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# Diversity of organism in the *Usnea longissima* lichen

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Lichens are self-supporting and ecologically obligate associations between symbiotic fungi, commonly an Ascomycete (mycobiont), and a photosynthesising organism (photobiont). Samples of *Usnea longissima* lichen were collected from *Abies georgei* in the Pudacuo National Park, Shangri-la County, Yunan Province, China, and their biological compositions were analysed by cultural and metagenomic approaches. We found that the *Usnea longissima* lichen symbiont contained a number of endolichenic fungi. The organisms identified included *Usnea*, *Sydowia*, *Alectoria*, *Punctelia*, *Penicillium*, *Mucor*, *Hypocrea*, *Trichoderma*, *Elaphocordyceps*, *Arthrinium* and *Cladosporium*. This is the first report of different genera of lichenized fungi, *Usnea*, *Alectoria* and *Punctelia*, co-existing in the same symbiont. This may be related to multiple origins for the fungi lichen symbiosis. Using algal-specific ITS rDNA primers, the photobiont of the *Usnea longissima* lichen was identified as *Trebouxia*. However, we also found that *Poterioochromonas* was present in the *Usnea longissima* lichen, through 18S rDNA clonal analysis of the metagenome. To date, there has been no report of two cross-phylum algae co-existing in a single lichen. This study provides new insight into the biological composition of the lichen *Usnea longissima*, highlighting its microbial diversity.

**Key words:** *Usnea longissima* lichen, biological composition, cultural and metagenomic approach, *Poterioochromonas*.

## INTRODUCTION

Lichens are self-supporting and ecologically obligate associations between symbiotic fungi, commonly an Ascomycete (mycobiont), and a photosynthesising organism (photobiont), which can either be a cyanobacterium or a eukaryotic green alga, usually a member of the genus *Trebouxia* (Trebouxiophyceae) (Ahmadjian, 1960). Lichens have been shown to produce a number of secondary metabolites that may protect them against physical stresses or biological attack (Kahng et al., 2004). Some lichen species and their compounds have been utilised for medicinal and industrial purposes (Muller, 2001). Among the medicinal lichens, *Usnea longissima* lichen is edible and is utilised in the preparation of traditional foods and medicines in both Eastern and Western countries. It is pale green or silvery-yellowish-green in color, fruticose and pendulous.

Its main branches are cylindrical and can reach up to 3 meters or more in length. The main branches are rarely divided but they have numerous short perpendicular side branches and fibrils of approximately equal length (3-40 mm). Papillae are lacking but soredia or isidia are occasionally formed on the side branches. Apothecia are extremely rare. When present, they are disc-shaped, 1-3 (22) mm across, and terminate on the ends of the side branches, with numerous fibrils extending from the thalline margin (Keon, 2002). Extracts have been shown to exert anti-bacterial, anti-inflammatory and detoxifying effects (Lauterwein et al., 1995; Kim and Lee, 2006). Many constituents have been identified (Mallavadhani et al., 2004) and these have a broad range of applications.

There have been many reports describing *Usnea longissima* lichen characteristics, distribution, histology, medicine, pharmacology and chemical components. However, little information is currently available about the symbiotic relationships in the *Usnea longissima* lichen. Now, molecular studies have provided new insights into the nature of symbiotic microorganisms. The molecular

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**Figure 1.** *Usnea longissima* Lichen was collected from *Abies georgei* in Pudacuo National Park, Shangri-la County, Yunnan Province, China.

systems of *Physcia* (Schreb.) Michaux lichens (photobiont and mycobiont) have been characterised in some detail using the internal transcribed spacer (ITS) of their nuclear ribosomal DNA (Helms et al., 2001). The advent of molecular tools to resolve community molecular diversity in culture-independent studies has produced evidence of far greater diversity than was previously appreciated by morphological and cultivation studies (Hugenholtz et al., 1998). A PCR study of Antarctic sandstone endoliths using the 'universal' 16S/18S rRNA gene primers has revealed complex communities of algae, fungi, and bacteria (De la Torre et al., 2003). It is generally accepted that combining cultural and metagenomic analysis leads to a better understanding of symbiosis. We conducted experiments to determine the biological composition of the *Usnea longissima* lichen using a combined cultural and metagenomic approach.

