

# Diversity of endophytic fungi associated with bryophyte in the maritime Antarctic (King George Island)

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**Abstract** Bryophytes comprise one of the richest microfungus microhabitats in the Antarctic environment. The maritime Antarctic is very vulnerable to rapid environmental change caused by global warming. The aim of this study was to investigate the importance of bryophytes as a microhabitat for fungal species in the maritime Antarctic by surveying endophytic fungal diversity from several bryophytes including *Andreaea* sp., *Barbilophozia hatcheri*, *Chorisodontium aciphyllum*, *Polytrichum alpinum*, *Polytrichum strictum*, *Sanionia uncinata*, and *Warnstorfia sarmentosa*. We collected 13 bryophyte samples at four localities on Barton Peninsula, King George Island. In total, 31 endophytic fungi morphotypes were isolated from bryophyte tissues by a thorough surface sterilization method. Using internal transcribed spacer sequence analysis, 16 endophytic fungal strains belonging to Ascomycota (12), Basidiomycota (1), Oomycota (1), and Zygomycota (2) phyla were obtained. Our results suggest the presence of a diverse range of fungal species even in a very limited area, and those bryophytes play an important

role in conserving fungal diversity in this harsh environment. Growth rate measurements at a wide range of temperatures confirmed that most of the fungal strains were both mesophilic and psychrotolerant. This is the first report of endophytic fungi in Antarctic moss tissue by fluorescence in situ hybridization.

**Keywords** Antarctic bryophyte · Biodiversity · Fungal endophytes · FISH · The maritime Antarctic

## Introduction

Antarctica, considered an extreme environment, is characterized by high-stress and high-disturbance conditions (Pugh 1980) that are amplified by low temperatures, high aridity, high incidence of ultraviolet radiation, low and sporadic availability of nutrients, and strong thermal excursions, particularly at the microhabitat level (Vishniac 1993). King George Island is the largest of the South Shetland Islands belonging to the maritime Antarctic zone (Lee et al. 2008), where the ameliorating effect of the ocean produces a milder climate (Kanda and Komárková 1997). King George Island is dominated by a diversity of bryophyte and lichen species with only two vascular plant species (Øvstedal and Lewis-Smith 2002). Microbial diversity and activity are also affected by extreme conditions, and it is generally accepted that microbial species richness is more restricted in such environments than in temperate regions (Wynn-Williams 1996).

The Southern Ocean represents a major barrier to the colonization and distribution of new species to Antarctica. Terrestrial organism propagules arrive in the Antarctic almost exclusively via wind dispersal, animals, or humans (Ellis-Evans and Walton 1990). This geographical isolation and the

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relatively low anthropogenic impact offer ideal conditions within which to study the fungal population composition in different substrates because airborne spores that could generate fungal flora in isolation experiments are relatively rare and the expected diversity is low when compared with temperate or tropical climates (Möller and Dreyfuss 1996).

In the past, mycological studies conducted in Antarctic regions have been generally restricted to soil (Tubaki and Asano 1965; Boyd et al. 1966; Heal et al. 1967; Bailey and Wynn-Williams 1982) or freshwater habitats (Ellis-Evans 1985; Montemartini-Corte 1991), and only plants, mosses, and other organic substrates have been used as sources for the isolation of Antarctic microfungi (Pugh and Allsopp 1982; Fletcher et al. 1985; Gamundi and Spinedi 1988; Caretta and Frate 1990; Baublis et al. 1991; Onofri et al. 1994; Hoshino et al. 1999; Bradner et al. 2000). The study of fungi in Antarctica is related to host distribution such as birds, invertebrates, and vegetation, with the latter consisting of bryophyte and lichen communities (Tosi et al. 2002) and focused on species that live in nonvascular plants. These include bryophytes (i.e., mosses, liverworts, and hornworts), which are functionally important producers (Upson et al. 2007; Hoshino et al. 2009; U'ren et al. 2010; Siciński et al. 2011; Zhang et al. 2013). Samples collected in mid-Victoria Land during 1988/1998 indicate that microfungi occur most frequently in mosses and the soil underneath them (Caretta and Frate 1990; Caretta et al. 1994). Mosses are a suitable substrate for the growth of some nematode-trapping fungi of the genus *Arthrobotrys* (Caretta et al. 1994). Within the Antarctic environment, mosses are one of the richest microfungi microhabitats, particularly in regard to indigenous psychrophilic species (Tosi et al. 2002).

Associations between mosses and fungi were long thought to be uncommon and rare (Grasso and Scheirer

1981). Although interactions between bryophilous ascomycetes and bryophytes have been investigated by Döbbeler (1997), endophyte and bryophyte interactions have remained largely unstudied (Davey and Currah 2006).

