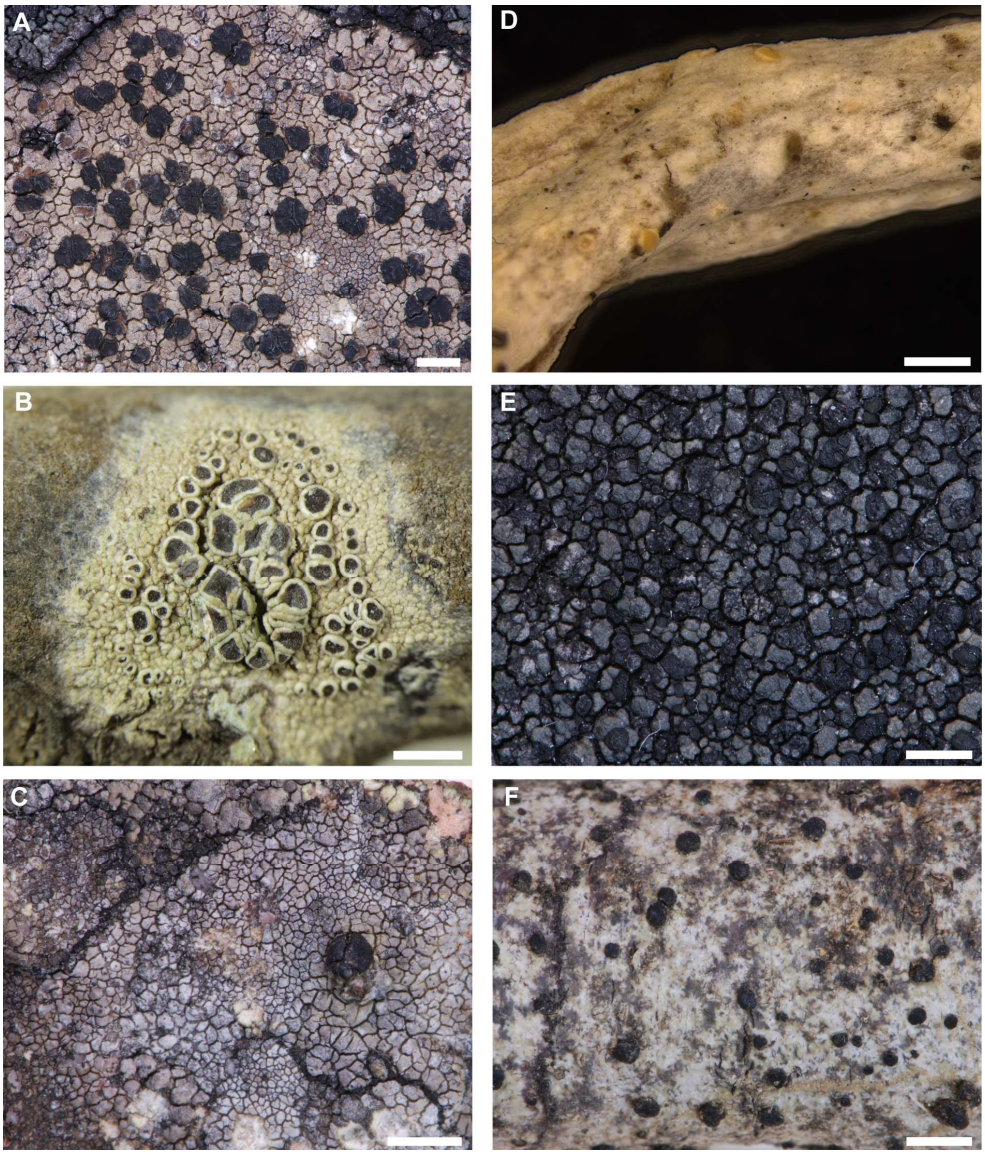

Figure 2. Genera belonging to Fuscideaceae *sensu* Eriksson & al. (2006). A – *Fuscidea mollis* (Wahlenb.) V. Wirth & Vězda (part of *T. Tønsberg 39940*; *BG-L-90300*), B – *Maronea constans* (Nyl.) Hepp (*HO:557799*), C –*Ropalospora lugubris* (*A.M. Fryday 8868*; *MSC0050548*), D –*Lettauia cladoniicola* D. Hawksw. & R. Sant. growing on *Cladonia ciliata* var. *ciliata* Stirt. (*J. Kocourková & K. Knudsen JK/7838*), E – *Orphniospora moriopsis* Körb. (part of *T. Tønsberg 39940*; *BG-L-101303*), F – *Sarrameana albidoplumbea* Vězda & P. James (*G. Kantvilas & J. Elix 78/08*; *HO:547319*). For *Hueidea* see Kantvilas & McCarthy, (2003; Fig. 1 on page 398). Scale A, C, E, & F = 2 mm; B = 1 mm; D = 0.5 mm. Photos: A– C, E, & F – A. Kurz; D – M. Zahradníková.

Main objectives of the thesis

As the taxonomy and systematics of the Fuscideaceae and *Fuscidea* are in need of revision and the knowledge about the identity of the photobiont in *Fuscidea* is poor, the main objectives of this thesis are to:

1) Investigate the placement of Fuscideaceae (Paper I).

2) Designate the genera that should be assigned to the family (Paper I).

3) Examine the phylogenetic relationships within the genus *Fuscidea* (Paper I).

4) Elucidate the taxonomy of the *F. lightfootii-F. pusilla* species complex (Paper II).

5) Assess the infraspecific taxonomy of *F. cyathoides* and the status of *F. fagicola* and *F. stiriaca* (Paper III).

6) Identify the photobiont in *Fuscidea* and clarify its systematic placement (Paper IV).

Material and methods

Taxon sampling

Specimens were obtained through fieldwork, loans from BM, H-Ach, HO, LD, MSC, UPS, S and TUR, from private collections and by personal visit to herbarium VER.

Chemical analysis

Thin-layer chromatography was carried on all specimens using solvents (A, B´ and C) according to the methods of Culberson & Kristinsson (1970), Culberson (1972) and Menlove (1974).

Morphometric analysis

The morphological variation between esorediate and sorediate saxicolous as well as corticolous forms of *F. cyathoides* was assessed using Principal Components Analysis (PCA) (Paper III).

DNA extraction, PCR amplification and sequencing

DNeasy Plant Mini Kit (Qiagen) was used for DNA extractions, following the plant leaf extraction protocol. The gene amplifications were performed for mtSSU, ITS, LSU rDNA of the mycobiont as well as for ITS and 18S rDNA of the photobiont (see Papers I-IV). The Polymerase Chain Reaction (PCR) mixture was adjusted according to the primers pair. PCR reactions were performed on a C1000^{TM} Touch thermal cycler (Bio-Rad Laboratories) using the following protocol: Initial denaturation at 94°C for 5 min, followed by a 63–55°C touchdown cycle depending on primers pair for the first 6 cycles, ending with 40 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 1 min 45 s and a final elongation at 72°C for 10 min. The PCR products were checked on a 1% RedGel-stained agarose gel under UV light and cleaned according to the manufacturer's instructions using Exo-Sap-IT (GE Heathcare). Sequencing reactions were carried out using a BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and run on an ABI Prism 3700XL DNA analyser (Applied Biosystems). The program SeqMan II version 4.05 (DNASTAR) was used to assemble the sequences.

Phylogenetic analyses

Sequence alignments were performed using Muscle (Edgar 2004a,b) implemented in the phylogenetic data editor PhyDE v.0.9971 (http://www.phyde.de/download.html) and Muscle (Edgar 2004a,b) or Geneious (Biomatters Ltd.) implemented in Geneious v.8.1.8 (Biomatters Ltd.), and followed by manual adjustment. Primer positions and ambiguous sites were excluded from the data matrices.

The Jukes-Cantor neighbor-joining model implemented in Geneious v.8.1.8 (Biomatters Ltd.) was used to assess the bootstrap scores in order to detect potential conflicts between individual data sets (Paper I).

The best-fit models for the individual and combined data sets were identified in the program jModelTest v.2.1.7 (Posada, 2008). The models with the lowest Akaike Information Criterion value were used in the analyses.

To obtain a 50% majority-consensus tree with branch supports shown as posterior probabilities, the program MrBayes v.3.2.1 (Ronquist & Huelsenbeck, 2003) was chosen to sample trees under a Markov chain Monte Carlo method. Significant posterior probabilities were considered to be equal to or above 0.95.

The maximum parsimony and maximum likelihood methods were carried out in PAUP* (Swofford, 2002) or RaxML v.7.2.8 (Stamatakis, 2006) to calculate a 50% majority-rule consensus trees with bootstrap supports. Significant posterior probabilities were considered to be equal or above 70%.

The program SATé-II v.2.2.7 (Liu & al., 2012) was used to analyse the non-aligned data matrix of ITS. The matrix was divided into subsets that were subsequently aligned by MAFFT (Katoh & al., 2005; Katoh & Toh, 2008) and combined by Muscle (Edgar 2004a,b) to conduct a new alignment. Ambiguous positions were identified and excluded by the use of Gblocks with stringent and relaxed masking (Talavera & Castresana, 2007) (Paper IV).
The secondary structures of the ITS2 sequences were folded using the RNAfold server (http://rna.tbi.univie.ac.at). The compensatory base pair changes (CBCs), i.e. nucleotide changes at both sides of paired bases and hemi-CBCs, i.e. nucleotide change at only one side of nucleotide pair, but still preserving pairing (e.g. Caisová & al., 2011), were located on the folded ITS2 operons according to Coleman (2000, 2003) in order to support the recognition of different species retrieved in the ITS phylogeny. The conserved parts of ITS2 were identified according to Coleman (2007) (Paper IV).

Results and discussion

Taxonomic placement of Fuscideaceae and its genera

The taxonomic position of Fuscideaceae, represented by *Fuscidea* and *Maronea*, remains unsure even when large molecular data sets have been applied. Reeb & al. (2004) concluded that Umbilicariaceae formed a robustly supported sister-group to Fuscideaceae and proposed to recognize these groups as a new order called Umbilicariales. Wedin & al. (2005) indicated that Fuscideaceae may be related to Umbilicariaceae, but without a significant support. Miadlikowska & al. (2006) proposed to classify the Fuscideaceae-Ophioparmaceae-Umbilicariaceae group as a separate order Umbilicariales within Lecanoromycetidae, but the order was not formally introduced. Bylin & al. (2007) found Fuscideacea as sister to Umbilicariales, although lacking support. Ropalosporaceae was reintroduced and placed with uncertain position tentatively within Umbilicariales. Bendiksby & Timdal (2013) did not support the inclusion of Fuscideaceae in Umbilicariales, even though they found an ascus type in *Umbilicaria* Hoffm. like that of *Fuscidea*, i.e. amyloid inner and outer layer with a non-amyloid layer in between (see Fig. 3). Miadlikowska & al. (2014) retrieved Fuscideaceae as a paraphyletic group within Umbilicariales and suggested that *Fuscidea mollis* (Wahlenb.) V. Wirth & Vězda should be recognized as a distinct genus.

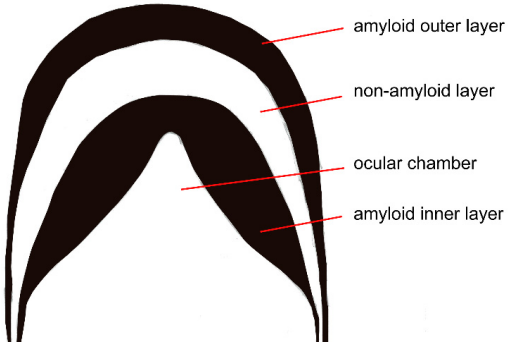

Figure 3: Structure of the *Fuscidea*-type ascus apex.

The taxonomic placement of Fuscideaceae and its associated genera within Lecanoromycetes were investigated using a 5-gene concatenated data set (Paper I). The family is nested within Umbilicariales (Fig. 4; Paper I: Figs. 1, S4). These results are similar to those of Miadlikowska & al. (2006, 2014) who determinated Fuscideaceae as sister to Ophioparmaceae. In Miadlikowska & al. (2014) the Ropalosporaceae was sister

to Fuscideaceae and nested as the first diverging lineage within Umbilicariales, while in the present study, *Ropalospora* is also located at the base of Fuscideaceae but is included in Fuscideaceae. The family Ropalosporaceae is synonymized with Fuscideaceae as in Eriksson & al. (2006) and Kantvilas (2004).

The circumscription of Fuscideaceae agrees with Hafellner (1984), but two more genera, *Ropalospora* and *Printzeniella* Palice, Tønsberg & Zahradn. gen. nov. ined., are included in the family. The similar ascus structure in Fuscideaceae and Teloschistales was found to be homoplastic by Miadlikowska & al. (2006). The members of the family have in common a crustose rimose-cracked to areolate thallus (in *Printzeniella* obsolete or poorly developed), a brownish hypothallus (sometimes inconspicuous in *Maronea* or invisible in *Printzeniella*), a coccoid green alga as the photobiont, lecideine or lecanorine apothecia with a brownish pigmentation (see Fryday, 2008), 8- or in *Maronea* and *Ropalospora*, multispored, slightly tapering above or cylindrical-clavate asci of the ± *Fuscidea*-type (Fig. 3), containing simple or septate ascospores and short bacilliform conidia. The secondary chemical compounds are heterogenous within Fuscideaceae comprising, e.g. anthraquinones, benzyl esters, depsides, depsidones, higher aliphatic acids and usnic acid (Tønsberg, 1992; Ekman, 1993; Kantvilas, 2004; Gilbert & al., 2009).

Figure 4 shows that *Fuscidea* is paraphyletic; this agrees with Miadlikowska & al. (2014). To make the genus monophyletic, it is possible to synonymize *Fuscidea* with *Maronea*, a genus introduced by Massalongo (1856) before *Fuscidea* by Wirth & Vězda (1972). As species of *Fuscidea* are more numerous than *Maronea*, conservation of the name *Fuscidea* over *Maronea* will be preferred. Another solution is to transfer the members of the *F. pusilla*-clade to *Maronea*, thus in the present phylogeny, *Fuscidea* will still be paraphyletic. It is also possible to recognize species in the *F. pusilla*-clade as a phylogenetically distinct genus, but *Fuscidea* will remain paraphyletic. It is worth conducting a new phylogenetic reconstruction with additional data from *Maronea* and *Fuscidea* as the backbone of the *Fuscidea*-clade is poorly resolved, in the hopes of obtaining a phylogeny with better node resolution.

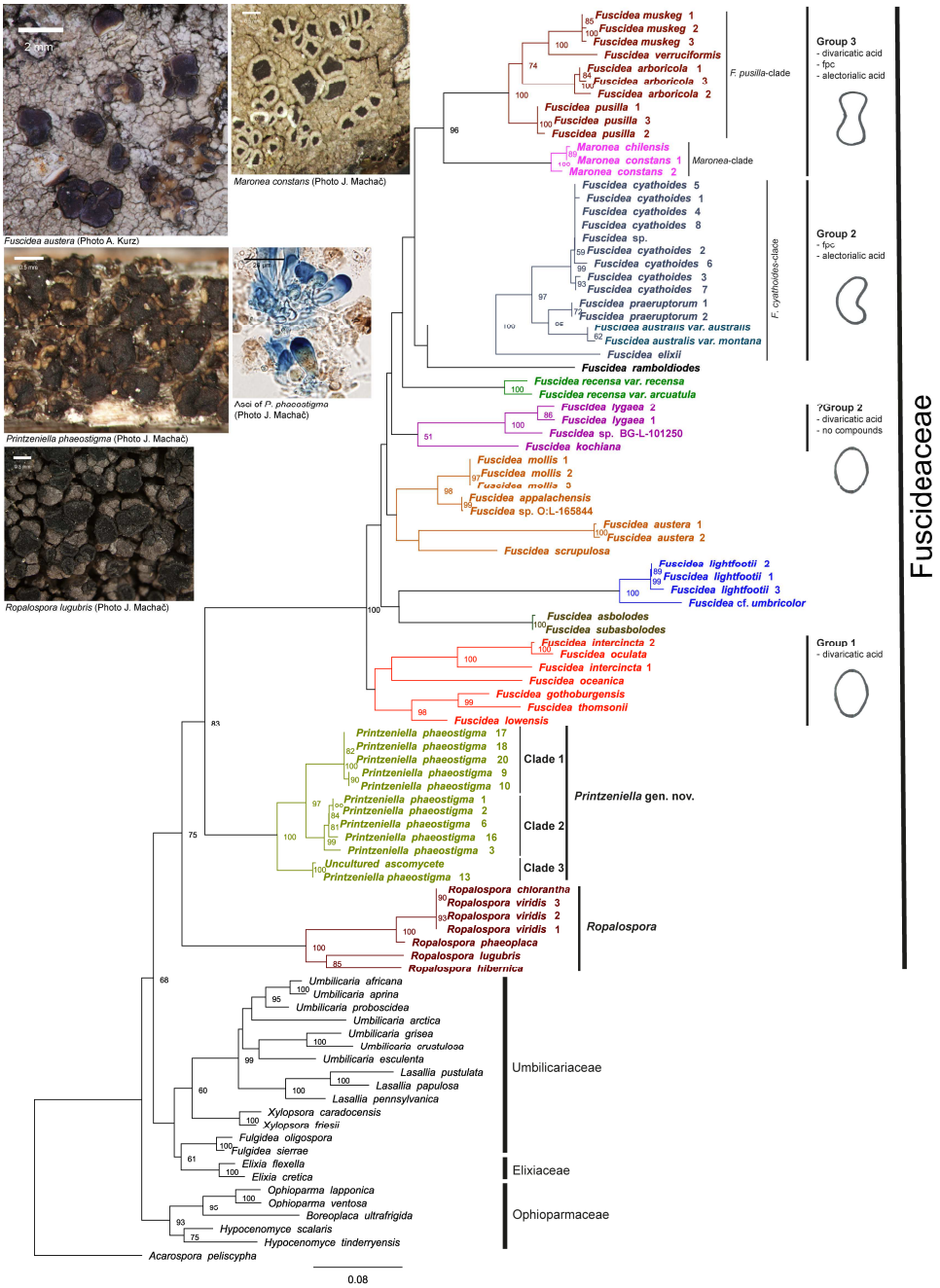

Figure 4. Phylogeny of Umbilicariales with emphasis on the genus *Fuscidea*, based on a 5-gene concatenated data set shown as a 50% majority-rule consensus tree obtained from the maximum likelihood analysis. Only bootstrap support values above 50% are given. The shapes of the spores and the chemical constituents are added for the groups of *Fuscidea*. Fpc = fumarprotocetraric acid.

