

1 Category of paper: Short note

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3 Microfungi associated with withering willow wood in ground contact near Syowa

4 Station, East Antarctica for 40 years

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22 **Abstract**Data are rather lacking on the diversity of microfungi associated with
23 exotic plant substrates transported to continental Antarctica. We examined the
24 diversity and species composition of microfungi associated with withering woody
25 shoots of saplings of *Salix* spp. (willows) transplanted and in ground contact
26 near Syowa Station, East Antarctica for more than 40 years. The willow
27 saplings originated from Hokkaido, Northern Japan, and were experimentally
28 transplanted in 1967-1968, but died within a few years. Dead willow shoots,
29 unbranched and standing on bare ground for approximately 50 years, were used
30 for the isolation of fungi with the surface disinfection method. A total of 43
31 isolates were retrieved from 32 (78%) of the 41 shoots tested. The fungal isolates
32 were classified into 18 molecular operational taxonomic units (MOTUs) based on

the similarity of rDNA ITS sequences at the 97% criterion. Leotiomycetes was the most common class in terms of the number of isolates and MOTUs, followed by Dothidiomycetes, Sordariomycetes, and Eurotiomycetes. Molecular phylogenetic affinities suggested that the closest relatives of the MOTUs were saprobic and root-associated fungi. The result of the present study suggested that *Cadophora luteo-olivacea* is widespread in soils throughout Antarctica and likely indigenous.

Keywords Continental Antarctica • Fungi • Root endophyte • *Salix* • Syowa Station

Introduction

Ice-free regions of continental Antarctica, comprising only about 2% of the continent, are cold and arid, and strong selection pressures are imposed on plant establishment and soil development. Despite the harsh environment, previous

studies have reported the occurrence of free-living fungi in soils and in association with bryophytes in coastal outcrops of continental Antarctica (e.g. Azmi and Seppelt 1997; Tosi et al. 2002, 2005; Newsham et al. 2009). Recent studies have examined fungal populations in historically-introduced exotic materials and found a significant overlap of fungi isolated from these materials and fungi isolated from environmental samples in pristine locations (Farrell et al. 2011). A significant effect of exotic substrates on indigenous soil fungi has also been found (Arenz et al. 2011). However, data are still lacking regarding the diversity of microfungi associated with exotic plant substrates transported to continental Antarctica. The purpose of the present study is to examine microfungi associated with withering woody shoots of saplings of *Salix* spp. (willows) in ground contact in Syowa Station, East Antarctica for 40 years.

Materials and methods

Study site and sample collection

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66 Samples were collected near Syowa Station on East Ongul Island, Lützow-Holm
67 Bay, East Antarctica (60°00'47"S, 39°34'57"E, 16 m a.s.l.). In February 1967,
68 saplings of dwarf deciduous shrubs *Salix pauciflora* and *S. reinii*, 10-20 cm in
69 height and originating from Hokkaido, Northern Japan, were transplanted at
70 experimental sites near Syowa Station by Dr. T. Hoshiai of the 8th Japanese
71 Antarctic Research Expedition (JARE-8) to test their growth and survivorship.
72 These saplings endured through winter, sprouted, and bloomed in the next
73 summer of 1968, but not all sprouted in the summer of 1969 (Hoshiai 1970).
74 Additional saplings were transplanted by Dr. Y. Endo of JARE-9 in 1968, giving
75 a similar result of the sapling producing leaves the next year but dying within a
76 few years because of the adverse environment of Antarctica (Hoshiai 1970).
77 During JARE-51 in 2009-2010, we found dead willow shoots still standing on the
78 experimental site. In February 2010, a total of 41 withering shoots (aboveground
79 parts without leaves, soil, or belowground parts, approximately 3 cm in height,
80 and 1-3 mm in basal diameter) were collected with tweezers, preserved in paper

81 bags, stored at 2°C, and taken back to the laboratory in Japan.

82

83 Fungal isolation

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85 Fungi were isolated from shoots using the surface disinfection method according
86 to Osono et al. (2012). The surface-disinfected shoots were plated on 9-cm Petri
87 dishes containing 2% lignocellulose agar (LCA) modified as described by Miura
88 and Kudo (1970) (glucose 0.1%, KH_2PO_4 0.1%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.02%, KCl 0.02%,
89 NaNO_3 0.2%, yeast extract 0.02%, and agar 2% (w/v)), two shoots per plate. Note
90 that the modified LCA of Miura and Kudo (1970) does not contain lignin or other
91 recalcitrant compounds. The modified LCA was used because its low glucose
92 content suppresses the overgrowth of fast-growing fungal species (Osono and
93 Takeda 1999). The plates were incubated in darkness at 10°C and observed for 4
94 weeks after the disinfection. Any fungal hyphae or spores appearing on the
95 plates were subcultured onto fresh LCA plates, incubated, and observed
96 micromorphologically. Isolates were then used for molecular analysis as

described below.