In this study, we isolated fungal endophytes from Antarctic bryophytes and conducted a phylogenetic study. Moreover, we tested the growth rate of the endophytic fungi isolated at a range of temperatures. Finally, we attempted to detect fungal endophyte hyphae in *Polytrichum strictum* using selective staining and in-tube fluorescence in situ hybridization (FISH) coupled with confocal laser scanning microscopy (CLSM).

## Materials and methods

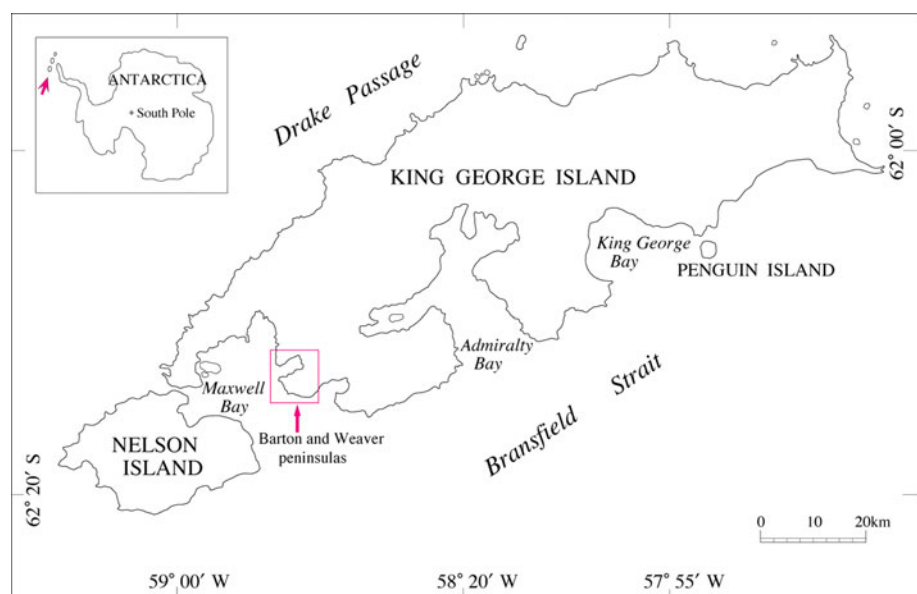
### Sample collection

We analyzed 13 bryophytes belonging to seven species collected from different locations in the maritime Antarctic, King George Island (Fig. 1; Table 1). Bryophyte samples devoid of apparent fungal infection were collected under aseptic conditions into sterile polyethylene tubes and preserved at 4 °C.

### Fungal endophytic isolation

Isolation of the internal fungus portion was performed as previously described by Li et al. (2007). Briefly, moss fragments were washed for 3 h in streaming water and immersed in 75 % ethanol for 1 min, in 2 % sodium hypochlorite for 3 min, and then in 75 % ethanol for 30 s. Each fragment was then gently rinsed with sterilized

