Bleaching of leaf litter and associated microfungi in subboreal and subalpine forests

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Bleaching of leaf litter and associated microfungi in subboreal and subalpine forests

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Abstract: Fungal decomposition of lignin leads to the whitening, or bleaching, of leaf litter, especially in temperate and tropical forests, but less is known about such bleaching in forests of cooler regions, such as boreal and subalpine forests.

The purposes of the present study were to examine the extent of bleached area on the surface of leaf litter and its variation with environmental conditions in subboreal and subalpine forests in Japan and microfungi associated with the bleaching of leaf litter by isolating fungi from the bleached portions of the litter.

Bleached area accounted for 21.7% to 32.7% and 2.0% to 10.0% of total leaf area of *Q. crispula* and *B. ermanii*, respectively, in subboreal forests, and for 6.3% and 18.6% of total leaf area of *B. ermanii* and *P. jezoensis* var. *hondoensis*, respectively, in a subalpine forest. In subboreal forests, elevation, C/N ratio and pH of FH layer, and slope aspect were selected as predictor variables for the bleached leaf area. Leaf mass per area and lignin content were consistently lower in the bleached area than in the nonbleached area of the same leaves, indicating that the selective decomposition of acid unhydrolyzable residue (AUR,
recalcitrant compounds such as lignin, tannins, and cutins) enhanced the mass loss of leaf tissues in the bleached portions. Isolates of a total of 11 fungal species (six species of Ascomycota and five of Basidiomycota) exhibited leaf-litter-bleaching activity under pure culture conditions. Two fungal species (*Coccomyces* sp. and *Mycena* sp.) occurred in both subboreal and subalpine forests which were separated from each other by approximately 1100 km.

*Key words*: decomposition, elevational gradient, fungal diversity, leaves, lignin, ligninolytic fungi.

**Introduction**

Fungi play central roles in the decomposition of lignin and other recalcitrant compounds (often registered as acid unhydrolyzable residues) in leaf litter of forest trees (van der Wal et al. 2013). Fungal decomposition of lignin is mediated by the activity of such extracellular enzymes as lignin peroxidases,
manganese peroxidases, phenol oxidases, and laccases (Eriksson et al. 1990) and often leads to the whitening, or bleaching, of leaf litter (Osono 2007). The fungal bleaching of leaf litter has been reported mainly from temperate and tropical forests (Osono 2006; Osono et al. 2008a, 2009), and less is known about it in forests of cooler regions, such as boreal and subalpine forests. The report of Hintikka (1970) is one of the milestone papers about the diversity and functioning of macrofungi (mainly in the Basidiomycota) associated with the bleaching of forest litter in Finnish boreal forests. More recently, Miyamoto et al. (2000) and Osono (2015c) showed that a suite of macrofungi in the Basidiomycota were capable of actively decomposing lignin and bleaching leaf litter in subboreal and subalpine forests of Japan. Compared to information about macrofungi, however, information is still lacking in boreal and subalpine forests regarding microfungi associated with the bleaching of leaf litter (Osono 2011) and possible climatic and environmental factors affecting their abundance.

The purposes of the present study were to examine (i) the extent of
bleached area on the surface of leaf litter and its variation with environmental
conditions and geographic locations and (ii) microfungi associated with the
bleaching of leaf litter by isolating fungi from the bleached portions as well as
surrounding nonbleached portions of the litter. We selected two study sites, one
in subboreal forest in northern Japan and another in subalpine forest in central
Japan, separated by 1100 km from each other, but having similar climatic
conditions and vegetation. Special attention was paid to an elevational gradient
on a mountain slope in the subboreal forest, in which sampling was conducted at
five elevational classes from 200 m to 1000 m. Several environmental factors
were measured at sampling sites of different elevational classes to test the
relative importance of these factors affecting bleaching on leaf litter. Fungi were
isolated from bleached areas, as well as nonbleached areas, and were then
tested for their bleaching activity under a pure culture condition, and subjected
to DNA base sequence analysis for taxonomic identity and assignment.

