Diversity of microfungi in ornithogenic soils from Beaufort Island, continental Antarctic

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Abstract

This paper presents the results of a biodiversity study of microfungi in ornithogenic soils from Beaufort Island (Ross Sea, continental Antarctic). During the 2004/05 austral summer, we sampled a wide range of soil habitats from an abandoned penguin rookeries to examine the biodiversity of soil microfungi. Beaufort Island is predominantly ice and snow covered, isolated, difficult to access and known to have been visited only infrequently. Warcup's soil plating method was used for fungal cultivation. A total of ten fungal taxa were isolated, consisting of seven ascomycetes, two anamorphic fungi and one yeast. In terms of their thermal classes, a total of four psychrophilic, five psychrotolerant and 1 mesophilic fungi were isolated. *Thelebolus microsporus, Geomyces* sp. and *Thelebolus* sp. were the most common isolated fungi. Internal Transcribed Spacer (ITS) and 18S rDNA sequences were obtained from 17 fungal isolates, confirming their identification as *Thelebolus microsporus*, *Thelebolus* sp., *Phoma herbarum* and *Geomyces* sp.

Key words: Antarctica, soil fungi, biodiversity, ornithogenic

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Introduction

Antarctic microfungal diversity

Studies of microfungal diversity in Antarctic have generally focused on the continental (Sugiyama et al. 1967, Wicklow 1968, Atlas et al. 1978, Friedmann 1982, Martin 1988, Del Frate et Carretta 1990, Onofri et Tosi 1992, Moller et Gams 1993, Smith 1994, Azmi et Seppelt 1998, Cheryl et Seppelt 1999, Selbmann et al. 2005, Krishnan et al. 2011) rather than the maritime Antarctic (Dennis 1968, Gray et al. 1982, Pugh et Allsopp 1982, Weinstein et al. 1997). Studies have taken place both in relatively easily accessible and more benign locations (Azmi et Seppelt 1990, Cheryl et Seppelt 1999) and in more environmentally challenging areas such as the Victoria Land Dry Valleys (Friedmann et al. 1985, Cameron et al. 1971). The occurrence of fungi associated with human-associated habitats and artifacts, such as in historical huts, have been studied extensively by Tubaki (1961), Martin (1988), Blanchette (2000), Blanchette et al. (2004) and Held et al. (2005).

The indigenous fungi of Antarctica have been studied in soils (Kerry 1990a, b, Finotti 1993, Azmi et Seppelt 1998, Hughes et al. 2003, Krishnan et al. 2011), ice cores and permafrost (Gilichinsky et al. 2005), lake sediment (Sugiyama et al. 1967) and also in the air column (Marshall 1997). Airborne spores originating from South America have also been detected in the Antarctic Peninsula region (Marshall 1997). Sugiyama et al. (1967) reported *Aspergillus* spp. and *Penicillium* sp. as the only identifiable fungi from water, sediments and soils of Lake Vanda, with more isolates being recorded from sediments compared to water and soil samples. They also detected greater diversity of fungi in culture at 25°C compared to 10°C. Gilichinsky et al. (2005) isolated 40 fungal strains representing 12 different taxa from cryopegs of Don Juan Pond, including *Geomyces* sp., *Penicillium* spp., *Alternaria* sp., *Aureobasidium* sp. and *Verticillium* spp., again noting very little growth at 10°C. Tubaki (1968) highlighted that the ability to continue slow growth at low temperature (*i.e.* near 0°C) might be more ecologically relevant than rapid growth rates at higher optimum culture temperatures.

The relationship between vertebrate presence and microfungal diversity in Antarctica has been reported by Wicklow (1968), Kerry (1990a, b) and Azmi et Seppelt (1998). *Thelebolus* spp. and *Phoma* sp. have been recorded in such vertebrate associated soils, for example, from the Windmill Islands (Azmi et Seppelt 1998, Cheryl et Seppelt 1999, Alias et al. 2005), Vestfold Hills and MacRobertson Island (Kerry 1990a).