**Fig. 1** Study area on Barton Peninsula and Weaver Peninsula, King George Island. (Color figure online)



**Table 1** Endophytic fungi isolated from the Antarctic bryophyte samples used in this study

| Endophytic fungi | Most closely related fungi (GenBank accession No.) | Similarity (%) | Bryophyte (bryophyte sample No.)     | Area, GPS   |
|------------------|--|----------------|--------------------------------------|---|
| EFOMIA 01        | Uncultured Clathrospira (HQ212305)                 | 97             | <i>Andreaea</i> sp. (1)              | Barton Peninsula (Penguin Village), S 62°14'11.1", W 58°46'16.6"  |
| EFOMIA 02        | <i>Lecytophora hoffmannii</i> (AB231012)           | 100            | <i>Andreaea</i> sp. (2)              | Barton Peninsula (Penguin Village), S 62°14'11.1", W 58°46'16.6"  |
| EFOMIA 03        | Uncultured ectomycorrhizal fungus (EU645617)       | 99             | <i>Warnstorfia sarmentosa</i> (3)    | Barton Peninsula, S 62°14'31.3", W 58°43'42.3"                    |
|                  |  |                | <i>P. strictum</i> (4)               | Barton Peninsula, S 62°14'31.3", W 58°43'42.3"                    |
|                  |  |                | <i>P. alpinum</i> (7)                | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
|                  |  |                | <i>Sanionia uncinata</i> (11)        | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 04        | Fungal sp. (HM123665)                              | 100            | <i>Barbilophozia hatcheri</i> (5)    | Barton Peninsula, S 62°14'27.3", W 58°44'36.2"                    |
| EFOMIA 05        | <i>Massarina rubi</i> (AF383963)                   | 96             | <i>Chorisodontium aciphyllum</i> (6) | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 06        | Uncultured Ascomycete (AM901846)                   | 98             | <i>C. aciphyllum</i> (6)             | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 07        | Uncultured fungus (FN610881)                       | 97             | <i>P. strictum</i> (4)               | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 08        | <i>Geomyces</i> sp. (JX171173)                     | 99             | <i>P. strictum</i> (4)               | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 09        | <i>Mortierella parvispora</i> (EU484297)           | 97             | <i>P. strictum</i> (4)               | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 10        | Fungal sp. (EU240043)                              | 98             | <i>P. strictum</i> (4)               | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 11        | Uncultured Ascomycota (FJ475721)                   | 98             | <i>P. strictum</i> (8)               | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 12        | <i>Coniochaeta ligniaria</i> (AY198390)            | 99             | <i>Andreaea</i> sp. (9)              | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 13        | <i>Pythium</i> sp. (AB299389)                      | 99             | <i>P. alpinum</i> (10)               | Barton Peninsula, S 62°14'31.3", W 58°43'42.3"                    |
| EFOMIA 14        | Uncultured fungus (FJ820588)                       | 99             | <i>S. uncinata</i> (11)              | Barton Peninsula (Mt. Kwan-ak Peak), S 62°13'31.0", W 58°46'41.4" |
| EFOMIA 15        | <i>Antarctomyces psychrotrophicus</i> (AM489755)   | 100            | <i>P. strictum</i> (12)              | Barton Peninsula (Penguin Village), S 62°14'11.1", W 58°46'16.6"  |
| EFOMIA 16        | Uncultured Coniochaetaaceae (FJ552932)             | 98             | <i>C. aciphyllum</i> (13)            | Barton Peninsula (Penguin Village), S 62°14'11.1", W 58°46'16.6"  |

distilled water, which was subsequently analyzed by polymerase chain reaction to check for fungal contamination of the thallus surface. Sterilized samples were then dried with sterile paper towels, after which the rinsed fragments were dried with sterile filter papers and then plated on potato dextrose agar with 0.01 % streptomycin and incubated at 15 °C. Fungi growing from each fragment were isolated from pure cultures, purified on 2 % MY solid medium (Malt Extract Broth; BD Difco, Sparks, MD, USA), and then deposited in the Korea Lichen and Allied Bioresources Center (KOLABIC), Korea Lichen Research Institute (KoLRI), Sunchon National University.

DNA extraction, amplification, sequencing, and phylogeny analysis

All isolates were grouped into different morphospecies based on the following morphological characteristics: colony color, texture, and growth rate on MY solid medium. All isolates were subjected to molecular identification. Fungal DNA extraction was performed using a DNeasy<sup>®</sup> Plant Mini Kit according to the manufacturer's protocols (Qiagen, Hilden, Germany). The internal transcribed spacer (ITS) region of the rDNA gene was amplified with the universal primers ITS4 (5'-TCCGCTTATTGATATGC-3') and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al. 1990). The amplified DNA was then concentrated and purified using a PCR quick-spin<sup>™</sup> PCR Product Purification Kit (INTRON Biotechnology, Seongnam, Korea). For the species-level identification by rDNA gene sequencing, the consensus sequence was aligned with all related species sequences retrieved from the GenBank database. The consensus sequences were then deposited into GenBank under the accession numbers HQ335293–HQ335308. Phylogenetic analysis was performed using the ITS region sequences of the endophytic fungi retrieved from the GenBank database (Supplementary Table 1) and the 16 endophytes isolated in this study. Sequence alignment was conducted in BioEdit, and a phylogenetic tree was generated by the neighbor-joining (NJ), minimum evolution (ME), and maximum likelihood (ML) analysis methods performed in MEGA 5.0 (Tamura et al. 2011) with the number of bootstrap trials set to 1,000.

Temperature preference

Temperature preference was tested using 15 representative fungal isolates. Samples were inoculated on MY medium plates (90 × 15 mm diameter) and incubated at 4 ± 1, 10 ± 1, 15 ± 1, 25 ± 1, and 30 ± 1 °C. Fungal growth was recorded daily for 1 month, and the mean growth rate was expressed in millimeter per day. All analyses were conducted in triplicate.

Selective fungal staining

Sterilized *P. strictum* (sample no. 4) were directly embedded in Tissue-Tek OCT compound (Sakura Fintek, Torrance, CA, USA), which prevents tissue damage. The samples were then rapidly frozen at –22 °C and cut into 8 µm-thick sections on a cryomicrotome (Microm HM 520, Labcon, Germany). The cryosections were then directly placed into a 1.5 ml microcentrifuge tube, after which they were cleared with 10 % KOH for 10 min at 90 °C and washed three times in deionized water. The sections were then treated with H<sub>2</sub>O<sub>2</sub> for 3 min and 2 % HCl for 4 min, after which they were stained with 0.5 % trypan blue for 12 h at 50 °C. Finally, the sections were placed on a regular microscopic glass slide and immediately mounted with 50 % glycerol and cover slipped.

