Selective lignin decomposition and nitrogen mineralization in forest litter colonized by *Clitocybe* sp.

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1 Running title: Bleached litter decomposition by *Clitocybe* sp.

2

3 ## Abstract

4

5 Fruiting bodies of *Clitocybe* sp. were encountered in association with partly
6 decomposed litter materials that were bleached due to colonization by mycelia of
7 this fungus. We clarified the impact of this fungus on the chemical and fungal
8 properties of litter materials and quantitatively evaluated the potential ability of
9 this fungus to cause selective decomposition of recalcitrant compounds such as
10 lignin. The content of acid-unhydrolyzable residues (AUR) was lower, and the
11 contents of soluble carbohydrates and nutrients (N, P, K, Ca, Mg) and net
12 mineralization rate of N were higher in bleached litter materials (BLM) than in
13 adjacent nonbleached litter materials (NBL). Total hyphal length was 5.4 times
14 greater in BLM than in NBL. A total of 49 fungal taxa were isolated, 30 from BLM
15 and 42 from NBL. In pure culture decomposition tests, *Clitocybe* sp. caused
16 greater mass loss in partly decomposed leaves than in freshly fallen leaves, which
17 was attributed to the greater mass loss of AUR and more selective decomposition

of AUR in partly decomposed leaves. Fourier transform infrared (FT-IR) spectroscopy showed that *Clitocybe* sp. was responsible for the selective transformation of the chemical structure of lignin. These results showed that *Clitocybe* sp. had a marked ability to remove AUR and lignin selectively from partly decomposed leaves and to enhance N mineralization, contributing to small-scale heterogeneity of the decomposition within the forest floor.

decomposition / forest floor material / FT-IR / fungi / litter-decomposing basidiomycetes

1. Introduction

Litter-decomposing basidiomycetes (LDB) are major components of saprobic fungal assemblages in forest soils and play central roles in decomposition of recalcitrant compounds in litter, such as lignin, that often limit carbon and nutrient cycling in the soils [3, 25]. A suite of LDB with abilities to selectively remove lignin and other recalcitrant compounds from litter materials are of

particular importance because these fungi have been shown to attack these compounds at a low or minimal expense of polymer carbohydrates. This often leads to the whitening (denoted here as bleaching) of litter materials and significant variations in physical and chemical properties of the litter within the forest floor [12, 13, 40]. Activities of ligninolytic enzymes produced by LDB have been studied recently [e.g. 43, 44, 45], and activities of LDB that might have the potential to cause mass loss of recalcitrant compounds have been investigated in pure culture decomposition tests [e.g. 20, 22]. However, their impacts on the composition of organic chemical constituents and nutrients, nutrient flux, and fungal abundance and diversity in litter materials have not been fully evaluated. Here we raise two particular questions regarding the functions of LDB causing selective decomposition of recalcitrant compounds in forest soils. First, how are decomposing abilities of LDB causing selective decomposition different from those of other major basidiomycetes and ascomycetes in a particular forest soil? Secondly, what are the roles of LDB in the transformation of partly decomposed materials enriched in recalcitrant compounds on the forest floor, taking into consideration the regular observations that mycelia of LDB are often associated

with partly decomposed materials beneath freshly fallen litter [13, 22]?

In a cool temperate forest of central Japan, fruiting bodies of *Clitocybe* sp. were encountered in association with partly decomposed litter materials that were bleached due to the colonization by mycelia of this fungus (Fig. 1). The purposes of the present study were to clarify the impact of this fungus on the transformation of litter materials and to quantitatively evaluate the ability of this fungus to cause selective decomposition of recalcitrant compounds, especially lignin. First, we determined the chemical composition, nutrient flux, and fungal abundance and diversity of litter materials bleached by *Clitocybe* sp. to confirm that this fungus is the primary agent responsible for the bleaching and transformation of litter materials. Second, we evaluated the ability of *Clitocybe* sp. to cause selective decomposition of recalcitrant compounds in partly decomposed leaves, in comparison with freshly fallen leaves, under pure culture decomposition tests and compared it with the decomposing activities of other major saprobic fungi found in the study site. Finally, we applied Fourier transform infrared (FT-IR) spectroscopy to the analysis of chemical structure of lignin and carbohydrate components in litter materials colonized by *Clitocybe* sp. FT-IR

spectroscopy measures the absorbance versus wavenumber (or equivalently, wavelength) of light to detect the vibration characteristics of chemical functional groups in a sample (Table 1) [23] and has been used to characterize chemical changes in wood decomposed by fungi and other microbes [9, 10, 11, 37, 38].

2. Materials and methods

2.1. Study area

Samples were collected in Ashiu Experimental Forest of Kyoto University (35°18'N and 135°43'E), Kyoto, Japan. Over the past 29 years the mean annual temperature has been 11.7°C and the mean monthly temperature has ranged from 0.4°C in January to 25.5°C in August at the office of Ashiu Experimental Forest about 5 km from the study site. The mean annual precipitation over the past 29 years is 2353 mm. The study area is covered with snow from December to April. The study site (altitude 660 m) is located in a mountainous area of a cool temperate natural forest dominated by *Fagus crenata* Bl. and *Quercus crispula* Bl. The site has been intact since at least 1898.

In the study site, fruiting bodies of *Clitocybe* sp. were occasionally encountered. Field surveys conducted nine times during the growing season between May and November 2001 indicated that fruiting bodies of this fungus were encountered at 2 out of 125 grids (2 x 2 m) in the study site, with a frequency of occurrence of 1.6% (T. Osono, unpublished data). Fruiting bodies were regularly associated with litter materials which were located at the boundary between the litter layer and the mineral soil layer, and which were bleached due to colonization by the mycelia of this fungus (Fig. 1). These litter materials are denoted here as bleached litter material (BLM). In contrast, adjacent brown litter materials were not bleached by any macrofungi and are denoted as nonbleached litter material (NBL). The boundary between BLM and NBL was sharp, and could be distinguished easily.

