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ARTICLE *in* NEW PHYTOLOGIST · JANUARY 2014

Impact Factor: 7.67 · DOI: 10.1111/nph.12678 · Source: PubMed

Molecular phylogeny and symbiotic selectivity of the green algal genus *Dictyochloropsis* s.l. (Trebouxiophyceae): a polyphyletic and widespread group forming photobiont-mediated guilds in the lichen family Lobariaceae

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Received: *17 October 2013*
Accepted: *5 December 2013*

New Phytologist (2014)
doi: 10.1111/nph.12678

Key words: *18S*, *Dictyochloropsis*, internal transcribed spacer (ITS), lichen, photobiont, *rbc*L, Lobariaceae, microsatellite.

Summary

• *Dictyochloropsis* s.l. is an ecologically important, common but little-studied genus of green algae. Here, we examined the diversity and host selectivity of algae attributed to this genus at both species-to-species and species-to-community levels.

• We conducted a molecular investigation of 15 cultured strains and several lichen photobionts, using *18S* rRNA, *rbc*L and ITS sequence data. We further used seven alga-specific microsatellite markers to study algal sharing among fungi of the family Lobariaceae in two populations in Madeira and Taiwan (454 lichens).

• We found that the genus *Dictyochloropsis* s.l. is polyphyletic. *Dictyochloropsis* clade 1 comprises only free-living algae whereas *Dictyochloropsis* clade 2 includes lichenized algae as well as free-living algae. Fungal selectivity towards algae belonging to *Dictyochloropsis* clade 2 is high. Selectivity varies geographically, with photobionts being restricted to a single region. Finally, we showed that *Dictyochloropsis* clade 2 individuals are shared among different fungal hosts in communities of lichens of the Lobariaceae.

• As for other green algal lineages, there is a high amount of cryptic diversity in *Dictyochloropsis*. Furthermore, co-evolution between *Dictyochloropsis* clade 2 algae and representatives of the Lobariaceae is manifested at the community level, with several unrelated fungal species being horizontally connected by shared photobiont clones.

Introduction

Lichens are obligate symbiotic associations between a fungus (mycobiont) and a population of algae and/or cyanobacteria (photobionts) (Ahmadjian, 1993; Honegger, 2012). Although the symbiotic state is obligate for the formation of the fungal thallus, it is not clear whether green algal photobionts are also obligatorily symbiotic. Scattered observations support the hypothesis that members of *Trebouxia* and *Asterochloris*, the most common lichen photobionts, may grow in the free-living state (Bubrick *et al.*, 1984; Mukhtar *et al.*, 1994). Nevertheless, free-living, locally available symbiotic green algae seem to be mostly rare and inconspicuous (Wornik & Grube, 2010).

The genetic structure of the lichen symbiosis is profoundly influenced by the way photobionts are dispersed and transmitted to their fungal host (Fernández-Mendoza *et al.*, 2011; Dal Grande *et al.*, 2012). On the one hand, vertical transmission of photobionts occurs when fungal and algal symbionts are co-dispersed from one generation to the next, in the form of asexual propagules, mostly over short distances (Büdel & Scheidegger, 2008; Dal Grande *et al.*, 2012; Werth & Scheidegger, 2012). Vertical photobiont transmission leads to congruent mycobiont–photobiont genetic structures at the intrapopulation and, in some cases, continental levels (Dal Grande *et al.*, 2012; Werth & Scheidegger, 2012; Widmer *et al.*, 2012). Sexual propagation, on the other hand, breaks symbiosis and usually fungal spores are dispersed separately from their photobiont. Sexual dispersal results in the reshuffling of the symbiotic association – horizontal photobiont transmission – because the symbiosis has to be reconstituted. This process is called relichenization. It can significantly influence mycobiont–photobiont relations, even in predominantly vegetative species (Ott, 1987; Nelsen & Gargas, 2008,

2009; Dal Grande *et al.*, 2012). Several strategies have been proposed to explain how the process of relichenization can occur in nature. For example, spores may capture the photobiont from other lichens as reported by Friedl (1987), Rikkinen (1995) and Beck *et al.* (2002), especially from juvenile stages or senescent/damaged thalli of closely related species (DeBach, 1964; Beck *et al.*, 1998). Faecal pellets of lichenivorous orobatid mites or snails containing viable photobiont cells represent another source of algae for reconstituting the symbiosis (Meier *et al.*, 2002; Boch *et al.*, 2011).

It has been hypothesized that, in species with predominantly sexual reproduction, lichen symbiotic association is less specific, and mycobionts tend to associate with a wide range of photobionts. In the last decades, molecular markers and phylogenetic analyses have been applied extensively to determine the level of specialization in mycobiont–photobiont associations. Different association patterns have been described, from high host selectivity where the mycobiont accepts only a single algal strain (e.g. *Psoroglaena stigonemoides* associated with the green alga *Auxenochlorella protothecoides* and *P. epiphylla*/*Chlorella luteoviridis*; Nyati *et al.*, 2007), to generalism where the host accepts multiple photobionts (Beck *et al.*, 2002; Romeike *et al.*, 2002; Blaha *et al.*, 2006; Guzow-Krzeminska, 2006; Otálora *et al.*, 2010; Muggia *et al.*, 2013; O'Brien *et al.*, 2013). The degree of host selectivity (range of compatible photobionts for a given lichen fungus) can vary even among closely related species or within a species range (Yahr *et al.*, 2004, 2006; Fernández-Mendoza *et al.*, 2011). High specificity is found in lichen symbioses where both partners are strictly selective towards each other (Beck *et al.*, 2002). On the photobiont side, it has been shown that identical algal strains (green algae or cyanobacteria) can generally form symbiotic associations with several lichen-forming fungal species (Beck *et al.*, 1998; Rikkinen *et al.*, 2002; Wirtz *et al.*, 2003; O'Brien *et al.*, 2005; Honegger, 2008, 2012).

Interestingly, it has been suggested that fungal hosts may express their photobiont specificity at a community level (photobiont-mediated lichen guilds; Rikkinen *et al.*, 2002; O'Brien *et al.*, 2013). Lichen guilds are communities of lichens growing in the same habitat that are horizontally linked by sharing the same photobiont. Green algal lichen guilds have been reported for lichens of the genera *Lecidella* and *Xanthoria* associated with *Trebouxia* (Beck *et al.*, 1998) and more recently for *Lepraria* and *Stereocaulon* associated with the green algal genus *Asterochloris* (Peksa & Škaloud, 2011). However, due to the lack of highly variable molecular markers, the lichen guild hypothesis has thus far only been tested at the species level of mycobiont–photobiont interactions. Also, marker resolution becomes critical when studying highly clonal organisms such as lichens (Arnaud-Haond *et al.*, 2007; Dal Grande *et al.*, 2012). In a recent study on a green algal lichen symbiosis associated with the green algal genus *Dictyochloropsis*, however, highly variable microsatellite markers enabled us to track down the photobiont transmission to the individual/thallus level and to identify clonal thalli in a population/guild (Dal Grande *et al.*, 2012).

One of the most challenging aspects of lichen biology has been unveiling the identity of the photobionts and the reciprocal selectivity of the symbionts (Beck *et al.*, 2002; Rikkinen *et al.*, 2002; Rikkinen, 2003; Otálora *et al.*, 2010). Photobiont selectivity studies rely on the phylogenetic characterization of algal taxa involved in the symbiosis. However, there is a general consensus that the diversity of green microalgae at all levels is still far from fully understood. A number of recent studies revealed the polyphyly of many morphologically defined genera in Trebouxiophyceae, the most common class of lichenized green algae (Friedl & Büdel, 2008). Studies on green algal lichen photobionts have focused mainly on the genera *Trebouxia* and *Asterochloris* (Trebouxiophyceae), although some studies have begun investigating many of the other lineages of lichen-associated Trebouxiophyceae (*Diplosphaera*: Thüs *et al.*, 2011; Fontaine *et al.*, 2012; *Coccomyxa* and *Pseudococcomyxa*: Lohtander *et al.*, 2003; Zoller & Lutzoni, 2003; Muggia *et al.*, 2011). *Dictyochloropsis* is a common but less-studied, ecologically important genus of aerophytic and lichenized algae. The genus was introduced by Geitler (1966) with the free-living type species *D. splendida*, and later emended by Tschermak-Woess (1980, 1984) with the addition of lichenized taxa (from here on called *Dictyochloropsis* s.l.). Representatives of *Dictyochloropsis* s.l. have been reported to form associations with many lichens of the family Lobariaceae (Tschermak-Woess, 1984, 1988, 1995; Moncada *et al.*, 2013), Ramalinaceae, Catillariaceae and Megalosporaceae (Nakano *et al.*, 1991; Rambold *et al.*, 1998). Species of *Dictyochloropsis* s.l. have also been reported as free-living, for example, soil and epiphytic algae (Geitler, 1966). One species of *Dictyochloropsis* s.l., *D. reticulata*, is the photobiont of the frequently studied lichen species *Lobaria pulmonaria*, and has been extensively studied regarding its population genetics and mode of transmission (Werth *et al.*, 2006, 2007; Dal Grande *et al.*, 2012; Grube & Spribille, 2012; Scheidegger *et al.*, 2012; Widmer *et al.*, 2012). Previous investigations on a global scale have shown that its fungal host is highly selective towards this widely distributed algal species (Walser *et al.*, 2004; Widmer *et al.*, 2012). Conversely, *D. reticulata* was reported to form symbioses with different lichen-forming fungi. However, the bulk of literature concerning the degree of selectivity for this organism relies on morphological identifications of algal cultures (Tschermak-Woess, 1951, 1984, 1995). Recently, Widmer *et al.* (2010) showed with molecular methods that the photobiont of *L. pulmonaria* is found in other Lobariaceaen lichens (*L. macaronesica*, *L. tuberculata*) and that the culture isolated from the lichen *Brigantiaea ferruginea* (Müll. Arg.) Kashiw. & Kurok. (Takeshita *et al.*, 1991), used in many studies as a reference culture for *D. reticulata* (Friedl, 1995; Walser *et al.*, 2003), belongs to a different algal lineage. Recent studies presented evidence that *Dictyochloropsis* s.l. is polyphyletic (Škaloud *et al.*, 2007; Widmer *et al.*, 2010; Thüs *et al.*, 2011). Moreover, Škaloud *et al.* (2005) emphasized that the present taxonomic diversity of the genus does not cover the overall variability of its representatives. However, the scarcity of molecular data has so far prevented an accurate assessment of the taxonomy and phylogenetic position of the representatives of this genus.

