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# Endophytic and endolichenic fungal diversity in maritime Antarctica based on cultured material and their evolutionary position among *Dikarya*

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**Abstract:** Fungal endophytes comprise one of the most ubiquitous groups of plant symbionts. They live asymptomatically within vascular plants, bryophytes and also in close association with algal photobionts inside lichen thalli. While endophytic diversity in land plants has been well studied, their diversity in lichens and bryophytes are poorly understood. Here, we compare the endolichenic and endophytic fungal communities isolated from lichens and bryophytes in the Barton Peninsula, King George Island, Antarctica. A total of 93 fungal isolates were collected from lichens and bryophytes. In order to determine their identities and evolutionary relationships, DNA sequences of the nuclear internal transcribed spacer (ITS), nuclear ribosomal small subunit (nuSSU), nuclear large subunit (nuLSU), and mitochondrial SSU (mtSSU) rDNA were obtained and protein coding markers of the two largest subunit of RNA polymerase II (*RPB1* and *RPB2*) were generated. Multilocus phylogenetic analyses revealed that most of the fungal isolates were distributed in the following six classes in the phylum *Ascomycota*: *Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes, Pezizomycetes* and *Sordariomycetes*. For the first time we report the presence of subphylum *Mortierellomycotina* that may belong to an undescribed order in endophytic fungi. Taken together, our results imply that lichens and bryophytes provide similar niches and harbour a selection of these fungi, indicating generalists within the framework of evolutionary adaptation.

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## INTRODUCTION

The kingdom *Fungi* is comprised of a diverse range of organisms engaged in parasitic, saprophytic, symbiotic, endoparasitic and endophytic lifestyles (Mueller *et al.* 2004, Crespo *et al.* 2014). Current estimates for the global number of fungal species have risen from 2.2 million to as many as 3.8 million species (Hawksworth & Lucking 2017). Fungal endophytes are an ecologically diverse group, residing within plant tissues without causing any apparent symptoms of infection (Petrini 1991, Wilson 1995, Zhang *et al.* 2013). While most studies of fungal endophytes have focused on those species that live in vascular plants, endophytes also live in nonvascular plants including bryophytes (*i.e.*, mosses, liverworts, and hornworts), which are functionally important (Upson *et al.* 2007, Hoshino *et al.* 2009, U'Ren *et al.*, 2010, Sičinski *et al.* 2011, Zhang *et al.* 2013). These fungi affect the host in diverse ways: promoting greater tolerance to extreme pH, vegetative growth and resistance to pathogens (Narisawa *et al.* 2002, Davey and Currah, 2006). The habitat range of these fungi is also broad; they have been isolated from many different land plants from all terrestrial ecosystems ranging from the tropics to the Polar Regions (Arnold *et al.* 2009, Zhang *et al.* 2013, Yu *et al.* 2014). Lichen thalli can also harbour endolichenic fungi that are typically found as endophytes in plants (Girlanda *et al.* 1997, Li *et al.* 2007, Arnold *et al.* 2009, Kannangara *et al.* 2009, U'Ren *et al.* 2010). These fungi also live in close association with algal photobionts inside apparently healthy lichen thalli, forming persistent and symptomless infections.

The importance of these endolichenic fungi remains unknown. However, abundant endolichenic fungi are present within lichen thalli, and their presence is presumed to play an important ecological role, such as assisting lichen formation, growth and protecting against insect herbivores by producing bioactive substances (Li *et al.* 2007, Paranagama *et al.* 2007). In addition, fungal endophytes are a phylogenetically diverse group. The vast majority of known endophytic and endolichenic fungi belong to the phylum *Ascomycota*,

distributed among the *Arthoniomycetes, Sordariomycetes, Dothideomycetes, Lecanoromycetes, Leotiomycetes*, *Pezizomycetes*, and *Eurotiomycetes* (Arnold *et al.* 2009, Park *et al.* 2015).

King George Island is the largest island in the South Shetland Islands belonging to the maritime Antarctic zone where the climate is milder due to oceanic influences (Kanda & Komárková 1997, Sancho & Pintado 2004, Li *et al.* 2007). While invasive plant species have increased recently (Chown *et al.* 2012), only two native species of flowering plants, Antarctic hair grass (*Deschampsia antarctica*) and Antarctic pearlwort (*Colobanthus quitensis*), are found so far. Vegetation is predominantly made up of lichens and bryophytes, which are specially adapted to survive in this area. Furthermore, several performance indicators show that this region is an excellent habitat for lichens and bryophytes (Øvstedal & Lewis-Smith 2001, Kim *et al.* 2006, Green *et al.* 2012). In addition, several black meristematic fungi were reported in Antarctic lichens (Selbmann *et al*. 2013). Thus, we selected King George Island as a model to explore the diversity of endophytic and endolichenic fungal communities associated with lichens and bryophytes. Since they lack visible reproductive structures and other distinctive phenotypic traits for classification, DNA sequence-based sample identification is prerequisite for objective exploration of the species diversity. We gathered DNA sequences of the nuclear internal transcribed region (ITS), nuclear ribosomal short subunit (nuSSU) and large subunit (nuLSU), mitochondrial ribosomal short subunit (mtSSU) rDNA, and the two largest subunits of RNA polymerase II (*RPB1* and *RPB2*). Endolichenic fungi resemble fungal endophytes of plants in taxonomy, mode of transmission procedure, and evolutionary history (U'Ren *et al.* 2010). Then we pose the question: are endolichenic and endophytic fungal communities in Barton Peninsula, King George Island different from each other or overlapping, forming flexible symbiotic relationships in both bryophytes and lichens? And lastly, do these fungal communities flourish through a host-specific evolutionary process?

Here we compare the endolichenic fungi with endophytic fungal communities isolated from lichens and bryophytes at the same location on the Barton Peninsula, King George Island. Furthermore, in order to resolve the evolutionary relationships, we prepared a five-locus dataset (nuSSU, nuLSU, mtSSU, *RPB1* and *RPB2*) of selected taxa in phylum *Ascomycota*.