Seabird influence on Antarctic terrestrial ecosystems

Antarctica's terrestrial ecosystems are fragile and easily affected by human or animal activities (Chown et Gaston 2000, Selmi et Boulinier 2001, Frenot et al. 2005). Seabirds and marine mammals, such as penguins and seals, feed in the surrounding ocean and reproduce and rest on land. There, they deposit large quantities of excrement (guano) and other organic material such as feathers, eggs and carcasses, hence fertilizing the otherwise nutrient-poor terrestrial ecosystem (Allen et al. 1967, Smith 1985, Myrcha et al. 1985, Myrcha et Tatur 1991, Tatur 2002, Zmudczyńska et al. 2012). For instance, Tatur (2002) reported that the 30 - 50,000

pairs of *Pygoscelis penguins* nesting on the west shore of Admiralty Bay, King George Island, maritime Antarctic deposited about 6.35 tonnes of guano (dry mass equivalent) on the land daily. The runoff from penguin rookeries will fertilize the immediate surrounding area (Park et al. 2007), while strong winds will transport and redistribute fine particles of guano and volatilized ammonia to a wider area (Wodehouse et Parker 1981). The vegetation zonation around areas of vertebrate concentration can be very distinct (Smykla et al. 2007), and indicative of the degree of rookery impact.

Soil chemistry is also impacted by the vertebrate presence. Potassium and marine-derived salts have been detected (Rankin et Wolff 2000). Soils near penguin rookeries were also found to contain high concentrations of low molecular weight sugars and polyols (in the form of mono- and disaccharides) in areas characterized by visible algal growth (Roser et al. 1994). Within the occupied penguin rookeries soils can contain high levels of organic acids such as oxalic, acetic, propionic and succinic acids (Roser et al. 1994). The surface layer of soils of active penguin rookeries contains fresh and leached guano underlain by aluminium-iron phosphatic clay (Tatur et al. 1997). It is generally entirely devoid of vegetation because of toxic overmanuring and active trampling. In contrast, abandoned penguin rookery sites can progressively become covered by extensive vegetation (Smykla et al. 2007).

Beaufort Island

Beaufort Island, located in the Ross Sea, is mostly ice- or snow-covered, but includes a variety of ice-free terrains and habitats that support relatively diverse biota (Seppelt et al. 1999). The island is isolated, difficult to access, and is known to have been visited only infrequently. The earliest scientific report is that of Stonehouse (1966). Further ornithological studies were reported by Woehler (1993) and Woehler et Croxall (1996). The natural biota and ecosystem of Beaufort Island therefore provide an ideal reference for such systems in the Ross Sea region.

The island includes several relictual penguin rookeries, and areas are dominated by matted layers of pin feathers that are characteristic of molting sites (Seppelt et al. 1996, Emslie et al. 2007). The oldest known records for the Adélie penguin have been identified from here (Emslie et al. 2007), with the ages of some feathers and bones exceeding the range of radiocarbon dating (more than 44,000 years old).

In the absence of previous studies of the mycobiota of Beaufort Island our objectives were to isolate, obtain axenic cultures and identify microfungi from soils on the island, in particular focusing on the soils of relict areas of penguin activity. We include molecular phylogenetic analyses of the microfungi isolated using the Internal Transcribed Spacer (ITS) and 18S rDNA sequences.

Materials and Methods

Sampling Site - Beaufort Island

The study area was located in the northern part of Beaufort Island (76° 57' S, 166° 55' E). This site has a northerly aspect and is sheltered from the cold southerly winds, therefore experiencing relatively warm summer temperatures (*see* Fig. 1). The local microclimate, stability of substratum and supply of water from the nearby ice cliffs and snow fields provide conditions favourable for supporting a relatively diverse terrestrial biota. This site possesses the most extensive continuous stand of mosses yet known from the McMurdo Sound region. The field survey was carried out during the austral summer of 2004/05 JS. Twenty-three soil samples from 20 sites were obtained. The upper 10cm of soil were collected using a sterile spatula, placed in sterile polyethylene bags (Whirl-Pack), mixed thoroughly and frozen by reducing temperature over 48 h period from 1° to -20°C.

