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ORIGINAL ARTICLE

Algal and Fungal Diversity in Antarctic Lichens

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ABSTRACT

The composition of lichen ecosystems except mycobiont and photobiont has not been evaluated intensively. In addition, recent studies to identify algal genotypes have raised questions about the specific relationship between mycobiont and photobiont. In the current study, we analyzed algal and fungal community structures in lichen species from King George Island, Antarctica, by pyrosequencing of eukaryotic large subunit (LSU) and algal internal transcribed spacer (ITS) domains of the nuclear rRNA gene. The sequencing results of LSU and ITS regions indicated that each lichen thallus contained diverse algal species. The major algal operational taxonomic unit (OTU) defined at a 99% similarity cutoff of LSU sequences accounted for 78.7–100% of the total algal community in each sample. In several cases, the major OTUs defined by LSU sequences were represented by two closely related OTUs defined by 98% sequence similarity of ITS domain. The results of LSU sequences indicated that lichen-associated fungi belonged to the Arthoniomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes, and Sordariomycetes of the Ascomycota, and Tremellomycetes and Cystobasidiomycetes of the Basidiomycota. The composition of major photobiont species and lichen-associated fungal community were mostly related to the mycobiont species. The contribution of growth forms or substrates on composition of photobiont and lichen-associated fungi was not evident.

LICHENS are symbiotic organisms that are mainly composed of lichenized fungi (mycobiont) and photosynthetic algae and/or cyanobacteria (photobiont). The relationships between the mycobiont and the photobiont have a certain level of specificity, in that one species of mycobiont and one species of photobiont form a symbiotic relationship in a thallus. However, this assumption of specificity and selectivity of lichenized fungi for their algal partners has been challenged by the results of studies in which molecular methods were used to characterize photobiont genotypes (Kroken and Taylor 2000; Muggia et al. 2010). One species of lichenized fungi can make symbiotic partnerships with various photobiont species when they grow at geographically distant locations (Kroken and Taylor 2000). The variable partnership is even found among samples collected from small geographic areas or inhabiting the same substrate (Blaha et al. 2006; Ohmura et al. 2006). In addition, several studies have reported that several different algal genotypes can be present in a single lichen thallus, for example, in the lichens *Parmotrema tinctorum*, *Protoparmeliopsis muralis*, *Ramalina farinacea*, and *Evernia mesomorpha* (Casano et al. 2011; Grube and Muggia 2010; Guzow-Krzeminska 2006; Ohmura et al. 2006; Piercey-Normore 2006). The multiple algal genotypes and a hypothesized switch of algal species were proposed to play roles in adaptation to changing environmental conditions (Piercey-Normore 2006). Phylogenetic analysis of photobionts in *Lepraria* and *Stereocaulon* revealed that environmental factors such as rain, sun exposure, and substrate are important in selection of photobionts (Peksa and Škaloud 2011). Elvebakk et al. (2008) showed a diverse genetic pattern of cyanobionts within the lichen genus *Pannaria*, including both association with lichen taxonomy and lichen habitats and a wide distribution of most photobiont guilds.

Lichens contain diverse lichen-associated fungi such as lichenicolous, endolichenic, and epilichenic fungi. Lichenicolous fungi, a group of fungi that form obligate associations

with lichens as saprotrophs or parasites, have long been recognized by the macro- and microstructures produced on the lichen thallus (Andreev et al. 1996; Hawksworth 1975; Hawksworth and Iturriaga 2006; Lawrey and Diederich 2003). More than 1,500 species have been described in the Ascomycota and Basidiomycota. More than 95% of described lichenicolous fungi belong to the Ascomycetes, and are in diverse taxonomic groups. Very few known lichenicolous fungi are within the Basidiomycetes (less than 5%); these fungi can be divided into the Agaricales, sclerotial fungi, and heterobasidiomycetes (Lawrey and Diederich 2003). Endolichenic fungi have been identified via culture methods and diverse species have been recognized in the Dothideomycetes, Eurotiomycetes, Sordariomycetes, and Tremellomycetes (Arnold et al. 2009; Millanes et al. 2011; U'Ren et al. 2010). Recent metagenomic studies on lichen-associated eukaryotes suggested that Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Orbiliomycetes were the major lichen-associated fungal phylotypes in *Rhizoplaca* and *Umbilicaria* (Bates et al. 2012). It was suggested that endolichenic fungi are distinct from the mycobiont or any other previously recognized fungal association of lichens, and that endolichenism is an incubator for the evolution of endophytism (Arnold et al. 2009). In the same study, it was proposed that the infection frequency of lichen thalli by endolichenic fungi decreases from the tropics to high-latitude areas.

Most of the studies on lichen-associated fungi have been conducted using direct observations or culture methods. Although these approaches have provided much information about the diversity, evolution, and ecology of lichen-associated fungi, more comprehensive and unbiased data on lichen-associated fungi may be obtained from metagenomic analyses based on high-throughput sequencing technology. It is also expected that high-throughput sequencing technology will be one of the methods that provide important information on algal diversity and community structure in lichen thalli. This will in turn provide a basis for understanding the selective partnership between the mycobiont and photobiont, the effects of environmental conditions on photobiont selection, and the ecological roles of photobiont in adapting to specific environmental conditions. In the present study, we investigated algal and fungal diversity in Antarctic lichens through sequence information of the eukaryotic large subunit rRNA gene (LSU) and the algal internal transcribed spacer rRNA gene (ITS) determined by 454 pyrosequencing technology. We analyzed fruticose, foliose, and crustose lichens inhabiting mosses and rocks to understand which factor is the most important one among mycobiont species, substrates, and growth forms in determining algal and fungal communities in the lichen thalli.