Maronea afroalpina Brusse shows affinity with *Fuscidea* and differs from other *Maronea* species in the lecideine apothecia (*vs*. lecanorine in *Maronea*), the presence of the paradepside divaricatic acid (*vs*. the metadepsides sekikaic and submerochlorophaeic acids or an unidentified compound in *Maronea*) and in occurring on rock at high altitudes (ca. 3000 m) (Brusse, 1989b; Kantvilas, 2004; LaGreca, 2006). *Maronea afroalpina* apparently holds an intermediate position between *Fuscidea* and *Maronea* and Brusse (1989b) argued that *M. afroalpina* may be a genus of its own. Unfortunately, it was not possible to get this species included in the phylogenetic tree.

Purvis & al. (1992) treated *Ropalospora* as congeneric with *Fuscidea* based on the close resemblance between these two genera. They are similar in thallus morphology and pycnidial anatomy, but differ in the presence of thickened rectangular hyphae in the excipulum (not present in *Ropalospora*) and spore shape (acicular and multiseptate in *Ropalospora*). In addition, the asci in *Ropalospora* are 8- or 30-spored (only 8-spored in *Fuscidea*) and resemble the *Fuscidea*-type, but differ in being cylindrical-clavate (not slightly tapering above) and in lacking an ocular chamber in most species (Ekman, 1993). Several chemical constituents found in *Ropalospora* are rare or not present in *Fuscidea*, e.g. anthraquinone (parietin), depsides (gyrophoric and perlatolic acids, atranorin), usnic acid and higher aliphatic acids (Ekman, 1993; Kantvilas, 2001, 2004; Purvis & al., 2009).

Printzeniella (Fig. 5) is an exclusively epiphytic and epixylic genus growing on acid bark and wood, rarely found on other substrates such as polypores. It is similar to *Fuscidea* with its brown apothecial, pycnidial and thalline pigmentation, but differs in having a *Trebouxia* photobiont, biatorine to lecanorine apothecia, a reduced and poorly differentiated excipulum proprium and asci resembling those of *Ropalospora*. For *Printzeniella* the higher aliphatic compound, apinnatic acid, not detected in *Fuscidea*, is diagnostic. The three phylogenetic clades of *Printzeniella* were all treated as *Printzeniella phaeostigma* (Körb.) Palice, Tønsberg & Zahradn. ined., since they were not morphologically distinguishable (Paper I: Taxonomy).

Because fresh material for the sequencing was not available, *Hueidea* was tentatively placed in Fuscideaceae based on the morphological and anatomical similarities in thalli, the photobionts and the asci structures between *Hueidea* and *Fuscidea* (Kantvilas & McCarthy, 2003).

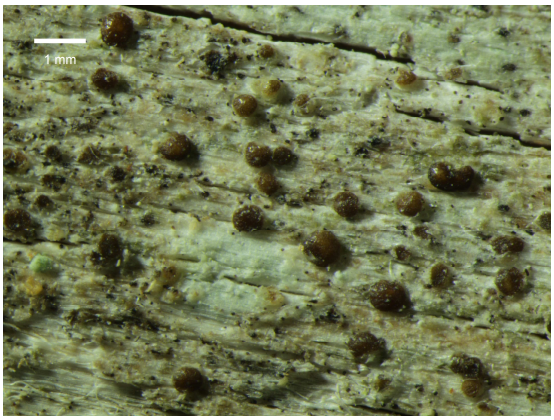

Figure 5. *Printzeniella phaeostigma* (Körb.) Palice, Tønsberg & Zahradn. ined. (*T. Tønsberg 46763*; *BG-L-101305*). Photo: A. Kurz.

Genera not closely related to Fuscideaceae

The genera *Lettauia*, *Loxospora*, *Orphniospora* and *Sarrameana* are not closely related to Fuscideaceae (Paper I: Fig. 1). Three of these genera are nested within the subclass Ostropomycetidae and one within the subclass Lecanoromycetidae.

The lichenicolous *Lettauia* is grouped with *Cryptodiscus* Corda in Stictidaceae within Ostropales. *Loxospora* with *Sarrameana* form the family Sarrameanaceae as sister to Ostropales. *Orphniospora* appears to be sister to the Lecideaceae *s. str.* within Lecideales, although lacking a significant support in the ML analysis. A broader taxon sampling is needed in order to assess the taxonomic positions of *Lettauia* and *Orphniospora*.

Phylogenetic relationships within *Fuscidea*

The reconstruction of the phylogenetic relationships of *Fuscidea* revealed three main groups, but some of the *Fuscidea* species remained unresolved (see Fig. 4).

Group 1 is unsupported in the phylogenetic tree, but possible to define by the presence of the lecideine, sessile (*F. lowensis* (H. Magn.) R.A. Anderson & Hertel) or immersed aspicilioid apothecia with a pseudothalline margin, i.e. the "*intercincta*-type – "halo" (Oberhollenzer & Wirth, 1984), subglobose to broadly ellipsoid spores becoming brownish when mature and the paradepside divaricatic acid. This group includes saxicolous species from Europe and North America such as *F. gothoburgensis* (H. Magn.) V. Wirth & Vězda, *F. intercincta* (Nyl.) Poelt, *F. lowensis*, *F. oceanica* Fryday & Coppins, *F. oculata* Oberholl. & V. Wirth and *F. thomsonii* Brodo & V. Wirth. The amyloid medulla in *F. gothoburgensis*

and *F. lowensis* may occasionally be non-amyloid (Oberhollenzer & Wirth, 1985; Fryday, 2008) and should therefore not be considered as a diagnostic character.

Group 2 is supported by the phylogenetic tree and includes usually fertile *Fuscidea* species with curved ascospores and the depsidone fumarprotocetraric acid (*F. australis* var. *australis* Kantvilas and var. *montana* Kantvilas, *F. cyathoides*) or the benzyl ester alectorialic acid (*F. elixii* Kantvilas and *F. praeruptorum* (Du Rietz & H. Magn.) V. Wirth & Vězda). *Fuscidea elixii*, endemic to Australia, appears as the first diverging lineage in Group 2 and morphologically resembles *F. australis* var. *australis* (Kantvilas, 2004). The taxonomy of *F. cyathoides* has been studied in detail in Paper III (see below). The mainly corticolous, esorediate and fertile *F. australis* resembles *F. cyathoides* in having sessile apothecia with a persistent margin and bean-shaped ascospores; it differs mainly in the ellipsoid conidia (bacilliform in *F. cyathoides*) (Kantvilas, 2001, 2004). Using this evidence, Kantvilas (2001) excluded *F. cyathoides* from Tasmania. The suggestion by Kantvilas (2001) that *F. australis* is distinct from *F. cyathoides* is confirmed here. The saxicolous taxon of *F. praeruptorum* is recognized as *F. praeruptorum* in the present study, while the corticolous one as *F. muskeg* Tønsberg & Zahradn. ined.. These species differ in the shape of the ascospores and both occur in Europe and North America (Santesson & al., 2004; Fryday, 2008; Gilbert & al., 2009).

Fuscidea ramboldioides is not included in Group 2 due to the lack of a significant support (Paper I: Figs. 1, S4). It is a fertile, esorediate and saxicolous taxon having a greyish-brown, sometimes olive-brown thallus, curved to medianly constricted ascospores and divaricatic acid (Kantvilas 2001, 2004).

The fertile and saxicolous species from Europe, i.e. *F. lygaea* (Ach.) V. Wirth & Vězda, the *Fuscidea* sp. from Norway (BG-L-101250) and *F. kochiana* (Hepp) V. Wirth & Vězda, share broadly ellipsoid to globose ascospores. They are located in one non-supported group, which is tentatively assigned to **Group 2** (see Paper I: Figs. 1, S4). The different outgroup and the fewer sequences in the alignment representing only the members of the Umbilicariales may cause the incongruence of their placement between Fig. 4 and Fig. 1 in Paper I. *Fuscidea lygaea* and *Fuscidea* sp. have no secondary compounds detected by TLC, but differ in thallus morphology (brown with purplish tinge in *F. lygaea vs*. pale-grey in *Fuscidea* sp.) and in the position of apothecia (sessile in *F. lygaea vs*. appressed with margin having pruina in young apothecia in *Fuscidea* sp.). *Fuscidea kochiana* has immersed apothecia, ascospores becoming red-brown when over-mature and divaricatic acid.

Group 3 includes corticolous *Fuscidea* and *Maronea* with a brown-green, green to olive-green thallus, a distinct dark brown prothallus, sessile apothecia and ascospores

with median constriction (rarely bean-shaped as in *F. verruciformis* or oblong in as *Maronea*). The *F. pusilla*-clade is comprised of *Fuscidea arboricola* Coppins & Tønsberg, *F. muskeg*, *F. pusilla* Tønsberg and *F. verruciformis* May. Inoue. Except for *F. verruciformis*, which is esorediate and confined to Japan, all the species are sorediate and occur in Europe and North America. The members of Group 3 differ in their secondary chemistry. *Fuscidea pusilla* contains paradepside divaricatic acid, *F. arboricola* and *F. verruciformis* contain depsidone fumarprotocetraric acid, whereas *F. muskeg* is characterized by the presence of benzyl ester alectorialic acid. The *Maronea*-clade includes *M. constans* and *M. chilensis* B. de Lesd. and is sister to the *Fuscidea*-clade. Based on the present results, *M. constans* may be conspecific with *M. chilensis*, but more data are needed to elucidate their taxonomy.

Some of the *Fuscidea* species have uncertain positions, including *F. austera* (Nyl.) P. James, which is synonym to the type species *F. aggregatilis* (Flot.) V. Wirth & Vězda.

The saxicolous *F. asbolodes* (Nyl.) Hertel & V. Wirth from Tasmania and *F. subasbolodes* Kantvilas from the Subantarctic islands have similar asci and ascospores, but differ in their chemistry, thallus colour and apothecial size (Kantvilas, 2004).

Two species that produce divaricatic acid are grouped in one supported clade, the corticolous *F. lightfootii* from Western Europe and the saxicolous *F.* cf. *umbricolor* (Nyl.) Hertel from northern South America. *Fuscidea lightfootii* may be confused with *F. pusilla*, whereas *F.* cf. *umbricolor* is similar to *F. lowensis*.

The saxicolous *F. appalachensis* Fryday, *F. austera*, *F. mollis* and *F. scrupulosa* (Eckfeldt) Fryday have similar ascospore morphology, i.e. broadly ellipsoid to globose, becoming brownish when over-mature (not in *F. mollis*). Most of them have divaricatic acid (*F. scrupulosa* has alectorialic acid) and European and North American distribution, but *F. austera* and *F. mollis* have also been reported from Asia (Inoue, 1981a). *Fuscidea appalachensis* resembles *F. kochiana* with a pale grey thallus, immersed apothecia and a position of ascospores in asci (uniseriate) (Fryday, 2008). More data are necessary to resolve the taxonomy of the sterile and sorediate specimen from Norway, R. Haugan 9194 (O:L-165844). In North America, *F. appalachensis* could be confused with *F. recensa* var. *arcuatula* (Arnold) Fryday (see Fryday, 2008). *Fuscidea mollis* is similar to *F. cyathoides*, but differs in the shape of the ascospores and the chemical constituent. The proposal by Miadlikowska & al. (2014) to assign *F. mollis* to a genus of its own was rejected, since *F. mollis* is clearly nested within *Fuscidea*.

The mainly saxicolous and sometimes sorediate *F. recensa* var. *recensa* (Stirt.) Hertel and occasionally corticolous, esorediate *F. recensa* var. *arcuatula* have ellipsoid to curved

ascospores and produce divaricatic acid (Fryday, 2008). *Fuscidea recensa* var. *recensa* occurs both in Europe (mostly sterile and sorediate) and North America (fertile and sorediate) and var. *arcuatula* from North America and Asia (Fryday, 2008; Moon, 2013).

Are *Fuscidea lightfootii* and *F. pusilla* conspecific?

The relationship between *F. lightfootii* (usually fertile) and *F. pusilla* (regarded as sterile only) was studied using a 5-gene data set. Since they are morphologically similar and chemically identical, Tønsberg & Johnsen (2008) suggested that they may be conspecific. As a result of DNA sequencing, *F. pusilla* was found fertile for the first time, having apothecia similar in morphology and anatomy to those of *F. lightfootii*. These two species are phylogenetically distinct and the hypothesis is therefore rejected. Although some morphotypes of *F. lightfootii* and *F. pusilla* appear to be distinguishable based on morphology, DNA sequencing is recommended for their identification.

They are sympatric in the British Isles and on the southwest coast of Norway (i.e. areas with an oceanic climate). *Fuscidea pusilla* also occurs in continental areas of Europe and throughout coastal Alaska (Paper II: Figs. 5–6). Reports of these species outside their distribution areas as defined in Paper II: Fig. 5, need revision.

Taxonomy of *Fuscidea cyathoides* in Europe

Fuscidea cyathoides is characterized by sessile apothecia, bean-shaped ascospores becoming brown when mature and the presence of fumarprotocetraric acid. Substrate ecology and the presence/absence of soredia have been used as important characters for the formal recognition of infraspecific taxa in *F. cyathoides* (Fries, 1831; Magnusson, 1925). In addition, Hafellner & Türk (2001) and Hafellner (2002) raised the corticolous form of *F. cyathoides* to the species level.

Fries (1831) suggested that the different thallus colour of saxicolous (grey when dry and umber-brown when wet) and corticolous specimens (black-brown when dry and greenish when wet) was significant and introduced var. *corticola* (as *Biatora rivulosa* b. *corticola* Fr.). This was not accepted by Oberhollenzer & Wirth (1984) and Gilbert & al. (2009), but was recognized by Inoue, (1981b) and Santesson & al. (2004).

Magnusson (1925) suggested the presence of soredia on the typical saxicolous form as the reason for the introduction of var. *sorediata* (H. Magn.) Poelt (as *Lecidea rivulosa* var.

sorediata H. Magn). This variety was commonly accepted by, for example, Gilbert & al. (2009).

Zschacke (1927) introduced the corticolous *Lecidea fagicola* Zschacke based on the absence of a black prothallus and probably the relatively large apothecia with pale brown margins (Paper III: Fig. 3C), later recognized as *F. fagicola* (Zschacke) Hafellner & Türk by Hafellner & Türk (2001). In describing the new corticolous species *Biatora stiriaca* A. Massal., Massalongo (1852) considered the bean-shaped ascospores and the presence of tuberculate apothecia as diagnostic. Hafellner (2002) transferred *Biatora stiriaca* to *Fuscidea* as *F. stiriaca* (A. Massal.) Hafellner and synonymized *F. fagicola* with *F. stiriaca*.

The taxonomic status of *F. cyathoides* was assessed by the use of chemical, morphometric and molecular methods. The variation in thallus morphology and colour, the presence of soredia, even the preferable substrate turned out not to be diagnostic for the varietal rank in *F. cyathoides*. All currently recognized varieties are therefore synonymized with the typical saxicolous form var. *cyathoides*. Similarly, the variation in apothecia and the presence of tuberculate apothecia were not significant for *F. fagicola* as well as *F. stiriaca* that should therefore be treated as synonyms of *F. cyathoides*.

Substrate specificity in *Fuscidea*

Fuscidea is comprised of approximately 40 species. Most saxicolous specimens (ca 75%) are restricted to siliceous vertical rock (i.e. *F. austera*, *F. intercincta* and *F. mollis*) and some corticolous specimens (ca 18%) are restricted to somewhat acidic smooth bark (i.e. *F. arboricola*, *F. lightfootii* and *F. muskeg* ined.); only a few species (ca 7%, i.e. *F. australis*, *F. cyathoides* and *F. recensa*) can inhabit both substrates (Tønsberg, 1992; Kantvilas, 2001; Gilbert & al., 2009). *Fuscidea* species generally have high substrate specificities occurring on rock or on bark only (see Fig. 6).

Among other genera within Lecanoromycetes, a strong substrate specificity is found in *Porpidia* Körb. According to Fryday & al. (2009), the genus *Porpidia* is represented by 20 species in the British Isles and all of them are exclusively saxicolous (mostly on siliceous rock), but some species such as *Porpidia crustulata* (Ach.) Hertel & Knoph, *P. macrocarpa* (DC.) Hertel & A.J. Schwab and *P. tuberculosa* (Sm.) Hertel & Knoph may be rarely found on hard-wood or on the bark of branches growing over a rock surface (Tønsberg, 1992; Z. Palice pers. com. 2017). On the contrary, the genus *Ochrolechia* A. Massal. displays a weak substrate specificity. Off the 11 species reported for the British

Isles, 3 species are corticolous and 3 species are both corticolous and saxicolous, 2 species grow on bryophytes, lichens and plant debris and 3 species inhabit all mentioned substrates (Fletcher & al., 2009).

Figure 6 shows the evolution of the substrate specificity in *Fuscidea* species. It is most likely that the common ancestor of *Fuscidea* was saxicolous and the corticolous taxa evolved several times.