The present study sets out to achieve three goals. The first is to identify genetically distinct monophyletic groups of strains of green algae assigned to the genus *Dictyochloropsis* s.l. The second

goal is to determine the degree of fungal selectivity within this algal lineage. To achieve these objectives, we used a molecular multi-locus phylogenetic approach to investigate the genetic variability of free-living as well as symbiotic algae collected across a broad geographical spectrum. The third goal is to test the existence of photobiont-mediated lichen guilds at a high-resolution level, using microsatellite fingerprinting to access the taxonomic range of compatible hosts and the amount of intrapopulation horizontal transmission for one *Dictyochloropsis* s.l. species. Studying patterns of symbiont diversity and selectivity is crucial for the understanding of fundamental ecological processes such as dispersal and establishment in the family Lobariaceae whose representatives are related to old-growth forest structures and are widely used as indicators of ecological continuity in conservation practice (Scheidegger & Werth, 2009).

Materials and Methods

Species sampling and algal cultures

Strains were sampled to represent the diversity of the algae attributed to the genus *Dictyochloropsis* s.l. Furthermore, algal symbionts were sequenced from lichen fungi in the Lobariaceae (genera *Crocodia*, *Dendriscosticta*, *Lobaria*, *Lobariella*, *Pseudocyphellaria*, *Ricasolia*, *Sticta* s.l.) collected in Europe, North, Central and South America, Russia, China, Taiwan and New Zealand. Details of the material, area of collection and GenBank accession numbers are presented in Table 1.

Algal cultures and uncultured lichen photobionts were sequenced at two loci: part of the nuclear small subunit ribosomal RNA gene (*18S* rRNA; 43 specimens) and part of the plastid-encoded large subunit of the ribulose-bisphosphate carboxylase-RuBisCO gene (*rbc*L; 52 specimens). For a subset of specimens and additional 19 specimens of lichens of the genera *Lobaria*, *Dendriscosticta* and *Ricasolia* (see Table 1), we also obtained sequences of the nuclear internal transcribed spacer (ITS). Most likely ITS2 secondary structures of the RNA transcript were determined by depicting the highly conserved start and end region of the four helices (Mai & Coleman, 1997). The structure of these sequence sections has been calculated using the RNAfold WebServer (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi).

Total lichen DNA was isolated from dried thallus material (35–50 mg) or from sterile algal cultures (50 mg) using the DNeasy 96 Plant Kit (Qiagen) according to the manufacturer's protocol. All amplifications were performed in a total volume of 50 µl with 2 µl DNA-extract using JumpStart™ REDTaq® ReadyMix™ (Sigma-Aldrich) and 100 nM of each primer. For primer sequences and PCR cycling conditions, see Table 2. All PCR products were purified with the MinElute PCR Purification Kit (Qiagen) and labeled with Big Dye Terminator v3.1 Kit (Life Technologies, Carlsbad, CA, USA). Cycle sequencing was performed as follows: 25 cycles of: 20 s 96°C, 5 s 50°C, 2 min 60°C. Post reaction cleanup was performed using Performa DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD, USA) following the manufacturer's protocol. Forward and reverse strand sequences were detected in an ABI PRISM 3100 Avant (Life Technologies) and checked with BLASTN (megablast) in GenBank. Sequence contigs were trimmed, assembled, aligned and manually edited in CLC DNA Workbench software (CLC-Bio, Mühltal, Germany). Regions containing gaps or poorly aligned sites were manually removed from the dataset.

Phylogenetic analyses

Each *18S* and *rbc*L sequence was BLASTed against the entire GenBank database. For each sequence, the top 100 BLAST hits belonging to the class Trebouxiophyceae or Ulvophyceae were included in the dataset. In total, our *18S* and *rbc*L datasets included 415 and 431 sequences, respectively. After the exclusion of identical sequences, datasets were reduced to 284 (*18S*) and 278 (*rbc*L) unique sequences. Datasets were analyzed separately. For each dataset, the nucleotide sequences were aligned with the program MAFFT v5 (Katoh & Standley, 2013) and introns were removed manually. Phylogenetic relationships and their confidence values were inferred using RAxML (1000 bootstrap pseudo-replicates), and Bayesian Markov chain Monte Carlo (BM) method (Metropolis *et al.*, 1953; Hastings, 1970) as implemented in MrBayes 3.1 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). All ML searches followed a GTRGAMMA model of molecular evolution. For BM analysis, the best-fit model was selected with the corrected Akaike Information Criterion and the Bayesian Information Criterion as implemented in jModelTest 0.1.1 (TrNef + I + G, $-\log_e = 10\,464.3541$ for *18S* rRNA; 012212 + I + G + F, $-\log_e = 23491.1044$ for *rbc*L; Guindon & Gascuel, 2003; Posada, 2006, 2008). All trees were rooted and BM phylograms including posterior probabilities were computed with five million generations, one out of every 100 trees was sampled and the first 12 500 trees were discarded as burn-in (likelihoods below stationary level). In all analyses, representatives of *Ulvales* were used as outgroup. ML trees of the two loci were graphically displayed with FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Several clades were collapsed for clarity of presentation.

We used alternative hypothesis testing to evaluate whether our data are sufficient to reject monophyly of *Dictyochloropsis* s.l. Using the GTR + I + G nucleotide substitution model, constrained and unconstrained trees were inferred, as implemented in Tree-PUZZLE 5.2 (Schmidt *et al.*, 2002). We used two tests to compare the different topologies: the Shimodaira–Hasegawa (SH) test (Shimodaira & Hasegawa, 1999) and the expected likelihood weight (ELW) test (Strimmer & Rambaut, 2002).

Haplotype networks were inferred for *18S* and *rbc*L alignments of the photobionts of the host genera *Crocodia*, *Dendriscosticta*, *Lobaria*, *Lobariella*, *Pseudocyphellaria*, *Ricasolia*, *Sticta* and for the ITS alignment of 24 photobionts of samples that amplified at seven microsatellite loci using statistical parsimony with multibase indels coded as single characters with TCS v1.21 (Clement *et al.*, 2000).

Analysis of specificity and microsatellite population genetics

Seven highly variable microsatellite markers (SSRs) specific for the algal strain AB06.006A2 (Dal Grande *et al.*, 2010; Widmer *et al.*, 2010) were tested on all samples.

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Table 1 Algal strains and lichen photobionts used in the present study and *18S*, *rbc*L and internal transcribed spacer (ITS) accession numbers

