RESEARCH LETTER

Cold adaptation of fungi obtained from soil and lake sediment in the Skarvsnes ice-free area, Antarctica

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Abstract

A total of 71 isolates were collected from lake sediment and soil surrounding lakes in the Skarvsnes area, Antarctica. Based on ITS region sequence similarity, these isolates were classified to 10 genera. Twenty-three isolates were categorized as ascomycetous fungi from five genera (*Embellisia*, *Phoma*, *Geomyces*, *Tetracladium* or *Thelebolus*) and 48 isolates were categorized as basidiomycetous fungi in five genera (*Mrakia*, *Cryptococcus*, *Dioszegia*, *Rhodotorula* or *Leucosporidium*). Thirty-five percent of culturable fungi were of the genus *Mrakia*. Eighteen isolates from eight genera were selected and tested for both antifreeze activity and capacity for growth under temperatures ranging from −1 to 25 °C. *Rhodotorula* sp. NHT-2 possessed a high degree of sequence homology with *R. gracialis*, while *Leucosporidium* sp. BSS-1 possessed a high degree of sequence homology with *Leu. antarcticum* (*Glaciozyma antarctica*), and these two isolates demonstrated antifreeze activity. All isolates examined were capable of growth at −1 °C. *Mrakia* spp., while capable of growth at −1 °C, did not demonstrate any antifreeze activity and exhibited only limited secretion of extracellular polysaccharides. Species of the genus *Mrakia* possessed high amounts of unsaturated fatty acids, suggesting that members of this genus have adapted to cold environments by increasing their membrane fluidity.

Introduction

Cold environments cover a large portion of our planet, with many ecosystems permanently exposed to temperatures below 5 °C (Feller & Gerday, 2003). Since microbes adapted to cold environments are capable of growth at temperatures below 0 °C, it is expected that these microbes employ unique physiological tools, such as cold-adapted enzymes and antifreeze proteins (AFP), in order to survive (Buzzini *et al.*, 2012). Antarctica is the southernmost landmass on Earth and has an area of *c.* 14 million km^2, making it the fifth-largest continent in the world. Approximately 98% of Antarctica is covered by ice and snow and temperatures in coastal areas usually range from 5 to −35 °C. Temperatures on Antarctic plateaus are much more extreme, ranging from *c.* −25 °C in summer to *c.* −70 °C in winter (Ravindra & Chaturvedi, 2011). The Skarvsnes ice-free area is located along the central Soya coast, East Antarctica, and contains many small oligotrophic freshwater lakes that constitute the only unfrozen water in the area (Imura *et al.*, 1999). The surface of these lakes is covered by 1–2 m of ice for 11 months of the year (Tanabe *et al.*, 2008); however, these lakes do not freeze below a depth of 3 m.

To our knowledge, over 700 fungal species have been isolated and recorded from Antarctica (Bridge *et al.*, 2010). Twelve ascomycetous and four basidiomycetous species were reported from around Syowa station, East Antarctica. (Soneda, 1961; Tsubaki, 1961a, b; Tsubaki & Asano, 1965). Despite previous research into the

mycoflora of the Skarvesnes ice-free area, knowledge of fungal biodiversity in this area remains incomplete. The present study aimed to assess the diversity of fungi isolated from soil collected around lakes as well as lake sediments in the Skarvsnes ice-free area, and to determine any relevant adaptations to cold environments.

Materials and methods

Ethics statement

All necessary permits were obtained for the field studies conducted and sample collection was performed with the permission of the Ministry of the Environment of Japan.

Sampling sites and sample collection

Samples were collected in and around lakes in the Skarvsnes ice-free area, which is located in the Lützow-Holm Bay, East Antarctica. Samples of surface soil samples collected around lakes and of lake sediments were obtained from lakes Abi-ike, Ageha-ike, Bosatsu-ike, Ebi-numa, Hyoutan-ike, Jizou-ike, Kuwai-ike, Kumogata-ike, Mago-ike, Naga-ike, Nisehyoutan-ike, Nyorai-ike, Ohgi-ike, Oyako-ike, Shimai-ike and Tokkuri-ike (Fig. 1). Sampling positions were recorded using a global positioning system (GPS). Collected samples were stored at −1 °C prior to use.