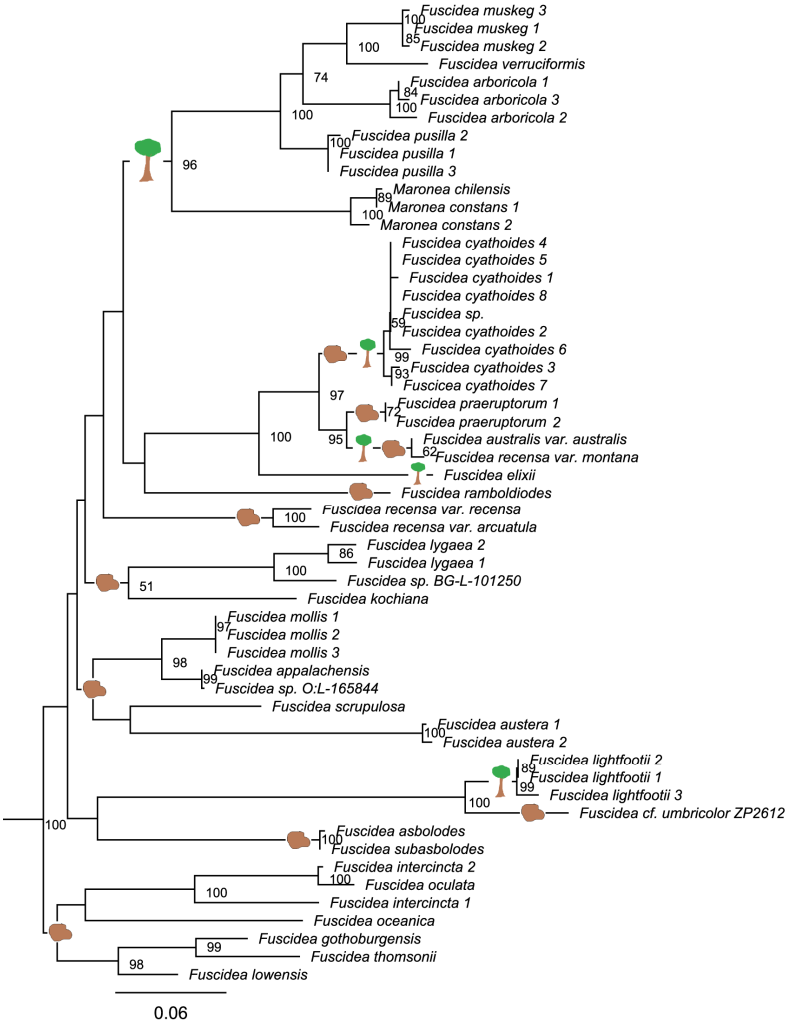

Figure 6. A part of Fig. 4. Marked substrate preference of the *Fuscidea* species. : saxicolous; : corticolous taxa.

A very interesting question is how and why the substrate preferences evolved among the *Fuscidea* species. Does the photobiont play any role in this ability? Do species colonizing more than one substrate have a higher genetic diversity of photobionts than exclusively saxicolous or corticolous species? The role of the substrate in the photobiont variation of *Fuscidea* species colonizing either rock, bark, or even both substrates is yet to be explained using more extensivetaxon sampling from different substrates.

Photobiont in the genus *Fuscidea*

The photobiont in the genus *Fuscidea* is identified as *Apatococcus* that is nested with uncertain position within the class Trebouxiophyceae where it is closely related to *Trebouxia* and/or *Myrmecia* Printz (Paper IV: Fig. 1). Two species of lichenized *Apatococcus* are found so far. Four of the five studied *Fuscidea* species are associated with *A. fuscideae*, but *F. lightfootii* (Sm.) Coppins & P. James has a photobiont of its own (Fig. 7, Paper IV: Figs. 1, 2). In addition, four different ITS groups were retrieved that possibly correspond to a distinct species of free-living *Apatococcus*, but this was not studied in detail.

Apatococcus is generally characterized by uninucleate cells with a single, parietal chloroplast without pyrenoids (Brand & Stockmayer, 1925). *Apatococcus lobatus* usually has a bi-lobed chloroplast in the mature cells, while *A. fuscideae* A.Beck & Zahradn. ined. has a reticulate, net like chloroplast (Paper IV: Fig. 4).

Using the Compensatory Base Changes (CBCs) species concept on the secondary structure of the ITS2 region, *A. lobatus* can be distinguished from *A. fuscideae* by three CBCs and one hemi-CBCs on helix I, one hemi-CBCs on helix II, four CBCs and six hemi-CBCs on helix III, from which three CBCs and five hemi-CBCs are on the conserved part of helix III. The photobiont in *F. lightfootii* differs from *A. fuscideae* in having two CBCs on helix I, seven CBCs and three hemi-CBCs on helix III, from which four CBCs and three hemi-CBCs are on the conserved part of helix III (Paper IV: Figs. 3, S2: A, E–F).

The resulting ML trees calculated from four individual ITS matrices contained different degrees of ambiguous sites, i.e. manually adjusted (MA), Gblocks with relaxed (R) and stringent masking (S) as well as non-aligned matrix. All of them showed almost identical topologies in the backbones of the ML trees and most of the recent nodes were recovered with only minor differences. The ML trees retrieved from the S matrix and SATé-II, for example, have different branching within group A and B than calculated from the MA and R matrices. Additionally, the S matrix restricted to the conserved alignment parts received

lower ML supports for most of the nodes than from the MA and R matrices, probably due to the short alignment. Although the aligning of the very variable ITS gene is difficult and time consuming, the alignment independent approach by SATé-II may provide reliable phylogenies faster than by traditional methods.

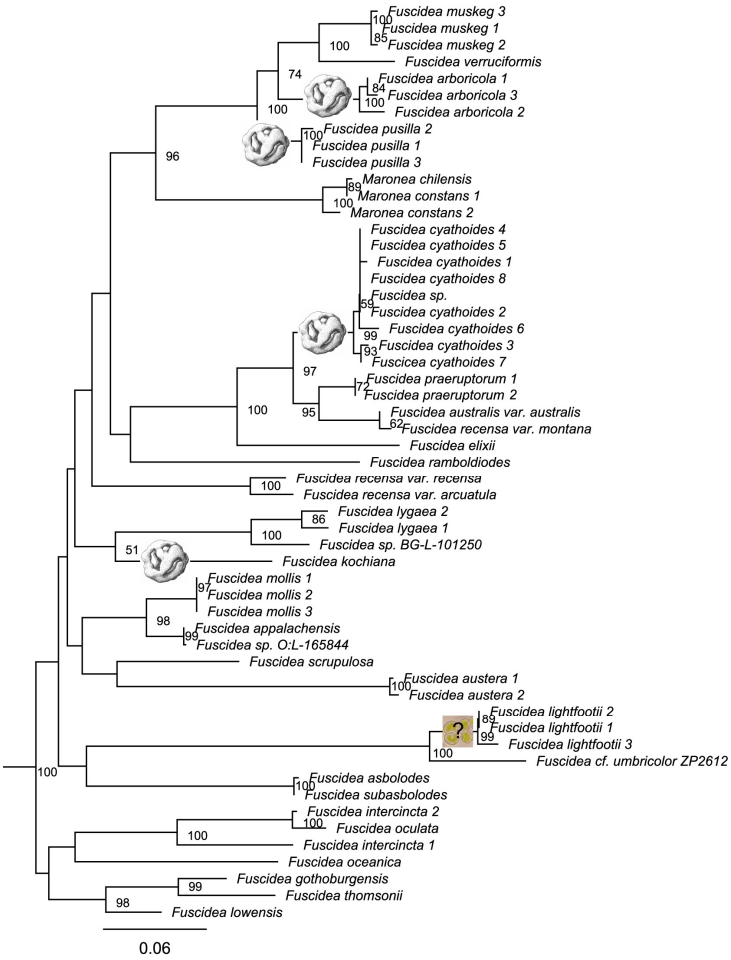

Figure 7. A part of Fig. 4. *Fuscidea* species where the photobiont is studied are marked ( ).

Conclusions

The family Fuscideaceae is assigned to Umbilicariales and presently accommodates *Fuscidea*, *Maronea*, *Ropalospora* and *Printzeniella* gen. nov., whereas *Hueidea* is only tentatively placed in Fuscideaceae. The *Fuscidea*-type ascus apex appears to be a diagnostic character for the family as suggested by Hafellner (1984) (Paper I). Although it is possible to identify some morphotypes of *F. lightfootii* and *F. pusilla*, DNA sequencing is recommended for their definitive identification (Paper II). The varieties of *F. cyathoides* are synonymized with the typical saxicolous form. Two corticolous species, *F. fagicola* and *F. stiriaca*, are found to be synonymous with *F. cyathoides* (Paper III). The photobiont in most of the studied *Fuscidea* species is *Apatococcus fuscideae* A. Beck & Zahradn. ined. and belongs to Trebouxiophyceae with uncertain position. *Fuscidea lightfootii* has a different photobiont (Paper IV).

Future perspectives

This close investigation of *Fuscidea* and Fuscideaceae gave new knowledge about this group, but it also revealed new challenges for further studies:

1) Is *Hueidea* phylogenetically related to Fuscideaceae?
2) How to make *Fuscidea* monophyletic?
3) How to resolve the phylogenetic relationships within *Fuscidea*?
4) Is *Fuscidea oculata* synonymous with *F. intercincta* as suggested by Wirth & al. (2013)?
5) Are *Fuscidea asbolode*s and *F. subasbolodes* distinct species as suggested by Kantvilas, (2001)?
6) Does the corticolous form of *Fuscidea recensa* var. *recensa* represent a new species?
7) Should *Maronea chilensis* be synonymized with *M. constans*?
8) Should the saxicolous form of the corticolous *Fuscidea australis* be recognized at the varietal rank?
9) Are *Fuscidea cyathoides* var. *japonica* May. Inoue & P. James and *F. cyathoides* var. *orientalis* (Zahlbr.) May. Inoue synonyms of *F. cyathoides* var. *cyathoides*?
10) Is *Fuscidea scrupulosa* conspecific with *F. circumflexa* (Nyl.) V. Wirth & Vězda as suggested by Fryday, (2008)?
11) Is *Fuscidea poeltii* Fryday a distinct species?
12) What are the distributional ranges of *Fuscidea lightfootii* and *F. pusilla*?
13) Is it possible to certainly solve the taxonomic position of *Apatococcus* in the systematics of green algae using protein-coding genes?
14) Will a genetic mapping of the *Fuscidea* photobionts be helpful in determining *Fuscidea* taxonomy and understanding systematic relationships among the various *Fuscidea* species?
15) Is the photobiont in *Fuscidea lightfootii* morphologically distinct from *Apatococcus fuscideae*?
16) Does the photobiont play any role in the substrate specificity of *Fuscidea*?

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IV

Paper IV

Molecular evidence of *Apatococcus,* including *A. fuscideae* sp. nov., as photobiont in the genus *Fuscidea*

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Abstract: The knowledge of the taxonomy and classification of algae (including lichenized) has recently increased rapidly, but there are still many gaps. We aimed to 1) identify the *Fuscidea* photobionts by locating their taxonomic positions in the green algal classification, and 2) resolve their interspecific relationships. The lichenized algae were examined based on morphological observations of axenic isolates as well as molecular studies of 18S and ITS nrDNA sequences. Analysis of the secondary structure of ITS2 operon complemented these investigations. We found that the *Fuscidea* photobionts were placed within the Trebouxiophyceae, related to *Apatococcus lobatus* (Chodat) J.B. Petersen. Phylogenetic

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analyses revealed one clade nesting free-living and lichenized *Apatococcus* F. Brand which comprised six different lineages in the ITS phylogeny. The lichenized alga associated with the investigated *Fuscidea* species, except for *F. lightfootii* (Sm.) Coppins & James, represents a hitherto unknown lineage within *Apatococcus*. *Fuscidea lightfootii* was lichenized with a separate lineage within *Apatococcus*, together with free-living members of which were already known from the Genbank sequences. All retrieved groups within *Apatococcus* were rather different in ITS sequence, thus most likely corresponding to different species. The most common photobiont of *Fuscidea* species, *Apatococcus fuscideae* A.Beck & Zahradn., was described as new to science.

Key words: lichenized algae; lichen; *Fuscidea lightfootii*; green algal systematics; Trebouxiophyceae; ITS2 secondary structure

Introduction

Lichens, known as symbiotic organisms, comprise at least two partners, the heterotrophic mycobiont (typically an Ascomycete) providing the water, nutrients and the shelter for its autotrophic partner called the photobiont (typically a green alga, a cyanobacterium, or sometimes both), which produces sugar alcohol for the mycobiont (Hawksworth 1988; Honegger 2000; Schwendener 1867). In many species, more than one photobiont species is involved (Högnabba et al. 2009; James and Henssen 1976). While the scientific name of lichens refers to the mycobiont, the photobiont has an independent scientific name. Rambold et al. (1998) argued that the photobiont genus can provide new and valuable information for lichen systematics due to the strong photobiont selectivity of the mycobiont.

The knowledge about the diversity of symbiotic green algae is increasing recently (e.g. Catalá et al. 2016; Leavitt et al. 2016; Sanders et al. 2016; Škaloud et al. 2016; Voytsekhovich and Beck 2016), but the identity of the photobionts is still unknown for many groups of lichens, with a general estimate of 97% of the lichens with unknown photobiont species (Voytsekhovich and Beck 2016). In British Isles 42% of lichen genera are associcated with an unidentified protococcoid or chlorococcoid green algae, 26% with trebouxiod green algae or *Trebouxia* de Puymaly, 20% with *Trentepohlia* Martius, 3% with *Coccomyxa* Schmidle and 9% with other known symbiotic green algae, as complied from Smith et al. (2009).

One of the lichen genera associated with an unidentified green alga is *Fuscidea* Wirth & Vězda. Its photobiont was identified as a protococcoid alga (Inoue 1981; Oberhollenzer and Wirth 1984), *Trebouxia* (Galloway 1985), *Apatococcus lobatus* (Chodat) J.B. Petersen (Watanabe et al. 1997), a chlorococcoid alga, probably *Chlorella* Beyerinck [Beijerinck] (Gilbert et al. 2009) and a coccoid green alga (Miadlikowska et al. 2014). The first record of *A. lobatus* from *F. cyathoides var. japonica* May. Inoue & P. James as a lichenized alga

made by Watanabe et al. (1997) was considered uncertain by Ettl and Gärtner (2014) because other algal species were described from this lichen by the same authors in the same publication, namely *Elliptochloris bilobata* Tschermak-Woess, *E. reniformis* H. Ettl & G. Gärtner (as *Palmellococcus reniformis* S.Watanabe), *E. subsphaerica* (Reisigl) Ettl & Gärtner (as *Chlorella reisiglii* S. Watanabe), and *Myrmecia biatorellae* J.B. Petersen.

Free-living *Apatococcus* algae F. Brand inhabit subaerial environments characterized by a high dosage of light irradiance and UV radiation and different anthropogenic substrates (e.g. Hallmann et al. 2016, Rindi 2007). Hallmann et al. (2016) listed several morphological adaptations such as thickened walls, the special growth form, and several layers of empty *Apatococcus* cells that may protect the free-living *Apatococcus* against harsh conditions.

To reveal the identity of the photobiont in *Fuscidea*, two different markers has been chose; the marker 18S rDNA is suitable for solving higher level systematics and has frequently been used for investigations of green algae at different, mainly higher taxonomic levels (Dal Grande et al. 2014; Friedl and Zeltner 1994; Friedl 1995, 1997; Škaloud et al. 2016), and ITS rDNA on the other hand is very variable, suitable for phylogenies at the species and intraspecific levels and commonly used for phylogenetic reconstructions, including lichenized algae (e.g. Beck et al. 1998, Catalá et al. 2016; Miadlikowska et al. 2014; Muggia et al. 2013; Nyati et al. 2014; Škaloud and Peksa 2010). ITS rDNA has also been advocated as a suitable marker for species level phylogenetics of algae (e.g. Gile et al. 2010; Pröschold et al. 2011) and used as an efficient barcode marker for green algae (Hadi et al. 2016). Škaloud and Peksa (2010) found better phylogenetic resolution for the photobiont genus *Asterochloris* when partitioning the ITS sequence into three regions: ITS1, 5.8 rDNA, and ITS2.

As there is no common agreement on lichenized *Apatococcus* and the identity of the photobiont associated with *Fuscidea*, we aim to 1) identify the *Fuscidea* photobionts by locating their taxonomic positions in the green algal classification, and 2) resolve their

interspecific relationships. In addition to the traditional alignment based approaches, two further methods were chosen to test for a possible influence of potential errors in ITS alignments, i.e. Gblocks conducting alignments with different amount of ambiguous sites (Talavera and Castresana 2007) and the alignment independent approach by SATé-II (Lie et al. 2012). The secondary structure of the ITS2 operon can provide additional characters in species delimitation (Buchheim et al. 2012; Caisová et al. 2013; Poulíčková et al. 2010; Rampersad 2014; Škaloud and Peksa 2010) when boosted by the application of the Compensatory Base Changes (CBCs) species concept (Coleman 2000; Müller et al. 2007). We therefore also tested the applicability of the CBC concept for species delimitation in *Apatococcus*.

Material and Methods

Specimen collection and identification: Voucher specimens were collected in Austria, Norway, and Scotland between 1998 and 2011 (Table 1) and deposited in the herbaria of BG and M (abbreviations according to Index Herbariorum; http://sweetgum.nybg.org/science/ih/).

Lichen substances: The secondary chemical compounds were analysed by thin-layer chromatography (TLC) using the methods of Culberson and Kristinsson (1970), Culberson (1972), and later modifications. All three solvents (A, B' and C) were used.

Culturing: The photobiont of *Fuscidea kochiana* (Hepp) V. Wirth & Vězda, specimen M-0154470, was isolated using a single cell manipulator and cultured on mineral medium following the protocol described by Beck and Koop (2001).

DNA sequencing: For identification of the *Fuscidea* photobionts, two nuclear ribosomal genes, 18S (SSU) for nine specimens and ITS for 13 specimens, were sequenced. The thalli of esorediate specimens were thoroughly washed by deionized water to reduce contamination

from free-living algae, and selected under a Carl Zeiss microscope to minimize the superficial grime. Except for the *F. kochiana* M-0154470 from which the culture AB98.122B1 was prepared, DNA was extracted directly from small lichen thalli areoles, using the DNeasy Plant Mini Kit (Qiagen) following the manufacturer's instructions.

Amplification of 18S was performed according to Dal Grande et al. (2014), and ITS was amplified using the green-algal specific forward primer AL1500bf (Helms et al. 2001) or AL1648 (Vargas and Beck 2012) in combination with ITS4 (White et al. 1990).

