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Revisiting photobiont diversity in the lichen family Verrucariaceae (Ascomycota)

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The Verrucariaceae (Ascomycota) is a family of mostly lichenized fungi with a unique diversity of algal symbionts, including some algae that are rarely or never associated with other lichens. The phylogenetic position of most of these algae has not yet been studied and, because morphology-based identifications can often be misleading, molecular data is necessary to revisit their identity and to explore patterns of association between fungal and algal partners. For this reason, the diversity of photobionts in this lichen family was investigated using molecular markers (*rbc*L and nuSSU) amplified from DNA extracts of lichen thalli and cultured isolates. Although a single algal genus, *Diplosphaera* (Trebouxiophyceae), was associated with 12 out of the 17 sampled genera of Verrucariaceae, representatives of eight other genera in five orders of the Chlorophyta and one genus in the Xanthophyceae also form lichen associations with members of the family. Fungal genera with simple crustose thalli (e.g. *Hydropunctaria*, *Wahlenbergiella, Bagliettoa*) use a high diversity and unusual selection of photobionts. In contrast, fungal genera with more complex thalli (e.g. *Placidium, Dermatocarpon*) tend to have lower photobiont diversity. Habitat requirements and phylogenetic histories are both partly reflected in the observed patterns of associations between lichenized fungi from the family Verrucariaceae and their photobionts.

Key words: Algal partners, Chlorophyta, *Dilabifilum*, *Diplosphaera*, *Heterococcus*, lichens, molecular identification, mycobiont, phycobiont, *Stichococcus*, Verrucariaceae, Xanthophyceae

Introduction

Eukaryotic algae and cyanobacteria are common components of symbioses, especially in mutualistic associations, where they provide a constant and stable source of carbohydrates to their heterotrophic symbionts. Although approximately 40 phototrophic genera have been recorded as symbionts (photobionts) of lichens, most lichenized fungi associate primarily with only two groups of chlorophyte algae, the trebouxiophycean genera *Trebouxia* and *Asterochloris* and the ulvophycean order Trentepohliales (genera *Trentepohlia*, *Phycopeltis* and *Cephaleuros*), as well as two genera of Cyanobacteria, *Nostoc* and *Rhizonema* (Tschermak-Woess, 1988; Friedl & Büdel, 2008; Lücking *et al*., 2009; Skaloud & Peksa, 2010). As a result, broad trends of associations between lichen mycobionts and photobionts can be discerned at a high phylogenetic level (Tschermak-Woess, 1988; Rambold *et al*., 1998; Persoh *et al*., 2004; Miadlikowska *et al*., 2006; Friedl & Büdel, 2008). For example, in Lecanorales and

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Teloschistales, the most species-rich orders of lichenized fungi, *Trebouxia* and *Asterochloris* are the predominant photobionts. Similarly, nearly all Peltigerales species associate with *Nostoc*, and tropical lichen orders such as Arthoniales, Ostropales, Pyrenulales and Trypetheliales tend to associate preferentially with Trentepohliales.

A very different trend in mycobiont–photobiont associations can be found in the family Verrucariaceae, where a remarkable number of algal genera can be found. Morphology-based studies have shown that the most common lichen photobionts mentioned above are only rarely or never reported as symbiotic partners of the Verrucariaceae (summarized in Tschermak-Woess, 1988). Instead, the Verrucariaceae form associations with a plethora of other algae. *Stichococcus*-like green algae ('*Protococcus* spp.', *Stichococcus* spp. and *Diplosphaera* spp., Trebouxiophyceae) seem to be the most common photobionts in this lichen family, but the morphological circumscription of these algal taxa is unclear and their genetic diversity has never been studied. Other Trebouxiophyceae that have been reported to associate with species of Verrucariaceae include *Auxenochlorella, Chlorella*, *Coccobotrys*, *Myrmecia* and *Trochiscia* (Tschermak, 1941*a*; Tschermak-Woess, 1988; Nyati *et al*., 2007). Amphibious Verrucariaceae from both saline and freshwater habitats have repeatedly been reported in association with *Dilabifilum*, a genus of Ulvophyceae (Tschermak-Woess, 1970, 1976; Thüs, 2002). In addition, the Verrucariaceae is the only mostly lichenized family to form associations with Xanthophyceae (*Heterococcus caespitosus* Vischer: Tschermak, 1941*b*; Zeitler, 1954; Parra & Redon, 1977; Tschermak-Woess, 1988; Thüs & Schultz, 2008), Phaeophyceae [*Petroderma maculiforme* (Wollny) Kuckuck: Moe, 1997; Peters & Moe, 2001; Sanders *et al*., 2004, 2005; *Ascophyllum nodosum* (Linnaeus) Le Jolis and *Pelvetia canaliculata* (Linnaeus) Decaisne & Thuret: Kohlmeyer & Volkmann-Kohlmeyer, 1998] and Rhodophyta (*Apophlaea* sp.: Kohlmeyer & Volkmann-Kohlmeyer, 1998). In most lichens, the symbiotic alga is embedded in a fungal tissue forming a lichen thallus that is dominated by the mycobiont. However, in the Verrucariaceae several fungal species are found in association with thalloid algae, such as the brown algae *Pelvetia* and *Ascophyllum* (Kohlmeyer & Volkmann-Kohlmeyer, 1998), and the green alga *Prasiola crispa* (Lightfoot) Kützing (Pérez-Ortega *et al*., 2010). In these associations the mycobiont grows within the independently formed algal thallus. Although the *Prasiola* photobiont of the species of Verrucariaceae *Mastodia tessellata* (Hooker f. & Harvey) Hooker f. & Harvey has recently been characterized (Pérez-Ortega *et al*., 2010), little is known about the photobiont identity of its close relatives found in typical fungus-dominated lichen thalli.

The factors responsible for the high photobiont phylogenetic diversity observed in the Verrucariaceae have been addressed only briefly and with contradictory conclusions. Following the observation that several amphibious species of Verrucariaceae are associated with *Dilabifilum*, Tschermak-Woess (1976) suggested that selection for the same photobiont could be a character reflecting phylogenetic relatedness between species of lichen-forming fungi. However, it has not been tested whether all Verrucariaceae species with a *Dilabifilum* photobiont belong to a monophyletic group. Whether photobiont selection in Verrucariaceae reflects coincident ecological requirements of the two symbionts or whether it results from clade-specific preferences of Verrucariaceae lineages for certain algae remains an open question.

Morphological identification of photobionts within intact lichen thalli is hampered by the fact that characters important for species or genus identification (e.g. cell size and shape, chloroplast and pyrenoid morphology) may be modified in the lichenized state (Tschermak-Woess, 1988; Nyati *et al*., 2007; Honegger, 2009). Furthermore, freshly collected material is needed for isolating living photobionts, isolation itself is often difficult, and many photobionts grow slowly in culture. It is not surprising, therefore, that the identity of many algal partners is still unknown. The application of molecular techniques has greatly facilitated the study of lichen photobionts in several ways. First, molecular data can provide quick and reliable identification of the algae living within the lichen thallus (Dahlkild *et al*., 2001; Helms *et al*., 2001; Lohtander *et al*., 2003). Second, the application of population genetics approaches to lichen-associated algae and cyanobacteria has allowed a revision of species concepts (Kroken & Taylor, 2000; O'Brien *et al*., 2005; Nelsen & Gargas, 2006; Yahr *et al*., 2006; Skaloud & Peksa, 2010; Fernández-Mendoza *et al*., 2011; Nelsen *et al*., 2011). Finally, DNA sequence data provide a phylogenetic framework for algal classification (Friedl & Büdel, 2008). In the Verrucariaceae, however, very few studies have included molecular data for the photobionts (Friedl & Zeltner, 1994; Peters & Moe, 2001; Nyati *et al*., 2007; Pérez-Ortega *et al*., 2010).

Most studies using molecular markers for the identification of lichen photobionts amplify algal genes directly from DNA extracts of the complete lichen thallus. These extracts however contain all

organisms co-existing with the principal symbionts, such as algae growing on the lichen thallus surface or in cracks or cavities within the lichen (accessory algae), and it may therefore be difficult to distinguish the main symbiotic algal component of a lichen thallus from opportunistic cohabitants (Helms *et al*., 2001; Honegger, 2009). These risks can be minimized if symbiotic algae are isolated from the thallus and DNA extracts prepared from unialgal photobiont cultures (Beck & Koop, 2001; Beck *et al*., 1998, 2002).

Here we explore the unique phylogenetic diversity of photobionts associated with the Verrucariaceae using molecular phylogenetics. In order to maximize our taxon sampling of the photobionts, we combined molecular data from isolated algal strains with a larger number of sequences generated from lichen-thallus DNA from herbarium specimens. These data are complemented with morphological observations from cultured algal isolates that were not sequenced. We analyse the phylogenies of photobionts and mycobionts in order to (1) establish or confirm the identity of the photobionts associated with main clades of the Verrucariaceae and (2) explore the patterns of association between algae and lichen-forming fungi in the context of evolution, thallus complexity and ecology.

Materials and methods

For the fungal partner, sequences were obtained using previously described protocols (Zoller *et al*., 1999; Zhou & Stanosz, 2001; Gueidan *et al*., 2007; Savić *et al*., 2008; Thüs & Nascimbene, 2008). Algal sequences produced in this study were obtained using two methods, one culture-independent, the other culture-dependent (see details below). In addition, the correspondence between obtained algal sequences and photobionts was verified by morphological identification of isolated algal strains from a total of 12 lichen species (see 'fungal associates' in Supplementary Table 1a (see supplementary material, which is available on the supplementary context tab of the article's online page at http://dx.doi.org/10.1080/09670262.2011.629788)). Specimens for which sequence data was missing were also used to confirm morphological identifications. The presence of cellular contacts between the fungus and the algal cells – either via haustoria or through envelopment of the algal cells by fungal hyphae – was used as the criterion for detecting lichenized algae (Tschermak-Woess, 1976). Thallus fragments with these types of contact between the symbionts were selected under the microscope and used to inoculate culture media.

Algal DNA extraction: culture-independent method

Total DNA was extracted from the whole lichen thallus (including both fungal and algal genomic DNA) and algal markers amplified using primers specific for the algae. This method was used for 38 lichen species representing all major clades of the Verrucariaceae (Supplementary Table 1a). Genomic DNA was extracted using a modified protocol from Zolan & Pukkila (1986) as detailed in Gueidan *et al*. (2007), or following Nelsen *et al*. (2011). The same DNA extracts were used to obtain sequences for the mycobionts.