## MATERIALS AND METHODS

### Study site and lichen sample collection

Sixty-one lichen samples growing on soil, rock and moss were collected from the Barton Peninsula, King George Island located in the Antarctic (Fig. 1) and preserved at -20 °C in sterile polyethylene tubes to prevent contamination from airborne fungal species (Supplementary Table S1). Lichen samples were identified by macro- and micro-morphological characteristics and chemical contents according to the species definition as described by Øvstedal & Lewis-Smith (2001).

### Isolation of endolichenic fungi

Isolation of the internal fungi was performed as previously described by Li *et al.* (2007). The surface of the lichen thalli was

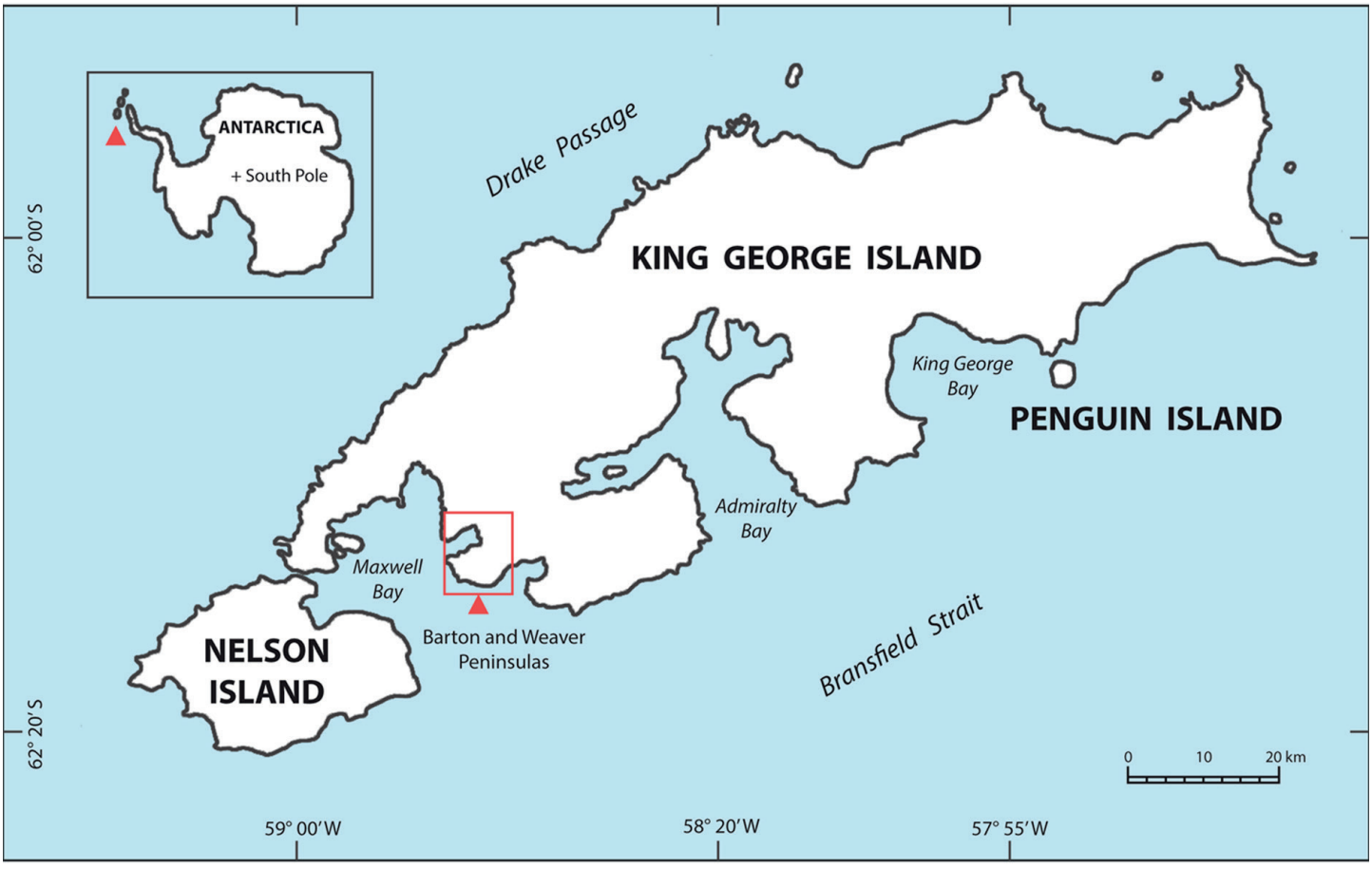


**Fig. 1.** Study area on Barton and Weaver Peninsula, King George Island in Antarctica (marked by red arrow).

cut into 0.5 $cm^2$ and the lichen thalli fragments were washed for 3 h in streaming water, then immersed in 75 % ethanol for 1 min, in 2 % sodium hypochlorite for 3 min and then in 75 % ethanol for 30 s. Finally, each fragment was gently rinsed with sterilised distilled water and the water was subsequently analysed by PCR to check for fungal contamination of the thalli surface. Sterilised samples were then dried with sterile paper towels and then plated on PDA with 0.01 % streptomycin and incubated at 15 °C. Fungi growing from each fragment were isolated into pure cultures on 2 % malt extract broth (ME, Difco, Sparks, USA) solid medium. All endolichenic fungi were grouped into different morphotypes based on the following culture phenotypic characteristics: colony colour, texture, growth rates and cell shape on ME solid medium. This is because endolichenic fungi rarely produce spores, therefore morphological features for identification is very limited (Choi *et al.* 1999). All fungal isolates were deposited at the Korea Lichen and Allied Bioresources Center (KOLABIC) at the Korea Lichen Research Institute (KoLRI) of Sunchon National University.

## DNA extraction, amplification and sequencing

Fungal DNA extraction was performed using a DNeasy Plant Mini Kit according to the manufacturer's protocols (Qiagen, Hilden, Germany). We amplified and sequenced the following six markers: nuSSU using primers NS1 (White *et al.* 1990) and NS24 (Gargas & Taylor 1992), nuLSU using primers LR0R (Rehner & Samuels 1994) and LR7 (Vilgalys & Hester 1990), mtSSU using mrSSU1 and mrSSU3R (Zoller *et al.* 1999), *RPB1* using RPB1-AFasc and RPB1-6R1asc2 (Hofstetter *et al.* 2007), *RPB2* using fRPB2-7cF and fRPB2-11aR (Liu *et al.* 1999), and ITS using ITS4 and ITS5 (White *et al.* 1990). In the case of endophytic fungal isolates from bryophytes living in King George Island, ITS sequences were used for analysis as described by Yu *et al.* (2014).