The PCR mixture consisted of 1× GeneAmp® 10× PCR Buffer II (Applied Biosystems), 2.5 µM $MgCl_2$ (Applied Biosystems), 20 µM dNTPs (Promega), 0.6 µM of each primer, 0.036U AmpliTaq® DNA Polymerase (Applied Biosystems), 5.0 µl of genomic DNA extract and 9.85 µl distilled water to a total volume of 25 µl. An initial denaturation at 94°C for 5 min was followed by 40 cycles of denaturation at 94°C for 30s, annealing with a 63–58°C touchdown procedure decreasing 1° per cycle, ending at 57°C for 30s, and polymerisation at 72°C in 1 min 45s with a final elongation at 72°C for 10 min.

The PCR products were visualized on a 1% RedGel-stained agarose gel under UV light, and purified according to the manufacturer's instructions using Exo-Sap-IT (GE Heathcare). The amplification primers were used for direct sequencing in both directions, with BigDye Terminator Cycle Sequencing kit (Applied biosystems) and run on an ABI Prism 3700XL DNA analyzer (Applied Biosystems) at the DNA Sequencing Lab, University of Bergen, Norway and LMU Munich, Germany. The GenBank accession numbers on the newly generated sequences from the DNA vouchers as well as the *F. kochiana* AB130 culture are given in Table 1.

Alignment and phylogenetic analyses: The phylogenetic analyses were based on two nrDNA molecular markers: 18S and ITS, analysed separately due to the very limited number of taxa for which both markers were available at Genbank.

Identifying the *Fuscidea* photobionts and inferring their taxonomic positions in the systematics of green algae

The 18S matrix comprised 337 sequences, including nine new sequences (Table 1), in addition to 328 sequences from GenBank (Supplementary material Table S1). The matrix was aligned using the program Muscle (Edgar 2004a,b) implemented in the phylogenetic data editor PhyDE version 0.9971 (http://www.phyde.de/download.html), followed by manual adjustment and is available in Supplementary material (Appendix B). Missing positions at the ends were coded as missing data (?). Two sequences of the Prasinophyceae, *Nephroselmis olivacea* Stein (GenBank Acc. No: FN562436) and *N. pyriformis* (N. Carter) Ettl (GenBank Acc. No: KF615768), were chosen as outgroup.

The substitution model for 18S was identified by the software jModelTest version 2.1.7 (Posada 2008). The model with the lowest Akaike information criterion (AIC) was used in the analyses (Table 2).

The Bayesian B/MCMC analysis (BI) was run for 20 million generations, sampling every 100th, with four parallel chains starting from a random tree using the default temperature of 0.2 in MrBayes version 3.2.1 (Ronquist and Huelsenbeck 2003). The equilibrium was inspected in Tracer version 1.6.0 (Rambaut et al. 2014) and resulted in a 10% "burn-in". A Bayesian 50% majority-rule consensus tree with average branch lengths was constructed from the resulting 180,000 trees and visualized using the program Geneious version 8.1.8. (Biomatters Ltd.). Posterior probabilities ≥ 0.9 were considered to be significant.

The maximum likelihood analysis (ML) was carried out using RAxML version 8 (Stamatakis 2014) with 1,000 bootstrap replicates and 10 random additions.

Resolving the interspecific relationships between the *Fuscidea* photobionts

The ITS matrix included 75 sequences, where off 13 new (Table 1), combined with 62 from GenBank (Table S1). Of the matrix with 664 characters, 176 were constant and 448

parsimony-informative. *Coccomyxa simplex* Mainx (GenBank Acc. No: HG972980), *Coccomyxa* sp. Schmidle (GenBank Acc. No: HG972980), and *Pseudococcomyxa simplex* (Mainx) Fott (GenBank Acc. No: HE586504) were chosen as outgroup based on a MegaBLAST search. *Symbiochloris* Škaloud, Friedl, A. Beck & Dal Grande from GenBank was included, as it may represent a sister group based on Hallmann et al. (2013a,b; 2016) and Neustupa et al. (2013). *Trebouxia* (lichenized) sequences were included due to the highest scores in a MegaBLAST search.

Substitution models with lowest AIC for ITS1, 5.8S, and ITS2 were estimated separately using jModelTest version 2.1.7 (Posada 2008) and used in the analyses (Table 2).

ITS has some alignment challenges in the hypervariable parts, and thus several methods were tested to adjust for these poorly aligned parts. Firstly, the ITS sequences were aligned with the Muscle algorithm implemented in Geneious version 8.1.8. (Biomatters Ltd.) with 65% similarity option on (Gap penalty=14.5, Gap extension penalty=5), followed by manual adjustments (called the MA matrix, available in Appendix B). To optimize the manual adjustments, information from the secondary structure of the ITS2 operon was also included. Secondly, the program Gblocks version 0.91b (Talavera and Castresana 2007) was used on the MA matrix and run under two different modes; relaxed and stringent masking. Under relaxed masking, smaller final blocks, gaps positions and less strict flanking positions were allowed resulting in a matrix called the R matrix, while under the stringent masking, ambiguous positions and gaps were not allowed resulting in a matrix called the S matrix (see Table 3). Missing positions at the ends were coded as missing data (?) in the MA and R matrices.

The partitioned MA and R matrices as well as the non-partitioned S matrix were analysed with the Bayesian B/MCMC approach for 15 million generations with four parallel chains, starting from a random tree, and using the default temperature of 0.2. Every 100th tree,

including branch lengths, was sampled. After inspection in Tracer version 1.6.0 (Rambaut et al. 2014), the first one and a half million generations were discarded as "burn-in". A Bayesian 50% majority-rule consensus tree with average branch lengths was constructed from the 135,000 resulting trees and visualized using Geneious version 8.1.8. (Biomatters Ltd.). Posterior probabilities ≥ 0.9 were considered to be significant.

PAUP*4.0b10 (Swofford 2002) was used to search for MP and ML trees for the MA, R and S matrices, not partitioned. The MP trees were found under a heuristic search using random sequence additions with 1,000 replicates and tree bisection-reconnection branch swapping (TBR). The MulTrees and steepest descent options were on, but the collapse zero-length branches option was off. A second heuristic search with 100 replicates, using the ML criterion with the empirical base frequencies as previously calculated in jModelTest (Posada 2008), was carried out to calculate the ML trees of the three ITS. Branch support for each ML tree was estimated by 100 bootstrap replicates with 10 random additions. High bootstrap support was considered to be equal or above 80%.

In addition, the non-aligned data matrix of ITS was analysed in SATé-II version 2.2.7 (Liu et al. 2012). This program automatically divides the matrix into subsets, which are subsequently aligned by MAFFT (Katoh et al. 2005; Katoh and Toh 2008) and merged using Muscle (Edgar 2004a,b) in order to produce a new alignment. The default settings were chosen, except for the iteration limit that was set to 50. This resulting alignment was analysed under the GTR CAT and the GTR+I+G evolution models using RAxML.

The secondary structures of the ITS2 sequences were reconstructed using RNAfold (http://rna.tbi.univie.ac.at) with folding temperature set to 25°C. Compensatory base pair changes (CBCs) are nucleotide changes at both sides of paired bases, while hemi-CBCs (e.g. G–U → G–C) have changes at only one side of nucleotide pairs that retain the base

pairing (e.g. Caisová et al. 2011). CBCs and hemi-CBCs were located according to Coleman (2000, 2003), the highly conserved regions were determined following Coleman (2007).

Results

Apatococcus is the *Fuscidea* photobiont and classified within the Trebouxiophyceae

The final 18S matrix consisted of 962 charaters of which 550 sites were constant and 284 parsimony-informative. The resulting 50% majority-rule consensus tree with posterior probabilities from the BI analysis (−ln = 10,686.02) is displayed in Fig. 1, combined with bootstrap supports retrieved from the ML analysis. The average standard deviation of split frequencies fell below 0.0063, which indicated convergence of the Markov chain when comparing two independent runs. The likelihood parameters of the MCMC analysis are listed in Supplementary material (Table S2).

Even though most of the deep relationships among major groups in the Trebouxiophyceae were poorly supported, the supported clades were in agreement with Dal Grande et al. (2014), Hallmann et al. (2016), Sanders et al. (2016), and Škaloud et al. (2016), which also lacked significant support at basal nodes. The incongruencies between BI and ML tree are indicated by asterisks in Fig. 1.

Within the Trebouxiophyceae, one distinct clade called the *Apatococcus*-clade with PP = 1.0/BS = 100% support included the *Fuscidea* photobionts in addition to 23 *Apatococcus* and two *Eukaryote* sequences, supporting the identity of the photobionts in question as members of *Apatococcus*. The *Apatococcus*-clade can be grouped into four supported clades based on the 18S data (see Fig. 1) and the *Fuscidea* photobiont appeared in two distinct groups. *Myrmecia* Printz and *Trebouxia* formed clades near *Apatococcus*, but lacking support.

One specimen of *A. lobatus*, strain CCALA 213 (GenBank Acc. No.: KF355939), appeared in the *Chlorella*-clade 2 (see discussion).

Resolving the interspecific relationships between the *Fuscidea* photobionts

The BI and ML resulting trees were examined for each ITS matrix separately and then compared. A Bayesian 50% majority-rule consensus tree conducted from the MA alignment with depicted PP and BS values from all phylogenetic analyses is given in Fig. 2.

The calculated parameters of all the BI approaches are given in Table S2 and the resulting tree statistics of ITS data matrices from the MP analysis are summarized in Table S3. In general, the tree resulting from the R matrix had higher PP and BS values than the others.

The resulting tree (Fig. 2) showed that *Apatococcus*, *Symbiochloris* and *Trebouxia* form three well-supported clades. The relationships between these groups remained unresolved.

The *Apatococcus*-clade, accommodating all lichenized as well as free-living *Apatococcus* specimens, was divided into six supported clades, the five clades reported by Hallmann et al. (2016) and a new clade. We entitled the supported clades alphabetically after Hallmann et al. (2016), but renamed their group B with group A (identified by the strain SAG 2145) and vice versa. This strain was isolated and identified as *A. lobatus* already by W. Vischer (Vischer, 1960) who also amended its description, and thus is a very suitable authentic culture of *Apatococcus lobatus*. The *Fuscidea* photobionts were included in two separate clades; F and E. In clade F with PP = 1.0/BS = 100%, most of the *Fuscidea* photobionts were included, while the photobiont of *F. lightfootii* (Sm.) Coppins & P. James was nested with free-living *Apatococcu*s specimens in clade E with 1.0/88% support.

The resulting phylogenetic trees from SATé-II revealed the same topology, i.e. six distinct groups.

The secondary structures of ITS2 operons were folded for all retrieved ITS groups (see Suppl. Fig S2), and the conserved features among Eukaryota and green algae (Coleman 2003, 2009;

Mai and Coleman 1997) were identified, i.e. four helices, a pyrimidine-pyrimidine mismatch near the base of helix II, AAA mononucleotide repeats between helices II and III, and a conserved section of the nucleotides GUUU and its modifications on the 5´side of helix III. Since the ITS2 secondary structures were very similar, the apical parts were also analysed. In total, 8 CBCs and 11 hemi-CBCs on the conservative part on helix III, and 1 hemi-CBCs on the conservative part on helix II were distinguished using group F, as the reference secondary structure for mapping the differences to the other five *Apatococcus* groups (Fig. 3 and Table 4). Both the CBCs and the hemi-CBCs observed on helix IV in group A, C, D, and E were not included, as these helices were not recognized by RNAfold (available at http://rna.tbi.univie.ac.at), but folded manually.

Taxonomy

***Apatococcus fuscideae* A. Beck & Zahradn. sp. nov. (Fig. 4)**

Description: Cells round to oval 9–30 × 9–15 µm in size. Cells duplicate by binary fission creating typical clusters of 2, 4 or 8 daughter cells, often flattened on one side, forming cubical packages. Chloroplast parietal, without distinctive pyrenoid, as typical for the genus *Apatococcus*. Cell wall up to 1 µm thick. No zoospores observed. Differing from *A. lobatus* by the typical reticulate, netlike chloroplast in mature cells, see Fig. 4.

Diagnostic ribosomal DNA sequences of strain AB98.122B1: 18S: GenBank Acc. No. KY587795; ITS: GenBank Acc. No. KY587804; for ITS2 secondary structure see Fig. 3 and in Suppl. Fig. S2 F.

Holotype: Permanently preserved strain SAG 2523.

Authentic strain: AB98.122B1 = Sammlung für Algenkulturen Göttingen (SAG) 2523.

Etymology: The specific epithet (*fuscideae*) refers to the lichen genus *Fuscidea*.

Type locality: Austria: Styria, 40 km SW of Graz; Koralpe Mountain range, Handalpe. 1730 m alt. 46°50'N 15°01'E. On a vertical, south-facing rock, about 1.5 m above the ground. Collected on 13.08.1998 by A. Beck, no. 130; M-0154470.

Host: *Fuscidea kochiana* (Hepp) V. Wirth & Vězda is a crustose lichen with grey to brown tinged, deeply cracked thallus. Apothecia are brown, lecideine, immersed having asci of the *Fuscidea*-type. Ascospores broadly ellipsoid, becoming brown when mature. Chemical constituent is divaricatic acid. It occurs on acid rock and is widely distributed (Gilbert et al. 2009).

Discussion

Apatococcus is the *Fuscidea* photobiont and placed within the Trebouxiophyceae

The *Fuscidea* photobionts are identified as *Apatococcus*, the most prominent epiphytic algae in temperate regions (Gärtner and Ingolic 1989). Except for the report by Watanabe et al. (1997), which is based on morphological methods only, *A. lobatus* has not been reported as photobiont in any lichen genus.

Apatococcus belongs to the Trebouxiophyceae with uncertain position. It might be closely related to *Symbiochloris* (Hallmann et al. 2013a,b; 2016; Neustupa et al. 2013) or to *Leptosira* Borzì (Škaloud et al. 2016), whereas in the present study, *Myrmecia bisecta* and/or the Trebouxiaceae are related to this genus. Yet, the exact phylogenetic position of *Apatococcus* remains uncertain.

The sequence of *A. lobatus* CCALA 213 (KF355939), nested in the *Chlorella*-clade 2, probably indicates a permutation of strains, because microscopic analysis of the strain (see photo at: http://ccala.butbn.cas.cz/en/apatococcus-lobatus-chodat-jb-petersen) is consistent with an identification as *Chlorella s.l.* Consequently, CCALA 213 should not be treated as *A. lobatus*.

To conclude, the genus *Apatococcus* is clearly a member of the Trebouxiophyceae, but its further classification remains unresolved.

Interspecific relationships between lichenized *Apatococcus*

Two different lichenized *Apatococcus* groups were found; group F with the sequences of *A. fuscideae* and group E with the photobiont of *F. lightfootii* with free-living *Apatococcus*. Their relationships differ between the genes, i.e. in the 18S phylogeny group F appears as sister to group A and group E as sister to group D, while in the ITS phylogenies group F is the first diverging lineage and group E is alike closely related to group D.

One can argue that the observed internal groups within *Apatococcus* in fact represent several distinct species, i.e. one already known, *A. lobatus* (group A), the one described here, *A. fuscideae* (group F), the photobiont in *F. lightfootii* together with free-living *Apatococcus* (group E), and three further free-living species (group B, C, D). Nevertheless, the hypothesis about the putative free-living species could not be verified, because this is outside the scope of this paper.

***Apatococcus fuscideae* (group F)**

The clade with the lichenized *A. fuscideae* receives significant support in all phylogenies. To facilitate further work we generated a reference culture from *F. kochiana* clone AB98.122B1 and deposited it in the Sammlung für Algenkulturen Göttingen as SAG 2523.

The species, *A. fuscideae* is not only delimited by molecular findings, but is also accompanied by morphological characters in its chloroplast, which is typical reticulate, thus netlike, in mature cells, while *A. lobatus* has uninucleate cells with single, often lobed, parietal chloroplast without pyrenoids (Brand and Stockmayer 1925; Ettl and Gärtner 2014). When using the secondary structure of the ITS2 operon (see Figs. 3 and S2 A, F), *A. lobatus* has three CBCs and one hemi-CBCs on helix I, one hemi-CBCs on helix II, four CBCs and six

hemi-CBCs on helix III (i.e. three CBCs and five hemi-CBCs on the conserved part of helix III) that are distinct from *A. fuscideae* (see Figs. 3 and S2 A, F).

Low genetic variation is observed between the photobiont sequences of group F (18S: 99.9% and ITS 99.5% pairwise identity). The uniformity of photobionts in *Fuscidea* distributed in distant localities may be due to the high mycobiont selectivity (e.g. Beck et al. 2002) to preserve their tight and ecological successful associations (e.g. Muggia et al. 2013). The reasons why algal partners are less variable in comparison with their compatible fungal partners could be also explained by the genotypic fixation in the algae caused by different evolutionary processes, e.g. a higher mutation rate in the mycobiont, or a longer time of divergence of the photobionts (Nuismer et al. 1999; Piercey-Normore and DePriest 2001).

***Apatococcus* in *F. lightfootii* (group E)**

The clade contains both free-living and lichenized *Apatococcus* associated with *F. lightfootii*, which is phylogenetically distinct from *Apatococcus fuscideae*. When using secondary structure of ITS2, the photobiont of *F. lightfootii* differs from *A. fuscideae* in having two CBCs on helix I, seven CBCs and three hemi-CBCs on helix III from which four CBCs and three hemi-CBCs on the conserved part of helix III (see Figs. 3 and S2 E, F).

When Hallmann et al. (2016) studied free-living *Apatococcus* from suburban surfaces, they recovered several fungal clones that might represent lichenized fungi. The free-living *Apatococcus* associated with *F. lightfootii* could therefore be lichenized. In order to describe this species in detail, its culture and a detailed study are needed.

Comparison of the individual ITS analyses

Due to variable ITS sequences, it was difficult to use them for determining phylogenies within Trebouxiophyceae. As no previous studies have targeted the issue about alignment depended and alignment independent analysis of ITS sequences among non-congeneric species within Trebouxiophyceae, we compare the resulting ML phylogenetic trees calculated from four individual matrices containg different amount of ambiguous sites, and thus investigate the errors of these matrices. Interestingly, all retrieved topologies show only minor differences, i.e. they are almost identical. Even in the R matrix, restricted to the conserved alignment parts with highest homology assessment, all the deep and most of the recent nodes are recovered, but their support are weak probably due to the low number of informative characters. The aligning of the variable ITS is difficult and time consuming especially when taking homology assessment by secondary structure analysis into account. In the phylogeny of the S matrix, group A and B are intermixed as the sequence of uncultured *Apatococcus* (GenBank Acc. No.: KX25118) appeared in group B. Thus the resolution of groups is slightly reduced in the most stringent matrix.