Algal DNA-extraction: culture-dependent method

Algal cells were isolated from the fresh lichen thallus and cultured on agar in Petri dishes. This approach was used for 12 lichen species, with a focus on amphibious taxa because for these species the amplification of algal sequences from lichen thallus extracts often produced ambiguous results or failed. Whenever possible, the isolation of photobionts was replicated using several specimens from different localities, so that the 12 species were represented by a total of 21 specimens (Supplementary Table 1a).

The specimens were collected from sites in Europe and North America (Supplementary Table 1a). Specimens from dry environments were kept in air-dried paper packets until inoculation, while those from coastal and freshwater sites were kept at 4°C in moist and opaque polyethylene bags and transferred to the laboratory within a week of collection. To reduce the risk of contamination by epiphytic algae, the upper thallus surface was removed with a sterile razor blade. A drop of sterile water was placed on the decorticated area and the lichen tissues macerated with a sterile lancet. A diluted suspension of algal cells and fungal hyphae was then transferred to different agar-based culture media (1.5% agar). Photobionts from coastal lichens were cultured using an artificial seawater medium modified after Starr & Zeikus (1993: protocol available at http://epsag.netcity.de) or filtered and pasteurized seawater (collected near Portsmouth, UK). Freshwater and terrestrial species were transferred to Bold Basal Medium (Bischoff & Bold, 1963), with addition of 2 ml l^{-1} soil extract (Ettl & Gärtner, 1995), and cultured at 16°C under a 12 : 12 hour light : dark regime. The photobiont isolates from *Bagliettoa* were cultured on *Trebouxia* medium (Ahmadjian, 1993) at 13–15°C in continuous light (Favero-Longo *et al*., 2009). After 1–3 weeks, algal colonies growing out of lichen thallus fragments were separated from the attached mycelia and transferred to fresh media. Cultures are kept at the Natural History Museum London (BM), the Department of Plant Biology of the University of Torino (TO) and EPSAG (SAG). Vouchers of lichen thalli have been deposited in the lichen herbaria at BG, BM, DUKE, F, GZU, LI, MARSSJ, NCU, NY, TO and TSB (Supplementary Table 1a). The algal genera and species investigated were identified morphologically using Ettl & Gärtner (1995). Nomenclature follows AlgaeBase (Guiry & Guiry, 2011) and, for the lichenized fungi, Index Fungorum (CABI, CBS & Landcare Research 2011) and Gueidan *et al*. (2009). For light microscopy, we used an Axioskop (Zeiss,

Oberkochen, Germany) equipped with differential interference contrast optics.

Amplification and sequencing of algal markers

Genomic DNA was obtained from cultured algal isolates using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). Two gene regions of the algal partner, the small subunit of the nuclear ribosomal RNA gene (nuSSU) and chloroplast-encoded large subunit of ribulose-bisphosphate carboxylase–oxygenase (*rbc*L), were amplified using published and newly designed primers (Table 1). One microlitre of undiluted, 1/10- or 1/100-diluted genomic DNA was added to the following PCR mix: 1× PCR buffer (buffer IV with 1.5 mM $MgCl_2$, Abgene, Rochester, NY), 2.5 µl dNTPs (0.2 mM each), BSA (1 mg ml^{-1}), primers (0.8 µM), 0.75 U Taq polymerase (Denville, South Plainfield, NJ), and water to a total volume of 25 µl. PCR was performed on a PTC-200 Peltier thermal cycler (MJ Research, Waltham, MA). For nuSSU, amplifications were run with an initial cycle of 3 min at 95°C, followed by 35 cycles of the following steps: 45 s at 95°C, 40 s at 52°C and 3 min at 72°C. For *rbc*L, one initial cycle of 5 min at 95°C preceded 35 cycles of the following steps: 45 s at 95°C, 90 s at 47°C and 2 min at 72°C, or alternatively with an annealing temperature of 50°C and 40 cycles. All amplifications ended with a final cycle at 72°C for 10 min.

After examination with gel electrophoresis or on a spectrophotometer (NanoDrop 2000, Thermo Fisher), PCR products were purified using the Microcon PCR cleaning kit (Millipore, Billerica, MA) or QIAquick Purification Kit (Qiagen, Hilden/Germany). Sequencing was carried out in 10 µl reactions using: 3 µl of purified PCR product, 1 µM of primer, 1 µl of Big Dye (Big Dye Terminator Cycle sequencing kit, ABI PRISM version 3.1; Perkin-Elmer, Applied Biosystems, Foster City, CA), 3 µl of Big Dye buffer, and 2 µl of double-distilled water. Automated reaction clean up and visualization was performed at the Duke IGSP Genome Sequencing & Analysis Core Facility and at the Nano-Bio Centre of the Technical University Kaiserslautern, using ABI 3730xl DNA analysers (Applied Biosystems).

Alignments and phylogenetic analyses

Sequences were assembled and edited using BioEdit (Hall, 1999) or Sequencher 4.2.2 (Gene Codes Corporation, Ann Arbor, MI). Four datasets (three algal with concatenated nuSSU and *rbc*L data and one fungal with concatenated *RPB*1, nuSSU, nuLSU and mtSSU data) were assembled. The first dataset (the *Heterococcus* dataset: Supplementary Table 1b) was assembled to investigate the phylogenetic placement of lichenized *Heterococcus* (Xanthophyceae, Heterokontophyta). It included 13 isolates of *Heterococcus* (including six newly sequenced photobionts), as well as three other xanthophyte species as outgroups. The second dataset (the Chlorophyta dataset: Supplementary Table 1c) was produced to examine the phylogenetic positions of chlorophyte photobionts, in particular within the two classes Ulvophyceae and Trebouxiophyceae. It included 100 ingroup taxa, including 76 Trebouxiophyceae, 20 Ulvophyceae and four Chlorophyceae. Three species of Prasinophytes were used as outgroups. The third dataset (the *Prasiola*-group dataset; Supplementary Table 1d) focused on a particular clade of trebouxiophyte algae, the Prasiolales and closely related taxa of the genera *Diplosphaera* and *Stichococcus*. It included 81 ingroup taxa and used three species of Chlorellales (Trebouxiophyceae) as outgroups. The Microthamiales might be more closely related to the *Prasiola*-group but the basal relationships between these groups are not supported. All sources of newly isolated or sequenced algae are summarized in Supplementary Table 1. Finally, the mycobiont dataset (Supplementary Table 1e) comprised 114 taxa, among which were 112 Verrucariaceae representing most of the main genera within this family, while two species of Chaetothyriales were used as an outgroup (TreeBase ID11790). The four different data matrices and trees are accessible on TreeBase (*Heterococcus* dataset, Treebase ID11791; Chlorophyta dataset, TreeBase ID11792; *Prasiola*-group dataset, TreeBase ID11793).

Manual alignments were performed using MacClade 4.06 (Maddison & Maddison, 2003). Ambiguous regions (*sensu* Lutzoni *et al*., 2000) and introns were delimited manually and excluded from the alignments. The phylogenetic signals from different amplified regions (*rbc*L and nuSSU for the algal datasets and nuLSU, nuSSU, mtSSU and *RPB*1 for fungal dataset) were tested for congruence using a 70% reciprocal bootstrap criterion (Mason-Gamer & Kellogg, 1996). The topology comparison was undertaken manually based on trees obtained with 500 bootstrap pseudoreplicates using RAxML VI-HPC (Stamatakis *et al*., 2005, 2008) on the Cipres Web Portal (http://www.phylo.org/sub_sections/portal/). Taxa or sequences responsible for incongruence were removed from the dataset (*Placidiopsis cinerascens* CG585b and *Verrucaria viridula* CG587 were removed from the algal datasets and also the four algal nuSSU sequences obtained from *Agonimia opuntiella* L623, *A. tristicula* L469, *Normandina acroglypta* L618 and *N. pulchella* L616, and one rbc*L* sequence obtained from *Stictis uceolatum* AAsn), and the two markers were combined. Phylogenetic relationships and confidence were inferred using RAxML on the Cipres Web Portal. The ML search followed a GTRMIX model of molecular evolution applied to the four partitions for the algal datasets (*rbc*L first, second and third codon positions, and nuSSU) and six partitions for the fungal dataset (*RPB*1 first, second and third codon positions, and nuLSU, nuSSU and mtSSU). Support values were obtained in RAxML with bootstrap analyses of 1000 pseudoreplicates.

Results

The photobionts of the Verrucariaceae we investigated belonged to the Xanthophyceae (Heterokontophyta) and the two green algal classes Trebouxiophyceae and Ulvophyceae (Chlorophyta).

Table 1. Amplification and sequencing primers used in this study to obtain *rbc*L and nuSSU sequences for Verrucariaceae photobionts. The abbreviations 'amp' and 'seq' indicate primers used for amplification and sequencing, respectively.

Marker	Target group	Primer name	Primer sequence	Direction	Use	Source
nuSSU	*Heterococcus*	18S SEQ 34f	GTC TCA AAG ATT AAG CCA TGC	forward	seq	Friedl (this study)
		18S SEQ 1122F	GGC TGA AAC TTA AAG GAA TTG	forward	seq	Friedl (this study)
		18S SEQ 1759R	CCT TGT TAC GAC TTC TCC TTC CTC	reverse	amp/seq	Friedl (this study)
		NS1	GTA GTC ATA TGC TTG TCT C	forward	amp	White *et al.* (1990)
	Trebouxiophyceae	NS1	GTA GTC ATA TGC TTG TCT C	forward	amp/seq	White *et al.* (1990)
		232F	CCC GAC TCG CGG TGA A	forward	amp/seq	Joneson (this study)
		NS5	AAC TTA AAG GAA TTG ACG GAA G	forward	seq	White *et al.* (1990)
		1006F	CCT AGT CTC AAC CAT AAA CG	forward	seq	Joneson (this study)
		18L	CAC CTA CGG AAA CCT TGT TAC GAC TT	reverse	amp/seq	Hamby *et al.* (1988)
		NS2	GGC TGC TGG CAC CAG ACT TGC	reverse	seq	White *et al.* (1990)
		NS4	CTT CCG TCA ATT CCT TTA AG	reverse	seq	White *et al.* (1990)
	Ulvophyceae	NS1	GTA GTC ATA TGC TTG TCT C	forward	amp	White *et al.* (1990)
		18L	CAC CTA CGG AAA CCT TGT TAC GAC TT	reverse	amp	Hamby *et al.* (1988)
		18S SEQ 1759R	CCT TGT TAC GAC TTC TCC TTC CTC	reverse	amp/seq	Friedl (this study)
		18S SEQ 20F	GTA GTC ATA TGC TTG TCT C	forward	amp/seq	Friedl (this study)
		18S SEQ 536	GAG CTG GAA TTA CCG CGG CTG CTG G	reverse	seq	Friedl (this study)
		18S SEQ 373F	TTC GAT TCC GGA GAG GGA G	forward	seq	Friedl (this study)
		18S SEQ 1122R	CAA TTC CTT TAA GTT TCA GCC	reverse	seq	Friedl (this study)
		18S SEQ 891	GTC AGA GGT GAA ATT CTT GGA	forward	seq	Friedl (this study)
		18S SEQ 422	CTA AGG GCA TCA CAG ACC TG	reverse	seq	Friedl (this study)
		18S SEQ 1122F	GGG GCT AAA TAG TAA GGG ATT G	forward	seq	Friedl (this study)
***rbc*L**	*Heterococcus*	1FL	AGT GAC CGT TAT GAA TCT G	forward	amp/seq	Negrisolo *et al.* (2004)
		2RL	GAG AGA ACG TTT CCT TTA CT	reverse	amp/seq	Negrisolo *et al.* (2004)
	Trebouxiophyceae	PRASF1	ATG GTT CCA CAA ACA GAA AC	forward	amp/seq	Sherwood *et al.* (2000)
		PRASR1	TTG TCA ATA GTA TCA AAT TC	reverse	amp/seq	Sherwood *et al.* (2000)
		a-ch-rbcL-203-5′-MPN	GAA TCW TCW ACW GGW ACT TGG ACW AC	forward	amp/seq	Nelsen *et al.* (2011)
		a-ch-rbcL-991-3′-MPN	CCT TCT ART TTA CCW ACA AC	reverse	amp/seq	Nelsen *et al.* (2011)
	Ulvophyceae	PRASF1	ATG GTT CCA CAA ACA GAA AC	forward	amp/seq	Sherwood *et al.* (2000)
		RH1	ATG TCA CCA CAA ACA GAA ACT AAA GC	forward	amp/seq	Manhart (1994)
		DilRBCL1R	TCC ATT TGC AAG CAG CAC GGA TAA	reverse	amp/seq	Gueidan (this study)
		1385r	AAT TCA AAT TTA ATT TCT TTC C	reverse	amp/seq	Manhart (1994)