Strain/ Isolate	Algal culture/Species[1], Clade	*18S*	*rbc*L	ITS/SSRs(*)	Host	Origin
SAG2098	*Dictyochloropsis asterochloroides*	**KC333460**	**KC333606**	–	Free living	Japan: Yokogawa, Hiroshima-City
SAG2073	*Dictyochloropsis asterochloroides*	**KC333459**	KC333605	–	Free living	Japan: Obayashi, Asakita-ku, Hiroshima-Pref.
CAUPH8602	*Dictyochloropsis reticulata*	**KC333479**	**KC333603**	KC333516	Free living	Malaysia: Hulu Kelantan
SAG2071	*Dictyochloropsis splendida*	**KC333456**	**KC333609**	KC333510	Free living	Japan: Nishi-kawaguchi-cho, Hiroshima-Pref.
SAG2097	*Dictyochloropsis splendida*	**KC333458**	**KC333608**	KC333511	Free living	Japan: Teramachi, Hiroshima-City
SAG244.80	*Dictyochloropsis splendida*	**KC333474**	**KC333599**	KC333514	*Chaenotheca brunneola*	Austria: near Graz
CAUPH8601	*Dictyochloropsis splendida*	**KC333457**	**KC333607**	KC333515	Free living	Czech Republic: České Středohoří Mts.
CAUPH8603	*Dictyochloropsis symbiontica*	**KC333473**	**KC333602**	KC333517	Free living	Malaysia: Tioman Island
CCHU5616	*Dictyochloropsis reticulata*	**KC333476**	**KC333590**	KC333524	*Brigantiaea ferruginea*	Japan
SAG2036	*Dictyochloropsis irregularis*	**KC333475**	**KC333600**	KC333522	Free living	Austria: Styria, Obdacher Sattel
SAG53.87	*Dictyochloropsis reticulata*, Sym5	KC333466	KF960688	KC333513/ *	*Lobaria macaronesica*	Spain: Teneriffe, Roque Chinotére
SAG46.85	*Dictyochloropsis symbiontica*, Sym4	**KC333470**	**KC333574**	KC333521	*Crocodia aurata*	New Zealand: North Island, North Auckland
SAG27.81	*Dictyochloropsis symbiontica*	**KC333480**	–	KC333512	*Chaenothecopsis consociata*	Austria: Lunz
AB06.006A2	*Dictyochloropsis reticulata*, Sym5	**KC333463**	**KC333577**	KC333520/ *	*Lobaria pulmonaria*	Spain: Pamplona
MP124	Uncultured photobiont	**KC333485**	**KC333595**	–	*Brigantiaea leucoxantha*	USA: Florida
MP167	Uncultured photobiont	**KC333486**	**KC333596**	–	*Megalospora sulphurata*	Brazil
MP168	Uncultured photobiont, Sym2	**KC333487**	**KC333582**	KC333538	*Lobariella* sp.	Brazil
MP169	Uncultured photobiont, Sym4	**KC333488**	**KC333575**	–	*Pseudocyphellaria* sp.	Brazil
MP668	Uncultured photobiont	–	**KC333591**	–	*Biatora* sp.	USA: Alaska
MP669	Uncultured photobiont	–	**KC333592**	–	*Biatora* sp.	USA: Alaska
MP737	Uncultured photobiont, Sym2	–	**KC333585**	–	*Sticta* sp.	Costa Rica
MP774	Uncultured photobiont, Sym1	–	**KC333584**	–	*Lobariaceae*	Costa Rica
MP775	Uncultured photobiont, Sym4	–	**KC333576**	–	*Pseudocyphellaria* sp.	Costa Rica
MP776	Uncultured photobiont, Sym1	–	**KC333583**	–	*Lobariella* sp.	Costa Rica
NZ1568	Uncultured photobiont, Sym7	**KC333490**	–	KC333541	*Pseudocyphellaria lividofusca*	New Zealand
NZ1570	Uncultured photobiont, Sym8	–	**KC333594**	KC333555	*Pseudocyphellaria lindsayi*	New Zealand
NZ1873	Uncultured photobiont	–	**KC333586**	–	*Crocodia aurata*	New Zealand
NZ5869	Uncultured photobiont, Sym9	–	**KC333598**	KC333550	*Pseudocyphellaria fimbriata*	New Zealand
NZ6001	Uncultured photobiont, Sym9	–	**KC333597**	KC333545	*Pseudocyphellaria homoeophylla*	New Zealand
NZ6006	Uncultured photobiont, Sym8	–	**KC333593**	–	*Sticta subcaperata*	New Zealand
NZ6009	Uncultured photobiont, Sym7	**KC333496**	–	KC333540	*Pseudocyphellaria multifida*	New Zealand
NZ6021	Uncultured photobiont, Sym7	**KC333498**	–	KC333539	*Sticta latifrons*	New Zealand
SA5417	Uncultured photobiont, Sym1	**KC333502**	**KC333620**	KC333528	*Lobariella pallidocrenulata*	Colombia
SA5420	Uncultured photobiont, Sym1	–	**KC333621**	KC333529	*Lobariella crenulata*	Colombia
SA5513	Uncultured photobiont, Sym1	**KC333503**	–	KC333530	*Lobariella pallidocrenulata*	Colombia
SA5514	Uncultured photobiont, Sym1	–	**KC333622**	KC333531	*Lobariella pallidocrenulata*	Colombia
SA5523	Uncultured photobiont, Sym3	**KC333505**	**KC333619**	–	*Sticta aff. neopulmonaria*	Colombia
SA5528	Uncultured photobiont, Sym2	–	**KC333617**	KC333533	*Sticta aff. neopulmonaria*	Colombia
SA5533	Uncultured photobiont, Sym2	**KC333504**	–	KC333534	*Sticta pulmonarioides*	Colombia
SA5534	Uncultured photobiont, Sym3	**KC333506**	**KC333618**	–	*Sticta aff. neopulmonaria*	Colombia

Table 1 (Continued)

Strain/ Isolate	Algal culture/Species[1], Clade	*18S*	*rbc*L	ITS/SSRs(*)	Host	Origin
SA5538	Uncultured photobiont, Sym2	**KC333507**	–	–	*Sticta* sp.	Colombia
SA5541	Uncultured photobiont, Sym3	–	**KC333616**	–	*Sticta* sp.	Colombia
SAG2069	*Dictyochloropsis splendida*	**KF960690**	**KC333601**	KC333509	Free living	Japan: Tojo-cho, Hiroshima-Pref.
SCH-12317	Uncultured photobiont, Sym4	KC333468	KC333572	–	*Crocodia aurata*	Madeira
SCH-AB08.002d	Uncultured photobiont, *Elliptochloris* sp. clade	KC333461	KC333610	–	*Catillaria chalybeia*	Switzerland
SCH-6057	Uncultured photobiont, Sym3	**KC333471**	**KC333580**	KC333527	*Sticta canariensis*	Spain: Tenerife
SCH-6058	Uncultured photobiont, Sym3	KC333472	KC333581	KC333523	*Sticta canariensis*	Spain: Tenerife
SCH-1069	Uncultured photobiont, Sym5	KC333465	KC333623	KC333557	*Dendriscosticta platyphylla*	Russia: South Baikal Lake
SCH-17084	Uncultured photobiont, Sym4	**KC333467**	KC333571	–	*Lobaria patinifera*	Ecuador: Galapagos
SCH-2021	Uncultured photobiont, Sym5	KC333464	KC333578	KC333558/*	*Dendriscosticta wrightii*	Canada: British Columbia
SCH-2339	Uncultured photobiont, Sym5	KC333508	KC333579	KC333559/*	*Ricasolia amplissima*	Greece: Peloponnese
SCH-1998	Uncultured photobiont	**KC333477**	KC333587	KC333525	*Lobaria oregana*	Canada: British Columbia
SCH-17733	Uncultured photobiont	KC333478	KC333588	–	*Lobaria oregana*	Canada: British Columbia
MP521	Uncultured photobiont	–	**KC333589**	–	*Lobaria oregana*	USA: Washington
SCH-22386	Uncultured photobiont	**KC333481**	**KC333566**	KC333518	*Sticta* sp.	Taiwan: Yilan County
SCH-22379	Uncultured photobiont	KC333482	KC333567	–	*Sticta* sp.	Taiwan: Yilan County
SCH-22290	Uncultured photobiont	KC333483	KC333568	KC333519	*Pseudocyphellaria* sp.	Taiwan: Yilan County
SCH-22297	Uncultured photobiont	KC333484	KC333569	–	*Pseudocyphellaria* sp.	Taiwan: Yilan County
SCH-6004	Uncultured photobiont	KC333462	KF960689	KC333526	*Crocodia aurata*	USA: North Carolina

Additional lichen specimens which were sequenced at the ITS region and amplified at seven alga-specific microsatellite loci (LPh1-LPh7; Dal Grande *et al.*, 2010)

Host	Genbank accession numbers	Voucher/ Strain	Country	Collector	Year
Ricasolia amplissima	KF960669	SCH-19902	Portugal	Scheidegger C & Werth S	2007
Lobaria crassior	KF960670	CT1/01c	TAIWAN: Hualien County	Scheidegger C	2010
L. crassior	KF960671	CT2/-1a	TAIWAN: Hualien County	Scheidegger C	2010
L. gyrophorica	KF960672	SCH-22531	TAIWAN: Hualien County	Scheidegger C	2010
L. immixta	KF960673	SCH-2156	SPAIN: Canary Islands	Nittinger F	2001
L. isidiophora	KF960674	SCH-22477	TAIWAN: Miaoli County	Dal Grande F & Scheidegger C	2010
L. japonica	KF960675	SCH-1502	RUSSIA: Sakhalin Island	Scheidegger C, Chabanenko S, Taran A	2002
L. kazawaensis	KF960676	SCH-1582	RUSSIA: Sakhalin Island	Scheidegger C, Chabanenko S, Taran A	2002
L. orientalis	KF960677	SCH-22463	TAIWAN: Miaoli County	Dal Grande F & Scheidegger C	2010
L. pindarensis	KF960678	SCH-18714	NEPAL	Scheidegger C	2009
	KF960679	SCH-18717	NEPAL	Scheidegger C	2009
L. sachalinensis	KF960680	SCH-1569	RUSSIA: Sakhalin Island	Scheidegger C, Chabanenko S, Taran A	2002
L. spathulata	KF960681	SCH-1524	RUSSIA: Sakhalin Island	Scheidegger C, Chabanenko S, Taran A	2002
L. sublaevis	KF960682	SCH-10154	PORTUGAL: Madeira Island	Scheidegger C & Werth S	2007
L. tuberculata	KF960683	SCH-1553	RUSSIA: Sakhalin Island	Scheidegger C, Chabanenko S, Taran A	2002
L. virens	KF960684	SCH-18432	UNITED KINGDOM: Scotland	Scheidegger C, Scheidegger D	2007
L. yunnanensis	KF960685	SCH-22499	TAIWAN: Miaoli County	Dal Grande F & Scheidegger C	2010
Dendriscosticta plathyphylloides	KF960686	SCH-18864	NEPAL: Rasuwa District	Scheidegger C	2009
D. praetextata	KF960687	SCH-19115	NEPAL: Rasuwa District	Scheidegger C	2009

Strains/isolates in bold are reported in Fig. 1 and Supporting Information Fig. S1.
[1]Names of the cultures refer to the most recent names given at the Culture Collection of Algae at Goettingen University (SAG).
*Samples which amplified at seven alga-specific microsatellite loci (Dal Grande *et al.*, 2010).