MATERIALS AND METHODS

Lichen samples

Lichen samples analyzed in this study were collected from various locations on Barton and Weaver Peninsulas in King George Island located in the maritime Antarctic (Table S1; Fig. S1). To avoid cross-contamination, samples were preserved and transported individually in plastic bags at −20 °C and preserved at the same temperature until analyzed. Species identity was determined based on the morphology and anatomy, and all the samples are deposited at TROM and KOPRI herbarium. Two *Cladonia borealis* S. Stenroos samples (CL1 and CL2) and a *Cladonia gracilis* (L.) Willd. sample (CL3) were growing on mosses. Three *Umbilicaria antarctica* Frey & I.M. Lamb samples (UM1, UM2, and UM3) were growing on rock substrates. The appearance of the UM3 sample was quite different from the other *Umbilicaria* samples because of the parasitic infection by *Arthonia rufidula* (Hue) D. Hawksw., R. Sant. & Øvstedal, which was determined by its characteristic spores in numerous black apothecia surrounded by necrotic tissue near the edges of the host. Two *Usnea aurantiaco-atra* (Jacq.) Bory samples (US1 and US2) were growing on rock substrate and the other *U. aurantiaco-atra* sample (US3) was growing on mosses. Three crustose samples that were included in this study for comparative studies with fruticose and foliose lichens were *Buellia granulosa* (Darb.) C.W. Dodge (CR1), *Amandinea coniops* (Wahlenb.) M. Choisy (CR2), and *Ochrolechia parella* (L.) A. Massal (CR3). They were all saxicolous. Upper parts of thalli of fruticose and foliose lichens and several apothecia of crustose lichens were subsampled for DNA extraction to avoid contamination from substrate materials. Subsamples for DNA extraction were examined with stereomicroscope to avoid contaminating materials.

DNA extraction, PCR, and sequencing

Lichen samples (approx. 0.02 g each lichen) were ground with a tissue lyser (Qiagen, Hilden, Germany) after freeze-drying. DNA was isolated using a Wizard® Genomic DNA Purification kit (Promega, Madison, WI). The DNA was further purified in one volume of CTAB buffer [2% (w/v) CTAB, 100 mM Tris–HCl, 20 mM EDTA, 1.42 M NaCl, 0.02% of 2-mercaptoethanol] by shaking at 200 *g* for 1 h at 37 °C and then extracted with chloroform–isoamyl alcohol (24:1, v/v). Genomic DNA was recovered by precipitation with isopropanol.

To determine phylogenetic relationships among lichenized fungi, amplification and sequencing of the partial SSU, ITS1-5.8S-ITS2, and partial LSU rDNA were conducted using the ITS1F, ITS4, LR0R, and LR5 primers by the procedures as described in a previous study (Park et al. 2012).

Pyrosequencing, sequence processing, and taxonomic assignment

Eukaryotic LSU was amplified with LSU26f and LSU657r barcode primers, which were designed by modifying LR0R (Rehner and Samuels 1994) and LR3 primers (Vilgalys and Hester 1990) to cover more diverse eukaryotic LSU sequences (Table S2). Algal ITSs were amplified with ITS1T and ITS4T barcode primers (Kroken and Taylor

2000), which were designed to amplify *Trebouxia* ITS sequences (Table S2). Eukaryotic LSU and algal ITS sequences were determined using a 454 GS-FLX Titanium sequencing machine. Sequences were read from both directions. Sequencing templates were prepared by pooling three independent PCR amplicons from 25-cycle reactions to reduce PCR biases. Sequences were processed to remove primer, linker, and barcode sequences. The 3′ end of sequences with low-quality values were trimmed when average quality scores were lower than 30 for a 5-bp window size. Sequences with ambiguous nucleotides or shorter than 250 bp were discarded. The preprocessing was conducted using PyroTrimmer (Oh et al. 2012). Chimeric reads were detected and discarded using the de novo chimera detection algorithm of UCHIME (Edgar et al. 2011). Sequence clustering was performed by TBC (Lee et al. 2012) with 99% similarity cutoffs for eukaryotic LSU. Algal ITS sequences were clustered by phylogenetic analyses. Monophyly and 98% sequence similarity criteria were applied to define algal ITS clusters. Taxonomic assignment of each operational taxonomic unit (OTU) was conducted for representative sequences of each cluster by phylogenetic analyses with reference sequences retrieved from the GenBank database.

Phylogenetic analyses

Sequences were aligned using ClustalW (Larkin et al. 2007) and manually adjusted with the aid of the PHYDIT program ver. 3.2 (http://plaza.snu.ac.kr/~jchun/phydit/). Phylogenetic trees were inferred by maximum parsimony (MP) using PAUP 4.0b10 (Swofford 2002) and maximum likelihood (ML) methods using PhyML 3.0 (Guindon and Gascuel 2003). MP analyses were performed with a heuristic search with 100 replicates of random addition, tree-bisection-reconnection branch swapping, and the "MulTrees" option not in effect. All gaps were treated as missing data. ML trees were obtained from the best of the NNIs and SPRs search options under the GTR model. BioNJ tree was used as a starting tree (Gascuel 1997). Bootstrap values were calculated from 1,000 resampled data sets for each phylogenetic method.

Sequence data for the LSU rDNA of lichenized fungi from 12 lichen samples were deposited in GenBank database with accession numbers JN863249 and KJ607895–KJ607905.

Sample clustering using FastUniFrac

The relatedness of samples based on the lichen-associated fungi was assessed by FastUniFrac analysis (Hamady et al. 2009). The phylogeny of representative sequences of each OTU was constructed by the Neighbor program in the Phylip package (Felsenstein 1995) based on the distance matrix calculated by pairwise sequence alignment using ClustalX (Larkin et al. 2007). The resulting phylogenetic tree was imported into the FastUniFrac server (http://bmf2.colorado.edu/fastunifrac/) along with the number of reads per OTU for each sample. Clustering of samples was conducted based on the UniFrac metric with the abundance weight option on. The support for each branch was calculated by Jackknife analysis with 1,000 permutations.

