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1 Colonization and lignin decomposition of pine needle litter by *Lophodermium pinastri*

2

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9

## 10 Summary

11

12 The colonization and lignin decomposition of pine (*Pinus thunbergii* and *P. densiflora*)

13 needle litter by an endophytic fungus, *Lophodermium pinastri,* was examined with field

14 observations, a field experiment, and laboratory experiments. In pine needles collected from

15 the field, needle mass per length and lignin content were lower in needle portions bearing

16 *Lophodermium* fruiting bodies than in the remaining needle portions, whereas total

17 carbohydrate content was not different between them. Total and live hyphal lengths were

greater in needle portions bearing *Lophodermium* fruiting bodies than in the remaining needle portions. *Lophodermium* fruiting bodies were not formed on sterilized needles after a six-month incubation on the forest floor, whereas they formed on 20% of nonsterilized needles, indicating that this fungus can only colonize live needles on the branch. In pure culture decomposition tests, mass loss of lignin was detected for several isolates of *L. pinastri,* but was variable among isolates and between the needles of two pine species. A comparison of the rDNA-ITS sequences between *L. pinastri* isolates of *P. thunbergii* and *P. densiflora* indicated that the two groups were phylogenetically separated. This study is the first to show that *L. pinastri* has the ability to decompose lignin in dead pine needles.

## 1 Introduction

*Lophodermium pinastri* (Schrad.) Chevall. is an ascomycete species in the Rhytismataceae and has intimate relationships with pines (*Pinus* spp.) as a supposed pathogen (Sakuyama 1993), as an endophyte (Hata and Futai 1996; Hata et al. 1998), and as a saprobe (Kendrick and Burges 1962; Soma and Saito 1979; Tokumasu 1996, 1998; Hirose and Osono 2006). In natural environments *L. pinastri* colonizes healthy needles latently as an endophyte, starts

active hyphal growth at the onset of needle senescence, and sporulates after needle abscission to infect live needles again to complete its life cycle. This fungus is a dominant colonizer of dead needles in early stages and takes part in needle decomposition as a saprobe (Hirose and Osono 2006). Sieber-Canavesi et al. (1991) showed in substrate utilization tests the production of cellulolytic enzymes by isolates of *Lophodermium* spp. from European conifers. Little is known, however, about its potential abilities to colonize fallen needles and to decompose litter and about its roles in the chemical changes in dead needles.

This study examined (i) the fungal biomass and chemical changes in needles senesced and previously colonized by *L. pinastri* under field conditions, (ii) the colonization of sterilized needles by *L. pinastri* on the forest floor, and (iii) the decomposition of needle litter by *L. pinastri* under pure culture conditions. Needles of *Pinus thunbergii* and *P. densiflora* were used as materials in the present study because the frequent occurrence of *L. pinastri* on these needles was previously documented (Tokumasu 1996, 1998; Hata et al. 1998). We hypothesized that the prior colonization of needles by endophytic *L. pinastri* enhanced the decomposition of needle tissues within their colonies, leading to heterogeneity of decomposition among single needles, and that *L. pinastri* is able to decompose lignin in needle tissues, as recent reports demonstrated that rhytismataceous fungi can cause mass loss

1 of lignin under field and laboratory conditions (Koide et al 2005a; Osono et al. 2008a).

2

3 **2 Materials and Methods**

4

5 **2.1 Study site and sample collection**

6 Samples were collected from a *P. thunbergii* plantation on a soil erosion control site at Mt.

7 Tanakami, Shiga, Japan (34°45'N, 135°56'E, 400 m above sea level). The details of the study

8 site were described in Hirose and Osono (2006). A study plot of 10 x 4 m was laid out at the

9 most representative location of the site and was divided into 10 subplots of 2 x 2m. The

10 overstory of the plot consisted only of mature *P. thunbergii*.