2.2. Sample collection

BLM and adjacent NBL (approx. 4 x 4 cm in area) were collected in the study site, a total of three pairs of BLM and NBL in November 1999 and an additional five pairs in December 2000. These samples were placed in paper bags

and taken to the laboratory. A portion of litter materials was dried at 40°C for one week to determine field moisture content and to be used for chemical analyses. The remaining samples were used for measurement of nitrogen (N) transformation, estimation of hyphal length, and isolation of fungi.

2.3. Chemical analyses

The dried samples of BLM and NBL collected in 1999 and 2000 were ground in a laboratory mill to pass through a 0.5-mm screen. The content of acid-unhydrolyzable residues (AUR) was estimated by gravimetry according to a standardized method using hot sulfuric acid digestion [17]. The amount of carbohydrates was estimated by means of the phenol-sulfuric acid method [5]. Contents of soluble carbohydrates and polyphenols were measured with the phenol-sulfuric acid method and Folin-Ciocalteau method [46], respectively. The methods were described in detail previously [35]. The AUR fraction contains not only true lignin but also cutin, tannin, and other secondary compounds and humic substances produced during decomposition [25]. In this study, the term AUR is used for the suite of these recalcitrant compounds for the sake of simplicity. The

holocellulose fraction was calculated as the difference between the total carbohydrate and the soluble carbohydrate.

Total N content was measured by automatic gas chromatography (NC analyzer SUMIGRAPH NC-900, Sumitomo Chemical, Osaka, Japan). After an acid wet oxidation in HNO_3 + $NClO_4$, the molybdate-ascorbic acid method was performed for P [24], flame photometry for K, and atomic absorption for Ca and Mg (Atomic absorption spectrophotometer 170-30S, Hitachi, Tokyo, Japan).

BLM and NBL collected in 2000 were used to estimate N transformation rates using an aerobic laboratory incubation method [14]. The samples were processed within 24 hours of sampling. Approximately 5 g (fresh weight) of each sample were incubated for 28 days at 30°C. Field moisture contents were used to convert fresh weight to dry weight. Both incubated and fresh samples were extracted with 2M KCl. The NH_4-N concentrations in extracts were measured by the indophenol blue method, and NO_3-N concentrations were measured colorimetrically after zinc reduction [16]. Net mineralization rates were calculated by subtracting the initial inorganic N (NH_4-N + NO_3-N) concentrations in extracts from the final concentrations. The net nitrification rate was calculated

by subtracting the initial NO_3-N concentration from the final NO_3-N concentration.

2.4. Hyphal length estimation

BLM and NBL collected in 1999 were used for hyphal length estimation by means of the agar-film method [15] but with several modifications [34]. The samples were processed within 24 hours of sampling. The methods were described in detail previously [35]. Total hyphal length was calculated as the sum of the length of hyaline hyphae stained with fluorescent brightener and that of darkly pigmented hyphae.

2.5. Isolation of fungi

A modified washing method was applied to BLM and NBL collected in 1999, and fungi were isolated following the methods described in detail previously [30]. Ten litter pieces (approx. 5 x 5 mm) were arbitrarily chosen from each BLM or NBL, making a total of 60 pieces (30 BLM and 30 NBL pieces) from three pairs of BLM and NBL. A modified lignocellulose agar (LCA) was used to isolate fungi.

LCA contains glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, and agar 1.3% (w/v) [21]. Note that the modified LCA does not contain lignin or other recalcitrant compounds. Plates were incubated at 20°C in the dark and observed at 3 days and at 1, 4, and 8 weeks after washing. Identification was based on micromorphological observations, with reference to the literature [4, 6, 7]. The frequency of individual species was calculated as a percentage of incidences based on the number of litter pieces with the species among the 30 pieces tested for BLM and NBL.

2.6. Pure culture decomposition test

Four isolates of four fungal species (Table 2) were used for the pure culture decomposition test [29]. An isolate of *Clitocybe* sp. was obtained from pileus tissue of a fruiting body collected in November 1999. An isolate of *Mycena polygramma* was obtained from the study site in November 1997 and deposited in a culture collection (IFO33011). Fruiting bodies of *M. polygramma* occurred with the second greatest frequency of LDB in the study site, with a frequency of occurrence of 15.2% (19 out of 125 grids) in the 2001 survey (T. Osono,

unpublished data). Isolates of *Xylaria* sp. (GS91) and *Geniculosporium* sp. (LS32) were obtained from *F. crenata* leaf litter collected in the study site in October 1996 and used in previous decomposition tests [26, 28]. *Xylaria* sp. and *Geniculosporium* sp. belong to the Xylariaceae, Ascomycetes and were major non-basidiomycetous fungi associated with the decomposition of AUR in freshly fallen leaves of *F. crenata* in the study site [27]. In the decomposition tests, therefore, we can compare the decomposing ability of *Clitocybe* sp. with those of major saprobic fungi associated with AUR decomposition in the study site.

Two types of leaf litter, freshly fallen and partly decomposed leaves of *F. crenata*, were used in the decomposition test. Freshly fallen leaves were collected from the forest floor around the study plot in November 1999. Freshly fallen leaves collected in November 1996 were exposed to natural microbial decomposition for 2 to 3 years in litterbags in the study site to prepare partly decomposed leaves [32]. The remaining mass of partly decomposed leaves was 53% to 61% of the original mass. The leaves were cut into pieces (approx. 1 x 1 cm), oven-dried at 40°C for 1 week, and preserved in a vinyl bag until the experiment started.

