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

Yusuke Hagiwara, Shunsuke Matsuoka, Satoru Hobara, Akira S. Mori, Dai Hirose, and Takashi Osono

Y. Hagiwara, S. Matsuoka, T. Osono. Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2113, Japan

S. Hobara. Department of Environmental Symbiotic Sciences, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

A. S. Mori. Graduate School of Environment and Information Sciences, Yokohama National University, Yokohama, Kanagawa 204-8501, Japan

D. Hirose. College of Pharmacy, Nihon University, Funabashi, Chiba 274-8555, Japan

Corresponding author: Takashi Osono (e-mail: tosono@ecology.kyoto-u.ac.jp)

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,

49 manganese peroxidases, phenol oxidases, and laccases (Eriksson et al. 1990) and
50 often leads to the whitening, or bleaching, of leaf litter (Osono 2007). The fungal
51 bleaching of leaf litter has been reported mainly from temperate and tropical
52 forests (Osono 2006; Osono et al. 2008a, 2009), and less is known about it in
53 forests of cooler regions, such as boreal and subalpine forests. The report of
54 Hintikka (1970) is one of the milestone papers about the diversity and
55 functioning of macrofungi (mainly in the Basidiomycota) associated with the
56 bleaching of forest litter in Finnish boreal forests. More recently, Miyamoto et al.
57 (2000) and Osono (2015c) showed that a suite of macrofungi in the
58 Basidiomycota were capable of actively decomposing lignin and bleaching leaf
59 litter in subboreal and subalpine forests of Japan. Compared to information
60 about macrofungi, however, information is still lacking in boreal and subalpine
61 forests regarding microfungi associated with the bleaching of leaf litter (Osono
62 2011) and possible climatic and environmental factors affecting their
63 abundance.
64 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

81

82 **Study sites**

83 Samples were collected from two sites in Japan: subboreal forests in
84 Hokkaido and a subalpine forest in Gifu. The subboreal forests were located on
85 the northwest slope of Mt. Rausu (44°04'N, 145°07'E) in Shiretoko Peninsula,
86 northeastern Japan. Mean annual temperature is 6.2 °C, and mean annual
87 precipitation is 1090 mm at Utoro Station of Automatic Meteorological Data
88 Acquisition System of Japan Meteorological Agency, located 3 m above sea level
89 and 10 km southwest of the study site. Dominant trees in the plots included
90 *Abies sachaliensis* F.Schmidt, *Quercus crispula* Bl., *Betula platyphylla* Suk.,
91 and *B. ermanii* Cham. (Mori et al. in press). Bleaching was noticeable on leaf
92 litter of such tree species as *Q. crispula*, *B. ermanii*, *Kalopanax pictus* (Thunb.)
93 Nakai, *Tilia maximowicziana* Shirasawa, *Acer japonicum* Thunb., and *A.*
94 *sachaliensis*. In 2010, 10 study plots (10 m × 10 m) each were established at 200,
95 400, 600, 800, and 1000 m above sea level, making a total of 50 plots (Mori et al.
96 in press), and used for the collection of leaf litter. The maximum distance

97 between the plots at each elevational class ranged between 311 and 435 m.
98 Further details of the study site were described in Ikeda et al. (2014).
99 The subalpine forest was located on the north slope of Mt. Ontake,
100 central Japan (35°56'N, 137°28'E, 2050 m above sea level). Mean annual
101 temperature is approximately 2 °C, and mean annual precipitation is
102 approximately 2500 mm (Osono 2015b). The study site contains mainly four
103 coniferous species [*Abies mariesii* Masters, *A. veitchii* Lindley, *Picea jezoensis*
104 var. *hondoensis* (Sieb. et Zucc.) Carrière, and *Tsuga diversifolia* (Maxim.)
105 Masters] and one hardwood species (*B. ermanii*). Bleaching was noticeable on
106 leaf litter of two tree species: *B. ermanii* and *P. jezoensis* var. *hondoensis*. A
107 study plot of 50 m × 10 m was laid out in 2008 and used for the collection of leaf
108 litter. Further details of the study site were given in Osono and Takeda (2007).
109
110 **Collection of leaves**
111 Sampling was conducted in the subboreal forests in July 2010. Leaves
112 of *Q. crispula* and *B. ermanii* of which more than half of the original leaf area

113 remained were collected from the surface of the forest floor beneath the canopy
114 of the respective tree species using a 15 cm × 15 cm quadrat. Leaves of *Q.*
115 *crispula* were present in 10 plots each of elevational classes 200, 400, and 600 m,
116 but not in the plots of elevational class 800 or 1000 m. Leaves of *B. ermanii* were
117 collected from 7, 4, 10, 10, and 10 plots of elevational classes 200, 400, 600, 800,
118 and 1000 m, respectively. Additionally, 20 leaves each of *Q. crispula* and *B.*
119 *ermanii* with evident bleaching were arbitrarily collected from the plots at 400
120 m and were used for fungal isolation.

