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

1	greater in needle portions bearing Lophodermium fruiting bodies than in the remaining needle
2	portions. Lophodermium fruiting bodies were not formed on sterilized needles after a
3	six-month incubation on the forest floor, whereas they formed on 20% of nonsterilized
4	needles, indicating that this fungus can only colonize live needles on the branch. In pure
5	culture decomposition tests, mass loss of lignin was detected for several isolates of L. pinastri,
6	but was variable among isolates and between the needles of two pine species. A comparison
7	of the rDNA-ITS sequences between L. pinastri isolates of P. thunbergii and P. densiflora
8	indicated that the two groups were phylogenetically separated. This study is the first to show
9	that L. pinastri has the ability to decompose lignin in dead pine needles.
10	
11	1 Introduction
12	
13	Lophodermium pinastri (Schrad.) Chevall. is an ascomycete species in the Rhytismataceae
14	and has intimate relationships with pines (Pinus spp.) as a supposed pathogen (Sakuyama
15	1993), as an endophyte (Hata and Futai 1996; Hata et al. 1998), and as a saprobe (Kendrick

- 16 and Burges 1962; Soma and Saito 1979; Tokumasu 1996, 1998; Hirose and Osono 2006). In
- 17 natural environments L. pinastri colonizes healthy needles latently as an endophyte, starts

1	active hyphal growth at the onset of needle senescence, and sporulates after needle abscission
2	to infect live needles again to complete its life cycle. This fungus is a dominant colonizer of
3	dead needles in early stages and takes part in needle decomposition as a saprobe (Hirose and
4	Osono 2006). Sieber-Canavesi et al. (1991) showed in substrate utilization tests the
5	production of cellulolytic enzymes by isolates of Lophodermium spp. from European conifers.
6	Little is known, however, about its potential abilities to colonize fallen needles and to
7	decompose litter and about its roles in the chemical changes in dead needles.
8	This study examined (i) the fungal biomass and chemical changes in needles
9	senesced and previously colonized by L. pinastri under field conditions, (ii) the colonization
10	of sterilized needles by L. pinastri on the forest floor, and (iii) the decomposition of needle
11	litter by L. pinastri under pure culture conditions. Needles of Pinus thunbergii and P.
12	densiflora were used as materials in the present study because the frequent occurrence of L.
13	pinastri on these needles was previously documented (Tokumasu 1996, 1998; Hata et al.
14	1998). We hypothesized that the prior colonization of needles by endophytic L. pinastri
15	enhanced the decomposition of needle tissues within their colonies, leading to heterogeneity
16	of decomposition among single needles, and that L. pinastri is able to decompose lignin in
17	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
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5	2.1 Study site and sample collection
6	Samples were collected from a <i>P. thunbergii</i> 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

1	Lophodermium spp. on P. thunbergii needles mostly consisted of L. pinastri but possibly
2	included other species, such as L. conigenus, with low frequency. In the present study, we
3	referred to Lophodermium fruiting bodies on pine needles as L. pinastri for the sake of
4	simplicity. The remaining needle portion without Lophodermium ascomata or conidiomata
5	was used for comparison. The remaining needle portions might have been colonized by
6	Lophodermium or other fungi, or be devoid of fungal colonization, but no fruiting bodies of
7	fungi were apparent.
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9	2.2 Measurement of needle mass per length, chemical analysis, and hyphal length
9 10	2.2 Measurement of needle mass per length, chemical analysis, and hyphal length estimation
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9 10 11 12	2.2 Measurement of needle mass per length, chemical analysis, and hyphal length estimation The length of needle portions bearing <i>Lophodermium</i> fruiting bodies and the remaining portions was measured under a binocular microscope with a magnification of 40x. Needle
9 10 11 12 13	2.2 Measurement of needle mass per length, chemical analysis, and hyphal length estimation The length of needle portions bearing <i>Lophodermium</i> fruiting bodies and the remaining portions was measured under a binocular microscope with a magnification of 40x. Needle portions bearing <i>Lophodermium</i> fruiting bodies and the remaining needle portions were then
9 10 11 12 13 14	2.2 Measurement of needle mass per length, chemical analysis, and hyphal length estimation The length of needle portions bearing <i>Lophodermium</i> fruiting bodies and the remaining portions was measured under a binocular microscope with a magnification of 40x. Needle portions bearing <i>Lophodermium</i> 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
<ul> <li>9</li> <li>10</li> <li>11</li> <li>12</li> <li>13</li> <li>14</li> <li>15</li> </ul>	2.2 Measurement of needle mass per length, chemical analysis, and hyphal length estimation The length of needle portions bearing <i>Lophodermium</i> fruiting bodies and the remaining portions was measured under a binocular microscope with a magnification of 40x. Needle portions bearing <i>Lophodermium</i> 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 <i>Lophodermium</i> fruiting bodies and the remaining needle
<ul> <li>9</li> <li>10</li> <li>11</li> <li>12</li> <li>13</li> <li>14</li> <li>15</li> <li>16</li> </ul>	2.2 Measurement of needle mass per length, chemical analysis, and hyphal length estimation The length of needle portions bearing <i>Lophodermium</i> fruiting bodies and the remaining portions was measured under a binocular microscope with a magnification of 40x. Needle portions bearing <i>Lophodermium</i> 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 <i>Lophodermium</i> fruiting bodies and the remaining needle portions were used to calculate the needle mass per length (mg/mm).

