RESEARCH ARTICLE

Photobiont association and genetic diversity of the optionally lichenized fungus *Schizoxylon albescens*

Lucia Muggia[1], Elisabeth Baloch[2], Edith Stabentheiner[1], Martin Grube[1] & Mats Wedin[3]

[1]Institute of Plant Sciences, Karl-Franzens-University Graz, Graz, Austria; [2]Jodrell Laboratory, Royal Botanic Gardens, Kew, Surrey, UK; and [3]Cryptogamic Botany, The Swedish Museum of Natural History, Stockholm, Sweden

Abstract

The fungus *Schizoxylon albescens* occurs both as lichen and as saprobe. Lichenized colonies grow on the bark of *Populus tremula*; saprotrophic morphs grow on dead *Populus* branches. We wanted to (1) test whether lichenized and saprotrophic *S. albescens* are genetically distinct, (2) investigate photobiont association and diversity, (3) investigate the interactions between fungi and algae that occur during co-cultivation and (4) test whether *Schizoxylon* shows algal selectivity during lichenization. Fungal and algal genetic diversity were investigated for three markers. Algae from lichenized thalli were isolated in axenic cultures, and isolate sequence diversity was compared with algae amplified directly from thallus fragments. Co-culture experiments of fungi and algae were performed to study the morphological interaction patterns. Two distinct phylogenetic units are revealed in *S. albescens*, which are interpreted as phenotypically cryptic species. The algae are related to *Coccomyxa* and *Pseudococcomyxa*, and form two distinct sister clades separating samples isolated in cultures from those amplified directly from thallus fragments, indicating that more easily cultured strains of algae are not necessarily major components of the lichens. *Schizoxylon albescens* interacts with isolated algal strains, similar to fungal–*Coccomyxa* symbioses in nature. As the system is maintained without difficulty in culture, it can potentially be an easily controlled lichen symbiosis study system under laboratory conditions.

Introduction

The lichen life style is one of the most well-known examples of symbiosis. Lichens represent the phenotype of nutritionally specialized fungi, the mycobionts, which derive carbon nutrition from the photobionts (green algae or cyanobacteria) located extracellularly within a matrix of fungal hyphae (Honegger, 1991; Hawksworth & Honegger, 1994). These mutualistic associations can be adapted to extreme environmental conditions, and are often able to colonize habitats where free-living fungi or algae would not survive. The lichen thallus, in which algae are protected by a fungal plectenchyma (Grube & Hawksworth, 2007), is a highly elaborated, exposed and long-living structure, and the morphological appearance is, to a high degree, genetically determined.

In some lichenized fungi, however, the fungal–algal associations are very loose, and only poorly distinguished thalli, if any at all, are formed. Such associations have been called 'primitive lichens' or 'borderline lichens' (Aptroot, 1991; Honegger, 1991; Hawksworth & Honegger, 1994; Kohlmeyer *et al.*, 2004; Grube, 2005). Here, the fungal partners often thrive in a loosely defined microbial consortium, which may include other fungi, various algae and bacteria. During a mainly taxonomic and phylogenetic study on the family *Stictidaceae* (Wedin *et al.*, 2005a, 2006), several species were found to represent borderline lichens. Moreover, they can live either as saprotrophs or as lichens depending on the substrate they grow on, a phenomenon named 'optional lichenization' by Wedin *et al.* (2004). The species showing optional lichenization are indeed very loosely lichenized. In these cases, the fungal life style depends on the substrate (bark or wood primarily of *Populus tremula*), rather than being inconsistent lichenization events on one substrate, as in other reported 'borderline lichens'. Wedin *et al.* (2004) interpreted this phenomenon as a way to
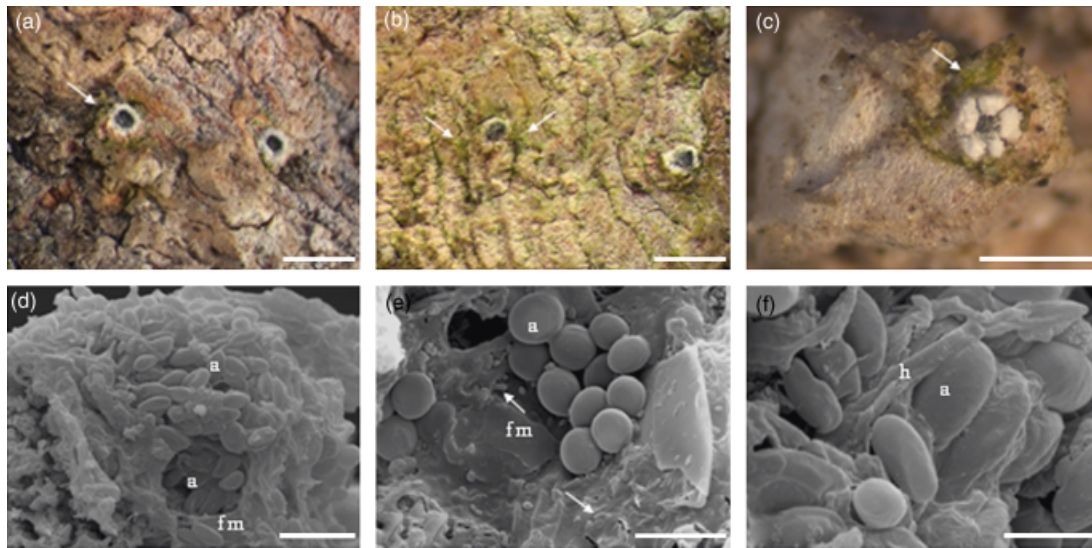
Fig. 1. Habitus of *Schizoxylon albescens* on *Populus* bark. Algal colonies are distributed around the fruit bodies (arrows in a–c; 'a' in d–f): (a) MW8235, (b) MW8249, (c) MW8251 in light microscopy; (d, e) MW8236, (f) MW8238 in SEM. (e) Bacterial (arrows) and algal (a) cells are entangled in the filamentous matrix (fm). (d, f) The filamentous matrix (fm) and the fungal hyphae (h) develop intermixed with the algae clumps (a). Scale bars = 1 mm (a–c), 5 μm (f), 10 μm (e), 20 μm (d).

increase the fungal ecological amplitude as a strategy in fungi adapted to the successional habitat, *Populus* stands in the boreal coniferous forests that regenerate after a major disturbance. In the *Stictidaceae* showing optional lichenization, the lichenized morphs look superficially distinctly different from their conspecific saprotrophic counterparts. When lichenized, the colonies grow on bark, forming whitish areas, with patches of lichenized algae around their ascomata. Alternatively, the saprotrophic forms grow on wood without any associated algae (Wedin *et al.*, 2006).

The recently described *Schizoxylon albescens*, a common, but much-overlooked species in Northern Sweden, was predicted to be an additional example of optional lichenization (Wedin *et al.*, 2006). Lichenized colonies typically grow in and around cracks in the rough bark of older *Populus* trunks (Fig. 1a–c), and saprotrophic morphs [initially interpreted as a distinct species by Wedin *et al.* (2005a)] grow on dead, decorticated *Populus*- and *Salix*-branches. *Schizoxylon* belongs to the *Stictidaceae* in the *Ostropales* (Sherwood, 1977a, b; Lumbsch *et al.*, 2004, 2007; Wedin *et al.*, 2005b; Lumbsch & Papong, 2009), a family with a considerable variation of fungal life styles – saprotrophic, parasitic and lichenized species are all represented (Gilenstam, 1969; Wedin *et al.*, 2004; Baloch *et al.*, 2009).

In this study, we estimate the taxonomic and genetic diversity of fungal and algal components in the *S. albescens* system, and identify the photobionts included in the lichenized populations. We further test whether lichenized and saprotrophic *S. albescens* correspond to two distinct phylogenetic species or whether they are indeed optional lichens,

as several related taxa are. We establish cultures of fungal and algal partners from this symbiotic system, to conduct initial co-cultivation experiments, and verify that interactions take place between the partners, and to study whether the cultures developed differently if wood or bark were added. Finally, co-cultivation experiments were also set up with additional potential photobionts to investigate the range of algal specificity in *Schizoxylon*.

Materials and methods

Sampling

The analysis included both freshly collected (spring and summer 2008) and herbarium material utilized in earlier studies (Wedin *et al.*, 2006). The material is mainly from Northern and Central Sweden; one specimen is from Northern Norway. The localities were grouped into four geographic areas: Lycksele lappmark (lpm), Lule-Pite lpm, Jämtland-Hälsingland and Västerbotten-Ångermanland (Table 1). Sixty-six lichenized samples of *S. albescens* were collected on trunks of *Populus*, and 11 nonlichenized samples on decaying wood. The samples (Table 1) are housed in the herbaria of The Swedish Museum of Natural History in Stockholm and The Museum of Evolution, Uppsala University, Uppsala.

Molecular analyses

In lichenized samples, fruiting bodies surrounded by algae were dissected using a sterile razor blade. DNA was extracted

Table 1. Herbarium, DNA extraction and NCBI accession numbers and geographic origin of the analysed samples of lichenized (bold) and saprotrophic thalli of *Schizoxylon albescens*