The alignment independent approach by SATé-II provides faster and obviously reliable results indicated by similar phylogenies as compared to the alignment based approach. In the present study, the ML tree calculated from SATé-II resolves six groups in the *Apatococcus*-clade and two main clusters in the *Trebouxia*-clade. The sequence of uncultured *Apatococcus* (GenBank Acc. No. KX025118) is nested in group B, and group C is the first diverging lineage within the *Apatococcus*-clade.

Conclusion

Based on our data presented here, *Apatococcus* is unequivocally identified as a lichenized alga of the lichen genus *Fuscidea* and placed within the Trebouxiophyceae, albeit with uncertain

position. All retrieved groups in *Apatococcus* are unique in ITS sequence and CBCs are observed in the secondary structure of ITS2. In the present study, two lichenized *Apatococcus* species are found, of which are found, of which *Apatococcus fuscideae* is described as new based on molecular and morphological characters.

Appendix A. Supplementary Data

Appendix B. Alignments of 18S and ITS manually adjusted (not included here)

Supplementary data associated may be found in the online version of this study at http://.....

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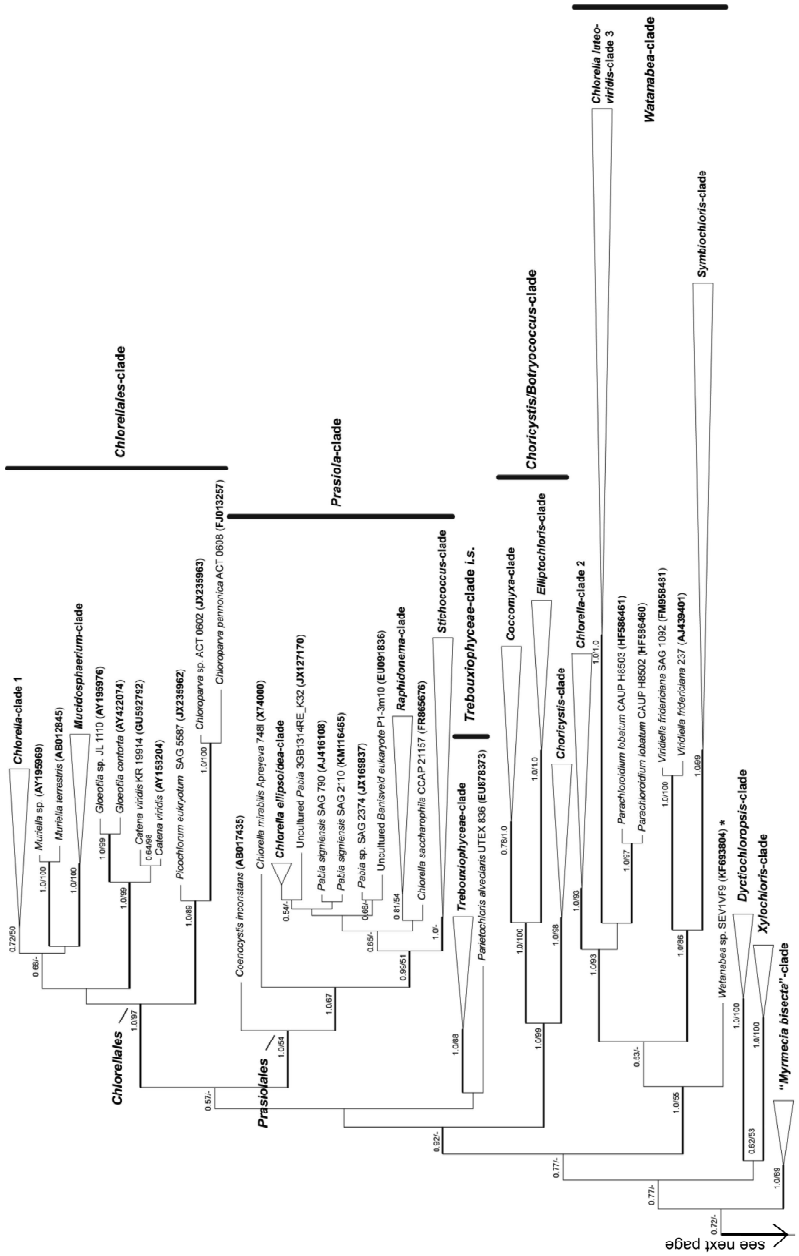

Chlorella-clade 1
Mucidosphaerium-clade
Chlorellales-clade
Prasiola-clade
Choricystis/Botryococcus-clade
Watanabea-clade
Chlorella lobophora-clade 3
Symbiochloris-clade
Chlorella-clade 2
Ellipsochloris-clade
Choricystis-clade
Coccomyxa-clade
Trebouxiophyceae-clade i.s.
Stichococcus-clade
Raphidonema-clade
Chlorella saccharophila CCAP 211/7 (FR865676)
Uncultured Bacillariaved eukaryote P1-3m10 (EU091836)
Pabia sp. SAG 2374 (JX165837)
Pabia asymmetrica SAG 2110 (KM116455)
Pabia asymmetrica SAG 780 (AJ416108)
Uncultured Pabia 3GB131-RE_K32 (JX127170)
Chlorella ellipsoidea-clade
Chlorella mirabilis Apreleva 7488 (X74080)
Coenocystis inconstans (AB917435)
Chloropanna sp. ACT 0602 (JX235963)
Chloropanna pennoidea ACT 0608 (FJ013257)
Ploochloron eukaryotum SAG 5587 (JX235962)
Catena viridis KR 19914 (GU592782)
Catena viridis (AY153204)
Gloeotila contorta (AY422074)
Gloeotila sp. JL 1110 (AY195976)
Mucidosphaerium eremitum (AB012645)
Mucidella sp. (AY195969)
Chlorella-clade 1
Chlorellales
Prasiolales
Paradoxichloris africana UTEX 836 (EU678373)
Trebouxiophyceae-clade i.s.
Coccomyxa-clade
Choricystis/Botryococcus-clade
Ellipsochloris-clade
Choricystis-clade
Chlorella-clade 2
Parachloroidium lobatum CAUP H6503 (HF586461)
Parachloroidium lobatum CAUP H8502 (HF586460)
Viridiella fridericiana SAG 1092 (FM958431)
Viridiella fridericiana 237 (AJ439401)
Chlorella lobophora-clade 3
Watanabea-clade
Symbiochloris-clade
Watanabea sp. SEV1VF9 (KF693804) *
Dyctiochloropsis-clade
Xylochloris-clade
"Myrmecia bisecta"-clade
see next page

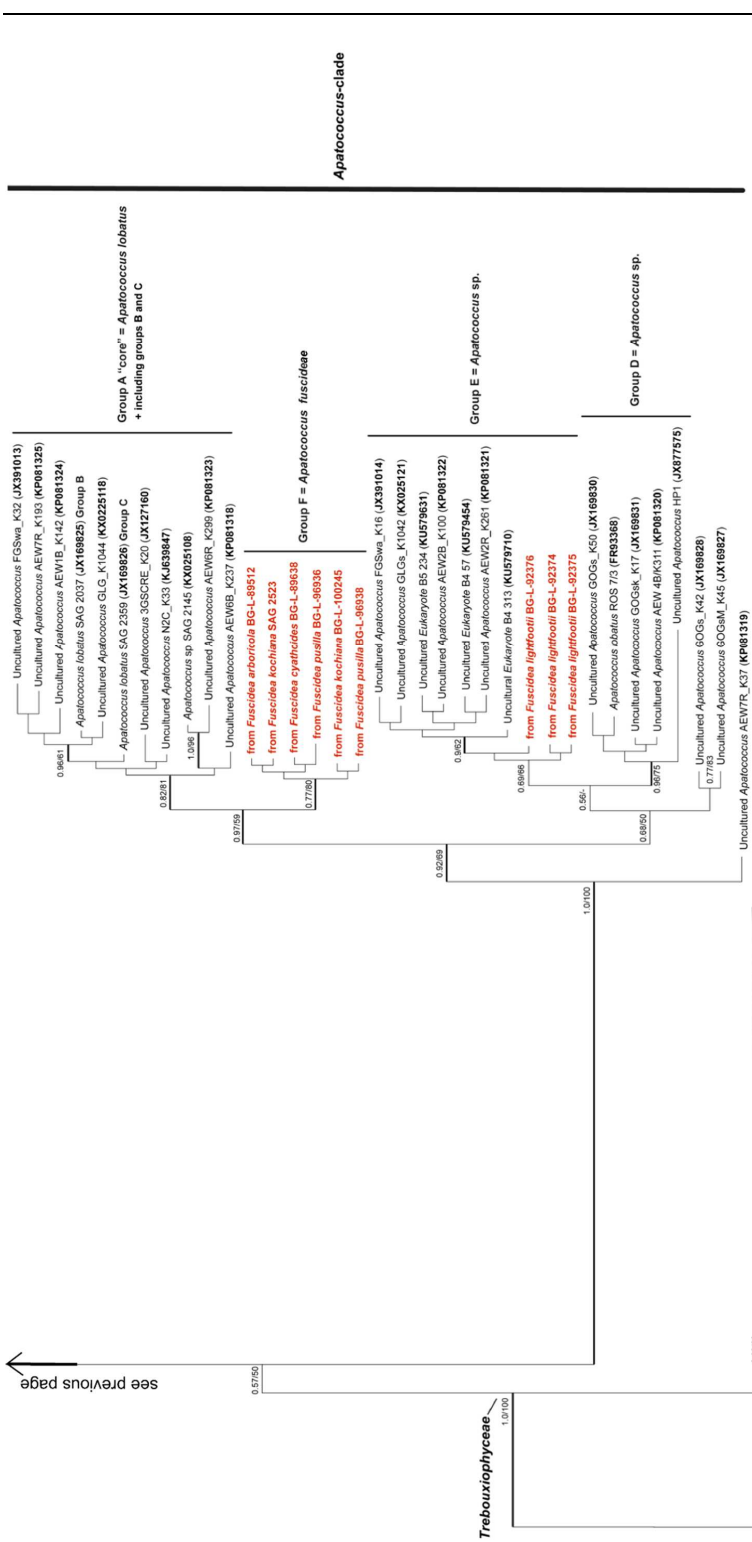
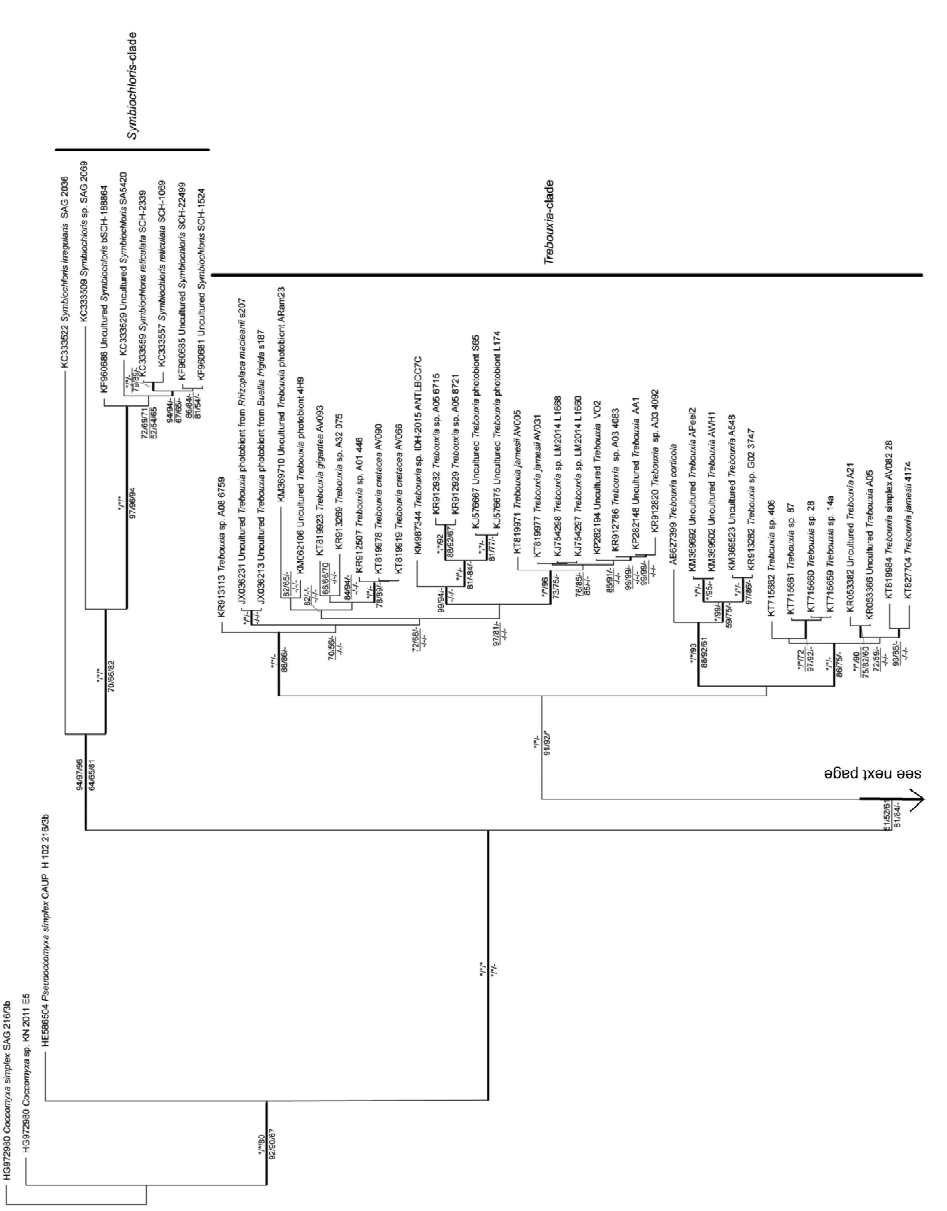


Figure 1. Phylogeny of the genus *Apatococcus* based on 18S shown as the 50% majority-rule consensus tree obtained from MrBayes (–lnL = 10,686.02). Support values are indicated above branches: posterior probabilities (PP) and below RaxML bootstrap values (BS). An asterisk indicates the incongruences between the BI and ML tree. Only values higher than 50% are shown.


Symbiochloris-clade
Trebouxia-clade
see next page

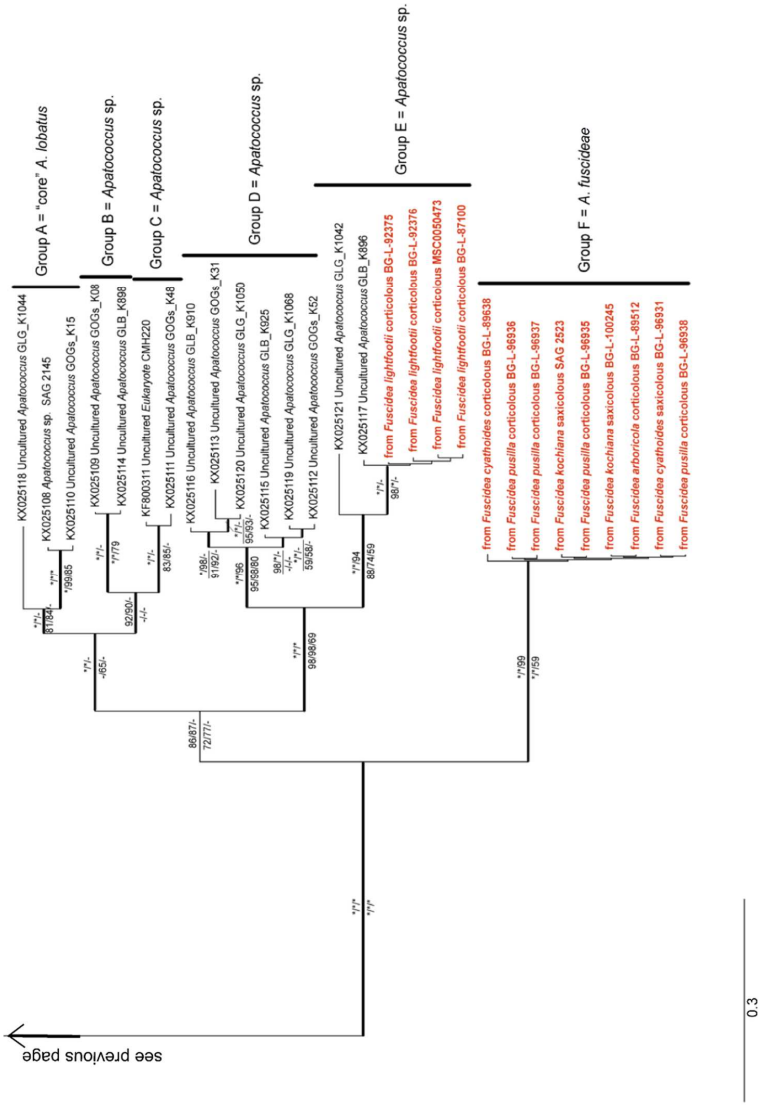


Figure 2. Phylogenetic relationships between members of *Apatococcus* and its closely related genera shown on the 50% majority-rule consensus tree (–lnL = 9,684.21) obtained with a Bayesian approach of ITS partitions aligned by Geneious. The values from the relaxed and stringent masking alignments are depicted here. Posterior probabilities (PP) are reported above branches and bootstrap values (BS) below in this order: aligned by Manually adjusted/Relaxed masking/Stringent masking. Robust support values (i.e. 1.0 of PP and/or 100% of BS) are indicated by asterisk on corresponding branches. Values lower than 50% are not shown.