Table 2 PCR cycling conditions and primer sequences for *18S*, *rbc*L and internal transcribed spacer (ITS) primers

Marker region	*18S*		*rbc*L				ITS
PCR[1]	CV1/CV2	a(treb)-nu-SSU-0078-5′-mpn, nu-SSU-0402-5′ (NS19UCB)/a(treb)-nu-SSU-0803-3′-mpn	rbcLa/b	rbcLAV/IN	a-ch- rbcL-203-5′-MPN/a-ch-rbcL-991-3′-MPN	nr-SSU-1780-5′ Algal/A-ITS-R	a-nu-ssu-1752-5′/ITS4T
Initial denaturation	94°C (2 min)	95°C (5 min)	94°C (2 min)	94°C (2 min)	95°C (5 min)	94°C (2 min)	95°C (5 min)
Number of cycles	30	35	30	30	40	30	10/25
Denaturation	94°C (30 s)	95°C (60 s)	94°C (30 s)	94°C (30 s)	95°C (60 s)	94°C (30 s)	95°C (60 s)
Annealing	55°C (30 s)	50°C (60 s)	55°C (30 s)	55°C (30 s)	50°C (60 s)	59°C (30 s)	62°C/53°C (60 s)
Extension	72°C (60 s)	72°C (60 s)	72°C (60 s)	72°C (60 s)	72°C (60 s)	72°C (60 s)	72°C (60 s)
Final extension	72°C (10 min)	72°C (7 min)	72°C (10 min)	72°C (10 min)	72°C (7 min)	72°C (10 min)	72°C (7 min)

Locus	Primers	Primer Sequence	Orientation	Reference
18S	CV1	TACCTGGTTGATCCTGCCAGTAG	Forward	Sawayama *et al.* (1995)
18S	CV2	CCAATCCCTAGTCGGCATCGT	Reverse	Sawayama *et al.* (1995)
18S	a(treb)-nu-SSU-0078-5′-mpn	CATGTCTAAGTATAAACTGCT	Forward	This study[2]
18S	nu-SSU-0402-5′ (NS19UCB)[3]	CCGGAGAAGGAGCCTGAGAAAC	Forward	Gargas & Taylor (1992)
18S	nu-SSU-0553-3′ (NS2)	GGCTGCTGGCACCAGACTTGC	Reverse	White *et al.* (1990)
18S	a(treb)-nu-SSU-0803-3′-mpn	TAGGCCAGAGTCCTATCGTGTTAT	Reverse	This study[2]
*rbc*L	rbcLa	ACAAAGGTCGCTGTTACGATATTG	Forward	Widmer *et al.* (2010)
*rbc*L	rbcLb	CTCGTTCGCCTTCTAGTTTACC	Reverse	Widmer *et al.* (2010)
*rbc*L	rbcLAV	TGCAGCTGAATCGTCTACAG	Forward	This study
*rbc*L	rbcLIN	GTTGCGATCTTTTTCGATGT	Reverse	This study
*rbc*L	a-ch-rbcL-203-5′-MPN	GAATCWTCWACWGGWACTTGG ACWAC	Forward	Nelsen *et al.* (2011)
*rbc*L	a-ch-rbcL-991-3′-MPN	CCTTCTARTTTACCWACAAC	Reverse	Nelsen *et al.* (2011)
ITS	nr-SSU-1780-5′ Algal	CTGCGGAAGGATCATTGATTC	Forward	Piercey-Normore & De Priest (2001)
ITS	A-ITS-R	GCGGGTGATCTTGCCTGAA	Reverse	Widmer *et al.* (2010)
ITS	a-nu-ssu-1752-5′	CTAGAGGAAGGAGAAGTCGT	Forward	Nelsen & Gargas (2006)
ITS	ITS4T	GGTTCGCTCGCCGCTACTA	Reverse	Kroken & Taylor (2000)

[1]Life Technologies Veriti thermal cycler (Life Technologies).
[2]Primer nomenclature for the newly described primers follows that of Gargas & DePriest (1996) and Nelsen & Gargas (2006), with primer locations corresponding to those of *Chlamydomonas reinhardtii* (GenBank Accession M32703).
[3]This primer was initially described as one which excludes algal amplification, while preferentially amplifying fungi (Gargas & DePriest, 1996); however, we were able to successfully use this primer to amplify algae from some lichen thalli. It is possible that with an increased annealing temperature, this primer would preferentially amplify fungi over algae. This primer was also used to amplify the *18S* from algae by Schmitt & Lumbsch (2001) and Muggia *et al.* (2011).

In order to test the existence of photobiont-mediated guilds sharing the algal strain AB06.006A2, we used SSR markers to further determine the algal genotype in two old-growth forests harbouring rich Lobariaceaen communities, that is, a primary laurel forest (laurisilva) in Madeira (Portugal; 32°45′37.0″N, −17°00′57.8″E, 824 m above sea level (a.s.l.)) and a subtropical mountainous broadleaved evergreen forest in Taiwan (Hualien County; 24°10′05.6″N, 121°17′29.8″E, 2938 m a.s.l.). Our sampling design was conceived to maximize the likelihood of collecting an identical photobiont genotype in different fungal host species. From the geographic center of the population, ten trees within an area of *c.* 900 square meters were checked for Lobariacean species. Thalli of *Lobaria* and *Dendriscosticta* species for each cardinal point were collected over the whole height of each tree. When several thalli of the same lichen were found at the same height of a tree, a maximum of three thalli per species was collected, for a total of 249 specimens for Madeira, and 205 for Taiwan.

Microsatellite genotyping was performed on total lichen DNA following Dal Grande *et al.* (2012). Fragment lengths were determined on a 3730 DNA Analyzer (Life Technologies), and electropherograms were analyzed with GENEMAPPER 3.7 (Life Technologies) using LIZ-500 as internal size standard. Microsatellite data were deposited in the Dryad Digital Repository: http://doi.org/10.5061/dryad.4q6b9. For each sampling site, photobiont populations were defined according to the fungal host (Table 3). For each population, the number of multi-locus genotypes (MLGs), unbiased genetic diversity (*h*) and number of alleles were calculated in GenAlex v6.5 (Peakall & Smouse, 2012). Samples having identical MLGs were treated as clones. The extent of interlocus recombination for the photobiont of each population was characterized using Multilocus 1.2 (Agapow

& Burt, 2001). The occurrence of identical algal MLGs in different lichen fungi was represented as I × J contingency tables using the function *visweb* in R, where each cell depicts the number of interactions recorded between a clonal algal MLG and a specific combination of at least two different lichen fungi. Algal genotype occurrences within fungal species (presence–absence matrices) were also represented as a network using the function *plotweb* (package *bipartite*; Dormann *et al.*, 2008, 2009) and the package *pheatmap* in R (R Development Core Team, 2012). Algal genotypes and fungal species correspond to the nodes of the network, and genotype occurrences in lichens correspond to the links between the nodes. Host specialization was further inferred by grouping photobiont MLGs by fungal host species and calculating pairwise linearized F_{ST} in Arlequin v3.01 (Excoffier *et al.*, 2005). Only groups with > 10 samples were included in the comparisons. Significance was assessed with permutation tests (9999 replicates). Rarefaction curves for the cumulative number of unique algal MLGs and the number of samples analyzed in the two sites were calculated in MOTHUR v1.22.2 (Schloss *et al.*, 2009).