## Sequence assembly and multiple sequence alignments

Sequences were assembled and edited using the software CodonCode Aligner (CodonCode Corp., Dedham, MA, USA). Sequence identity was assessed using the mega-BLAST search function in GenBank (Sayers *et al.* 2011). We used the program MAFFT v. 7 (Katoh & Toh 2008) to align DNA sequences of 418 samples (Supplementary Table S1 and S3) for each gene region. For all six loci, we applied the G-INS-I alignment algorithm (recommended for sequences with global homology), '200PAM/K = 2' scoring matrix, and offset value = 0.0, with the remaining parameters set to default values. To improve the accuracy of the ITS and *RPB2* alignments for downstream OTU (operational taxonomic units) delimitation, only the newly generated sequences of endophytic and endolichenic fungi isolated from bryophytes and lichens on the King George Island were included. Multiple sequence alignments were performed in MAFFT using the same parameters as described above. The program Gblocks v. 0.91b (Talavera & Castresana 2007) was used to remove ambiguously aligned regions, using options for a "less stringent" selection on the Gblocks web server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) for subsequent phylogenetic analyses.

## OTU delimitation analyses

Since endophytic and endolichenic fungi lack visible reproductive structures and other distinctive phenotypic traits, and moreover, because morphology-based species circumscriptions have been shown to be inadequate for characterisation of species-level diversity (Arnold *et al.* 2009), we used the Automatic Barcode Gap Discovery method (ABGD; Puillandre *et al.* 2012) to circumscribe OTUs representing candidate species. ABGD employs a genetic distance-based approach to detect a 'barcode gap', separating OTUs based on non-overlapping values of intra- and interspecific genetic distances and is independent of any topology (Hebert *et al.* 2003, Puillandre *et al.* 2012). This method infers a model-based confidence limit for intraspecific divergence and then detects the barcode gap as a first significant gap beyond this limit to infer primary partitions. The primary data partitions are then recursively split to obtain finer partitions using the same approach until no further gaps can be detected (Puillandre *et al.* 2012). We used the ABGD web server (http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html) to identify barcode gaps in the ITS of endophytic and endolichenic fungi as well as the *RPB1* data matrix. Puillandre *et al.* (2012) suggested that implementing a $P_{max}$ value of 0.01 provides the most accurate estimate for the number of groups based on empirical comparisons of groupings inferred using ABGD with data from previous studies where species groups are well-characterised. Genetic distances were calculated using the JC69 model (default parameter), and other model parameters were set using default parameter values as follows, with the exception of the $P_{max}$ value: $P_{min}$ = 0.001, $P_{max}$ = 0.01, steps = 10and Nb bins = 20. We implemented a range of values for the gap width (X) between 0.1 and 1.5, to assess the consistency of the inferred groups under varying gap width values.

## Phylogenetic analyses

Individual gene topologies were reconstructed using the program RAxML v. 8.1.11 (Stamatakis 2006, Stamatakis *et al.* 2008), as implemented on the CIPRES Web Portal, with the GTR-GAMMA model as described below. Support values were assessed using the "rapid bootstrapping" option with 1 000 replicates. We compared individual gene topologies to identify conflicting nodes, statistically supported (*i.e.* ≥ 70 % bootstrap). Incongruence among clades with bootstrap values < 70 % was considered statistically insignificant (Divakar *et al.* 2012, Wiens 1998). Without evidence of conflicting evolutionary histories, independent markers were combined to achieve maximum phylogenetic resolution and support. Two concatenated datasets were prepared: a two-gene (nuSSU and nuLSU) dataset of 362 samples representing *Dikarya* and member of *Mortierellales*, and a five-gene (nuSSU, nuLSU, mtSSU, *RPB1* and *RPB2*) dataset of 150 samples representing major groups of *Ascomycota*. As ITS data were impossible to align across *Dikarya* and *Zygomycota*, this locus was excluded from the concatenated dataset. In order to evaluate the phylogenetic relation of two samples (EFOMIA09 and EFOMIA10) grouping with *Mortierellomycotina* an additional two gene larger dataset published in Wagner *et al.* (2013) was used.

The ML analyses of all the three concatenated data sets were performed in RAxML with the GTR-GAMMA model, a parameter (Γ) for rate heterogeneity among sites and without a parameter for estimating the proportion of invariable sites. We used locus-specific model partitions treating all loci as separate partitions, and evaluated nodal support using 1 000 bootstrap pseudoreplicates. An alternative partition strategy was inferred via PartitionFinder v. 1.1.1 (Lanfear *et al.* 2012). The best-


**Fig. 2.** A total of 32 representative endophytic fungal cultures from 32 OTUs based on the *RBP2* gene sequences. The OTU number is in the upper left corner and the name of the fungus is on the bottom centre of the photographs. The endophytic fungi were cultured on potato dextrose agar media or malt-yeast extract media. The three endophytic fungi, EFOMIA09, EFOMIA10, and EFOMIA16, were cultured on PDA supplemented with 30 µg/mL of Rose Bengal to prohibit bacterial contamination.

fitting partition scheme was selected from a total of 16 initial pre-defined partitions, corresponding to the complete nuSSU region, the complete nuLSU region, the complete mtSSU region, the first, second and third codon positions of two coding region in the *RPB1*, two introns in the *RPB1*, two intronic regions in the *RPB1*, the first, second and third codon positions of the coding region in the *RPB2*, and an intron in the *RPB2*.

In order to validate the ability of ABGD to infer evolutionarily independent species-level lineages from ITS and *RPB2* sequences, we analysed sequence data from the nuclear and mitochondrial genomes within a phylogenetic framework to identify OTUs that exhibited genealogical exclusivity across independent loci (Avise & Ball 1990).