11 Needle litter of *P. thunbergii* was collected from litter layers in May, August, and

12 November 2003 and in February 2004. On each sampling occasion, litter layer material was

13 collected from 10 subplots using a 15x15cm quadrat. Ten needles were arbitrarily collected in

14 each quadrat. A total of 100 needles were used each month for estimation of the needle mass

15 per length and determination of the chemical composition of needles. In the present study, we

16 defined a colony of *Lophodermium* as bearing ascomata or conidiomata and being surrounded

17 by black zone lines (Hirose and Osono 2006). Hirose and Osono (2006) found that

*Lophodermium* spp. on *P. thunbergii* needles mostly consisted of *L. pinastri* but possibly included other species, such as *L. conigenus*, with low frequency. In the present study, we referred to *Lophodermium* fruiting bodies on pine needles as *L. pinastri* for the sake of simplicity. The remaining needle portion without *Lophodermium* ascomata or conidiomata was used for comparison. The remaining needle portions might have been colonized by *Lophodermium* or other fungi, or be devoid of fungal colonization, but no fruiting bodies of fungi were apparent.

## 2.2 Measurement of needle mass per length, chemical analysis, and hyphal length estimation

The length of needle portions bearing *Lophodermium* fruiting bodies and the remaining portions was measured under a binocular microscope with a magnification of 40x. Needle portions bearing *Lophodermium* fruiting bodies and the remaining needle portions were then cut separately, dried at 40˚C for one week, and measured for oven-dry mass. Needle length and mass of needle portions bearing *Lophodermium* fruiting bodies and the remaining needle portions were used to calculate the needle mass per length (mg/mm).

Needle portions bearing *Lophodermium* fruiting bodies and the remaining portions

1 were combined to make one sample for each month and each of needle portions with or
2 without *Lophodermium* fruiting bodies, ground in a laboratory mill so they could pass through
3 a 0.5-mm screen, and used for the determination of lignin and total carbohydrate contents.
4 Lignin content in the sample was estimated by gravimetry according to a standardized method
5 using hot sulfuric acid digestion (King and Heath 1967). Total carbohydrate content was
6 estimated by the phenol-sulfuric acid method (Dubois et al. 1956). The details of the methods
7 are described in Osono et al. (2008b).
8 In February 2004, needle litter of *P. thunbergii* was collected from litter layers from
9 five randomly chosen subplots, 30 to 50 needles per subplot. Needle portions bearing
10 *Lophodermium* fruiting bodies and the remaining portions of needles were then cut separately
11 and combined to make one sample for each subplot. Hyphal lengths of these samples were
12 estimated using the agar film method of Jones and Mollison (1948) but with several
13 modifications (Osono and Takeda 2001). Total and live hyphal lengths were estimated
14 separately within the same microscope fields by staining hyphae with fluorescent brightener
15 and acridine orange to visualize all hyaline and live hyphae, respectively. The details of the
16 methods are described in Osono and Takeda (2001). Samples were processed within 24 h of
17 sampling.

1 One-way analysis of variance was used to evaluate the difference in needle mass per
2 length, contents of lignin and total carbohydrates, and hyphal length between needle portions
3 bearing *Lophodermium* fruiting bodies and the remaining needle portions.
4
5 **2.3 Field incubation of sterilized litter**
6 In April 2004, dead needles attached to the branch were collected from standing-dead
7 individuals of *P. thunbergii* in the study site. The needles were taken to the laboratory and
8 air-dried at room temperature (ca. 15-20˚C) for one week. The needles (3 g) were enclosed in
9 a litterbag (15x15 cm) made of polypropylene shade cloth with a mesh size of approximately
10 2 mm. A total of 20 bags were prepared, and 10 of them were sterilized by exposure to
11 ethylene oxide gas at 60˚C for 6 h (denoted as sterilized needles), while the other 10 were not
12 exposed to ethylene oxide (denoted as nonsterilized needles). About 10 g of other needles was
13 used for the determination of oven-dry mass at 40˚C. Litterbags were then placed on the forest
14 floor in May 2005, one bag with sterilized and one bag with nonsterilized needles per subplot.
15 The bags were attached to the forest floor with metal pins to prevent movement and loss and
16 to ensure good contact between the bags and the litter layer. The bags were retrieved in
17 November 2005 at six months after placement. The bags were put into vinyl bags and taken to

1 the laboratory. Ten needles were extracted from each litterbag and observed for the presence

2 or absence of *Lophodermium* fruiting bodies under a binocular microscope with a

3 magnification of 40x. Frequency of occurrence of *Lophodermium* fruiting bodies was

4 calculated as the number of needles on which the colonies were observed divided by the total

5 number of needles tested in each litterbag (i.e., 10 needles), expressed as a percentage. A

6 paired t-test was used to evaluate the difference in remaining mass between sterilized and

7 nonsterlized needles.

