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Diversity of the Lichenized Fungi in King George Island, Antarctica, Revealed by Phylogenetic Analysis of Partial Large Subunit rDNA Sequences

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Lichens are predominant and important components of flora in the terrestrial ecosystem of Antarctica. However, relatively few researches on the phylogenetic position of Antarctic lichen-forming fungi have been accomplished. In this study, partial sequences of nuclear large subunit rDNAs from 50 Antarctic specimens were obtained and the phylogeny was reconstructed. Antarctic lichen species were distributed in 4 orders, including the monophyletic order *Agyrales*, paraphyletic orders *Pertusariales* and *Teloschistales*, and polyphyletic order *Lecanorales*. Species diversity was highest in the order *Lecanorales*, followed by *Teloschistales* and *Pertusariales*. Based on the phylogeny and sequence similarity analyses, it is proposed that the taxonomy of *Stereocaulon alpinum*, *Physcia caesia*, *Usnea aurantiacoatra*, and *Cladonia* species should be revised by careful examination of their phenotypic and molecular characteristics. Six species known to be endemic to Antarctica, *Catillaria corymbosa*, *Himantormia lugubris*, *Leptogium puberulum*, *Pertusaria pertusa*, *Rhizoplaca aspidophora*, and *Umbilicaria antarctica*, formed unique lineages, implying independent origins in the Antarctic area.

Keywords: Antarctica, diversity, King George Island, lichens, phylogeny

The Antarctic is defined geographically as all lands and adjoining ice shelves south of latitude 60°S. King George Island is the largest island in the South Shetland Islands belonging to the maritime Antarctic zone and is located about 120 km from the Antarctic continent. The island spans from the north east to the south west between latitudes 61°50' and 62°15'S and longitudes 57°30' and 59°01'W. Floristically, the island possesses two vascular plants and diverse bryophytes and lichens [27]. The island is one of the best places for studying the evolution of lichen-forming fungi of Antarctica because of its geographical locality and diversity of lichens [17].

Lichens are symbiotic organisms of mycobionts (fungi) and photobionts (algae or cyanobacteria). They constitute the main part of Antarctic flora, along with algae and bryophytes [16]. Some lichens are nature's pioneers that are the first to penetrate into the areas emerging from beneath the ice, and have a successful evolutionary strategy to adapt in extreme environmental conditions, and have resulted in diverse, abundant, and important flora in the terrestrial ecosystem of Antarctica [24].

The first comprehensive floristic study of Antarctic lichens was reported by Dodge [6]. The recent significant study of Antarctic lichens was carried out by Øvstedal and Lewis Smith [27], who provided descriptions and keys to identify genera and species. They recognized 427 species in Antarctica and South Georgia. The lichen diversity of King George Island has been reported by Redón [30], Andreev [1], Inoue [13], Olech [21, 24], Osyczka and Olech [25], and Kim *et al.* [17]. Olech [24] reported 294 species from the King George Island. Kim *et al.* [17] reported 62 lichen species around the Korean Antarctic Scientific Station at Barton Peninsula on King George Island.

Molecular phylogenetic studies have provided new insights to understanding the evolutionary relationships among phenotiypically recognized species. Recently, collaborative studies among classical taxonomists and molecular phylogeneticists have resulted in a revised classification system of fungi [4, 7, 10, 14, 20, 29, 31]. By extensive molecular phylogenetic studies, many new insights have been found on the matter of polyphyletic taxonomic groups, development of cryptic species, and morphological variation by genetic diversity, geographical distribution, and environmental adaptation [19, 22, 33, 35, 36, 42]. It is now generally accepted that combining morphological and molecular phylogenetic analyses leads to a better understanding of fungal relationships and biodiversity [9].

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Table 1. List of specimens used in this study.