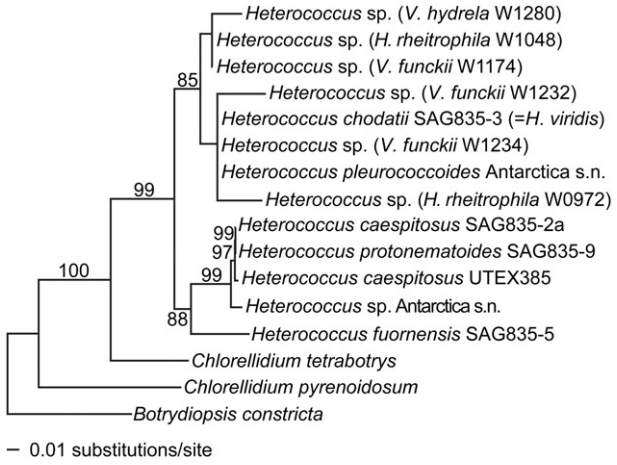


Fig. 1. Phylogenetic placement of the xanthophycean photobionts of Verrucariaceae. The tree was obtained using a maximum-likelihood analysis of a two-gene dataset (*rbc*L–nuSSU). Lichenized algal strains are indicated in parentheses with the name of the corresponding lichen-forming fungus and its collection number. Bootstrap values >70% are indicated above or below the branches.

Within the Xanthophyceae (Heterokontophyta), all of the lichen-associated algal isolates belonged to the same genus, *Heterococcus* (Fig. 1). In the Ulvophyceae, all the photobiont isolates belonged to the genus *Dilabifilum*. In the Trebouxiophyceae (Figs 2 and 3), the photobiont isolates belonged to several genera, which were delimited here according to Ettl & Gärtner (1995), Skaloud & Peksa (2010) and Guiry & Guiry (2011).

*Phylogeny of the xanthophycean photobionts (*Heterococcus *spp., Fig. 1)*

Sequences of all six photobiont isolates of *Heterococcus* clustered together with sequences of the authentic strain of *H. chodatii* Vischer (a synonym of *H. viridis* Chodat) and an isolate of *H. pleurococcoides* Pitschmann (85% bootstrap). *Heterococcus caespitosus*, the species thought to be the photobiont of the sampled species of Verrucariaceae belonged to a different well-supported clade, together with *H. protonematoides* Vischer and an unidentified specimen of *Heterococcus* (99% bootstrap). All our *Heterococcus* isolates were associated with freshwater Verrucariaceae from periodically or permanently submerged habitats. The mycobionts of these lichens [*Hydropunctaria rheitrophila* (Zschacke) C. Keller, *Verrucaria funckii* (Sprengel) Zahlbruckner and *V. hydrela* Acharius] do not belong to a unique lineage, but are members of three distantly related clades (Fig. 4).

The initial morphological characteristics in culture of these lichen-associated *Heterococcus* resembled *H. caespitosus*, with elongated and frequently branched filaments. However, after a few months our isolates underwent changes in cell morphology and thallus appearance: the filaments disintegrated and our isolates grew instead in short and weakly branched chains with rounded to ovoid cells. When transferred to fresh agar media, filaments with slender cells temporarily re-appeared for a few weeks (Fig. 5), but they never reached the lengths achieved by filaments of the young thalli when first isolated from wild-collected lichens. In liquid culture, cells always remained oval to spherical and grew in small aggregates or chains (Fig. 6). The morphology of the photobiont *Heterococcus* was therefore intermediate between *H. pleurococcoides*, which tends to have shorter chains or cell clusters, and *H. viridis* (syn. *H. chodatii*), which first develops branched thalli but soon separates into individual cells.

*Phylogeny of ulvophycean photobionts (*Dilabifilum *spp., Fig. 2)*

All our isolates of *Dilabifilum* from the Verrucariaceae belonged to a single well-supported clade within the Ulvophyceae (94% bootstrap; Fig. 2). This clade was sister to a lineage including the genera *Kornmannia* and *Blidingia* (94% bootstrap). It contained the type strain of *Dilabifilum arthopyreniae* (Vischer & Klement) Tschermak-Woess, an accessory alga isolated from the shell-colonizing lichen *Collemopsidium halodytes* (Nylander) Grube & B.D. Ryan (syn. *Arthopyrenia kelpii* Körber). The clade also contained algal strains isolated from periodically or permanently submerged Verrucariaceae from freshwater and coastal intertidal habitats. Species from at least three different clades of amphibious Verrucariaceae (*Hydropunctaria, Wahlenbergiella* and *Verrucaria aquatilis* Mudd) were associated with these species of *Dilabifilum* (Fig. 4). In culture, none of our algal strains produced zoospores, which prevented further identification. Other relevant diagnostic characters, such as the shape of the terminal cells and the dimensions of cells in the filaments, were variable even within single thalli of our isolates (Fig. 7).

Phylogeny of trebouxiophycean photobionts (Figs 2 and 3)

In our study, we found sequences of *Trebouxia sensu stricto* in only two species, *Bagliettoa marmorea* (Scopoli) Gueidan & Cl. Roux and *Hydropunctaria maura* (Wahlenberg) C. Keller, Gueidan & Thüs. It is very likely that the sequence of *Trebouxia* from *H. maura* represents an epiphyte instead of the actual photobiont, because this

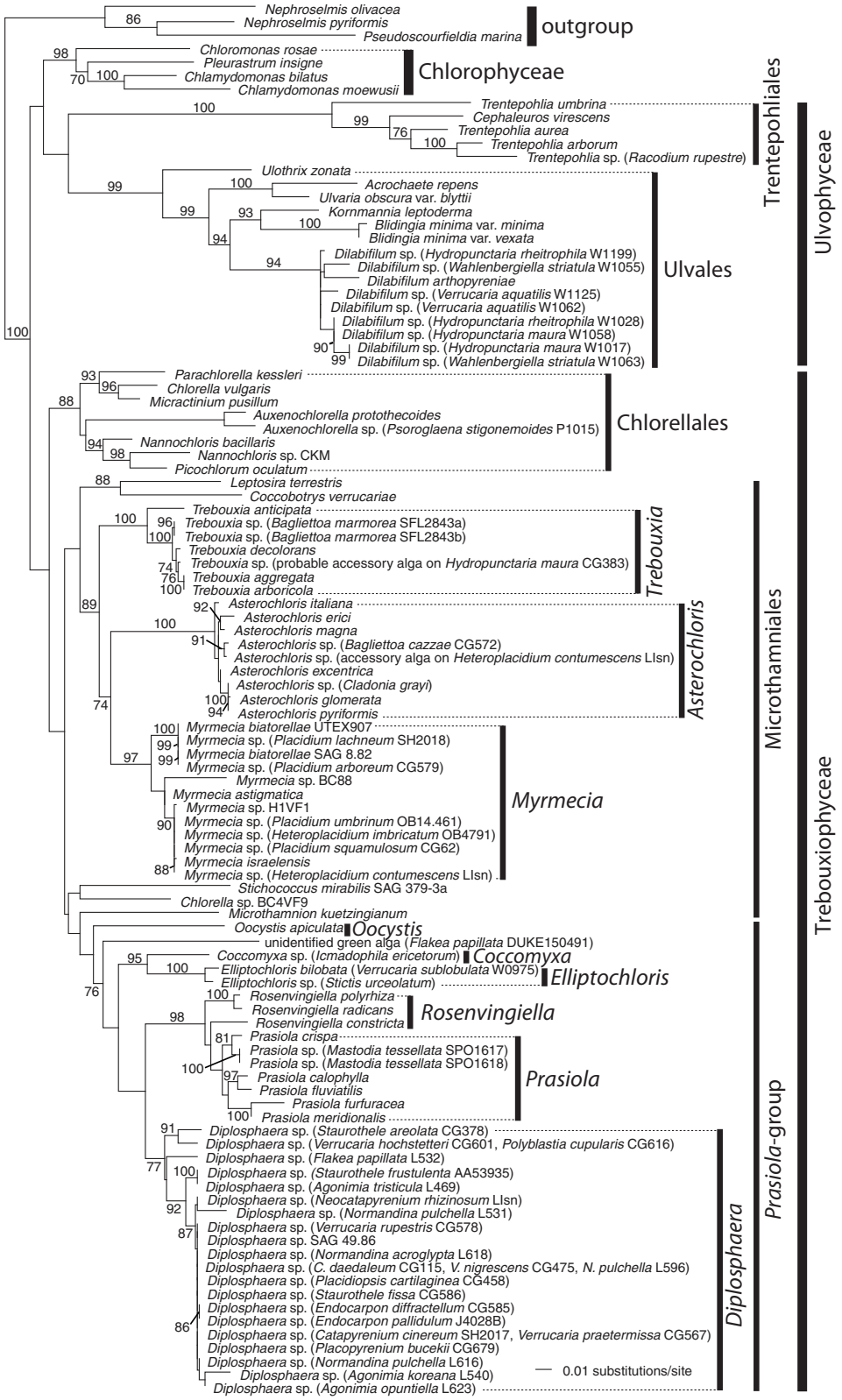


Fig. 2. Phylogenetic placement of chlorophyte photobionts of Verrucariaceae. The tree was obtained using a maximum-likelihood analysis of a two-gene dataset (*rbc*L–nuSSU). Lichenized algal strains are indicated by the addition, in parentheses, of the name of the corresponding lichen-forming fungus. They all belong to the family Verrucariaceae, except for *Cladonia grayi*, *Icmadophila ericetorum*, *Lobaria linita*, *Racodium rupestre*, *Stictis urceolatum* and *Xanthoria parietina*. For the Verrucariaceae, the collection numbers follow the fungal names. Bootstrap values >70% are indicated above or below the branches. Photobionts with identical sequences are represented by a single taxon in the phylogeny with the names and collection numbers of each lichen indicated.