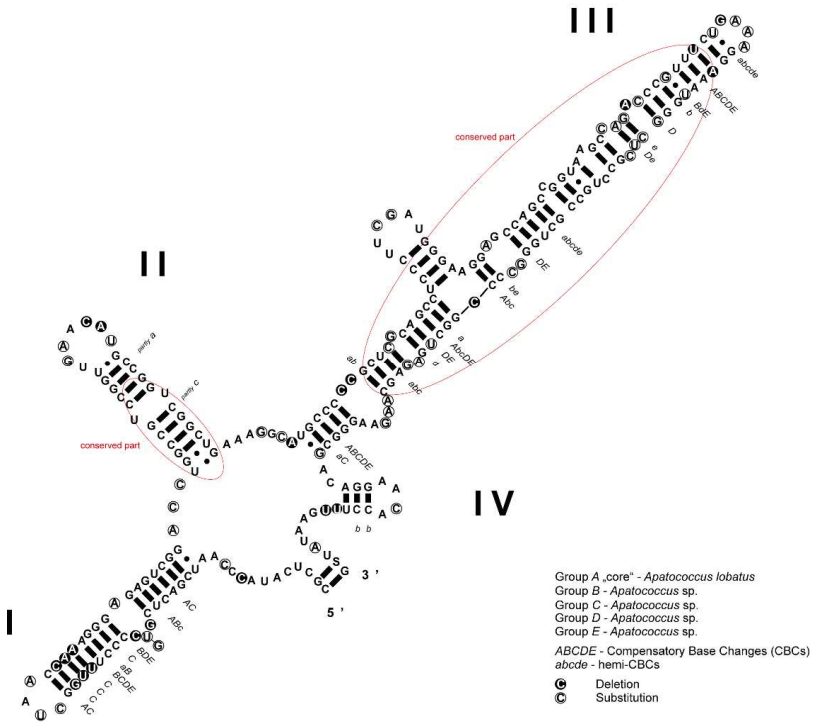


Figure 3. The secondary structure of ITS2 operon based on *Apatococcus fuscideae* A.Beck & Zahradn. Capitals indicate Compensatory base changes (CBCs), small letters hemi-CBCs when compared to the other five *Apatococcus* groups.

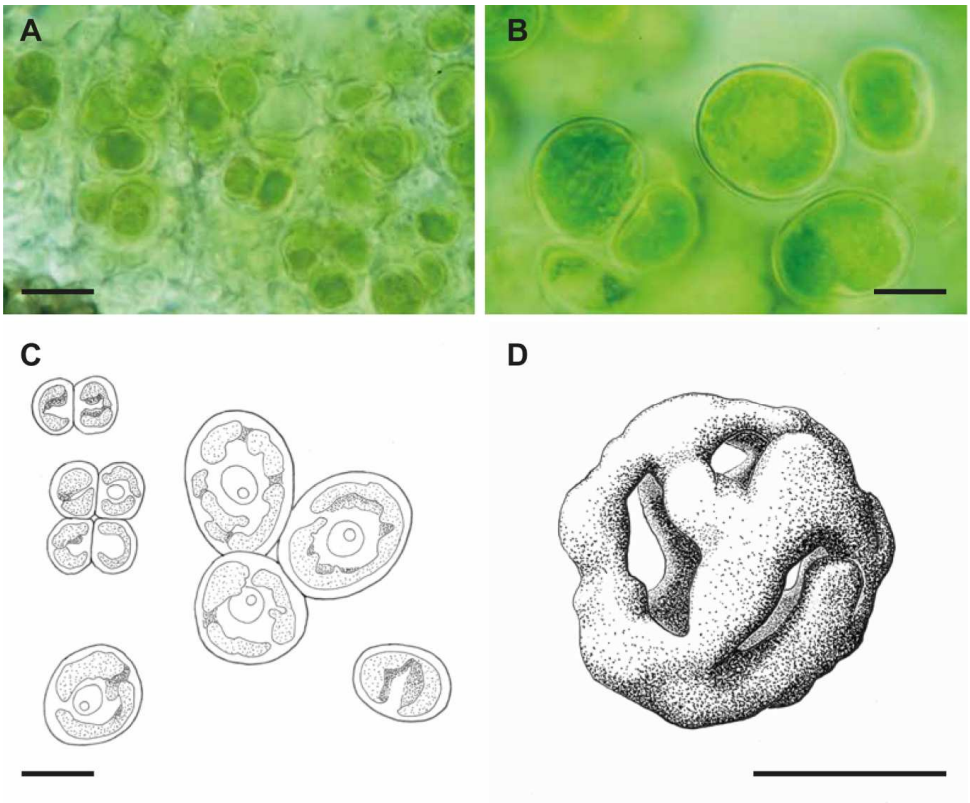


Figure 4. Light micrograph (A, B) and schematic drawings (C, D) of *Apatococcus fuscideae* A.Beck & Zahradn. (isolated from *Fuscidea kochiana* [AB130; M-0154470]). Scale bar: 10 µm. (A) Lichenized state. (B) Axenic culture: Optical median section (cell in the center) and surface view (cell on the left side). (C) Diads, Tetrads, and cells with reticulate chloroplast. (D) Magnified reticulate chloroplast.

Table 1. List of voucher specimens with their GenBank accession numbers.

Species	Authors	Locality	Substrate	Collection Date	DNA Vouchers	Herbarium Number	GenBank Accesion Number	
							18S	ITS
Fuscidea arboricola	Coppins & Tønsberg	Norway: Nordland	*Juniperus communis*	3.iix.2010	MZ11_230211	BG-L-89512	KY587790	KY587799
Fuscidea cyathoides	(Ach.) V. Wirth & Vězda	Norway: Rogaland	*Betula pubescens*	25.iix.2010	MZ29_070611	BG-L-89638	KY587791	KY587800
Fuscidea cyathoides	(Ach.) V. Wirth & Vězda	Norway: Hordaland	siliceous rock	15.xi.2010	MZ33_070611	BG-L-96931	–	KY587803
Fuscidea kochiana	(Hepp) V. Wirth & Vězda	Norway: Hordaland	siliceous rock wall	15.xi.2010	MZ32_070611	BG-L-100245	KY587792	KY587801
Fuscidea kochiana	(Hepp) V. Wirth & Vězda	Austria: Styria	rock	13.xiii.1998	– [a]	M-0154470	KY587795	KY587804
Fuscidea lightfootii	(Sm.) Coppins & P. James	Norway: Rogaland	*Alnus glutinosa*	12.ii.2011	MZ45_190112	BG-L-92374	KY587797	–
Fuscidea lightfootii	(Sm.) Coppins & P. James	Norway: Rogaland	*Betula* sp.	12.ii.2011	MZ46_190112	BG-L-92376	KY587796	KY587809
Fuscidea lightfootii	(Sm.) Coppins & P. James	Norway: Rogaland	*Betula* sp.	12.ii.2011	MZ47_190112	BG-L-92375	KY587798	KY587808
Fuscidea lightfootii	(Sm.) Coppins & P. James	Scotland: Mid-Lothian	*Salix* sp.	30.x. 2010	MZ31_070611	MSC0050473	–	KY587810
Fuscidea lightfootii	(Sm.) Coppins & P. James	Norway: Rogaland	*Salix caprea*	7.iii.2009	MZ38_020811	BG-L-87100	–	KY587811
Fuscidea pusilla	Tønsberg	Norway: Hedmark	*Betula* sp.	4.vi.2011	MZ39_020811	BG-L-96937	–	KY587806
Fuscidea pusilla	Tønsberg	Norway: Hedmark	*Betula* sp.	4.vi.2011	MZ40_020811	BG-L-96936	KY587793	KY587805
Fuscidea pusilla	Tønsberg	Norway: Hedmark	*Betula* sp.	4.vi.2011	MZ41_020811	BG-L-96935	–	KY587802
Fuscidea pusilla	Tønsberg	Norway: Hedmark	*Betula* sp.	4.vi.2011	MZ42_020811	BG-L-96938	KY587794	KY587807

[a] isolated photobiont culture only

Table 2. Best-fit models for each data matrix of 18S and partitioned ITS calculated in jModelTest. Three different approaches were applied in the alignment of ITS: Geneious, Relaxed and Stringent Gblocks masking.

Locus nrDNA	**Data matrix**	**No. of characters**	**Best fit model**	**Calculated -lnL**
18S	*Manually adjusted*	962	GTR+I+G	10,099.64
ITS1	*Manually adjusted*	267	GTR+I+G	5,731.89
	Relaxed masking	250	GTR+I+G	4,606.69
5.8 S	*Manually adjusted*	159	SYM+I	606.3083
	Relaxed masking	158	K80+G	570.4275
ITS2	*Manually adjusted*	238	GTR+G	3,983.89
	Relaxed masking	213	GTR+G	3,230.98
ITS	*Manually adjusted*	664	GTR+I+G	10,710.00
	Stringent masking	199	GTR+I+G	859.7838

Table 3. Calculated parameters of Gblocks masking. Asterisk indicates the percentage of selected base pairs by Gblocks masking from the manually adjusted data matrix.

Locus	**ITS1**		**5.8 S**		**ITS2**	
Masking	*Relaxed*	*Stringent*	*Relaxed*	*Stringent*	*Relaxed*	*Stringent*
Min. no. seq. for conserved positions	39	39	39	39	39	39
Min. no. seq. for flank positions	39	65	39	65	39	65
Max. no. seq. for nonconserved positions	8	4	8	4	8	4
Min. length of block	5	10	5	10	5	10
Alowed gap positions	with Half	none	with Half	none	with Half	none
Gblocks alignment	250 (93%*)	0 (0%*)	157 (100%*)	155 (98%*)	221 (92%*)	41 (17%*)

Table 4. Overview of CBCs and hemi-CBCs on the conservative parts of helix II (in brackets) and III within the six ITS groups of *Apatococcus*. Grey shade divides CBCs and hemi-CBCs.

		hemi-CBCs					
		Group A	Group B	Group C	Group D	Group E	Group F
CBCs	Group A		5	5 (1)	4	4	5
	Group B	x		1 (2)	6	6	6
	Group C	x	1		5 (1)	3 (1)	4 (1)
	Group D	2	4	3		2	2
	Group E	2	5	3	5		3
	Group F	3	2	1	6	4	

Appendix A: Supplementary material

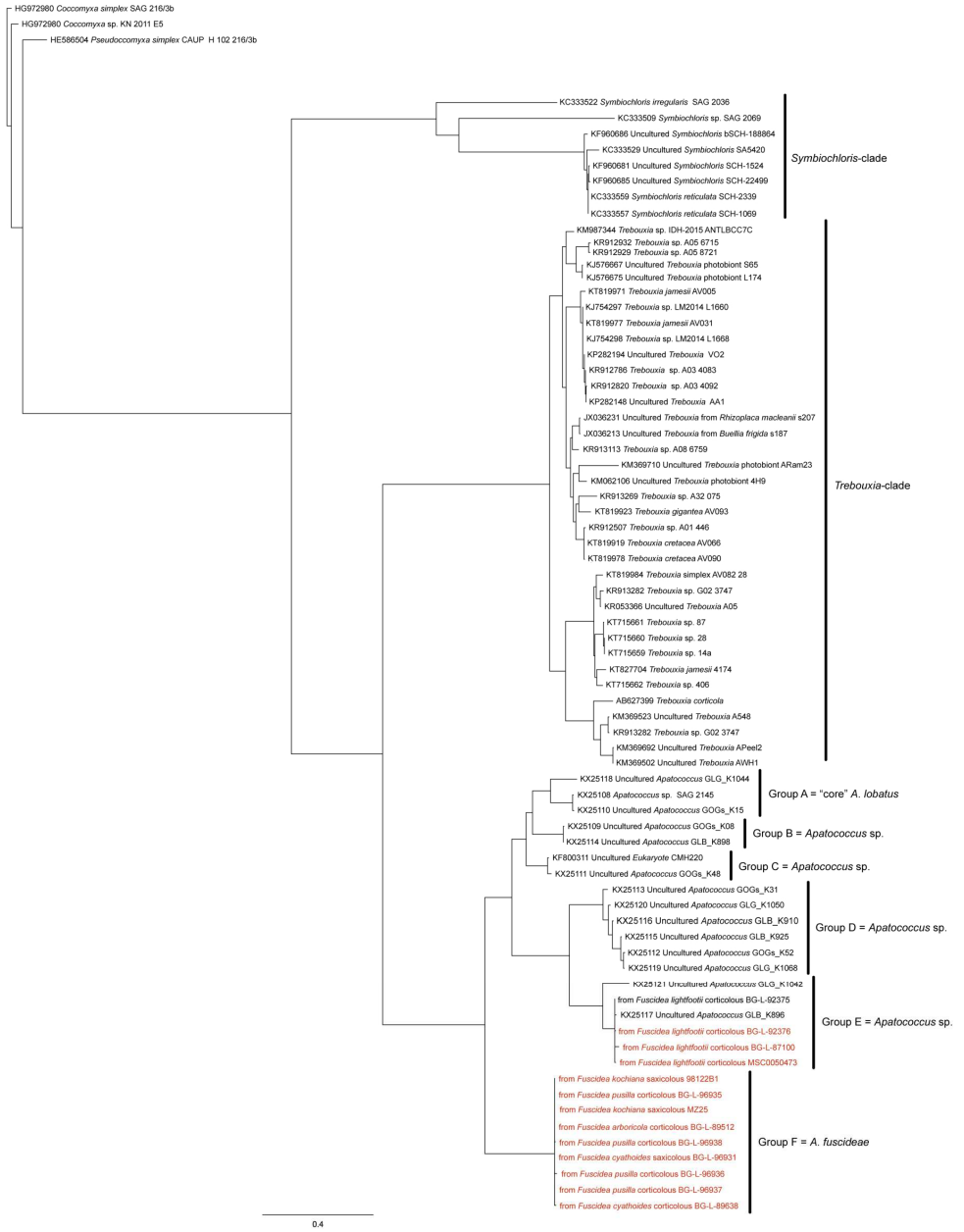


Figure S1. Phylogenetic relationships between members of *Apatococcus* and its closely related genera based on the most likely tree (–lnL = 9,753.9922) obtained with a SATé-II tree estimation under the GTR+I+G substitution model.

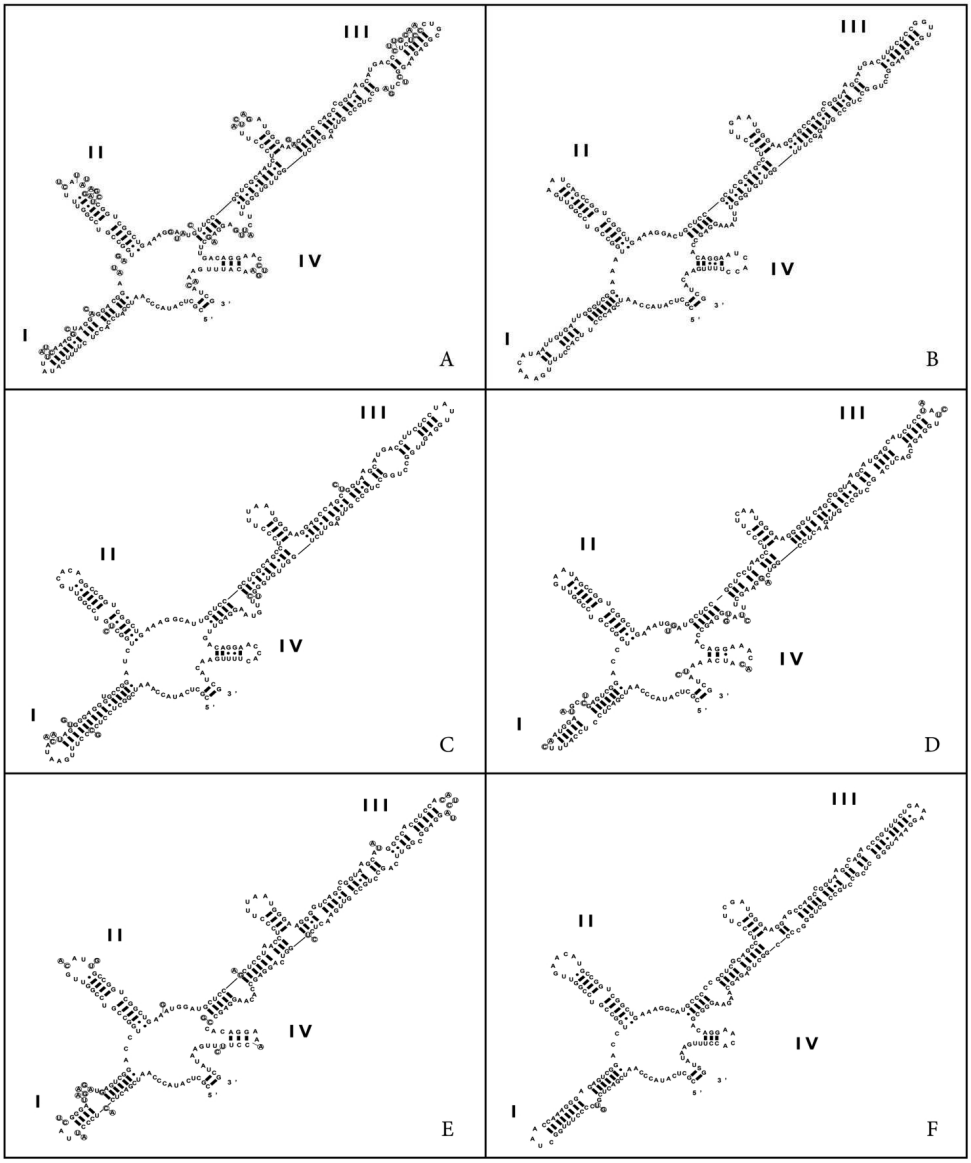


Figure S2. The overview of the secondary structures based on ITS2 operon of six ITS internal groups. The reference sequence of each ITS group was generated from or chosen from available sequences in GenBank: Group A – *Apatococcus lobatus* (GAN: KY587804), B – Uncultured *Apatococcus* clone GLB_K898 (GAN: KX0255114), C – Uncultured *Apatococcus* clone GOGs_K48 (GAN: KX025111), D – Uncultured *Apatococcus* clone GLB_K910 (GAN: KX025116), E – *Fuscidea lightfootii* MSC0050473 (GAN: KY587810), and F – *Fuscidea kochiana* AB130 (GAN: KY587801). The grey colour indicates the missing part of the reference sequence, which was substituted by another sequence from the same group.