The litter material (1 g) was sterilized by exposure to ethylene oxide gas at 60°C for 6 hours and used in pure culture tests. Prior to the experiments, the sterilized freshly fallen and partly decomposed leaves were placed on 2% MA, and after 8 weeks of incubation at 20°C in the dark, no microbial colonies had developed on the agar. Thus, the effectiveness of the sterilization method used in the present study was assured. The plates were incubated for 16 weeks at 20°C in the dark. Three plates were prepared for each isolate, and three uninoculated plates served as a control. Occurrence of bleaching on the surface of leaves was observed under a binocular microscope (40x). Mass loss of leaves was determined as a percentage of the original mass, taking the mass loss of leaves in the uninoculated and incubated control treatment into consideration, and the mean values were calculated for each isolate and each litter type. Chemical analyses were performed for those fungi that caused more than 3% loss of leaf mass. The duplicate leaves were then combined and used for analyses of AUR, total carbohydrates, and N as described above. Mass losses of AUR, total carbohydrate, and N in leaves were calculated using the same equation as used for the mass loss of leaves. AUR/carbohydrate loss ratio (AUR/Carb) is a useful index of the

1 substrate-use pattern of each fungal species [35]. AUR/Carb of each fungal species
2 was calculated according to the equation:
3 AUR/Carb = mass loss of AUR (% original AUR mass) / mass loss of total
4 carbohydrate (% original total carbohydrate mass)
5
6 *2.7. FT-IR analysis*
7 A total of 22 samples, 16 from the field (8 BLM and 8 NBL samples in
8 1999 and 2000) and 6 from the pure culture tests, were used for FT-IR analysis in
9 the solid phase. The six samples from the pure culture tests consisted of
10 undecomposed initial leaves (denoted as treatment IN), control leaves inoculated
11 with no fungi and incubated for 12 weeks (treatment CO), and leaves inoculated
12 with *Clitocybe* sp. and incubated for 12 weeks (treatment CL) for each of freshly
13 fallen and partly decomposed leaves of *Fagus crenata*. Each ground sample was
14 embedded in KBr and compacted into a disc using a bench press. FT-IR spectra
15 were recorded in the absorbance mode at a resolution of 4 cm^{-1} with wavenumber
16 range of 400-4000 cm^{-1}, using FT/IR-4100 (JASCO Co., Tokyo, Japan) Each
17 spectrum was composed of 100 scans.

2.8. Data analysis

A paired t-test was performed to evaluate the difference in moisture content, chemical properties, and hyphal lengths between BLM and adjacent NBL. Fisher's exact probability test was performed to evaluate the difference in the frequency of occurrence of fungal taxa between BLM and NBL. A t-test was performed to evaluate the difference in mass loss of sterilized litter caused by individual fungal isolates under the pure culture condition between freshly fallen and partly decomposed leaves.

FT-IR spectra were baseline-corrected and normalized using JASCO Spectra Manager, Version 2 (Jasco Co., Tokyo, Japan). A total of 15 peaks that reflect functional groups associated with the lignocellulose matrix in litter materials were selected in the fingerprint region (800-2000 cm^{-1}) (Table 1). Peak heights were expressed as relative heights using the largest peak, a peak at 3355 to 3394 cm^{-1} that was associated with a hydroxyl group. A paired t-test was performed to examine the difference in relative heights of peaks in FT-IR spectra between BLM and NBL. Pearson's correlation coefficients were calculated for the

linear relationship between relative heights of peaks in FT-IR spectra and contents of AUR.

3. Results

3.1. Moisture content and chemical composition

The moisture content of BLM was significantly lower than that of NBL collected in 1999, whereas the difference was not significant in 2000 (Table 3). The AUR content was significantly lower and the content of soluble carbohydrates was significantly greater in BLM than in NBL (Table 3). The contents of holocellulose and polyphenols were not significantly different between BLM and NBL (Table 3). The contents of N, P, K, Ca, and Mg in BLM were generally significantly greater than those in NBL (Table 3). The contents of ammonium and nitrate, net mineralization rate, and net nitrification rate in BLM were significantly greater than those in NBL (Table 3).

3.2. Hyphal length

The total hyphal length was 5.4 times greater in BLM than in NBL and the difference was statistically significant (Table 4). The darkly pigmented hyphal length and the percentage of darkly pigmented hyphal length relative to total hyphal length were not significantly different between BLM and NBL (Table 4).

3.3. Fungal assemblages

A total of 49 fungal taxa were isolated, 30 from BLM and 42 from NBL (Table 5). The frequencies of occurrence were significantly ($P<0.05$) greater in BLM than in NBL for four fungal taxa (*Trichoderma koningii*, *Chaetomium globosum*, *Penicillium miczynskii*, and *Geniculosporium* sp.), whereas the frequencies of occurrence were significantly ($P<0.05$) lower in BLM than in NBL for 11 taxa (*T. longibrachiatum*, *Mucor hiemalis*, *Mortierell verticillata*, *T. viride*, *Clonostachys rosea*, *Umbelopsis ramanniana*, *Absidia glauca*, *Cladosporium cladosporioides*, *P. velutinum*, *Mortierella* cf. *zicae*, and *Mortierella wuyshanensis*). The frequencies of occurrence of the other 34 fungal taxa were not significantly ($P>0.05$) different between BLM and NBL. *Clitocybe* sp. was isolated from BLM with a frequency of occurrence of 3.3% but was not isolated from NBL.