RESULTS

Phylogeny of mycobionts

The two *C. borealis* samples (CL1 and CL2) were closely related to each other and *C. gracilis* (CL3) was distantly related to *C. borealis* with 98.3% sequence similarity (Fig. 1). All of the *Umbilicaria* samples were closely related to each other and there was only one nucleotide difference among their LSU sequences. The three *U. aurantiaco-atra* samples had identical LSU sequences, even though they showed morphological and substrate differences. US1 and US2 were attached to rocks and had fruticose structures with many attenuated branches, while US3 was growing on mosses without any attachment structures. It had thin, smooth, and long curved branches with very few attenuated branches. The three crustose lichens were distantly related to each other. *Buellia granulosa* (CR1) and *A. coniops* (CR2) were grouped in the *Caliciaceae* clade, which was supported by molecular phylogeny by five genetic loci (Miadlikowska et al. 2006). The genera *Amandinea* and *Buellia* did not form a monophyletic group. *Ochrolechia parella* (CR3) belonged to the *Pertusariaceae* clade, comprising a family which is dominated by the polyphyletic genera *Pertusaria* and *Ochrolechia* (Miadlikowska et al. 2006).

Algal diversity

Sequencing of LSU amplicons produced 1,822–3,367 sequence reads. They were composed of fungal, algal, and protozoan sequences (Table 1). They contained 122 (4.9% of total LSU sequence reads) to 1,268 (45.2% of total LSU sequence reads) algal sequences. By the clustering with a 99% similarity cutoff, 1–17 OTUs were recognized for each sample. All of the lichen samples contained one major algal OTU, which accounted for 78.7–100% of total algal sequences. The major OTU (eLSU10) of *Cladonia* (CL1, CL2, and CL3) was related to *Asterochloris erici* (Fig. 2A). *Umbilicaria* (UM1, UM2, and UM3), *Usnea* (US1, US2, and US3), *B. granulosa* (CR1), and *O. parella* (CR3) shared the same algal OTU (eLSU04), which was related to *Trebouxia jamesii*. *Amandinea coniops* (CR2) contained *Trebouxia impressa* as the major OTU (eLSU09). In addition to the major OTUs, most of the lichen samples except one *Usnea* sample (US2) contained phylogenetically diverse minor OTUs. For example, one *Cladonia* sample (CL2) contained OTUs related to *T. impressa*, *T. jamesii*, and *Pseudochlorella* sp. and an undefined lineage. Among the minor OTUs, eLSU50 and eLSU78 accounted for 6.2% and 3.4% of algal sequence reads, respectively. Three *Umbilicaria* samples also contained diverse algal species including *A. erici* and diverse lineages related to *Pseudochlorella*. In the CR2 sample,

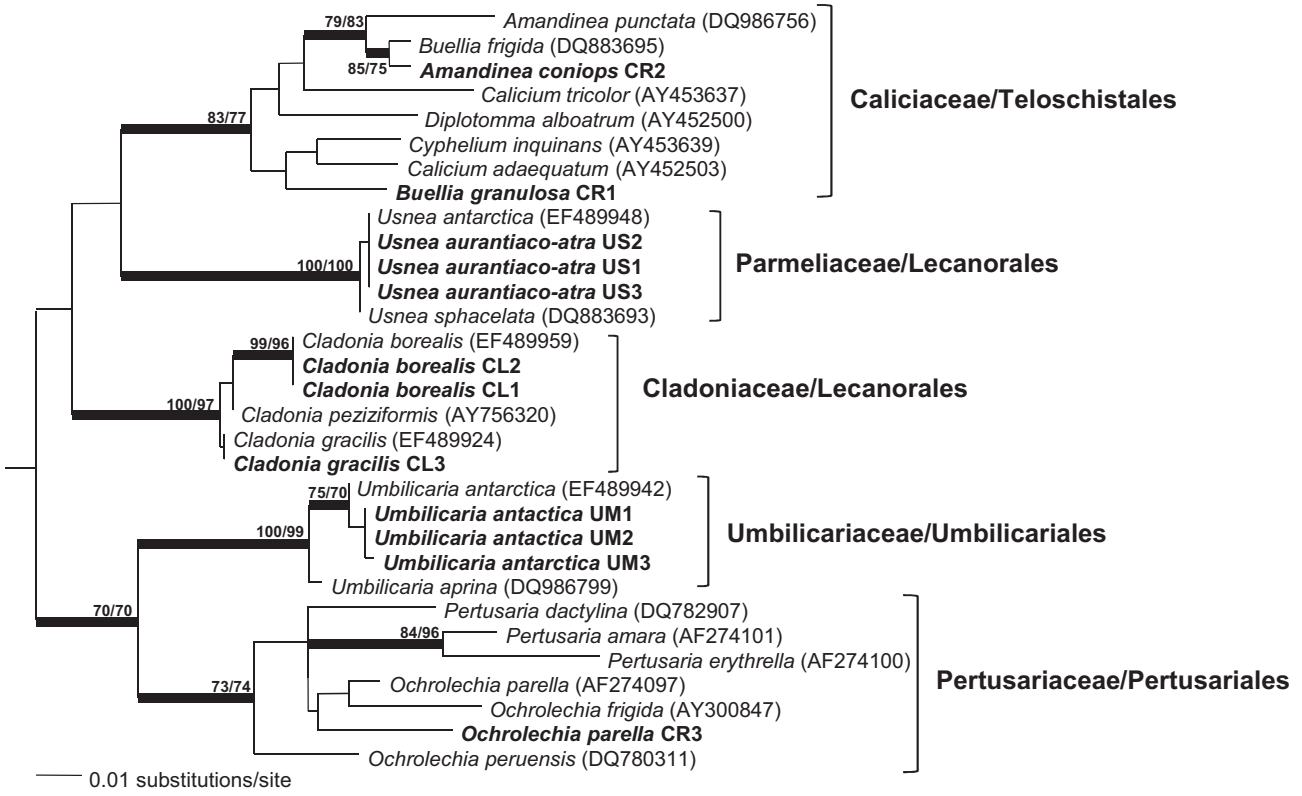

Figure 1 Maximum likelihood tree of LSU rDNA of lichenized fungi from 12 lichen samples. Thick branches indicate those maintained in both ML and MP trees. Bootstrap values for ML and MP trees (both 1,000 replicates) are shown on corresponding branches. The tree was rooted with *Ascocoryne sarcoides* (FJ176886, Leotiomycetes).

the second major OTU (eLSU14), which was related to *Pseudochlorella*, accounted for 14.2% of algal sequence reads.