Materials and Methods
Study sites

Samples were collected from two sites in Japan: subboreal forests in Hokkaido and a subalpine forest in Gifu. The subboreal forests were located on the northwest slope of Mt. Rausu (44°04’N, 145°07’E) in Shiretoko Peninsula, northeastern Japan. Mean annual temperature is 6.2 °C, and mean annual precipitation is 1090 mm at Utoro Station of Automatic Meteorological Data Acquisition System of Japan Meteorological Agency, located 3 m above sea level and 10 km southwest of the study site. Dominant trees in the plots included *Abies sachaliensis* F.Schmidt, *Quercus crispula* Bl., *Betula platyphylla* Suk., and *B. ermanii* Cham. (Mori et al. in press). Bleaching was noticeable on leaf litter of such tree species as *Q. crispula*, *B. ermanii*, *Kalopanax pictus* (Thunb.) Nakai, *Tilia maximowicziana* Shirasawa, *Acer japonicum* Thunb., and *A. sachaliensis*. In 2010, 10 study plots (10 m × 10 m) each were established at 200, 400, 600, 800, and 1000 m above sea level, making a total of 50 plots (Mori et al. in press), and used for the collection of leaf litter. The maximum distance
between the plots at each elevational class ranged between 311 and 435 m.

Further details of the study site were described in Ikeda et al. (2014).

The subalpine forest was located on the north slope of Mt. Ontake, central Japan (35°56’N, 137°28’E, 2050 m above sea level). Mean annual temperature is approximately 2 °C, and mean annual precipitation is approximately 2500 mm (Osono 2015b). The study site contains mainly four coniferous species \[\textit{Abies mariesii} \text{Masters}, \textit{A. veitchii} \text{Lindley}, \textit{Picea jezoensis} \text{var. hondoensis} \text{(Sieb. et Zucc.) Carrière}, \text{and} \textit{Tsuga diversifolia} \text{(Maxim.) Masters}\] and one hardwood species (\textit{B. ermanii}). Bleaching was noticeable on leaf litter of two tree species: \textit{B. ermanii} and \textit{P. jezoensis} var. \textit{hondoensis}. A study plot of 50 m × 10 m was laid out in 2008 and used for the collection of leaf litter. Further details of the study site were given in Osono and Takeda (2007).

**Collection of leaves**

Sampling was conducted in the subboreal forests in July 2010. Leaves of \textit{Q. crispula} and \textit{B. ermanii} of which more than half of the original leaf area
remained were collected from the surface of the forest floor beneath the canopy of the respective tree species using a 15 cm × 15 cm quadrat. Leaves of *Q. crispula* were present in 10 plots each of elevational classes 200, 400, and 600 m, but not in the plots of elevational class 800 or 1000 m. Leaves of *B. ermanii* were collected from 7, 4, 10, 10, and 10 plots of elevational classes 200, 400, 600, 800, and 1000 m, respectively. Additionally, 20 leaves each of *Q. crispula* and *B. ermanii* with evident bleaching were arbitrarily collected from the plots at 400 m and were used for fungal isolation.

Sampling of leaves of *B. ermanii* and *P. jezoensis* var. *hondoensis* was conducted in the subalpine forest in June and August 2008, respectively. Leaves of *B. ermanii* of which more than half of the original leaf area remained, or needles of *P. jezoensis* var. *hondoensis* were collected from the surface of the forest floor beneath the canopy of 10 individuals of each tree species using a 15 cm × 15 cm quadrat. Additionally, 20 leaves each of *B. ermanii* and *P. jezoensis* var. *hondoensis* with evident bleaching were arbitrarily collected from the plot and were used for fungal isolation. Trees are referred to by their genus names in
the present study for the sake of simplicity.

Measurement of leaves

The leaves for leaf area measurement were placed in paper bags and taken back to the laboratory at ambient temperature. The leaves of Quercus and Betula were pressed between layers of plywood and paper and oven-dried at 40 °C for one week. The leaves were then photocopied and scanned with a photoscanner (EPSON GT-8000). Total leaf area and the proportion of bleached area were measured with an image software (NIH image, Windows version v.4.0.3, Scion), according to the method described in Hagiwara et al. (2012). Ten Picea needles were randomly selected for each quadrat, oven-dried at 40 °C for one week, and measured for total length and the length of needle portions with bleaching under a binocular microscope with magnification of 40× (Hirose and Osono 2006). The bleached area or bleached length of leaves was defined as the bleached percentage of the total leaf area or length. The bleached length of Picea needles was denoted here as the bleached area for the sake of simplicity.
mean value of the bleached area was calculated for each elevational class in subboreal forests and for the plot in the subalpine forest.