Herbarium number		Fungus			Algae		Origin	
		GPDH	Btub	IntTrSp	rbcL	nuSSU		
GG2443a (UPS)	–	–	–	AY661690			Northern Sweden	Lule-Pite lpm
GG2696a (UPS)	CO271	HQ287405	–	DQ401144			Northern Sweden	Lycksele lpm
MW7575 (UPS)	UM21	–	HQ287354	HQ287297			Central Sweden	Jämtland-Hälsingland
PI1473 (UPS)	UM23	–	–	HQ287298			Northern Sweden	Norrbotten
MW7345 (UPS)	UM24	–	–	HQ287299			Northern Sweden	Västerbotten-Ångermanland
MW7919 (UPS)	UM27	HQ287406	HQ287355	HQ287300			Northern Sweden	Lycksele lpm
MWsn04 (UPS)	UM41	HQ287407	HQ287356	HQ287301			Northern Sweden	Västerbotten-Ångermanland
MW7104 (UPS)	UM42	–	HQ287357	HQ287302			Northern Sweden	Västerbotten-Ångermanland
MW7576 (UPS)	UM43	–	–	HQ287303			Central Sweden	Jämtland-Hälsingland
GG2667 (UPS)	UM44	HQ287408	HQ287358	HQ287304			Northern Sweden	Lycksele lpm
MW7199 (UPS)	UM45	HQ287409	HQ287359	HQ287305			North Norway	Norway
GG2646 (UPS)	UM46	HQ287410	HQ287360	HQ287306			Northern Sweden	Lycksele lpm
GG2534a (UPS)	UM48	–	HQ287361	HQ287307			Northern Sweden	Lycksele lpm
MW7645 (UPS)	UM57	HQ287411	HQ287362	HQ287308			Northern Sweden	Lule-Pite lpm
MW7922 (UPS)	EB99	HQ287412	HQ287363	HQ287309			Northern Sweden	Lycksele lpm
MW7898 (UPS)	EB109	–	HQ287364	HQ287310			Northern Sweden	Lule-Pite lpm
MW7664b (UPS)	EB119	HQ287413	HQ287365	HQ287311			Northern Sweden	Lule-Pite lpm
MW6742 (UPS)	EB122	HQ287414	HQ287366	HQ287312			Northern Sweden	Lycksele lpm
MW6735 (UPS)	EB123	–	–	HQ287313			Northern Sweden	Lycksele lpm
MW7675 (UPS)	EB124	HQ287415	HQ287367	HQ287314			Northern Sweden	Lule-Pite lpm
MW7666b (UPS)	EB125	HQ287416	–	–			Northern Sweden	Lule-Pite lpm
GG2448b (UPS)	EB127	HQ287417	–	–			Northern Sweden	Lule-Pite lpm
GG2361 (UPS)	EB128	HQ287418	–	–			Northern Sweden	Lule-Pite lpm
MW7067 (UPS)	EB129	HQ287419	HQ287368	HQ287315			Northern Sweden	Lycksele lpm
MW7036 (UPS)	EB130	HQ287420	HQ287369	HQ287316			Northern Sweden	Lycksele lpm
MW7075 (UPS)	EB131	HQ287421	HQ287370	HQ287317			Northern Sweden	Lycksele lpm
MW7077 (UPS)	EB132	HQ287422	HQ287371	HQ287318			Northern Sweden	Lycksele lpm
MW7078 (UPS)	EB133	HQ287423	HQ287372	HQ287319			Northern Sweden	Lycksele lpm
MW7028 (UPS)	EB134	HQ287424	HQ287373	HQ287320			Northern Sweden	Lycksele lpm
MW7034 (UPS)	EB135	HQ287425	HQ287374	HQ287321			Northern Sweden	Lycksele lpm
MW7020 (UPS)	EB136	HQ287426	HQ287375	HQ287322			Northern Sweden	Lycksele lpm
MW7026b (UPS)	EB137	HQ287427	HQ287376	HQ287323			Northern Sweden	Lycksele lpm
MW7030 (UPS)	EB138	HQ287428	HQ287377	HQ287324			Northern Sweden	Lycksele lpm
GG2691 (UPS)	EB139	HQ287429	HQ287378	HQ287325			Northern Sweden	Lycksele lpm
GG2453 (UPS)	EB140	HQ287430	HQ287379	HQ287326			Northern Sweden	Lycksele lpm
GG2434 (UPS)	EB141	HQ287431	HQ287380	HQ287327			Northern Sweden	Lycksele lpm
GG2354 (UPS)	EB142	HQ287432	HQ287381	HQ287328			Northern Sweden	Lycksele lpm
GG2616 (UPS)	EB143	HQ287433	HQ287382	HQ287329			Northern Sweden	Lule-Pite lpm
GG2622 (UPS)	EB144	HQ287434	HQ287383	–			Northern Sweden	Lule-Pite lpm
GG2365 (UPS)	KK012	HQ287435	HQ287384	AY661689			Northern Sweden	Lule-Pite lpm

Table 1. Continued

Herbarium number		Fungus			Algae		Origin	
		GPDH	Btub	IntTrSp	rbcL	nuSSU		
MW8229 (S)	L541	–	–	–	HQ287461		Central Sweden	Jämtland-Hälsingland
MW8230 (S)	L542	HQ287436	–	HQ287330	HQ287462		Central Sweden	Jämtland-Hälsingland
MW8231 (S)	L543	HQ287437	–	HQ287331	–		Central Sweden	Jämtland-Hälsingland
MW8232 (S)	L544	–	–	HQ287332	HQ287463		Central Sweden	Jämtland-Hälsingland
MW8233 (S)	L545	HQ287438	HQ287385	HQ287333	HQ287464		Central Sweden	Jämtland-Hälsingland
MW8234 (S)	L546	HQ287439	–	HQ287334	HQ287465		Central Sweden	Jämtland-Hälsingland
MW8235 (S)	L547	HQ287440	HQ287386	HQ287335	HQ287466		Central Sweden	Jämtland-Hälsingland
MW8236 (S)	L548	–	–	HQ287336	HQ287467		Central Sweden	Jämtland-Hälsingland
MW8237 (S)	L549	HQ287441	HQ287387	–	–		Central Sweden	Jämtland-Hälsingland
MW8238 (S)	L550	HQ287442	–	HQ287337	HQ287468		Central Sweden	Jämtland-Hälsingland
MW8239 (S)	L551	–	HQ287388	HQ287338	HQ287469		Central Sweden	Jämtland-Hälsingland
MW8240 (S)	L552	HQ287443	–	HQ287339	HQ287470		Central Sweden	Jämtland-Hälsingland
MW8243 (S)	L553	HQ287444	HQ287389	HQ287340	HQ287471		Central Sweden	Jämtland-Hälsingland
MW8245 (S)	L554	HQ287445	HQ287390	HQ287341	–		Central Sweden	Jämtland-Hälsingland
MW8246 (S)	L555	HQ287446	HQ287391	–	–		Central Sweden	Jämtland-Hälsingland
MW8247 (S)	L556	HQ287447	HQ287392	HQ287342	–		Central Sweden	Jämtland-Hälsingland
MW8249 (S)	L557	–	HQ287393	HQ287343	–		Central Sweden	Jämtland-Hälsingland
MW8250 (S)	L558	HQ287448	HQ287394	–	HQ287472		Central Sweden	Jämtland-Hälsingland
MW8251 (S)	L559	HQ287449	HQ287395	HQ287344	HQ287473		Central Sweden	Jämtland-Hälsingland
MW8252 (S)	L560	HQ287450	HQ287396	HQ287345	–		Central Sweden	Jämtland-Hälsingland
MW8253 (S)	L561	HQ287451	HQ287397	HQ287346	HQ287474		Central Sweden	Jämtland-Hälsingland
MW8254 (S)	L562	HQ287452	HQ287398	HQ287347	–		Central Sweden	Jämtland-Hälsingland
MW8207 (S)	L563*	–	–	–	HQ287475	HQ287270	Northern Sweden	Västerbotten-Ångermanland
MW8207 (S)	L564	–	–	–	HQ287476	HQ287271	Northern Sweden	Västerbotten-Ångermanland
MW8207 (S)	L565	–	–	–	HQ287477	HQ287272	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L566	–	–	–	HQ287478	HQ287273	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L567*	–	–	–	HQ287479	HQ287274	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L568*	–	–	–	HQ287480	HQ287275	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L569	–	–	–	HQ287481	HQ287276	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L570	–	–	–	HQ287482	HQ287277	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L571	–	–	–	HQ287483	HQ287278	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L572	–	–	–	HQ287484	HQ287279	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L573	–	–	–	–	HQ287280	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L574	–	–	–	HQ287485	HQ287281	Northern Sweden	Västerbotten-Ångermanland
MW8207/2 (S)	L575	–	–	–	HQ287486	–	Northern Sweden	Västerbotten-Ångermanland
MW8220 (S)	L587*	–	–	–	–	HQ287282	Northern Sweden	Västerbotten-Ångermanland
MW8220 (S)	L588*	–	–	–	–	HQ287283	Northern Sweden	Västerbotten-Ångermanland
MW8220 (S)	L589	–	–	–	–	HQ287284	Northern Sweden	Västerbotten-Ångermanland
MW8220 (S)	L590	–	–	–	–	HQ287285	Northern Sweden	Västerbotten-Ångermanland
MW8224 (S)	L591	–	–	–	–	HQ287286	Northern Sweden	Västerbotten-Ångermanland

Sample	Label						Origin	Region
MW8224 (S)	L592	–	–	–	–	HQ287287	Northern Sweden	Västerbotten-Ångermanland
MW8224 (S)	L593	–	–	–	–	HQ287288	Northern Sweden	Västerbotten-Ångermanland
MW8324 (S)	L598	HQ287453	–	HQ287348	HQ287487		Northern Sweden	Västerbotten-Ångermanland
MW8327 (S)	L599	–	–	HQ287349	HQ287488		Northern Sweden	Västerbotten-Ångermanland
MW8328 (S)	L600	–	–	–	HQ287489		Northern Sweden	Västerbotten-Ångermanland
MW8330 (S)	L601	HQ287454	HQ287399	–	HQ287490		Northern Sweden	Västerbotten-Ångermanland
MW8355 (S)	L602	HQ287455	HQ287400	–	HQ287491		Northern Sweden	Västerbotten-Ångermanland
MW8356 a (S)	L603	HQ287456	HQ287401	–	HQ287492		Northern Sweden	Västerbotten-Ångermanland
MW8356 b (S)	L604	HQ287457	HQ287402	HQ287350	HQ287493		Northern Sweden	Västerbotten-Ångermanland
MW8358 (S)	L605	HQ287458	–	–	–	–	Northern Sweden	Västerbotten-Ångermanland
MW8359 (S)	L606	HQ287459	HQ287403	HQ287351	HQ287494		Northern Sweden	Västerbotten-Ångermanland
MW8360 (S)	L607	–	–	–	HQ287495		Northern Sweden	Västerbotten-Ångermanland
MW8361 (S)	L608	–	–	–	HQ287496		Northern Sweden	Västerbotten-Ångermanland
MW8362 (S)	L609	–	–	–	HQ287497		Northern Sweden	Västerbotten-Ångermanland
MW8363 (S)	L610	–	–	–	HQ287498		Northern Sweden	Västerbotten-Ångermanland
MW8364 (S)	L611	HQ287460	–	HQ287352	–		Northern Sweden	Västerbotten-Ångermanland
MW8365 (S)	L612	–	HQ287404	HQ287353	HQ287499	–	Northern Sweden	Västerbotten-Ångermanland
MW8233 (S) f	L625	–	–	–	HQ287500	HQ287289	Central Sweden	Jämtland-Hälsingland
MW8233 (S) i	L626	–	–	–	HQ287501	HQ287290	Central Sweden	Jämtland-Hälsingland
MW8233 (S) l	L627	–	–	–	HQ287502	HQ287291	Central Sweden	Jämtland-Hälsingland
MW8233 (S) m	L628	–	–	–	HQ287503	HQ287292	Central Sweden	Jämtland-Hälsingland
MW8233 (S) n	L629	–	–	–	HQ287504	HQ287293	Central Sweden	Jämtland-Hälsingland
MW8233 (S) p	L630	–	–	–	HQ287505	HQ287294	Central Sweden	Jämtland-Hälsingland
MW8238 (S) a	L631*	–	–	–	HQ287506	HQ287295	Central Sweden	Jämtland-Hälsingland
MW8238 (S) b	L632	–	–	–	HQ287507	HQ287296	Central Sweden	Jämtland-Hälsingland

Herbarium acronyms are reported in parentheses.

*Label samples for which ITS sequences were also obtained (not reported).