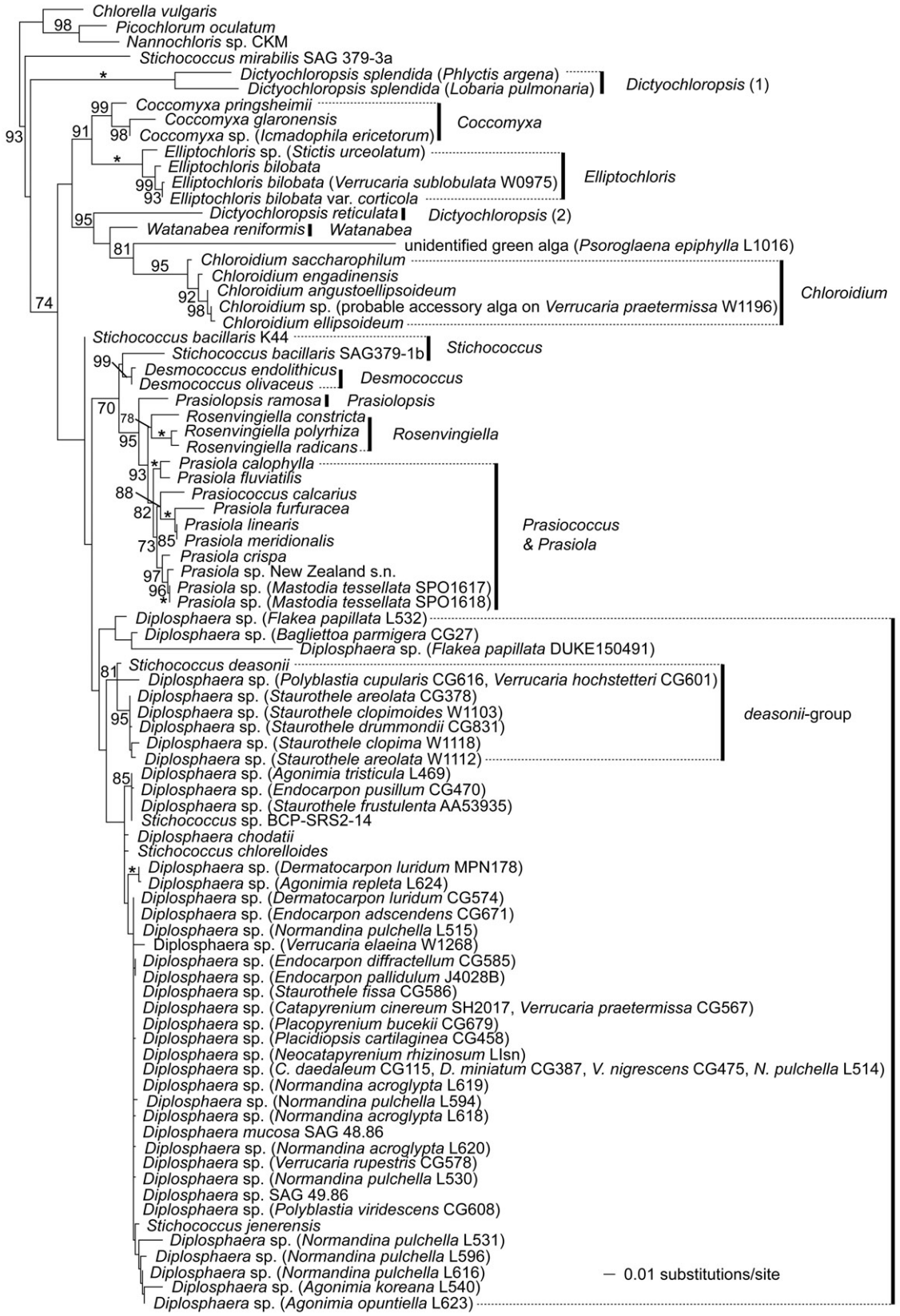


Fig. 3. Phylogenetic placement of the photobionts of Verrucariaceae related to the *Prasiola*-group. The tree was obtained using a maximum-likelihood analysis of a two-gene dataset (*rbc*L–nuSSU). Lichenized algal strains are indicated by the addition, in parentheses, of the name of the corresponding lichen-forming fungus. They all belong to the family Verrucariaceae, except for *Icmadophila ericetorum*, *Lobaria pulmonaria*, *Phlyctis argena* and *Stictis urceolatum*. For the Verrucariaceae, the collection numbers follow the fungal names. Bootstrap values >70% are indicated above or below the branches, except for values of 100%, which are indicated by a star. Photobionts with identical sequences are represented by a single taxon in the phylogeny with the names and collection numbers of each lichen indicated.

lichen species has repeatedly been found associated with a filamentous alga using morphological methods (including in this present study), and not with coccal *Trebouxia*-like algae. The coccal *Trebouxia sensu stricto* from *B. marmorea* has been found in two specimens (Favero-Longo *et al.*, 2009; Fig. 2). Other sampled species of *Bagliettoa* were associated with different photobionts: *B. cazzae* (Zahlbruckner) Vězda & Poelt was found with *Asterochloris* sp. and *B. parmigera* (J. Steiner) Vězda & Poelt with *Diplosphaera* sp. (Figs 2 and 3).

Sister to *Trebouxia* were the genera *Myrmecia* and *Asterochloris* (Fig. 2), which include both lichenized and free-living species. According to our taxon sampling, two species of *Myrmecia*, *M. biatorellae* J.B. Petersen and *M. israelensis* (S. Chantanachat & H. Bold) T. Friedl, can be found as photobionts of the Verrucariaceae. They are exclusively associated with the two closely related lichen genera *Placidium* and *Heteroplacidium* (Fig. 4), and are found in terricolous, saxicolous and corticolous species. In *Asterochloris*, our culture-independent method yielded one algal genotype found as an accessory alga on *H. contumescens* (Nylander) Breuss and involved in symbiosis with the fungal species *Bagliettoa cazzae* (Fig. 2). However, the identity of the photobiont of *B. cazzae* needs confirmation as our results are based on the observation of a single specimen.

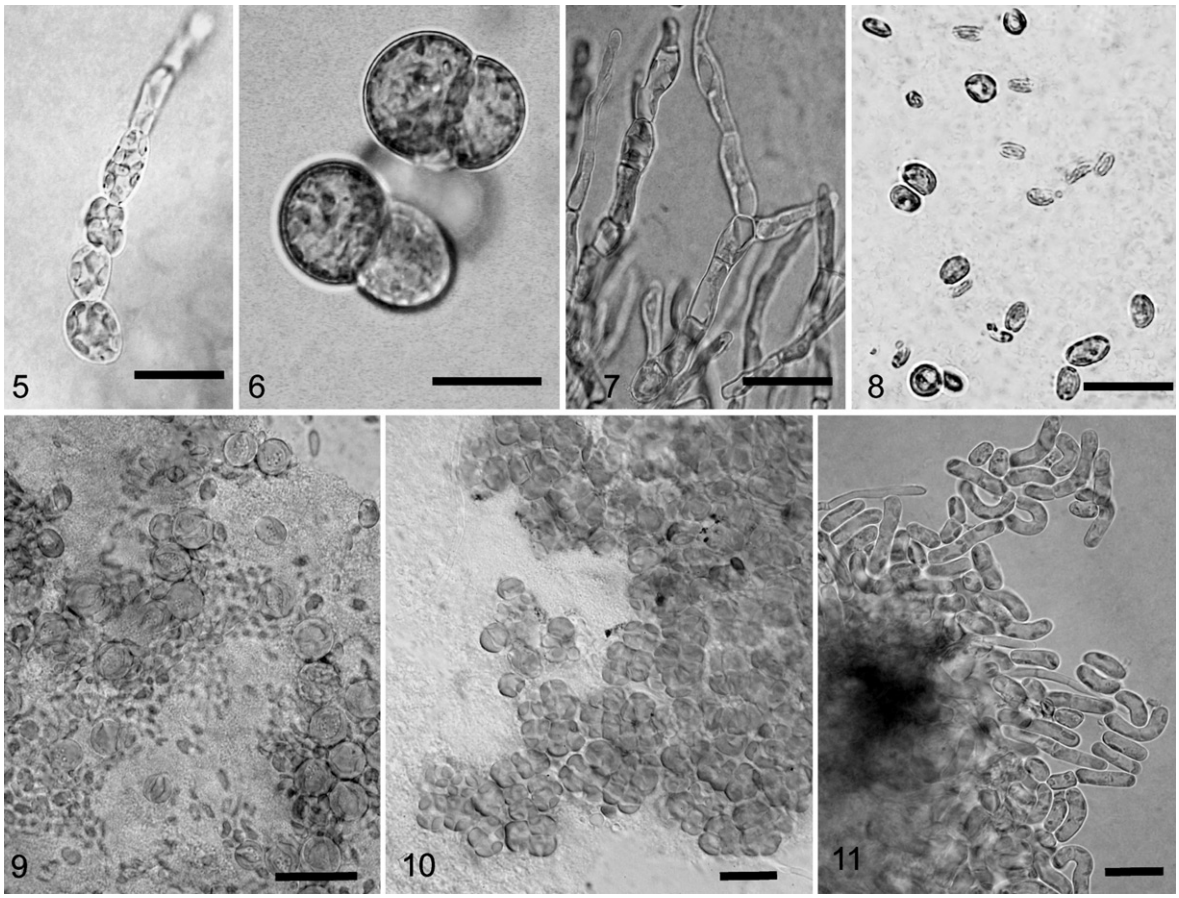
The majority of trebouxiophycean photobionts were within the *Prasiola*-group (Figs 2 and 3). The sequence of one of our photobiont isolates, which came from the amphibious lichen *Verrucaria praetermissa* (Trevisan) Anzi, was nested within the genus *Chloroidium* Nadson (Fig. 3). The minute ellipsoidal cells with a single parietal chloroplast observed in our isolates of *Chloroidium* fit well the description of *C. ellipsoideum* (Gerneck) Darienko, Gustavs, Mudimu, C.R. Menendez, R. Schumann, U. Karsten, Friedl & Pröschold. However, our morphological observations suggested that *Chloroidium* in this lichen species may represent an accessory alga, and not the principal photobiont. Although we have isolated *Chloroidium* repeatedly from thalli of *V. praetermissa*, only in one case were the algal cells directly connected to fungal hyphal walls from the lichen thallus (Fig. 8). However, such cellular contacts were often observed with *Diplosphaera* cells in the same thalli.