A 6-mm-diameter cork borer was used to excise leaf disks, avoiding the primary vein, from the bleached area and surrounding nonbleached area of the same leaves of *Q. crispula* and *B. ermanii* from subboreal and subalpine forests. Three to 61 disks (mean: 11 disks) were punched out for each quadrat. The disks were oven-dried again at 40 °C for one week and weighed to calculate leaf mass per area (LMA). To compare the chemical composition, the disks were combined to make one sample each of bleached and nonbleached leaf area for each tree species and each study site, ground in a laboratory mill, and used for proximate chemical analyses. Content of acid unhydrolyzable residue (AUR) was measured with hot sulfuric acid digestion, according to the method described in Osono (2015c).

We measured five environmental variables for each study plot of subboreal forests that were expected to influence the occurrence of bleached portions on leaves: carbon to nitrogen (C/N) ratio, pH (KCl), and gravimetric
water content of all materials in FH layer, inclination of the slope, and the slope aspect. Methods of measurement of these variables were described in Mori et al. (in press). Briefly, total carbon and nitrogen contents were determined with combustion method by automatic gas chromatography (NC analyzer SUMIGRAPH NC-900, Sumitomo Chemical, Osaka, Japan). The pH was measured in a 1 N potassium chloride solution with Docu-pH meter+ (Sartorius, Goettingen, Germany).

**Isolation and screening of bleaching fungi**

The leaves for fungal isolation were placed in paper bags and preserved at 4 °C for no longer than three days before the isolation of fungi. Single leaf disks were excised from the bleached portion of individual leaves of *Quercus* and *Betula*, using a sterile 6-mm-diameter cork borer, and other single disks were excised from the nonbleached portion of the same leaves. One bleached and one nonbleached portion (5-10 mm in length) were cut separately and aseptically for individual *Picea* needles. This produced a total of 20 bleached and 20
nonbleached disks or needle portions for each tree species from each study site. Fungi were isolated from bleached and nonbleached disks or needle portions using the surface disinfection method according to Hirose et al. (2014). The surface-disinfected materials were plated on 9-cm Petri dishes containing 2% lignocellulose agar (LCA) modified as described by Miura and Kudo (1970) [glucose 0.1%, KH$_2$PO$_4$ 0.1%, MgSO$_4$·7H$_2$O 0.02%, KCl 0.02%, NaNO$_3$ 0.2%, yeast extract 0.02%, and agar 2% (w/v)], two disks or needle portions per plate. Note that the modified LCA of Miura and Kudo (1970) does not contain lignin or other recalcitrant compounds. The modified LCA was used because its low glucose content suppresses the overgrowth of fast-growing fungal species (Osono and Takeda 1999). The plates were incubated in darkness at 10 °C and observed for 4 weeks after the disinfection. Any fungal hyphae or spores appearing on the plates were subcultured onto fresh LCA plates, incubated, and observed micromorphologically. Isolates were tested for their ability to bleach leaf litter under a pure culture condition. Newly shed leaves of Quercus and Betula without obvious
fungal or faunal attack were collected from the forest floor of subboreal forests in July 2010, and those of *Betula* were collected from the subalpine forest in September 2008. These leaves were oven-dried at 40°C for one week, cut into pieces (1 cm × 1 cm), and autoclaved at 120°C for 20 min. The sterilized leaf pieces were placed on the surface of Petri dishes (6 cm diameter), one piece per plate, containing 20 ml of 2% malt extract agar [malt extract 2% and agar 2% (w/v)] previously inoculated with fungal isolates and incubated at 20°C for two weeks. *Quercus* isolates were inoculated with sterilized *Quercus* leaf pieces, and *Betula* and *Picea* isolates were inoculated with *Betula* leaf pieces. The plates were sealed firmly with laboratory film so that moisture did not limit the bleaching on the agar medium and incubated at 20°C for 24 weeks in the dark. After incubation, the leaf pieces were retrieved and examined under a binocular microscope with magnification of 40× for the occurrence of bleaching on the leaf surface. The occurrence of bleaching was scored visually into six classes: 0, no bleached portion; 1, bleached in 1% to 20% of leaf area; 2, bleached in 21% to 40%; 3, bleached in 41% to 60%; 4, bleached in 61% to 80%; and 5, bleached in
81% to 100%. The visual class was transformed into the percentage bleached area with respect to the total leaf area by applying the median for each of class 1 to 5. Three control plates with the sterilized leaves but no fungal inoculation were also incubated under the same conditions. No microbial colonies or bleaching developed on the control plates, indicating the effectiveness of the sterilization method used in the present study. Fungal isolates with the score of 1 to 5 were regarded as possessing bleaching activity. We obtained a total of 180 fungal isolates from 160 leaf disks (four litter types × two portions × 20 leaves), of which 40 isolates were judged to be bleaching fungi and used for molecular analysis as described below.