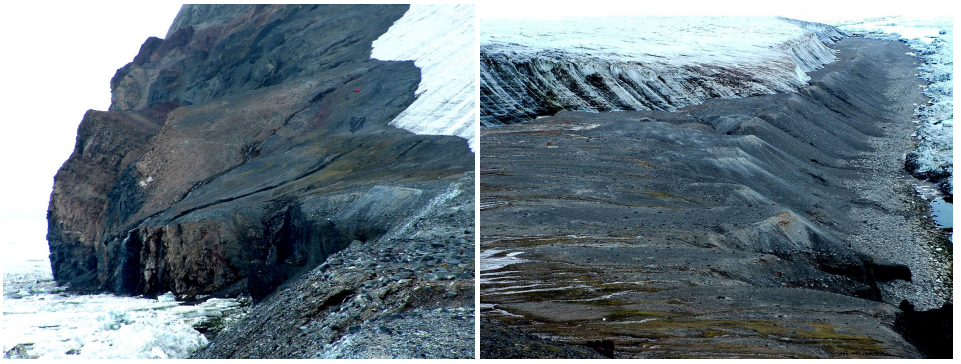

Fig. 1. Sampling sites on Beaufort Island.

Isolation of microfungi

Fungi were isolated from the soil samples using a modification of the soil plate method (Warcup 1950). Approximately 0.1g of soil was placed on the agar surface and distributed using 1 ml of sterile distilled water and rotating the plate. Five replicates were plated on isolation media obtained from OXOID (Potato dextrose agar – PDA) and incubated at 4°C or 25°C. Isolated fungi were categorized into standard thermal classes – psychrophilic fungi only growing at 4°C, psychrotolerant growing at both 4° and 25°C, and mesophilic growing only at 25°C. PDA was chosen as the growth media for its ability to encourage growth of many genera of fungi. All plates were examined daily to determine numbers of colonies and to avoid overgrowth of one fungal colony by another. Fungal isolates were sub-cultured for identification. Filamentous fungi were identified to species wherever possible, coupled with the aid of DNA sequencing techniques. Percentage of occurrence of fungi was determined by dividing the frequency of occurrence by the total number of replicates.

DNA extraction

DNA was extracted using a modification of the method of Jin *et al*. (2004). Briefly, homogenized mycelia were incubated in DNA extraction buffer (100mM Tris-HCl, 50mM EDTA, 500mM NaCl, 10mM â-mecaptoethanol [pH8.0], Proteinase-K (50 µg/mL) and SDS (200 g/L [pH 7.2]) at 65°C for 2 hrs, then cooled on ice before 5 M potassium acetate was added. The mixture was kept on ice for another 20 min and then centrifuged at 20,800 *g* for 10 min. The supernatant was recovered and purified twice with phenol: chloroform:isoamyl alcohol (PCIA) [25: 24:1]. The genomic DNA was precipitated with ice-cold absolute ethanol, then washed in 70% ethanol and resuspended in Tris-EDTA buffer [pH 8.0]. The genomic DNA was treated with RNase A (5 mg/ mL) at 65°C for 1 h and stored at -20°C. The genomic DNA was visualized on 1.0% (w/v) TBE agarose gel.