In contrast, the molecular identification of the photobiont of *Verrucaria sublobulata* Servít as *Elliptochloris bilobata* Tschermak-Woess was reliable, as microscopical observations confirmed the presence of cellular contacts between the symbionts. Our isolates showed the characteristic morphology of *E. bilobata*, with a deeply divided parietal chloroplast, the formation of rod-like autospores, and numerous transparent droplets in the centre of the cell (Fig. 9). In our phylogeny, this photobiont was part of a well-supported clade together with lichenized and non-lichenized strains of *Elliptochloris* (100% bootstrap; Fig. 3) and its sequence was almost identical to the epiphytic *E. bilobata* var. *corticola* Eliáš, Neustupa & Škaloud (1 bp difference in the nuSSU sequence; Fig. 3).

A large number of Verrucariaceae species were associated with coccoid algae with a parietal unlobed chloroplast, which only covered up to two-thirds of the cell wall. These algal cells often formed characteristic three- to four-celled clusters in early growth stages after their isolation from the lichen thallus. A representative strain of this photobiont was our isolate W1269 from *Verrucaria elaeina* Borrer (Fig. 10). The sequence generated from this photobiont strain fell within a large unsupported group of taxa within the *Prasiola* group (Fig. 3), which can be assigned to the genus *Diplosphaera sensu lato* (including some species of *Stichococcus*). This group also included a large number of sequences from lichen thallus DNA-extracts of various Verrucariaceae. A lineage formed by the genera *Desmococcus*, *Rosenvingiella* and *Prasiola* was sister to the *Diplosphaera* clade, although this relationship was without statistical support. The type species of *Stichococcus*, *S. bacillaris* Nägeli, represented here by two published strains SAG 379-1b and K44, was not nested within *Diplosphaera*: one strain (SAG 379-1b) was sister to *Desmococcus* and the other (K44) lay at the base of a lineage including *Desmococcus*, *Diplosphaera*, *Prasiococcus*, *Prasiola*, *Prasiolopsis*, *Rosenvingiella* and *S. bacillaris* SAG 379-1b. The phylogenetic position of *S. bacillaris* is still problematic, because neither of the placements of these two strains was significantly supported in our phylogeny. The authentic strains of *S. deasonii* Neustupa, Eliáš & Šejnohová, *S. jenerensis* Neustupa, Eliáš & Šejnohová and *Diplosphaera mucosa* Broady were nested within *Diplosphaera*, as well as a strain of *S. chlorelloides* Grintzesco & Péterfi.

A high bootstrap support (95% bootstrap; Fig. 3) was obtained in our phylogeny for a group of photobionts morphologically similar to *Stichococcus mirabilis* Lagerheim. This group was nested among lichenized strains of *Diplosphaera* and sister to *S. deasonii* and a genotype of *Diplosphaera* obtained from the lichen thalli of *Polyblastia cupularis* A. Massalongo and *Verrucaria hochstetteri* Fries. Photobiont cells isolated from *Staurothele* had rod-like cells at first, becoming more or less curved in culture, and one or more deeply lobed chloroplasts (Fig. 11). Based

Fig. 4. Distribution of photobionts across a phylogeny of the Verrucariaceae obtained using a maximum-likelihood analysis of a four-gene dataset (*RPB*1–nuLSU–nuSSU–mtSSU). Lichen specimens for which molecular data are available for the photobionts are indicated in bold. For these specimens, the identity of the photobiont is shown after the taxon label using the following abbreviations: Ast = *Asterochloris*, Dil = *Dilabifilum*, Dip = *Diplosphaera*, Dip/dea = *Diplosphaera deasonii*-group (*Stichococcus* aff. *mirabilis*), Ell = *Elliptochloris*, Het = *Heterococcus*, Myr = *Myrmecia*, Pet = *Petroderma maculiforme*, Pra = *Prasiola*, Tre = *Trebouxia*. *For *Hydropunctaria rheitrophila*, two other specimens not included in this tree (W1048 and W0972) were associated with *Heterococcus* sp. (see Fig. 1).



Figs 5–11. Light photomicrographs of algae isolated from crustose lichens of the family Verrucariaceae. **5.** Filamentous growth in young culture of *Heterococcus* sp. isolated from *Verrucaria funckii* and cultured on 1.5% BBM-agar (BM H. Thüs W1174). **6.** Cell aggregates of *Heterococcus* sp. isolated from *V. funckii* and cultured in liquid BBM medium (BM H. Thüs W1174). **7.** *Dilabifilum* sp. isolated from *V. aquatilis* (BM H. Thüs W1125). **8.** *Chloroidium* sp. isolated from *V. praetermissa* (BM H. Thüs W1198). **9.** *Elliptochloris bilobata* isolated from *V. sublobulata* (BM H. Thüs W0975). **10.** *Diplosphaera* sp. isolated from *V. elaeina* (BM H. Thüs W1269). **11.** *Stichococcus* cf. *mirabilis* isolated from *Staurothele clopima* (BM H. Thüs W1118). Scale bars: 10 μm.

on their lichen associates and their morphology, this lineage most likely corresponds to *S. mirabilis sensu* Ahmadjian & Heikkila (1970) although it is distinct from the nuSSU-sequence of the non-lichenized strain of *S. mirabilis* kept as SAG 379-3a, which forms here the earliest branching in the *Prasiola*-group (Figs 2 and 3).

Sequences of algae from lichen DNA-extracts of the species *Normandina pulchella* (Borrer) Nylander and *N. acroglypta* (Norman) Aptroot here represented by multiple specimens, nested within the *Diplosphaera* clade. Algal sequences obtained from the thallus of *Flakea papillata* O.E. Eriksson, a membranous-foliose lichen recently shown to belong to Verrucariaceae (Muggia *et al*., 2009), were also nested within this clade (Fig. 3). Because of an unusually thin sterile thallus composed of only one cell layer and the presence of rhizoids, this lichen has been attributed in the past to algal colonies, bryophytes or prothalli of ferns (Eriksson, 1992). Algal sequences from our lichen extracts of *Flakea papillata* are related to those of the *Diplosphaera*-group in our analysis (Fig. 3). The photobionts of both specimens of *Flakea papillata* (DUKE150491 and L532) belong to a clade also including the photobiont of *Baglietoa parmigera*, although support is lacking for this relationship (Fig. 3). Further work will be necessary to confirm whether any of the two sequences obtained in our study truly belong to the photobiont of *Flakea papillata*.

Phylogeny of the lichen family Verrucariaceae (Fig. 4)

The multigene phylogeny (*RPB*1–nuLSU–nuSSU–mtSSU) was similar to previous studies (Gueidan *et al*., 2007, 2009; Savić *et al*., 2008; Muggia *et al*., 2009, 2010; Pérez-Ortega *et al*., 2010). Additionally, the phylogenetic position of several freshwater species was demonstrated here: *Verrucaria elaeina* was sister to *V. praetermissa*,

and *V. funckii* to *V. submersella* Servít (Fig. 4); *V. hydrela* appeared as sister to *V. dolosa* Hepp. *Verrucaria sublobulata* is sister to a clade including *Atla*, *Henrica*, *Sporodictyon* and *V. rupestris* Schrader, although without support. The species *V. aquatilis*, represented here by two specimens, was on a long branch and its relationship to other Verrucariaceae was not supported.

Discussion

The family Verrucariaceae has long been of great interest to lichenologists and phycologists in determining the breadth of lichen photobiont diversity. Microscopical observations have produced a particularly long list of compatible photobionts for this lichen family (Tschermak-Woess, 1988). More recently, some studies have used molecular data to confirm the identity of some of these photobionts (Friedl & Zeltner, 1994; Peters & Moe, 2001; Nyati *et al*., 2007; Pérez-Ortega *et al*., 2010; Gueidan *et al*., 2011), but our study is the first to sample a large number of species across this family. The new data have allowed us to check the identity and phylogenetic position of photobionts from the Verrucariaceae and investigate the patterns of associations between the symbionts.

Patterns of photobiont associations in Verrucariaceae

The most common photobionts in the Verrucariaceae are from the genus *Diplosphaera*. They are associated with many lichen-forming fungal species, irrespective of their phylogenetic placement in the Verrucariaceae tree (Fig. 4). Both early diverging Verrucariaceae (e.g. *Normandina*) and more recently diverging ones (e.g. *Endocarpon*, *Staurothele*) have strains of *Diplosphaera* as photobionts. The algal genus *Myrmecia*, on the other hand, is restricted to a single lineage within the Verrucariaceae, which includes the two squamulose genera *Heteroplacidium* and *Placidium* (Figs 2 and 4). Although this lineage includes species from different substrata (bark, rock and soil), all sampled species have *Myrmecia* as a photobiont. It is therefore likely that photobiont associations in this group of lichens with complex thalli result mostly from common ancestry. Similarly, in the foliose genus *Dermatocarpon*, an amphibious species (*D. luridum*) and a species from exposed rock surfaces (*D. miniatum*) both have *Diplosphaera* as a photobiont (Fig. 4).

In contrast to lichens with complex thalli, species with simple crustose thalli (e.g. *Wahlenbergiella*, *Hydropunctaria*, *Bagliettoa*) and often lacking an upper cortex, are characterized by a high diversity of photobionts. In particular, the amphibious lineage that includes *Wahlenbergiella* and *Mastodia* is characterized by unusual photobionts: *Mastodia tessellata* is associated with *Prasiola* sp., *W. tavaresiae* with *Petroderma maculiforme* and *W. striatula* (Wahlenberg) Gueidan & Thüs with *Dilabifilum* sp. (Fig. 4). Other amphibious species, such as *H. rheitrophila*, are known to associate with different photobionts depending on the ecological conditions (Thüs & Schultz, 2008). Moreover, endolithic species of *Bagliettoa* are among the few Verrucariaceae associated with *Trebouxia*. For these amphibious and endolithic lineages, switches in photobionts are probably common and photobiont associations most likely result from ecological conditions.