Isolation of fungi

Each 0.1 g soil sample was placed on either a potato dextrose agar (PDA) or a basidiomycetes selective agar (BSA) (15 g L^{-1} malt extract, 2 g L^{-1} yeast extract, 10 μg L^{-1} benomyl, 2 mL L^{-1} lactic acid and 15 g L^{-1} agar) containing 50 μg mL^{-1} chloramphenicol at 4 °C for a period of up to 2 weeks. Yeast samples were chosen for isolation based on colony morphology. Each colony of a different

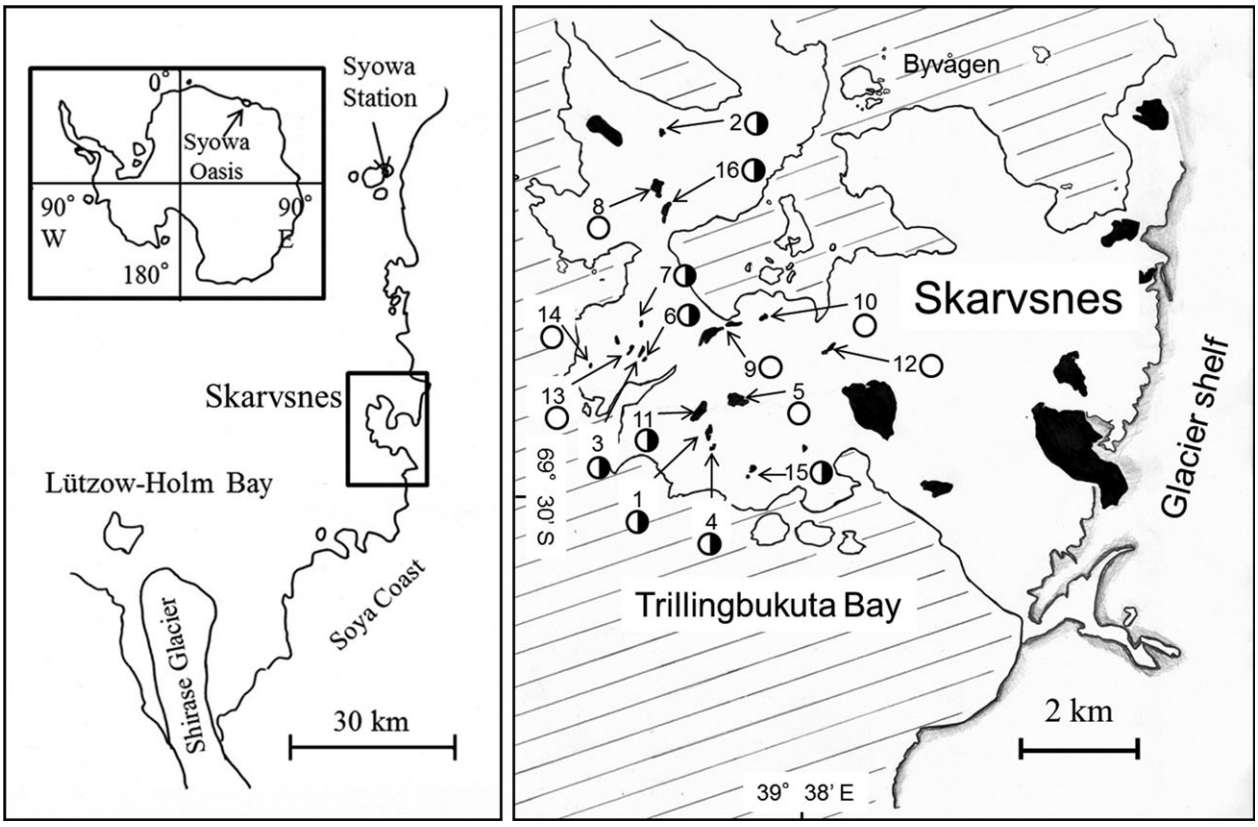

Fig. 1. Location of the study site. (From left) The location of the Skarvsnes ice-free area, Antarctica, and the locations of sampling sites within that area. Numbers and arrows indicate the locations of lakes in Skarvsnes, Syowa Oasis, Antarctica, that were examined in the present study (1. Abi-ike, 2. Ageha-ike, 3. Bosatsu-ike, 4. Ebi-numa, 5. Hyoutan-ike, 6. Jizou-ike, 7. Kuwai-ike, 8. Kumogata-ike, 9. Oyako-ike, 10. Mago-ike, 11. Naga-ike, 12. Nisehyoutan-ike, 13. Nyorai-ike, 14. Ohgi-ike, 15. Shimai-ike, 16. Tokkuri-ike). Open circles (○) indicate sites from which samples were collected from the soil surrounding lakes, and half-filled circles (◑) indicate sites from which samples were taken from both the soil and lake sediment.