We address the question as to whether one fungal taxon always associates with a particular algal taxon in the *Usnea longissima* lichen. The findings from this study may further the understanding of biological diversity in symbiosis.

## MATERIALS AND METHODS

### Lichen collection

Fresh thalli of *U. longissima* were collected in June, 2009 from Pudacuo National Park, Shangri-la County, Yunnan Province, China (27°49'N, 99°59'E). Where the mean annual temperature is 5.4°C and the mean annual precipitation is approximately 580 mm. The collected lichens were located at an altitude of ca. 3640 m above sea level. The collected samples (Figure 1) from *Abies georgei* were identified by their morphological characteristics and lyophilised for storage.

### Isolation and culture of free-living microbes

We isolated endolichenic fungi within 72 h of sample collection. After washing in tap water for 30 s, the samples were cut into 2 mm<sup>2</sup> pieces and the surfaces were sterilised by sequential immersion in 96% ethanol for 10 s, 0.5% NaOCl for 2 min, and 70% ethanol for 2 min (Stocker-Wörgötter, 2002). After this treatment, the lichen pieces were pressed lightly for 10 s against 2% malt extract agar (MEA) and 2% beef extract peptone agar (BEPA) to harvest any fungi and bacteria (Gams et al., 2007) that remained on the external surfaces and to check the efficiency of the surface sterilisation. The samples were then lightly crushed under sterile

**Table 1.** Primers for molecular identification.

Species	Primer	Reference
Bacterial	27f: 5'-AGAGTTTGATCCTGGCTCAG-3'	Ikner et al. (2007)
16S rDNA	1492r: 5'-TACCTTGTACGACTT-3'	
Cyanobacteria	517CyanF: 5'-TTATTGGGCCTAAAGCRT-3'	Lane (1991)
16S rDNA	1492ABR: 5'-GGTTACCTTGTACGACTT-3'	Clementino et al. (2008)
Eukaryote	18SF: 5'-CCTGGTTGATCCTGCCAG-3'	Anne et al. (2011)
18S rDNA	18SR: 5'-TTGATCCTTCTGCAGGTTCA-3'	
Fungi	ITS1: 5'-TCCGTAGGTGAACCTGCGG-3'	Gardes and Bruns (1993)
ITS	ITS4: 5'-TCCTCCGCTTATTGATATGC-3'	White et al. (1990)
Trebouxia	AL1500bf: 5'-GATGCATTCAACGAGCCTA-3'	Helms et al. (2001)
ITS	LR3: 5'-CCGTGTTTCAAGACGGG-3'	Friedl and Rokitta (1997)

conditions. Broken fragments of the lichen thalli were transferred to 2% MEA plates to harvest cultivatable fungi, and transferred to 2% BEPA plates to harvest cultivatable bacteria from the interior of the tissues.

#### DNA extraction, PCR amplification and sequence alignment of isolates

The pure microbial cell cultures were dispersed in a 400  $\mu$ L saline-EDTA buffer (150 mM NaCl and 10 mM EDTA at pH 8.0). To the bacterial suspension, 5  $\mu$ L lysozyme solutions (10 mg/mL) was added and 5  $\mu$ L snail enzyme solution (100 mg/mL) was added to the fungal suspension. The samples were then incubated at 37°C for 30 min. Afterwards, 5  $\mu$ L of proteinase K (15 mg/mL) and 10  $\mu$ L of 25% SDS were added, followed by incubation at 55°C for 30 min. The DNA was extracted with equal volumes of phenol and chloroform. After centrifugation, the DNA was recovered from the aqueous phase using a PCR-Mi Clean-up System (Viogene, Sunnyvale, CA, USA). PCR amplification of the 16S rDNA or 18S rDNA fragments was performed using primer pairs (Table 1) in a Biometra thermocycler. The PCR mixtures contained 1 U Taq DNA polymerase in the manufacturer/s buffer, 1.5 M MgCl<sub>2</sub>, 200  $\mu$ M dNTPs and 0.3  $\mu$ M of each primer. The samples were first denatured at 98°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The amplification was completed with a final extension step at 72°C for 7 min. The PCR products were verified in a 1% agarose gel and then sequenced by Sangon (Shanghai, China).