In Verrucariaceae, genera with complex thalli seem to have a higher specificity for their photobionts than taxa with crustose thalli, which is consistent with observations in other groups of lichens (Helms, 2003; Blaha *et al*., 2006; Honegger, 2009). A complex thallus with a highly structured and developed cortex may prevent lichen-forming fungi from interacting with and adopting other photobionts from their environment, whereas a simple thallus lacking a cortex may perhaps allow greater freedom to develop alternative associations. Except for a few lineages of squamulose species most Verrucariaceae have simple crustose thalli and many lack a cortex. A simple morphology and a diverse ecology might therefore explain the high photobiont diversity found in this lichen family.

Revision of the identity of photobionts associated with Verrucariaceae

Heterococcus. In the past, *H. caespitosus* was the only species of this xanthophyte genus reported as a photobiont of Verrucariaceae, being described from *Verrucaria elaeomelaena* (A. Massalongo) Arnold, *V. funckii* (Tschermak, 1941*b*; Zeitler, 1954; Tschermak-Woess, 1988) and *V.* cf. *praetermissa* (Zeitler, 1954, under the older synonym *V. laevata* auct.). It was also described from *Hydropunctaria maura* (Parra & Redon, 1977), but this record is most probably based on specimens representing an undescribed lichen species and not *H. maura*, because the reported ascospore size falls outside the usual range for this species. Our results demonstrate that the identity of *Heterococcus* species associated with amphibious Verrucariaceae has to be revised. None of our studied specimens of *H. maura* and *V. praetermissa* had *Heterococcus* as a photobiont. More importantly, none of the *Heterococcus* isolates sequenced

here form a monophyletic clade with *Heterococcus caespitosus*, but they are more closely related to two other species, *H. pleurococcoides* and *H. chodatii*.

The confusion between *H. caespitosus* and the actual *Heterococcus* photobionts may have arisen because of the morphological changes that occur with time when the photobionts are grown in culture. The longer cells and branched filaments present in the initial isolation phase led Thüs & Schultz (2008) to identify *H. caespitosus* as a photobiont of Verrucariaceae, but we show here that the same strains do not in fact belong to this algal species. The challenges caused by changes in cell morphology in *Heterococcus* have been noted previously (e.g. Darling *et al.*, 1987; Lokhorst, 1992; Ettl & Gärtner, 1995). The genetic distances between our isolates and either *H. pleurococcoides* or *H. chodatii* are small and further studies with several more rapidly evolving markers (e.g. ITS rDNA) are required in order to find out if this well-supported clade consists of more than one species or represents a single but morphologically variable taxon. Given the problematic morphological circumscription of species in the genus *Heterococcus*, the earlier record of *H. caespitosus* from *Verrucaria elaeomelaena sensu stricto* (Tschermak, 1941*b*) will require confirmation by molecular data.

Dilabifilum. In a previous study, Friedl & Büdel (2008) showed that *Dilabifilum arthopyreniae* belonged to the Ulvales (Ulvophyceae). Our results confirm this phylogenetic placement with eight additional strains of *Dilabifilum*. There is considerable genetic diversity within the *Dilabifilum* clade but neither the habitat (freshwater vs. saline environments) nor the association with specific lichen taxa corresponds to the observed phylogenetic structure. The morphology of the isolated strains changed considerably over time. Characters affected by these changes, such as the width: length ratio of the cells and the form of terminal cells of the filaments, are regarded as diagnostic at the species level (Johnson & John, 1990). A revision of the genus *Dilabifilum*, including a molecular study of authentic strains of *D. incrustans* (Vischer) Tschermak-Woess, *D. printzii* (Vischer) Tschermak-Woess and *D. prostratum* Broady & Ingerfeld, is needed before a name can be attributed to our isolates.

Auxenochlorella. The photobiont of the granulose *Psoroglaena stigonemoides* (Orange) Henssen has previously been shown to belong to the genus *Auxenochlorella* (Nyati *et al.*, 2007). This is the only representative of the Chlorellales associated with any of the Verrucariaceae.

Asterochloris and *Trebouxia*. Algae from the genera *Asterochloris* and *Trebouxia* are among the most common symbionts in most lichenized fungi but are rare in the Verrucariaceae. In addition to the *Myrmecia rbc*L sequence, a sequence of *Asterochloris* was obtained for *Heteroplacidium contumescens* with the culture-independent method. Because all other members of the *Heteroplacidium–Placidium* lineage associate with *Myrmecia*, this record most likely corresponds to an accessory alga. Our results suggest that species of *Bagliettoa* associate with three different photobiont genera, *Asterochloris*, *Trebouxia* and *Diplosphaera* (Figs 2 and 3). Further studies are needed in order to confirm these records and whether there is low photobiont specificity at the generic level for the endolithic genus *Bagliettoa*.

Myrmecia. *Myrmecia biatorellae* has previously been isolated from various squamulose Verrucariaceae and the amphibious crust *Verrucaria submersella* (Zeitler, 1954). Our data confirm *M. biatorellae* as a photobiont of squamulose Verrucariaceae (*Heteroplacidium* and *Placidium*) and, for the first time, *M. israelensis* has been identified as another photobiont from this lichen family.

Elliptochloris. *Elliptochloris* had been reported as an occasional photobiont in the Verrucariaceae (Thüs & Schultz, 2008). Although most of the amphibious species of *Staurothele* cited in earlier reports of *Elliptochloris* have been included, we failed to find it in any of these lichens. It is therefore likely that *E. bilobata* is an occasional epi- or endophyte but not the principal photobiont of *Staurothele*. However, in our study *E. bilobata* was found in our study associated with *Verrucaria sublobulata* and this record suggests that *Elliptochloris* may have a limited inundation tolerance when occurring in lichens; all other reports of *E. bilobata* from Verrucariaceae refer to collections from rather shaded and humid or temporarily inundated habitats (Tschermak-Woess, 1985; Trembley *et al.*, 2002; Thüs & Schultz, 2008).

Prasiola sp. and *Petroderma maculiforme*. Although our taxon sampling covered several species of the genus *Wahlenbergiella*, the very closely related genus *Mastodia* remains the only lichen with *Prasiola* as a photobiont. In *M. tessellata*, the fungus lives within the thallus of the alga. *Wahlenbergiella*, however, is often associated with various strains of *Dilabifilum* (Ulvales), which occur inside a typical crustose lichen thallus. Despite the fact that *W. striatula* often grows side by side with *Mastodia tessellata* in the Southern Hemisphere, and may sometimes even become

overgrown by free-living *Prasiola*, its photobiont is always *Dilabifilum*. *Verrucaria tavaresiae* R.L. Moe, a species associated with the brown alga *Petroderma maculiforme*, was recently shown to belong to the genus *Wahlenbergiella* (Gueidan *et al*., 2011). In this association, the algal filaments are also embedded within a thallus formed by the fungus (Sanders *et al*., 2004, 2005).

Stichococcus deasonii group. With the exception of *Staurothele fissa* (Taylor) Zwackh and *S. frustulenta* Vainio, all other sampled species of *Staurothele sensu stricto* (species with an epilithic thallus, Gueidan *et al*., 2009) share a genetically and morphologically similar photobiont (Figs 3 and 4). Like *Endocarpon* the genus *Staurothele* is characterized by the presence of algal cells within the fruiting bodies. These algal cells are small (<7 µm) and either cube- or rod-like, whereas algal cells from the thallus are larger (usually >10 µm) and spherical. Ahmadjian & Heikkila (1977) showed using culture experiments that the algae in the fruiting bodies of *Staurothele clopima* (Wahlenberg) Th. Fries are the same as the larger rounded cells from the thallus and they identified them as *Stichococcus mirabilis*. This alga differs from the closely related *S. deasonii* in the absence of a pyrenoid, the longer and often slightly curved cells, a lobed chloroplast, and the absence of filaments when cultured on solid media. Our isolates, which include collections from Europe and North America, fit this description, although some cells are strongly curved. However, our isolates do not cluster with a North American strain of *S. mirabilis* (SAG 379-3a) for which a sequence is available in GenBank, so further work will be necessary to confirm the identity of the elongated photobionts of *Staurothele sensu stricto*.

Diplosphaera. Our molecular and morphological observations indicate that earlier reports of photobionts from the poorly defined genera *Diplosphaera*, *Protococcus* and *Stichococcus* all seem to correspond in fact to members of the genus *Diplosphaera*. Historically, and particularly in the context of lichen symbiosis, the genus *Protococcus* has been used to accommodate species lacking clear diagnostic characters (Stahl, 1874; Winter, 1875; Zeitler, 1954). Moreover, the genera *Stichococcus* and *Diplosphaera* have always been difficult to differentiate by morphological characters. *Diplosphaera* differs from *Stichococcus* by the formation of two-celled clusters (Ettl & Gärtner, 1995). Both species and genus delimitations in this group of algae are poorly known.

Řeháková (1968) regarded *S. chlorococcoides* and most of the strains reported as *Protococcus* spp. by earlier authors as synonyms of *Diplosphaera chodatii*, attributing to this species a wide concept. Her illustrations make it clear that she included strains producing large amounts of mucilage, which would usually be referred to as *Diplosphaera mucosa*. Based on her own culture experiments, Řeháková (1968) reported *Diplosphaera chodatii* as the photobiont of *Dermatocarpon arnoldianum* auct., *D. luridum* (Dillenius *ex Withering*) J.R. Laundon, *D. miniatum* (Linnaeus) W. Mann and *D. rivulorum* (Arnold) Dalla Torre & Sarnthein. She added *Polyblastia nigella* Krempelhuber, *Staurothele caesia* (Arnold) Arnold, *S. clopima*, *S. clopimoides* Baglietto & Carestia) J. Steiner, *S. fissa*, *S. frustulenta*, *S. rufa* (A. Massalongo) Zschacke, *Verrucaria acrotelloides* A. Massalongo, *V. dufourii* De Candolle, *V. myriocarpa* Lönnroth and *V. tristis* (A. Massalongo) Krempelhuber to her list of compatible mycobionts, based on illustrations from Winter (1875), Stahl (1874) and Zeitler (1954). These observations agree with our results, as our sequences of *Diplosphaera* were found associated with the same groups of mycobionts as those cited by Řeháková (1968). As in other related algal genera (e.g. Rindi *et al*., 2007), the genetic variation among the strains of the *Diplosphaera* for the nuSSU and *rbc*L gene regions is low, but the morphological and ecological variations are important. Faster evolving markers are required in order to study species delimitation in this morphologically diverse group of lichen photobionts and free-living algae.