8

9 **2.4 Pure culture decomposition test**

10 Five isolates of *L. pinastri* and five isolates of other species obtained from *P. thunbergii*

11 needles collected in the study site in May 2003 (Hirose and Osono 2006) were used for the

12 pure culture decomposition test (Koide et al. 2005b). Senesced needles of *P. thunbergii* fallen

13 on the forest floor were collected in the study site in November 2007 and oven-dried at 40˚C

14 for one week. The needles were preserved in a vinyl bag until the experiment began.

15 Needles were cut into pieces 2 cm in length and sterilized by exposure to ethylene

16 oxide gas at 60˚C for 6 h. Needles (0.3 g) were placed on the surface of Petri dishes (9 cm in

17 diameter) containing 20 ml of 2% agar. Fungal isolates were inoculated, and the plates were

1 incubated in darkness at 20°C for 12 weeks, according to the method described in Koide et al.
2 (2005b). Four plates were prepared for each isolate, and four uninoculated plates served as a
3 control. The mass loss of decomposed needles was determined as a percentage of the original
4 mass, taking the mass loss of control needles into consideration. Chemical analyses were
5 performed for isolates that caused mass loss of more than 3.0%. The needles from four plates
6 were combined to make one sample for each isolate and used for analyses of lignin and total
7 carbohydrates as described previously.

8 Additional pure culture tests were performed using *P. densiflora* needles and *L.*
9 *pinastri* isolated from *P. densiflora*. Four isolates of *L. pinastri* were obtained from *P.*
10 *densiflora* collected in Ueda, Nagano, in May 2007. Recently dead needles of *P. densiflora*
11 were collected in Sanada in November 2007. The fungal isolates and needles were used for
12 pure culture decomposition tests as described above.

13 Lignin/weight loss ratio (L/W) and lignin/carbohydrate loss ratio (L/C) are useful
14 indices of the substrate utilization pattern of individual fungal isolates (Osono et al. 2006).
15 L/W and L/C were calculated according to the following equations:

16 L/W = mass loss of lignin (% original mass of lignin) / mass loss of needle litter (%
17 original mass of litter)

L/C = mass loss of lignin (% original mass of lignin) / mass loss of total carbohydrate (% original mass of total carbohydrates)

## 2.5 DNA methods and phylogenetic analysis

DNA of fourteen isolates used in the pure culture tests was extracted from mycelia cultured on 2.5% malt extract liquid medium following the modified CTAB method described by Matsuda and Hijii (1999). The rDNA ITS region was amplified with primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). Polymerase chain reactions were performed using HotStarTaq Plus Master Mix (Qiagen, Ontario, Canada). Each PCR tube contained a 50 $\mu$l mixture (16 $\mu$l of distilled water, 25 $\mu$l of master mix, 3 $\mu$l of template DNA, 5 $\mu$l of coral load and 0.5 $\mu$l of each primer (final, 0.25 $\mu$M)). Each DNA fragment was amplified using a PCR thermal cycler (DNA engine; Bio-Rad, USA). The thermal cycling schedule was as follows: the first cycle consisted of 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s at 58°C for annealing, 1 min at 72°C, and the final cycle of 10 min at 72°C. The reaction mixture was then cooled at 4°C for 5 min. PCR products were purified with a QiAquick PCR Purification Kit (Qiagen, Ontario, Canada).

Purified PCR products were sequenced by Macrogen Japan Inc. (Tokyo, Japan).

1 Sequencing reactions were performed on a PTC-225 Peltier Thermal Cycler (MJ Research,
2 MA, USA) using ABI PRISMR BigDyeTM Terminator Cycle Sequencing Kits with
3 AmpliTaqR DNA polymerase (FS enzyme) (Applied Biosystems, CA, USA).
4 The sequences determined in this study were deposited in DDBJ
5 (AB511811-AB511819, AB519646-AB519648, AB247947, AB247949). The base sequences
6 of five isolates other than *Lophodermium* were compared with those of known species using
7 BLAST searching, and the closest fungal taxa for each isolate was determined (Table 1). The
8 following phylogenetic analysis was performed for the data of 10 *Lophodermium* isolates.
9 Twenty-six sequences of *Lophodermium* spp. used in Ortiz-Garcia et al. (2003) were also
10 included in the phylogenetic analysis.
11 MAFFT ver. 6 (Katoh et al. 2008) was used for preliminary multiple alignments of
12 nucleotide sequences. Final alignments were manually adjusted. Alignment gaps were treated
13 as missing data, and ambiguous positions were excluded from the analysis. Phylogenetic
14 analyses were conducted using the Neighbor-Joining method (Saitou and Nei 1987). The
15 evolutionary distances were computed using the Maximum Composite Likelihood method
16 (Tamura et al. 2004). To estimate clade support, the bootstrap procedure of Felsenstein (1985)
17 was employed with 1000 replicates. These analyses were carried out using MEGA4 (Tamura

1 et al. 2007).