Molecular methods

Genomic DNA was extracted from mycelia that had been cultured on 2.5% malt extract agar overlaid with a cellophane membrane following the modified CTAB method described by Matsuda and Hijii (1999). Polymerase chain reactions (PCR) were performed using a Quick Taq HS DyeMix (Toyobo, Osaka, Japan). Each PCR reaction contained a 50 µl mixture (21 µl distilled water, 25 µl master mix, 3 µl ca. 0.5ng/µl template DNA, and 0.5 µl of each primer (final, 0.25 µM)). To PCR amplify the region including the rDNA ITS and 28S rDNA D1-D2 domain, the primer pair ITS1f (Gardes and Bruns 1993) and LR3 (Vilgalys and Hester 1990) was used. Each DNA fragment was amplified using a PCR thermal cycler (DNA engine; Bio-Rad, Hercules, CA, USA) using the following thermal cycling schedule. The first cycle consisted of 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C for annealing, 1 min at 72°C, and a final cycle of 10

113 min at 72°C. The reaction mixture was then cooled at 4°C for 5 min. PCR
114 products were purified with a QiAquick PCR Purification Kit (Qiagen,
115 Germany) according to the manufacturer's instructions.

116 Purified PCR products were sequenced by FASMAC Co., Ltd.
117 (Kanagawa, Japan). Sequencing reactions were performed in a Gene Amp PCR
118 System 9700 (Applied Biosystems, USA) using a BigDye Terminator V3.1
119 (Applied Biosystems), following the protocols supplied by the manufacturer. The
120 fluorescent-labeled fragments were purified from the unincorporated
121 terminators using an ethanol precipitation protocol. The samples were
122 resuspended in formamide and subjected to electrophoresis in an ABI 3730xl
123 sequencer (Applied Biosystems).

124 The sequences determined in this study were deposited in the DNA
125 Data Bank of Japan (DDBJ) (AB752244-AB752287). The rDNA ITS sequences
126 were compared with available rDNA sequences in the GenBank database using
127 BLASTN searches (Altschul et al. 1990). For phylogenetic analysis, MAFFT ver.
128 6 (Kato and Toh 2008) was used for preliminary multiple alignments of

nucleotide sequences. Final alignments were manually adjusted using BioEdit (Hall 1999). Alignment gaps were treated as missing data, and ambiguous positions were excluded from the analysis. The phylogenetic tree was conducted by maximum likelihood (ML) methods (Felsenstein 1981) with the best fit nucleotide substitution model based on the lowest Bayesian Information Criterion (BIC) score. To estimate clade support, the bootstrap procedure of Felsenstein (1985) was employed with 1000 replicates. These analyses were carried out using MEGA5 (Tamura et al. 2011).

The isolates were grouped into molecular operational taxonomic units (MOTUs) according to the similarity of rDNA ITS sequences at the 97% criterion. The frequency of occurrence of MOTU was calculated as a percentage of the number of shoots from which a MOTU was detected compared with the total number of shoots tested (i.e. 41).

Results

Fungi were isolated from 32 (78%) of the 41 shoots tested for isolation. A total of 43 isolates were obtained, and these were classified into 18 MOTUs (Table 1, Fig. 1). Leotiomyces was the most frequent class, including 29 isolates of 10 MOTUs, followed by Dothidiomyces (9 isolates, 4 MOTUs), Sordariomyces (3 isolates, 2 MOTUs), and Eurotiomyces (2 isolates, 2 MOTUs) (Fig. 2). The most frequent MOTUs were MOTU1 in the Leotiomyces that had 100% sequence match of the ITS region to *Cadophora luteo-olivacea* (7 isolates), MOTU9 in Leotiomyces (7 isolates), and MOTU18 in Dothidiomyces (5 isolates) (Table 1, Fig. 2).