## RESULTS

### Endolichenic fungal isolation and OTU delimitation

A total of 61 endolichenic fungal isolates were collected from 45 Antarctic lichen samples. Among these, 21 lichen species were identified, belonging to 10 families: *Candelariaceae, Cladoniaceae, Lecanoraceae, Parmeliaceae, Physciaceae, Pilocarpaceae, Ramalinaceae, Sphaerophoraceae, Stereocaulaceae,* and *Teloschistaceae* (Supplementary Table S1). In addition, 32 endophytic fungal isolates were obtained, including 16 that have been previously described (Yu *et al.* 2014), were isolated from 13 bryophytes (Supplementary Table S1). Representatives of endolichenic and endophytic fungal isolates are shown in Fig. 2. The sample identities were confirmed by analyses of the ITS1-5.8S and ITS2 rDNA region (ITS region) sequences.

*Sequences*: Endolichenic and endophytic isolates were grouped into 33 OTU in ABGD analyses of the ITS region and 34 OTUs from analysing a single copy gene *RPB2* (Fig. 3 and Supplementary Table S1). Since results of both datasets were similar, only the cluster of the *RPB2* marker is shown in Fig. 3. Of these, only seven OTUs were closely related to known fungal species with higher than 97 % sequence similarity. They were identified as *Anthostomella leucospermi*, *Chaetomium globosum*, *Peziza varia*, *Phoma herbarum*, and *Phoma violacea* with 98 % sequence similarity cut-off (Supplementary Table S2), ABGD clustering and monophyletic relationship. For OTU validation, nuSSU, nuLSU and mtSSU loci exhibited significantly less variability than the ITS region, and the two protein coding markers *RPB1* and *RPB2*. The comparison between OTUs inferred from the ITS and *RPB2* sequences revealed high levels of genealogical concordance between the ITS and the protein coding markers. Relationships among OTUs are shown in maximum likelihood (ML) topology in Fig. 4 and Supplementary Fig. S1.

### Molecular phylogeny

A total of 508 sequences were newly generated for this study, including 73 ITS, 92 nuSSU, 92 nuLSU, 91 mtSSU, 72 *RPB1* and 88 *RPB2* sequences (Supplementary Table S1). The two gene (nuSSU and nuLSU) data matrix contained 362 taxa with 2 185 unambiguously aligned nucleotide positions (Supplementary Table S1 and S3). The five gene data matrix contained 150 taxa with 4 643 unambiguously aligned nucleotide positions (Supplementary Table S3). Topologies of single-locus analyses did not conflict and hence combined analyses were performed. The ML phylogeny estimated from the concatenated two-gene and five-gene data matrixes are depicted as a cartoon tree in Fig. 4 (full tree in Supplementary Fig. S1) and Fig. 5, respectively. Of the 93 isolates from the studied area, almost all were in phylum *Ascomycota*, two were in *Basidiomycota* and another two belonged to *Mortierellales* (*Mortierellomycotina*). In *Basidiomycota*, isolates clustered only in the order *Boletales* of *Agaricomycetes*. However, in *Ascomycota* they were spread throughout the tree. Within *Ascomycota*, the largest number of isolates grouped with *Leotiomycetes*, followed by *Sordariomycetes* and *Dothideomycetes*. Three isolates belonged to *Eurotiomycetes* whereas one isolate each were assigned to *Lecanoromycetes* and *Pezizomycetes*. All the OTUs discovered in ABGD analysis were found to be monophyletic in multilocus phylogenies.

## DISCUSSION

Lichens and bryophytes are important components of current ecosystems, particularly in the Antarctic King George Island. Many genera of fungi commonly found as endophytes also occur within asymptomatic lichens and bryophytes (Kannangara *et al.* 2009, U'Ren *et al.* 2010, U'Ren *et al.* 2012, Zhang *et al.* 2013, Yu *et al.* 2014). Endophytic fungi largely lack reproductive structures and other visible phenotypic features, therefore traditional morphology-based species circumscriptions have shown to be inadequate to objectively characterise species-level diversity in this group of fungi (Arnold *et al.* 2009, Wagner *et al.* 2013, Oono *et al.* 2014, Chen *et al.* 2015). Here we used multilocus DNA sequence data for accurate sample identification and applied the barcode gap detection approach (Puillandre *et al.* 2012) to objectively circumscribe candidate species of endophytic fungi.

In this study, we reveal the endolichenic and endophytic fungal diversity in dominant lichen and bryophyte species in the Barton Peninsula of King George Island. Sixty-one endolichenic fungal isolates (numbered ELXXXXXX) were successfully obtained from 44 lichen samples belonging to 21 lichen species. The isolation frequency and diversity of 61 endolichenic fungi were compared with their host lichen family. Interestingly, endolichenic fungal isolation frequency was not related with the diversity of host lichen species. Namely, the number of lichen species in *Parmeliaceae* and *Stereocaulaceae* was higher than in other families but the isolation frequency of endolichenic fungi was not significantly different among the families.

In ABGD analyses, we circumscribed 34 candidate species (OTUs) for the 93 samples isolated from common lichen and bryophytes species of Antarctic King George Island. The results of endophytic fungi isolated from bryophytes species have been published in our previous study and here we focus on the endolichenic fungi (Yu *et al.* 2014). Since the obtained sequences are from axenic cultures of isolated fungi, these could be used as reference sequences for identification of environmental and soil fungi and also for detection of cryptic species. The species-level OTUs detected in this study were numbered OTU1 to OTU34 (Fig. 3). It is interesting to note that most of the OTUs from the isolates of Antarctic King George Island represent undescribed species. Candidate species level OTU9 was the most common taxon in Antarctic King George Island, followed by OTU26 and OTU29 (Fig. 3).