Algal ITS sequences amplified with *Trebouxia*-specific primers were clustered into 2–13 OTUs at a 98% similarity cutoff, depending on the sample. Three *Cladonia* samples contained *Asterochloris* as the major OTU. *Umbilicaria* (UM1, UM2, and UM3), *Usnea* (US1, US2, and US3), *B. granulosa* (CR1), and *O. parella* (CR3) contained *T. jamesii* as the major OTU. *Amandinea coniops* (CR2) contained a major OTU related to *T. impressa*, *Trebouxia potteri*, and *Trebouxia flava*. These results on the major OTUs of algal ITS sequences were consistent with the results of LSU sequencing results (Fig. 2B). An interesting observation from the algal ITS sequence analyses was that in many cases, the major OTU defined by LSU sequences consisted of two closely related ITS OTUs. UM3 contained two related *Trebouxia* OTUs accounting for 73.9% (aITS01) and 26.1% (aITS04) of algal sequence reads. CR1 contained two related OTUs in the *T. jamesii* lineage accounting for 82.9% (aITS01) and 7.6% (aITS04) of algal sequence reads. CR2 contained two related OTUs in the *T. flava* lineage accounting for 77.8% (aITS03) and 19.2% (aITS06) of sequence reads. CR3 contained aITS01 (72.4%) and aITS04 (25.1%) as the two major OTUs.

Fungal diversity

Fungal sequences constituted 54.8–95.1% of total LSU sequence reads. Depending on the sample, there were 26–66 fungal OTUs defined by monophyly and 99% similarity cutoff criteria. The major OTU for each sample was related to the mycobiont sequences that were determined by Sanger sequencing (Fig. 1). These constituted 53.7–93.2% of total LSU sequence reads (Table 1). The other fungal sequences were regarded as lichen-associated fungi, which included lichenicolous, endolichenic, and epilichenic fungi. They were minor components in most of the samples, accounting for only 0.2–4.5% of total LSU sequence reads except for one *Umbilicaria* sample (UM3), in which 24.4% of the total sequence reads were for lichen-associated fungi. The major lichen-associated fungal OTUs in the UM3 sample belonged to the Arthoniomycetes (eLSU11, 42.6% of total lichen-associated fungal sequence reads), Lecanoromycetes (eLSU12, 24.7%), and Eurotiomycetes (eLSU13, 14.3%) (Table 2).

The taxonomic affiliations of the lichen-associated fungi were determined by phylogenetic analysis with reference sequences (Fig. 3). They belonged to the Arthoniomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes, and Sordariomycetes of the Ascomycota, and Cystobasidiomycetes and Tremellomycetes of the Basidiomycota. Several

Table 1. Summary of sequencing results

Sample	CL1	CL2	CL3	UM1	UM2	UM3	US1	US2	US3	CR1	CR2	CR3
	C. borealis	*C. borealis*	*C. gracilis*	*Um. antartica*	*Um. antartica*	*Um. antartica*	*Us. aurantico-atra*	*Us. aurantico-atra*	*Us. aurantico-atra*	*B. granulosa*	*A. coniops*	*O. parella*
Growth forms	Fruticose	Fruticose	Fruticose	Foliose	Foliose	Foliose	Fruticose	Fruticose	Fruticose	Crustose	Crustose	Crustose
Substrate	Moss	Moss	Moss	Rock	Rock	Rock	Rock	Rock	Moss	Rock	Rock	Rock
Eukaryotic LSU rDNA												
Fungal sequence reads												
Mycobiont	2,150 (90.0)	2,981 (93.1)	1,909 (86.8)	1,674 (62.6)	1,649 (76.2)	1,371 (64.5)	1,507 (53.7)	2,965 (88.1)	2,319 (93.2)	1,368 (75.1)	1,986 (71)	2,308 (90.7)
LAF	5 (0.2)	36 (1.1)	47 (2.1)	50 (1.9)	88 (4.1)	519 (24.4)	31 (1.1)	2 (0.06)	47 (1.9)	81 (4.4)	20 (0.8)	7 (0.3)
Algal sequence reads	232 (9.7)	178 (5.6)	244 (11.1)	944 (35.3)	426 (19.7)	237 (11.1)	1,268 (45.2)	400 (11.9)	122 (4.9)	373 (20.5)	782 (27.9)	230 (9.0)
Protozoan sequence reads	1 (0.04)	8 (0.25)	0 (0.0)	6 (0.21)	1 (0.05)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.04)	0 (0.0)	10 (0.36)	0 (0.0)
Total sequence reads	2,388	3,203	2,200	2,674	2,164	2,127	2,806	3,367	2,489	1,822	2,798	2,545
Algal ITS rDNA												
Total sequence reads	712	802	560	253	251	92	284	774	183	618	621	395

LAF, Lichen-associated fungi. The values within parentheses are in percentage.