Discussion

Some of the microfungi associated with dead willow shoots in the present study are classed as saprobic fungi (Table 1). For example, *Cadophora luteo-olivacea* (MOTU1) is a saprobe occurring in many habitats including wood, soil, and plants (Gramaje et al. 2011). Several *Cadophora* species, including *C.*

161 *luteo-olivacea*, have also been isolated from soils and historic wood along the
162 Ross Sea region of Antarctica (Arenz et al. 2006) and have the potential to cause
163 soft rot in wood (Blanchette et al. 2004). Similarly, *Phialocephala lagerbergii*,
164 which had 99% sequence match of the ITS region to MOTU3, is known to be a
165 wood-inhabiting fungus (Grünig et al. 2009). *Geomyces vinaceus*, an anamorph
166 of *Pseudogymnoascus roseus* and which had 100% sequence match of the ITS
167 region to MOTU8, is associated with wood, soil, and roots (Rice and Currah
168 2006). *Coniochaeta lignaria*, which had 99% sequence match of the ITS region to
169 MOTU13, has been shown to have lignocellulose-degrading enzymes (Lopez et al.
170 2007), which can facilitate growth and energy acquisition in dead willow shoots
171 consisting of structural lignin and cellulose polymers.

172 We noted that root-associated microfungi were isolated frequently from
173 the dead willow shoots (Table 1). For example, *Ilyonectria robusta* and *Phoma*
174 *sclerotioides*, which had 99% sequence match of the ITS region to MOTU14 and
175 MOTU17, respectively, are root-rot fungi (Wunsch and Bergstrom 2011; Cabral
176 et al. 2012). *Phialocephala fortinii*, which had 99% sequence match of the ITS

177 region to MOTU5, and also possibly MOTU2 in *Phialocephala*, is a common
178 endophyte of plant roots and is widespread in sub-Antarctic ecosystems and also
179 present in continental Antarctica (Grünig et al. 2008; Newsham et al. 2009).
180 Jumpponen et al. (2003) detected a DNA sequence with 99% similarity to *P.*
181 *fortinii* in a rhizoid of the liverwort *Cephaloziella varians* on the Antarctic
182 Peninsula.

183 It is unclear whether these fungi were widespread or localized in their
184 distribution in Antarctica and whether they were indigenous to Antarctica or
185 introduced along with the saplings in soil from Japan. MOTU1, one of the most
186 frequent taxa (Table 1), had 99% to 100% sequence match (with query coverage
187 between 89% and 97%) of the ITS region to *Cadophora luteo-olivacea* isolated
188 from wood and soil in the Ross Sea Region (DQ317327, Arenz et al. 2006;
189 GU212374, Blanchette et al. 2010) and along the Antarctic Peninsula (FJ911899,
190 Rosa et al. 2010; HQ438025, Gonçalves et al. 2012). This result suggested that
191 this fungus is widespread in soils throughout Antarctica and likely indigenous.
192 Similarly, *Geomyces vinaceus* (OTU8) was isolated from moss samples in

193 Victoria Land on the west coast of the Ross Sea (Tosi et al. 2002), but the
194 distribution of this fungus in Antarctica remains unknown and deserve further
195 researches.

196 It is unclear whether the fungi isolated in the present study were active
197 or dormant in dead shoots. However, the supply of exotic woody substrates, such
198 as dead willow shoots, can contribute to fungal abundance, as the natural lack of
199 organic material in Antarctica limits the densities of fungal populations (Arenz
200 et al. 2011). To exist in Antarctica, fungi need to be able to tolerate the harsh
201 environment, and Antarctic fungi have a variety of physiological traits that
202 enable them to survive under cold and dry conditions (Robinson 2001), including
203 cold tolerance, accumulation of intercellular trehalose and polyols, secretion of
204 antifreeze proteins, and enzymes active at low temperatures. Future studies will
205 include physiological evaluations of these fungal isolates and measurements of
206 activity at low temperatures.

207

208 **Acknowledgements** We thank Dr. Y. Motoyoshi and members of JARE-51

209 for their assistance during the expedition. This study was partially supported by
210 the National Institute of Polar Research through General Collaboration Projects
211 no.23-35, by a JSPS KAKENHI Grant (No. 70370096), and by the Nihon
212 University Multidisciplinary Research Grant for 2012 to H.D.