2

3 **3 Results**

4

5 **3.1 Needle mass per length, chemical composition, and hyphal length of** needle portions

6 bearing *Lophodermium* fruiting bodies

7 Needle mass per length was significantly lower in needle portions bearing *Lophodermium*

8 fruiting bodies than in the remaining needle portions (Table 2). Mean value of lignin content

9 was significantly lower in needle portions bearing *Lophodermium* fruiting bodies than in the

10 remaining needle portions, whereas mean value of total carbohydrate content was not

11 significantly different between needle portions bearing *Lophodermium* fruiting bodies and the

12 remaining needle portions (Table 2). Total hyphal length, live hyphal length, and the

13 percentage live hyphal length relative to total hyphal length were significantly greater in

14 needle portions bearing *Lophodermium* fruiting bodies than in the remaining needle portions

15 (Table 2).

16

17 **3.2 *Lophodermium* fruiting bodies on sterilized needles incubated on the forest floor**

1 The remaining mass of sterilized needles after six months of field incubation was 75±2%

2 (mean ± standard error, n=10) of the original mass and was not significantly (P>0.05, paired

3 t-test) different from that of nonsterilized needles (77±1%, mean ± standard error, n=10). No

4 *Lophodermium* fruiting bodies were formed on sterilized needles after six months of

5 incubation on the forest floor (i.e., frequency of occurrence equaled to 0%), whereas the

6 frequency of occurrence on nonsterilized needles was 20±4% (mean ± standard error, n=10).

7

8 **3.3 Pure culture decomposition test**

9 Lignin and total carbohydrate contents of initial needles were 373 mg/g and 420 mg/g,

10 respectively, for *P. thunbergii* and 374 mg/g and 407 mg/g, respectively, for *P. densiflora*.

11 The mass loss of pine needles caused by 14 isolates under the pure culture conditions

12 ranged from -0.4% to 19.9% (Table 3). The mass loss of needles decomposed by *L. pinastri*

13 was lower for *P. thunbergii* (-0.4% to 7.8%) than for *P. densiflora* (8.5% to 19.9%). The mass

14 loss of needles decomposed by five other fungi ranged from 0.3% to 6.4%. Mass loss of

15 lignin was detected for five isolates of *L. pinastri* (TL10, DHL2, DHL4, DHL3, and DHL1)

16 and was greater in *P. densiflora* than in *P. thunbergii*. Mass loss of lignin was negative for *L.*

17 *pinastri* TF3, *Pestalotiopsis* sp. O5, and Xylariales sp. O3 (Table 3). L/W for five ligninolytic

isolates of *L. pinastri* ranged from 1.31 to 1.96 and L/C ranged from 0.69 to 2.34.

### 3.4 Genetic variation of *Lophodermium pinastri*

The neighbor-Joining tree showed that nine strains identified as *L. pinastri* by their morphological characters were divided into two close clades (Fig. 1). These clades corresponded to their host species: one consisted of strains isolated from *P. densiflora* (95% of bootstrap value) and another from *P. thunbergii* (100% of bootstrap value). The percentage of nucleotide differences was 6.3% to 7.3% between *L. pinastri* isolates associated with *P. densiflora* and *P. thunbergii*.