OTUs showed close relationships with known species. They include *Usnea antarctica* or *U. aurantiaco-atra* (eLSU01) detected from *Cladonia* and *Umbilicaria* samples (CL2, CL3, UM2, and UM3), *Epibryon diaphanum* or *Pseudocladosporium hachijoense* (eLSU20) from *Umbilicaria* and *Usnea* samples (UM1, UM2, UM3, and US1), *Infundichalara microchona, Chalara constricta*, *Xenopolyscytalum pinea*, *Cistella acuum*, or *Dicocistella grevillei* (eLSU25) from *Usnea* samples (US1 and US3), *Catenulifera brachynonia* (eLSU60) from *Cladonia* and *Usnea* samples (CL2 and US3), *Alatospora acuminata, Alatospora constricta*, *Gorgomyces honrubiae*, or *Flagellospora leucorhynchos* (eLSU87) from Cladonia and Umbilicaria samples (CL2, CL3, and UR3), and *Psoroma hypnorum, Psoroma paleaceum*, or *Psoroma fruticulosum* (eLSU90) from *Usnea* sample (US3). However, most of the OTUs were distantly related to reference sequences and it was difficult to assign specific genus or family names.

We conducted FastUniFrac analysis based on the relative abundance of lichen-associated fungal OTUs for nine lichen samples. The samples CL1, US2, and CR3 were excluded from this analysis as they contained very few lichen-associated fungal sequences. The resulting dendrogram showed a close relationship between the two *Cladonia* samples (CL2 and CL3) and between the two *Umbilicaria* samples (UM1 and UM2) (Fig. 4). The UM3 sample in the genus *Umbilicaria* was distantly related to the other *Umbilicaria* samples. The relatedness of lichen-associated fungal OTU abundance did not appear to be correlated with growth forms or substrate.

Two *Cladonia* samples (CL2 and CL3) shared OTUs in the Lecanoromycetes, Leotiomycetes, and Tremellomycetes (Table 2). They lacked lichen-associated fungal OTUs in the Dothideomycetes, Eurotiomycetes, and Sordariomycetes. In contrast, three *Umbilicaria* samples shared OTUs in the Cystobasidiomycetes, Eurotiomycetes, and Tremellomycetes. One of the *Umbilicaria* samples (UM3) contained abundant OTUs in the Dothideomycetes and Lecanoromycetes. Two *Usnea* samples (US1 and US3) shared OTUs in the Cystobasidiomycetes, Eurotiomycetes, and Leotiomycetes. OTUs in the Lecanoromycetes, Sordariomycetes, and Tremellomycetes were very rare. One of the crustose samples (CR1) contained abundant OTUs in the Sordariomycetes, but none of the OTUs was shared among the three crustose samples. The occurrence of common lichen-associated fungal OTUs in CL2 and CL3 in the genus *Cladonia*, in UM1 and UM2 in the genus *Umbilicaria*, and in US1 and US3 samples in the genus *Usnea* may explain the close relatedness in the FastUniFrac analysis (Fig. 4).

DISCUSSION

The presence of several genotypes of algal species in a single thallus has been reported from morphological and genotyping analyses (Casano et al. 2011; Guzow-Krzeminska 2006; Piercey-Normore 2006). To investigate the prevalence of this phenomenon of multiple algal genotypes in a thallus, we examined algal diversity by sequencing

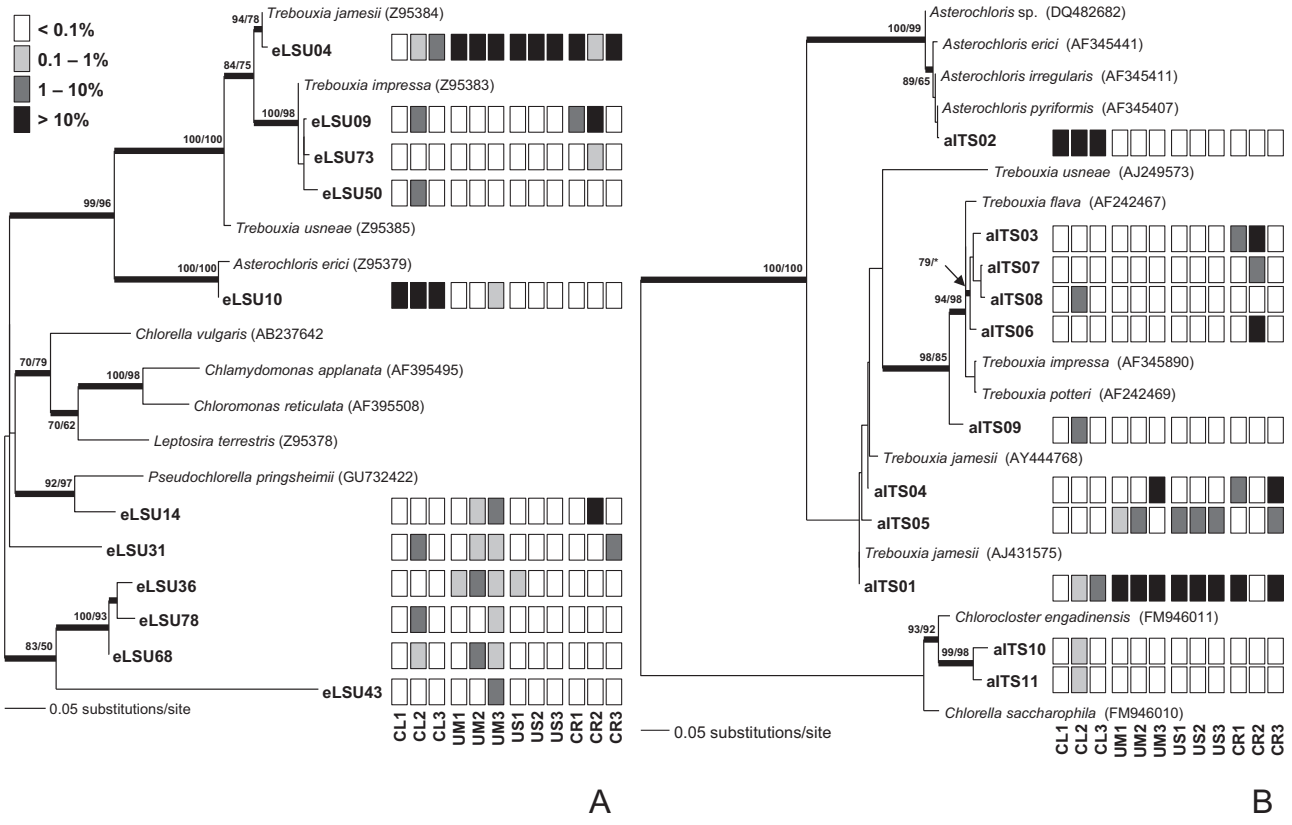

Figure 2 Maximum likelihood trees of photobiont based on sequences of **A.** LSU rDNA and **B.** algal ITS rDNA. Thick branches indicate those maintainded in both ML and MP trees. Bootstrap values for ML and MP tree are shown on corresponding branches. The tree was midpoint-rooted. Intensity of shading rectangle indicates ratio (%) of the sequences.