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214 **References**

215

216 Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local
217 alignment search tool. J Mol Biol 215:403-410

218 Arenz BE, Held BW, Jurgens JA, Farrell RL, Blanchette RA (2006) Fungal
219 diversity in soils and historic wood from the Ross Sea region of
220 Antarctica. Soil Biol Biochem 38:3057-3064

221 Arenz BE, Held BW, Jurgens JA, Blanchette RA (2011) Fungal colonization of
222 exotic substrates in Antarctica. Fun Div 49:13-22

223 Azmi OR, Seppelt RD (1997) Fungi of the Windmill Islands, continental
224 Antarctica. Effect of temperature, pH and culture media on the growth

225 of selected microfungi. Polar Biol 18:128-134

226 Blanchette RA, Held BW, Jurgens JA, McNew DL, Harrington TC, Duncan SM,
 227 Farrell RL (2004) Wood-destroying soft rot fungi in the historic
 228 expedition huts of Antarctica. Appl Environ Microbiol 70:1328-1335

229 Blanchette RA, Held BW, Arenz BE, Jurgens JA, Baltes NJ, Duncan SM, Farrell
 230 RL (2010) An Antarctic hot spot for fungi as Shackleton's historic hut on
 231 Cape Royds. Microb Ecol 60:29-38

232 Cabral A, Groenewald JZ, Rego C, Oliveira H, Crous PW (2012) Cyllindrocarpon
 233 root rot: multi-gene analysis reveals novel species within the
 234 *Ilyonectria radicicola* species complex. Mycol Progr 11:655-688

235 Farrell RL, Arenz BE, Duncan SM, Held BW, Jurgens JA, Blanchette RA (2011)
 236 Introduced and indigenous fungi of the Ross Island historic huts and
 237 pristine areas of Antarctica. Polar Biol 34:1669-1677

238 Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum
 239 likelihood approach. J Mol Evol 17:368-376

240 Felsenstein J (1985) Confidence limits on phylogenies: an approach using the

241 bootstrap. Evolution 39:783-791
 242 Gardes M, Bruns TD (1993) ITS primers with enhanced specificity for
 243 basidiomycetes: application to the identification of mycorrhizae and
 244 rusts. Mol Ecol 21:113-118
 245 Gonçalves VN, Caz ABM, Rosa CA, Rosa LH (2012) Diversity and distribution of
 246 fungal communities in lakes of Antarctica. FEMS Microbiol Ecol
 247 82:459-471
 248 Gramaje D, Mostert L, Armengol J (2011) Characterization of *Cadophora*
 249 *luteo-olivacea* and *C. melinii* isolates obtained from grapevines and
 250 environmental samples from grapevine nurseries in Spain. Phytopathol
 251 Mediterr 50:S112-S126
 252 Grünig CR, Queloz V, Sieber TN, Holdenrieder O (2008) Dark septate
 253 endophytes (DSE) of the *Phialocephala fortinii* s.l. - *Acephala*
 254 *applanata* species complex in tree roots: classification, population
 255 biology, and ecology. Botany 86:1355-1369
 256 Grünig CR, Queloz V, Duò A, Sieber TN (2009) Phylogeny of *Phaeomollisia*

257 *piceae* gen. sp. nov.: a dark, septate, conifer-needle endophyte and its
 258 relationships to *Phialocephala* and *Acephala*. Mycol Res 113:207-221
 259 Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and
 260 analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser
 261 41:95-98
 262 Hoshiai T (1970) Ongul Island: willow. Polar News 10:30-31 (in Japanese)
 263 Jumpponen A, Newsham KK, Neises DJ (2003) Filamentous ascomycetes
 264 inhabiting the rhizoid environment of the liverwort *Cephaloziella*
 265 *varians* in Antarctica are assessed by direct PCR and cloning.
 266 Mycologia 95:457-466
 267 Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence
 268 alignment program. Brief Bioinform 9:286-298
 269 Kimura M (1980) A simple method for estimating evolutionary rate of base
 270 substitutions through comparative studies of nucleotide sequences. J
 271 Mol Evol 16:111-120
 272 Lopez MJ, Vargas-Gracia MC, Suárez-Estrella F, Nichols NN, Dien BS, Moreno

273 J (2007) Lignocellulose-degrading enzymes produced by the ascomycete
 274 *Coniochaeta ligniaria* and related species: application for a
 275 lignocellulosic substrate treatment. Enzyme Microb Technol 40:794-800
 276 Matsuda Y, Hijii N (1999) Characterization and identification of *Strobilomyces*
 277 *confusus* ectomycorrhizas on momi fir by RFLP analysis of the
 278 PCR-amplified ITS region of the rDNA. J For Res 4:145-150
 279 Miura K, Kudo M (1970) An agar-medium for aquatic hyphomycetes. Trans
 280 Mycol Soc Japan 11:116-118
 281 Newsham KK, Upson R, Read DJ (2009) Mycorrhizas and dark septate root
 282 endophytes in polar regions. Fun Ecol 2:10-20
 283 Osono T, Takeda H (1999) A methodological survey on incubation of fungi on leaf
 284 litter of *Fagus crenata*. Ap For Sci Kansai 8:103-108 (in Japanese with
 285 English abstract)
 286 Osono T, Tateno O, Masuya H (2012) Diversity and ubiquity of xylariaceous
 287 endophytes in live and dead leaves of temperate forest trees.
 288 Mycoscience 54:54-61