3.4. Pure culture decomposition test

The initial AUR content of freshly fallen and partly decomposed leaves was 428 and 482 mg/g dry litter; the initial content of total carbohydrates was 239 and 208 mg/g; and the initial N content was 17 and 16mg/g, respectively.

The mass loss of freshly fallen and partly decomposed leaves caused by *Clitocybe* sp., *Mycena polygramma*, *Xylaria* sp., and *Geniculosporium* sp. ranged from 0.7% to 17.9% (Table 2). *Clitocybe* sp. caused the greatest mass loss of the leaves. Bleaching was noticeable on freshly fallen leaves inoculated with the four isolates, whereas on partly decomposed leaves it was noticeable only for *Clitocybe* sp. *Clitocybe* sp. caused a significantly greater mass loss in partly decomposed leaves than in freshly fallen leaves. For the other three fungi, on the other hand, the mass loss of partly decomposed leaves was significantly lower than that of freshly fallen leaves.

All four fungal isolates had the abilities to decompose both AUR and total carbohydrates (Table 2). The mass loss of AUR was greater in *Clitocybe* sp. than in the other three fungi. *Clitocybe* sp. caused greater mass loss of AUR in partly

decomposed leaves than in freshly fallen leaves, whereas *M. polygramma* and *Geniculosporium* sp. caused greater mass loss of AUR in freshly fallen leaves. The mass loss of total carbohydrates was generally lower in *Clitocybe* sp. and *M. polygramma* than in *Xylaria* sp. and *Geniculosporium* sp. The mass loss of total carbohydrates was similar between freshly fallen and partly decomposed leaves for *Clitocybe* sp. and *M. polygramma* and was greater in freshly fallen leaves than in partly decomposed leaves in *Geniculosporium* sp. The mass loss of N was greater in *Clitocybe* sp. than in the other three fungi, and was greater in partly decomposed leaves than in freshly fallen leaves for *Clitocybe* sp. *Clitocybe* sp. caused selective decomposition of AUR, with AUR/Carb of 2.3 to 4.2, whereas the other three fungi caused selective decomposition of total carbohydrates, with AUR/Carb between 0.2 and 0.6 (Table 2).

3.5. FT-IR analysis

The FT-IR spectra in the fingerprint region of BLM and NBL (field samples) and freshly fallen and partly decomposed leaves of *Fagus crenata* (pure culture samples) showed close similarities in overall appearance, with major

peaks at 1658 cm^{-1} and at 1033 cm^{-1} (Fig. 2). The relative heights of the 15 peaks were not significantly different between BLM and NBL collected in 1999, probably due to the low number of samples (Table 6). In 2000, the relative heights of seven peaks at 1593, 1505, 1462, 1266, 1227, 835, and 817 cm^{-1}, all resulting from lignin, were significantly lower in BLM than in NBL, whereas no significant differences were found for the relative heights of the other eight peaks, some of which were most probably related to carbohydrates (Fig. 2, Tables 1, 6). Combining the data of 1999 and 2000 yielded significant differences between BLM and NBL in the relative heights of the same seven peaks as in the data of 2000 (Table 6). Partly decomposed leaves in treatment CL had lower heights of the same seven peaks at 1593, 1505, 1462, 1266, 1227, 835, and 817 cm^{-1} than those in treatments IN and CO, whereas such differences were less obvious in the spectra of freshly fallen leaves between the treatments IN, CO, and CL (Table 7).

The AUR content in BLM and NBL collected in 1999 (n=6) and 2000 (n=10) was significantly and positively correlated with the relative heights of peaks at 835 and 817 cm^{-1} (Table 8). When the data of 1999 and 2000 were combined (n=16), AUR content was significantly and positively correlated with

1 the relative heights of peaks at 1505, 1266, 1227, 835, and 817 cm^{-1} (Table 8). No
2 significant correlations were found between the content of AUR and the relative
3 heights of peaks for the freshly fallen or partly decomposed leaves in treatments
4 IN, CO, or CL used in pure culture tests (n=6) (Table 8). When all data of BLM,
5 NBL, and freshly fallen and partly decomposed leaves were used for calculation
6 (n=22), AUR content was significantly and positively correlated with the relative
7 heights of peaks at 1593, 1505, 1462, 1422, 1367, 1329, 1266, 1227, 835, and 817
8 cm^{-1} (Table 8), the seven of which (1593, 1505, 1462, 1266, 1227, 835, and 817)
9 result from lignin (Table 1).
10
11 **4. Discussion**
12
13 The lower AUR content in BLM associated with fruiting bodies of
14 *Clitocybe* sp. in the field (Table 3) and the potential ability of this fungus to cause
15 bleaching and selective decomposition of AUR in leaves under the pure culture
16 condition (Table 2) indicated that *Clitocybe* sp. was the primary agent responsible
17 for the bleaching and AUR decomposition in forest floor materials. BLM served as

'hot-spots' of fungal activity and N mobilization within the forest floor, as indicated by the greater hyphal abundance (Table 4) and the greater net N mineralization rate (Table 3) compared to NBL. The lower moisture content in BLM compared to NBL in 1999 was possibly attributable to the greater amount of hydrophobic mycelia in BLM. A number of fungal taxa were encountered associated with BLM (Table 5), but previous studies showed that many of such microfungi as *Trichoderma* spp., *Penicillium* spp., and Zygomycetes (e.g., *Umbelopsis*, *Mortierella*, *Mucor*, and *Absidia*) were incapable of attacking plant cell wall components and of causing significant mass loss of litter materials [28]. The exceptions were *T. hamatum* and *Pestalotiopsis* spp., which can be regarded as cellulose decomposers [28, 31], and *Geniculosporium* sp., which were shown to cause mass loss of AUR but utilized carbohydrates more selectively (Table 2). However, their contributions to the reduction of AUR content in BLM might have been minimal [1].