Nannochloris normandinae. Tschermak-Woess (1988) described the isolated photobiont of *Normandina pulchella* as *Nannochloris normandinae* Tschermak-Woess, which differs from species of *Stichococcus* and *Diplosphaera* in the presence of a pyrenoid. In our study, none of the photobionts isolated from the ten specimens of *Normandina pulchella* and *N. acroglypta* belonged to *Nannochloris*. Instead, they were *Diplosphaera* species. Moreover, our phylogeny shows that *Stichococcus jenerensis*, another species with a well-developed pyrenoid, is part of the large *Diplosphaera* clade. The authentic strain of *Nannochloris normandinae* could not be located, so new culture experiments will be required in order to verify if *N. normandinae* belongs in fact to *Diplosphaera* or if species of *Normandina* have different photobionts than previously reported.

Culture-dependent versus culture-independent methods

According to our results, amphibious lichens seem to be particularly liable to produce erroneous records of photobionts when using PCR methods

directly on environmental samples of thalli. All our attempts to amplify *rbc*L or nuSSU of species of *Dilabifilum* and *Heterococcus* from lichen thallus extracts (using the culture-independent method) have consistently failed, even with specific primers. With general primers, these markers were more easily amplified for other algae occurring as epiphytes or as photobionts of neighbouring lichens. This amplification bias in the culture-independent method can lead to confusing results. For example, in coastal intertidal environments, *Dilabifilum*-associated lichens often grow side by side with *Caloplaca* species, and are sometimes overgrown by them, such as *C. microthallina* Weddell on *Hydropunctaria maura*. Using the culture-independent method, we have recovered a sequence of *Trebouxia* from *H. maura*, whereas the culture-dependent method confirms that *H. maura* is associated with *Dilabifilum*. Similarly, a previous record of *Trebouxia* from *Verrucaria praetermissa* (Thüs, 2002) is refuted by our new data.

Fast-growing algae are occasionally found on the surface and in the cracks of the lichen thallus. These accessory algae can also contaminate photobiont cultures obtained from thallus fragments and can easily be mistaken for the photobiont unless the fragments are checked for the connection between algal cells and fungal hyphae. Accessory algae have been found in lichen thalli of various families of lichen-forming fungi (Beck *et al*., 1998, 2002; Helms *et al*., 2001; Thüs, 2002; Lakatos *et al*., 2004; Honegger, 2009) and their involvement in the lichen symbiosis merits further studies.

Conclusion

Our study provides the first molecular confirmation of the identity of photobionts from a large number of species within the Verrucariaceae and helps to identify areas of conflict between morphology- and molecular-based identifications. It also documents the value of complementing the fast and easy culture-independent method with the more time consuming culture-dependent method. Moreover, the use of cultures is essential for differentiating photobionts from accessory algae. Our results show that the link between ecology and photobiont patterns is strong for crustose Verrucariaceae, in particular in amphibious habitats where the diversity of associated algae is the highest. For Verrucariaceae with more complex thalli, the diversity of associated algae is lower and is best explained by common ancestry. Ecological preferences and phylogenetic relatedness are therefore both involved in shaping the patterns of photobiont associations in the lichen family Verrucariaceae.

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Supplementary material

The following supplementary material is available for this article, accessible via the Supplementary Content tab on the article's online page at http://dx.doi.org/10.1080/09670262.2011.629788

References

Ahmadjian, V. (1993). *The Lichen Symbiosis*. 2nd ed. New York: John Wiley.

Ahmadjian, V. & Heikkila, H. (1970). The culture and synthesis of *Endocarpon pusillum* and *Staurothele clopima*. *Lichenologist*, **4**: 259–267.

Beck, A. & Koop, H.-U. (2001). Analysis of the photobiont population in lichens using a single-cell manipulator. *Symbiosis*, **31**: 57–67.

Beck, A., Friedl, T. & Rambold, G. (1998). Selectivity of photobiont choice in a defined lichen community: inferences from cultural and molecular studies. *New Phytol*., **139**: 709–720.

Beck, A., Kasalicky, T. & Rambold, G. (2002). Mycophotobiontal selection in a Mediterranean cryptogam community with *Fulgensia fulgida*. *New Phytol*., **153**: 317–326.

Bischoff, H.W. & Bold, H.C. (1963). Phycological studies IV. Some soil algae from Enchanted Rock and related algal species. *Univ. Texas Publ*., **6318**: 1–95.

Blaha, J., Baloch, E. & Grube, M. (2006). High photobiont diversity associated with the euryoecious lichen-forming ascomycete *Lecanora rupicola* (Lecanoraceae, Ascomycota). *Biol. J. Linn. Soc*., **99**: 283–293.

CABI, CBS & Landcare Research (2011). *Index Fungorum*. http// www.indexfungorum.org, searched on 27/04/2011

Dahlkild, Å., Källersjö, M., Lohtander, K. & Tehler, A. (2001). Photobiont diversity in Physciaceae (Lecanorales). *Bryologist*, **104**: 527–536.

Darling, R.B., Friedmann, E.I. & Broady, P.A. (1987). *Heterococcus endolithicus* sp. nov. (Xanthophyceae) and other terrestrial *Heterococcus* species from Antarctica: morphological changes during life history and response to temperature. *J. Phycol*., **23**: 598–607.

Eriksson, O. E. (1992). *Psoroglaena cubensis* and *Flakea papillata* gen. et sp. nov., two corticolous lichens with a pantropical distribution. *Systema Ascomycetum*, **11**: 11–27.

Ettl, H. & Gärtner, G. (1995). *Syllabus der Boden-, Luft- und Flechtenalgen*. Stuttgart: Gustav Fischer.

Favero-Longo, S.E., Borghi, A., Tretiach, M. & Piervittori, R. (2009). *In vitro* receptivity of carbonate rocks to endolithic lichen-forming aposymbionts. *Mycol. Res*., **113**: 1216–1227.

Fernández-Mendoza, F., Domaschke, S., García, M.A., Jordan, P., Martín, M.P. & Printzen, C. (2011). Population

structure of mycobionts and photobionts of the widespread lichen *Cetraria aculeata*. *Mol. Ecol.*, **20**: 1208–1232.

Friedl, T. & Büdel, B. (2008). Photobionts. In *Lichen Biology*. 2nd ed. (Nash, T. H. III, editor), 7–26. Cambridge University Press, Cambridge.

Friedl, T. & Zeltner, C. (1994). Assessing the relationships of some coccoid green lichen algae and the Microthamniales (Chlorophyta) with 18S ribosomal RNA gene sequence comparisons. *J. Phycol.*, **30**: 500–506.

Gueidan, C., Roux, C. & Lutzoni, F. (2007). Using a multigene analysis to assess generic delineation and character evolution in the Verrucariaceae (Eurotiomycetes, Ascomycota). *Mycol. Res.*, **111**: 1147–1170.

Gueidan, C., Savić, S., Thüs, H., Roux, C., Keller, C., Tibell, L., Prieto, M., Heimarsson, S., Breuss, O., Orange, A., Fröberg, L., Amtoft Wynns, A., Navarro-Rosinés, P., Krzewicka, B., Pykälä, J., Grube, M. & Lutzoni, F. (2009). Generic classification of the Verrucariaceae (Ascomycota) based on molecular and morphological evidence: recent progress and remaining challenges. *Taxon*, **58**: 184–208.

Gueidan, C., Thüs, H. & Pérez-Ortega, S. (2011). Phylogenetic position of the brown algae-associated lichenized fungus *Verrucaria tavaresiae* (Verrucariaceae). *Bryologist*, DOI: 10.1639/0007-2745-114.3.xxx.

Guiry, M.D. & Guiry, G.M. (2011). *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org; searched on 27 April 2011.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acid Symp. Ser.*, **41**: 95–98.

Hamby, R.K., Sim, L.E. & Zimmer, E.A. (1988). Direct RNA sequencing: optimisation of extraction and sequencing techniques for work with higher plants. *Plant. Mol. Biol. Rep.*, **6**: 179–197.

Helms, G. (2003). *Taxonomy and symbiosis in associations of Physciaceae and* Trebouxia. Dissertation zur Erlangung des Doktortitels, Mathematisch-naturwissenschaftliche Fakultäten, Georg-August-Universität Göttingen.

Helms, G., Friedl, T., Rambold, G. & Mayrhofer, H. (2001). Identification of photobionts from the lichen family Physciaceae using algal-specific ITS rDNA sequencing. *Lichenologist*, **33**: 73–86.

Honegger, R. (2009). Lichen-forming fungi and their photobionts. In *Plant relationships V, The Mycota*. 2nd ed. (Deising, H.B., editor), 305–333. Springer-Verlag, Berlin.

Johnson, L.R. & John, D.M. (1990). Observations on *Dilabifilum* (Class Chlorophyta, order Chaetophorales sensu stricto) and allied genera. *Eur. J. Phycol.*, **25**: 53–61.

Kohlmeyer, J. & Volkmann-Kohlmeyer, B. (1998). *Mycophycias*, a new genus for the mycobionts of *Apophlaea*, *Ascophyllum* and *Pelvetia*. *Systema Ascomycetum*, **16**: 1–7.

Kroken, S. & Taylor, J.W. (2000). Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with the fungal genus *Letharia*. *Bryologist*, **103**: 645–660.

Lakatos, M., Lange-Bertalot, H. & Büdel, B. (2004). Diatoms living inside the thallus of the green algal lichen *Coenogonium linkii* in neotropical lowland rain forests. *J. Phycol.*, **40**: 70–73.

Lohtander, K., Oksanen, I. & Rikkinen, J. (2003). Genetic diversity of green algal and cyanobacterial photobionts in *Nephroma* (Peltigerales). *Lichenologist*, **35**: 325–339.

Lokhorst, G.M. (1992). Taxonomic studies in the genus *Heterococcus*: (Tribophyceae, Tribonematales, Heteropediaceae); a combined cultural and electron microscopy study. *In Cryptogamic Studies*, vol 3 (Jülich, W., editor). Fischer, Stuttgart.

Lücking, R., Lawrey, J.D., Sikaroodi, M., Gillevet, P.M., Chaves, J.L., Sipman, H.J.M. & Bungartz, F. (2009). Do lichens domesticate photobionts like farmers domesticate crops? Evidence from a previously unrecognized lineage of filamentous cyanobacteria. *Am. J. Bot.*, **96**: 1409–1418.

Lutzoni, F., Wagner, P., Reeb, V. & Zoller, S. (2000). Integrating ambiguously aligned regions of DNA sequences in phylogenetic analyses without violating positional homology. *Syst. Biol.*, **49**: 628–651.

Maddison, W.P. & Maddison, D.R. (2003). *MacClade: Analysis of Phylogeny and Character Evolution*. Version 4.6. Sinauer, Sunderland, MA.

Manhart, J.R. (1994). Phylogenetic analysis of green plant rbcL sequences. *Mol. Phylogenet. Evol.*, **3**: 114–127.