Fixation

The endophytic fungal strain EFOMIA 03 cultured in MY liquid medium and sterilized *P. strictum* thalli were fixed with freshly prepared 3.7 % paraformaldehyde solution in PBS for 12 h at 4 °C and then washed three times in ice-cold phosphate-buffered saline (PBS) before storage at –20 °C in 1:1 ice-cold PBS/96 % ethanol.

Probe design and evaluation

All 28S rDNA fungal endophyte sequences were aligned with the 28S rDNA-specific probe sequences (Baschien et al. 2008), and an NME-2 of 13-mer probe was designed to detect fungal endophytes (5'-GTTTCAGCGGGTAT-3'). The NME-2 probes were labeled with fluorescein maleimide (Vector Laboratories, Burlingame, VT, USA) using a FastTag R Basic labeling kit (Vector Laboratories), and fluorescein labeling efficiency was confirmed using dot blot (Vector Laboratories). The fluorescein-labeled NME-2 probe was stored in the dark at –20 °C.

In-tube FISH

Small fragments (10–15 mm length) of fixed *P. strictum* and EFOMIA 03 were embedded in Tissue-Tek<sup>®</sup> O.C.T. Compound (Sakura Fintek), rapidly frozen at –22 °C, and cut into 8 µm-thick sections on a cryotome. The cryosections were then placed directly in a 1.5 ml microcentrifuge tube. To decrease the inherent autofluorescence signals, the cryosections were treated with 100 mM Tris–HCl buffer (pH 8) with 50 mM sodium borohydride (NaBH<sub>4</sub>) for 30 min. The samples were subsequently embedded in permeabilization buffer [1 × PBS, 1 % sodium dodecyl sulfate (SDS), pH 5.5] and lysing enzymes from *Trichoderma harzianum* (Sigma-Aldrich, St. Louis, MO, USA) at a final

concentration of 1 mg of lysing enzymes per milliliter of buffer. The samples were then incubated at 30 °C for 1 h, after which they were rinsed twice with ice-cold PBS. An ethanol series (50–70–85–96 % ethanol solutions, 3 min each) was carried out in the same tube. The samples were then quickly rinsed and washed for 3 min at room temperature. All hybridizations were performed at 42 °C for 12 h in a buffer containing 0.9 M NaCl, 20 mM Tris–HCl (pH 8), 0.01 % SDS, and 40 % formamide, and the fluorescent probes were gently mixed to give a final oligonucleotide concentration of 5 ng/μl. The hybridization buffer was immediately removed, and the samples were rinsed twice with prewarmed washing buffer [20 mM Tris–HCl (pH 8) and 31.4 mM NaCl] for 2–3 h at 42 °C. The washing buffer was then removed, and the samples were rinsed with ice-cold water. The section embedded with 1 × ISH blocking solution was subsequently warmed for 30 min at 37 °C and incubated with 10 μg of biotinylated anti-fluorescein of 1 × blocking solution ml for 30 min at room temperature, after which it was washed twice for 3 min each in 1 × blocking solution to remove the unconjugated biotinylated anti-fluorescein. Next, the section was incubated with fluorescein-avidin DCS solution (10 μg/ml in 1 × blocking solution) for 30 min at room temperature and washed twice for 5 min in 4 × SSC + 0.1 % Tween 20. The sections were finally placed on a regular glass microscope slide and quickly dried with compressed air. The sections were immediately mounted with Vectashield® HardSet™ Mounting Medium (Vector Laboratories), incubated for 12 h at 4 °C as recommended by the manufacturer, and observed by CLSM.

## Results

### Phylotype identification and phylogeny

In total, 31 morphologically distinct fungal endophytes were obtained from 13 different Antarctic bryophytes and grouped into 16 different fungal endophyte strains by ITS sequence analysis (Table 1). Of these endophytes, three strains (EFOMIA 02, 04, and 15) were 100 % homologous with fungi retrieved from the GenBank database. EFOMIA 02 was 100 % homologous with *Lecythophora hoffmannii* (GenBank: AB231012). EFOMIA 04 showed 100 % similarity to a fungal sp. (GenBank: HM123665), and EFOMIA 15 was 100 % homologous with *Antarctomyces psychrotrophicus* (GenBank: AM489755) isolated from the larvae of the introduced chironomid *Eretmoptera murphyi* on Signy Island.