The colonization of litter materials by mycelia of *Clitocybe* sp. led to significant changes in the chemical properties of BLM (Table 3). Our findings that *Clitocybe* sp. caused reduction of the AUR content and enrichment of the

nutrients in BLM were consistent with those of chemical analyses of BLM produced by other ligninolytic LDB [12, 13, 40]. The higher values of soluble carbohydrate content in BLM (Table 3) were possibly attributable to the decomposition of AUR by *Clitocybe* sp., as the selective decomposition of AUR during leaf litter decomposition can result in the production of soluble sugars [18, 41]. The increased contents of N, P, K, Ca, and Mg in BLM were partly attributable to the accumulation of fungal hyphae enriched in these nutrients (Table 4). For example, fungal hyphae often accumulate Ca in the form of calcium oxalate. The greater pool size of inorganic-N and the enhanced N mineralization in BLM (Table 3) were already reported in boreal forests of Finland [13]. The selective decomposition of AUR by *Clitocybe* sp. accounted for the enhanced N mobilization in the field (Table 3) and in the pure culture (Table 2). Previous studies have shown that N release from litter is closely associated with AUR decomposition. For example, a series of litterbag experiments in the field showed that the release of N from decomposing leaf litter began in the middle of the course of decomposition when mass loss of AUR had started [2]. In a field incubation experiment of *Camellia japonica* leaf litter, N release was faster in leaf

1 portions bleached by ligninolytic ascomycetes than in those colonized by
2 cellulolytic fungi [18]. Also in laboratory tests, AUR decomposition in pine needles
3 by LDB led to significant N mineralization [19]. The selective delignification of
4 *Larix leptolepis* needles by LDB was related to the decreased final concentration
5 of N in the remaining needles [31].
6 Many fungal taxa encountered in association with BLM and NBL (Table
7 5) were incapable of attacking plant cell wall components, as discussed above, and
8 their growth appeared to depend on readily available carbon compounds, such as
9 soluble carbohydrates and delignified cellulose [28, 31]. The four fungal taxa (*T.*
10 *koningii*, *Chaetomium globosum*, *Penicillium miczynskii*, and *Geniculosporium*
11 sp.) with increased frequency of occurrence in BLM can be regarded as
12 competitive secondary colonizers that favored litter materials with increased
13 availability of readily available carbohydrates; possible associations with such
14 resources are: *T. koningii* and *P. miczynskii* with soluble carbohydrates, and
15 cellulolytic *C. globosum* and *Geniculosporium* sp. with delignified cellulose [4].
16 *Trichoderma koningii*, as well as *Calcarisporium arbuscula*, may also be
17 mycoparasites of *Clitocybe* sp. or other microfungi. In contrast, the 11 fungal taxa

with decreased frequency of occurrence in BLM may be less competitive for these resources. Interestingly, six of these 11 taxa belong to the Zygomycota, and five of these zygomycetes (i.e., *Mucor hiemalis*, *Mortierella verticillata*, *Absidia glauca*, *Mortierella*. cf. *zicae*, and *Mortierella wuyshanensis*) were not isolated from BLM (Table 5). The reasons for the decrease of some Zygomycetes from BLM remain unclear, but may be related to their adaptation to low availability of simple carbon sources due to the accumulation of recalcitrant humic substances in NBL. Other possible explanations for the increase or decrease of fungal taxa in BLM include changes in moisture content, availability of inorganic-N and nutrients, structural disintegration of litter materials, or food web structure through interactions with other microfungi and soil animals.

The AUR/Carb values for *Clitocybe* sp. (2.3 and 4.2 for freshly fallen and partly decomposed leaves, respectively; Table 2) were at the highest part of the range previously reported for LDB in temperate forests [20, 22, 28, 31, 33, 44, 45], and similar to the values for ligninolytic ascomycetes with bleaching activity [18, 33]. Using partly decomposed leaves as a substratum for pure culture tests, the present study explicitly demonstrated that *Clitocybe* sp. was able to cause greater

mass loss of AUR and N and more selective decomposition of AUR in partly decomposed leaves than in freshly fallen leaves (Table 2). This result was in accordance with our observation that its fruiting bodies were regularly associated with partly decomposed litter materials at the boundary between the litter layer and the mineral soil layer and suggested that this fungus was physiologically adapted to the partly decomposed materials enriched in AUR.

The ability of *Clitocybe* sp. to remove AUR from partly decomposed leaves appeared unique as it contrasted with the abilities of *Mycena polygramma*, *Xylaria* sp., and *Geniculosporium* sp., which were major ligninolytic fungi in the study site but caused lower mass loss in partly decomposed leaves than in freshly fallen leaves (Table 2). In the study site, these three fungal taxa colonized and decomposed *F. crenata* leaf litter successively, leading to the accumulation of N and recalcitrant compounds such as lignin in partly decomposed leaves [27]. Despite the potential ability of *Clitocybe* sp. to bleach the litter, the contribution of this fungus to the overall decomposition of forest floor materials in the forest stand appears to be small as a result of the low frequency of occurrence of mycelia of this fungus. However, the bleaching of litter materials by *Clitocybe* sp. plays

important roles in contributing to the small-scale heterogeneity of decomposition of recalcitrant compounds and N mineralization within the forest floor of the study site.