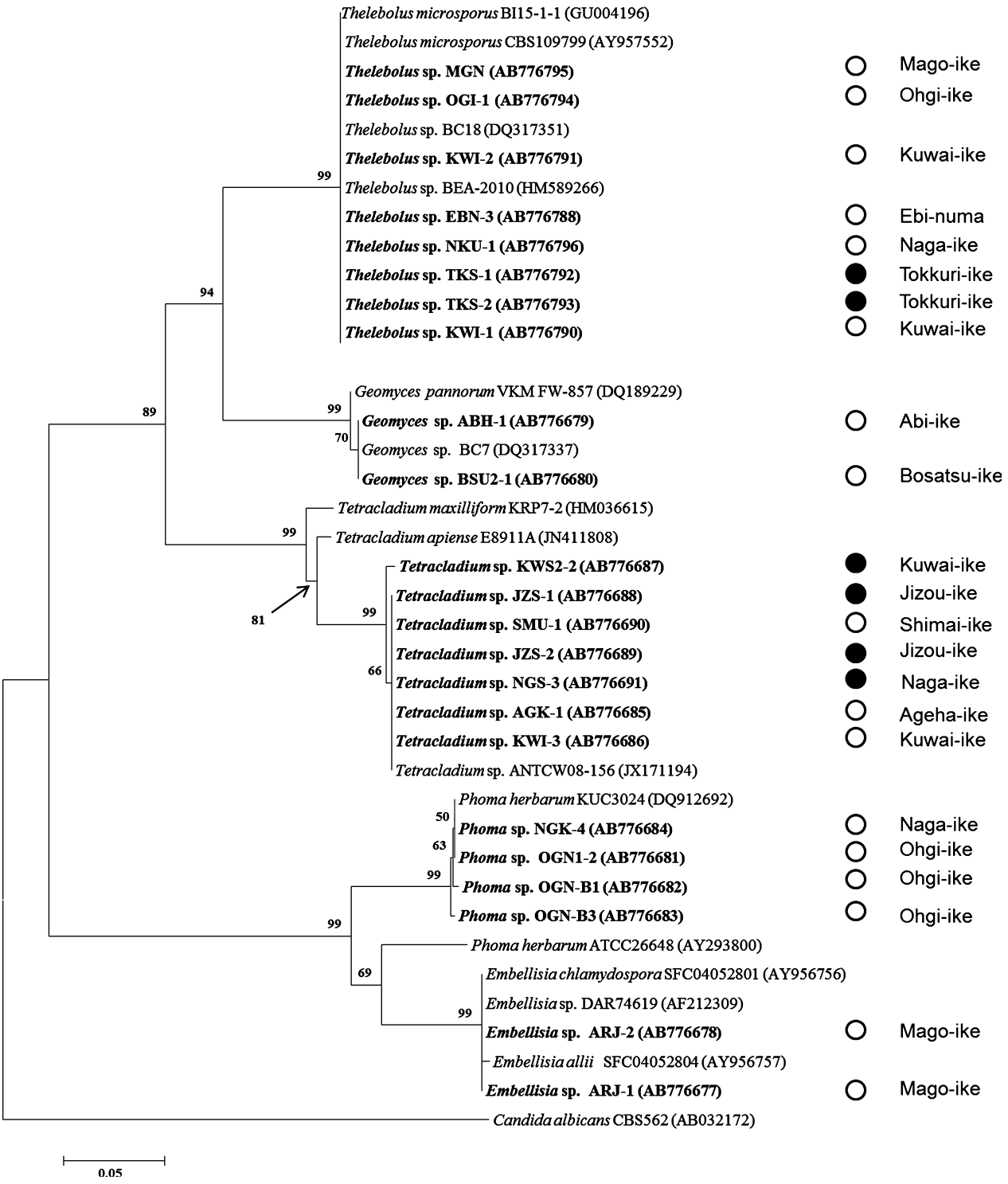

Fig. 2. Phylogenetic tree of *Ascomycetes* obtained from lakes in the Skarvsnes ice-free area, Antarctica. Neighbor-joining tree based on the ITS region containing the 5.8S rRNA gene sequence for all *Ascomycetes* species collected. Bootstrap percentages based on 1000 replications are displayed over branches. *Candida albicans* CBS562 was used as an outgroup. Open circles (○) indicate samples obtained from soil surrounding lakes, and closed circles (●) indicate samples obtained from lake sediment. Lake names on the far right of the diagram indicate the sampling location of each species.

morphology was purified by repeated streaking on PDA or BSA plates.

Sequencing analysis

DNA was extracted from colonies using an Isoplant II kit (Wako Pure Chemical Industries, Osaka, Japan) in accordance with protocols provided by the manufacturer. Extracted DNA was amplified through PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan). The ITS region of DNA samples was amplified using the following primers: ITS1F (5′-GTA ACA AGG TTT CCG T) (Gardes & Bruns, 1993) and ITS4 (5′-TCC TCC GCT TAT TGA TAT GC) (White *et al.*, 1990). Sequences were determined using an ABI prism 3100 Sequencer (Applied Biosystems, Life Technologies, Tokyo, Japan). ITS region sequences of isolates were deposited into the DNA Data Bank of Japan (DDBJ) (Figs 2 and 3). Alignment was achieved using CLUSTAL W (\http://clustalw.ddbj.nig.ac.jp/) and corrected manually. Phylogenetic analyses with neighbor-joining analyses of ITS regions containing 5.8S rRNA gene were performed using MEGA software version 5.0 (Tamura *et al.*, 2011). Bootstrap analysis employing 1000 replicates was also performed.