two genetic loci. We found that all of the lichen samples except one *Usnea* sample (US2) contained multiple algal genotypes, as reflected by the sequences of the LSU and ITS loci. However, all of the samples had one major LSU genotype that accounted for 78.7–100% of total algal sequences. The major algal genotype was shared among the same lichen genera. In the case of the ITS locus, the major genotype consisted of two closely related genotypes in several cases. Because of the presence of fungal species included in Lecanoromycetes in lichen thalli, it could be suspected that multiple algal genotypes were originated from contaminated lichen tissues. However, it does not seem appropriate to explain high proportion of minor algal species or genotypes by contaminating lichen tissues considering very low abundance of LAF included in Lecanoromycetes (Fig. S2). The presence of multiple algal genotypes and the dominance of one or a few major genotypes may imply that lichens have ample opportunities to encounter and retain diverse algal genotypes. In addition, there might be selection mechanisms for a particular algal genotype by the fungal host or under particular environmental conditions. Piercey-Normore (2006) hypothesized that algal genotypes might be switched to adapt to changing environmental conditions. Recently, it was shown that environmental factors are important in photobiont selection (Peksa and Škaloud 2011).

In the current study, we compared algal diversity in twelve lichens with various mycobionts, growth forms, and substrates. *Usnea aurantiaco-atra* growing on mosses (US3) did not share major algal species with the *Cladonia* samples growing on mosses. Some of the lichens growing on rocks contained *T. jamesii*, but the other contained *T. impressa* as the major algal species. *Umbilicaria antarctica* with foliose thalli, *U. aurantiaco-atra* with fruticose thalli, and *B. granulosa* and *O. parella* with crustose apothecia contained *T. jamesii* as the major algal species. However, *A. coniops* with crustose apothecia contained *T. impressa* as the major photobiont. In this case, growth forms or substrate might not be an important factor in deciding photobiont selectivity. The only factor that seems to be important in selection of the major algal species was the mycobiont species in the sense that the same mycobiont species always had the same major algal species as the symbiotic partner. The effects of climate could not be investigated in the present study because the lichen samples were collected from a small geographic area.

Sequencing of the LSU revealed that all of the lichen samples contained various lichen-associated fungi in the lichen thalli. One of the interesting findings from the sequence analyses was that many of the lichen samples contained lichen-associated fungi affiliated with the Lecanoromycetes, a fungal class mostly composed of liche-

Table 2. Relative abundance of major lichen-associated fungal OTUs (> 0.5%)

Class	OTU (Sequence reads)	CL1 (5)	CL2 (36)	CL3 (47)	UM1 (50)	UM2 (88)	UM3 (519)	US1 (31)	US2 (2)	US3 (47)	CR1 (81)	CR2 (20)	CR3 (7)	Total (933)
Arthoniomycetes	eLSU11	0	0	0	0	0	42.6	0	0	0	0	0	0	23.7
	eLSU41	0	0	0	0	0	3.1	0	0	0	0	0	0	1.7
	eLSU51	0	0	0	0	0	2.1	0	0	0	0	0	0	1.2
	eLSU76	0	0	0	0	0	0	0	0	12.8	0	0	0	0.6
Eurotiomycetes	eLSU13	0	0	0	16.0	36.0	14.3	0	0	0	0	0	0	12.6
	eLSU20	0	0	0	16.0	11.0	0.2	38.7	0	0	0	0	0	3.4
	eLSU59	0	0	0	0	0	1.2	6.5	0	2.1	0	0	0	1.0
	eLSU86	0	0	0	0	0	1.0	0	0	0	0	0	0	0.5
	eLSU89	0	0	0	0	0	0.6	0	0	4.3	0	0	0	0.5
	eLSU91	0	0	0	0	0	0	0	0	0	6.2	0	0	0.5
Lecanoromycetes	eLSU01	0	16.7	25.5	0	1.0	1.0	0	0	0	0	0	0	2.3
	eLSU12	0	0	0	0	0	24.7	0	0	0	0	0	0	13.7
	eLSU40	0	0	0	0	0	0	0	0	0	0	85.0	0	1.8
	eLSU55	0	13.9	10.6	0	0	0	0	0	0	0	0	0	1.1
	eLSU64	0	0	0	0	0	1.5	0	0	0	0	0	0	0.9
	eLSU75	0	0	0	0	4.0	0.4	0	0	0	0	0	0	0.6
	eLSU90	0	0	0	0	0	0	0	0	10.6	0	0	0	0.5
Leotiomycetes	eLSU25	0	0	0	0	0	0	35.5	0	27.7	0	0	0	2.6
	eLSU60	0	2.8	0	0	0	0	0	0	17.0	0	0	0	1.0
	eLSU87	0	8.3	2.1	0	0	0	0	0	2.1	0	0	0	0.5
Sordariomycetes	eLSU37	0	0	0	0	0	0	0	0	0	22.2	0	0	1.9
	eLSU56	0	0	0	0	0	0	0	0	0	12.3	0	0	1.1
	eLSU77	0	0	0	0	0	0	0	0	2.1	6.2	0	0	0.6
Cystobasidiomycetes	eLSU21	0	2.8	0	4.0	17.0	1.7	0	0	0	0	0	0	3.3
	eLSU63	0	0	8.5	0	1.0	0	3.2	50.0	2.1	0	0	0	0.9
Tremellomycetes	eLSU23	0	0	0	0	0	0	0	0	0	35.8	0	0	3.1
	eLSU35	0	0	14.9	10.0	2.0	0.8	0	0	0	0	0	0	1.9
	eLSU39	0	0	0	28.0	1.0	0.4	0	0	0	0	0	0	1.8
	eLSU47	20.0	2.8	19.1	0	0	0	3.2	0	0	1.2	0	0	1.4
	eLSU92	80.0	2.8	0	0	0	0	0	0	0	0	0	0	0.5