PCR, cloning and DNA sequencing

PCR amplification was performed in 15µL reactions that contained 0.2 µM forward and reverse primers (combination of ITS5, ITS2, ITS86 and ITS4 primers (White *et al*. 1990, Turenne *et al*. 1999) for the ITS1-5.8S-ITS2 region; EF4 and EF3 primers (Smith *et al*. 1999) for the 18S rDNA region), 0.2 mM dNTPs, 1x DyNAzyme™ II DNA Polymerase Reaction buffer, 2 U DyNAzyme™ II DNA Polymerase Recombinant Enzyme (Finnzymes; Finland), 5% DMSO and 10 ng of genomic DNA. PCR for the ITS1-5.8S-ITS2 region was performed in a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad Laboratories Inc.; USA) with an initial denaturation of 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 45-50°C for 40 s, and 72°C extension for 1 min, with final extension at 72°C for 15 min. PCR for the 18S rDNA region followed a similar programme but used 54°C as the annealing temperature. PCR products were visualized on 2% (w/v) TBE agarose gel. The PCR products were purified from the agarose gels using PureLink™ Quick Gel Extraction Kit (Invitrogen; USA) and then cloned into pGEM®-T Easy Vector (Promega; USA). White colonies were randomly selected and the presence of the ITS or 18S rDNA inserts were confirmed by PCR amplification. Colony PCR reaction (15µL) contained 0.2 µM M13 forward and reverse primers, 0.2 mM dNTPs, 1x DyNAzyme™ II DNA Polymerase Reaction buffer and 2 U DyNAzyme™ II DNA Polymerase Recombinant Enzyme (Finnzymes; Finland). Colony PCR was performed in a Bio-Rad MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc.; Hercules, USA) with an initial denaturation of 94°C for 10 min followed by 25 cycles of 94°C for 30 s, 60°C for 40 s, and 72°C extension for 1-2 min, with final extension at 72°C for 15 min. PCR products were visualized on 2% (w/v) TBE agarose gel. PCR products from the colony PCR were purified using the *AccuPrep*® PCR Purification Kit (Bioneer; South Korea). DNA sequencing was outsourced and performed by SolGent Incorporated Sequencing Services (Daejeon; South Korea) using M13 forward and reverse primers. Sequence chromatograms were checked for ambiguity and edited in Chromas 2.33 (Technelysium Pt. Ltd.; Australia), before being submitted to the GenBank database.

Sequence alignment

Multiple sequence alignment was performed separately for the ITS and 18S rDNA sequences using Clustal W in MEGA 5.2 (Tamura *et al*. 2011), which included also sequences from Basic Local Alignment Search Tool (BLAST) comparisons and other fungi reference sequences obtained from GenBank. Alignments were inspected, corrected and trimmed manually to achieve maximum homology.

Phylogenetic analysis

The best evolutionary model was chosen separately for the ITS and 18S rDNA aligned datasets using jModelTest 2.1.3 (Darriba *et al*. 2012) based on the corrected Akaike Information Criterion (AICc). Phylogenetic analyses to construct Neighbour Joining (Maximum likelihood distance) and Maximum Parsimony (heuristic search with 10 replicates of random sequence addition; unordered nucleotide changes with accelerated transformation (ACCTRAN); Tree Bisection Reconnection (TBR) algorithm for branch swapping) trees were performed with 1000 bootstrap replicates in PAUP 4.0b10 (Swofford 2002). Bayesian analyses were computed in MrBayes 3.1.2 (Ronquist et Huelsenbeck 2003) whereby trees with the highest posterior probabilities were sampled by Metropolis-coupled Markov Chain Monte Carlo (MCMCMC) from four chains of 1,000,000 generations at every 100 generations. The initial 25% of the trees were discarded as "burn-in" to ensure the chains reached stationarity. Only branches with over 70% bootstrap values or posterior probabilities were considered in all phylogenetic trees.

Results

No.	Species	4°C	25°C	Total frequency of occurrence (%)
1.	*Thelebolus microsporus*	12.5	-	**12.5**
2.	*Geomyces* sp.	2.33	8.43	**10.76**
3.	*Thelebolus* sp.	11.34	0.29	**11.63**
4.	Asco JBI8	-	9.59	**9.59**
5.	*Phoma herbarum*	8.43	0.87	**9.30**
6.	JBIsp5	2.91	4.94	**7.85**
7.	*Thelebolus globosus*	3.49	-	**3.49**
8.	Asco sp. 5	1.74	-	**1.74**
9.	White yeast	2.91	-	**3.49**
10.	Pink yeast	1.16	1.16	**2.41**

Table 1. Fungi isolated from relictual penguin rookery soils, Beaufort Island and their overall frequency of occurrence.