#### Extraction of the metagenomic DNA of the symbiotic microbes

After the lichen pieces were surface sterilised and rinsed in sterile water, they were frozen in liquid nitrogen, crushed, and then mixed with 20 mL of a DNA extraction buffer (100 mM Tris, 100 mM EDTA, 200 mM NaCl, and 3% CTAB, pH 9.0). Snail enzyme, cellulose and lysozyme were added to final concentrations of 300  $\mu$ g/mL, 100  $\mu$ g/mL and 50  $\mu$ g/mL, respectively, and the mixture was incubated at 37°C for 1 h. Sodium dodecyl sulphate (2 mL of a 20% solution) was added, and the mixture was incubated at room temperature for 1 h. Subsequently, 1 volume of phenol was added, and the mixture was mixed and centrifuged for 10 min at 10,000 g. The aqueous phases were extracted with 1 volume of chloroform. After centrifugation for 10 min at 10000 g, 0.1 volume of 5 M potassium acetate was added to the aqueous phase and precipitated for 15 min at room temperature. The samples were centrifuged for 10 min at 10000 g. The supernatant was amended

with 0.1 volume of 5 M NaCl and 0.7 volume of isopropanol and incubated for 30 min at room temperature to precipitate the DNA. The DNA was obtained by centrifugation for 10 min at 10000 g and the resulting pellet was washed with 70% ethanol, dried, and resuspended in 100  $\mu$ L of TE (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA), then further purified using a DNA Purification Kit (GENMED Scientifics INC. U.S.A). In most cases, passage through two columns was needed to remove all PCR-inhibiting substances.

#### 16S rRNA 18S rRNA and ITS gene clone libraries

Fungal and bacterial clone libraries were prepared from the metagenomic DNA of the lichen. The bacterial and cyanobacterial 16S rDNA, fungal ITS and 18S rDNA were amplified by PCR (as described above) using the primers listed in Table 1. The PCR amplicons were purified using the Quick Mini Purification Kit (TaKaRa, Japan) and subsequently cloned into a pMD18-T (TaKaRa, Japan) vector using *Escherichia coli* TOP10 as the host. The transformed *E. coli* cells were grown overnight on LB agar plates containing 50  $\mu$ g/mL ampicillin. Randomly-selected colonies were transferred to an LB broth containing 50  $\mu$ g/mL ampicillin and grown overnight at 37°C. PCR screened for the presence of inserts of the appropriate size using M13F and M13R primers (Sangon, Shanghai China). Each plasmid that contained an appropriately-sized insert was bi-directionally sequenced using the M13F and M13R primers.

#### Sequence and phylogenetic analyses

The colonisation rate (CR) was calculated as the total number of lichen segments infected by the fungi divided by the total number of segments incubated (Petri et al., 1982). As in other phylogenetic studies, the CR was then expressed as a percentage. The abundance was based on the number of clones whose sequence was  $\geq$ 98% identical to that of the clone listed.

The 16S rDNA, 18S rDNA or ITS sequences were manually edited using BioEdit (Hall, 1999). Sequences were checked for chimeras with MALLARD (Ashelford et al., 2006). After removing low-quality sequences, the remaining sequences representing distinct phylotypes (a 98% sequence similarity was used as the criterion for being in the same phylogenetic clade) and their sequences were assigned to taxonomic groups using the RDP-II (<http://www.rdp.cme.msu.edu>) classifier with a confidence threshold of 80%. The sequences have been deposited in GenBank under the accession numbers JN397366 - JN397382.