Results

Photobiont phylogenies

We obtained 15 *18S* rRNA and 14 *rbc*L sequences of green algal cultures attributed to the genus *Dictyochloropsis* s.l. and 28 *18S* rRNA and 38 *rbc*L lichen photobiont sequences (Table 1). Sequencing reactions of uncultured lichen photobionts produced clean reads at all loci, indicating that a single photobiont genotype was predominant in each thallus. The final *18S* rRNA alignment contained 1022 sites, whereas the *rbc*L alignment contained

Table 3 (a) Information on two photobiont populations (Madeira, Taiwan) structured by mycobiont species (b) Pairwise F_{ST} values for photobionts, structured by mycobiont species, for two populations in Madeira and Taiwan (only species with > 10 specimens are included)

	Sample Size	No. of MLGs	*h*	No. of alleles	Private alleles (PA)	Mean Frequency PA	Value for all isolates		Value for unique MLGs only	
							I_A	r_D	I_A	r_D
(a)										
Madeira	249	79	0.614	6.114			0.47	0.088	0.13*	0.024*
Lobaria immixta	78	21	0.623	8.000	10	0.128205128	0.82	0.171	0.34*	0.071*
Lobaria macaronesica	43	10	0.663	6.714	4	0.093023256	1.09	0.186	0.23**	0.039**
Lobaria pulmonaria	23	7	0.584	3.429	15	0.652173913	3.57	0.715	0.96	0.202
Lobaria sublaevis	85	34	0.657	8.714	5	0.058823529	0.59	0.122	0.17	0.037
Lobaria virens	20	7	0.544	3.714	0	–	1.16	0.237	−0.26**	−0.069**
Taiwan	205	107	0.756	9.457			0.94	0.169	0.39	0.073
Lobaria crassior	41	24	0.740	10.571	18	0.036585366	0.84	0.153	0.38	0.072
Lobaria isidiophora	34	16	0.706	8.714	12	0.032085561	1.74	0.302	0.16**	0.030**
Lobaria japonica	26	7	0.788	7.714	3	0.038461538	2.05	0.351	−0.44**	−0.076**
Dendriscosticta platyphylloides	81	46	0.831	14.429	38	0.024691358	1.21	0.214	0.77	0.139
Dendriscosticta praetextata	16	14	0.717	5.857	0	–	1.02	0.183	0.57	0.105
Lobaria orientalis	2	–	–	–	–	–	–	–	–	–
Lobaria spathulata	1	–	–	–	–	–	–	–	–	–
Dendriscosticta platyphylla	4	–	–	–	–	–	–	–	–	–

(b) Madeira	*L. immix.*	*L. macar.*	*L. pulm.*	*L. subl.*	*L. vir.*
L. immixta (*n* = 78)					
L. macaronesica (*n* = 43)	0.05				
L. pulmonaria (*n* = 23)	0.15	0.16			
L. sublaevis (*n* = 85)	0.03	0.04	0.13		
L. virens (*n* = 20)	0.09	0.09	0.21	0.06	

Taiwan	*L. crass.*	*L. isidiop.*	*L. japon.*	*D. platyph.*	*D. praetex.*
L. crassior (*n* = 41)					
L. isidiophora (*n* = 34)	0.07				
L. japonica (*n* = 26)	0.06	0.07			
D. platyphylloides (*n* = 81)	0.03	0.05	0.04		
D. praetextata (*n* = 16)	0.05	0.07	0.07	0.03	

*, $P = 0.01$; **, $P > 0.05$.
MLG, multi-locus genotypes; *h*, unbiased genetic diversity; I_A, Index of Association ($P < 0.01$); r_D, rBarD ($P < 0.01$).
(b) All values are significant ($P < 0.001$) as assessed by Bonferroni corrected permutation tests.

994 sites (data available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.4q6b9). Although the position of some clades varied in the phylograms inferred from the two loci data, the overall topological differences of highly supported clades (ML > 75%, PP > 0.95) did not represent significant conflict (Fig. 1, Supporting Information Fig. S1). Many backbone nodes of the *18S* and *rbc*L trees were poorly supported, as already reported in Nakada *et al.* (2008) and Leliaert *et al.* (2012). The nuclear internal transcribed spacer (ITS) sequences were too divergent to produce a reliable alignment large enough for phylogenetic analysis. However, ITS2 secondary structure analysis may be used for the discrimination of algal strains at the subgeneric level (Fig. S2).

Alternative hypothesis test using both ELW and SH-tests showed that the hypothesis of *Dictyochloropsis* s.l. to be monophyletic can be rejected ($P < 0.001$). This genus is partitioned into two independent and well-supported trebouxiophyceaen clades which likely represent distinct genera (ML = 98%, PP = 1 for *18S*, ML = 88%, PP = 0.99 for *rbc*L; Figs 1, S1), and are here referred with the names *Dictyochloropsis* clade 1 and clade 2. *Dictyochloropsis* clade 2 grouped as sister to *Viridiella fridericiana* with moderate support in the *18S* phylogeny (ML = 78%, PP = 0.96), whereas it is currently impossible to determine a sister group for *Dictyochloropsis* clade 1.

Dictyochloropsis clade 1 is composed of free-living, aerophytic strains only. *Dictyochloropsis* clade 2 is represented by lichen photobionts (Figs 1, S1; in green) and by epiphytic, free-living strains from Austria (SAG2036), Japan (NIES-378, SAG2069, SAG2154) and Malaysia (CAUPH603 and CAUPH8602). Among the lichenized strains, *Dictyochloropsis* clade 2 showed a low selectivity for lichen genera, being associated with members of the Lobariaceae (*Crocodia*, *Dendriscosticta*, *Lobaria*, *Lobariella*, *Pseudocyphellaria*, *Ricasolia*, *Sticta*), Mycocaliciaceae (*Chaenothecopsis*, SAG 27.81), Brigantiaeaceae (*Brigantaea*, e.g. CCHU 5616), Megalosporaceae (*Megalospora*), Coniocybaceae (*Chaenotheca*, SAG 244.80), Phlyctidaceae (*Phlyctis*, UTEX LB 2599/2612) and Ramalinaceae (*Biatora*) (Fig. 1).

The photobiont of a specimen of *Catillaria chalybeia* collected near Zürich (Switzerland) belonged to the *Elliptochloris* clade which contains, amongst others, symbiotic algae of lichens and sea anemones (*Zoochlorellae*), whereas a specimen collected near Lunz (Austria) was reported to be lichenized with *Dictyochloropsis reticulata* sensu Tschermak-Woess (1984).

Photobiont selectivity in the Lobariaceae

All *18S* and *rbc*L photobiont alleles of the studied lichens of the Lobariaceae belonged to *Dictyochloropsis* clade 2 except for the photobiont of two unknown species of *Sticta* and *Pseudocyphellaria* (Taiwan, unknown alga).

According to *18S* rRNA and *rbc*L data, the same photobiont was found in the following lichen fungi: *Sticta canariensis*/*S. aff. neopulmonaria* (Sym3), *Lobariella* sp./*Ll. pallidocrenulata* (Sym1), *Crocodia aurata*/*Lobaria patinifera* (Sym4), *Dendriscosticta platyphylla* /*D. wrightii* /*L. macaronesica* /*L. pulmonaria* /*R. amplissima* (Sym5), *P. lividofusca*/*P. multifida*/*S. latifrons* (Sym7), *P. lindsayi*/*S. subcaperata* (Sym8), *P. fimbriata*/*P. homeophylla* (Sym9), and *Sticta* sp./*Pseudocyphellaria* sp. (Taiwan, Yilan County, Ming Ch'ih forest) (Figs 1–3).