It should be borne in mind that AUR in the present study analyzed with sulfuric acid hydrolysis can include not only true lignin of plant origin but also cutin, tannin, humic substances, and melanin, which are metabolized by ligninolytic enzymes or different suites of extracellular enzymes of LDB [25]. Using FT-IR spectroscopy, the present study demonstrated that the activity of *Clitocybe* sp. to remove recalcitrant compounds (as AUR) selectively from forest floor materials was associated with its ability to cause selective transformation of lignin in litter materials that the fungus colonizes (Tables 6, 7, 8). Pandey and Pitman [37] also reported that *Phanerochaete chrysosporium*, a wood-decay fungus causing selective delignification, decreased in the intensity of lignin bands at 1505 and 1245 cm^{-1} in wood samples. *Clitocybe* sp. caused greater changes in FT-IR spectra in partly decomposed leaves than in freshly fallen leaves (Table 7), suggesting that ligninolytic enzymes of this fungus were more adapted to the decomposition of lignin in partly decomposed materials. Because FT-IR

spectroscopy requires minimal sample preparation and fewer quantities of samples than conventional proximate analysis, and is much less time-consuming than proximate analysis, this technique is one of potential alternative techniques to proximate analysis for studying the chemistry of leaf litter decomposed by fungi and deserves further researches.

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Osono et al. Table 1

Table 1. Band origins of the 15 peaks detected in FT-IR spectra. The assignment followed Schultz and Glasser (1986), Faix (1991), Pandey (1998), and Rodrigues et al. (1998) [8, 36, 39, 42].

Wavenumber (cm^{-1})	Band origin
1735	C=O stretch in unconjugated ketones, carbonyls and in ester groups (frequently of carbohydrate origin); conjugated aldehydes and carboxylic acids
1658	C=O stretch; in conjugated p-subst. aryl ketones; strong electronegative substituents lower the wavenumber
1593	aromatic skeletal vibrations plus C=O stretch; S>G; G condensed > G etherified
1505	aromatic skeletal vibrations; G>S
1462	C-H deformations; assymetry in $-CH_3$ and $-CH_2-$
1422	aromatic skeletal vibrations combined with C-H in-plane deformations
1367	aliphatic C-H stretch in CH_3, not in OCH_3; phenolic OH
1329	S ring plus G ring condensed; (i.e., G ring substituted in position 5)
1266	G ring plus C=O stretch
1227	C-C plus C-O plus C=O stretch; G condensed > G etherified
1126	aromatic C-H in-plane deformation (typical for S units); plus secondary alcohols plus C=O stretch
1033	aromatic C-H in-plane deformation, G > S; plus C-O deformation in primary alcohols; plus C=O stretch (unconjugated)
925	C-H out-of-plane; aromatic
835	C-H out-of-plane in positions 2 and 6 of S, and in all positions of H units
817	C-H out-of-plane in positions 2, 5, and 6 of G units

Osono et al. Table 2

Table 2. Mass loss (% original mass) of freshly fallen and partly decomposed leaves of *Fagus crenata*, mass loss of AUR, total carbohydrates, and nitrogen in these leaves, and AUR to carbohydrate loss ratio (AUR/Carb) caused by four fungal species under pure culture conditions. Values indicate means ± standard errors (n=3). Results of t-test for comparison between freshly fallen and partly decomposed leaves are shown. *** P<0.001, * P<0.05, + P<0.10. nd not determined.

Fungus	Litter type	Litter		AUR	Total carbohydrates	Nitrogen	AUR/Carb
Clitocybe sp.	Freshly fallen	9.0±1.4	+	25.0	11.1	3.2	2.3
	Partly decomposed	17.9±1.3		43.7	10.4	9.6	4.2
Mycena polygramma	Freshly fallen	10.7±0.4	*	8.1	13.2	0.6	0.6
	Partly decomposed	5.7±0.4		7.0	15.9	-2.9	0.4
Xylaria sp.	Freshly fallen	9.0±0.4	***	6.2	30.8	-5.1	0.2
	Partly decomposed	0.7±0.3		nd	nd	nd	nd
Geniculosporium sp.	Freshly fallen	10.6±0.1	*	9.2	32.4	0.0	0.3
	Partly decomposed	3.5±0.5		4.6	12.3	2.0	0.4

Osono et al. Table 3

Table 3. Water content (%), contents of organic chemical constituents and nutrients (mg/g dry litter), inorganic-N pool size (mg N/kg dry litter), and N transformation rate (mg N/kg dry litter/30d) in bleached litter material (BLM) produced by *Clitocybe* sp. in comparison with adjacent nonbleached litter material (NBL) collected in 1999 and (or) 2000. Values indicate mean ± standard errors. The results of paired *t*-test are shown. *** P<0.001, ** P<0.01, * P<0.05, + P<0.10, ns non significant. nd not determined.

	1999 (n=3)			2000 (n=5)		
	BLM	NBL	P	BLM	NBL	P
Moisture content	186±10	243±10	*	371±27	342±12	ns
Organic chemical constituents						
AUR	324±7	422±3	**	355±17	455±5	**
Holocellulose	229±7	202±12	ns	168±9	163±2	ns
Soluble carbohydrates	29±0	19±1	**	37±2	22±1	**
Polyphenols	7±0	7±0	ns	9±1	9±0	ns
Nutrients						
N	24.5±0.2	22.1±0.5	+	22.3±0.4	20.1±0.6	+
P	1.5±0.0	1.0±0.0	**	1.1±0.1	0.8±0.0	*
K	1.0±0.0	0.8±0.1	ns	2.6±0.1	1.9±0.1	**
Ca	8.6±0.3	6.2±1.0	+	6.9±0.7	5.2±0.5	**
Mg	1.6±0.0	1.2±0.0	*	1.3±0.2	1.0±0.1	+
Inorganic-N						
NH_4-N	nd	nd	nd	416±185	109±16	*
NO_3-N	nd	nd	nd	8±1	3±0	***
Net mineralization rate	nd	nd	nd	4655±2065	1128±273	*
Net nitrification rate	nd	nd	nd	14±7	5±2	+

Osono et al. Table 4

Table 4. Hyphal length (m/g dry litter) in bleached litter material (BLM) produced by *Clitocybe* sp. in comparison with adjacent nonbleached litter material (NBL) collected in 1999. Values indicate mean ± standard errors (n=3). The results of paired *t*-test are shown. ** P<0.01, ns not significant.