**Table 2.** Summary of 18S rDNA identified from cultured fungal community.

Clone no.	% Abundance <sup>a</sup>	Putative group	Closest BLAST match (GenBank accession no.)	% Identity	GenBank accession no.
1	17.2	Pezizomycotina	<i>Hypocrea koningii</i> (EU722404.1)	98	JN397379
2	12.1	Pezizomycotina	<i>Trichoderma viride</i> (FJ598872.1)	99	JN397381
3	7.1	Pezizomycotina	<i>Elaphocordyceps jezoensis</i> (AB027365.1)	99	JN397382
4	13.2	Pezizomycotina	<i>Arthrinium marii</i> (AB220231.1)	99	JN397380
5	35.6	Pezizomycotina	<i>Cladosporium bruhnei</i> (AY251096.2)	98	JN397376
6	15.2	Pezizomycotina	<i>Penicillium freii</i> (AJ005446.1)	97	JN397377
7	2.0	Zygomycotina	<i>Mucor hiemalis</i> (AF113428.1)	98	JN397378
8	1.0	Betaproteobacteria	<i>Burkholderia sordicola</i> (AF512827)	99	JN397375

<sup>a</sup>Abundance is based on the number of clones whose sequence was ≥98% identical to that of the clone listed. Approximately 98 individual cultures were screened.

**Table 3.** Summary of 18S rDNA and ITS clone library analysis.

	ITS library		18S rDNA library		Putative group
	% Abundance	GenBank accession no	% Abundance	GenBank accession no	
<i>Usnea longissima</i>	62	JN397371	-	-	Lecanoromycetes
<i>Sydowia polyspora</i>	16	JN397374	-	-	Dothideomycetes
<i>Alectoria sarmentosa</i>	18	JN397372	34	JN397367	Lecanoromycetes
Penicillium	4	JN397373	8	JN397370	Eurotiomycetes
<i>Punctelia hypoleucites</i>	-	-	30	JN397368	Lecanoromycetes
<i>Cladosporium bruhnei</i>	-	-	24	JN397369	Dothideomycetes
Poteriochromonas	-	-	4	JN397366	Chrysophyta

<sup>a</sup>Abundance is based on the number of clones whose sequence was ≥98% identical to that of the clone listed. Approximately, 50 clones were screened in ITS and 18S rDNA clone library, respectively.

## RESULTS

### Recovery and characterisation of symbiotic microbes

The *Usnea longissima* lichen sampled in this study harboured endolichenic fungi and bacteria. No fungi or bacteria were isolated from the exterior surfaces of the surface-sterilised host tissues, showing that the microorganisms were