Moreover, the candidate species-level OTUs numbers 1, 2, 15 and 19 were present in both lichen and bryophyte samples

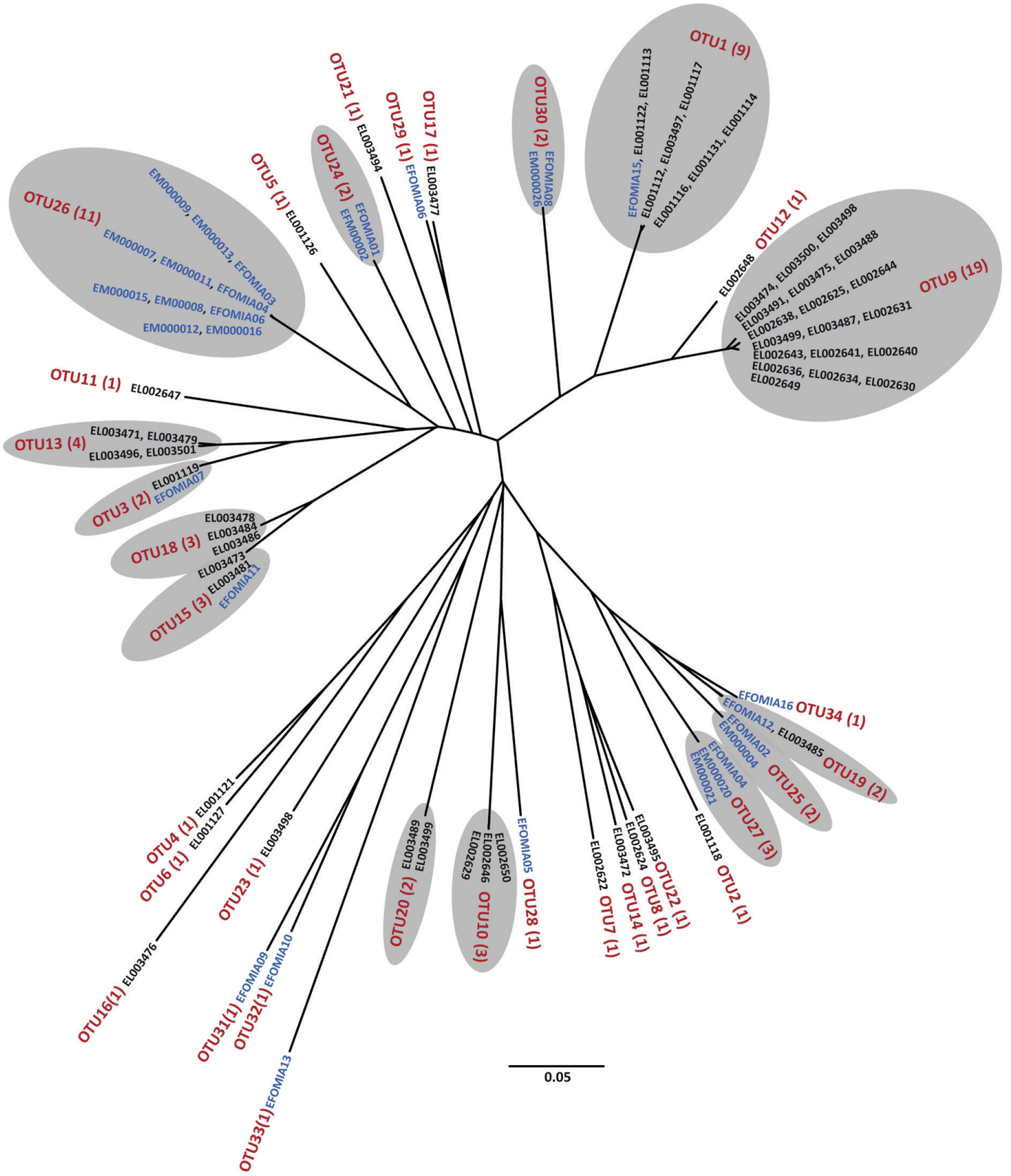


**Fig. 3.** Candidate species-level OTUs inferred from Automatic Barcode Gap Discovery (ABGD) analysis of the *RPB2* dataset. OTUs are numbered from 1 to 34 and the numbers in parentheses represents isolates clustered in each OTU. Endolichenic fungi isolated from lichen thalli are indicated in black, and endophytic fungi isolated from bryophytes are marked in blue.

collected from the same area, indicating generalists in the same ecological niches. Judging from the estimated total of 1.5 million (Hawksworth 1991) to as many as 5.1 million fungal species (Blackwell 2011, Rosling *et al.* 2011), our results demonstrate that also in the Antarctic, a high percentage of endophytic (endolichenic) fungal species remain undescribed. Similar results have been reported in tropical endophytes (Arnold & Lutzoni 2007). A detailed morphological study of the