Molecular methods

Genomic DNA was extracted from mycelia that had been cultured on 2.5% malt extract agar overlaid with a cellophane membrane following the modified CTAB method described by Matsuda and Hijii (1999). Polymerase chain reaction (PCR) was performed using a Quick Taq HS DyeMix (Toyobo,
Osaka, Japan). Each PCR reaction contained a 50 µl mixture [21 µl distilled water, 25 µl master mix, 3 µl ca. 0.5 ng/µl template DNA, and 0.5 µl of each primer (final, 0.25 µM)]. To PCR amplify the region including the rDNA ITS and 28S rDNA D1-D2 domain, the primer pair ITS1f (Gardes and Bruns 1993) and LR3 (Vilgalys and Hester 1990) was used. Each DNA fragment was amplified using a PCR thermal cycler (DNA engine; Bio-Rad, Hercules, CA, USA) using the following thermal cycling schedule. The first cycle consisted of 5 min at 94ºC, followed by 35 cycles of 30 s at 94ºC, 30 s at 50ºC for annealing, 1 min at 72ºC, and a final cycle of 10 min at 72ºC. The reaction mixture was then cooled at 4ºC for 5 min. PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Germany) according to the manufacturer’s instructions.

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

The sequences determined in the present study were deposited in the
DNA Data Bank of Japan (DDBJ) (LC014885 to LC014897). The rDNA ITS and
28S sequences were compared with available rDNA sequences in the GenBank
database using BLASTN searches (Altschul et al. 1990). For phylogenetic
analysis, MAFFT ver. 7 (Katoh and Standley 2013) was used for preliminary
multiple alignments of nucleotide sequences. Final alignments were manually
adjusted using BioEdit (Hall 1999). Alignment gaps were treated as missing
data, and ambiguous positions were excluded from the analysis. Phylogenetic
tree was conducted by Maximum Likelihood (ML) methods (Felsenstein 1981)
with the best fit nucleotide substitution model based on the lowest BIC score
(Bayesian Information Criterion). To estimate clade support, the bootstrap
procedure of Felsenstein (1985) was employed with 1000 replicates. These
analyses were carried out using MEGA6 (Tamura et al. 2013).
Based on the phylogenetic tree, the isolates were manually grouped into operational taxonomic units according to the similarity of 28S rDNA D1-D2 sequences with a criterion of 98%. Based on the results of molecular barcoding and micromorphological observation, we determined the identity and taxon of fungal isolates with bleaching activity.

**Statistical analysis**

Generalized linear models (GLMs) were used to evaluate the difference in bleached area of *B. ermanii* and *Q. crispula* leaf litter in subboreal forests with a Gaussian distribution using the elevational class, C/N ratio, pH, and water content of FH layer, the inclination of the slope, and the slope aspect as predictor variables. The GLMs were performed with the `glm` function of R version 3.0.2 for Mac (http://www.r-project.org) and with the `glht` function of the R multcomp package for multiple comparisons with Tukey's test. To find the most parsimonious models we performed automatic forward stepwise model selections with the Akaike information criterion (AIC), using the minimum AIC.
as the best-fit estimator. The model selections were performed with the stepAIC 273 function of R. The GLMs were used to evaluate the difference in LMA of *B. ermanii* and *Q. crispula* leaf litter in subboreal forests with a Gaussian 275 distribution using the leaf portions (bleached vs nonbleached) and the 276 elevational class as predictor variables.