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

129 the present study for the sake of simplicity.

130

131 **Measurement of leaves**

132 The leaves for leaf area measurement were placed in paper bags and
133 taken back to the laboratory at ambient temperature. The leaves of *Quercus* and
134 *Betula* were pressed between layers of plywood and paper and oven-dried at
135 40 °C for one week. The leaves were then photocopied and scanned with a
136 photoscanner (EPSON GT-8000). Total leaf area and the proportion of bleached
137 area were measured with an image software (NIH image, Windows version
138 v.4.0.3, Scion), according to the method described in Hagiwara et al. (2012). Ten
139 *Picea* needles were randomly selected for each quadrat, oven-dried at 40 °C for
140 one week, and measured for total length and the length of needle portions with
141 bleaching under a binocular microscope with magnification of 40× (Hirose and
142 Osono 2006). The bleached area or bleached length of leaves was defined as the
143 bleached percentage of the total leaf area or length. The bleached length of *Picea*
144 needles was denoted here as the bleached area for the sake of simplicity. The

145 mean value of the bleached area was calculated for each elevational class in
146 subboreal forests and for the plot in the subalpine forest.
147 A 6-mm-diameter cork borer was used to excise leaf disks, avoiding the
148 primary vein, from the bleached area and surrounding nonbleached area of the
149 same leaves of *Q. crispula* and *B. ermanii* from subboreal and subalpine forests.
150 Three to 61 disks (mean: 11 disks) were punched out for each quadrat. The disks
151 were oven-dried again at 40 °C for one week and weighed to calculate leaf mass
152 per area (LMA). To compare the chemical composition, the disks were combined
153 to make one sample each of bleached and nonbleached leaf area for each tree
154 species and each study site, ground in a laboratory mill, and used for proximate
155 chemical analyses. Content of acid unhydrolyzable residue (AUR) was measured
156 with hot sulfuric acid digestion, according to the method described in Osono
157 (2015c).
158 We measured five environmental variables for each study plot of
159 subboreal forests that were expected to influence the occurrence of bleached
160 portions on leaves: carbon to nitrogen (C/N) ratio, pH (KCl), and gravimetric

161 water content of all materials in FH layer, inclination of the slope, and the slope
162 aspect. Methods of measurement of these variables were described in Mori et al.
163 (in press). Briefly, total carbon and nitrogen contents were determined with
164 combustion method by automatic gas chromatography (NC analyzer
165 SUMIGRAPH NC-900, Sumitomo Chemical, Osaka, Japan). The pH was
166 measured in a 1 N potassium chloride solution with Docu-pH meter+ (Sartorius,
167 Goettingen, Germany).
168
169 **Isolation and screening of bleaching fungi**
170 The leaves for fungal isolation were placed in paper bags and preserved
171 at 4 °C for no longer than three days before the isolation of fungi. Single leaf
172 disks were excised from the bleached portion of individual leaves of *Quercus* and
173 *Betula*, using a sterile 6-mm-diameter cork borer, and other single disks were
174 excised from the nonbleached portion of the same leaves. One bleached and one
175 nonbleached portion (5-10 mm in length) were cut separately and aseptically for
176 individual *Picea* needles. This produced a total of 20 bleached and 20

177 nonbleached disks or needle portions for each tree species from each study site.
178 Fungi were isolated from bleached and nonbleached disks or needle portions
179 using the surface disinfection method according to Hirose et al. (2014). The
180 surface-disinfected materials were plated on 9-cm Petri dishes containing 2%
181 lignocellulose agar (LCA) modified as described by Miura and Kudo (1970)
182 [glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_20$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%,
183 yeast extract 0.02%, and agar 2% (w/v)], two disks or needle portions per plate.
184 Note that the modified LCA of Miura and Kudo (1970) does not contain lignin or
185 other recalcitrant compounds. The modified LCA was used because its low
186 glucose content suppresses the overgrowth of fast-growing fungal species (Osono
187 and Takeda 1999). The plates were incubated in darkness at 10 °C and observed
188 for 4 weeks after the disinfection. Any fungal hyphae or spores appearing on the
189 plates were subcultured onto fresh LCA plates, incubated, and observed
190 micromorphologically.
191 Isolates were tested for their ability to bleach leaf litter under a pure
192 culture condition. Newly shed leaves of *Quercus* and *Betula* without obvious