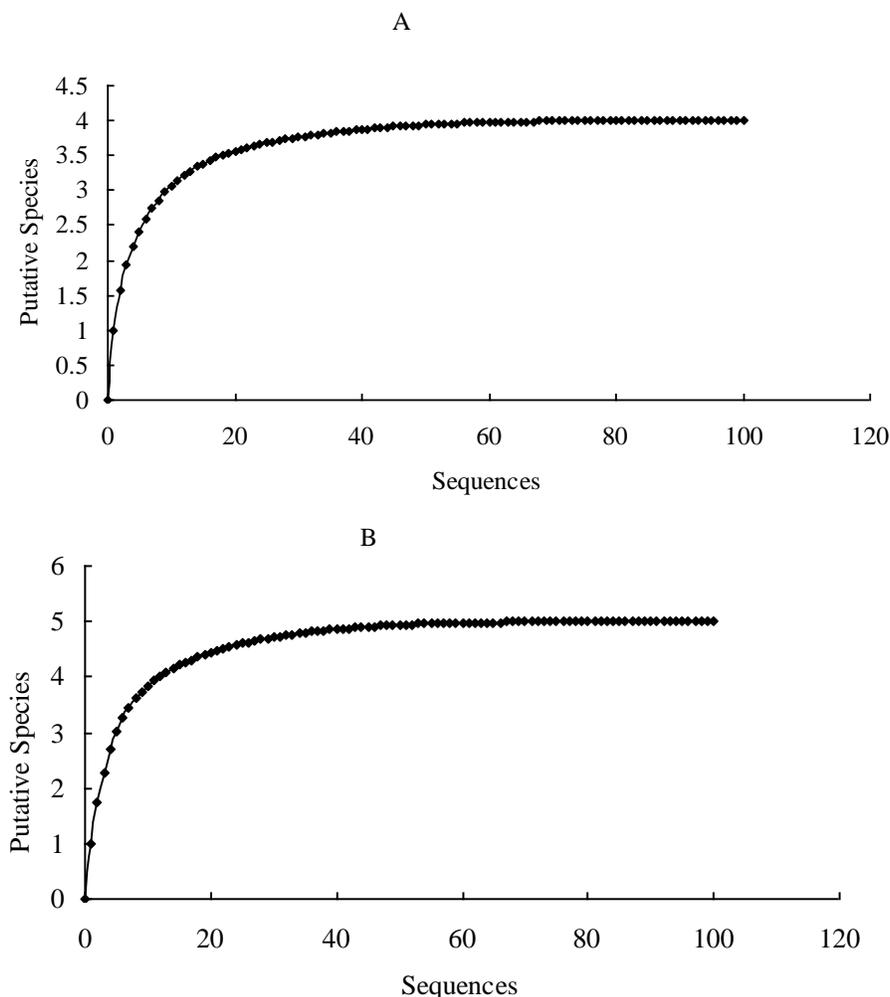
isolated from the symbiote interior. Endolichenic fungi were recovered regardless of the sampling position, and the CR was 91.5%.

A total of 98 endolichenic fungi isolates were recovered from 107 lichen thallus segments. Of these, eight taxa were identified to the genus level; (Table 2) six of these were *Pezizomycotina* (*Ascomycete*), one was *Zygomycotina* (*Ascomycete*), and one was *Burkholderia* (*Betaproteobacteris*). The genus in highest

abundance (35.6%) was *Cladosporium*, followed by *Hypocrea koningii*, *Penicillium*, *Arthrinium* and *Trichoderma*, with abundances of 17.2, 15.2, 13.2 and 12.1%, respectively. We harvested *Burkholderia* (1%) from tissue interiors.

### Metagenomic analysis

The metagenomic DNA recovered from lichens



**Figure 2.** Species accumulation curves, 95% confidence intervals, and bootstrap estimates of richness based on ITS (A) and 18S rDNA (B) OTU (95% sequence similarity) for a 50 clone from metagenome of *Usnea longissima* Lichen.

was amplified with bacterial and cyanobacterial 16S rRNA-specific primers. Although, *Burkholderia* bacteria were recovered in culture, there were no amplification products with either the bacterial or cyanobacterial primers. Although, the experimental specimens were fresh and the symbiotic structure intact, the assumption was that there were too few bacteria to be detected using molecular techniques. Dense surface mycelium, giving the bacteria few opportunities for invasion, and antibiotics, such as Usnic Acid, produced by the symbionts may have inhibited survival of the bacteria. We analysed the fungi-ITS clone library.

Table 3 shows the composition of the ITS clone libraries. Our cloning effort was statistically sufficient to capture the total OTU richness (Figure 2A). Of the 50 randomly-chosen clones, the *Usnea longissima* species (with 100% identity) was the most abundant with 62% of the total sequences. Only a few (4%) *Penicillium* spp.-related sequences (with 100% identity) were detected.

The other *Pezizomycotina*-related sequences were represented mainly by *Alectoria sarmentosa* relatives (99% identity and 18% abundance) and *Sydowia polyspora* (99% identity and 16% abundance).

To supplement the data and find more lineages, a total of 50 18S rRNA gene clones were randomly selected and sequenced (richness curves, Figure 2B).

Of the 18S rDNA clones (Table 3), the most abundant sequences (34, 30 and 24%) were homologous to *Alectoria sarmentosa* (99% identity), *Punctelia hypoleucites* (97% identity) and *Cladosporium bruhnei* (99% identity), respectively.