17 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.

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## 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
10 11	Five isolates of <i>L. pinastri</i> and five isolates of other species obtained from <i>P. thunbergii</i> needles collected in the study site in May 2003 (Hirose and Osono 2006) were used for the
10 11 12	Five isolates of <i>L. pinastri</i> and five isolates of other species obtained from <i>P. thunbergii</i> needles collected in the study site in May 2003 (Hirose and Osono 2006) were used for the pure culture decomposition test (Koide et al. 2005b). Senesced needles of <i>P. thunbergii</i> fallen
10 11 12 13	Five isolates of <i>L. pinastri</i> and five isolates of other species obtained from <i>P. thunbergii</i> needles collected in the study site in May 2003 (Hirose and Osono 2006) were used for the pure culture decomposition test (Koide et al. 2005b). Senesced needles of <i>P. thunbergii</i> fallen on the forest floor were collected in the study site in November 2007 and oven-dried at 40°C
10 11 12 13 14	Five isolates of <i>L. pinastri</i> and five isolates of other species obtained from <i>P. thunbergii</i> needles collected in the study site in May 2003 (Hirose and Osono 2006) were used for the pure culture decomposition test (Koide et al. 2005b). Senesced needles of <i>P. thunbergii</i> fallen on the forest floor were collected in the study site in November 2007 and oven-dried at 40°C for one week. The needles were preserved in a vinyl bag until the experiment began.
<ol> <li>10</li> <li>11</li> <li>12</li> <li>13</li> <li>14</li> <li>15</li> </ol>	Five isolates of <i>L. pinastri</i> and five isolates of other species obtained from <i>P. thunbergii</i> needles collected in the study site in May 2003 (Hirose and Osono 2006) were used for the pure culture decomposition test (Koide et al. 2005b). Senesced needles of <i>P. thunbergii</i> fallen on the forest floor were collected in the study site in November 2007 and oven-dried at 40°C for one week. The needles were preserved in a vinyl bag until the experiment began. Needles were cut into pieces 2 cm in length and sterilized by exposure to ethylene
<ol> <li>10</li> <li>11</li> <li>12</li> <li>13</li> <li>14</li> <li>15</li> <li>16</li> </ol>	Five isolates of <i>L. pinastri</i> and five isolates of other species obtained from <i>P. thunbergii</i> needles collected in the study site in May 2003 (Hirose and Osono 2006) were used for the pure culture decomposition test (Koide et al. 2005b). Senesced needles of <i>P. thunbergii</i> fallen on the forest floor were collected in the study site in November 2007 and oven-dried at 40°C for one week. The needles were preserved in a vinyl bag until the experiment began. Needles were cut into pieces 2 cm in length and sterilized by exposure to ethylene oxide gas at 60°C for 6 h. Needles (0.3 g) were placed on the surface of Petri dishes (9 cm in

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)

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## 2.5 DNA methods and phylogenetic analysis

- 5 DNA of fourteen isolates used in the pure culture tests was extracted from mycelia cultured 6 on 2.5% malt extract liquid medium following the modified CTAB method described by 7 Matsuda and Hijii (1999). The rDNA ITS region was amplified with primers ITS1F (Gardes 8 and Bruns 1993) and ITS4 (White et al. 1990). Polymerase chain reactions were performed 9 using HotStarTaq Plus Master Mix (Qiagen, Ontario, Canada). Each PCR tube contained a 50 10  $\mu$ 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 11 load and 0.5  $\mu$ l of each primer (final, 0.25  $\mu$ M)). Each DNA fragment was amplified using a 12 PCR thermal cycler (DNA engine; Bio-Rad, USA). The thermal cycling schedule was as 13 follows: the first cycle consisted of 5 min at 94°C, followed by 45 cycles of 30 s at 94°C, 30 s 14 at 58°C for annealing, 1 min at 72°C, and the final cycle of 10 min at 72°C. The reaction 15 mixture was then cooled at 4°C for 5 min. PCR products were purified with a QiAquick PCR 16 Purification Kit (Qiagen, Ontario, Canada).
- 17 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	

**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 $\pm$ 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 $\pm$ 1%, mean $\pm$ 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 $20\pm4\%$ (mean $\pm$ 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 <i>P. thunbergii</i> and 374 mg/g and 407 mg/g, respectively, for <i>P. densiflora</i> .
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 <i>P. thunbergii</i> (-0.4% to 7.8%) than for <i>P. densiflora</i> (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 <i>P. densiflora</i> than in <i>P. thunbergii</i> . Mass loss of lignin was negative for <i>L</i> .
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.