nized fungi. In particular, the *Cladonia* samples (CL2 and CL3) and two *Umbilicaria* samples (UM2 and UM3) contained *U. antarctica* or related species as the lichen-associated fungus (Table 2). We cannot exclude the possibility of contamination by propagules or fragments of *U. antarctica* that were attached to the surfaces of *Cladonia* and *Umbilicaria* samples because the specimens were not washed before DNA extraction. However, we could not detect observable structures by stereomicroscopy and it is unlikely that the same sequence would be detected from four independent samples as a result of such a random process. In addition, *U. antarctica* is frequently found as an epiphyte on old thalli of *U. antarctica* and *Umbilicaria kappeni* (Øvstedal and Lewis Smith 2001). We also observed that *Usnea* species produced thalli growing on *Cladonia* and *Umbilicaria* specimens among field samples collected from King George Island (unpublished data). These findings raise the question of whether lichenized fungi maintain their life form solely as major symbionts in lichens or whether they can switch to lichenicolous or endolichenic forms on other lichens. So far, we have not clearly answered this question, but further studies on the structure of lichen thalli with specific staining of each component by FISH technique and phylogenetic and population genetics studies of lichenized and lichen-associated fungi will provide further information on this topic.

Another remarkable observation from the analysis of lichen-associated fungi was that the morphologically different sample (UM3) of the genus *Umbilicaria* contained abundant lichen-associated fungal sequences. Its major lichen-associated fungal OTUs, eLSU11, eLSU12, and eLSU13, were in the Arthoniomycetes, Lecanoromycetes, and Eurotiomyces, respectively (Table 2). The OTU eLSU13 was shared by the two other *Umbilicaria* samples, but eLSU11 and eLSU12 were observed only in UM3. As the LSU rRNA gene sequence of the UM3 mycobiont differed at one nucleotide site from that of the other two *Umbilicaria* samples (UM1 and UM2) and they were collected from different localities, there are several possible explanations for the relationships among the different mycobiont genotypes, the lichen-associated fungal community structure, and the different morphologies: (1) the genetic difference of the mycobiont might have driven differences in morphology and the lichen-associated fungal community; (2) infection by a large number of lichen-associated fungi may have caused the difference in morphological development, as is the case in pathogen-infected plants or in other symbiotic relationships with microorganisms (Hématy et al. 2009; Nap and Bisseling 1990); or (3) there are no specific relationships among