Nine strains exhibited 98–99 % similarity to fungi retrieved from the GenBank database, most of which were homologous with uncultured fungi. The remaining strains

shared less than 97 % similarity to fungi retrieved from the GenBank database. Almost all of the endophytes were previously unidentified.

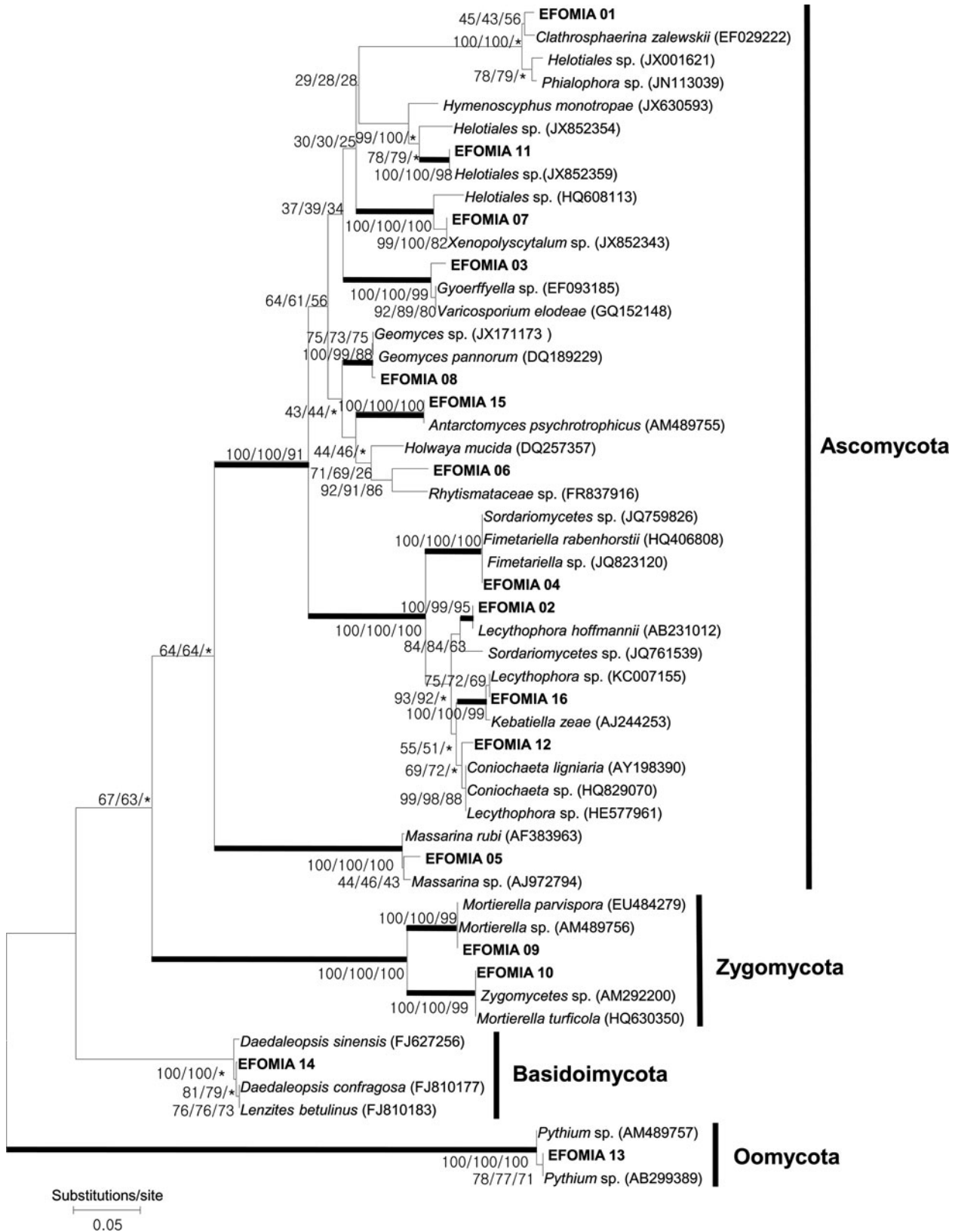
All of the endophytic isolates were grouped into 16 different strains and identified by molecular analysis. Phylogenetic analysis revealed that 12 strains belonged to Ascomycota, two strains to Zygomycota, and one strain each to Basidiomycota and Oomycota (Fig. 2).