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**Results**

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**Bleached area on leaf litter**

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Bleached area accounted for 21.7% to 32.7% and 2.0% to 10.0% of the 282 mean total leaf area of *Q. crispula* and *B. ermanii*, respectively, in subboreal 283 forests and for 6.3% and 18.6% of total leaf area of *B. ermanii* and *P. jezoensis* 284 var. *hondoensis*, respectively, in subalpine forest (Fig. 1a). In subboreal forests, 285 elevational class, C/N ratio and pH of FH layer, and/or slope aspect were 286 selected as predictor variables for the bleached leaf area of *Q. crispula* and *B. ermanii*, of which the elevational class and C/N ratio of FH layer had significant
\( \chi^2 \) values (P<0.05) for *Q. crispula* and the elevational class had a significant \( \chi^2 \) value (P<0.05) for *B. ermanii* (Table 1, Fig. 1b). The bleached area on *Q. crispula* leaves was significantly (P<0.05) greater at 600 m than at 200 and 400 m, and that on *B. ermanii* leaves was significantly (P<0.05) greater at 600 m than at 800 and 1000 m. The water content of FH layer and the inclination of the slope were not selected as predictor variables for the bleached leaf area of *Q. crispula* or *B. ermanii*.

Leaf mass per area (LMA) was consistently lower in the bleached area than in the nonbleached area of the same leaves for *Q. crispula* and *B. ermanii* from subboreal and subalpine forests (Table 2). The difference in LMA between the bleached and nonbleached area was statistically significant (GLM, P<0.001) for the two tree species from subboreal forests (Table 2). The LMA of *Q. crispula* was also significantly (GLM, P<0.001) lower at the elevational classes of 400 and 600 m than at 200 m (Table 2). AUR content was lower in the bleached leaf area than in the nonbleached leaf area for *Q. crispula* and *B. ermanii* from subboreal forests (Table 2).
Fungi with bleaching activity

A total of 11 fungal species (six in Ascomycota and five in Basidiomycota) were isolated from bleached and/or nonbleached portions and exhibited bleaching activity under pure culture conditions (Table 3, Fig. 2). Five of the six ascomycete species belonged to Rhytismataceae and the other to Xylariaceae; two basidiomycete species belonged to Tricholomataceae and the others to Mycenaceae, Russulaceae, or Phanerochaetaceae (Fig. 2). The bleaching activity was lower in Xylariaceae sp. (which caused mean bleaching of less than 10% of the leaf area) than in rhytismataceous and basidiomycete species (which causing bleaching of more than or equal to 50% of the leaf area) (Table 3).

Three, three, five, and four fungal species were detected as fungi with bleaching activity toward Q. crispula, B. ermanii of subboreal forest, B. ermanii of subalpine forest, and P. jezoensis var. hondoensis, respectively (Table 3). Coccomyces sp. and Mycena sp. occurred on leaves of three tree species in both
subboreal and subapline forests (Table 3).

Discussion

The proportions of the bleached area of *Q. crispula* leaves were at the highest part of the range previously reported for tree leaves in temperate and tropical forests, whereas those of *B. ermanii* leaves were at the low to middle part of the range (summarized in Osono 2006; Hagiwara et al. 2012). The higher bleached area of *Q. crispula* leaves than in that of *B. ermanii* leaves was possibly attributed to the lower content of AUR (Table 2) and nitrogen (discussed further below) in *Q. crispula* leaves. Few data have been available on the bleached portion of conifer needles comparable to the needles of *P. jezoensis* var. *hondoensis* in the subalpine forest. The lower LMA and the lower AUR content on the bleached area than on the nonbleached area are consistent with previous data (Osono 2006) and indicate that the selective decomposition of AUR enhanced the mass loss of leaf tissues in the bleached portions which
ligninolytic fungi colonized, compared to the nonbleached portions of the same leaves.