Species	Voucher No.	PCR products size (kb)	GenBank Acc. No.
Caloplaca sublobulata (Nyl.) Zahlbr. bloulata	ANT050943	1.5	EF489950
Catillaria corymbosa* (Hue) I.M. Lamb	**ANT050867**	**1.5**	**EF489937**
	ANT050872	1.5	EF489940
Cetraria aculeata (Schreb.) Fr.	ANT050760	1.5	EF489911
	ANT050812	1.5	EF489921
	ANT050957	1.5	EF489956
Cladonia borealis Stenroos	ANT050813	2.1	EF489922
	ANT050855	2.1	EF489933
	ANT050965	2.1	EF489959
Cladonia chlorophaea (Flörke ex Sommerf.) Spreng.	ANT050826	1.5	EF489927
	ANT050833	1.8	EF489928
Cladonia furcata (Huds.) Schrad.	ANT050856	2.1	EF489934
	ANT050857	2.1	EF489935
Cladonia gracilis (L.) Willd.	ANT050816	1.8	EF489924
	ANT050834	2.1	EF489929
	ANT050964	2.1	EF489958
Cladonia pleurota (Flörke) Schaer.	ANT050858	2.1	EF489936
Cladonia pyxidata (L.) Hoffm.	ANT050825	1.8	EF489926
Himantormia lugubris* (Hue) I.M. Lamb	**ANT050762**	**1.5**	**EF489912**
	ANT050774	**1.5**	**EF489915**
	ANT050851	**1.5**	**EF489931**
	ANT050928	**1.5**	**EF489947**
Leptogium puberulum* Hue	**ANT050958**	**1.5**	**EF489957**
Ochrolechia frigida (Sw.) Lynge	ANT050769	1.5	EF489913
Pertusaria excludens* Nyl.	**ANT050905**	**2.1**	**EF489944**
Physcia caesia (Hoffm.) Fürnr.	ANT050907	1.9	EF489945
Placopsis contortuplicata I.M. Lamb	ANT050773	1.5	EF489914
	ANT050823	1.5	EF489925
	ANT050946	1.5	EF489951
Pseudephebe pubescens (L.) M. Choisy	ANT050779	1.7	EF489916
	ANT050954	1.7	EF489953
Ramalina terebrata Hook. f. & Taylor	ANT050809	1.5	EF489919
	ANT050901	1.5	EF489943
Rhizoplaca aspidophora* (Vain.) Redón	**ANT050836**	**1.5**	**EF489930**
Rinodina olivaceobrunnea C.W. Dodge & G.E. Baker	ANT050811	2.1	EF489920
Sphaerophorus globosus (Huds.) Vain.	ANT050793	1.5	EF489917
	ANT050887	1.5	EF489941
	ANT050935	1.5	EF489949
	ANT050956	1.5	EF489955
Stereocaulon alpinum Laurer	ANT050947	1.5	EF489952
Umbilicaria antarctica* Frey & I.M. Lamb	**ANT050868**	**1.5**	**EF489938**
	ANT050895	1.5	EF489942
Umbilicaria decussata (Vill.) Zahlbr.	ANT050969	1.5	EF489960
Usnea antarctica Du Rietz	ANT050815	1.8	EF489923
	ANT050852	1.8	EF489932
	ANT050870	1.9	EF489939
	ANT050931	1.9	EF489948
Usnea aurantiacoatra (Jacq.) Bory	ANT050955	1.9	EF489954
Xanthoria candelaria (L.) Th. Fr.	ANT050803	1.5	EF489918
	ANT050915	1.5	EF489946

*Endemic species to Antarctica [27].

Despite extensive floral investigation in the Antarctic area, molecular phylogenetic studies are restrictive in a few taxa. In this study, 50 Antarctic lichen-forming fungi reported by Kim *et al.* [17] were analyzed using the sequences of nuclear large subunit rDNAs (LSU rDNAs) to provide a general perspective on the phylogenetic biodiversity of lichen-forming fungi in the Antarctic area.

Materials and Methods

Specimens and DNA Extraction

Among 62 lichen collections from King George Island [17], 50 macrolichen specimens were examined in this study (Table 1). Information for the modern systematics and the latest scientific names was obtained from the CABI Bioscience Database of Fungal Name (http://www.indexfungorum.org) and the CBS database (http://www.cbs.knaw.nl). Information for the fungal classification system was also acquired from proposals by Eriksson [7], Hibbett *et al.* [10], and Miadlikowska *et al.* [21]. The lichen materials were ground using a freeze-crusher (SK200; Tokken, Japan), and genomic DNAs were extracted using the Plant DNA mini-kit (Qiagen, Germany) according to the manufacturer's guide.