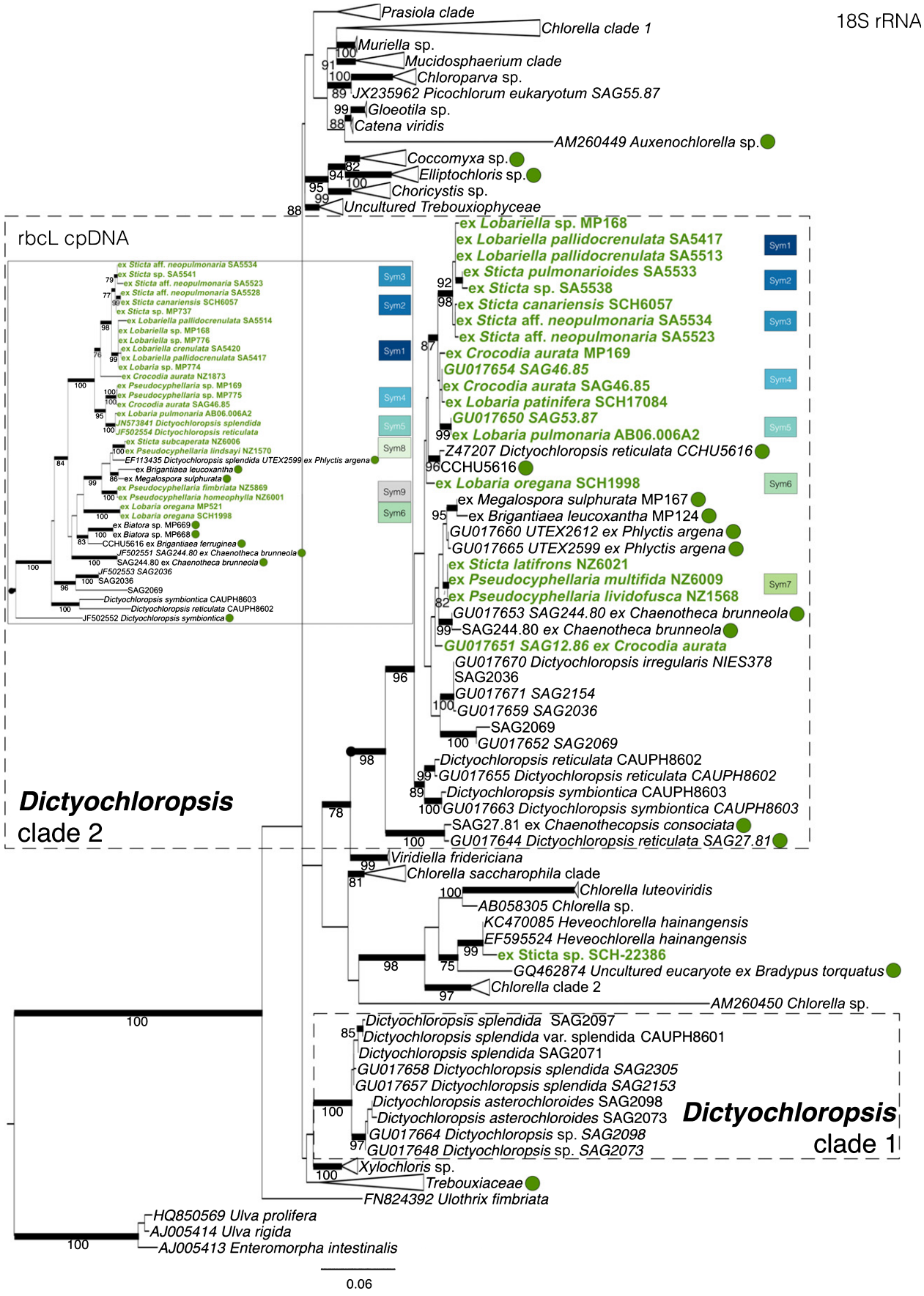
Microsatellite analysis

The seven alga-specific microsatellite markers amplified DNA of photobiont Sym5 only, for a total of four *Dendriscosticta*, 16 *Lobaria* and one *Ricasolia* species. Their allele lengths were in the same range or matched the allele lengths of the photobiont of *L. pulmonaria* (Widmer *et al.*, 2010, 2012; Dal Grande, 2011; Dal Grande *et al.*, 2012).

A 97–100% identity in ITS sequence of the photobiont of these samples further indicated that Sym5 is a single, coherent species.

The 7-loci microsatellite analysis of Sym5 photobionts from 249 (Madeira) and 205 (Taiwan) thalli of Lobariaceaen lichens resulted in a total of 79 (Madeira) and 110 (Taiwan) photobiont multi-locus genotypes (MLGs). Microsatellite markers showed considerable variation at the population level (h Madeira = 0.614, h Taiwan = 0.756; Table 3). Of these, 35 (Madeira) and 78 (Taiwan) were unique MLGs, occurring only once in the population. Nineteen MLGs in 72 thalli (Madeira) and eleven MLGs in 32 thalli (Taiwan) occurred multiple times with the same fungal host (i.e. were clonal within the same lichen being the result of potential vertical transmission). Twenty-one MLGs for a total of 142 (Madeira) and 25 MLGs for a total of 95 thalli (Taiwan) were shared among different fungal hosts (Figs 4, S3). When we compared photobionts associated with different fungal hosts using F_{ST}, none of the species had significantly different photobiont populations from all other species (F_{ST} Madeira = 0.03−0.09; F_{ST} Taiwan: 0.03−0.07), except for *L. pulmonaria* in Madeira which displayed the highest number of private alleles (PA = 15, Table 3) and F_{ST} = 0.13−0.21. No identical algal MLGs were found between the two localities. Algal rarefaction curves computed for the whole dataset of Madeira (249 samples) and Taiwan (205 samples) did not reach saturation (Fig. 4a), suggesting that further genotyping would have revealed more algal MLGs in each population.

Fig. 1 Maximum-likelihood (ML) tree of the green algae inferred from *18S* rRNA sequences. Some of the clades of the tree were collapsed for clarity of presentation. Numbers below branches indicate ML bootstrap proportions (> 75%). Thickened branches indicate Bayesian Markov chain Monte Carlo (BM) posterior probabilities > 0.95. Phylogenetic positions of algae associated with species of Lobariaceae are in bold and green; lichenized taxa or clades which include lichen photobionts are indicated with a green circle. GenBank sequences are in italics and start with the accession number. An expanded version of this tree is available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.4q6b9.



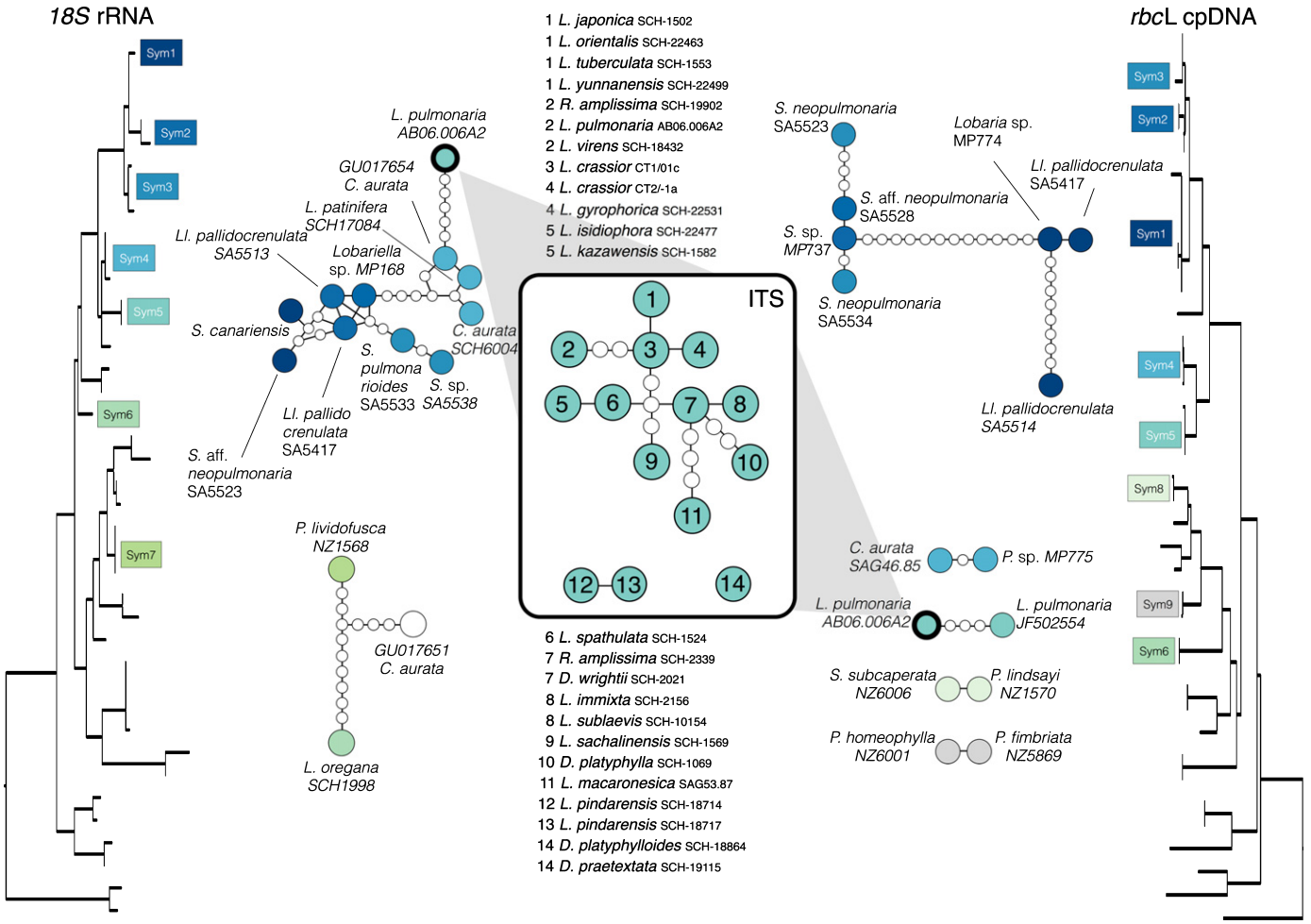


Fig. 2 Haplotype networks of *18S* rRNA, *rbc*L cpDNA and internal transcribed spacer (ITS) alleles of photobionts associated with lichens of the genera *Crocodia*, *Dendriscosticta*, *Lobaria*, *Pseudocyphellaria*, *Ricasolia* and *Sticta* s.l.