289 Rice AV, Currah RS (2006) Two new species of *Pseudogymnoascus* with
 290 *Geomyces* anamorphs and their phylogenetic relationship with
 291 *Gymnostellatospora*. Mycologia 98:307-318

292 Robinson CH (2001) Cold adaptation in Arctic and Antarctic fungi. New Phytol
 293 151:341-353

294 Rosa LH, Vieira MLA, Santiago LF, Rosa CA (2010) Endophytic fungi
 295 community associated with the dicotyledonous plant *Colobanthus*
 296 *quitensis* (kunth) Bartl. (Caryophyllaceae) in Antarctica. FEMS
 297 Microbiol Ecol 73:178-189

298 Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5:
 299 molecular evolutionary genetics analysis using maximum likelihood,
 300 evolutionary distance, and maximum parsimony methods. Mol Biol
 301 Evol 28:2731–2739

302 Tosi S, Casado B, Gerdol R, Caretta G (2002) Fungi isolated from Antarctic
 303 mosses. Polar Biol 25:262-268

304 Tosi S, Onofri S, Brusoni M, Zucconi L, Vishniac H (2005) Response of Antarctic

305 soil fungal assemblages to experimental warming and reduction of UV
306 radiation. Polar Biol 28:470-482

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

310 Wunsch MJ, Bergstrom GC (2011) Genetic and morphological evidence that
311 Phoma sclerotioides, causal agent of brown root rot of alfalfa, is
312 composed of a species complex. Phytopathology 101:594-610

1 Hirose et al. Table 1.

2

3

4 **Table 1** The number of isolates and blast identity results (in percentage) of fungal molecular operational taxonomic units
5 (MOTUs) isolated from withering willow shoots and sequence accession number for the closest relative found at GenBank.

Class	MOTU	Number of isolates	Closest match at Genbank	
			(Accession number)	Sequence similarity %
Leotiomycetes	1	7	<i>Cadophora luteo-olivacea</i> (GU128589)	100
	9	7	Leotiomycetes sp. (JQ759481)	99
	2	4	<i>Phialocephala</i> sp. (FM999988)	99
	7	3	Leotiomycetes sp. (JQ758759)	99
	5	2	<i>Phialocephala fortinii</i> (EU888625)	99
	8	2	<i>Geomyces vinaceus</i> (AJ608972)	100
	3	1	<i>Phialocephala lagerbergii</i> (AB190400)	99
	4	1	Helotiales sp. (AB598096)	92
	6	1	<i>Clathrosporium intricatum</i> (EF029192)	95
	10	1	<i>Tetracladium</i> sp. (AJ890435)	99
Eurotiomycetes	11	1	<i>Exophiala salmonis</i> (GU586858)	99
	12	1	<i>Penicillium turbatum</i> (AY213679)	100
Sordariomycetes	14	2	<i>Ilyonectria robusta</i> (JF735265)	99

	13	1	<i>Coniochaeta ligniaria</i> (AY198390)	99
Dothidiomycetes	18	5	Dothideomycetes sp. (JQ759636)	98
	15	2	Leptosphaeria sp. (GU934537)	99
	16	1	<i>Phoma</i> sp. (HM589351)	100
	17	1	<i>Phoma sclerotioides</i> (FJ179158)	99

1 Figure legend

2

3 **Fig. 1** Maximum-likelihood (ML) phylogeny inferred from rDNA ITS sequences
4 including 18 fungal molecular taxonomic units (MOTUs) isolated from withering
5 willow shoots. The evolutionary model used was the Kimura 2-parameter model
6 (Kimura 1980) with a discrete Gamma distribution (+G, parameter = 0.7952)
7 and a proportion of Invariant sites (+I, 34.3127% sites) to allow for
8 non-uniformity of rates among sites. Bootstrap values for the ML analysis are
9 indicated for corresponding branches.

10

11 **Fig. 2** Rank-abundance relationship of fungal molecular taxonomic units
12 (MOTUs) isolated from withering willow shoots. Black bar, Leotiomycetes; blank
13 bar, Eurotiomycetes; shaded bar, Sordariomycetes; gray bar, Dothidiomycetes.

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1 Hirose et al. Fig. 1

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