	BLM	NBL	P
Total hyphae	37484±3012	6927±273	**
Darkly pigmented hyphae	1222±80	1001±308	ns
% darkly pigmented hyphae	3.3±0.4	12.7±4.3	ns

Osono et al. Table 5

Table 5. Frequency of fungi (%) associated with bleached litter material (BLM) produced by *Clitocybe* sp. in comparison with adjacent nonbleached litter material (NBL) collected in 1999. The results of Fisher's exact probability test are shown. *** P<0.001, ** P<0.01, * P<0.05, + P<0.10, ns not significant. na not applicable.

Fungus	BLM	NBL	
Trichoderma koningii	53.3	33.3	+
Chaetomium globosum	23.3	0.0	**
Penicillium miczynskii	20.0	3.3	*
Geniculosporium sp.	13.3	0.0	+
Trichoderma longibrachiatum	20.0	66.7	***
Mucor hiemalis	0.0	56.7	***
Mortierella verticillata	0.0	46.7	***
Trichoderma viride	30.0	46.7	+
Clonostachys rosea	10.0	46.7	**
Umbelopsis ramanniana	16.7	43.3	*
Absidia glauca	0.0	33.3	***
Cladosporium cladosporioides	0.0	26.7	**
Penicillium veltinum	0.0	20.0	*
Mortierella cf. *zicae*	0.0	16.7	*
Mortierella wuyshanensis	0.0	13.3	+
Trichoderma hamatum	50.0	53.3	ns
Penicillium citrinum	13.3	16.7	ns
Mucor sp.	13.3	6.7	ns
Gliocladium virens	10.0	13.3	ns
Gliomastix felina	10.0	3.3	ns
Umbelopsis isabellina	6.7	20.0	ns
Penicillium sclerotiorum	6.7	10.0	ns
Trichoderma pseudokoningii	6.7	10.0	ns
Calcarisporium arbuscula	6.7	6.7	ns
Trichoderma harzianum	6.7	6.7	ns

Lechanicillium suchlasporia	6.7	6.7	ns
Mortierella sp.1	6.7	3.3	ns
Pestalotiopsis sp.1	6.7	0.0	ns
Paecilomyces carneus	3.3	6.7	ns
Acremonium sp.1	3.3	3.3	ns
Arthrinium state of *Apiospora montagnei*	3.3	3.3	ns
Mucor piriformis	3.3	3.3	ns
Phoma sp.	3.3	3.3	ns
Lechanicillium psalliotae	3.3	3.3	ns
Acremonium sp.2	3.3	0.0	ns
Arthrinium phaeospermum	3.3	0.0	ns
Clitocybe sp.	3.3	0.0	ns
Mortierella globurifera	0.0	10.0	ns
Penicillium waksmanii	0.0	10.0	ns
Penicillium thomii	0.0	6.7	ns
Umbelopsis angulispora	0.0	3.3	ns
Penicillium chrysogenum	0.0	3.3	ns
Penicillium glabrum	0.0	3.3	ns
Penicillium janthinellum	0.0	3.3	ns
Penicillium verrucosum	0.0	3.3	ns
Scedosporium sp.	0.0	3.3	ns
Trichoderma polysporum	0.0	3.3	ns
Zygorrhynchus heterogamus	0.0	3.3	ns

1

Osono et al. Table 6

Table 6. Relative height (%) of peaks in FT-IR spectra for bleached litter materials (BLM) and nonbleached litter materials (NBL) collected in the study site in 1999 and 2000. Values are means±standard errors. Results of paired t-test are shown. *** P<0.001, ** P<0.01, * P<0.05, ns non significant.

Wavenumber	1999 (n=3)			2000 (n=5)			Total (n=8)		
(cm^{-1})	BLM	NBL	P	BLM	NBL	P	BLM	NBL	P
1593	73±1	75±2	ns	70±3	81±2	*	71±2	79±2	*
1505	52±1	56±3	ns	53±2	67±3	*	53±1	63±3	**
1462	53±1	55±2	ns	54±2	63±2	*	53±1	60±2	*
1266	58±1	62±2	ns	62±2	68±1	*	61±1	66±1	*
1227	60±1	64±2	ns	65±2	71±1	*	63±2	68±2	*
835	4±0	7±1	ns	3±0	5±0	**	3±0	6±0	***
817	6±0	7±1	ns	5±0	7±0	*	5±0	7±0	**

Results of eight wavenumbers (1735, 1658, 1422, 1367, 1329, 1126, 1033, and 925 cm^{-1}) on which no significant differences were detected between BLM and NBL are not shown in the table.

Osono et al. Table 7

Table 7. Relative height (%) of peaks in FT-IR spectra for freshly fallen and partly decomposed leaves of *Fagus crenata* used in pure culture tests for three treatments: undecomposed initial (IN), uninoculated and incubated control (CO), and inoculated with *Clitocybe* sp. (CL).