Table 2. Primers used in PCR and sequencing reactions

	Locus amplified	Primer name	Primer sequence	Type	References
Fungus	β-Tubulin gene	BT3LM5	GAACTGCTACTTCAACGAG	PCR, sequencing	Myllys *et al.* (2001)
		BT1910R	TCTGGTCCTCRACCTCCTTCA	PCR, sequencing	Myllys *et al.* (2001)
	Glyceraldehyde-3-phosphate dehydrogenase gene (GPDH)	GPD_schizF	ATTGTTCGCAGCATTGAGCA	PCR, sequencing	In this study
		GPD_schizR	CAATGTCGTCCTCGGTGTAG	PCR, sequencing	In this study
	Internal transcribed spacer (ITS)	ITS1F	CTTGGTCATTTAGAGGAAGTAA	PCR, sequencing	Gardes & Bruns (1993)
		ITS4	TCCTCCGCTTATTGATATGC	PCR, sequencing	White *et al.* (1990)
	Ribulose bisphosphate carboxylase large subunit gene (*rbcL*)	rbcL320	TATTCGAAGAAGGTTCAGTAAC	PCR, sequencing	Nozaki *et al.* (1995)
		rbcL803	TCGTGCATAATAATAGGTACAC	PCR, sequencing	Nozaki *et al.* (1995)
Algae	Nuclear small subunit ribosomal DNA (*nuc*SSU)	NS8	TCCGCAGGTTCACCTACGGA	PCR, sequencing	White *et al.* (1990)
		NS19	CCGGAGAAGGAGCCTGAGAAAC	PCR, sequencing	White *et al.* (1990)
		NS1680schizF	ATTAAGAGGGACAGTCGGGGGC	Sequencing	In this study
		NS3033schizR	GGCAACCACCAACTAAGAACGGC	Sequencing	In this study
	Internal transcribed spacer (*ITS*)	ITS1T	GGAAGGATCATTGAATCTATCGT	PCR, sequencing	Kroken & Taylor (2000)
		ITS4T	GGTTCGCTCGCCGCTACTA	PCR, sequencing	Kroken & Taylor (2000)

using the DNAeasy Plant Mini Kit (Qiagen, Vienna, Austria). DNA extractions of isolated algal cultures followed Cubero *et al.* (1999). The genetic diversity was investigated in three loci both for the fungi and for the algae.

The fungal markers used were the β-tubulin gene, the glyceraldehyde-3-phosphate dehydrogenase (GPDH) gene and the internal transcribed spacers (ITS) and 5.8S rRNA gene (Table 2).

The photobiont markers used were the chloroplast gene for the ribulose bisphosphate carboxylase large subunit (*rbcL*), the small nuclear subunit of the rDNA (*nuc*SSU) and the ITS and 5.8S rRNA gene (Table 2). Two sequencing primers were designed in this study for the sequencing of the algal *nuc*SSU (Table 2).

PCR reactions were prepared for a 30 μL final volume containing double-distilled water, $10 \times Taq$ polymerase reaction buffer (10 mM Tris pH 8.3), MgCl$_2$ (1 mM), 2.5 mM of each dNTP, 0.1 U of *Taq* DNA polymerase and 10 μM primers. PCR amplifications were performed with an initial heating step of 2 min at 94 °C, linked to 37 cycles 45 s at 94 °C, of which the first seven cycles were touchdown for 45-s annealing conditions alternative at 57–51 °C for GPDH or 58–52 °C for the photobiont loci *rbcL* and *nuc*SSU. The annealing temperature in the remaining 30 cycles was 50 °C for GPDH and 52 °C for the photobiont loci. Extensions were run for 1 min 45 s at 72 °C in the 37 cycles and one extension step of 7 min at 72 °C was performed at the end. ITS and β-tubulin primers did not require touchdown and the amplifications were run in 30 cycles with 53 °C and 58 °C annealing, respectively.

PCR products were cleaned using Qiaquick spin columns (Qiagen) or enzymatically (ExoSAP-IT, GE Healthcare, UK). Both strands were sequenced using the BigDye Cycle

Sequencing Ready Reaction Kit (Applera, Austria) and sequences were run on an ABI310 automated sequencer (Applera).

Alignment and phylogenetic analysis

Separate alignments for each gene locus were produced automatically with CLUSTALW as implemented in BIOEDIT 5.0.6 (Hall, 1999; http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) and then adjusted manually.

Haplotype networks for the fungal markers were constructed under the 95% parsimony probability criterion (Templeton *et al.*, 1992) using the TCS program (Clement *et al.*, 2000). The haplotypes are shaded differently (Fig. 2a–c) following the geographic provenance according to Table 1.

For each fungal marker, phylogenetic analyses were also performed using MRBAYES 3.1.2 (Huelsenbeck & Ronquist, 2003). The optimal nucleotide substitution model, the GTR+I+G model (Rodriguez *et al.*, 1990), was estimated with MRMODELTEST v3.7 (Posada & Crandall, 1998) using the Akaike information criterion and the hierarchical likelihood ratio test (Posada & Crandall, 1998), and was used for likelihood calculations. The Bayesian Markov Chain Monte Carlo (B/MCMC) analyses were run for 2 million generations starting from a random tree, with six incrementally heated chains using the default temperature of 0.2, and every 100th tree was sampled. To ensure that all chains had converged at a single level, the burn-in sample of 5K trees was discarded for each run and a majority rule consensus tree was calculated for the remaining 15.001 trees. The burn-in period was determined after testing for the stationarity of likelihood values, i.e. by plotting numbers of generation vs. the log probability and checking for the convergent
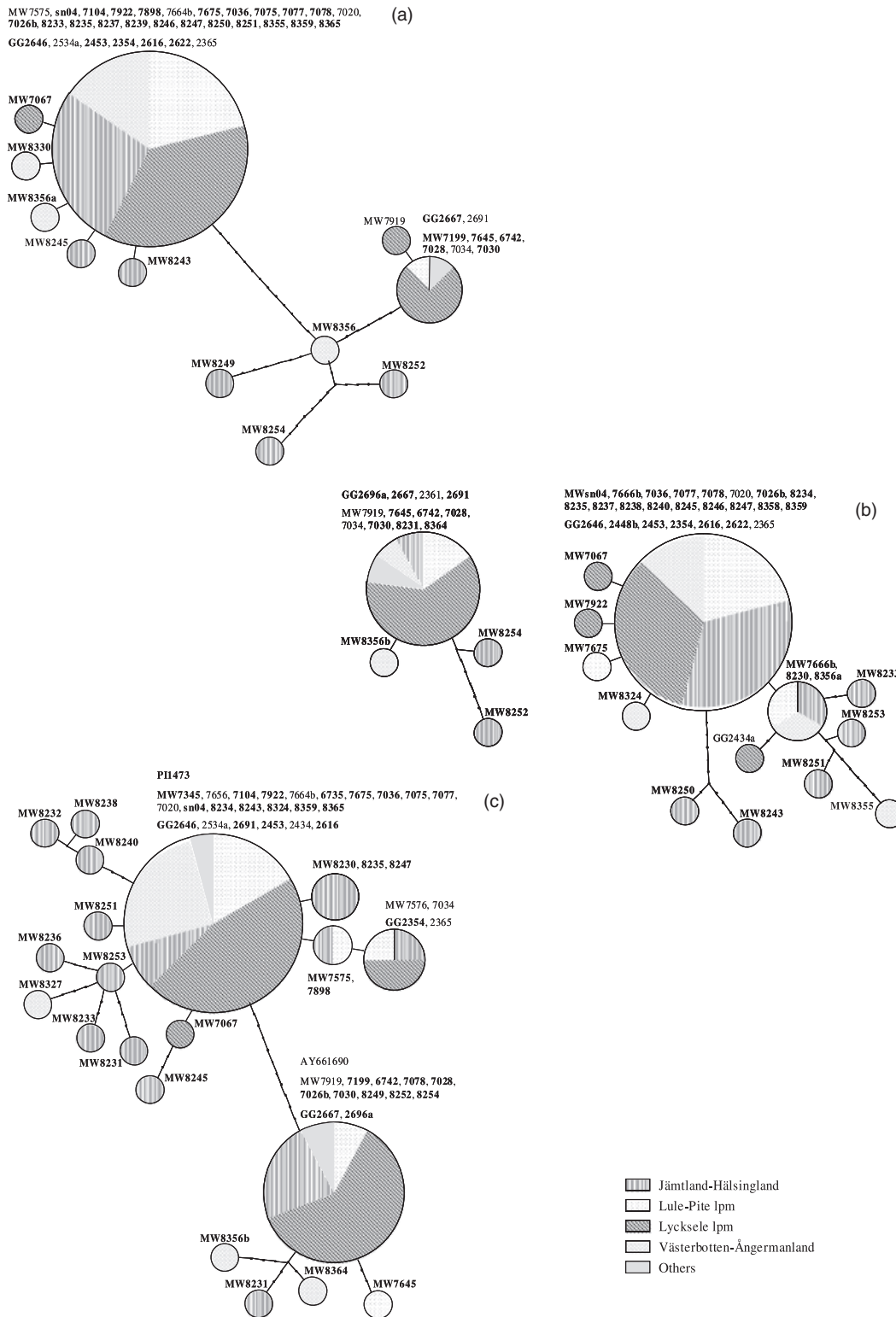
Fig. 2. Haplotype analyses: 95% probability haplotype networks for β-tubulin (a), GPDH (b) and ITS (c) gene of lichenized (bold) and saprotrophic thalli of *Schizoxylon albescens* in Central and Northern Sweden. The geographical origin of the samples is mapped on the haplotype networks and listed in the legend.

diagnostic factor, which approached 1 (Ronquist *et al.*, 2005). The program TRACER 1.4 (Rambaut & Drummond, 2004) was used to determine chain convergence and the appropriate burn-in.

The ITS, *nuc*SSU and *rbcL* photobiont datasets were analysed separately; relevant sequences retrieved from Gen-Bank were included. To identify the group of algae to which the *Schizoxylon* photobiont(s) belongs, we first performed phylogenetic analyses including different representatives of *Chlorophyceae* (not shown) and then restricted the datasets to the most closely related taxa. Two sequences of *Chlorella* sp. were selected as outgroup for each dataset. All phylogenetic analyses were carried out according to the same methods and parameters used for the fungal markers. The phylogenetic trees were visualized in TREEVIEW (Page, 1996).

Isolation of algal cells in culture

Eleven samples, those presenting the most abundant algal colonies around the fruiting bodies (MW7645, 7919, 8207, 8220, 8230, 8233, 8234, 8235, 8238, 8249, 8251), were selected to isolate algal cells in axenic cultures. In order to minimize bacterial contaminations, the first inocula were carried out on media containing antibiotics (ampicillin and tetracyclin). We applied three different strategies to facilitate the isolation of the right photobionts: (1) one fruiting body was taken with a sterile razor blade and the isolations followed the method described by Yamamoto *et al.* (2002), with modifications from Stocker-Wörgötter (2002); (2) algal clumps were removed with a sterile needle and transferred directly on the agar medium; and (3) algae clumps were diluted in sterile water and plated by pipetting. If an isolation event resulted in a diversity of colonies, each colony was subcultured on a new Petri dish. The algae were grown on *Trebouxia* medium (TM; Ahmadjian, 1967) and Malt Yeast (MY; Ahmadjian, 1967) medium and algal colonies were maintained alive by subculturing them every 2 months. The identity of the isolates was finally checked by DNA sequence analysis of the *nuc*SSU and *rbcL* loci.