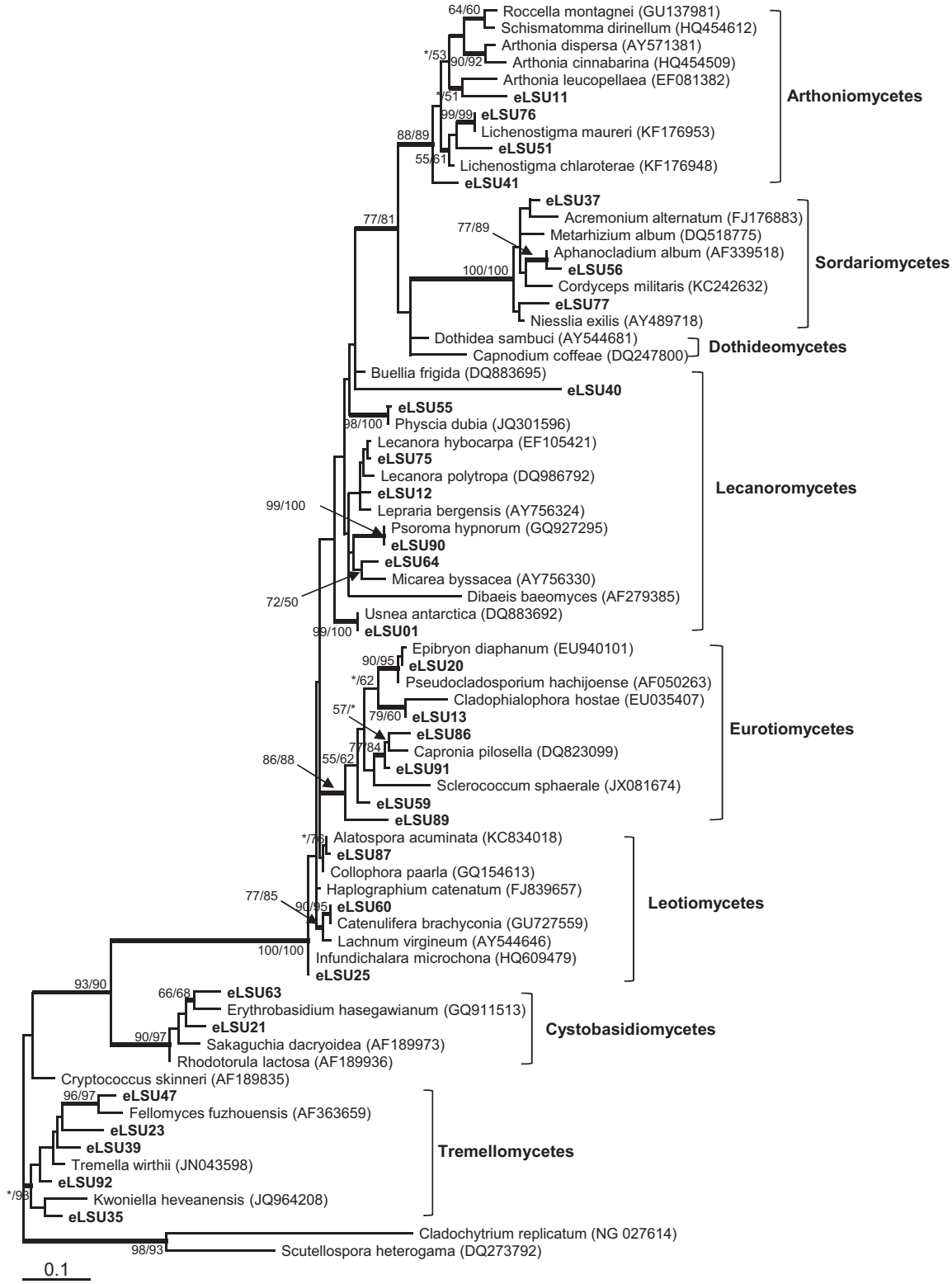

Figure 3 Maximum likelihood tree of LSU rDNA of lichen-associated fungi. Thick branches indicate those maintained in both ML and MP trees. Bootstrap values for ML and MP trees (both 1,000 replicates) are shown on corresponding branches. Tree was rooted with *Scutellospora heterogama* (DQ273792, Glomeromycota) and *Cladochytrium replicatum* (NG027614, Chytridiomycota).

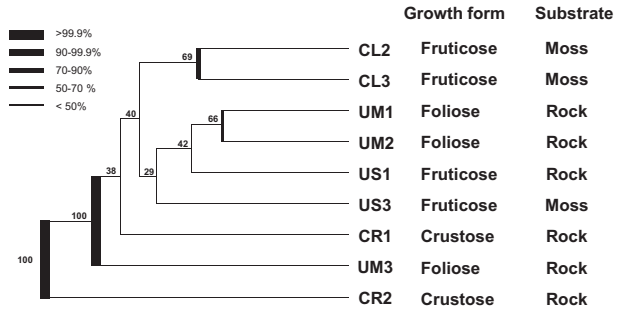

Figure 4 Jackknife sample clustering analysis of all lichen-associated fungi OTUs based on distance matrix generated by calculating pairwise FastUniFrac values. Sample clustering was based on UniFrac metric with abundance weight option on. Support for each branch was calculated by Jackknife analysis with 1,000 permutations.

mycobiont genotypes or infection by parasites, instead different environmental conditions affected the morphology. Because of the limited number of samples for comparison in the present study, we could not determine which explanation is most likely to be correct. However, the necrotic thallus around mature apothecia of *A. rufidula*, considered to be a common parasite of *U. antarctica* in Antarctica (Øvstedal and Lewis Smith 2001), obviously explains its deviating appearance. However, in the present sample *A. rufidula* also produces its own, brownish 0.1-mm large thallus granules. Thus, this species is obviously a juvenile parasite, later probably developing into an autonomous lichenicolous lichen, a change in relationship with age also known from other species (Ihlen 1998).

We believe that the Arthoniomycetes sequence eLSU11 represents *A. rufidula*. However, this lichen also includes several other fungi, so it might be that a necrotic thallus is more vulnerable to invasion by other fungi in addition to the one that initially caused the thallus damage. In future studies, this could be addressed by including more samples with similar genetic backgrounds and different morphologies. Defining the contribution of lichen-associated fungi or environmental conditions to lichen morphogenesis may help explain the highly variable morphological features of lichen species.

Another interesting finding from the analysis of lichen-associated fungi was that the lichens harbored diverse species related to basidiomycetous yeast taxa in the Cystobasidiomycetes and Tremellomycetes, but no ascomycetous yeast taxa. These observations together with the FastUniFrac result, which revealed closely related lichen-associated fungal community structures among the related lichen samples, implied that each lichen thallus provides a specific environment that only certain fungal species are allowed to inhabit. In the case of bacterial communities, the selective presence of bacterial species was explained by diverse secondary metabolites such as usnic acid (Boustie and Grube 2005; Francolini et al. 2004). Likewise, selective control of fungal species might be explained by specific antagonism of secondary metabolites produced by lichens (Halama and Van Haluwin 2004; Schmeda-Hirschmann et al. 2008).

We examined algal and fungal community structures in Antarctic lichens with different mycobiont species, growth forms, and substrates. All of them contained a high biodiversity, with numerous genotypes in addition to the major photobiont and mycobiont. Even in such an extreme environment as in Antarctica lichens can generally be called miniature ecosystems rather than simple symbiotic individuals. Most of the samples contained multiple algal species in a single thallus, and even different strains of the dominant photobionts. All of the lichen samples contained major algal genotypes and the selection of algal genotypes was dependent on the mycobiont species. We could not detect specific relationships between algal genotype and growth forms or substrates. We hope the observation of multiple algal species in a thallus and selection of specific genotypes as the major one lead to the studies on how lichenized fungi meet several different photobionts, how diverse algal species are maintained in lichen thallus, and what affects the selection of the major photobionts. The analyses on the lichen-associated fungal community provided many interesting results. Although we could not provide concrete answers with limited number of samples and limited methodological approaches to understand their relationships, this study on lichen-associated fungi raised several interesting issues, such as whether there is a switch between lichenized and lichen-associated life forms, and how the mycobiont controls the lichen-associated fungal community. These questions can be addressed in further research including structural, phylogenetic, metabolomics, and comparative genomics studies.

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SUPPORTING INFORMATION

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

Table S1. Species, locality, and habitat information of the samples.
Table S2. PCR primers used in this study.
Figure S1. Sampling sites on Barton and Weaver Peninsulas in King George Island.
Figure S2. Relationship between relative abundances of lichen-associated Lecanoromycetes among whole fungal sequences and minor photobionts among whole algal sequences.