PCR Amplification and Sequencing

The ITS1-5.8S-ITS2 and partial LSU rDNA were amplified using the primers ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3') [8] and LR5 (5'-ATC CTG AGG GAA ACT TC-3') [40]. In some cases, semi-nested PCR reactions were applied with the primer pair ITS5 (5'-GGA AGT AAA AGT CGT AAC AAG G-3') [41] and LR5 for improved PCR reactions. PCR was performed using Quick PCR Premix (Bioneer, Korea). Amplifications were performed in a T-gradient thermocycler (Biometra, Germany) with the following cycling parameters: 5 min initial denaturation at 94°C, 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 52°C, and 1 min 30 sec extension at 72°C, and 10 min final extension at 72°C. Semi-nested PCR was carried out in 20-μl reaction mixtures using a nested primer set, ITS5 and LR5, and 1 μl of 1/100 diluted 1st PCR products. The PCR product sizes were determined by comparison with the 1-kb DNA ladder (SolGent, Korea), and PCR products were purified with a PCR purification kit (Bioneer, Korea). Sequences of the LSU rDNA regions were determined by overlapping sequencing reactions on complementary DNA strands with primers LR0R (5'-GTA CCC GCT GAA CTT AAG C-3') [32] and LR5, using the ABI 3730XL automated sequencer (Applied Biosystems Company, U.S.A.). In the case of long PCR products, additional sequencing reactions were conducted with primers LR3 (5'-GGT CCG TGT TTC AAG AC-3') [40] and LR3R (5'-GTC TTG AAA CAC GGA CC-3') [40]. The sequences were proofread, edited, and aligned using the jPHYDIT program [15].

Phylogenetic Analysis

Fifty sequences generated in this study and 57 LSU rDNA sequences retrieved from the GenBank database were aligned by the program CLUSTALX ver. 1.83 [38] (gap opening penalty=15.0; gap extension penalty=6.66). The alignment was manually adjusted. Ambiguously aligned sites were excluded from the following analyses. Phylogenetic trees were inferred from the data sets by the neighbor-joining (NJ) method, maximum parsimony (MP), and Bayesian inference using likelihood criterion. The NJ tree was reconstructed using PAUP 4.0b10 [37] under the Kimura's 2-parameter model [18]. Parsimony analysis was conducted using PAUP 4.0b10 with tree bisection reconnection (TBR) branch swapping and MAXTREES set to auto-increase. All gaps were treated as missing data. Supports for internal branches in distance and parsimony analyses were tested by the bootstrap analyses of 1,000 replications. Bayesian inference was carried out using MrBayes ver. 2.01 [12]. Metropolis-coupled Markov chain Monte Carlo (MCMCMC) algorithm was used within a Bayesian framework to estimate the posterior probability of phylogenetic trees. The Tamura-Nei (TrN) model with an estimated proportion of invariable sites and an estimated gamma distribution shape parameter was selected as the best-fit model of sequence evolution using the program MODELTEST ver. 3.7 [28]. The Markov chains were run for 2,000,000 generations, sampling every 10 generations. The first 30,000 samples were removed to avoid including any trees that might have been sampled before convergence of the Markov chain. The posterior probability of ancestral nodes based on the consensus was calculated. *Schizosaccharomyces pombe* (Z19578) was used as an outgroup for rooting the tree.

Results

PCR Amplification and Sequencing

The rDNA sequences were obtained from 50 Antarctic lichen-forming fungi, which belonged to 26 species, 19 genera, 14 families, and 4 orders of *Lecanoromycetes*. PCR products by primers ITS1F and LR5 ranged between 1.5 and 2.1 kb (Table 1). In contrast to the approximately 1.5-kb PCR products from 29 samples, PCR products ranging between 1.7 and 2.1 kb were obtained from *Cladonia borealis*, *Cladonia chlorophaea*, *Cladonia furcata*, *Cladonia gracilis*, *Cladonia pleurota*, *Cladonia pyxidata*, *Pertusaria excludens*, *Physcia caesia*, *Pseudephebe pubescens*, *Rinodina*