Isolation of fungi in culture

Schizoxylon albescens was isolated from the two non-lichenized MW7919 and MW7664b, and from the lichenized MW7645 samples following Yamamoto *et al.* (2002), with some modification outlined in Stocker-Wörgötter (2002). The fungi were grown on MY (Ahmadjian, 1967) and Lilly Barnett Medium (LBM; Lilly & Barnett, 1951) media. Fungal thalli grown up to 1 cm in diameter were subcultured every 3–4 months. The identity of the isolates was checked by DNA analysis of the ITS locus.

Algal and fungal isolates are stored as cryostock culture in the Institute of Plant Sciences, Graz.

Co-culture experiments of fungi and algae

The establishment of lichenized structures was studied by setting up mixed cultures of the fungus with the algae. Different combinations of the three isolated fungi, MW7645 (lichenized), MW7664b and MW7919 (nonlichenized), with 10 selected algal isolates (Supporting Information, Table S1) were grown on LBM (Lilly & Barnett, 1951), Lichen Medium (LM; Honegger, 1993), MY and TM (Ahmadjian, 1967) media.

Fungi and algae were also subcultivated on Petri plates to which sterile *Populus* wood and bark pieces were added, to test whether the fungus and algae would behave differently when affected by wood or bark.

We finally set up co-cultivation experiments with three additional lichen photobionts available in the culture collection of our laboratory (Graz) to test whether *Schizoxylon* shows any selectivity towards different photobionts. These were a *Trebouxia arboricola* (*Trebouxiophyceae*) isolated from the lichen *Tephromela atra*, a *Chlorella*-like photobiont (*Trebouxiophyceae*) from *Flakea papillata* and a *Trentepohlia* sp. (*Ulvophyceae*) from *Cystocoleus ebeneus*.

Scanning electron microscopy (SEM) analyses

Scanning electron microscope observations were performed on the two lichenized samples MW8236 and MW8238 (original thalli) and on five well-developed mixed cultures (Table S1). The samples were fixed in 2% paraformaldehyde/ 2.5% glutaraldehyde in 0.1 M cacodylate buffer with 0.1 M sucrose at pH 7.2 for 4 h (Karnovsky, 1965) at room temperature. After rinsing the cells twice in buffer with 0.1 M sucrose (15 min), the samples were gradually infiltrated with acetone and dried at the CO_2-critical point (CPD030, Baltec). The samples were sputtered with gold and analysed using a Philips XL30ESM.

Results

Fungal genetic diversity

We obtained a total of 51 new β-tubulin, 56 GPDH and 57 ITS rDNA sequences. One haplotype network was recovered for the β-tubulin and ITS loci (Fig. 2a and c), whereas the GPDH haplotypes formed two distinct networks (Fig. 2b). Jämtland-Hälsingland and Västerbotten-Ångermanland are the regions in which, on average, the highest allelic richness was detected among the individuals. In particular, the highest genetic diversity is observed in the ITS region of samples coming from Jämtland-Hälsingland: 15 different haplotypes are found among 20 specimens. Jämtland-Hälsingland and Lycksele lpm are the regions from which most material was available. Lycksele lpm is also the region where we obtained the most complete dataset for the three fungal markers and where we recovered the lowest genetic diversity (Table S1).

The most frequent β-tubulin haplotype is shared by 26 lichenized and five nonlichenized samples from all the regions of Sweden studied (Fig. 2a). A second haplotype is detected only in three regions and shared by six lichenized and two nonlichenized samples. Ten single β-tubulin haplotypes are recovered, two for Lycksele lpm, of which one belongs to a nonlichenized specimen, three for Västerbotten-Ångermanland and five for Jämtland-Hälsingland (Fig. 2a).

The most common GPD haplotype comprises 24 samples (two nonlichenized), originating from all the Swedish regions studied (Fig. 2b). The second most common haplotype (smaller network) occurs in all Swedish regions (the majority are samples from Lycksele lpm) and from Norway. Fourteen different haplotypes are found in single individuals from Jämtland-Hälsingland (seven), Lule-Pite lpm (one) and Lycksele lpm (three) – one of which is a nonlichenized specimen – and Västerbotten-Ångermanland (three) (Fig. 2b).

Two ITS haplotypes are shared by lichenized (30) and non-lichenized (six) samples coming from all regions in Sweden and Norway (Fig. 2c). Three less common haplotypes are shared by samples coming from Jämtland-Hälsingland (five), Lule-Pite lpm (two) and Lycksele lpm (two). The more frequent of these haplotype (shared by four individuals) is present in three nonlichenized individuals, one from each of these Swedish regions (Fig. 2c). Fifteen haplotypes are found individually in Jämtland-Hälsingland (10), Lule-Pite lpm (one) and Västerbotten-Ångermanland (three). Eight specimens (MWsn04, 7020, 7036, 7077, 8359, and GG2453, 2646, 2616) share the most common haplotype of each marker.

The phylogenetic inferences obtained for each individual marker do not segregate lichenized samples from the saprobes: samples representing both life styles constitute two main groups, distinguished as sp. 1 and sp. 2, in which they are intermixed (Fig. S1a–c). However, members of the sp. 1 and of the sp. 2 are, respectively, unresolved in the GPD and β-tubulin analyses.

Algal genetic diversity

Six ITS, 27 *nuc*SSU and 20 *rbcL* sequences were obtained from the algae isolated in culture (Table 1). Only *rbcL* sequences could be obtained out of 27 specimens growing on bark. For these specimens, several attempts to amplify the ITS and *nuc*SSU rDNA algal regions were unsuccessful, possibly due to poor primer matching. We could not obtain any algal sequences directly from DNA extractions of the two samples MW8207 and MW8219 (Table 1). However, for these samples, we gained data for the three fungal loci and we could isolate the algal strains.

All algal sequences, obtained from the amplification of the ITS, *nuc*SSU and *rbcL* loci, were similar to the sequences of *Coccomyxa* and *Pseudococcomyxa* (*Chlorophyceae*) when conducting BLAST searches in GenBank (BLAST score: 97–99% maximum identity).

The *rbcL* phylogeny groups all *Schizoxylon*-associated photobionts into one large clade in which two fully supported subclades are distinguished (Fig. 3). Clade A comprises samples exclusively coming from cultured algae isolated from the richly lichenized *Schizoxylon* samples selected. The sequence of *Pseudococcomyxa simplex* (EF589155) is nested in clade A. Clade B groups sequences amplified directly from lichenized thalli and three sequences of photobionts isolated in culture. For the specimens MW8233 and MW8238, we generated *rbcL* algal sequences both from the DNA extractions from the original thalli on bark and from the photobionts that were isolated in cultures. The *rbcL* sequences (Fig. 3) from MW8233 are present either in clade A (two sequences, L625, L630) or in clade B (three sequences, L626, L628, L629), whereas the algae isolated from MW8238 (two sequences, L631, L632) group are present only in clade A.

The *nuc*SSU contains a very small amount of genetic difference (we detected among 1350 bp only six variable sites and some scattered indels), and our sequences group into one single fully supported, but unresolved clade (Fig. 4), including four *Coccomyxa* sequences from GenBank. The ITS sequences likewise showed high similarity (BLAST score: 99% maximum identity) to published *Coccomyxa* sequences from lichen photobionts (Zoller & Lutzoni, 2003). The ITS sequences were not used further in our phylogenetic studies.

Isolation in axenic cultures and co-culture experiments

The fungus *S. albescens* was successfully isolated in culture from all the three selected samples (MW7645, MW7664 and MW7919). The *Schizoxylon* fungi formed white-pink colonies, which usually developed three-dimensionally with aerial hyphae.

Twenty-five algal isolates were obtained from four lichenized samples (MW8207, 8220, 8233, 8238). Initially, 40 algal inoculates were set; 15 were discarded due to fungal and bacterial contaminations. Of the remaining isolates, 10 were selected to set up co-culture experiments with the fungi (Table S2). Three algal strains correspond to the cultivated algae in clade B and six correspond to samples present in clade A of Fig. 3; *rbcL* sequence data were not obtained for L573 PL3-3 (Table S2). Generally, the *Schizoxylon* fungus and algae grew better on MY and LBM media. We observed no difference between the samples grown directly on the media (Fig. 5b–d) and those partially grown on the bark or the wood pieces (Fig. 5a). The algal cells and colonies show the diagnostic characteristics described by Jaag (1931) for
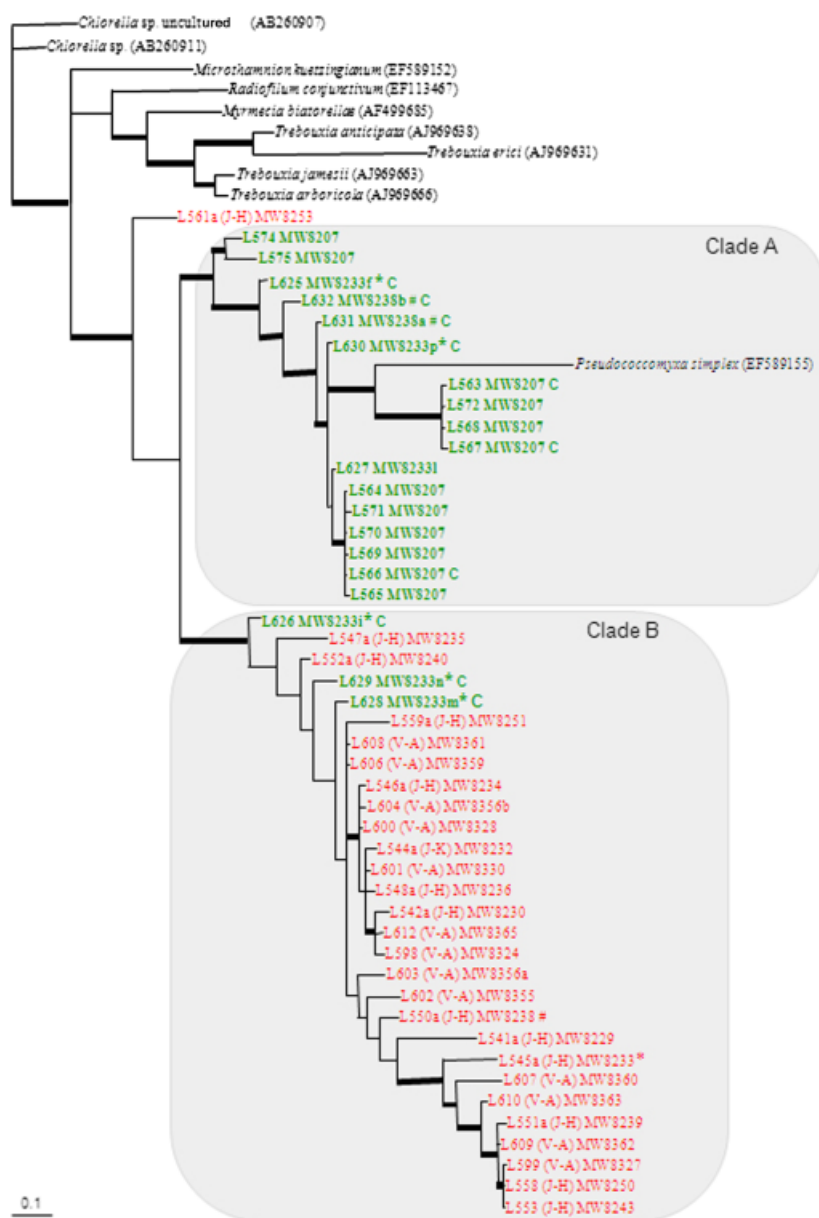
Fig. 3. Phylogeny of *rbcL* genes: 50% majority-rule consensus tree of a B/MCMC sampling procedure. Bold branches are supported by posterior probabilities > 95%. Algal strains isolated in cultures are marked in bold; those additionally co-cultivated with the mycobiont are labelled with 'C'. J-H (Jämtland-Hälsingland) and V-A (Västerbotten-Ångermanland) indicate the geographical provenance. Isolated algal strains from MW8233 and MW8238 and their own original thallus are marked by * and #, respectively. Samples retrieved from GenBank are reported with the NCBI accession numbers.