Wavenumber	Freshly fallen leaves			Partly decomposed leaves		
(cm^{-1})	IN	CO	CL	IN	CO	CL
1593	78	83	81	89	81	72
1505	67	69	67	69	60	52
1462	61	68	66	67	61	56
1266	67	73	71	73	69	63
1227	69	75	73	75	71	66
835	9	9	6	7	7	3
817	8	7	5	7	7	4

Results of seven wavenumbers on which significant differences were detected between BLM and NBL (Table 6) are shown in the table.

Osono et al. Table 8

Table 8. Pearson's correlation coefficients for linear relationship between relative height of peaks of FT-IR spectra and contents of acid-unhydrolyzed residue (AUR). *** P<0.001, ** P<0.01, ** P<0.05, ns non significant.

	Bleached and nonbleached litter materials									
Wavenumber	1999		2000		1999+2000		Pure culture test		All data	
(cm^{-1})	(n=6)	P	(n=19)	P	(n=16)	P	(n=6)	P	(n=22)	P
1593	0.29	ns	0.40	ns	0.38	ns	0.63	ns	0.53	*
1505	0.50	ns	0.54	ns	0.56	*	0.37	ns	0.59	**
1462	0.45	ns	0.45	ns	0.49	ns	0.47	ns	0.59	**
1422	-0.13	ns	0.31	ns	0.32	ns	0.37	ns	0.45	*
1367	0.24	ns	0.40	ns	0.43	ns	0.38	ns	0.54	**
1329	0.15	ns	0.36	ns	0.39	ns	0.35	ns	0.49	*
1266	0.62	ns	0.51	ns	0.58	*	0.58	ns	0.67	***
1227	0.54	ns	0.48	ns	0.54	*	0.59	ns	0.64	**
835	0.89	*	0.83	**	0.62	**	0.58	ns	0.69	***
817	0.73	ns	0.67	*	0.51	*	0.69	ns	0.51	*

Results of five wavenumbers (1735, 1658, 1126, 1033, and 925 cm^{-1}) on which no significant correlations were detected are not shown in the table.

1 Figure legend

2

3 **Figure 1.** Bleached litter material produced by *Clitocybe* sp. in a cool

4 temperate forest in Kyoto, Japan. Bar = 1 cm.

5

6 **Figure 2.** FT-IR spectra of bleached litter materials (BLM, black lines) and

7 nonbleached litter materials (NBL, gray lines) collected in 2000. Arrows indicate

8 a total of 15 peaks in the fingerprint region (Table 1). Open arrows indicate the

9 peaks for which the relative heights were significantly different between BLM

10 and NBL by the paired t-test; filled arrows indicate the peaks for which the

11 relative heights were not significantly different (Table 6).

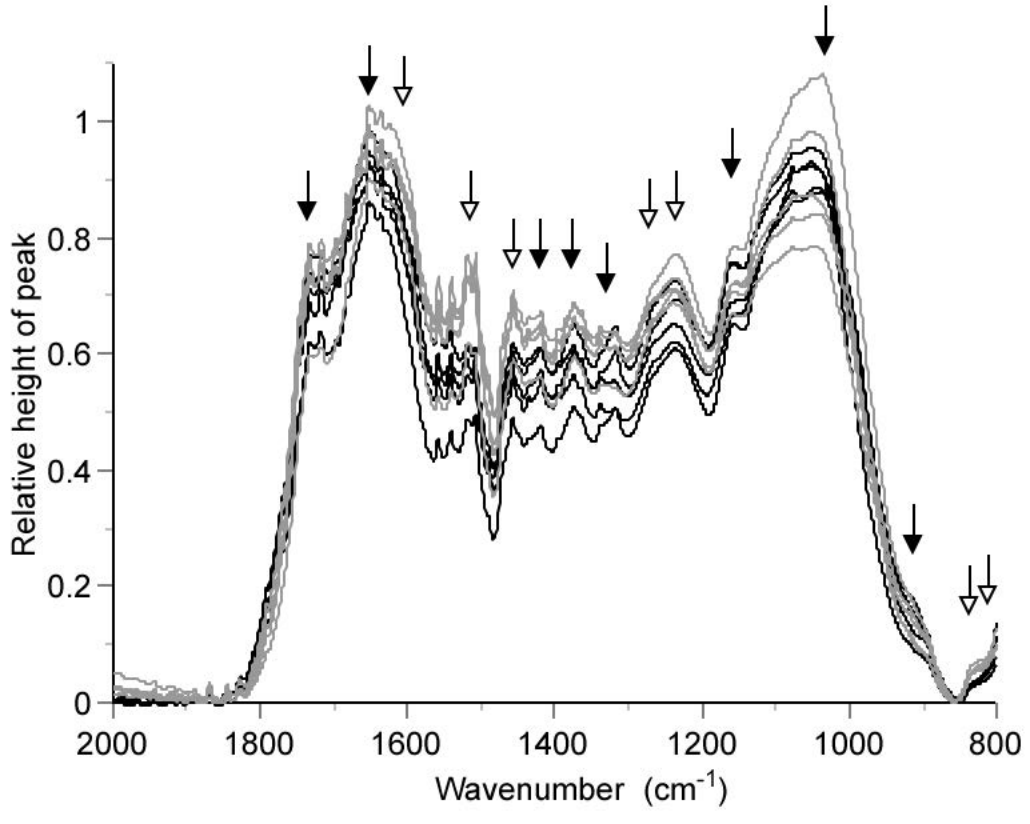
12

13

1 Osono et al. Fig. 1

2

3


Relative height of peak
0
0.2
0.4
0.6
0.8
1
2000
1800
1600
1400
1200
1000
800
Wavenumber (cm-1)