### Temperature preference

The temperature preferences of 15 fungal endophytes are presented in Table 2. The strain EFOMIA 07 isolated from *P. strictum* (sample no. 4) was not included because of its very slow growth. Most of the fungal strains tested grew at the relatively low temperature of 4 °C, but EFOMIA 05 could only grow between 10 and 30 °C, indicating that it is not psychrotolerant. EFOMIA 03, 06, and 10 grew at temperatures in the range of 4–15 °C, while optimum EFOMIA 10 growth occurred at 15 °C. EFOMIA 08, 09, 11, and 15 grew at temperatures in the range of 4–25 °C, and EFOMIA 11 displayed different morphotypes under different temperatures. Specifically, its color changed from white to orange, and the texture of its hyphae appeared cottonless from 4 to 10 °C. However, the hyphae produced a dendritic pattern from 15 to 25 °C (data not shown). Optimum EFOMIA 09 and 10 growth occurred at 15 °C and they also grew well at 4 °C, indicating that they are likely psychrophilic. EFOMIA 01, 04, 13, and 14 grew at 4–30 °C, with EFOMIA 04 and 14 exhibiting very good growth at 25–30 °C, indicating that they are mesophilic. EFOMIA 01, 12, and 16 grew relatively well at 30 °C, and these three strains were relatively more thermotolerant than the other strains investigated.

### Selective staining and FISH of fungal hyphae on Antarctic moss

Selective staining of vertical *P. strictum* (sample no. 4) sections clearly highlighted EFOMIA 03 hyphae (blue) within moss tissues (Fig. 3a). The endophytic fungus hyphae extended across the cell wall of the moss, but were not observed in the cell-wall interspaces. Prior to in-tube FISH application, the NME-2-labeled probe concentrations of 200 ng/μl were confirmed by dot blot analysis (data not shown), indicating that complete labeling was accomplished. To check the applicability of the in-tube FISH method and ability of the NME-2 probe to detect EFOMIA 03, we conducted the in-tube FISH experiment on EFOMIA 03 directly. The EFOMIA 03 hyphae were detected based on the NME-2 probe FISH signal upon fluorescence microscopy (data not shown). Negative controls without FISH probes consistently exhibited no signals.



**Fig. 2** Phylogenetic tree of fungal taxa recovered from Antarctic bryophytes and their closest relatives. The tree was constructed based on the rDNA gene sequence (ITS1-5.8S-ITS2) by the NJ, ME, and

ML analysis methods in MEGA 5.0 with 1,000 bootstrap trials. The bootstrap values are presented near the corresponding branch (NJ/ME/ML), and those above 95 % are indicated by bold lines

**Table 2** Endophytic fungi temperature preferences

| Endophytic fungal isolate | Temperature (°C) |    |     |     |     |
|---------------------------|------------------|----|-----|-----|-----|
|                           | 4                | 10 | 15  | 25  | 30  |
| EFOMIA 01                 | +                | +  | +   | +   | +   |
| EFOMIA 02                 | +                | +  | +   | +   | ++  |
| EFOMIA 03                 | +                | +  | +   | -   | -   |
| EFOMIA 04                 | +                | +  | ++  | +++ | +++ |
| EFOMIA 05                 | -                | +  | +   | +   | +   |
| EFOMIA 06                 | +                | +  | +   | -   | -   |
| EFOMIA 08                 | +                | +  | +   | +   | -   |
| EFOMIA 09                 | ++               | ++ | +++ | +   | -   |
| EFOMIA 10                 | ++               | ++ | +++ | -   | -   |
| EFOMIA 11                 | +                | +  | +   | +   | -   |
| EFOMIA 12                 | +                | +  | ++  | ++  | ++  |
| EFOMIA 13                 | +                | +  | +   | ++  | +   |
| EFOMIA 14                 | +                | ++ | ++  | +++ | +++ |
| EFOMIA 15                 | +                | +  | ++  | +++ | -   |
| EFOMIA 16                 | +                | +  | +   | ++  | ++  |

(0–2: +, 2–4: ++, 4 ≤ :+++ , mm/day)

The moss cryosections were treated with sodium borohydride to lower the mosses' inherent autofluorescence. The optimal concentration of formamide for the NME-2 probe was 40 % and a hybridization temperature 42 °C, under which endophytic fungal hyphae could be detected based on FISH signals (Fig. 3b).

## Discussion

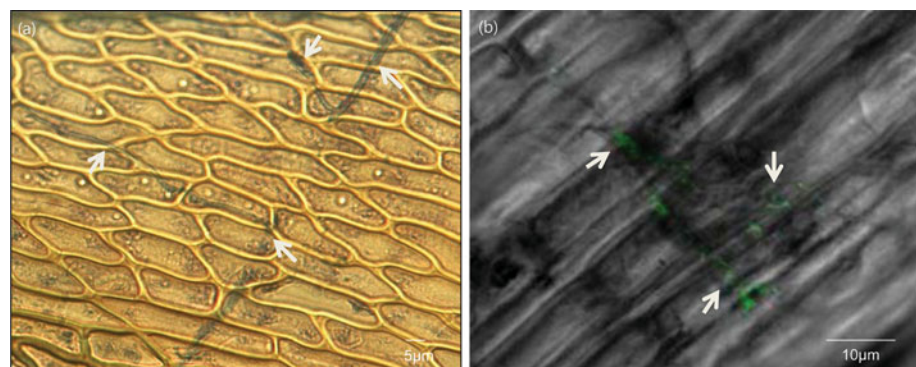
According to Ruisi et al. (2007), geographical isolation combined with environmental stress makes Antarctica a good place to search for new endemic fungal species. Studies of endophytic fungi in relatively extreme environments and from phylogenetically distinct plant lineages are promising sources of novel species and will contribute to our understanding of fungal diversity, the cryptic