We also detected the presence of *Penicillium chrysogenum* (99% identity and 8% abundance). In the 18S rDNA clone library, we did not find *Trebouxia*-related sequence, but a few (4%) *Poterioochromonas*-related sequences were detected with a 99% identity. The *Poterioochromonas*-related sequences were identified as Ochromonadaceae, Ochromonadales, Synurophyceae,

Stramenopiles, and Chrysophyta.

## DISCUSSION

### Genetic diversity of endolichenic fungi

Using a combination of cultural and metagenomic analysis, we found that the *Usnea longissima* lichen symbiont contained a number of different endolichenic fungi. The organisms identified were *Usnea*, *Sydowia*, *Alectoria*, *Punctelia*, *Penicillium*, *Mucor*, *Hypocrea*, *Trichoderma*, *Elaphocordyceps*, *Arthrinium* and *Cladosporium*. Studies have identified the mycobiont in different lichens as *Usnea* (Mallavadhani et al., 2004), *Alectoria* (Johansson et al., 2011) or *Punctelia* (Romagni and Gries, 2000). However, this is the first report of different genera of lichenized fungi, namely *Usnea*, *Alectoria* and *Punctelia*, co-existing in the same symbiont. Among the lichenized fungi, the 18S rDNA of *Alectoria sarmentosa* (JN397367) and *Punctelia hypoleucites* (JN397368) was found to be 95.01% homologous. The difference is an extra 85 base inserted into *Alectoria sarmentosa* (JN397367). This difference provides evidence that the symbiosis may have accelerated the horizontal transfer of genes (Andrea et al., 1995). The co-existence of different lichenized fungi is an interesting phenomenon. Andrea et al. (1995) showed indirect evidence of multiple origins for the fungi lichen symbiosis, while our results provide direct evidence. The selection of the fungus-alga partnership is not a random process (Dahlkild et al., 2001; Piercey-Normore, 2004). Selectivity is defined as a preferential interaction between two organisms (Gams et al., 2007). High selectivity occurs when a free-living alga associates with a lichen-forming fungus, even when other algae are more common in the same habitat. Low selectivity occurs when a lichen-forming fungus associates with the more common alga species in a habitat. For example, it has been suggested that the low selectivity of *Umbilicaria* allows the lichen to adapt to harsh or changing environmental conditions. A change in environmental conditions can result in the selection of a different species or genotype of algal symbiont (Romeike et al., 2002). This is also the underlying concept in the adaptive bleaching hypothesis for algae partners in coral symbioses. When environmental conditions change, some of the associated fungus does not become the mycobiont. Instead, new consortiums of different algal partners which are more suited to the environmental conditions take over (Kinzie et al., 2001; Rowan, 2004). The assumption is that the other small amounts of lichenized fungi within the asymptomatic lichen thalli may play alternative roles.

In our *Usnea longissima* lichen specimen, although *Usnea* may be the more common and dominant mycobiont, the lichen-forming fungi, *Alectoria* and *Punctelia* were also present. One of the reasons that the

fungi and algae did not form a very specific symbiont (that is, low selectivity) may be because they are in an earlier symbiotic evolutionary status (samples from a primitive forest, no human disturbance). Another reason for the presence of multiple lichenized fungi may be to provide complementary alternatives to form a new consortium in order to adapt to a new environment.