Determination of antifreeze activity

One loopful of pure culture was inoculated into 50 mL autoclaved PDB (potato dextrose broth) and incubated at −1°C and left standing without shaking for 2 months. Culture broth samples of 5 μL were then collected and observed under 50× magnification using a Leica DMLB 100 photomicroscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a Linkam LK600 temperature controller (Linkam, Surrey, UK) to observe antifreeze activity. Samples were instantly frozen at *c.* −25 °C, and then warmed up to 0 °C on the sample stage to create ice crystal seeds in the solutions. Resulting solutions were then cooled to a temperature ranging from *c.* −1 to −5 °C and the growth of ice crystal seeds monitored. Antifreeze activity of the isolate was determined by the shape of ice crystals. Hexagonal crystals indicated positive and rounded shape indicated negative activity (Griffith *et al.*, 1992).

Determination of extracellular polysaccharide content and the effect of culture temperatures on growth

One loopful of each pure culture was inoculated onto PDA and incubated at −1, 4, 10, 20 or 25 °C for 2 weeks. Colonies of isolates were checked and the effect of temperature on each culture was determined.

To determine the presence of extracellular polysaccharides (EPS), one loopful of pure culture biomass was inoculated into 50 mL autoclaved PDB (potato dextrose broth) and incubated at 4 °C for 4 weeks at 120 r.p.m. Biomasses were removed by centrifugation at 3500 ***g*** for 15 min at 4 °C. The extracted supernatants assumed to contain crude EPS and the crude EPS, were combined with 99% (v/v) ethanol to form solutions four times the volume of the original supernatants. The resulting mixtures were then centrifuged at 5000 ***g*** for 10 min at 4 °C. The extracellular polysaccharide content of the resulting insoluble samples was determined using the phenol-sulfuric acid method (Dubois *et al.*, 1956).

Analysis of whole-cell fatty acids

Whole-cell fatty acids were extracted from 100 mg freeze-dried biomass using acid methanolysis and analyzed by gas chromatography equipped with a flame ionization detector (model GC353, GL Science, Japan) at an initial temperature of 140 °C for 15 min. Temperature was then increased to 240 °C at a rate of 4 °C min^{-1}. Fatty acids were identified by comparison using fatty acid methyl ester (FAME) mix as a standard (Supelco 37 component FAME mix).

Results and discussion

Samples were collected from 16 sites in the Skarvsnes ice-free area (Fig. 1). A total of 71 isolates were obtained and classified to 10 genera based on sequences covering the ITS region (Figs 2 and 3). The average length of the ITS region reads was 518 bp, with a maximum length of 616 bp and minimum length of 400 bp. Twenty-three isolates were classified as ascomycetous fungi and assigned to five genera (*Embellisia*, *Phoma*, *Geomyces*, *Tetracladium*, *Thelebolus*). Forty-eight isolates were classified as basidiomycetes and assigned also to five genera [*Mrakia*, *Cryptococcus*, *Dioszegia*, *Rhodotorula*, *Leucosporidium* (*Glaciozyma*)]. Dominant isolates belonged to the genera *Mrakia* (35.2%), *Cryptococcus* (22.5%), *Thelebolus* (11.3%) and *Tetracladium* (9.9%). These genera were collected from soils around lakes and lake sediments. Genera *Rhodotorula*, *Leucosporidium* and *Geomyces* were also collected from both the soil around lakes and lake sediments. Fungi of the genera *Phoma*, *Embelisia* and *Dioszegia* were isolated from soil surrounding lakes. No fungal species were exclusively isolated from the lake sediments.

Species of the genus *Embellisia* have generally been isolated from higher plants, often as endophytes or pathogens, in areas of moderate temperature (Li & Nan, 2007). Brander *et al.* (2000) previously isolated an *Embellisa* sp.