Mason-Gamer, R. & Kellogg, E. (1996). Testing for phylogenetic conflict among molecular datasets in the tribe Triticeae (Graminae). *Syst. Biol.*, **45**: 524–545.

Miadlikowska, J., Kauff, F., Hoftstetter, V., Fraker, E., Grube, M., Hafellner, J., Reebe, V., Hodkinson, B.P., Kukwa, M., Lücking, R., Hestmark, G., Otalora, M.G., Rauhut, A., Büdel, B., Scheidegger, C., Timdal, E., Stenroos, S., Brodo, I., Perlmutter, G.B., Ertz, D., Diederich, P., Lendemer, J.C., May, P., Schoch, C.L., Arnold, A.E., Gueidan, C., Tripp, E., Yahr, R., Robertson, C. & Lutzoni, F. (2006). New insights into classification and evolution of the Lecanoromycetes (Pezizomycotina, Ascomycota) from phylogenetic analyses of three ribosomal RNA- and two protein-coding genes. *Mycologia*, **98**: 1088–1103.

Moe, R. (1997). *Verrucaria tavaresiae* sp. nov., a marine lichen with a brown algal photobiont. *Bull. California Lichen Soc.*, **4**: 7–11.

Muggia, L., Gueidan, C., Perlmutter, G.B., Eriksson, O.E. & Grube, M. (2009). Molecular data confirm the position of *Flakea papillata* in the Verrucariaceae. *Bryologist*, **112**: 538–543.

Muggia, L., Gueidan, C. & Grube, M. (2010). Phylogenetic placement of some morphologically unusual members of Verrucariales. *Mycologia*, **102**: 835–846.

Negrisolo, E., Maistro, S., Incarbone, M., Moro, I., Dalla Valle, L., Broady, P.A. & Andreoli, C. (2004). Morphological convergence characterizes the evolution of Xanthophyceae (Heterokontophyta): evidence from nuclear SSU rDNA and plastidial *rbc*L genes. *Mol. Phylogenet. Evol.*, **33**: 156–170.

Nelsen, M.P. & Gargas, A. (2006). Actin type I introns offer potential for increasing phylogenetic resolution in *Asterochloris* (Chlorophyta: Trebouxiophyceae). *Lichenologist*, **38**: 435–440.

Nelsen, M.P., Rivas Plata, E., Andrew, C.J., Lücking, R. & Lumbsch, H.T. (2011). Phylogenetic diversity of trentepohlialean algae associated with lichen-forming fungi. *J. Phycol.*, **47**: 282–290.

Nyati, S., Beck, A. & Honegger, R. (2007). Fine structure and phylogeny of green algal photobionts in the microfilamentous genus *Psoroglaena* (Verrucariaceae, lichen-forming ascomycetes). *Plant Biol.*, **9**: 390–399.

O'Brien, H., Miadlikowska, J. & Lutzoni, F. (2005). Assessing host specialization in symbiotic cyanobacteria associated with four closely related species of the lichen fungus *Peltigera*. *Eur. J. Phycol.*, **40**: 363–378.

Parra, O.O. & Redon, J. (1977). Aislamento de *Heterococcus caespitosus* Vischer ficobionte de *Verrucaria maura* Wahlenb. *Bol. Soc. Biol. Concepción*, **51**: 219–224.

Pérez-Ortega, S., de los Ríos, A., Crespo, A. & Sancho, L.G. (2010). Symbiotic lifestyle and phylogenetic relationships of the bionts of *Mastodia tessellata* (Ascomycota, *incertae sedis*). *Am. J. Bot.*, **97**: 738–752.

Persoh, D., Beck, A. & Rambold, G. (2004). The distribution of ascus types and photobiontal selection in Lecanoromycetes (Ascomycota) against the background of a revised SSU nrDNA phylogeny. *Mycol. Prog.*, **3**: 103–121.

Peters, A.F. & Moe, R.L. (2001). DNA sequences confirm that *Petroderma maculiforme* (Phaeophyceae) is the brown algal phycobiont of the marine lichen *Verrucaria tavaresiae* (Verrucariales, Ascomycota) from central California. *Bull. California Lichen Soc.*, **8**: 41–43.

Rambold, G., Friedl, T. & Beck, A. (1998). Photobionts in lichens: possible indicators of phylogenetic relationships? *Bryologist*, **101**: 392–397.

Řeháková, H. (1968). *Lisejnikové rasy z rodu* Trebouxia, Diplosphaera *a* Myrmecia (*Flechten der Gattungen* Trebouxia, Diplosphaera *und* Myrmecia). Candidate dissertation, Katedra Bot., University of Karlova, Praha.

Rindi, F., McIvor, L., Sherwood, A.R., Friedl, T., Guiry, M.D. & Sheath, R.G. (2007). Molecular phylogeny of the green algal order Prasiolales (Trebouxiophyceae, Chlorophyta). *J. Phycol.*, **43**: 811–822.

Sanders, W.B., Moe, R.L. & Ascaso, C. (2004). The intertidal marine lichen formed by the Pyrenomycete fungus *Verrucaria tavaresiae* (Ascomycotina) and the brown alga *Petroderma maculiforme* (Phaeophyceae): thallus organization and symbiont interaction. *Am. J. Bot.*, **91**: 511–522.

Sanders, W. B., Moe, R. L. & Ascaso, C. (2005). Ultrastructural study of the brown alga *Petroderma maculiforme* (Phaeophyceae) in the free-living state and in lichen symbiosis with the intertidal marine fungus *Verrucaria tavaresiae* (Ascomycotina). *Eur. J. Phycol.*, **40**: 353–361.

Savić, S., Tibell, L., Gueidan, C. & Lutzoni, F. (2008). Molecular phylogeny and systematics of *Polyblastia* (Verrucariaceae, Eurotiomycetes) and allied genera. *Mycol. Res.*, **112**: 1307–1318.

Sherwood, A.R., Garbary, D.J. & Sheath, R.G. (2000). Assessing the phylogenetic position of the Prasiolales (Chlorophyta) using rbcL and 18S rRNA gene sequence data. *Phycologia*, **39**: 139–146.

Skaloud, P. & Peksa, O. (2010). Evolutionary inferences based on ITS rDNA and actin sequences reveal extensive diversity of the common lichen alga *Asterochloris* (Trebouxiophyceae, Chlorophyta). *Mol. Phylogenet. Evol.*, **54**: 36–46.

Stahl, E. (1874). Beiträge zur Entwicklungsgeschichte der Flechten (vorläufige Mittheilung). *Bot. Zeitung*, **1874**: 177–180.

Stamatakis, A., Ludwig, T. & Meier, H. (2005). RAxML-III: A fast program for maximum likelihood-based inference of large phylogenetic trees. *Bioinformatics*, **21**: 456–463.

Stamatakis, A., Hoover, P. & Rougemont, J. (2008). A rapid bootstrap algorithm for the RAxML web-servers. *Syst. Biol.*, **75**: 758–771.

Starr, R.C. & Zeikus, J.A. (1993). UTEX – The culture collection of algae at the University of Texas at Austin. *J. Phycol.*, Suppl. 29.

Thüs, H. (2002). Taxonomie, Verbreitung und Ökologie silicoler Süsswasserflechten im ausseralpinen Mitteleuropa. *Bibl. Lichenol.*, **83**: 1–214.

Thüs, H. & Nascimbene, J. (2008). Contributions toward a new taxonomy of Central European freshwater species of the lichen genus *Thelidium* (Verrucariales, Ascomycota). *Lichenologist*, **40**: 499–521.

Thüs, H. & Schultz, M. (2008). Freshwater Flora of Central Europe, Vol. 21/1: Fungi, Part 1: Lichens. Heidelberg: Spektrum.

Trembley, M.L., Ringli, C. & Honegger, R. (2002). Morphological and molecular analysis of early stages in the resynthesis of the lichen *Baeomyces rufus*. *Mycol. Res.*, **106**: 768–776.

Tschermak, E. (1941a). Beitrag zur Entwicklungsgeschichte und Morphologie der Protococcale *Trochiscia granulata*. *Österr. Bot. Z.*, **90**: 67–73.

Tschermak, E. (1941b). Untersuchungen über die Beziehungen von Pilz und Alge im Flechtenthallus. *Österr. Bot. Z.*, **90**: 233–307.

Tschermak-Woess, E. (1970). Über wenig bekannte und neue Flechtengonidien V. Der Phycobiont von *Verrucaria aquatilis* und die Fortpflanzung von *Pseudopleurococcus arthopyreniae*. *Österr. Bot. Z.*, **118**: 443–455.

Tschermak-Woess, E. (1976). Algal taxonomy and the taxonomy of lichens: the phycobiont of *Verrucaria adriatica*. In *Lichenology: Progress and Problems* (Brown, D.H., Hawksworth, D.L. & Bailey, R.H., editors), 79–88. Academic Press, London.

Tschermak-Woess, E. (1985). *Elliptochloris bilobata* kein ganz seltner Phycobiont. *Herzogia*, **7**: 105–116.

Tschermak-Woess, E. (1988). The algal partner. In *CRC Handbook of Lichenology*, Vol. I (Galun, M., editor), 39–92. CRC Press, Boca Raton.

White, T.J., Bruns, T., Lee, S.B. & Taylor, J.W. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *A Guide to Methods and Applications, PCR Protocols* (Gelfand, M.A., Sninsky, J.J. & White, T.J., editors), 315–322. Academic press, San Diego.

Winter, G. (1875). Ueber die Gattung *Sphaeromphale* und Verwandte. Ein Beitrag zur Anatomie der Krustenflechten. *Pringsh. Jahrb. wiss. Bot.*, **10**: 245–274.

Yahr, R., Vilgalys, R. & DePriest, P.T. (2006). Geographic variation in algal partners of *Cladonia subtenuis* (Cladoniaceae) highlights the dynamic nature of a lichen symbiosis. *New Phytol.*, **171**: 847–860.

Zeitler, I. (1954). Untersuchungen uber die Morphologie, Entwicklungsgeschichte und Systematik von Flechtengonidien. *Österr. Bot. Z.*, **101**: 453–487.

Zhou, S. & Stanosz, G.R. (2001). Primers for the amplification of mtSSU rDNA, and a phylogenetic study of *Botryosphaeria* and associated anamorphic fungi. *Mycol. Res.*, **105**: 1033–1044.

Zolan, M.E. & Pukkila, P.J. (1986). Inheritance of DNA methylation in *Coprinus cinereus*. *Mol. Cell. Biol.*, **6**: 195–200.

Zoller, S., Scheidegger, C. & Sperisen, C. (1999). PCR primers for the amplification of mitochondrial small subunit ribosomal DNA of lichen-forming Ascomycetes. *Lichenologist*, **31**: 511–516.