Coccomyxa cultures: the algae grew in circular colonies *c.* 1–2 mm above the medium, with well-delimited margins, dark green colour and a glossy surface (partially visible in Fig. 5d), but became paler when old. The algal cells are rather small and regularly elliptic/ovoid.

In the co-cultures with the *Coccomyxa*-like algae, the fungus often covered the algal colonies with a thin white mat of hyphae (Fig. 5b and c). Under the light microscope, fungal hyphae were observed to be surrounding and closely adhered to algal cells. Five well-grown co-cultures were selected for SEM analyses (Table S2).

In co-culture with *Trebouxia* sp., the algae developed healthy colonies, but the fungus did not grow together with

them (Fig. 6a). *Trentepohlia* sp. grew very poorly and slowly whereas the fungus formed a rather well-developed mycelium (Fig. 6b). In culture with the *Chlorella*-like photobiont of *F. papillata*, both the alga and the fungus developed very small and independent colonies (Fig. 6c). Because only the cultures set with *Trentepohlia* sp. and with the *Chlorella*-like photobiont were more conspicuous, they were investigated using SEM.

Analyses of symbiotic interactions with SEM

Algal cells associated with the fungus in the environmental samples of lichenized thalli of MW8236 and MW8238 are
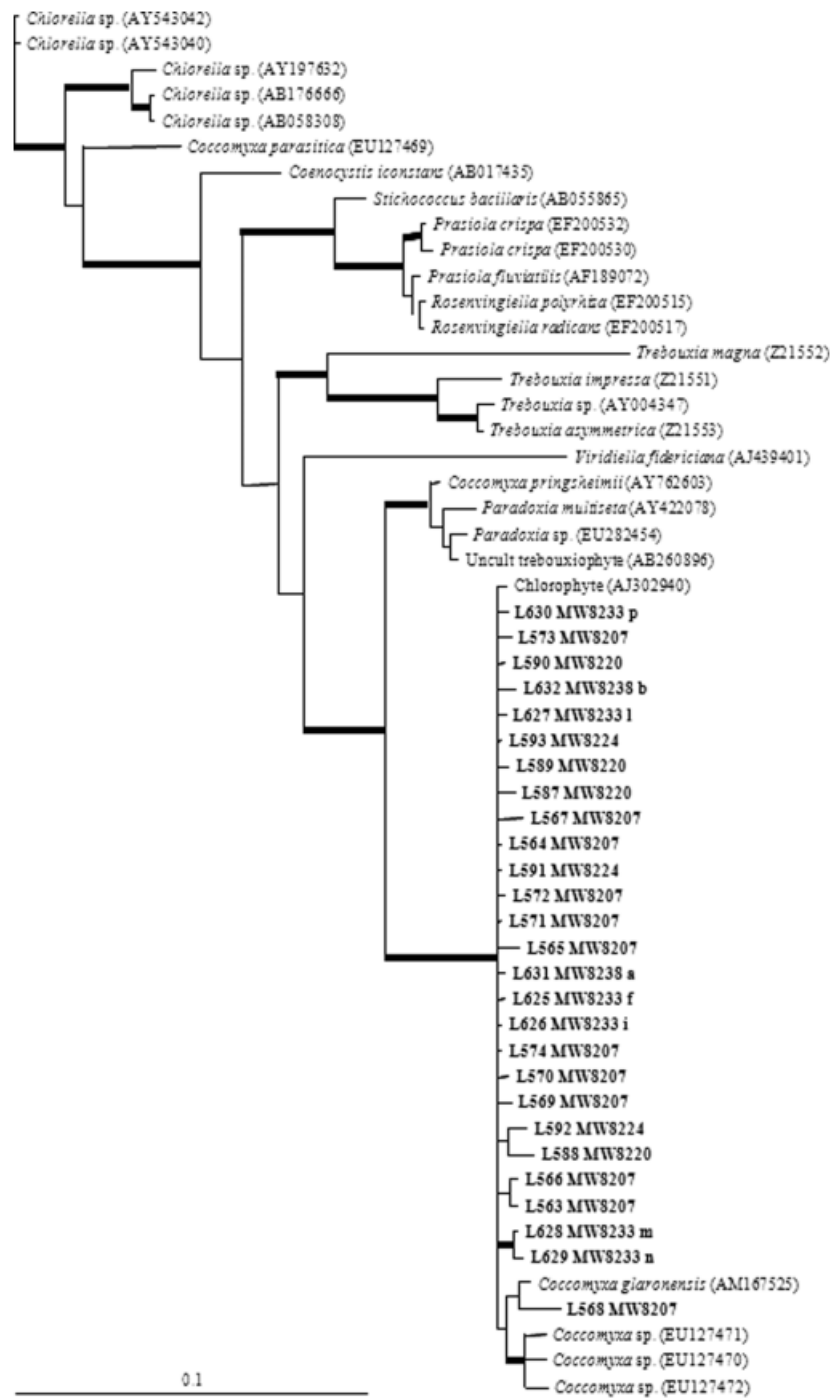
Fig. 4. Phylogeny of *nuc*SSU genes: 50% majority-rule consensus tree of a B/MCMC sampling procedure. Bold branches are supported by posterior probabilities > 95%. Algal strains isolated in cultures are marked in bold. Samples retrieved from GenBank are reported with the NCBI accession numbers.

spherical to elliptical in shape and group in small relatively dense packets (Fig. 1d–f). The fungal hyphae grow intermixed with the algae clumps, and occasionally, the hyphae cover the algal cells with a secreted, unidentified filamentous matrix. This matrix fuses the hyphae to the algal cell wall and keeps the algae together in packets (Fig. 1d). Bacteria and fungal spores are also entangled in the lichenized thallus (Fig. 1e) and occasionally fungal hyphae are adhering to the algal cell walls (Fig. 1f).

In the co-cultures, the arrangement of the algae and the hyphae resembles the pattern observed both in the environmental samples and with the stereomicroscope for the cultures. A relatively thick algal layer, conspicuous in the transversal section, grew beneath the fungal hyphae.
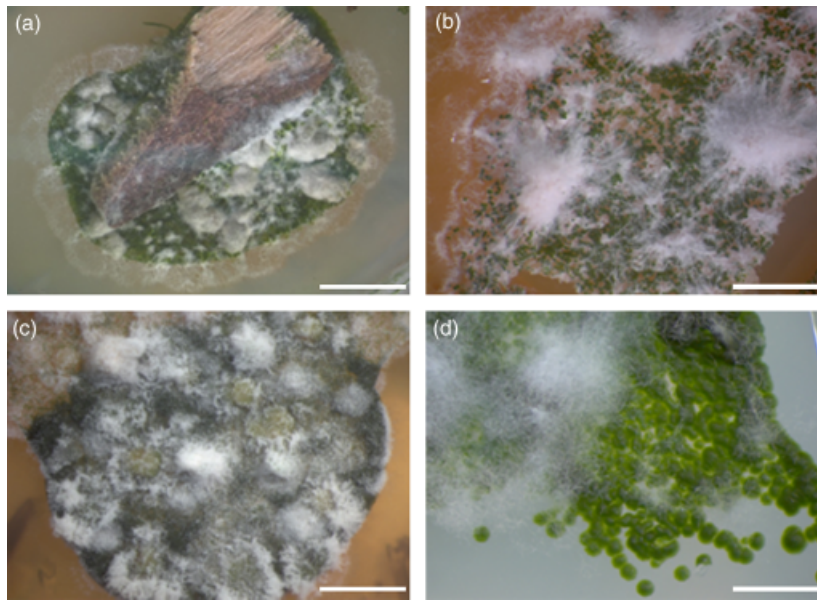
Fig. 5. Co-cultures of the fungus *Schizoxylon albescens* MW7919 together with isolated algal strains (*Coccomyxa*) PL2.1 (a), PL4.1 (b), MW8233n (c) and MW8238a (d), on LBM medium (a, d) and on MY medium (b, c). Scale bar = 0.5 cm.
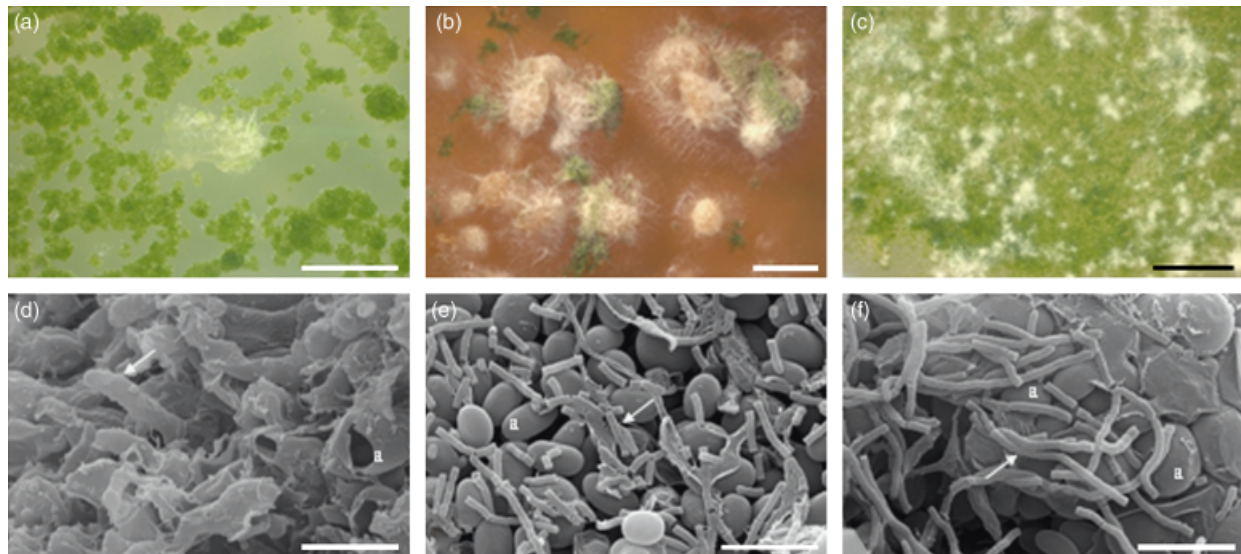

Fig. 6. Additional co-cultivation experiments: *Schizoxylon* fungus co-cultivated with *Trebouxia* sp. (a), *Trentepohlia* sp. (b, d) and the *Chlorella*-like photobiont of *Flakea papillata* (c, e, f), in light microscopy (a–c) and SEM (d–f). Fungal hyphae (arrows) and algal cells (a) are indicated. Scale bars = 1 mm (a), 2 mm (b), 0.5 mm (c), 10 μm (d, e) and 4 μm (f).