## 4 Discussion

Differences in needle mass per length and lignin content between needle portions bearing *Lophodermium* fruiting bodies and the remaining needle portions (Table 2) indicated small-scale heterogeneity of decomposition among single needles that were associated with their colonization by fungi. It is not probable that the lower needle mass per length and lignin content in needle portions bearing *Lophodermium* fruiting bodies reflected some original

1 variation among undecomposed needles; rather, it was probably due to the more active
2 decomposition of needle tissues and lignin by *L. pinastri*. In fact, the greater lengths of total
3 and live hyphae in needle portions bearing *Lophodermium* fruiting bodies than in the
4 remaining needle portions (Table 2) indicated the active ingrowth and respiration of *L.*
5 *pinastri* within the needles. Live hyphae are located at the periphery of mycelia and account
6 for no more than 10% of the total hyphal length in general (Kjøller and Struwe 1982), and
7 their abundance is correlated with the respiration rate of fungi (Ingham and Klein 1984; Bååth
8 and Söderström 1988). The results of pure culture decomposition tests (Table 3) also provided
9 positive support for the potential activity of *L. pinastri* to decompose lignin in needle tissues.
10 Another possible explanation for the lower needle mass per length and lignin content
11 in needle portions bearing *Lophodermium* fruiting bodies is the prior colonization of dead
12 needles by *L. pinastri*. *Lophodermium* species are one of the dominant endophytes of
13 healthy-looking needles of *Pinus thunbergii* and *P. densiflora* in Japan (Hata et al. 1998). The
14 observation that *Lophodermium* fruiting bodies did not occur on previously sterilized,
15 endophyte-free needles indicated that *L. pinastri* can only colonize live needles on the branch.
16 This life-style gives *L. pinastri* an advantage for colonizing needle tissues and utilizing
17 available resources for growth in advance of other saprobic fungi that colonize needles after

1 needle fall. The prior colonization of needle tissues at the onset of needle senescence has been

2 described in detail for rhytismataceous endophytes (Stone 1987; Osorio and Stephan 1991).

3 Prior colonization and lignin decomposition by endophytes was also demonstrated for

4 *Coccomyces* sp. on *Camellia japonica* leaves (Koide et al. 2005a; Osono and Hirose 2009).

5 The observed L/W of 1.31 to 1.96 and L/C of 0.69 to 2.34 for ligninolytic *L. pinastri*

6 suggest that they have a potential ability to decompose lignin selectively, according to the

7 criteria of Worrall et al. (1997). These values are among the highest in the range previously

8 reported for litter-decomposing fungi from temperate forests (Osono and Takeda 2002, 2006;

9 Osono et al. 2003, 2006, 2008a, 2008b, 2009) and are comparable to those reported for

10 ligninolytic basidiomycetes, but are slightly lower than those reported for *Coccomyces* species

11 in the Rhytismataceae, such as those on *Camellia japonica* (L/C of 4.2 to 5.6, Koide et al.

12 2005b) and on *Gaultheria shallon* (L/W of 2.6 and L/C of 4.7 to 6.5, Osono et al. 2008a). The

13 negative values of mass loss of lignin for a few isolates can be due to the formation of

14 acid-insoluble substances that are registered as 'lignin' fractions (Oosno et al. 2006).

15 It should be noted that not all of *L. pinastri* isolates from *Pinus thunbergii* and *P.*

16 *densiflora* needles were ligninolytic, and that the ability to decompose needles was highly

17 variable among the isolates (Table 3). Similar variation in mass loss has been reported, for

example *Xylaria* sp. on *Fagus crenata* leaves (ranging from 5.5% to 9.9% for 9 isolates, Osono and Takeda 2002) and for *Coccomyces* sp. on *Camellia japonica* leaves (from 7.1% to 14.8% for 3 isolates, Koide et al. 2005b); the variation in needle mass loss for *L. pinastri* on pine was, however, among the greatest so far reported. The variation in the base sequence of the rDNA internal transcribed spacer (ITS) region was relatively small when compared among the isolates from a single host tree species (Fig. 1). The among-isolate variation in decomposing ability cannot be related to ITS variability and thus remains unclear. The variation, however, may imply further heterogeneity in decomposition among needle portions bearing *Lophodermium* fruiting bodies on single needles that reflects the among-isolate variation in decomposing abilities.