In subboreal forests, the proportions of the bleached area on leaves of both *Q. crispula* and *B. ermanii* reached the highest value at the elevational class of 600 m, and that of *Q. crispula* was significantly affected by the C/N ratio of FH layer (Fig. 1, Table 1). These results suggested that the bleached area did not respond linearly with the temperature lapse with the elevation and possible effects of other local factors. For example, previous studies demonstrated difference in microfungal assemblages at different elevational classes (van Maanen et al. 2000; Gourbière et al. 2001; Osono and Hirose 2009b). There is also some evidence to suggest that high N content could inhibit colonization by ligninolytic fungi. For example, N could cause a biochemical suppression of AUR-degrading enzymes of fungi (Keyser et al. 1978; Fenn et al. 1981). Osono et al. (2002) reported that avian excreta rich in N suppressed the colonization of litter by ligninolytic fungi. Similarly, Hagiwara et al. (2012) found that the bleached area of *Acacia mangium* leaf litter was negatively correlated with N
content of the litter, supporting the notion that the colonization of leaves by ligninolytic fungi could be inhibited by higher N content. The unimodal pattern of bleached area on *B. ermanii* leaves along the elevational gradient (Fig. 1a) implied that not only N level, pH, and the slope aspect but also other microenvironmental factors, such as the composition of tree and ground vegetation, singly or in combination, might affect the occurrence of bleached area on the litter. Two other possible factors affecting the occurrence of bleached portions on leaf litter along the elevational gradient include (i) different leaf fall time between the study sites and (ii) competition between bleaching fungi and other microbes.

The present results of proximate chemical composition analysis of leaf tissues, the isolation of fungi, and the pure culture bleaching assay demonstrated that 11 fungal species were responsible for AUR decomposition and bleaching of leaf litter in the subboreal and subalpine forests. The taxonomic composition of bleaching fungi of subboreal and subalpine leaves was generally consistent with that reported for other temperate and tropical regions.
Regarding Ascomycetes, species in the Rhytismataceae have been reported to bleach the leaf litter and decompose the AUR fraction of *Camellia japonica* (Koide et al. 2005b; Osono and Hirose 2009a), *Gaultheria shallon* (Osono et al. 2008b), and *Pinus* spp. (Osono and Hirose 2011). These rhytismataceous species can be endophytic on live leaf tissues and take part in lignin decomposition in early stages of decomposition (Koide et al. 2005a). Fungi in the Xylariaceae in Ascomycetes are well known for their ligninolytic activity, especially in the tropics (e.g. Osono et al. 2011, 2013). Basidiomycetes are major components of ligninolytic fungal assemblages in temperate and tropical forests (Boddy et al. 2008; Osono 2015a). *Mycena* sp. isolated as a bleaching fungus in the present study was also found fruited on the forest floor of the subalpine forest (Osono 2015a) and caused the selective delignification of *Betula* leaves (Osono 2015d).

The present study demonstrated the occurrence of bleaching on leaf litter of subboreal and subalpine forests in Japan, and the association of fungi with it. The occurrence of the same fungal species (*Coccomyces* sp. and *Mycena* sp.) at the two locations, despite their distance of approximately 1100 km from...
each other, can be partly attributed to the similarity in the climatic conditions and vegetation between these subboreal and subalpine forests. Similarly, fruiting bodies of such ligninolytic macrofungi as *M. aurantiidisca* were observed in both a subboreal forest in Hokkaido (Miyamoto et al. 1998) and the subalpine forest of the present study (Osono 2015a). It remains unclear to what extent the bleaching fungi contribute to the loss of AUR in decomposing litter on the forest floor of these forest stands. Tian et al. (2000), for example, found that the decomposition of AUR was slower than that of carbohydrates in leaf litter during a 3.5-year incubation in subalpine forest, suggesting relatively minor roles of ligninolytic activity compared to cellulolytic activity of the whole fungal assemblages. Further studies will be needed to follow simultaneously the changes in bleached area, the succession of ligninolytic fungi, and the loss of AUR in decomposition processes to quantify the role of fungi associated with the bleaching.

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Fig. 1. Bleached area (% total leaf area or length) on leaves of subboreal and subalpine forests of different elevations (a) and as related to C/N ratio of FH layer in subboreal forests (b). Values are means (n=10) and bars indicate standard errors. Numbers indicate the elevations. Gray columns and open squares, *Betula ermanii*; blank columns and filled circles, *Quercus crispula*; striped columns, *Picea jezoensis* var. *hondoensis*.