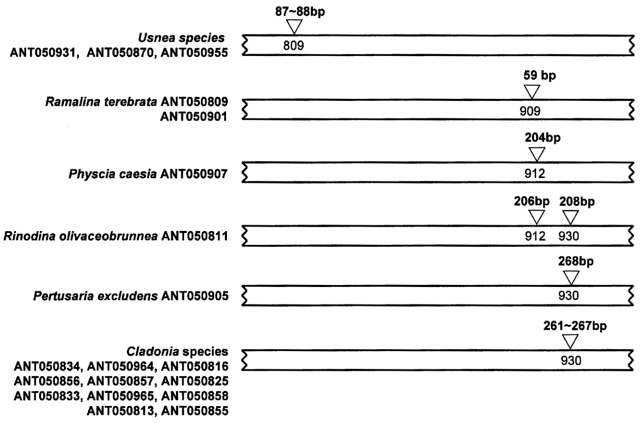

Fig. 1. Intron distribution pattern in LSU rDNA of Antarctic lichen-forming fungi.
Intron insertional positions (*Saccharomyce cerevisiae* numbering) are indicated in the box, and the length of the intron is presented over the insertional position.

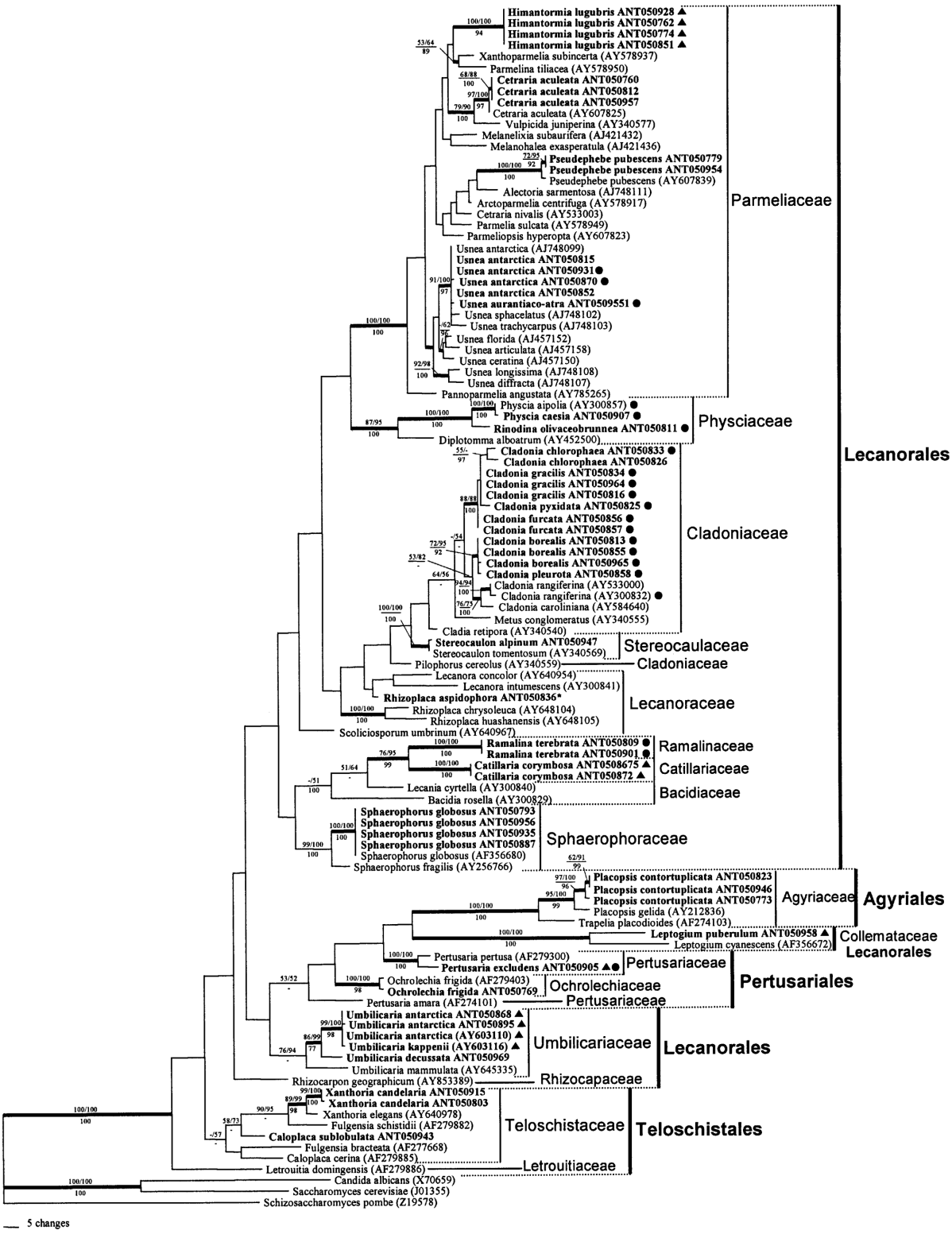

Fig. 2. The most parsimonious tree of the lichen-forming fungi derived from nuclear large subunit ribosomal RNA gene sequences. Branches maintained in three different analyses (MP, NJ, and B-MCMCMC analyses) are presented by bold lines. Numbers above branches that are before the slash are MP bootstrap proportions and those that are after the slash are NJ bootstrap proportions. Values below branches are B-MCMCMC posterior probabilities. One Ascomycetes species, *Schizosaccharomyces pombe* (Z19578), was used as an outgroup. Antarctic lichen-forming fungi are indicated by bold type. Black triangle (▲) represents the species known to be endemic to Antarctica. The presence of introns in the LSU rDNA is indicated with black circles (●).

olivaceobrunnea, *Usnea antarctica*, and *Usnea aurantiacoatra*. Size variation among samples of the same species was observed in *C. chlorophaea*, *C. gracilis*, and *U. antarctica*. These results implied the presence of introns or multiple insertions in the rDNA regions. From the sequence analysis of LSU rDNA, it was revealed that they contained introns of 59–268 bp in four nucleotide positions (Fig. 1). In the case of *C. chlorophaea* ANT050833, *C. gracilis* ANT050816, and *C. pyxidata* ANT050825, the presence of approximately 260 bp introns could explain the size difference from intron-missing 1.5-kb PCR products. However, the presence of introns in the LSU rDNA was not enough to explain the PCR size difference in most sequences, implying additional introns or multiple insertions in the 18S and/or ITS rDNA regions. Among samples with large PCR products, introns were not found in the LSU rDNA of *P. pubescens*. Small introns of 59 bp were recovered from LSU rDNA of *Ramalina terebrata* ANT050809 and ANT050901.