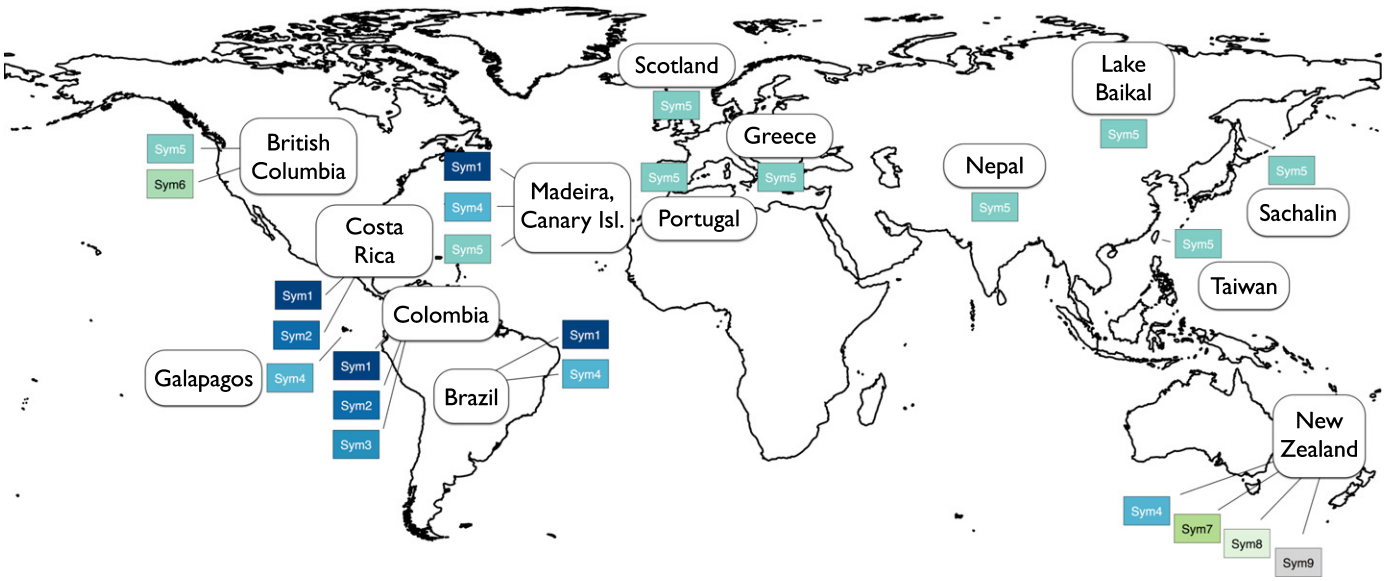


Fig. 3 Location of the main lineages of *Dictyochloropsis* clade 2 associated with fungi of Lobariaceae (Sym1–9).

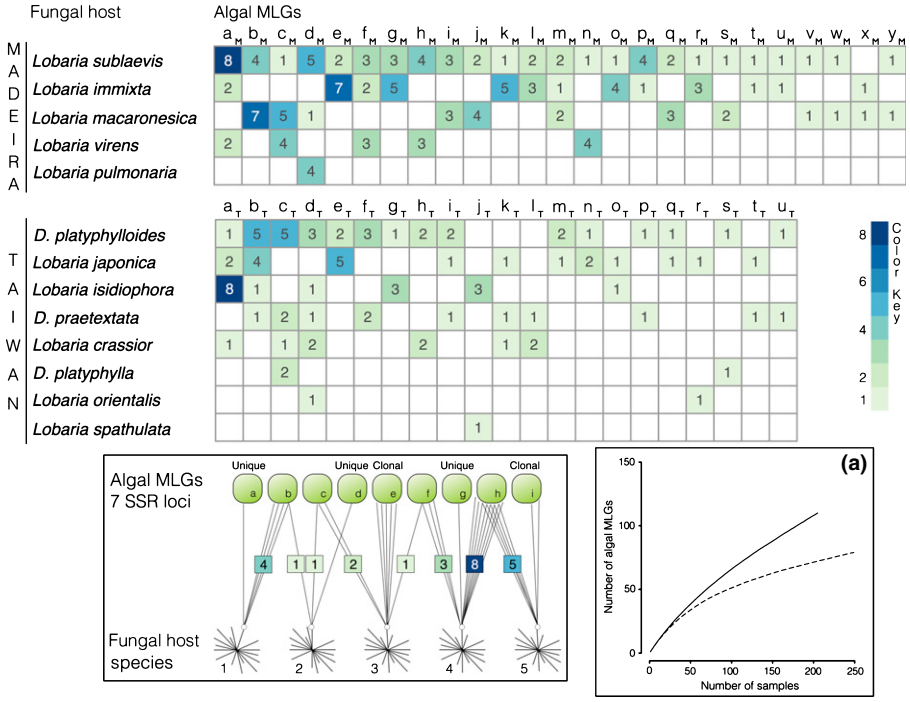


Fig. 4 Heat map of the Interaction Network for shared Sym5 algal clones (identical multilocus genotypes at seven SSR loci; a_M–y_M: Madeira, a_T–u_T: Taiwan) and their fungal host species. Schematic representation of the algal clonal sharing among fungal hosts is provided (bottom left). Colors represent the number of occurrences of the algal clone in the given lichen species, from absent (white) to abundant (dark blue). Data are presented for two lichen communities sampled from 10 trees in Madeira (249 thalli) and Taiwan (205 thalli). (a) Rarefaction curves of algal genotypes (MLGs) in the populations of Madeira (dashed line) and Taiwan (continuous line).

Discussion

Polyphyly of the genus *Dictyochloropsis* s.l.

The taxonomy of algae of the genus *Dictyochloropsis* s.l. has been questioned based on recent morphological studies and molecular data (Škaloud *et al.*, 2005; Widmer *et al.*, 2010; Thüs *et al.*, 2011). Partial nuclear *18S* rRNA and *rbc*L cpDNA sequences were used to infer phylogenetic relationships among several lineages of both free-living and lichenized green algae attributed to this genus. Our results indicate that the genus *Dictyochloropsis* s.l., as currently circumscribed, is polyphyletic. The genus is split into two distinct, highly supported lineages. The morphology of algae of one lineage, *Dictyochloropsis* clade 1, is congruent with the original description of the genus, and includes the type species, *D. splendida* (Geitler, 1966; Škaloud *et al.*, 2007). This lineage is composed of free-living, aerophytic algae that have a peculiar chloroplast ontogeny with a distinct parallel arrangement of lobes, and reproduce only via autospores (Škaloud *et al.*, 2007). By contrast, *Dictyochloropsis* clade 2 is composed of free-living as well as of lichenized algae. These algae have one evenly perforated chloroplast and produce both aplanospores as well as zoospores with a typical insertion of the flagella (Tschermak-Woess, 1980, 1984; Nakano & Isagi, 1987; Škaloud *et al.*, 2007). The high support from both nuclear and plastid loci and the differences in chloroplast morphology indicate that these clades most likely represent different genera.

Both *Dictyochloropsis* clade 1 and clade 2 contain several undescribed taxa whose phylogenetic position cannot be fully resolved with the loci used. Considering the small number of isolates available for each species, a more extensive taxon sampling and sequencing data of more variable loci is needed before drawing taxonomic conclusions on the taxa of the two genera. Preliminary analyses of ITS rRNA sequencing data show that molecular signatures based on unique ITS secondary structures can be used for delimiting particular algal species, as recently shown for other algal groups (e.g. *Asterochloris*, Škaloud & Peksa, 2010; see Fig. S2; Coleman, 2009). In particular, the surprising diversity of photobionts of *Sticta* and *Pseudocyphellaria* s.l. species collected from South America and New Zealand is an issue certainly worthy of further study.

Our results are in concordance with molecular data for other algal groups that have revealed extreme polyphyly and cryptic diversity of morphologically simple genera of diatoms (Lundholm *et al.*, 2006; Amato *et al.*, 2007; Sato *et al.*, 2008; Vanormelingen *et al.*, 2008), free-living green algae (Lewis & Flechtner, 2004; Vanormelingen *et al.*, 2007) and lichenized algae (*Trebouxia*, Kroken & Taylor, 2000; *Coccomyxa*, Friedl *et al.*, 2007).

Patterns of association of lichenized algae

In *Dictyochloropsis* clade 2, many lineages were lichenized. Algae of this genus are found in association with members of the Lobariaceae (*Crocodia*, *Dendriscosticta*, *Lobaria*, *Pseudocyphellaria*, *Ricasolia*, *Sticta*), Mycocaliciaceae (*Chaenothecopsis*), Brigantiaeaceae (*Brigantaea*), Megalosporaceae (*Megalospora*), Coniocybaceae (*Chaenotheca*), Phlyctidaceae (*Phlyctis*) and Ramalinaceae (*Biatora*).

Several species of Lobariaceae associate with *Dictyochloropsis* clade 2 photobionts (Tschermak-Woess, 1984). Two undescribed *Pseudocyphellaria* (SCH22290/97) and *Sticta* (SCH22386/79) species from Taiwan associate with a photobiont from a different algal group related to an endophyte of tropical trees, *Heveochlorella hainangensis*. It is unlikely that these

divergent sequences represent contaminating epibionts, as the sequencing of DNA extracts of isolated photobiont layers obtained by scraping the lichens using a sterile scalpel resulted in identical sequences.

It has been suggested for lichens as well as for other symbiotic systems that reproductive strategies and ecological factors are the driving forces leading to specificity (Yahr *et al.*, 2006; Elvebakk *et al.*, 2008; Rikkinen & Virtanen, 2008; Piercey-Normore, 2009; Otálora *et al.*, 2010; O'Brien *et al.*, 2013). Lichen fungi of Lobariaceae are generally associated with old-growth forests and display both dispersal mechanisms – that is, asexual dispersal leading to vertical photobiont transmission, and sexual dispersal leading to horizontal photobiont transmission (Scheidegger & Werth, 2009; Dal Grande *et al.*, 2012; Werth & Scheidegger, 2012). For algal species with wide distributions, we did not find any case of high reciprocal selectivity between algal haplotype groups and single fungal species. Therefore, even in predominantly vegetative species such as *L. pulmonaria*, low-frequency sexual reproduction can effectively contribute to photobiont switching (Ohmura *et al.*, 2006; Dal Grande *et al.*, 2012).