Fig. 2. Maximum-likelihood (ML) phylogeny inferred from 28S rDNA partial sequences including 41 bleaching fungi isolated from bleached and nonbleached portions of leaf litter in subboreal and subalpine forests. The evolutionary model used was the Kimura 2-parameter model (Kimura 1980) with a discrete Gamma distribution (+G, parameter = 0.5624). Bootstrap values for the ML analysis are indicated for corresponding branches. GenBank accession numbers are given in
parentheses.

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Table 1. Summary of generalized linear models for bleached leaf area in subboreal forests and selected predictor variables. ns, not selected. *** P<0.001, * P<0.05. The water content of FH layer and the inclination of the slope were not selected as predictor variables

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<tr>
<td></td>
<td>Deviance</td>
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<td>Elevational class</td>
<td>772.0</td>
<td>0.02*</td>
<td>463.7</td>
<td>&lt;0.001***</td>
</tr>
<tr>
<td>C/N ratio of FH layer</td>
<td>436.3</td>
<td>0.04*</td>
<td>47.3</td>
<td>0.08</td>
</tr>
<tr>
<td>pH of FH layer</td>
<td>120.4</td>
<td>0.28</td>
<td>ns</td>
<td></td>
</tr>
<tr>
<td>Slope aspect</td>
<td>ns</td>
<td></td>
<td>31.1</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Table 2. Leaf mass per area (LMA, mg/cm$^2$) and the content (mg/g) of acid unhydrolyzable residue (AUR) in bleached (BL) and nonbleached (NB) portions of leaf litter from subboreal and subalpine forests. Values indicate means ± standard errors (n=4-10). nd, no data. na, not analyzed because of insufficient amount of samples for analysis.

<table>
<thead>
<tr>
<th></th>
<th>Subboreal</th>
<th></th>
<th>Betula ermanii</th>
<th></th>
<th>Subalpine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quercus crispula</td>
<td>Betula ermanii</td>
<td></td>
<td>Betula ermanii</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>BL NB</td>
<td>BL NB</td>
<td>BL NB</td>
<td></td>
<td>BL NB</td>
<td></td>
</tr>
<tr>
<td>LMA in elevational class</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 m</td>
<td>5.3 ± 0.3</td>
<td>7.4 ± 0.4</td>
<td>3.8 ± 0.3</td>
<td>5.3 ± 0.3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>400 m</td>
<td>4.4 ± 0.3</td>
<td>6.4 ± 0.3</td>
<td>3.9 ± 0.2</td>
<td>5.5 ± 0.2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>600 m</td>
<td>4.1 ± 0.3</td>
<td>6.1 ± 0.3</td>
<td>4.4 ± 0.3</td>
<td>6.3 ± 0.8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>800 m</td>
<td>nd</td>
<td>nd</td>
<td>4.5 ± 0.6</td>
<td>5.6 ± 0.8</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>1000 m</td>
<td>nd</td>
<td>nd</td>
<td>4.4 ± 0.4</td>
<td>7.3 ± 0.7</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>2050 m</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>4.4 ± 0.1</td>
<td>5.3 ± 0.1</td>
</tr>
</tbody>
</table>

**Chemical content**

<table>
<thead>
<tr>
<th></th>
<th>AUR</th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>297.0</td>
<td>442.3</td>
<td>398.2</td>
<td>497.3</td>
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</tr>
</tbody>
</table>
Table 3. Number of isolates of bleaching fungi isolated from bleached and nonbleached portions of leaf litter and their bleaching activity (as % bleached leaf area caused by fungal isolates, with respect to total leaf area).

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Subboreal</th>
<th>Subalpine</th>
<th>Total number of isolates</th>
<th>Bleaching activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quercus</td>
<td>Betula</td>
<td>Betula</td>
<td>Picea</td>
</tr>
<tr>
<td><strong>Ascomycota</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coccomyces sp.</td>
<td>3</td>
<td>1</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Rhytismataceae sp.1</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Xylariaceae sp.</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rhytismataceae sp.2</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Rhytismataceae sp.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Lophodermium sp.</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Basidiomycota</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycena sp.</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Clitocybe sp.</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Peniophora sp.</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Ceriporia sp.</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Tricholomataceae sp.</td>
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<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td><strong>Number of species</strong></td>
<td>3</td>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>