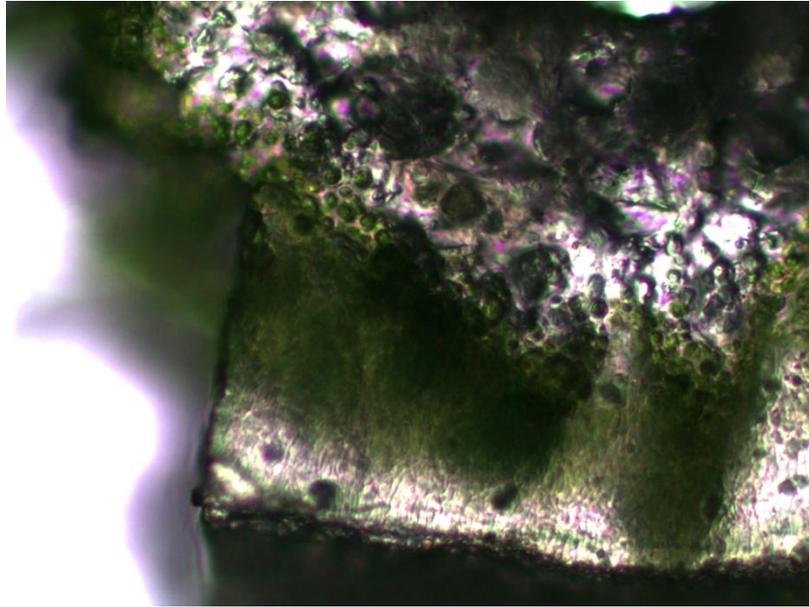
An important finding was *Penicillium* captured in the symbiont. This provides indirect evidence that major *Ascomycota* lineages of non-lichen-forming species are derived from lichen-forming ancestors (Helms et al., 2001).

### Photobiont

Algal-specific ITS rDNA primers (Table 1) were used to identify the photobiont of the *Usnea longissima* lichen (Helms et al., 2001). The photobiont ITS lineage was classified as a species of *Trebouxia*. In contrast to the lichenized fungi, almost no genetic variation was found in the photobiont. Studies using direct PCR amplification of the 18S rDNA gene through general eukaryotic primers have led to the discovery of previously unknown lineages (Massana et al., 2004; Not et al., 2007). Therefore, the 18SF/18SR primers were used to explore the possibility that other algae might co-exist within the lichen. An 18S rDNA sequence of *Poterioochromonas* spp. was detected instead of the *Trebouxia*, although we obtained several fungal clones of the 18S rDNA sequence.

*Poterioochromonas* spp. is a Chrysophyte. Chrysophytes (Wujek and Bicudo, 2004) are found as single cells or sometimes in clusters of up to 30 cells, with the posterior end of the cell sitting in a hemispherical or cone-to-goblet-shaped lorica that has a short or long stalk for attaching to surfaces (cell clusters are joined by the bases of the stalks). Chrysophyte cells are Ochromonas-like, with the anterior end protruding from lorica and the posterior end not attached to the lorica via a protoplasmic strand. The cells are easily freed when disturbed and have 2 unequal flagella, 1-3 chloroplasts (occasionally without chloroplast), no eyespot, 1 (-2) anterior contractile vacuole(s), no stomatocysts, and phototrophic and phagotrophic nutrition. They are common in freshwater and reproduce by longitudinal division, with one daughter cell remaining in the parental lorica and the other escaping and forming a new lorica.

Although, identified in this study by a metagenomic approach, we were unable to detect *Poterioochromonas* using an inverted microscope (Figure 3). The occurrence of more than one algal genotype in a single thallus has previously been reported by several authors (Nelsen and Gargas, 2009; Martin et al., 2010) and the algae were identified as members of several *Asterochloris* or *Trebouxia* lineages. Friedl (1989) and Friedl and Rokitta (1997) also found six species of Parmeliaceae from tree bark at different locations in Europe. Based on



**Figure 3.** Inverted microscope image of algal layer of *Usnea longissima* Lichen.

morphological studies of the cultured algal strains each was found to be associated with two or three different species of *Trebouxia*. However, the co-existence of cross-phylum algae has not been previously reported. This makes our findings particularly interesting. High specificity occurs when one fungal taxon or genotype always associates with a particular algal taxon or genotype, and low specificity occurs when more than one fungal taxon or genotype associates with more than one algal taxon or genotype (Franc ois et al., 2001; Laure et al., 2011). Perhaps, the presence of different algae in the *Usnea longissima* lichen resulted in co-existence of multiple lichen fungal lineages. This could be a factor contributing to increased lichenized fungal diversity. Further studies should include proving the existing site and function of *Poterioochromonas* and other lichenized fungi, *Alectoria* and *Punctelia*.

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