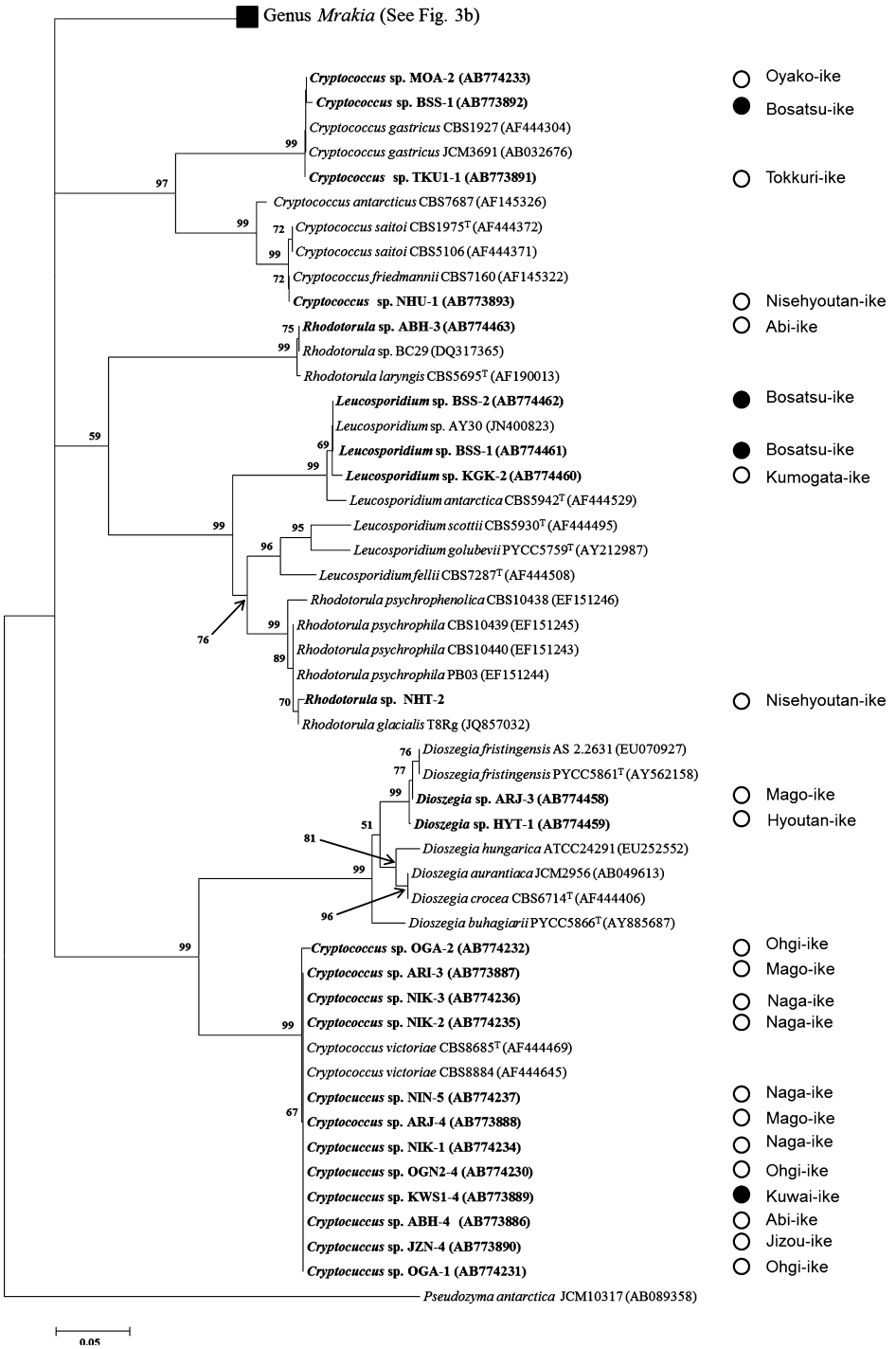

Fig. 3. Phylogenetic tree of *Basidiomycetes* obtained from lakes in the Skarvsnes ice-free area, Antarctica. Neighbor-joining tree based on the ITS region containing the 5.8S rRNA gene sequence for all *Basidiomycetes* species collected. Bootstrap percentages based on 1000 replications are displayed over branches. *Psudozyme antarctica* JCM10317 was used as an outgroup. Open circles (○) indicate samples obtained from soil surrounding lakes, while closed circles (●) indicate samples obtained from lake sediment. Lake names on the far right of the diagram indicate the sampling location of each species. Phylogenetic tree of Basidiomycetous yeast *Mrakia* obtained from lakes in the Skarvsnes ice-free area, Antarctica. Neighbor-joining tree based on the ITS region containing the 5.8S rRNA gene sequence for all *Mrakia* species collected. Bootstrap percentages based on 1000 replications are displayed over branches. *Mrakiella aquatic* DBVPG4990 was used as an outgroup. Closed squares (■) indicates that the link to the closed square in Fig. 3a. Open circles (○) indicate samples obtained from soil surrounding lakes, and closed circles (●) indicate samples obtained from lake sediment. Lake names on the far right of the diagram indicate the sampling location of each species.