ecology of microfungal symbionts, and the evolution of plant–fungal symbioses (Higgins et al. 2007).

In 2000, *Penicillium* sp., *Trichoderma* sp., and *Embelisia* sp. isolated from the Antarctic moss *Bryum argenteum* were collected from Southern Victoria Land (Bradner et al. 2000). Furthermore, in 2002, 19 Antarctic mosses comprising eight species were collected from 17 sites in Victoria Land; the moss samples primarily consisted of *Bryum pseudotriquetrum*, *Ceratodon purpureus*, and *Syntrichia princeps*. Overall, 120 fungi were isolated from the mosses comprising 18 genera and 28 Ascomycota, Basidiomycota, and Zygomycota species (Tosi et al. 2002). In 1996, 10 unidentified mosses were collected at Arctowski and Jubany on King George Island, and 31 endophytic fungi were identified from them. Overall, nine of the endophytic fungi were previously unidentified and assigned to the Ascomycota, while one belonged to the Zygomycota (Möller and Dreyfuss 1996).

In this study, 16 endophytic fungi were isolated from 13 bryophytes belonging to seven species collected at the Korea Antarctic Research Station on Barton Peninsula, King George Island. When compared with previous reports by Möller and Dreyfuss (1996), we identified a smaller number of endophytic fungal isolates, but several fungal species from one bryophyte species. Among them, two endophytes of the *Geomyces* and *Mortiella* genera were also found in our study. Moreover, the two genera of our endophytes exhibited similar temperature preferences to those of the same genus isolated in 1996. The isolated endophytic fungi fell into four phyla, Ascomycota, Zygomycota, Basidiomycota, and Oomycota, which suggests that the endophytic fungi isolated in this study are highly diverse on Barton Peninsula, with the moss *P. strictum* (sample no. 4) exhibiting the richest fungal diversity. Five endophytic fungi from the phyla Ascomycota and Zygomycota were harbored in the bryophyte. The harsh surface sterilization treatment used in this study can affect moss cells in thin thalli and subsequently damage fungal endophytes living in and/or between the cells. Thus, it is highly

**Fig. 3** Colonization of EFOMIA 03 hyphae within *P. strictum* (4) visualized by selective fungal hyphae staining and in-tube FISH. **a** The arrow indicates the EFOMIA 03 hyphae pattern. The hyphae (stained blue) extended across the moss cell wall but was not observed in the cell-wall spaces or the sectioned moss tissue. **b** In-tube FISH with *P. strictum* (4) using NME-2 probes (CLSM)



likely that the majority of isolates came from *P. strictum* (sample no. 4) because this species has thick tissues compared with those of the other bryophytes examined in this study.

Phylogenetic analysis revealed that EFOMIA 01, EFOMIA 07, and EFOMIA 11 belong to the phylum Ascomycota (Fig. 2). EFOMIA 01 was closely related to *Clathrosphaerina zalewskii* (GenBank: EF029222). EFOMIA 07 and EFOMIA 11 were closely related to *Xenopolyscytalum* sp. (GenBank: JX852343) and *Helotiales* sp. (GenBank: JX852359), respectively, both of which were isolated from the Antarctic region. These findings suggest that the fungal lineage might be adapted to polar regions. Specifically, EFOMIA 11 exhibited different phenotypes depending on growth temperature.

EFOMIA 03, found in several Antarctic mosses, also belonged to the phylum Ascomycota (Fig. 2) and was closely related to *Gyoerffyyella* sp. (GenBank: EF093185) and *Varicosporium elodeae* (GenBank: GQ152148). Therefore, EFOMIA 03 has a wide range of moss hosts and was the most common endophyte found in the study area. Moreover, EFOMIA 03 grew from 4 to 15 °C and was very sensitive to high temperatures. These findings suggest that the endophyte has adapted to Antarctic environmental conditions over a long time and may be sensitive to future temperature increases in the region.