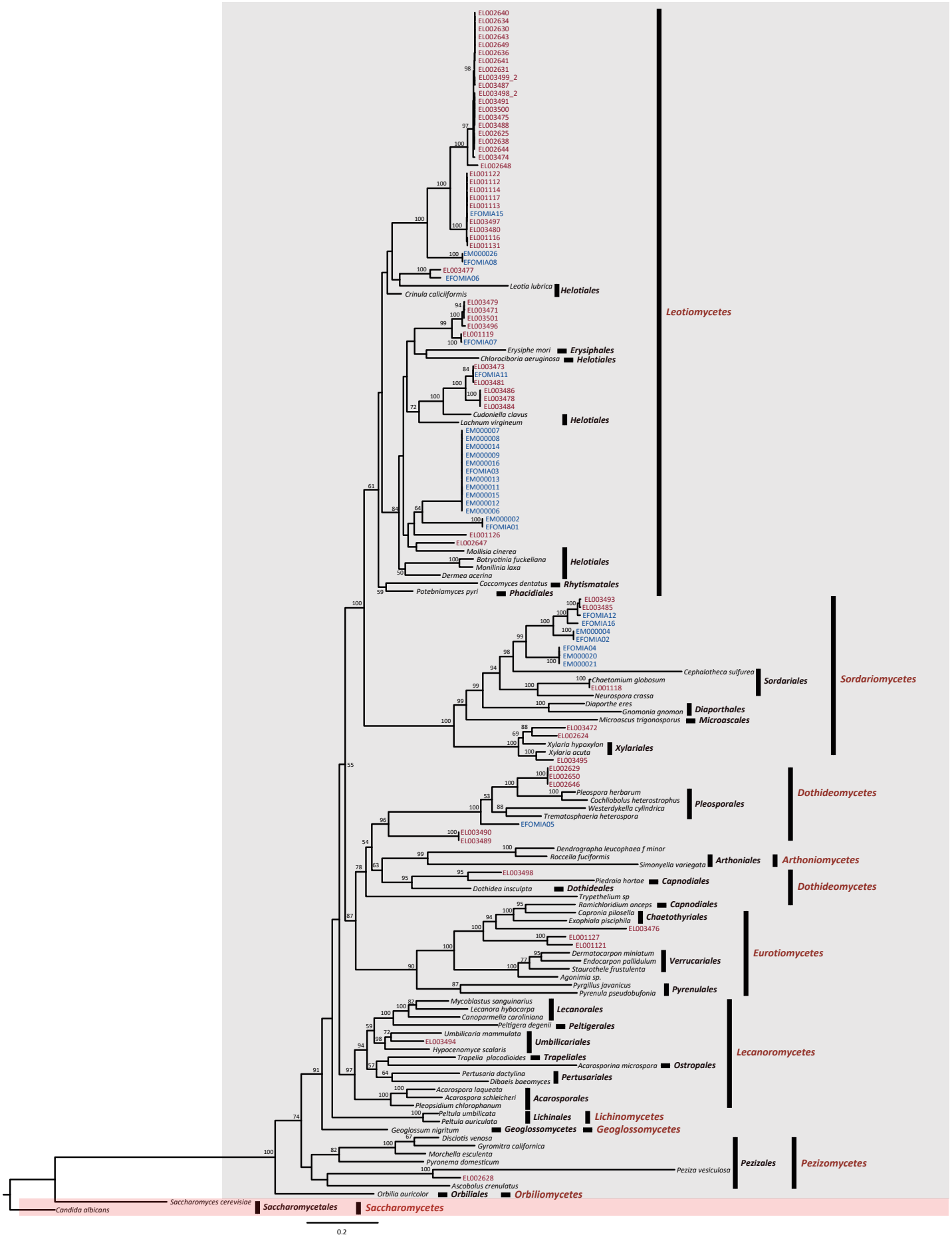


**Fig. 4.** Maximum Likelihood analysis based on concatenated five-locus dataset of small and large subunit (nuSSU and nuLSU) rDNA, mitochondrial small subunit (mtSSU) rDNA, and protein coding *RPB1* and *RPB2* markers of 62 taxa representing major lineages of *Ascomycota*. Two taxa of *Saccharomycetes* are used as outgroup. Node support ≥ 70 % is given on the branches. Taxon labels starting with "EL" in red represents endolichenic fungal isolates from lichen, and endophytic fungal isolates from bryophytes are labelled starting with "EF" or "EM" in blue.

cultures may aid in the formal description of these taxa in an integrative framework. However, developing robust hypotheses of species identification continues to be a 'work-in-progress' for examining species diversity in an unexplored area. Here, we assessed evolutionary independence of OTUs inferred from the ITS and *RPB2* markers, using mitochondrial and protein coding loci. Results from independent and concatenated datasets supported to large extent monophyly of OTUs inferred from ITS and *RPB2* sequences (Fig. 4 and Supplementary Fig. S1). This validation approach suggests that species level diversity assessed in the ABGD program likely provides a reasonable estimate of species diversity in the studied area. Moreover, the method implemented in our study for discovering species-level diversity based on OTUs is routinely used for organisms where morphological features are scarce or absent, such as bacteria (reviewed in Yarza *et al.* 2014). Similar to the previous studies (Arnold *et al.* 2009, U'Ren *et al.* 2012, Chen *et al.* 2015), our results demonstrate that most of the endophytic and endolichenic fungal isolates from the Antarctic King George Island belonged to classes *Dothideomycetes, Eurotiomycetes, Lecanoromycetes, Leotiomycetes, Pezizomycetes* and *Sordariomycetes* of *Ascomycota*. Only two samples belonged to *Basidiomycota* and another two to *Mortierellomycotina* (Fig. 4 and Supplementary Fig. S1). A detailed analysis of *Mortierellomycotina* including a larger dataset revealed that the two isolates EFOMIA09 and EFOMIA10, belong to a sister clade of *Mortierellales* (Supplementary Fig. S1, 2). This relationship was strongly supported (bootstrap 90 %). Currently with six genera belonging to the *Mortierellaceae* family, they are accepted members of *Mortierellomycotina*, and these fungi are commonly found as soil inhabiting saprobic organisms on decaying organic matter (Wagner *et al.* 2013). This is the first report of endophytic fungi in *Mortierellomycotina* and the sister relation of our two isolates to the order *Mortierellales* suggest that these samples may belong to an undescribed order within this group. A detailed morphological study of the cultures is needed to formally describe this lineage as a new order within *Mortierellomycotina*.

Using a five-locus dataset phylogeny, we establish the evolutionary relation of 89 Ascomycete endophytic and endolichenic fungi isolated from common bryophytes and lichen species of Antarctic King George Island. Our results demonstrate that these fungi were distributed in 10 orders in *Pezizomycotina* (Fig. 4). In accordance with previous studies, endophytic fungi isolated from different hosts and geographic regions such as arctic, boreal, temperate and tropical, were mostly grouped with *Pezizomycotina* (Arnold *et al.* 2009, Gazis *et al.* 2012, U'Ren *et al.* 2012, Chen *et al.* 2015). While *Leotiomycetes* and *Sordariomycetes* predominated the studied area, the *Pezizomycetes* and *Lecanoromycetes* were the least common, with only a single isolate each. Although, Antarctic endophyte (including endolichenic) assemblages were especially dominated by species belonging to the order *Helotiales* (*Leotiomycetes*), orders *Sordariales* and *Xylariales* (*Sordariomycetes*), these were least common in tropical and temperate areas (see *e.g.* Arnold & Lutzoni 2007). Indeed, *Lecanoromycetes* included the major lineages of lichen forming fungi (Miadlikowska *et al.* 2014, Jaklitsch *et al*. 2016). Our results demonstrate that of the 61 endolichenic fungal isolates from lichen thalli just one was grouped as *Umbilicariales* (*Lecanoromycetes*), suggesting no host specificity. These data are in agreement with a recent metabarcoding study, in which authors showed low specificity of endolichenic fungi segregated from lichen taxa growing in an alpine habitat (Fernández-Mendoza *et al.* 2017). While we establish the phylogenetic relations of most of the isolates in different orders of *Pezizomycotina*, the relationship of the isolates EL001127 and EL001121 in *Eurotiomycetes*, and EL003489 and EL003490 in *Dothideomycetes* remains unclear. These may belong to undescribed orders and a detailed study focusing especially on these two classes is needed in order to fix their systematic positions.