For *Nostoc*-containing lichen symbioses it has been suggested that geographic patterns do not guide the evolution of specificity in lichens (O'Brien *et al.*, 2005, 2013; Stenroos *et al.*, 2006; Elvebakk *et al.*, 2008; Otálora *et al.*, 2010). In our study, we have evidence to both support and reject this hypothesis, depending on the algal clade analyzed. On the one hand, we found that lichens growing in different geographic regions may share identical *18S* and/or *rbc*L photobiont haplotypes**.** This is evident for the alga Sym4 found in one *Crocodia* species from New Zealand, Europe and North America and one *Lobaria* species sampled on the Galápagos islands, and for the alga Sym5 found in three *Lobaria*, two *Dendriscosticta* and one *Ricasolia* species sampled in Europe, as well as East Asia and North America (Figs 2, 3, Table 1). On the other hand, we found unique photobiont haplotypes in lichens with limited distributions. For example, algae Sym2 and Sym3 were only found in *Lobariaceae* of Central and South America (Costa Rica, Colombia, Brazil). We also identified several photobiont clades that were exclusive to New Zealand (Sym7, Sym8 and Sym9) or the distinct photobionts from Taiwan.

Interestingly, we did not find any generalist Lobariaceaen taxa, that is, fungi associated with more than one photobiont haplotype group/species, except for *C. aurata*. The ability to associate with diverse photobionts, possibly adapted to different ecological niches may allow the mycobiont to colonize a wider range of habitats (Yahr *et al.*, 2006; Nelsen & Gargas, 2008). It has been shown in other lichen systems (e.g. Antarctic cyanolichens, Wirtz *et al.*, 2003; *Protoparmeliopsis muralis*, Muggia *et al.*, 2013) that mycobionts having broader and varied ecological distribution have low photobiont selectivity. However, the high selectivity for certain *Dictyochloropsis* clade 2 strains presented in this study is consistent with the fact that fungi of the Lobariaceae have narrow ecological niches. Representatives of this family are known to be susceptible to high light (Gauslaa & Solhaug, 1999) and to favor habitats with high air humidity (Pannewitz *et al.*, 2003), and they colonize forest areas of high ecological continuity (Scheidegger, 1995).

Photobiont-mediated guild hypothesis

The results of microsatellite fingerprinting show that the alga Sym5 is a common photobiont among Lobariacean lichens of the genera *Lobaria* (16 species) and *Dendriscosticta* (4 species) and *Ricasolia* (1 species, see Table 1). Rikkinen *et al.* (2002) hypothesized that co-occurring lichen fungi may exhibit a high level of selectivity at the community level. This was shown at the species level for lichens associated with *Asterochloris* and *Nostoc* photobionts (Paulsrud *et al.*, 1998; Škaloud & Peksa, 2010). In this study, we used highly variable microsatellite markers developed for Sym5 to show that high selectivity at the community level can be linked to horizontal transmission of photobiont clones among lichens with similar microhabitat requirements.

Population genetics analyses using microsatellites in two long-lived forests harboring rich Lobariaceaen communities (Madeira, Taiwan) showed a very high genetic diversity of the photobiont species. Rarefaction analysis of algal MLGs suggested the presence of high levels of intraspecific cryptic diversity. A denser sampling is therefore needed in order to reveal the full algal diversity associated with fungi of the Lobariaceae in these communities.

Overall, the genetic structure of the photobiont at both sites was that of a clonal organism (I_A Madeira = 0.13, $P = 0.01$; I_A Taiwan = 0.39, $P < 0.01$). Analyses at the level of the single algal clones (i.e. identical multi-locus genotypes at seven SSR loci) indicate a surprising amount of symbiont switching (probably involving dissociation and relichenization). All fungal species analyzed associate with more than one genotype shared between at least two fungal hosts. In many cases, we observed recurrent photobiont clones shared between two or more host species, for example, algal clones a_M, b_M, c_M, d_M found in three *Lobaria* species in the population of Madeira, and clones a_T, b_T, c_T, d_T associated with two *Lobaria* and two *Dendriscosticta* species in the population of Taiwan (see Figs 4, S3). Thus, horizontal interspecific transfer of the photobiont was common in both green-algal lichen communities, as recently found for cyanobacterial lichen communities (O'Brien *et al.*, 2013). This suggests that a diverse range of fungal partners plays a role in increasing the ecological range of the alga. Thus, like mycobionts, switching partners is a way for the alga to widen its habitat range and to colonize different ecological niches, thus becoming able to survive under new selective pressures. On the fungal side, when algal shifts are common enough, the selection–drift balance should attenuate the effect of detrimental algal mutations on fungal fitness (Buschbom & Mueller, 2006; Mikheyev *et al.*, 2007; Nelsen & Gargas, 2008, 2009; Queller & Strassmann, 2009). Furthermore, according to the photobiont-mediated guild hypothesis (Rikkinen *et al.*, 2002), the dispersal ecology of the guild may be shaped by different reproductive strategies of its members. Ideally, a guild has two kinds of members: predominantly asexual core species and predominantly sexual fringe species. Core species (*L. immixta*, *L. macaronesica*, *L. pulmonaria* in Madeira, and *L. isidiophora*, *L. spathulata* and *Dendriscosticta praetextata* in Taiwan) can effectively co-disperse photobionts at the local scale by producing large numbers of symbiotic diaspores. These photobionts, as

shown in our study, can be incorporated via horizontal transmission in fringe species (*L. sublaevis*, *L. virens* in Madeira; *L. crassior*, *L. orientalis*, *Dendriscosticta platyphylla* and *D. platyphylloides* in Taiwan). The common benefit for the guild members relies on the fact that, so long as even a few photobiont cells survive, they can significantly promote the re-colonization of the area by the whole fungal guild (Piercey-Normore & De Priest, 2001; Rikkinen *et al.*, 2002; Piercey-Normore, 2006, 2009; Rikkinen, 2009). The guild concept was recently challenged for cyanobacterial lichen symbioses by the discovery that fungal hosts (*Peltigera* spp.) previously thought to be highly specialized on one cyanobacterial cluster, are in fact associated with at least two divergent groups of *Nostoc* (O'Brien *et al.*, 2013). In our study, however, the most extensively sampled species, *L. pulmonaria*, associates with a single species of *Dictyochloropsis* clade 2 (Sym5), and all other species associated with Sym5 were never found associated with other photobiont species, too. The investigated lichen communities therefore likely represent a horizontally linked system of fungi highly specialized on a single, shared photobiont.

In conclusion, our results support the hypothesis that, from the fungal perspective, co-evolution between photobionts and their fungal hosts occurs at a deeper phylogenetic level. Thus by ignoring the guild scale, the extent of interactions and co-evolution in lichens may be underestimated. The photobiont-mediated lichen communities described in our study were in accordance with a model in which the algal photobiont acts as a landscape for a community of potentially competing fungi. Such patterns have been observed in other symbioses, for example, the intracellular bacterial genus *Wolbachia* and aphids, fungus-growing insects, corals and root symbioses (Chapela *et al.*, 1994; Okuma & Kudo, 1996; Wulff, 1997; Rowan, 1998, 2004; Herre *et al.*, 1999; Stanton, 2003; Pochon & Pawlowski, 2006; Aanen *et al.*, 2007; Mikheyev *et al.*, 2007; Porras-Alfaro & Bayman, 2007). Future studies should aim to clarify the role of different reproductive strategies of guild members (core and fringe species) in the evolution of photobiont-mediated guilds.

Acknowledgements

We thank Bibiana Moncada and Robert Lücking for specimens from South America, Soili Stenroos and Filip Högnabba (Helsinki) for specimens from New Zealand, Frank Bungartz (Charles Darwin Research Station, CDRS) and Karen Dillman (Tongass National Forest Service) for specimens from Galapagos and Alaska, Jeng-Tze Yang and Jeng-Long Tsai National Chung Hsing (University of Taichung, Taiwan) for organizing collecting permits in Taiwan and for field assistance, Steve Leavitt for specimens from North America, Carrie Andrew and Robert Lücking for sequences, and Philipp-André Schmidt for rarefaction analysis. This work was supported by the Swiss National Science Foundation (projects 31003A-105830 and 31003A-127346 to C.S.), the Botanical Society of America and the University of Chicago (to M.P.N.), a fellowship from the German Academic Exchange Service (DAAD) (to G.S.).

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 ML tree of the green algae inferred from *rbc*L sequences.

Fig. S2 Predicted secondary structures of ITS2 transcripts of *Dictyochloropsis* clade 1 and clade 2.

Fig. S3 Bipartite networks for the presence of clonal Sym5 algal multi-locus genotypes associated with fungal species from a single population of lichen-forming fungi from Madeira and Taiwan.

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