Phylogenetic Analysis

The aligned dataset of 107 sequences was composed of 794 nucleotide sites after excluding ambiguously aligned sites. Among them, 447 sites were constant, 82 sites were variable but non-informative, and 265 sites were parsimony-informative. The likelihood parameters of the Bayesian analysis had the following average values: base frequencies freqA=0.2143, freqC=0.2628, freqG=0.3281, and freqT=0.1947; rate matrix R[A-C]=1, R[A-G]=2.6188, R[A-T]=1, R[C-G]=1, R[C-T]=7.3981, and R[G-T]=1; and gamma distribution shape parameter α=0.4616 and proportion of invariable sites (I)=0.3215.

From the heuristic search based on parsimony criterion, one most-parsimonious tree (TL=1538 steps, CI=0.375, RI=0.751) was retrieved (Fig. 2). Lineages that were supported by high bootstrap values in the parsimony analysis were recovered in the neighbor-joining and Bayesian analyses. Branches supported in three different analyses are presented by bold lines in the tree (Fig. 2). In this tree, the order *Agyrales* formed a monophyletic group. The orders *Pertusariales* and *Teloschistales* formed paraphyletic groups with low bootstrap supports. The relationships were not maintained in the Bayesian tree (data not shown). The order *Lecanorales* formed a polyphyletic group, as proposed by Reeb *et al*. [31]. The Antarctic members of *Lecanorales* were distributed in the *Catillariaceae*, *Cladoniaceae*, *Collemataceae*, *Lecanoraceae*, *Parmeliaceae*, *Physciaceae*, *Ramalinaceae*, *Sphaerophoraceae*, *Stereocaulaceae*, and *Umbilicariaceae* clades.