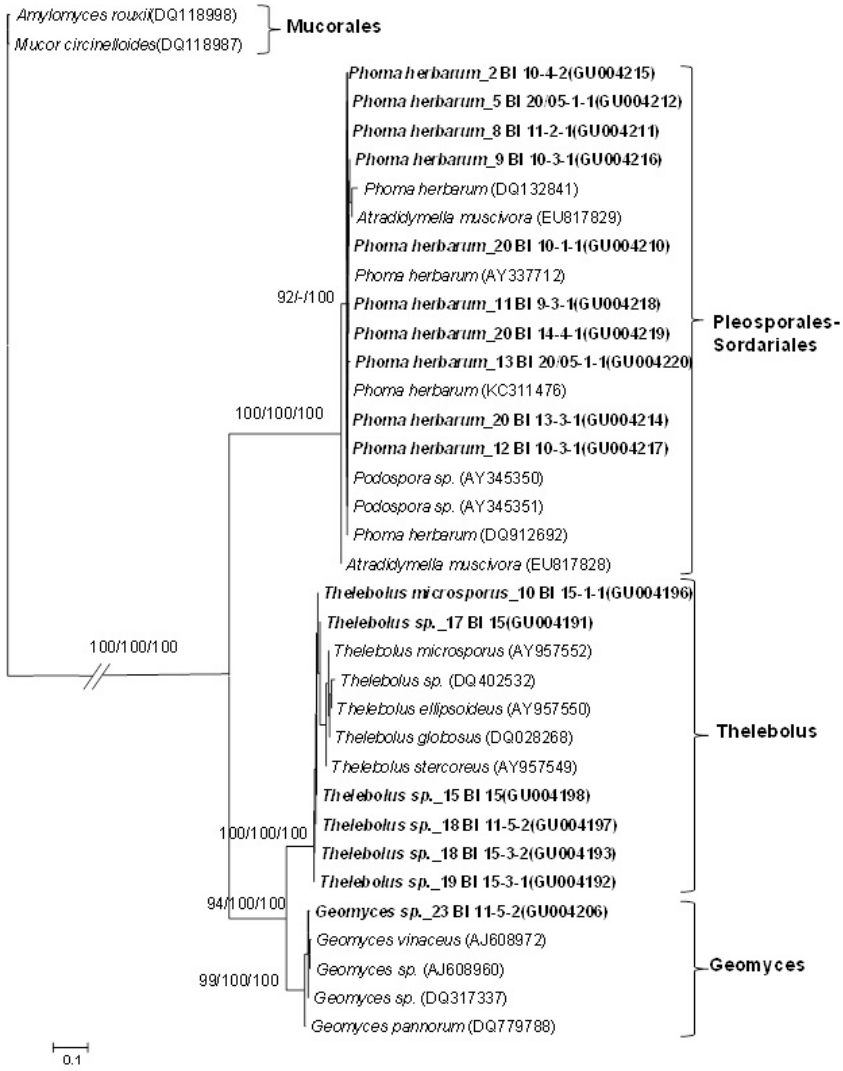

Fig. 2. Neighbour Joining phylogram of fungal isolates from Beaufort Island soil samples (in bold) using ITS1-5.8S-ITS2 sequences (564 bp). Tree is based on maximum likelihood distance (AICc best-fit model selected by jModelTest is TrN+G: gamma=0.8360; substitution rates *r*AC=1.0000, *r*AG=3.8962, *r*AT=1.0000, *r*CG= 1.0000, *r*CT=5.8151, *r*GT=1.0000; base frequencies *f*A=0.2591, *f*C=0.2248, *f*G=0.2214, *f*T=0.2947). Bootstrap values for Neighbour Joining, Maximum Parsimony and posterior probability values for Bayesian analysis are shown at the branches, respectively. Sequences from BLAST results and reference sequences were also included and the tree is rooted with Mucorales fungi. GenBank accession numbers of sequences are shown in parentheses.