The host lichens are *Usnia antarctica*, *Cladonia borealis*, and *Psilolechia lucida*, mainly growing on moss mats in the island. Therefore, it is highly possible that some endophytes can facultatively select their hosts between lichens and bryophytes at a given location. This result is consistent with a previous study (Furbino *et al.* 2014). If it is true, we might rule out the hypothesis that in Antarctica, these endophytes colonise lichen thalli to obtain their carbon sources from photobionts (symbiotic algae). Rather, lichens could be serving a more important function as a shelter for the endophytes in extreme environmental conditions.

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## REFERENCES

Arnold AE, Lutzoni F (2007). Diversity and host range of foliar fungal endophytes: are tropical leaves biodiversity hotspots? *Ecology* **88**: 541–549.

Arnold AE, Miadlikowska J, Higgins KL, *et al*. (2009). A phylogenetic estimation of trophic transition networks for ascomycetous fungi: are lichens cradles of symbiotrophic fungal diversification? *Systematic Biology* **58**: 283–297.

Avise JC, Ball RM (1990). Principles of genealogical concordance in species concepts and biological taxonomy. *Oxford Surveys in Evolutionary Biology* **7**: 45–67.

Blackwell M (2011). The fungi: 1, 2, 3 ... 5.1 million species? *American Journal of Botany* **98**: 426–438.

Chen KH, Miadlikowska J, Molnar K, *et al*. (2015). Phylogenetic analyses of eurotiomycetous endophytes reveal their close affinities to *Chaetothyriales, Eurotiales*, and a new order - *Phaeomoniellales*. *Molecular Phylogenetics and Evolution* **85**: 117–130.

Choi YW, Hyde KK, Ho WH (1999). Single spore isolation of fungi. *Fungal Diversity* **3**: 29–38.

Chown SL, Huiskes AH, Gremmen NJ, *et al*. (2012). Continent-wide risk assessment for the establishment of nonindigenous species in Antarctica. *Proceedings of the National Academy of Science USA* **109**: 4938–4943.

Crespo A, Divakar PK, Lumbsch HT (2014). *Fungi: hyperdiversity closer to animals than to plants*. Sunderland, MA: Sinauer Associates, Inc.

Davey ML, Currah RS (2006). Interactions between mosses (Bryophyta) and fungi. *Canadian Journal of Botany* **84**: 1509–1519.

Divakar PK, Del-Prado R, Lumbsch HT, *et al*. (2012). Diversification of the newly recognized lichen-forming fungal lineage *Montanelia* (*Parmeliaceae, Ascomycota*) and its relation to key geological and climatic events. *American Journal of Botany* **99**: 2014–2026.

Furbino LE, Godinho VM, Santiago IF, *et al*. (2014). Diversity patterns, ecology and biological activities of fungal communities associated with the endemic macroalgae across the Antarctic Peninsula. *Microbial ecology* **67**: 775–787.

Gargas A, Taylor JW (1992). Polymerase chain reaction (PCR) primers for amplifying and sequencing nuclear 18S rDNA from lichenized fungi. *Mycologia* **84**: 589–592.

Gazis R, Miadlikowska J, Lutzoni F, *et al*. (2012). Culture-based study of endophytes associated with rubber trees in Peru reveals a new class of *Pezizomycotina*: *Xylonomycetes. Molecular Phylogenetics and Evolution* **65**: 294–304.

Girlanda M, Isocrono D, Bianco C, *et al*. (1997). Two foliose lichens as microfungal ecological niches. *Mycologia* **89**: 531–536.

Green TGA, Brabyn L, Beard C, *et al*. (2012). Extremely low lichen growth rates in Taylor Valley, Dry Valleys, continental Antarctica. *Polar Biology* **35**: 535–541.

Hawksworth DL (1991). The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycological Research* **95**: 641–655.

Hawksworth DL, Lucking R (2017). Fungal Diversity Revisited: 2.2 to 3.8 Million Species. *Microbiology Spectrum* **5**(4):FUNK-0052-2016. doi:10.1128/microbiolspec.FUNK-0052-2016.

Hebert PDN, Cywinska A, Ball SL (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **270**: 313–321.

Hofstetter V, Miadlikowska J, Kauff F, *et al*. (2007). Phylogenetic comparison of protein-coding versus ribosomal RNA-coding sequence data: a case study of the *Lecanoromycetes* (*Ascomycota*). *Molecular Phylogenetics and Evolution* **44**: 412–426.

Hoshino T, Xiao N, Tkachenko OB (2009). Cold adaptation in the phytopathogenic fungi causing snow molds. *Mycoscience* **50**: 26–38.

Kanda H, Komárková V (1997). *Antarctic terrestrial ecosystems.* Amsterdam: Elsevier.

Kannangara BT, Rajapaksha RS, Paranagama PA (2009). Nature and bioactivities of endolichenic fungi in *Pseudocyphellaria* sp., *Parmotrema* sp. and *Usnea* sp. at Hakgala montane forest in Sri Lanka. *Letters in Applied Microbiology* **48**: 203–209.

Katoh K, Toh H (2008). Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics* **9**: 286–298.

Kim JH, Ahn IY, Hong SG, *et al.* (2006). Lichen flora around the Korean Antarctic Scientific Station, King George Island, Antarctic. *Journal of Microbiology* **44**: 480–491.

Lanfear R, Calcott B, Ho SY, *et al*. (2012). Partitionfinder: combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution* **29**: 1695–1701.

Li WC, Zhou J, Guo SY, *et al*. (2007). Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. *Fungal Diversity* **25**: 69–80.