The hyphae are branched and spread on and among the algae and cover the upper surface of the algal layer with a very thin sheet of the filamentous matrix (Fig. 7a, c and f). In some cases, hyphae and the filamentous matrix are so tightly appressed to the algal cell walls that it is almost impossible to identify the boundary between them (Fig. 7d, g and h). In some areas, the matrix is distributed continuously on the algae, whereas in other parts, it seems to tear, probably due to the overgrowth of the algae (Fig. 7b, e, g and h). We did not observe fungal penetration hyphae (pegs or haustoria, hyphal structures penetrating the algal cell walls,

produced in many lichen interactions with other algae) in any specimens or co-cultures.

In co-cultures with *Trentepohlia* (Fig. 6d) or with the coccoid photobiont of *F. papillata* (Fig. 6e and f), we noticed neither a thick net of fungal hyphae or filamentous matrix nor interactions among hyphae and algal cells. In both cases, the hyphae grow inconspicuously among the algal cells. Particularly in the co-culture with the coccoid photobiont of *F. papillata*, *S. albescens* develops extremely thin hyphae, which break into small fragments and do not form any continuous net (Fig. 6e and f).
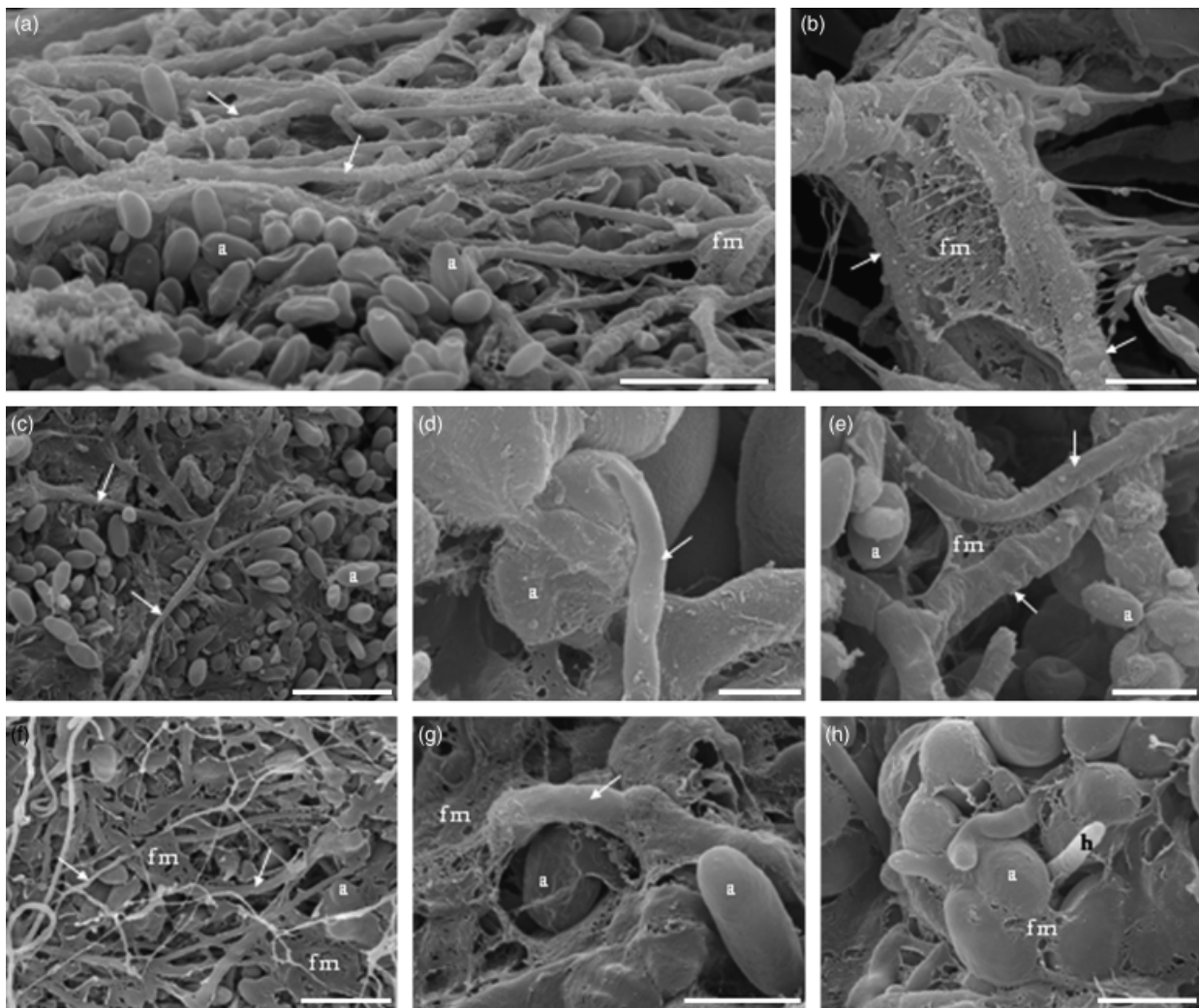
Fig. 7. SEM analyses of co-cultures of *Schizoxylon albescens* and isolated algal (*Coccomyxa*) strains. (a–c, f, h) MW7919 with algal strain MW8238a; (d) MW7919 with algal strain MW8233n; (e) MW7919 with algal strain PL2.1; (g) MW7664 with algal strain MW8233n. Fungal hyphae (arrows), algal cells (a) and filamentous matrix (fm) are indicated. Scale bars = 20 μm (a, c), 10 μm (f), 5 μm (b, e), 4 μm (g, h), 2 μm (d).

Discussion

Mycobiont genetic diversity: species concept and life styles

The lichenized and the saprotrophic morphs of *S. albescens* do not correspond to separate phylogenetic species. Our haplotype analyses do not show any genetic differentiation between the two life styles, nor is the genetic difference correlated with geographical origin in any of the markers. Further, the most commonly found haplotypes are shared among individuals belonging to different geographic origins. Up to 15 single haplotypes are recovered for each genetic marker among samples of the same geographic origin, thus indicating a certain degree of genetic diversity. In comparison, a high degree of genetic difference – described by increased rates of nucleotide substitutions – was demonstrated to correlate to the transition between lichenization and mutualism among closely related species of the basidiomycete genus *Omphalina* (Lutzoni & Pagel, 1997). Our study, however, focuses on the within-species genetic diversity of an optionally lichenized fungus, *S. albescens*. Here, we do not find any correlation between the allelic richness and the different life styles. Similarly, Wedin *et al.* (2004) could not find any genetic difference within optionally lichenized *Stictis* sp., which are closely related to *Schizoxylon*. However, later, the same authors (Wedin *et al.*, 2006) could correlate a substantial morphological within-species variation to the fungal life styles,

concluding that optionally lichenization apparently affects the anatomy and morphology of these fungi.

Schizoxylon albescens clearly forms two genetically distinct units in the haplotype analyses, which cannot be separated on morphology alone. These results are further supported by the phylogenetic inferences reconstructed for each marker (Fig. S1): here, both units are monophyletic in the ITS rDNA, sp. 2 without significant support; however, sp. 1 is further supported as monophyletic in the GPDH tree and sp. 2 is supported in the β-tubulin phylogeny. Although the information present in these markers is somehow limited for phylogenetic analyses, and the resolution in these phylogenies is thus not impressive, the overall congruent patterns between single-marker phylogenies and haplotype analyses suggest that two distinct genetic units are present. We are tempted to interpret these two units as representing morphologically cryptic species, identified here through haplotype analyses and concordance of gene genealogies (Grube & Kroken, 2000; Taylor *et al.*, 2000). In lichen fungal systematics, the phylogenetic species concept has been applied in several species groups including the difficult *Letharia vulpina/columbiana* sp. complex (Kroken & Taylor, 2001) and the heavy-metal-accumulating *Acarospora smaragdula* complex (Wedin *et al.*, 2009). *Schizoxylon albescens* s.l. is thus another example in the *Stictidaceae* of two morphologically extremely similar sister lineages showing optional lichenization (as in *Stictis confusa/Stictis populorum*; Wedin *et al.*, 2004).

Algae strains in the optional lichenization and their genetic diversity

Lichenized algal colonies are normally present in varying amounts around the fungal fruiting bodies on bark (Fig. 1a–c). It can be argued that the molecular data obtained for the photobionts appear fragmentary and unsuitable for phylogenetic analyses due to our failure to amplify all the markers for all the samples. By contrast, they are very valuable for an approximate identification of the algae and for comparing how easily different genotypes grow in cultures. The *nuc*SSU and the ITS rDNA sequences show a high similarity to *Coccomyxa* lichen photobionts when performing BLAST searches in GenBank. The most similar sequences come from a unique algal endophyte of *Ginkgo* trees (*Coccomyxa glaronensis*, Tremouillaux-Guiller & Huss, 2007) and from a *Coccomyxa* parasitizing mussels (Rodríguez *et al.*, 2008). Both the diagnostic morphological characteristics observed and the sequence data obtained thus indicate that these algae are closely related to *Coccomyxa*. This genus is still very poorly known taxonomically, which is indicated by the limited number of *Coccomyxa* photobiont sequences available in public databases. In the phylogenetic analysis of the *nuc*SSU, all the algae isolated in

culture form one unresolved group, but this analysis lacks data from thalli of samples from the field. In the tree based on the *rbcL* locus, however, which includes DNA data both from the algal isolates and from direct thallus extractions, the algae form two distinct clades corresponding to clade A and to clade B, respectively. Only three algal cultures, L626, L628 and L629, fall into the clade B. The results clearly show that the *nuc*SSU rDNA does not contain enough genetic diversity to distinguish species in this group, but that the *rbcL* locus is more variable and can be considered a more appropriate marker to estimate genetic variation. The *rbcL* analysis indicates that there is a substantial diversity of algal strains and the detection of two main distinct clades may be explained in two ways. The dominant strain in the thallus, which would be the most important photobiont, is not the best competitor under the culture conditions provided. The clade A includes no algal sequences obtained directly from the thalli, indicating that these algae may not be the major components of the lichen association, but rather represent admixed algae present on the bark that just happen to grow better in culture while others lack growth factors present in the natural habitat. Under these circumstances, the culture-dependent approach underestimates the diversity of involved photobionts and may even select algae that are not necessarily involved as photobionts. The clade A groups together with a sample of *Pseudococcomyxa*, which is closely related to *Coccomyxa*, of which no *rbcL* sequences are available at present (2009–2010) in GenBank. An alternative explanation would be that the *rbcL* locus of a certain group of algae amplifies better due to primer biases. The main symbiotic partner could be represented by those cultured strains, but in environmental samples, the *rbcL* primers would anneal better with the DNA of algae that are present in minor amounts in the thalli. This finding could have implications for culture-based studies of potential lichen photobionts, especially in cases where the photobionts are commonly found to be free-living.