Table S1. List of used taxa with their GenBank accession numbers. ITS groups are indicated for the *Apatococcus* sequences generated by Hallmann et al. (2016).

Species Name	GenBank Accession Number		ITS group
	18S	ITS	
Chlorella luteoviridis	AB006045	–	
Chlorella agustoellipsoidea	AB006047	–	–
Chlorella trebouxioides	AB006048	–	–
Pseudochlorella sp. CCAP 264-2	AB006049	–	–
Pseudochlorella subsphaerica	AB006050	–	–
Muriella terrestris	AB012845	–	–
Coenocystis inconstans	AB017435	–	–
Stichococcus bacillaris D10-1	AB055865	–	–
Stichococcus bacillaris K4-4	AB055866	–	–
"*Chlorella*" *saccharophila* MBIC10067	AB058306	–	–
Uncultured *Chlorophyta* DA-09	AB257664	–	–
Uncultured *Chlorella* 1660/10	AB260895	–	–
Uncultured *Trebouxiophyceae*	AB260896	–	–
Pseudochlorella sp. (*Chlorella ellipsoidea*) CCAP 211/1ANIES-2150	AB488583	–	–
Choricystis sp. NIES-2342	AB488587	–	–
Chlorella saccharophila NIES-640	AB488790	–	–
Chlorella saccharophila NIES-2352	AB488791	–	–
Parachlorella beijerinckii	AB517729	–	–
Trebouxia corticola	–	AB627399	–
Uncultured freshwater *eukaryote* RW5_2010	AB721029	–	–
Coccomyxa sp. KGU-D001	AB742451	–	–
Prasiola fluviatilis	AF189072	–	–
Prasiola mexicana Mex 12	AF189075	–	–
Prasiola mexicana CR24	AF189076	–	–
Raphidonema nivale	AF448477	–	–
Stichococcus sp. B2VFF10	AF513370	–	–
Coccomyxa sp. SAG 2325	AJ302939	–	–
Raphidonema nivale CCAP 470/4	AJ306532	–	–
Raphidonema sempervirens CCAP 470/6	AJ309939	–	–
Stichococcus mirabilis CCAP 379/3	AJ311638	–	–
Raphidonema pyrenoidifera CCAP 470/5	AJ311640	–	–
Prasiola crispa SAG 43.96	AJ416106	–	–
Stichococcus bacillaris SAG 397-1b	AJ416107	–	–
Pabia signensis SAG 7.90	AJ416108	–	–
Raphidonemopsis sessilis UTEX 1711	AJ431667	–	–
Elliptochloris bilobata SAG 245.80	AM422984	–	–
Pseudochlorella pyrenoidosa SAG 18.95	AM422985	–	–
Coccomyxa sp. CPCC 508	AM981206	–	–
Catena viridis	AY158204	–	–
Muriella sp. AS 2-4	AY195969	–	–
Choricystis sp. AS 5-1	AY195970	–	–
Choricystis sp. AS-29	AY195972	–	–
Meyerella planktonica Itas 2/24 S-12w	AY195973	–	–

Gloeotila sp. JL11-10	AY195976	–	–
Chlorella sp. Mary 9/21BT-10w	AY197620	–	–
Choricystis sp. MDL1/12-8	AY197623	–	–
Chlorella sp. NDem 9/21T-13d	AY197628	–	–
Choricystis sp. Pic8/18P-11w	AY197629	–	–
Chlorella sp. MDL5-18	AY197632	–	–
Diacanthos belenophorus	AY323837	–	–
Stichococcus sp. BCP-SRS2-14	AY377441	–	–
Gloeotila contorta	AY422074	–	–
Choricystis sp. Itas 9/21S-1w	AY543052	–	–
Prasiolopsis ramosa SAG 26.83	AY762600	–	–
Myrmecia incisa SAG 2007	AY762602	–	–
Trebouxiophyte sp. UR47/4	AY762604	–	–
Choricystis minor SAG 17.98	AY762605	–	–
Trebouxiophyte sp. UR55/3	AY762606	–	–
"*Chlorella*" ellipsoidea	D13324	–	–
Stichococcus deasonii UTEX 1706	DQ275460	–	–
Stichococcus jenerensis D 4	DQ275461	–	–
Uncultured *marine eukaryote* FV23_CilD6	DQ310282	–	–
Elliptochloris bilobata SAG 245.80	DQ530055	–	–
Uncultured *Chlorodendraceae* Amb_18S_460	EF023169	–	–
Salicaceae Amb_18S_571	EF023266	–	–
Phytolaccaceae Amb_18S_747	EF023408	–	–
Rosenvingiella radicans Rrad4	EF200518	–	–
Rosenvingiella polyrhiza Rpol2	EF200519	–	–
Prasiola calophylla Pcal1	EF200521	–	–
Prasiola sp. GALW015488	EF200522	–	–
Rosenvingiella sp. GALW014367	EF200523	–	–
Prasiola stipitata Psti2	EF200526	–	–
Rosenvingiella constricta Rco1	EF200529	–	–
Prasiola crispa Pcri3	EF200532	–	–
Uncultured *Eukaryote* rtCF18sti	EF591011	–	–
Heveochlorella hainangensis FGG01	EF595524	–	–
Elliptochloris bilobata var. *corticola*	EF688289	–	–
Uncultured *Banisveld eukaryote* P1-3m10	EU091836	–	–
Uncultured *Banisveld eukaryote* P1-3m11	EU091837	–	–
Uncultured *Banisveld eukaryote* P1-5m3	EU091846	–	–
Uncultured *Banisveld eukaryote* P2-3m7	EU091854	–	–
Coccomyxa sp. Flensburg fjord 1	EU127470	–	–
Coccomyxa sp. Flensburg fjord 2	EU127471	–	–
Coccomyxa sp. Kragero	EU127472	–	–
Chlorella sp. 594-GA375	EU282453	–	–
Parietochloris alveolaris UTEX 836	EU878373	–	–
Chloroparva pannonica ACT0608	FJ013257	–	–
Elliptochloris MR-L2009 ZC113	FJ217361	–	–
Elliptochloris MRL-2009 ZC102	FJ217366	–	–
Elliptochloris MRL-2009 ZC108	FJ217367	–	–
Uncultured *Trebouxiophyceae* C_41	FJ490210	–	–
Uncultured *Trebouxiophyceae* C_45	FJ490214	–	–

Uncultured *Trebouxiophyceae* C_46	FJ490215	–	–
Uncultured *Trebouxiophyceae* C_48	FJ490217	–	–
Uncultured *Trebouxiophyceae* E_45	FJ490221	–	–
Uncultured *Trebouxiophyceae* H_25	FJ490228	–	–
Uncultured *Trebouxiophyceae* H_26	FJ490229	–	–
Uncultured *Trebouxiophyceae* H_28	FJ490231	–	–
Uncultured *Trebouxiophyceae* H_29	FJ490232	–	–
Uncultured *Trebouxiophyceae* H_30	FJ490233	–	–
Uncultured *Trebouxiophyceae* H_31	FJ490234	–	–
Uncultured *Trebouxiophyceae* H_33	FJ490236	–	–
Uncultured *Trebouxiophyceae* H_34	FJ490237	–	–
Coccomyxa mucigena SAG 216-4	FJ648513	–	–
Elliptochloris sp. SAG 2117	FJ648515	–	–
Elliptochloris sp. SAG 2201	FJ648516	–	–
Elliptochloris bilobata SAG 245.80	FJ648517	–	–
Elliptochloris subsphaerica SAG 2202	FJ648518	–	–
Uncultured *Trebouxiophyceae* QE17	FJ790649	–	–
Uncultured *Trebouxiophyceae* QE29	FJ790655	–	–
Trebouxiophyceae sp. SC2-2	FJ946881	–	–
Trebouxiophyceae sp. EO7-4	FJ946882	–	–
Chlorella sp. VI2	FJ946883	–	–
Chlorella sp. VI11	FJ946884	–	–
Chlorella sp. WO10-1	FJ946886	–	–
Lobosphaeropsis lobophora SAG 37.88	FM205833	–	–
Didymogenes palatina SAG 30.92	FM205840	–	–
Chlorella lewinii CCAP 211/90	FM205861	–	–
Actinastrum hantzschii CCAP 200/3	FM205884	–	–
Chloroidium ellipsoideum SAG 211	FM946017	–	–
Chlorella angustoellipsoidea CCAP 211/108	FM946021	–	–
Pseudochlorella sp. CCAP211/1A	FM958479	–	–
Viridiella fridericiana SAG 10.92	FM958481	–	–
Chlorella variabilis CCAP 211/84	FN298923	–	–
Pseudococcomyxa simplex SAG 216-9a	FN298926	–	–
Coccomyxa sp. CCAP 211/97	FN298928	–	–
Choricystis sp. SAG 211-40c	FN298929	–	–
Nephroselmis olivacea NIES-483	FN562436	–	–
Apatococcus lobatus ROS 7/3	FR693368	–	D
Stichococcus bacillaris CCAP379/1A	FR717539	–	–
Coccomyxa sp. 216-25	FR850476	–	–
Chlorella reisiglii CCAP 11/8	FR865615	–	–
"*Chlorella*" *luteoviridis* CCAP 211/10A	FR865652	–	–
"*Chlorella*" *luteoviridis* CCAP 211/10E	FR865653	–	–
"*Chlorella*" *luteoviridis* CCAP 211/3	FR865663	–	–
Chloroidium saccharophilum CCAP 211/31	FR865664	–	–
"*Chlorella*" *luteoviridis* CCAP 211/4	FR865668	–	–
Chloroidium ellipsoideum CCAP 211/40	FR865669	–	–
"*Chlorella*" *saccharophila* CCAP 211/57	FR865676	–	–
"*Chlorella*" *luteoviridis* CCAP 211/5B	FR865678	–	–
Chlorella saccharophila CCAP 211/60	FR865679	–	–

Chlorella vulgaris CCAP 211/79	FR865683	–	–
Phlanophila sp. CCAP 462/1	FR865753	–	–
Uncultured *Eukaryote* BT57_1	GQ462874	–	–
Kalenjinia gelatinosa CCAP 222/8	GQ477061	–	–
Mucidosphaerium pulchellum ACOI 755	GQ487198	–	–
Mucidosphaerium pulchellum CCAP 222/2a	GQ487199	–	–
Mucidosphaerium palustre CB 2008/6	GQ487216	–	–
Mucidosphaerium sphagnale CB 2008/19	GQ487218	–	–
Mucidosphaerium sphagnale CB 2008/44	GQ487219	–	–
Dictyosphaerium sp. CCAP 211/86	GQ487242	–	–
Parachlorella sp. CCAP 206/1	GQ502287	–	–
Hindakia tetrachotoma CCAP 222/69	GQ867590	–	–
Symbiochloris symbiontica SAG 27.81	GU017644	–	–
Asterochloris phycobiontica SAG 26.81	GU017647	–	–
Dictyochloropsis sp. SAG 2073	GU017648	–	–
Symbiochloris reticulata SAG 53.87	GU017650	–	–
Symbiochloris symbiotica SAG 12.86	GU017651	–	–
Symbiochloris sp. SAG 2069	GU017652	–	–
Symbiochloris splendida SAG 244.80	GU017653	–	–
Symbiochloris symbiontica SAG 46.85	GU017654	–	–
Symbiochloris reticulata CAUPH 8602	GU017655	–	–
Dictyochloropsis splendida SAG 2153	GU017657	–	–
Dictyochloropsis splendida SAG 2305	GU017658	–	–
Symbiochloris irregularis SAG 2036	GU017659	–	–
Symbiochloris splendida UTEX 2612	GU017660	–	–
Symbiochloris symbiontica CAUPH 8603	GU017663	–	–
Dictyochloropsis asterochloroides SAG 2098	GU017664	–	–
Symbiochloris splendida UTEX 2599	GU017665	–	–
Symbiochlori irregularis NIES-378	GU017670	–	–
Symbiochloris irregularis SAG 2154	GU017671	–	–
Uncultured *Choricystis* ESS220206.010	GU067789	–	–
Catena viridis KR 1991/4	GU592792	–	–
Pseudococcomyxa simplex CAUP H 102	HE586504	HE586504	–
Pseudococcomyxa simplex CAUP H 103	HE586505	–	–
Coccomyxa sp. KN-2011-C4	HE586508	–	–
Coccomyxa sp. KN-2011-C15	HE586512	–	–
Coccomyxa sp. KN-2011-T2	HE586514	–	–
Coccomyxa sp. KN-2011-T4	HE586516	–	–
Coccomyxa sp. KN-2011-U2	HE586517	–	–
Choricystis sp. GSE4G	HE586518	–	–
Coccomyxa sp. KN-2011-E5	–	HE586504	–
Leptochlorella corticola I2e	HE984579	–	–
Parachloroidium lobatum CAUP H8502	HF586460	–	–
Parachloroidium lobatum CAUP H8503	HF586461	–	–
Coccomyxa simplex SAG 216-3b	–	HG972980	–
Mucidosphaerium sphagnale CB 2008/15	HM066006	–	–
Mucidosphaerium sphagnale KR 2009/1	HM066007	–	–
Chlorella chlorelloides CB 2008/1101	HQ111432	–	–
Chlorella volutis CB 2008/691	HQ111434	–	–

Uncultured *Eukaryote* N1TE_01	HQ143746	–	–
Uncultured *Chlorophyta* PA2009C7	HQ191364	–	–
Coccomyxa sp. KR 1988/12	HQ287928	–	–
Elliptochloris reniformis SAG2200	HQ317305	–	–
Compactochlorella kochii CCAP 222/61	HQ322125	–	–
Compactochlorella kochii CB 2008/104	HQ322126	–	–
Trebouxiophyceae sp. A42	HQ418418	–	–
Chlorella saccharophila KMMCC FC-29	HQ702277	–	–
Chlorella vulgaris KMMCC EC-5	HQ702321	–	–
Chlorella saccharophila var. *saccharophila* KMMCC FC-5	HQ702322	–	–
Pseudochlorella prigsheimii KMMCC FC-6	HQ702324	–	–
Chlorella vulgaris KMMCC FC-41	HQ702325	–	–
Ulva prolifera LYG-HT	HQ850569	–	–
Myrmecia irregularis CCAP 221/8	HQ902935	–	–
Uncultured *Eukaryote* CA-1-6-2d	HQ999103	–	–
Heveochlorella roystonensis ITBB A3-8	JN003601	–	–
Stichococcus bacillaris siva2011	JN168788	–	–
Stichococcus minutus NJ-17	JN400256	–	–
Elliptochloris sp. Amtoft s.n.	JN573866	–	–
Elliptochloris sp. W0975	JN573884	–	–
Diplosphaera sp. W1196	JN573885	–	–
Stichococcus bacillaris KMMCC 20	JQ315605	–	–
Stichococcus bacillaris KMMCC 36	JQ315606	–	–
Stichococcus bacillaris KMMCC 47	JQ315608	–	–
Stichococcus bacillaris KMMCC 169	JQ315611	–	–
Stichococcus bacillaris KMMCC 199	JQ315613	–	–
Stichococcus bacillaris KMMCC 1480	JQ315614	–	–
Stichococcus sp. KMMCC 293	JQ315616	–	–
Stichococcus sp. KMMCC 313	JQ315617	–	–
Stichococcus sp. KMMCC 364	JQ315618	–	–
Stichococcus sp. KMMCC 246	JQ315619	–	–
Stichococcus sp. KMMCC 881	JQ315620	–	–
Stichococcus bacillaris KMMCC 18	JQ315623	–	–
Stichococcus sp. KMMCC 365	JQ315624	–	–
Uncultured *Chlorophyta* B1_4_1E_66	JQ627438	–	–
Uncultured *Chlorophyta* A2_4b_1E_31	JQ627445	–	–
Uncultured *Chlorophyta* A2_4b_1E_35	JQ627446	–	–
Coccomyxa sp. XDL-2012	JQ946088	–	–
Uncultured *Xylochloris* AEW2R-K255	JQ988938	–	–
Uncultured *Xylochloris* HEG9B-K2617	JQ988940	–	–
Xylochloris sp. SAG 2382	JQ988942	–	–
Uncultured *Trebouxia* photobiont *Buellia frigida* s187	–	JX036213	–
Uncultured *Trebouxia* photobiont *Rhizoplaca macleanii* s207	–	JX036231	–
Chlorella sp. ZJU0205	JX097055	–	–
Chlorella sp. ZJU0204	JX097056	–	–
Chlorella sp. ZJU0208	JX097060	–	–
Chlorella sp. ZJU0209	JX097061	–	–
Uncultured *Apatococcus* 3GSCRE_K20	JX127160	–	A
Uncultured *Stichococcus* 3GB18_K125	JX127161	–	–