Stereocaulon alpinum from the Antarctic was closely related to *Stereocaulon tormentosum* from Sweden. Sequences of the 28S rDNA region (nucleotide positions 1–927, *S. cerevisiae* numbering) of the two species had only one base difference. *Physcia caesia* from Antarctic was closely related to *Physcia aipolia* from Sweden. They showed 1 base difference in the exon regions of 28S rDNA (nucleotide positions 1–947, *S. cerevisiae* numbering). The two sequences contained introns between nucleotide positions 911 and 912 (*S. cerevisiae* numbering). Intron sequences were different at 3 nucleotide sites among 203 nucleotide sites. *Usnea aurantiacoatra* was not distinguished from *U. antarctica* in the phylogenies reconstructed by parsimony, distance, and Bayesian methods. *Usnea aurantiacoatra* showed 1–2 bp difference from *U. antarctica*, 4 bp difference from *U. sphacelatus*, and 7 bp difference from *U. trachycarpus*. Introns found between nucleotide positions 808 and 809 (*S. cerevisiae* numbering) were shared by two *U. antarctica* samples and *U. aurantiacoatra*. Intron sequences were identical except for 1-bp insertion or deletion. *Cladonia* species from the Antarctic were divided into two groups. *Cladonia chlorophaea*, *C. gracilis*, *C. pyxidata*, and *C. furcata* formed a monophyletic group with 88% bootstrap supports by parsimony and distance analyses, and 100% posterior probability by the Bayesian analysis. *Cladonia borealis* and *C. pleurota* formed a monophyletic group with 72% and 95% bootstrap supports by parsimony and distance analyses, and 92% posterior probability by the Bayesian analysis. Among fifteen *Cladonia* sequences included in the analyses, twelve contained introns between nucleotide positions 929 and 930 (*S. cerevisiae* numbering). The intron-containing sequences did not form a monophyletic group.

Among six species known to be endemic to Antarctica (Table 1, marked with a bold letter and *), *Catillaria corymbosa*, *Himantormia lugubris*, *Leptogium puberulum*, and *Rhizoplaca aspidophora* formed unique and independent lineages. *Pertusaria excludens* was closely related to *Pertusaria pertusa* from Germany. They showed 98.62% sequence similarity in LSU partial sequences (11 bases difference among 798 sites). The Antarctic species *P. excludens* was distinguished from *P. pertusa* in that it contained an intron between nucleotide positions 929 and 930 (*S. cerevisiae* numbering). *Umbilicaria antarctica* and *Umbilicaria kappenii*, which are known to be endemic to Antarctica, formed a monophyletic lineage with high bootstrap support (MP 99% and NJ 100%). Among four LSU rDNA sequences of *U. antarctica* and *U. kappenii*, only one or two nucleotide positions were different. These results support the previous research by Ott *et al*. [26], who proposed that *U. kappenii* should be reduced to synonymy with *U. antarctica* based on the phylogenetic analyses.

DISCUSSION

From the PCR amplification of rDNA regions encompassing the 3' domain of 18S, ITS1, 5.8S, and ITS2, and the 5' domain of 28S rDNA, size variations in PCR products were observed. In some cases, the size difference could be

explained by the presence of introns in the LSU rDNA regions. However, the presence of introns in the LSU rDNA was not enough to explain the size difference in most cases (Table 1 and Fig. 1). These results imply that additional introns or multiple insertions are present in the DNA regions, sequences of which were not determined in this study. Intron-possessing sequences are distributed in *Cladoniaceae*, *Physciaceae*, *Parmeliaceae*, *Pertusariaceae*, and *Ramalinaceae* (Fig. 2). The intron insertional position and size of introns were variable among introns of different clades (black circles in Fig. 2), implying that they had independent origins. However, intron sequences of the same clade were homologous, and the phylogenies of intron sequences of *Cladonia* species were consistent with the LSU rDNA phylogeny (data not shown). These results imply that introns of *Cladonia* species might have a single origin, and some sequences lost introns during evolution. The heterogeneity of intron presence/absence in rRNA genes of lichen fungi has been reported in Ascomycota [3], among different species of the genus *Cladonia* [5], and among *Physcia* species [34]. The explanation by recent origin and frequent insertion/deletion events of introns were proposed in those studies and it is consistent with the results obtained in this study.