The mass loss of *Pinus densiflora* needles was greater than that of *P. thunbergii* needles when inoculated with *L. pinastri* isolates from the respective needles (Table 3). Two explanations may account for this difference between these two pine species. First, the physical and chemical qualities of needles can be different between two pine species. *Pinus thunbergii* needles have a more rigid structure, are generally harder and stronger, and appear to be more resistant to fungal decomposition than *P. densiflora* needles. The lignin and total carbohydrate contents of needles used in the pure culture decomposition tests were similar

1 between these pines, but other chemical properties such as secondary compounds, wax and
2 resin, and nutrient contents might be different. Secondly, the potential abilities to grow and
3 decompose needles may be different between *L. pinastri* isolates obtained from the two pine
4 species. This is possible because a comparison of the rDNA-ITS sequences between the two
5 groups indicated that they are phylogenetically separated (Fig. 1). Future cross-inoculation
6 and decomposition experiments (two needles x two groups of isolates) will be of help to test
7 the two possibilities mentioned above.

8 Ortiz-Garcia et al. (2003) indicated that the intraspecific percentage of nucleotide
9 differences was 5.6 % between *L. pinastri* isolates from *P. sylvestris* and *P. ponderosa*
10 (AY100649 and AY100650, respectively; Fig. 1) and ranged from 0% to 7.6% between *L.*
11 *baculiferum* isolates from three different pine species. The higher nucleotide differences
12 between *L. pinastri* isolates from *P. densiflora* and *P. thunbergii* in the present study were
13 consistent with the previous studies and suggested that *L. pinastri* has potentially high ITS
14 intraspecific variations as well as *L. baculiferum*. The intraspecific nucleotide divergence in *L.*
15 *pinastri* could not exactly correlate with the phylogenetic relationships of the host, because
16 ITS sequences of *L. pinastri* isolates from *P. densiflora* in section *Pinus* were similar to *P.*
17 *ponderosa* in section *Trifolius* (Farjon 2005) and formed a clade with high bootstrap support

(95%) (Fig. 1).

Previous studies on fungal assemblages and their succession have shown that the major fungi potentially responsible for lignin decomposition in pine needles are basidiomycetes such as *Marasmius* species occurring in early stages of decomposition (Tokumasu 1996, 1998) and *Gymnopus* species in late stages (Soma and Saito 1979). On the other hand, ascomycete and zygomycete species have been regarded as cellulolytic or sugar fungi, including *L. pinastri,* the cellulolytic activity of which has been demonstrated in substrate utilization tests (Sieber-Canavesi et al. 1991) and in field observations (Mitchell and Millar 1978; Ponge 1991). Therefore, the present study is the first to demonstrate that *L. pinastri* in the Ascomycetes has the ability to decompose lignin in pine needles *in vitro* and *in vivo,* but with significant variation of ligninolytic abilities among isolates. The lignin decomposition caused by *L. pinastri* in early stages of decomposition will provide a unique situation for subsequent decomposition processes of pine needles. Koide et al. (2005a, 2005b) reported a similar situation in which the colonization of *Camellia japonica* leaves by an endophyte *Coccomyces* sp. led to selective delignification in the initial stage of decomposition; Osono and Hirose (2009) demonstrated that the prior delignification by *Coccomyces* sp. influenced the subsequent decomposition by fungal colonizers. Further

studies will be necessary on the relative importance of environmental, litter quality, and genetic factors on the ligninolytic activity of *L. pinastri* on pine needles and its ecological relevance to fungal succession and needle decomposition. Use of specific PCR primers to detect mycelia of *L. pinastri* will be practical and promising to prove the colonization of this fungus within needles.

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13

1 Osono and Hirose Table 1

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4

5 *Table 1*. DNA sequence analysis results for the rDNA ITS region based on BLAST searches
6 for five isolates of species other than *Lophodermium pinastri* obtained from *Pinus thunbergii*
7 needles.

| Strain Code | DDBJ accession number | BLAST Match Taxon (Accession number) | Sequence similarity (%) | Score (Expected value) | Estimate of taxonomic affinities |
|---|---|---|---|---|---|
| O1 | AB511811 | *Phoma* sp. (FJ228203) | 98 | 970 (0.0) | Leotiomycete sp. |
| O2 | AB511812 | *Phoma* sp. (FJ228203) | 99 | 985 (0.0) | Leotiomycete sp. |
| O3 | AB511813 | Leaf litter ascomycete (AF502740) | 97 | 928 (0.0) | Xylariales sp. |
| O4 | AB511814 | *Scleroconidioma sphagnicola* (DQ182416) | 99 | 1046 (0.0) | *Scleroconidioma* sp. |
| O5 | AB511815 | *Pestalotiopsis lespedezae* (EF055200) | 99 | 1099 (0.0) | *Pestalotiopsis* sp. |