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## 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*.

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## 4 Discussion

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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 <i>Pinus thunbergii</i> and <i>P. densiflora</i> 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

1	example Xylaria sp. on Fagus crenata leaves (ranging from 5.5% to 9.9% for 9 isolates,
2	Osono and Takeda 2002) and for Coccomyces sp. on Camellia japonica leaves (from 7.1% to
3	14.8% for 3 isolates, Koide et al. 2005b); the variation in needle mass loss for L. pinastri on
4	pine was, however, among the greatest so far reported. The variation in the base sequence of
5	the rDNA internal transcribed spacer (ITS) region was relatively small when compared
6	among the isolates from a single host tree species (Fig. 1). The among-isolate variation in
7	decomposing ability cannot be related to ITS variability and thus remains unclear. The
8	variation, however, may imply further heterogeneity in decomposition among needle portions
9	bearing Lophodermium fruiting bodies on single needles that reflects the among-isolate
10	variation in decomposing abilities.
11	The mass loss of Pinus densiflora needles was greater than that of P. thunbergii
12	needles when inoculated with L. pinastri isolates from the respective needles (Table 3). Two
13	explanations may account for this difference between these two pine species. First, the
14	physical and chemical qualities of needles can be different between two pine species. Pinus
15	thunbergii needles have a more rigid structure, are generally harder and stronger, and appear
16	to be more resistant to fungal decomposition than P. densiflora needles. The lignin and total
17	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 <i>L. baculiferum</i> . The intraspecific nucleotide divergence in <i>L</i> .
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

1 (95%) (Fig. 1).

2 Previous studies on fungal assemblages and their succession have shown that the 3 major fungi potentially responsible for lignin decomposition in pine needles are 4 basidiomycetes such as Marasmius species occurring in early stages of decomposition 5 (Tokumasu 1996, 1998) and Gymnopus species in late stages (Soma and Saito 1979). On the 6 other hand, ascomycete and zygomycete species have been regarded as cellulolytic or sugar 7 fungi, including L. pinastri, the cellulolytic activity of which has been demonstrated in 8 substrate utilization tests (Sieber-Canavesi et al. 1991) and in field observations (Mitchell and 9 Millar 1978; Ponge 1991). Therefore, the present study is the first to demonstrate that L. 10 pinastri in the Ascomycetes has the ability to decompose lignin in pine needles in vitro and in 11 vivo, but with significant variation of ligninolytic abilities among isolates. The lignin 12 decomposition caused by L. pinastri in early stages of decomposition will provide a unique 13 situation for subsequent decomposition processes of pine needles. Koide et al. (2005a, 2005b) 14 reported a similar situation in which the colonization of Camellia japonica leaves by an 15 endophyte Coccomyces sp. led to selective delignification in the initial stage of decomposition; Osono and Hirose (2009) demonstrated that the prior delignification by 16 17 Coccomyces sp. influenced the subsequent decomposition by fungal colonizers. Further

1	studies will be necessary on the relative importance of environmental, litter quality, and
2	genetic factors on the ligninolytic activity of L. pinastri on pine needles and its ecological
3	relevance to fungal succession and needle decomposition. Use of specific PCR primers to
4	detect mycelia of L. pinastri will be practical and promising to prove the colonization of this
5	fungus within needles.
6	
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8	
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15	
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12	
13	

- 1 Osono and Hirose Table 1
- 2
- 3
- 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	DDBJ	BLAST Match	Sequence	Score	Estimate of
Code	accession	Taxon	similarity	(Expected	taxonomic affinities
	number	(Accession number)	(%)	value)	
01	AB511811	<i>Phoma</i> sp. (FJ228203)	98	970 (0.0)	Leotiomycete sp.
02	AB511812	<i>Phoma</i> 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)	<i>Scleroconidioma</i> sp.
05	AB511815	Pestalotiopsis lespedezae (EF055200)	99	1099 (0.0)	Pestalotiopsis sp.

- 1 Osono and Hirose Table 2

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

	N	Needle portions bearing <i>Lophodermium</i> fruiting bodies	Remaining needle portion	Р
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.

- J

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	L/W	L/C
					carbohydrates		
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	$0.7\pm0.4$	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	05	$6.4\pm0.4$	-5.3	22.4	-0.83	-0.24
	sp.						
	Xylariales sp.	O3	3.9±0.4	-3.7	8.9	-0.95	-0.41
	Leotiomycete sp.	01	2.2±0.4	nd	nd	nd	nd
	Scleroconidioma	O4	$0.7\pm0.2$	nd	nd	nd	nd
	sp.						
	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

- 1 Figure legend

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.
10	



0.0\*