Liu YJ, Whelen S, Hall BD (1999). Phylogenetic relationships among Ascomycetes: evidence from an RNA polymerse II subunit. *Molecular Biology and Evolution* **16**: 1799–1808.

Mueller GM, Schmitt JP, Leacock PR, *et al*. (2004). Global diversity and distribution of macrofungi. *Biodiversity and Conservation* **16**: 37–48.

Narisawa K, Kawamata H, Currah RS, *et al*. (2002). Suppression of Verticillium wilt in eggplant by some fungal root endophytes. *European Journal of Plant Pathology* **108**: 103–109.

Oono R, Lutzoni F, Arnold AE, *et al*. (2014). Genetic variation in horizontally transmitted fungal endophytes of pine needles reveals population structure in cryptic species. *American Journal of Botany* **101**: 1362–1374.

Øvstedal DO, Lewis-Smith RI (2001). *Lichens of Antarctica and South Georgia: A Guide to their Identification and Ecology*. Cambridge, UK: Cambridge University Press.

Paranagama PA, Wijeratne EM, Burns AM, *et al*. (2007). Heptaketides from *Corynespora* sp. inhabiting the cavern beard lichen, *Usnea cavernosa*: first report of metabolites of an endolichenic fungus. *Journal of Natural Products* **70**: 1700–1705.

Park CH, Kim KM, Elvebakk A, et al. (2015). Algal and fungal diversity in Antarctic lichens. *The Journal of Eukaryotic Microbiology* **62**: 196-205.

Petrini O (1991). *Fungal endophytes of tree leaves*. Springer New York.

Puillandre N, Lambert A, Brouillet S, *et al*. (2012). ABGD, Automatic Barcode Gap Discovery for primary species delimitation. *Molecular Ecology* **21**: 1864–1877.

Rehner SA, Samuels GJ (1994). Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycological Research* **95**: 625–634.

Rosling A, Cox F, Cruz-Martinez K, *et al*. (2011). *Archaeorhizomycetes*: unearthing an ancient class of ubiquitous soil fungi. *Science* **333**: 876–879.

Sancho LG, Pintado A (2004). Evidence of high annual growth rate for lichens in the maritime Antarctic. *Polar Biology* **27**: 312–319.

Sayers WE, Barrett T, Benson DA, *et al*. (2011). Data resources of the National Center for Biotechnology Information. *Nucleic Acids Research* **39**: D38–D51.

Siciński J, Jażdżewski K, Broyer CD, *et al*. (2011). Admiralty bay benthos diversity - A census of a complex polar ecosystem. *Deep Sea Research Part II* **58**: 30–48.

Stamatakis A (2006). RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Stamatakis A, Hoover P, Rougemont J (2008). A rapid bootstrap algorithm for the RAxML Web servers. *Systematic Biology* **57**: 758–771.

Talavera G, Castresana J (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematic Biology* **56**: 564–577.

U'ren JM, Lutzoni F, Miadlikowska J, *et al*. (2010). Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. *Microbial Ecology* **60**: 340–353.

U'ren JM, Lutzoni F, Miadlikowska J, *et al*. (2012). Host and geographic structure of endophytic and endolichenic fungi at a continental scale. *American Journal of Botany* **99**: 898–914.

Upson R, Read DJ, Newsham KK (2007). Widespread association between the ericoid mycorrhizal fungus *Rhizoscyphus ericae* and a leafy liverwort in the maritime and sub-Antarctic. *The New phytologist* **176**: 460–471.

Vilgalys R, Hester M (1990). Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* **172**: 4238–4246.

Wagner L, Stielow B, Hoffmann K, *et al*. (2013). A comprehensive molecular phylogeny of the *Mortierellales* (*Mortierellomycotina*) based on nuclear ribosomal DNA. *Persoonia* **30**: 77–93.

White TJ, Bruns TD, Lee SB, *et al*. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics In: *PCR Protocols: a guide to methods and application* (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds.): 315–322. San Diego, California: Academic Press.

Wiens JJ (1998). Combining data sets with different phylogenetic histories. *Systematic Biology* **47**: 568–581.

Wilson D (1995). Endophyte: the evolution of a term, and clarification of its use and definition. *Oikos* **73**: 274–276.

Yarza P, Yilmaz P, Pruesse E, *et al*. (2014). Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature Reviews Microbiology* **12**: 635–645.

Yu NH, Kim JA, Jeong MH, *et al*. (2014). Diversity of endophytic fungi associated with bryophyte in the maritime Antarctic (King George Island). *Polar Biology* **37**: 27–36.

Zhang T, Zhang YQ, Liu HY, *et al*. (2013). Diversity and cold adaptation of culturable endophytic fungi from bryophytes in the fildes region, King George Island, maritime Antarctica. *FEMS Microbiology Letters* **341**: 52–61.

Zoller S, Scheidegger C, Sperisen C (1999). PCR primers for the amplification of mitochondrial small subunit ribosomal DNA of lichen-forming ascomycetes. *Lichenologist* **31**: 511–516.

**Supplementary Material: http://fuse-journal.org/**

**Table S1.** Endolichenic and endophytic fungal isolated from the Antartic lichen and moss samples.

**Table S2.** Blast search results from endolichenic fungal isolates using ITS region sequences.

**Table S3.** A total 324 taxa and the retrieved nuSSU, nuLSU, mtSSU, RPB1, and RPB2 sequences from GenBank.

**Fig. S1.** Maximum Likelihood analysis based on concatenated two-locus dataset of small and large subunit (nuSSU and nuLSU) rDNA of 272 taxa (2 and 10 ingroup taxa of *Dikarya* and *Mortierellomycotina*, respectively) and 1 outgroup taxon *Umbelopsis* as member of the *Mucorales*); representing major lineages. Node support equal and or above 70 % is given on the branches. Taxon labels starting with "EL" in red represents endolichenic fungal isolates from lichen thalli, while labels starting with "EF" or "EM" in blue indicate endophytic fungal isolates of bryophytes.

**Fig. S2.** Maximum Likelihood analysis of the *Mortierellomycotina* dataset published in Wagner *et al*. (2013), showing phylogenetic relation of the isolates EFOMIA09 and EFOMIA10. Node support equal and or above 70 % is given on the branches.