Coccomyxa is a relatively common unicellular green alga in the class *Trebouxiophyceae*, and is distantly related to other lichen photobiont genera, for example *Trebouxia* or *Stichococcus* (Karsten *et al.*, 2005). The genus is polyphyletic (Rodríguez *et al.*, 2008) and includes free-living species, parasitic species of marine echinoderms, epiphytic or endophytic species of higher plants and lichen thalli, and lichen photobionts (Jaag, 1931; Honegger, 1991; Gray *et al.*, 1999; Rodríguez *et al.*, 2008). Also in our analysis of the *nuc*SSU gene (Fig. 4), *Coccomyxa* is polyphyletic. *Coccomyxa* is known to lichenize with Ascomycetes in the *Peltigerales* (i.e. *Nephroma*, *Peltigera*, *Solorina*), with species of the genus *Icmadophila* (Jaag, 1931), and with species of the basidiomycetous genus *Omphalina* (Jaag, 1931; Zoller & Lutzoni, 2003).

As we know nothing about the within-species variation of the *rbcL* in these algae, we cannot state whether more than

one *Coccomyxa* sp. takes part in the optional lichenization. There is still a considerable genetic variation both between algal isolates from different *Schizoxylon* samples and between the different algal isolates from the sample MW8233. Even those isolates (L626, L628 and L629) grouping in the clade B are not identical to the sequences in the same clade obtained from the DNA extractions directly from the original thallus. This indicates that a number of comparatively closely related algal strains, possibly several species, may be involved in the lichenization of *Schizoxylon*.

Symbiotic interactions in culture

Although *in vitro* studies focusing on the initial stages of the lichenization process have shown that axenically isolated lichen mycobionts and photobionts can reform lichenized structures in culture (Ahmadjian *et al.*, 1980; Ahmadjian & Jacobs, 1981; Bubrick *et al.*, 1985; Ott, 1987; Galun & Garty, 1988; Stocker-Wörgötter, 1995, 2001; Schaper & Ott, 2003), we did not observe any penetration peg or haustorium – a typical structure of lichenization in which fungal hyphae enter the photobiont cells to extract nutrients (Honegger & Brunner, 1981; Tschermak-Woess, 1988; Honegger, 1991, 1993) – in our culture experiments. Only fungal hyphae adhering tightly to the algal cells were observed. This, however, is the expected situation in symbioses involving *Coccomyxa* and there are a couple of alternative explanations of why no penetrating hyphae are formed in such interactions. Plessl (1963) stated that *Coccomyxa* cells might be too small to be penetrated by fungal hyphae, but Honegger & Brunner (1981) and Honegger (1984) provided a more likely explanation by suggesting that the absence of fungal penetrating hyphae is due to the characteristic structure of the cell wall in *Coccomyxa*. In both free-living and lichenized species of *Coccomyxa*, the outermost cell wall layer contains sporopollenin, which is a highly efficient barrier against fungal parasitism (Honegger & Brunner, 1981; Honegger, 1991). This layer would just allow wall-to-wall apposition and very tight adhesion of the algal and fungal cells. For these reasons, in our experiments, it is also more difficult to determine where hyphae would terminate their linear growth when coming in contact with one or more algal cells, and start to embrace and surround the algae completely, as observed in typical symbiotic interactions (Joneson & Lutzoni, 2009). Consequently, SEM observations made at the very early stages of development would probably not be sufficiently convincing and interpretable in this case. The contacts between algae and hyphae are more evident on the uppermost layers of the cultures, where the fungus builds a densely branched hyphae mat and a filamentous matrix, and the hyphae grow densely among the algae (Fig. 7a, c and f). The lower layers are indeed composed mainly by algal cells, the fungal hyphae do not penetrate much into the algal

colonies, and in transversal sections, a net of hyphae is not as well detectable as on the surface of the cultures. Nevertheless, the matrix we observed should not be mistaken for the gelatinous sheet typically produced by *Coccomyxa* cells. Free-living *Coccomyxa* usually produce a mucilaginous substance, which keeps the algal cells together, sometimes also including different species, and maintains a colony structure (Jaag, 1931; Tschermak-Woess, 1988). In cultures, the filaments of the matrix clearly originate from the fungal hyphae and spread over the algal cells, and also between hyphae that have not yet come into contact with the algae (Fig. 7b, d, e, g and h). *Coccomyxa* cells usually produce no gelatinous matrix when they are in a lichenized state (Tschermak-Woess, 1988). The lack of a matrix in both the natural samples and the cultures is thus a further confirmation that the fungus and algae establish an association.

In lichens, mycobionts and photobionts are considered compatible if they enter into the second stage of development, characterized by the increased branching of the hyphae around the algal cells (Ahmadjian *et al.*, 1978; Galun, 1988; Joneson & Lutzoni, 2009). As this was indeed observed in our co-cultures, we interpret this as evidence for considering the algae compatible photobionts. The samples analysed by scanning microscopy were co-cultures established with two algal strains, L628 and L629, belonging to clade B, which group sequences obtained directly from the DNA extraction from the thalli, and one strain, L631, belonging to clade A. If strains of the clade A would be just algae that represent better competitors in culture, we would expect that L631 would not interact with the mycobiont as well as L628 and L629. However, we did not observe any difference in the interactions, and the mycobiont developed the same thalline growth structures in all cases. This might be explained by the fact that our preliminary co-culture experiments selected the best-growing algae rather than the 'best' photobionts, and that growth conditions were maintained constant for all the co-cultures. Our observations may thus be interpreted as that *Schizoxylon* may lichenize with algae that are closely related to, but not identical to the main photobiont as long as they are compatible. A certain lack of specificity is also known from certain other lichens: *Lecanora rupicola*, *Protoparmeliopsis muralis*, *Cladonia subulata*, *Cladonia macilenta* and *Evernia mesomorpha* form thalli with different photobiont species of the same genus (Guzow-Krzemińska, 2006; Doering & Piercey-Normore, 2009; O. Peksa & P. Škaloud, unpublished data).

Brunauer *et al.* (2007) noticed similar tight parietal adhesion between *Coccomyxa* cells co-cultivated with a lichenicolous fungus, but the same fungus developed haustoria when co-cultured with *Trebouxia* photobionts. However, *S. albescens* does not seem to interact with any of the other photobionts tested. Cultures with *Trebouxia* sp., *Trentepohlia* sp. and the *Chlorella*-like photobionts revealed

no signs of interaction between the fungus and the algae. If *S. albescens* would have also interacted with the other lichen photobionts tested, any specificity for certain algal partners could have been stated from our observations. The preference of the fungus to interact in culture with certain algal strains of the *Coccomyxa/Pseudococcomyxa*-type strengthens the molecular results in which only *Coccomyxa/Pseudococcomyxa*-like sequences are detected.

Conclusions

We have investigated the *S. albescens* fungal and algal partners in detail. *Schizoxylon albescens* was shown to represent two distinct, but cryptic fungal species: both form optional lichenization and grow very well in culture. *Schizoxylon* forms loosely lichen-structured symbioses with photobionts related to the known lichen photobiont *Coccomyxa*. The photobionts isolated in culture show substantial genetic variation, and three fungal isolates did not show preference between algal isolates originating from thallus fragments.

The study of lichen symbiosis has long suffered from a lack of model organisms and easily cultivable lichens in which the lichenization does not collapse under controlled laboratory conditions. As the *Schizoxylon*–'*Coccomyxa*' system is common in the northern Scandinavian boreal forests, and our first attempts indicate that it is easily established and maintained in culture, we suggest that it should be explored further in functional studies of lichen fungal–algal interactions under controlled laboratory conditions. We suggest that it could potentially become a model system for such interactions including simple lichen associations. If further cultivation experiments prove successful, relichenization with these fungi *in vitro* could provide us with a valuable insight into the biology of lichens and of the understanding of symbiotic systems in general.

Acknowledgements

References

Ahmadjian V (1967) A guide to the algae occurring as lichen symbionts: isolation, culture, cultural physiology, and identification. *Phycologia* **6**: 127–160.

Ahmadjian V & Jacobs JB (1981) Relationship between fungus and alga in the lichen *Cladonia cristatella* Tuck. *Nature* **289**: 169–172.

Ahmadjian V, Jacob JB & Russell LA (1978) Scanning electron microscope study of early lichen symbiosis. *Science* **200**: 1062–1064.

Ahmadjian V, Russell LA & Hildreth KC (1980) Artificial reestablishment of lichens. I. Morphological interactions between the phycobionts of different lichens and the mycobionts of *Cladonia cristatella* and *Lecanora chrysoleuca*. *Mycologia* **72**: 73–89.

Aptroot A (1991) A monograph of the Pyrenulaceae (excluding *Anthracothecium* and *Pyrenula*) and the Requienellaceae, with notes on the Pleomassariaceae, the Trypetheliaceae and *Mycomicrothelia* (lichenized and non-lichenized Ascomycetes). *Bibl Lichenol* **44**: 1–178.

Baloch E, Gilenstam G & Wedin M (2009) Phylogeny and classification of *Cryptodiscus*, with a taxonomic synopsis of the Swedish species. *Fungal Divers* **38**: 51–68.

Brunauer G, Blaha J, Hager A, Türk R, Stocker-Wörgötter E & Grube M (2007) An isolated lichenicolous fungus forms lichenoid structures when co-cultured with various coccoid algae. *Symbiosis* **44**: 127–136.

Bubrick P, Frensdorff A & Galun M (1985) Selectivity in the lichen symbiosis. *Lichen Physiology and Cell Biology* (Brown DH, ed), pp. 319–334. Plenum Press, New York.

Clement M, Posada D & Crandal KA (2000) TCS: a computer program to estimate gene genealogies. *Mol Ecol* **9**: 1657–1659.

Cubero OF, Crespo A, Fatehi J & Bridge PD (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium stored and lichenized fungi. *Plant Syst Evol* **217**: 243–249.

Doering M & Piercey-Normore MD (2009) Genetically divergent algae shape an epiphytic lichen community on jack pine in Manitoba. *Lichenologist* **41**: 69–80.

Galun M (1988) Lichenization. *CRC Handbook of Lichenology*, Vol. II (Galun M, ed), pp. 153–169. CRC Press Inc., Boca Raton, FL.

Galun M & Garty J (1988) Soredia formation of compatible and incompatible lichen symbionts. *Cell to Cell Signals in Plant, Animal and Microbial Symbiosis* (Scannerini S, Smith D, Bonfante-Fasolo P & Gianinazzi-Pearson V, eds), pp. 207–217. Springer-Verlag, Berlin.