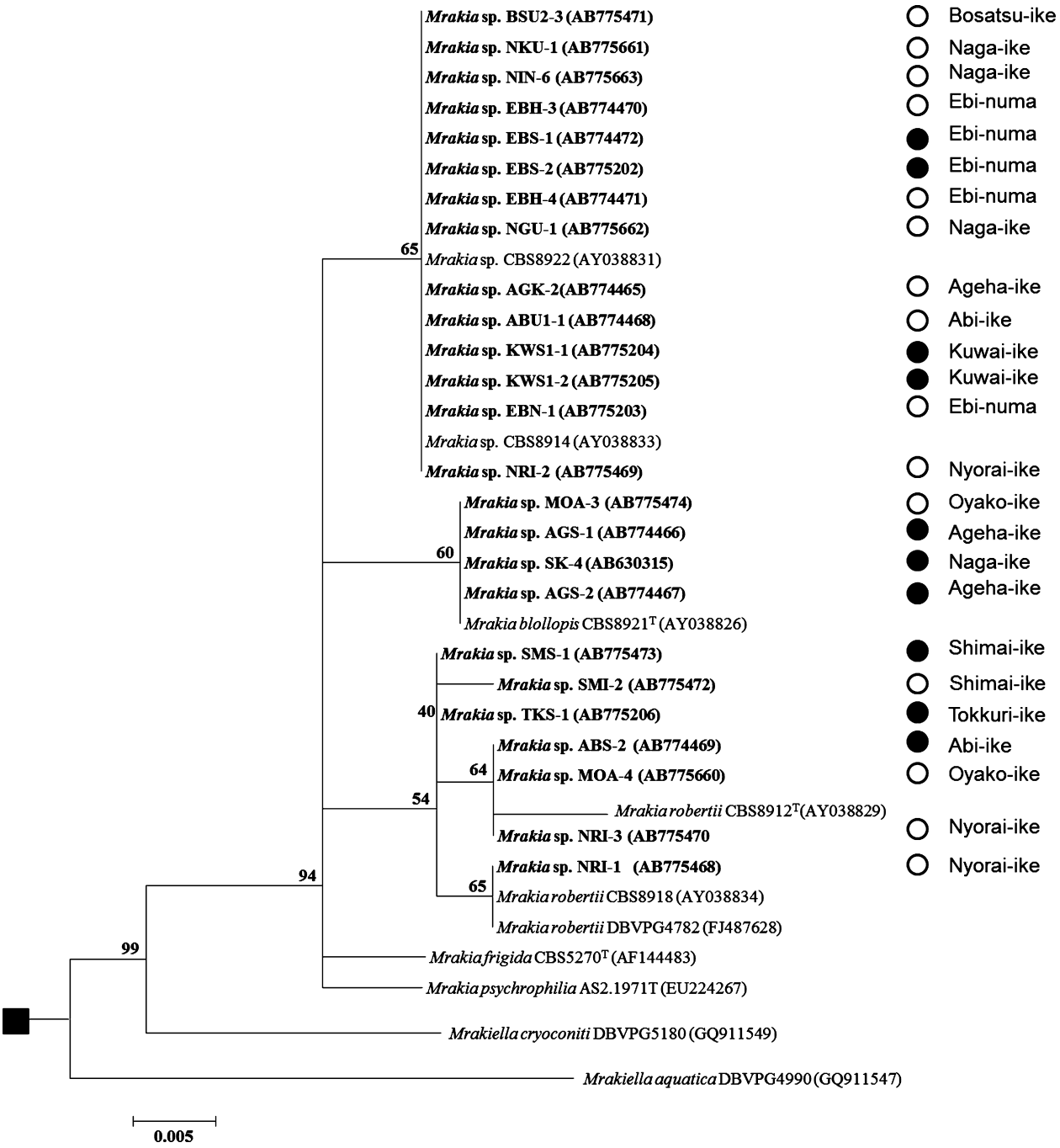

Fig. 3. Continued.

from moss in Victoria Land, Antarctica. In the present study, two isolates of *Embellisia* sp. were collected from surface soil surrounding Lake Mago-ike. The genus *Phoma* has been reported from various cold environments (Fletcher *et al.*, 1985). In Antarctica, this genus was isolated from Bunger Hill (Barker, 1977), MacRobertson Land (Fletcher *et al.*, 1985) and Victoria Land (Toshi *et al.*, 2002). The majority of these samples were isolated from moss, which led researchers to believe that the genus preferred moss as a substrate; however, Gonçalves *et al.* (2012) reported that a *Phoma* sp. was collected from water in a King George Island lake. The present study identified four isolates of *Phoma* sp. that were collected from soil surrounding lakes Ohgi-ike and Naga-ike. Iso-