The lichen species analyzed in this study were distributed among 4 orders of the *Lecanoromycetes*, *viz*, *Agyriales*, *Lecanorales*, *Pertusariales*, and *Teloschistales*. Species of *Lecanorales* were classified in 10 families, *Catillariaceae*, *Cladoniaceae*, *Collemataceae*, *Lecanoraceae*, *Parmeliaceae*, *Physciaceae*, *Ramalinaceae*, *Sphaerophoraceae*, *Stereocaulaceae*, and *Umbilicariaceae*. In the revised classification system proposed by Miadlikowska *et al*. [21], these families are classified in 5 orders, *Lecanorales* (*Cladoniaceae*, *Lecanoraceae*, *Parmeliaceae*, *Ramalinaceae*, *Sphaerophoraceae*, *Stereocaulaceae*), *Peltigerales* (*Collemataceae*), *Rhizocarpales* (*Catillariaceae*), *Teloschistales* (*Physciaceae*), and *Umbilicariales* (*Umbilicariaceae*). *Ochrolechiaceae* was merged to *Pertusariaceae* in the classification system by Miadlikowska *et al.* [21]. When the system was applied for the lichen species included in this study, species diversity was highest in *Lecanorales*, containing 9 genera and 15 species classified in 6 families. *Teloschistales* contained 4 genera and 4 species in 2 families. *Pertusariales* contained 2 genera and 2 species in a single family. *Umbilicariales* contained 1 genus and 3 species. *Agyriales*, *Peltigerales*, and *Rhizocarpales* contained single species, respectively.

Close relationships between lichen species from Antarctica and Europe were revealed in *Physia* and *Stereocaulon*. *Physcia caesia* and *Physcia aipolia* were collected in the Antarctic and Sweden, respectively. They contained very similar sequences in the large subunit rDNA region and shared very similar intron sequences. Both of the species are known to be circumpolar species and have similar morphological and chemical characteristics [39]. *Stereocaulon alpinum* and *Stereocaulon tomentosum* were collected in the Antarctic and Sweden, respectively. They contained very similar large subunit rDNA sequences. In the molecular phylogenetic study of the genus *Stereocaulon* based on the ITS and β-tubulin gene sequences [11], *S. alpinum* collected in Italy, Argentina, and Austria formed polyphyletic groups and one of the lineages showed close relationship with the monophyletic group of *S. tomentosum*. *Stereocaulon alpinum* is known as a widely distributed species, exhibiting much morphological variation and having an unclear species boundary [11]. From these results, it is concluded that delineating the monophyletic group and defining the species boundary are prerequisites to discussing geographical distribution and evolution. *Cladonia* and *Usnea* showed species diversification among Antarctic species. The *Cladonia* species included in this study were separated into two monophyletic groups. *Cladonia chlorophaea*, *C. gracilis*, and *C. pyxidata* formed a monophyletic group and shared similar LSU rDNA sequences. *Cladonia borealis*, *C. furcata*, and *C. pleurota* formed a second monophyletic group and shared similar LSU rDNA sequences. The grouping of two monophyletic lineages was supported by the phylogenetic study based on the ITS and β-tubulin sequences [35]. *Usnea antarctica* and *U. aurantiacoatra* were not clearly distinguished in this study (Fig. 2). This result was supported by ITS and RPB1 sequence analyses [2, 33], in which the two species were intermixed. *Usnea sphacelatus* and *Usnea trachycarpus*, which were grouped with *U. antarctica* and *U. aurantiacoatra* in the *Neuropogon* group, have been collected in the Antarctic and neighboring regions. These morphologically highly variable species appeared to form polyphyletic groups, and most of the morphological characteristics that have been used to delineate species were not conserved in the same phylogenetic lineage [33]. Considering the complicated relationships among Antarctic *Usnea* species, it is suggested that development of highly informative phylogenetic markers such as mitochondrial genome sequences, fingerprint markers including AFLP and ISSR, and ultrastructural characteristics are required to define species boundary clearly.

Phylogenetic relationships of six lichen species that are known to be endemic to Antarctica were analyzed in this study. Most of them formed unique lineages distinguished from lineages of other continents. *Pertusaria excludens*, which has close relatives from other continent, was differentiated by significant sequence difference and intron possession. Although all of the related species were not included in this study, it is proposed that many of the lichen species from Antarctica have unique origins. The understanding on the origin and the evolution of Antarctic lichen species would be improved by phylogenetic studies including lichen species from neighboring continents including Australia, South America, and Southern Africa.

In this study, the phylogenetic information of 50 lichen-forming fungi from King George Island, Antarctica, was

provided. Although the relationships of many Antarctic lichen-forming fungi were somewhat ambiguous, this study is the first comprehensive molecular phylogenetic study on the diversity of lichen-forming fungi in King George Island. Among them, some formed unique lineages and some were closely related to the lichens from other continents. Although this study has limits in diversity of reference species and in sequence information compared with the big scope of taxonomic groups, it will provide important information for further studies on the evolution of the Antarctic lichen species.

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