Uncultured *Stichococcus* 3GSG1RE_K41	JX127162	–	–
Uncultured *Myrmecia* 3GSG3RE_K13	JX127163	–	–
Uncultured *Trebouxiophyceae* 3GB14_K3762	JX127167	–	–
Uncultured *Pabia* 3GB1314RE_K32	JX127170	–	–
Apatococcus lobatus SAG 2037	JX169825	–	A
Apatococcus lobatus SAG 2359	JX169826	–	A
Uncultured *Apatococcus* GOGsM_K45	JX169827	–	D
Uncultured *Apatococcus* GOGs_18S_K42	JX169828	–	D
Uncultured *Apatococcus* GOGs_18S_K50	JX169830	–	D
Uncultured *Apatococcus* GOGsk_K17	JX169831	–	D
Coccomyxa sp. GOGrp_K07	JX169832	–	–
Radiococcaceae sp. SAG 2384	JX169833	–	–
Coenochloris signiensis CCAP 176/3	JX169834	–	–
Radiococcaceae sp. SAG 2375	JX169835	–	–
Pabia sp. SAG 2374	JX169837	–	–
Chloroidium ellipsoideum	JX169838	–	–
Chloroidium sp. GOGrp_K10	JX169839	–	–
Chloroidium sp. GOGrp_K11	JX169840	–	–
Heterochlorella sp. GOGrp_K46	JX169841	–	–
Heterochlorella sp. GOGrp_K01	JX169842	–	–
Uncultured *Trebouxia* GOGs_18S_K02	JX169843	–	–
Uncultured *Trebouxia* GOGsk_K6	JX169844	–	–
Trebouxia sp. GOGre_K46	JX169845	–	–
Uncultured *Trebouxia* GOGsM_K51	JX169846	–	–
Pseudochloris wilhelmii SAG 5587	JX235962	–	–
Chloroparva sp. ACT 0602	JX235963	–	–
Uncultured *Stichococcus* FGSsan_K35	JX391005	–	–
Uncultured *Apatococcus* FGSwa_K32	JX391013	–	A
Uncultured *Apatococcus* FGSwa_K16	JX391014	–	E
Uncultured *Apatococcus* HP1	JX877575	–	D
Chlorella sorokiniana	JX910111	–	–
Coccomyxa sp. AC1	KC155323	–	–
Coccomyxa sp. AH4	KC155324	–	–
Dictyochloropsis splendida SAG 2071	KC333456	–	–
Dictyochloropsis splendida CAUP H8601	KC333457	–	–
Dictyochloropsis splendida SAG 2097	KC333458	–	–
ex *Catillaria chalybeia* SCH-AB08.002d	KC333461	–	–
ex *Lobaria pulmonaria* AB06.006A2	KC333463	–	–
ex *Lobaria patinifera* SCH-17084	KC333467	–	–
ex *Crocodia aurata SAG* 46.85	KC333470	–	–
ex *Sticta canariensis* SCH-6057	KC333471	–	–
Symbiochloris symbiontica CAUPH 8603	KC333473	–	–
ex *Chaenotheca brunneola* SAG 244.80	KC333474	–	–
Symbiochloris reticulata CCHU5616	KC333476	–	–
ex *Lobaria oregana* SCH-1998	KC333477	–	–
Symbiochloris reticulata CAUPH 8602	KC333479	–	–
ex *Chaenothecopsis consociata* SAG 27.81	KC333480	–	–
ex *Sticta* sp. SCH-22386	KC333481	–	–
ex *Brigantiaea leucoxantha* MP124	KC333485	–	–

ex *Megalospora sulphurata* MP167	KC333486	–	–
ex *Lobariella* sp. MPN168	KC333487	–	–
ex *Crocodia aurata* MP169	KC333488	–	–
ex *Pseudocyphellaria lividofusca* NZ1568	KC333490	–	–
ex *Pseudocyphellaria multifida* NZ6009	KC333496	–	–
ex *Sticta latifrons* NZ6021	KC333498	–	–
ex *Lobariella pallidocrenulata* SA5417	KC333502	–	–
ex *Lobariella pallidocrenulata* SA5513	KC333503	–	–
ex *Sticta pulmonarioides* SA5533	KC333504	–	–
ex *Sticta* aff. *neopulmonaria* SA5523	KC333505	–	–
ex *Sticta* aff. *neopulmonaria* SA5534	KC333506	–	–
ex *Sticta* sp. SA5538	KC333507	–	–
Symbiochloris sp. SAG 2069	–	KC333509	–
Symbiochloris irregularis SAG 2036	–	KC333522	–
Uncultured *Symbiochloris* SA5420	–	KC333529	–
Symbiochloris reticulata SCH-1069	–	KC333557	–
Symbiochloris reticulata SCH-2339	–	KC333559	–
Heveochlorella hainangensis SAG 2360	KC470085	–	–
Apatococcus lobatus CCALA 213	KF355939	–	–
Chlorella sorokiniana KU-1019	KF444207	–	–
Nephroselmis pyriformis CCMP 717	KF615768	–	–
Watanabea sp. BCP-SEV1VF9	KF693804	–	–
Uncultured *Eukaryote* CMH 220	–	KF800311	C
Uncultured *Symbiochloris* SCH-1524	–	KF960681	–
Uncultured *Symbiochloris* SCH-22499	–	KF960685	–
Uncultured *Symbiochloris* bSCH-18864	–	KF960686	–
Dictyochloropsis splendida SAG 2069	KF960690	–	–
Uncultured *Trebouxia* photobiont S65	–	KJ576667	–
Uncultured *Trebouxia* L174	–	KJ576675	–
Uncultured *Apatococcus* N2C_K33	KJ639847	–	A
Trebouxia sp. LM2014 L1660	–	KJ754297	–
Trebouxia sp. LM2014 L1668	–	KJ754298	–
Chloroidium ellipsoideum FG2/4.5E	KM020071	–	–
Trochisciopsis tetraspora SAG 19.95	KM020112	–	–
Uncultured *Trebouxia* photobiont 4H9	–	KM062106	–
Coccomyxa sp. SAG 2040	KM116459	–	–
Stichococcus sp. SAG 2119	KM116460	–	–
Heterochlorella luteoviridis SAG 2133	KM116461	–	–
Heterochlorella luteoviridis SAG 2213	KM116462	–	–
Chloroidium angustoellipsoideum SAG 2041	KM116463	–	–
Pabia signiensis SAG 2110	KM116465	–	–
Uncultured *Trebouxia* AWH1	–	KM369502	–
Uncultured *Trebouxia* A548	–	KM369523	–
Uncultured *Trebouxia* APeel2	–	KM369692	–
Uncultured *Trebouxia* photobiont ARam23	–	KM369710	–
Trebouxia sp. IDH-2015 ANTLBCC7C	–	KM987344	–
Uncultured *Apatococcus* AEW6B_K237	KP081318	–	A
Uncultured *Apatococcus* AEW7R_K37	KP081319	–	D
Uncultured *Apatococcus* AEW4B_K311	KP081320	–	D

Uncultured *Apatococcus* AEW2R_K261	KP081321	–	E
Uncultured *Apatococcus* AEW2B_K100	KP081322	–	E
Uncultured *Apatococcus* AEW6R_K299	KP081323	–	A
Uncultured *Apatococcus* AEW1B_K142	KP081324	–	A
Uncultured *Apatococcus* AEW7R_K193	KP081325	–	A
Uncultured *Trebouxia* AA1	–	KP282148	–
Uncultured *Trebouxia* VO2	–	KP282194	–
Uncultured *Trebouxia* A05	–	KR053366	–
Uncultured *Trebouxia* A21	–	KR053382	–
Trebouxia sp. OTU A01 ID 446	–	KR912507	–
Trebouxia sp. OTU A03 ID 4083	–	KR912786	–
Trebouxia sp. OTU A03 ID 4092	–	KR912820	–
Trebouxia sp. OTU A05 ID 8721	–	KR912929	–
Trebouxia sp. OTU A05 ID 6715	–	KR912932	–
Trebouxia sp. OTU A08 ID 6759	–	KR913113	–
Trebouxia sp. OTU A32 ID 075	–	KR913269	–
Trebouxia sp. OTU G02 ID 3747	–	KR913282	–
Trebouxia sp. 14a	–	KT715659	–
Trebouxia sp. 28	–	KT715660	–
Trebouxia sp. 87	–	KT715661	–
Trebouxia sp. 406	–	KT715662	–
Trebouxia cretacea AV066	–	KT819919	–
Trebouxia gigantea AV093	–	KT819923	–
Trebouxia jamesii AV005	–	KT819971	–
Trebouxia jamesii AV031	–	KT819977	–
Trebouxia cretacea AV090	–	KT819978	–
Trebouxia simplex AV082	–	KT819984	–
Trebouxia jamesii 4174	–	KT827704	–
Uncultured *Eukaryote* B4_57	KU579454	–	E
Uncultured *Eukaryote* B5_234	KU579631	–	E
Uncultured *Eukaryote* B4_313	KU579710	–	E
Apatococcus sp. SAG 2145	KX025108	KX025108	A
Uncultured *Apatococcus* GOGs_K08	–	KX025109	B
Uncultured *Apatococcus* GOGs_K15	–	KX025110	A
Uncultured *Apatococcus* GOGs_K48	–	KX025111	C
Uncultured *Apatococcus* GOGs_K52	–	KX025112	D
Uncultured *Apatococcus* GOGs_K31	–	KX025113	D
Uncultured *Apatococcus* GOGs_K31	–	KX025113	D
Uncultured *Apatococcus* GLB_K898	–	KX025114	B
Uncultured *Apatococcus* GLB_K925	–	KX025115	D
Uncultured *Apatococcus* GLB_K910	–	KX025116	D
Uncultured *Apatococcus* GLB_K896	–	KX025117	E
Uncultured *Apatococcus* GLG_K1044	KX025118	KX025118	A
Uncultured *Apatococcus* GLG_K1068	–	KX025119	D
Uncultured *Apatococcus* GLG_K1050	–	KX025120	D
Uncultured *Apatococcus* GLG_K1042	KX025121	KX025121	E
Chlorella saccharophila SAG 211-9a	X63505	–	–
Chlorella luteoviridis SAG 211-2a	X73998	–	–
Chlorella mirabilis Adreyeva 748I	X74000	–	–

Myrmecia biatorellae UTEX	Z28971	–	–
Dictyochloropsis reticulata	Z47207	–	–
Myrmecia bisecta	Z47209	–	–
Trebouxia usneae UBT-87.019A1	Z68702	–	–
Trebouxia arboricola SAG 219-1a	Z68705	–	–

Table S2: Calculated parameters of the 18S and individual partitioned ITS data matrices from the Bayesian inference. In the columns, the values without and with brackets indicate mean and variance, respectively.

Parameters	**18S**	**ITS1**		**5.8 S**		**ITS2**		**ITS**
Data matrix		***Manually adjusted***	***Relaxed masking***	***Manually adjusted***	***Relaxed masking***	***Manually adjusted***	***Relaxed masking***	***Stringent masking***
Frequency A	0.2347 (0.0001)	0.1785 (0.0001)	0.1825 (0.0002)	0.3092 (0.0011)		0.1986 (0.0002)	0.1817 (0.0002)	0.2396 (0.0007)
Frequency C	0.2358 (0.0001)	0.2981 (0.0002)	0.3030 (0.0003)	0.2357 (0.0009)		0.2885 (0.0003)	0.2895 (0.0004)	0.2728 (0.0007)
Frequency G	0.2661 (0.0001)	0.2966 (0.0003)	0.2926 (0.0003)	0.2305 (0.0009)		0.2729 (0.0003)	0.2857 (0.0004)	0.2731 (0.0007)
Frequency T	0.2634 (0.0001)	0.2268(0.0002)	0.2219 (0.0002)	0.2246 (0.0008)		0.2401 (0.0003)	0.2431 (0.0003)	0.2144 (0.0006)
Gamma shape (G)	0.1916 (0.0002)	3.3108 (0.4160)	3.3481 (0.5083)	0.1121 (0.0001)	0.1235 (0.0001)	1.2100 (0.0219)	1.2590 (0.0285)	0.1053 (0.0000)
Proportion of invariant sites (I)	0.2777 (0.0006)	0.0467 (0.0002)	0.0487 (0.0003)					0.3187 (0.0038)
R-matrix [A-C]	0.1102 (0.0001)	0.1433 (0.0003)	0.1525 (0.0003)			0.1138 (0.0003)	0.1149 (0.0004)	0.0486 (0.0004)
R-matrix [A-G]	0.2621 (0.0004)	0.2196 (0.0004)	0.2075 (0.0004)			0.2726 (0.0008)	0.3074 (0.0010)	0.2223 (0.0024)
R-matrix [A-T]	0.1111 (0.0001)	0.2048 (0.0004)	0.2031 (0.0004)			0.1894 (0.0005)	0.1822 (0.0006)	0.1643 (0.0019)
R-matrix [C-G]	0.0908 (0.0001)	0.0509 (0.0001)	0.0538 (0.0001)			0.0444 (0.0001)	0.0424 (0.0001)	0.1021 (0.0008)
R-matrix [C-T]	0.3680 (0.0004)	0.2730 (0.0004)	0.2749 (0.0005)			0.2748 (0.0006)	0.2591 (0.0008)	0.3873 (0.0029)
R-matrix [G-T]	0.0577 (0.0001)	0.1083 (0.0002)	0.1082 (0.0002)			0.1050 (0.0002)	0.0940 (0.0002)	0.0754 (0.0011)
Kappa (K)					2.7198 (0.5376)			

Table S3. Tree statistics of individual ITS data matrices calculated by maximum parsimony method.

	ITS Data matrix		
Parameters	***Manually adjusted***	***Relaxed***	***Stringent***
No. of total characters	664	621	199
No. of constant characters	176	174	141
No. of parsimony-informative characters	448	408	45
Tree length	2002	1700	109
Consistency index	0.4995	0.5039	0.7431
Homoplasy index	0.5005	0.4951	0.2569
Retention index	0.8739	0.8771	0.9548
Rescaled retention index	0.4365	0.4428	0.7096

Errata for
Taxonomy and phylogeny of the family Fuscideaceae (Umbilicariales, Ascomycota) with special emphasis on *Fuscidea*.

Martina Zahradníková

Thesis for the degree philosophiae doctor (PhD)
at the University of Bergen

(signature of candidate) (signature of faculty)

30.08.2017

Errata

Page 91: Johnson → Johnsen

Page 92: to examine their phylogenetic relationships → for species delimitation
supported by 80% bootstrap value → with high support
sorediate and fertile taxa → sorediate and fertile taxa the so-called species pairs

Page 94: the sentence is corrected: A concatenated data set of ITS region divided in four partitions, i.e. ITS1, 5.8S, ITS2, and LSU, and mtSSU was used to...

Page 95: equal to or above 0.9 → equal to or above 0.95
equal to or above 80% → equal to or above 70%
Individual fragments were inspected... → Individual trees were inspected...
the same settings as described. → the same settings as described. No significant conflicts were detected.

Page 96: equal to or above 0.9 → equal to or above 0.95
raxmlGUI → raxmlGUI version 1.3
22 taxa → 12 taxa
added: 17 sequences were newly acquired.

Page 97: occurred together → was related
Fuscidea cyathoides (Ach.) V. Wirth & Vězda. → *Fuscidea cyathoides* (Ach.) V. Wirth & Vězda, although lacking support.
82 taxa → 13 taxa
placement of *F. cyathoides* → placement of *F. pusilla* BG-L-98625
with moderate support PP=0.62/ML=57% → unsupported lineage

Page 100: *Fuscidea gothoburgensis* BG-L-96934 → *Fuscidea gothoburgensis* BG-L-100245

Page 101: *Fuscidea gothoburgensis* BG-L-96934 → *Fuscidea gothoburgensis* BG-L-100245

Page 113: Johnson → Johnsen
CZECH REPUBLIC. W Bohemia. ... S Bohemia. ..... → **CZECH REPUBLIC**. S Bohemia. ..... W Bohemia. ...
IRELAND. Co. Waterford. ... Co. Kildare. ... → **IRELAND**. Co. Kildare. ... Co. Waterford. ...

Page 114: *T. Tønsberg* 44870 (BG-L-98665) → *T. Tønsberg* 44871 (BG-L-98666)

Page 119: …Zschacke, 1927). → …Zschacke, 1927) (see Table 1).

Page 121: the colour of the thalli. The ratio ... (Table 2). → the colour of the thalli (Table 2). The ratio...

Page 142, Figure 2: *Fuscidea gothoburgensis* BG-L-96934 → *Fuscidea gothoburgensis* BG-L-100245
Fuscidea pusilla BG-L-96935 → *Fuscidea pusilla* BG-L-96938

Page 146, Table 2: the column "Variety" was added
the values of the standard deviation were corrected for some calculations as were a few values of the mean of the given parameters, numbers of single measurements per one parameter were added

Page 147, Table 3: Herbarium/Collector number → Collection/Accession number.
Fuscidea pusilla BG-L-96935 → *Fuscidea pusilla* BG-L-96938
Fuscidea austera E. Timdal 4174 → *Fuscidea austera* E. Timdal 4177

Page 149: in Abstract, the first sentence: Knowledge about recognition and delimitation of lichenized algae and consequently algal systematics… → The knowledge of the taxonomy and classification of algae (including lichenized)...

Page 150: in Abstract, the last sentence: The photobiont of most *Fuscidea* species, *Apatococcus fuscideae* A. Beck & Zahradn., was circumscribed... → The most common photobiont of *Fuscidea* species, *Apatococcus fuscideae* A.Beck & Zahradn., was described as new to science.

Page 151: (Smith et al. 2009) → as complied from Smith et al. (2009)

Page 152: the citation of Piercey-Normore 2006 was deleted

Page 153: in Material and Methods: explanation was added: (abbreviations according to Index Herbariorum; http://sweetgum.nybg.org/science/ih/)
in Material and Methods: TLC → thin-layer chromatography (TLC)

Page 159: Visher → Vischer

Page 160: *Description*: with Cells → *Description*: Cells

Page 161: in Taxonomy: with grey, to tinged brown → grey to brown tinged
in Taxonomy: ..., occurring on acid rock in Europe. → It occurs on acid rock and is widely distributed (Gilbert et al. 2009).

in Discussion: morphological methods → morphological methods only, genera → genus
in Discussion: the following sentence was deleted: A new alga species, *Apatococcus fuscideae*, is described for the photobionts of selected *Fuscidea* species, except for *F. lightfootii*.

Page 162: in *Apatococcus fuscideae*: "Group F only consists of ..." → "The clade with …"

Page 163: In *Apatococcus* in *F. lightfootii*: This group ... distinct from the lichenized photobionts nested in group F. → The clade ... distinct from *Apatococcus fuscideae*.

Page 164: the third sentence was changed: ...are almost identical with only minor differences... → ...show only minor differences, i.e. they are almost identical.
in the fourth sentence, two words were changed: shallow → recent, lowest → weak
the fifth sentence was corrected: Aligning the variable ITS... → The aligning of the variable ITS...

Page 165: in Conclusion, the last sentence was shortened: is described. Its delimitation is based on... → ...is described as new based on...

Page 167: microthamniales (chlorophyte) → Microthamniales (Chlorophyte)
Green Algae → green algae

Page 173: *Apatococcus fuscideii* → *Apatococcus fuscideae*

Page 175: *Apatococcus fuscideii* → *Apatococcus fuscideae*

Page 176: four hemi-CBCs were added to the terminal loop of Helix III

Page 179, Table 4: in parenthesis → in brackets
added: Grey shade divides CBCs and hemi-CBCs.