lates of the genus *Geomyces* have been obtained numerous times from soil samples taken from Kay Land, Terra Nova, and Cape King, Antarctica (Frate & Caretta, 1990). In the present study, only two isolates of *Geomyces* sp. were isolated from the Skarvsnes ice-free area and eight isolates of *Thelobolus* sp. were isolated from both the soil around lakes and lake sediments in the Skarvsnes ice-free. Ruisi *et al.* (2007) determined that a *Thelebolus* sp. was one of the fungi most frequently isolated from the Antarctic continent. *Thelebolus* strains have been isolated from both littoral mats and under ice mats in Ace Lake, Lake Druzhby and Highway Lake (Vestfold Hills); Organic Lake, and Red Lake on Manning Island (Larsemann Hills); and Lake Fryxell and Lake Hoare (McMurdo Dry Valleys) (Göttlich *et al.*, 2003). Seven isolates of *Tetracladium* sp. were isolated from soil and lake sediment taken from the Skarvsnes region. *Tetracladium* sp. had been previously isolated from soil taken from West Antarctica and plant matter taken from Admiralty Bay (Bridge & Newsham, 2009; Rosa *et al.*, 2009). We obtained a total of 23 ascomycetous isolates from soil surrounding lakes and lake sediments. From lake sediments, we collected a total of six isolates from Tokkuri-ike, Kuwai-ike, Jizou-ike and Naga-ike. These isolates belonged to the genera *Thelebolus* spp. and *Tetracladium* spp.

These results indicate a low biodiversity of ascomycetous fungus in lake sediments at the Skarvsnes ice-free area, as compared with the soil surrounding the lakes.

Sixteen of the 71 isolates obtained belong to *Cryptococcus*; of those 16 isolates, 12 possessed high sequence similarity with *Cryptococcus victoriae*, three were close related to *Cry. gastricus* and one was related to *Cry. friedmannii*. *Cryptococcus victoriae* is a cosmopolite yeast and has been isolated from cold environments around the world. *Crypto-*

Table 1. Antifreeze activity, presence of EPS and growth at various culture temperatures of all isolates

Isolates	Antifreeze activity	EPS	Culture temperature				
			−1 °C	4 °C	10 °C	20 °C	25 °C
Mrakia sp. AGK-2	–	w	+	+	+	w	–
Mrakia sp. EBN-1	–	w	+	+	+	w	–
Mrakia sp. KWS1-1	–	w	+	+	+	w	–
Mrakia sp. SK-4	–	w	+	+	+	w	–
Mrakia sp. SMS-2	–	w	+	+	+	w	–
Mrakia sp. TKS-1	–	w	+	+	+	w	–
Cryptococcus sp. OGA-1	–	w	+	+	+	+	–
Cryptococcus sp. NHU-1	–	w	+	+	+	+	–
Cryptococcus sp. MOA-2	–	w	+	+	+	+	–
Rhodotorula sp. ABH-3	–	w	+	+	+	+	–
Rhodotorula sp. NHT-2	+	w	+	+	+	+	–
Leucospridium sp. BSS-1	+	+	+	+	+	–	–
Dioszegia sp. ARJ-3	–	w	+	+	+	+	–
Phoma sp. NGK-4	–	w	+	+	+	+	+
Tetracladium sp. AGK-1	–	w	+	+	+	+	+
Tetracladium sp. JZS-2	–	w	+	+	+	+	+
Thelebolus sp. EBN-3	–	w	+	+	+	+	+
Thelebolus sp. TKS-1	–	w	+	+	+	+	+

–, indicates a lack of antifreeze activity, a lack of EPS or the inability to grow at a given temperature; +, indicates positive antifreeze activity, considerable amounts of EPS (> 0.05 mg mL^{-1}) or considerable growth at a given temperature; w, indicates weak antifreeze activity, the presence of EPS (< 0.05 mg mL^{-1}) or a capacity for weak growth at a given temperature.

Table 2. Fatty acid composition of *Mrakia* spp., *Rhodotorula glacialis, Cryptococcus victoriae* and *Leucosporidium* sp. Fatty acid analysis results for *M. blollopis* SK-4 and other species are shown. Fatty acid data were taken from Thomas-Hall *et al.* (2002, 2010), Rossi *et al.* (2009) and this study

Fatty acids	*M. blollopis* SK-4	*M. blollopis* CBS 8921^T	*M. frigida* CBS5270^T	*M. robertii* CBS8912^T	*R. glacialis* DBVPG4785	*Cry. victoriae* CBS8920	*Leu.* sp. BSS-1
Palmitic acid (C16:0)	8	19	13	16	14.5	10	12
Palmitoleic acid (C16:1)	8	2	0	1	1.4	ND	3
Oleic acid (C18:1n9)	22	16	8	30	39.4	50	58
Linoleic acid (C18:2)	55	33	52	35	21.2	30	13
Linolenic acid (C18:3n3)	2	30	27	18	15.4	7	5

coccus victoriae, in Antarctica, has also been collected from soil samples taken from Victoria Land, Lichen Valley, Vestfold Hills and Davis Base (Montes *et al.*, 1999; Thomas-Hall *et al.*, 2002; Vaz *et al.*, 2011). Two isolates of *Rhodotorula* spp., three isolates of *Leucosporidium* spp. (recently *Leu. antarcticum* was redefined as *Glaciozyma antarctica*; Turchetti *et al.*, 2011) and two isolates of *Dioszegia* spp. were also collected during the present study. Species of these genera have been identified in South Victoria Land and various other cold temperature environments (Fell *et al.*, 1969; Buzzini *et al.*, 2012). Furthermore, a *Leucospridium* sp. (*Glaciozyma* sp.) has also been isolated from meltwater stream sediment, glacial meltwater stream sediment and sea water (Vishniac & Klingler, 1986; Klingler & Vishniac, 1988; Turchetti *et al.*, 2011).