Gardes M & Bruns TD (1993) ITS primers with enhanced specificity for basidiomycetes. Application for the identification of mycorrhizae and rust. *Mol Ecol* **2**: 113–118.

Gilenstam G (1969) Studies in the genus *Conotrema*. *Ark Bot* **7**: 149–179.

Gray AP, Lucas IAN, Seed R & Richardson CA (1999) *Mytilus edulis chilensis* infested with *Coccomyxa parasitica* (Chlorococcales, Coccomyxaceae). *J Mollus Stud* **65**: 289–294.

Grube M (2005) *Frigidopyrenia* – a new genus for a peculiar subarctic lichen, with notes on similar taxa. *Phyton* **45**: 305–318.

Grube M & Kroken S (2000) Molecular approaches and the concept of species and species complexes in lichenized fungi. *Mycol Res* **104**: 1284–1294.

Grube M & Hawksworth DL (2007) Trouble with lichen: the re-evaluation and re-interpretation of thallus form and fruit body types in the molecular era. *Mycol Res* **111**: 1116–1132.

Guzow-Krzemiñska B (2006) Photobiont flexibility in the lichen *Protoparmeliopsis muralis* as revealed by ITS rDNA analysis. *Lichenologist* **38**: 469–476.

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

Hawksworth DL & Honegger R (1994) The lichen thallus: a symbiotic phenotype of nutritionally specialized fungi and its response to gall producers. *Plant Galls. Organisms, Interactions, Populations. The Systematics Association Special Volume* (Williams MAJ, ed), pp. 77–98. Clarendon Press, Oxford.

Honegger R (1984) Cytological aspects of the mycobiont–phycobiont relationship in lichens. Haustorial types, phycobiont cell wall types, and the ultrastructure of the cell surface layers in some cultured and symbiotic mycobionts and phycobionts. *Lichenologist* **16**: 111–127.

Honegger R (1991) Functional aspects of the lichen symbiosis. *Annu Rev Plant Phys* **42**: 553–578.

Honegger R (1993) A simple outdoor culturing system for the foliose macrolichens *Xanthoria parietina* (L.) Th. Fr. and *Parmelia sulcata* Taylor. *Bot Helv* **103**: 223–229.

Honegger R & Brunner U (1981) Sporopollenin in the cell walls of *Coccomyxa* and *Myrmecia* phycobionts of various lichens: an ultrastructural and chemical investigation. *Can J Botany* **59**: 2713–2734.

Huelsenbeck JP & Ronquist F (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572–1574.

Jaag O (1931) Morphologische und physiologische Untersuchungen über die zur Gattung *Coccomyxa* gehörenden Flechtengonidien. *Verh Schwei Nat Gesell* **1931**: 331–332.

Joneson S & Lutzoni F (2009) Compatibility and thigmotropism in the lichen symbiosis: a reappraisal. *Symbiosis* **47**: 109–115.

Karnovsky MJ (1965) A formaldehyde–glutaraldehyde fixative of high osmolarity for use in electron microscopy. *J Cell Biol* **27**: 137A–138A.

Karsten U, Friedl T, Schumann R, Hoyer K & Lembcke S (2005) Mycosporine-like amino acids and phylogenies in green algae: *Prasiola* and its relatives from the Trebouxiophyceae (Chlorophyta). *J Phycol* **41**: 557–566.

Kohlmeyer J, Hawksworth DL & Volkmann-Kohlmeyer B (2004) Observation of two marine and maritime 'borderline' lichens: *Mastodia tesellata* and *Collemopsidium pelvetiae*. *Mycol Prog* **3**: 51–56.

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

Kroken S & Taylor JW (2001) A gene genealogical approach to recognize phylogenetic species boundaries in the lichenzed fungus *Letharia*. *Mycologia* **93**: 38–53.

Lilly VG & Barnett HL (1951) *Physiology of Fungi*. McGraw-Hill, New York.

Lumbsch HT & Papong K (2009) *Ocellularia gyrostomoides* belongs to the genus *Schizoxylon* (Stictidaceae, Ascomycota). *Mycotaxon* **109**: 319–322.

Lumbsch HT, Schmitt I, Palice Z, Wiklund E, Ekman S & Wedin M (2004) Supraordinal phylogenetic relationships of Lecanoromycetes based on a Bayesian analysis of combined nuclear and mitochondrial sequences. *Mol Phylogenet Evol* **31**: 822–832.

Lumbsch HT, Schmitt I, Wiklund E & Wedin M (2007) The phylogenetic placement of Ostropales within Lecanoromycetes (Ascomycota) revisited. *Mycol Res* **111**: 257–267.

Lutzoni F & Pagel M (1997) Accelerated evolution as a consequence of transitions to mutualism. *Proc Nat Acad Sci* **94**: 11422–11427.

Myllys L, Lohtander K & Tehler A (2001) Beta-tubulin, ITS and group I intron sequences challenge the species pair concept in *Physcia aipolia* and *P. caesia*. *Mycologia* **93**: 335–343.

Nozaki H, Ito M, Sano R, Uchida H, Watanabe MM & Kuroiwa T (1995) Phylogenetic relationships within the colonial Volvocales (Chlorophyta) inferred from *rbcL* gene sequence data. *J Phycol* **31**: 970–979.

Ott S (1987) The juvenile development of lichen thalli from vegetative diaspores. *Symbiosis* **3**: 57–74.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**: 357–358.

Plessl A (1963) Über die Beziehungen von Haustorienstypus und Organisationshohe bei Flechten. *Österr Bot Z* **110**: 1994–1269.

Posada D & Crandall KA (1998) Modeltest – testing the model of DNA substitution. *Boinformatics* **14**: 817–818.

Rambaut A & Drummond A (2004) Tracer 1.4. Available from http://tree.bio.ed.ac.uk/software/tracer/ (Accessed February 2010).

Rodriguez F, Oliver JL, Marin A & Medina JR (1990) The general stochastic model of nucleotide substitution. *J Theor Biol* **142**: 485–501.

Rodríguez F, Feist SW, Guillou L, Harkestad LS, Bateman K, Renault T & Mortensen S (2008) Phylogenetic and morphological characterisation of the green algae infesting blue mussel *Mytilus edulis* in the North and South Atlantic oceans. *Dis Aquat Organ* **81**: 231–240.

Ronquist F, Huelsenbeck JP & van der Mark P (2005) *MyBayes 3.1 Manual*. Available from http://mrbayes.csit.fsu.edu/mb3. 1_manual.pdf (Accessed June 2009).

Schaper T & Ott S (2003) Photobiont selectivity and interspecific interactions in lichen communities. I. Culture experiments with the mycobiont *Fulgensia bracteata*. *Plant Biology* **5**: 441–450.

Sherwood MA (1977a) The ostropalean fungi. *Mycotaxon* **5**: 1–277.

Sherwood MA (1977b) The ostropalean fungi II: *Schizoxylon*, with notes on *Stictis*, *Acarosporina*, *Coccopeziza*, and *Carestiella*. *Mycotaxon* **6**: 215–260.

Stocker-Wörgötter E (1995) Experimental cultivation of lichens and lichen symbionts. *Can J Botany* **73**: S579–S589.

Stocker-Wörgötter E (2001) Experimental lichenology and microbiology of lichens: culture experiments, secondary chemistry of cultured mycobionts, resynthesis, and thallus morphogenesis. *Bryologist* **104**: 576–581.

Stocker-Wörgötter E (2002) Investigating the production of secondary compounds in cultured lichen mycobionts. *Protocol in Lichenology, Culturing Biochemistry, Ecophysiology and Use in Biomonitoring* (Kanner I, Beckett RP & Varma AK, eds), pp. 296–306. Springer, Berlin, Germany.

Taylor JW, Jacobson DJ, Kroken S, Kasuga T, Geiser DM, Hibbett DS & Fisher MC (2000) Phylogenetic species recognition and species concepts in fungi. *Fungal Genet Biol* **31**: 21–32.

Templeton AR, Crandall KA & Sing CF (1992) A cladistic analysis of phenotypic associations with haplotype inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**: 619–633.

Tremouillaux-Guiller J & Huss VA (2007) A cryptic intracellular green alga in *Ginkgo biloba*: ribosomal DNA markers reveal worldwide distribution. *Planta* **226**: 553–557.

Tschermak-Woess E (1988) The algal partner. *Handbook of Lichenology, Vol. I* (Galun M, ed), pp. 39–91. CRC Press Inc., Boca Raton, FL.

Wedin M, Döring H & Gilenstam G (2004) Saprotrophy and lichenization as options for the same fungal species on different substrata: environmental plasticity and fungal lifestyles in the *Stictis–Conotrema* complex. *New Phytol* **164**: 459–465.

Wedin M, Döring H, Könberg K & Gilenstam G (2005a) Generic delimitations in the family Stictidaceae (Ostropales, Ascomycota) – the *Stictis–Conotrema* problem. *Lichenologist* **37**: 67–75.

Wedin M, Wiklund E, Crewe A, Döring H, Ekman S, Nyberg Å, Schmitt I & Lumbsch T (2005b) Phylogenetic relationships of the Lecanoromycetes (Ascomycota) as revealed by analyses of mtSSU and nLSU rDNA sequence data. *Mycol Res* **109**: 159–172.

Wedin M, Döring H & Gilenstam G (2006) *Stictis* s. lat. (Ostropales, Ascomycota) in northern Scandinavia, with a key and notes on morphological variation in relation to lifestyle. *Mycol Res* **110**: 773–789.

Wedin M, Westberg M, Crewe AT, Tehler A & Purvis OW (2009) Species-delimitation and evolution of mineral bioaccumulation in the lichenized *Acarospora smaragdula* (Ascomycota, Fungi) complex. *Cladistics* **25**: 161–172.

White TJ, Burns TD, Lee S & Taylor J (1990) Amplification and direct sequencing of fungal ribosomal DNA genes for phylogenies. *PCR Protocols, a Guide to Methods and Applications* (Innis MA, Gelfand DH, Snisky JJ & White TJ, eds), pp. 315–322. Academic Press, San Diego.

Yamamoto Y, Kinoshita Y & Yoshimura I (2002) Culture of thallus fragments and re-differentiation of lichens. *Protocol in Lichenology, Culturing Biochemistry, Ecophysiology and Use in Biomonitoring* (Kranner I, Beckett RP & Varma AK, eds), pp. 34–46. Springer, Berlin, Germany.

Zoller S & Lutzoni F (2003) Slow algae, fast fungi: exceptionally high nucleotide substitution rate differences between lichenized fungi *Omphalina* and their symbiotic green algae *Coccomyxa*. *Mol Phylogenet Evol* **29**: 629–640.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Schematic representation of the phylogenetic inferences obtained for each fungal marker: β-tubulin (a), GPD (b), and ITS (c).

Table S1. Estimation of haplotype diversity in the β-tubulin, GPD, and ITS loci in *Schizoxylon albescens*.

Table S2. Co-culture experiments of *Schizoxylon albescens* and algal strains.

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