EFOMIA 09, 10, and 15 were isolated from *P. strictum* (sample nos. 4 and 12). Phylogenetic analysis indicated that EFOMIA 09 and 10 belonged to the genus *Mortierella*, Zygomycota. EFOMIA 09 and 10 are psychrophilic fungi because they grew well at 4 °C. EFOMIA 15 was assigned to the Ascomycota (Fig. 2) and is similar to a fungus (GenBank: AM489755) previously isolated from an insect (*E. murphyi*) found on Signy Island, Antarctica (Fig. 2). Of the fungi isolated from the Antarctic mosses, many live in association with other organisms, nematodes, insects, and other fungi (Tosi et al. 2002).

Phylogenetic analysis indicated that EFOMIA 02, 04, 12, and 16 were closely related (Fig. 2) and belong to the class *Sordariomycetidae*. EFOMIA 02, 12, and 16 grew at 4–30 °C, and their optimum growth occurred at 30 °C. Their temperature preferences imply that they are tolerant to a wide range of temperatures and that they might have originated from a temperate zone and adapted to cold regions.

EFOMIA 08 closely matched *Geomyces pannorum* (GenBank: DQ189229) isolated from the mosses *B. pseudotriquetrum*, *C. purpureus*, *S. princeps*, *B. argentum*, *Schistidium antarctici*, and *Campylopus pyriformis* in Antarctic Victoria Land and one unidentified moss from King George Island (Möller and Dreyfuss 1996; Tosi et al. 2002). These results indicate that EFOMIA 08 is an endophytic fungus of Antarctic bryophytes. Furthermore,

the results of the EFOMIA 08 growth–temperature relationships were the same as those reported for *G. pannorum* var. *pannorum* (Tosi et al. 2002).

EFOMIA 05 and 06 were isolated from *C. aciphyllum* (sample no. 6) and belong to the phylum Ascomycota (Fig. 2). EFOMIA 05 is similar to *Massarina* sp. (GenBank: AJ972794) in the order *Pleosporales* (Table 1), which was isolated from boreal forest bryophytes (Kause-rud et al. 2008). EFOMIA 05 did not grow at 4 °C, but did so at 10–30 °C, which suggests that EFOMIA 05 might have adapted to the maritime Antarctic as an endophyte.

EFOMIA 14 is the only endophyte included in the phylum Basidiomycota and is similar to the plant pathogen *Daedaleopsis confragosa* (GenBank: FJ810177) (Fig. 2).

EFOMIA 13 was highly homologous with *Pythium* sp. (GenBank: AB299389), which was isolated from moss in the Arctic and Antarctic regions. *Pythium* (Oomycota) has been widely observed in Antarctica (Bridge and Denton 2007), and *Pythium tenue* has been reported from the Dry Valleys of Victoria Land in continental Antarctica (Knox and Paterson 1973).

Our investigation into the growth–temperature relationships revealed that most fungal isolates had a maximum growth rate within the range of 15–30 °C (Table 2) and that all endophytes, except for EFOMIA 05, grew at 4 °C. These results are similar to previously reported data from numerous studies on Antarctic fungi. Endophytic fungi isolated from Antarctic bryophytes are mesophilic and psychrotolerant (Bailey and Wynn-Williams 1982; Caretta and Frate 1990; Möller and Dreyfuss 1996).

Like tracheophytes, bryophytes harbor endophytic fungi that have no apparent detrimental effects on their hosts (Jakucs et al. 2003). Endophytic fungi in the roots of vascular plants, e.g., *Heteroconium chaetospora*, may imbue their host plants with resistance to pathogens such as *Verticillium dahliae* and *Plasmiodiophora brassicae* (Narisawa et al. 1998; Narisawa et al. 2002). The presence of fungi isolated from *Brachythecium rutabulum* allows this plant to grow over a much wider pH range than when the fungi are absent (During and Van Tooren 1990). Endophytic fungi may provide bryophyte hosts with greater tolerance to extreme pH or promote vegetative growth (Davey and Currah 2006). It is also possible that the presence of endophytic fungi in Antarctic bryophytes is one of the evolutionary strategies that enabled adaptation to the extreme environment of the region.

In this study, EFOMIA 01, 03, 04, 06, 07, 10, 11, 14, and 16 exhibited high levels of sequence similarity to uncultured fungi sequences (Table 1), which suggests that these endophytic fungi isolated from Antarctic bryophytes are novel species. We found that diverse endophytic fungi are associated with Antarctic bryophytes with a wide range of temperature preferences (Table 2). We have also developed a



novel FISH method to visualize the presence of endophytic fungi in Antarctic moss tissue. This method can be applied to monitor cold-adapted endophyte communities, which are highly vulnerable to increasing temperatures caused by global warming, in response to changes in the terrestrial ecosystem in the maritime Antarctic.

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