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1 Osono and Hirose Table 2

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5 *Table 2*. Needle mass per length, contents of lignin and total carbohydrates, and total and live
6 hyphal length in needle portions bearing *Lophodermium* fruiting bodies and remaining
7 portions of *Pinus thunbergii* needle litter at Mt. Tanakami. Paired t-test, *** P<0.001, **
8 P<0.01, * P<0.05, ns non significant.

| | N | Needle portions bearing *Lophodermium* fruiting bodies | Remaining needle portion | P |
|---|---|---|---|---|
| **Physical and chemical properties** | | | | |
| Needle mass per length (mg/mm) | 4 | 12.4±0.0 | 16.7±0.0 | *** |
| Lignin content (mg/g) | 4 | 362±2 | 392±10 | * |
| Total carbohydrate content (mg/g) | 4 | 528±17 | 494±10 | ns |
| **Hyphal length** | | | | |
| Total (m/g) | 5 | 3393±181 | 1821±105 | *** |
| Live (m/g) | 5 | 233±42 | 22±12 | * |
| % live hyphae | 5 | 7±1 | 1±1 | ** |

9 % live hyphae = live hyphal length / total hyphal length x 100.

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1 Osono and Hirose Table 3

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5 *Table 3*. Mass loss (% original mass) of needle litter, lignin, and total carbohydrates of *Pinus*
6 *thunbergii* and *P. densiflora* needle litter, and lignin/needle mass loss ratio (L/W) and
7 lignin/carbohydrate loss ratio (L/C) caused by *Lophodermium pinastri* and other needle
8 microfungi. Values indicate means ± standard errors (N=4). nd not determined.

| Substratum | Fungus | Code | Needle | Lignin | Total carbohydrates | L/W | L/C |
|---|---|---|---|---|---|---|---|
| *P. thunbergii* | *L. pinastri* | TL10 | 7.8±1.0 | 10.2 | 14.7 | 1.31 | 0.69 |
| | *L. pinastri* | TF3 | 3.6±0.7 | -3.1 | 7.6 | -0.85 | -0.40 |
| | *L. pinastri* | TL4 | 0.7±0.4 | nd | nd | nd | nd |
| | *L. pinastri* | TL3 | 0.1±0.5 | nd | nd | nd | nd |
| | *L. pinastri* | TL1 | -0.4±0.6 | nd | nd | nd | nd |
| | *Pestalotiopsis* sp. | O5 | 6.4±0.4 | -5.3 | 22.4 | -0.83 | -0.24 |
| | Xylariales sp. | O3 | 3.9±0.4 | -3.7 | 8.9 | -0.95 | -0.41 |
| | Leotiomycete sp. | O1 | 2.2±0.4 | nd | nd | nd | nd |
| | *Scleroconidioma* sp. | O4 | 0.7±0.2 | nd | nd | nd | nd |
| | Leotiomycete sp. | O2 | 0.3±0.4 | nd | nd | nd | nd |
| *P. densiflora* | *L. pinastri* | DHL2 | 19.9±1.5 | 31.7 | 20.9 | 1.59 | 1.52 |
| | *L. pinastri* | DHL4 | 14.4±1.5 | 25.2 | 18.1 | 1.75 | 1.40 |
| | *L. pinastri* | DHL3 | 13.4±0.9 | 18.6 | 10.7 | 1.39 | 1.74 |
| | *L. pinastri* | DHL1 | 8.5±1.7 | 16.7 | 7.1 | 1.96 | 2.34 |

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1 Figure legend

2

3 *Fig. 1*. Neighbour-joining tree based on the ITS1-5.8s-ITS2 rDNA sequences of

4 *Lophodermium* spp. including the strains examined in this study (see Table 2). GenBank

5 accession numbers are shown in parentheses. Bootstrap values above 50% are given adjacent

6 to the corresponding node. TL3, TL4, TF3, TL10, and TL1 are the isolates from *Pinus*

7 *thunbergii*, and DHL1, DHL2, DHL3, and DHL4 are from *P. densiflora*. DDBJ accession

8 number: TL10, AB519647; TF3, AB519646; TL4, AB247949; TL3, AB520991; TL1,

9 AB519648; DHL2, AB511817; DHL4, AB511819; DHL3, AB511818; DHL1, AB511816.

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1 Osono and Hirose Fig. 1.

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