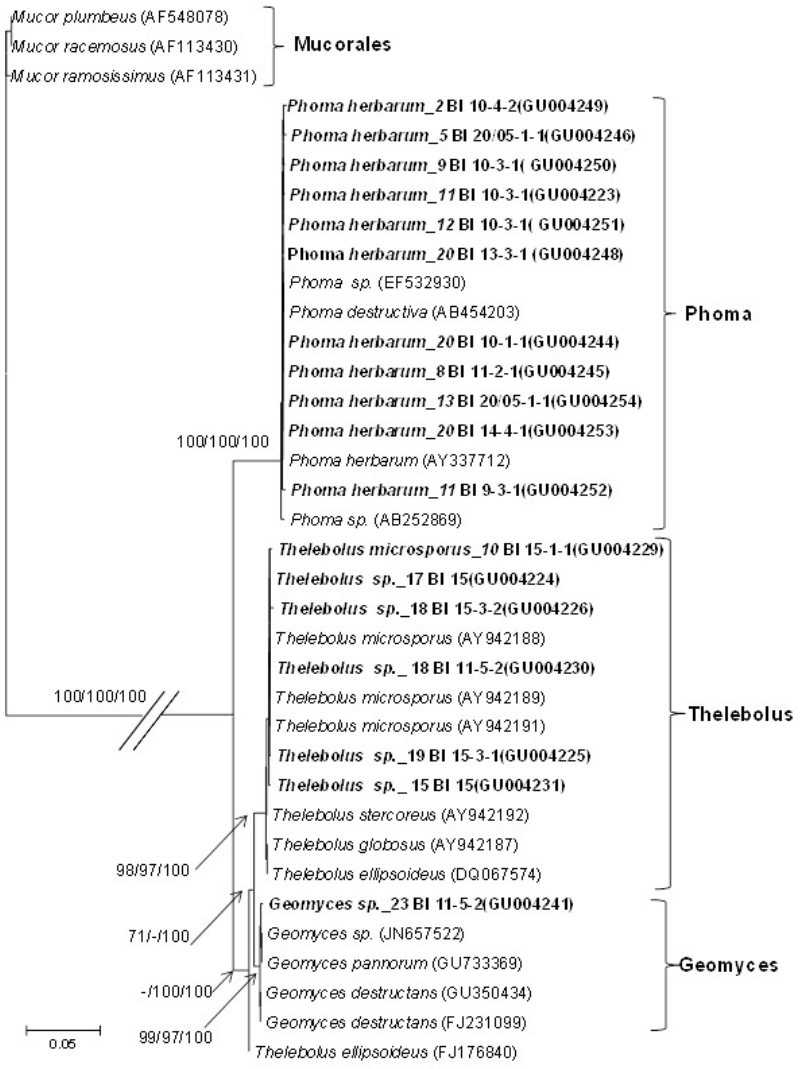

Fig. 3. Neighbour Joining phylogram of fungal isolates from Beaufort Island soil samples (in bold) constructed using 18 rDNA sequences (1561bp). Tree is based on maximum likelihood distance (AICc best-fit model selected by jModelTest is TIM1+I: rate=equal; I =0.6210, substitution rates *r*AC= 1.0000, *r*AG= 2.7871, *r*AT= 0.4272, *r*CG= 0.4272 , *r*CT= 6.6625, *r*GT=1.0000; base frequencies *f*A= 0.2629, *f*C= 0.2030, *f*G= 0.2602, *f*T= 0.2739). Bootstrap values for Neighbour Joining, Maximum Parsimony and posterior probability values for Bayesian analysis are shown at the branches, respectively. Sequences from BLAST search results and reference sequences are also included and the tree is rooted with Mucorales fungi. GenBank accession numbers of sequences are shown in parentheses.