Di Menna (1966) reported that *c.* 24% of culturable yeasts from soil in Antarctica were *Mrakia* spp. *Mrakia* spp. have also been identified previously in various cold regions such as the Arctic, Siberia, the Alps, Patagonia and Antarctica (Morgesin *et al.*, 2005; de Garcia *et al.*, 2007, 2012; Pathan *et al.*, 2010; Thomas-Hall *et al.*, 2010; Singh & Singh, 2012; Tsuji *et al.*, 2013). *Mrakia* spp. were the major mycoflora and some of the most adaptive fungi in the lake sediments at Skarvsnes ice-free area, East Antarctica.

Some fungi secrete extracellular AFP to prevent freezing of the cell when exposed to extreme cold conditions. Fungal AFPs have been found in snow mold fungi and have been identified as phytopathogens in both wheat and grass (Snider *et al.*, 2000; Hoshino *et al.*, 2003, 2009). Xiao *et al.* (2010a) tested 23 fungal species from Antarctica for antifreeze activity and determined antifreeze activity in only two species. Xiao *et al.* (2010b) also reported that 13 of a total of 145 eukaryotic microorganisms (fungi and stramenopile) isolates obtained in Antarctica demonstrated antifreeze activity. Nine of the 13 isolates that demonstrated antifreeze activity were basidiomycetous yeast, of which seven were *Leu. cantarcticum* (*Glaciozyma antarctica*) and two were *Rhodotorula glacialis* (Xiao *et al.*, 2010b). In the present study, 18 isolates of eight genera were chosen from 71 total isolates and tested for antifreeze activity and capacity for growth at temperatures ranging from −1 to 25 °C. *Rhodotorula* sp. NHT-2 demonstrated high ITS region sequence homology with *R. glacialis*, and *Leucosporidium* sp. BSS-1 demonstrated high ITS region sequence homology with *Leu. antarcticum* (*Glaciozyma antarctica*) –only these two isolates demonstrated antifreeze activity. Results of the present study as well as those of Xiao *et al.* (2010a, b) indicate that Antarctic fungi rarely demonstrated antifreeze activity and that almost all fungi employed other strategies to survive in these extreme environments. Eighteen of the isolates tested in the present study were capable of growth at −1 °C. *Leucosporidium* sp BSS-1 was not capable of growth at 20 °C, although *Phoma* sp. NGK-4, *Tetracladium* spp. AGK-1 and JZS-2 and *Thelebolus* sp. EBN-3 were capable of growth at 25 °C (Table 1).

Robinson (2001) reported that physiological characteristics such as cryoprotectant sugars, polyols, fatty acids, AFPs and cold-active enzymes are advantageous to soil organisms for survival during the polar winters. *Leucosporidium antarcticum* (*Glaciozyma antarcticum*), a cryophilic yeast, (Hoshino & Matsumoto, 2012), has been known to form colonies within frost columns and secrete AFP under low temperature conditions (Lee *et al.*, 2010). Moreover, the species has been shown to secrete both AFP and extracellular polysaccharides (EPS) when exposed to low temperatures (Fujiu 2010; Master's thesis, Graduate School of Science, Hokkaido University). *Leucosporidium antarcticum* (*Glaciozyma antarcticum*) are known to have the capacity to survive at low temperatures, but although species of this genus have not demonstrated antifreeze activity, and very few of them have been known to secrete EPS, *Mrakia* spp. have been identified as the dominant culturable fungi in lake sediments at the Skarvsnes ice-free area, East Antarctic, Pathan *et al.* (2010) determined that cold-adapted yeasts commonly contained high concentrations of unsaturated fatty acids, such as C18:1 and C18:2, and that these fatty acids were considered essential for survival at low temperatures. Results of fatty acid composition indicated that *Mrakia* spp. and *Cry. victoriae* had adapted to low temperature conditions by achieving high membrane fluidity (Turk *et al.*, 2011; Singh *et al.*, 2013). Moreover, *Mrakia blollopis* SK-4 has high concentrations of C18:2 and totals of C18:1 and C18:2, compared with those of *M. blollopis* CBS8921^T (Table 2). It was therefore concluded that strain SK-4 was more highly adapted for survival in cold lake environments than the other *Mrakia* spp. analyzed.

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