Table 1 shows the frequency of occurrence of isolated fungi growing at the different temperatures of 4°C and 25°C. In total, ten taxa were isolated from relictual rookeries. The majority of these were ascomycetes (seven species), with two anamorphic fungi and one yeast. Phylogenetic analysis of the ITS and 18S rDNA sequences confirmed the isolates as *Thelebolus microsporus*, *Thelebolus* sp., *Phoma herbarum* and *Geomyces* sp. (*see* Fig. 2, 3). *Thelebolus microsporus*, *Thelebolus* sp. and Asco BI8. were the most commonly isolated fungi, with frequencies of occurrence of >10% (Table 1). Of the fungi obtained, four taxa grew only at 4°C, four at both 4 and 25°C, and one only at 25°C.

Discussion

Azmi et Seppelt (1998) reported only seven taxa isolated from penguin-associated soils from the Windmill Islands, but also noted high occurrence of sterile mycelia and *Mortierella* sp. In the present study, the mycoflora of Beaufort Island was dominated by ascomycetes. *Thelebolus microsporus* was found at various sites in the Windmill Islands region (Azmi et Seppelt 1998), but most frequently at sites under seabird influence. Similarly, where sites are strongly influenced by relictual penguin colonies on Beaufort Island, *T. microsporus* also occurred at a very high percentage.

Anamorphic fungi are known to be able to colonise extreme environments, and are also able to utilise toxic substances that are otherwise lethal to other groups of fungi (Thomas et Hill 1976, Jongmans *et al*. 1997). As the toxicity reduces, other groups of fungi such as ascomycetes can start to colonise an area, and the presence of hyphomycetes will proportionately reduce. Hyphomycetes are known to survive in xeric environments (Gunde-Cimerman *et al*. 2003), some can help degrade petroleum (Kirk et Gordon 1988), while others are pathogenic to rotifers and tardigrades in Antarctic lakes (McInnes 2003) or are nematophagous (Gray *et al.* 1982). The numbers of taxa of psychrophilic and psychrotolerant fungi recorded on Beaufort Island were 9 and 5, respectively. Robinson (2001) highlighted the predominance of psychrotolerance in the mycobiota of the Arctic and Antarctic noting that, although the most of fungi isolated from the Antarctic are able to grow at around 0°C, the temperature of substrata in some periods of the year can be much higher than the air temperature (*see* also Möller et Dreyfuss 1996).

Azmi et Seppelt (1998) observed that fungal diversity was low in guaniferous soil in the centre of active penguin colonies. In the present study, no fungi were isolated from active penguin rookeries where the soils were waterlogged with sea water and fresh penguin guano. This most likely is due to the high ammonia concentration in fresh guano (Legrand *et al.* 1998, Rankin et Wolff 2000), and also to the presence of acrylic acid, a microbial antagonist that is often detected in ornithogenic soils from active penguin rookeries (Fletcher *et al*. 1985, Roser *et al*. 1994).

Geomyces spp. are widely distributed in Antarctica (Fletcher *et al.* 1985, Kerry 1990a, b, Azmi et Seppelt 1998, Gilichinsky *et al.* 2005), occurring under varying environmental conditions (Kerry 1990a, b). Although capable of producing cellulase at 1°C (Hurst *et al.* 1983) and psychrotolerant, members of the genus are thought to be poor competitors (Ivarson 1974). Kerry (1990a, b) noted the absence or low frequency of occurrence *Geomyces pannorum* when other fungal species such as *Phoma herbarum* were present. In contrast, in the current study, the mycobiota was dominated by *Thelebolus* spp., *Geo-*

myces sp. and Asco JBI8. This suggests that the community observed here shows a clear signal of the founder effect. Although members of the genus *Geomyces* are no longer categorised as hyphomycetes, they are amongst the best-known examples of primary colonising microfungi (Ivarson 1974).

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