193 fungal or faunal attack were collected from the forest floor of subboreal forests
194 in July 2010, and those of *Betula* were collected from the subalpine forest in
195 September 2008. These leaves were oven-dried at 40°C for one week, cut into
196 pieces (1 cm × 1 cm), and autoclaved at 120°C for 20 min. The sterilized leaf
197 pieces were placed on the surface of Petri dishes (6 cm diameter), one piece per
198 plate, containing 20 ml of 2% malt extract agar [malt extract 2% and agar 2%
199 (w/v)] previously inoculated with fungal isolates and incubated at 20°C for two
200 weeks. *Quercus* isolates were inoculated with sterilized *Quercus* leaf pieces, and
201 *Betula* and *Picea* isolates were inoculated with *Betula* leaf pieces. The plates
202 were sealed firmly with laboratory film so that moisture did not limit the
203 bleaching on the agar medium and incubated at 20°C for 24 weeks in the dark.
204 After incubation, the leaf pieces were retrieved and examined under a binocular
205 microscope with magnification of 40× for the occurrence of bleaching on the leaf
206 surface. The occurrence of bleaching was scored visually into six classes: 0, no
207 bleached portion; 1, bleached in 1% to 20% of leaf area; 2, bleached in 21% to
208 40%; 3, bleached in 41% to 60%; 4, bleached in 61% to 80%; and 5, bleached in

81% to 100%. The visual class was tranformed 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,

225 Osaka, Japan). Each PCR reaction contained a 50 µl mixture [21 µl distilled
226 water, 25 µl master mix, 3 µl ca. 0.5 ng/µl template DNA, and 0.5 µl of each
227 primer (final, 0.25 µM)]. To PCR amplify the region including the rDNA ITS and
228 28S rDNA D1-D2 domain, the primer pair ITS1f (Gardes and Bruns 1993) and
229 LR3 (Vilgalys and Hester 1990) was used. Each DNA fragment was amplified
230 using a PCR thermal cycler (DNA engine; Bio-Rad, Hercules, CA, USA) using
231 the following thermal cycling schedule. The first cycle consisted of 5 min at 94ºC,
232 followed by 35 cycles of 30 s at 94ºC, 30 s at 50ºC for annealing, 1 min at 72ºC,
233 and a final cycle of 10 min at 72ºC. The reaction mixture was then cooled at 4ºC
234 for 5 min. PCR products were purified with a QIAquick PCR Purification Kit
235 (Qiagen, Germany) according to the manufacturer's instructions.
236 Purified PCR products were sequenced by FASMAC Co., Ltd.
237 (Kanagawa, Japan). Sequencing reactions were performed in a Gene Amp PCR
238 System 9700 (Applied Biosystems, USA) using a BigDye Terminator V3.1
239 (Applied Biosystems), following the protocols supplied by the manufacturer. The
240 fluorescent-labeled fragments were purified from the unincorporated

241 terminators using an ethanol precipitation protocol. The samples were
242 resuspended in formamide and subjected to electrophoresis in an ABI 3730xl
243 sequencer (Applied Biosystems).

244 The sequences determined in the present study were deposited in the
245 DNA Data Bank of Japan (DDBJ) (LC014885 to LC014897). The rDNA ITS and
246 28S sequences were compared with available rDNA sequences in the GenBank
247 database using BLASTN searches (Altschul et al. 1990). For phylogenetic
248 analysis, MAFFT ver. 7 (Katoh and Standley 2013) was used for preliminary
249 multiple alignments of nucleotide sequences. Final alignments were manually
250 adjusted using BioEdit (Hall 1999). Alignment gaps were treated as missing
251 data, and ambiguous positions were excluded from the analysis. Phylogenetic
252 tree was conducted by Maximum Likelihood (ML) methods (Felsenstein 1981)
253 with the best fit nucleotide substitution model based on the lowest BIC score
254 (Bayesian Information Criterion). To estimate clade support, the bootstrap
255 procedure of Felsenstein (1985) was employed with 1000 replicates. These
256 analyses were carried out using MEGA6 (Tamura et al. 2013).

257 Based on the phylogenetic tree, the isolates were manually grouped into
258 operational taxonomic units according to the similarity of 28S rDNA D1-D2
259 sequences with a criterion of 98%. Based on the results of molecular barcoding
260 and micromorphological observation, we determined the identity and taxon of
261 fungal isolates with bleaching activity.
262
263 **Statistical analysis**
264 Generalized linear models (GLMs) were used to evaluate the difference
265 in bleached area of *B. ermanii* and *Q. crispula* leaf litter in subboreal forests
266 with a Gaussian distribution using the elevational class, C/N ratio, pH, and
267 water content of FH layer, the inclination of the slope, and the slope aspect as
268 predictor variables. The GLMs were performed with the *glm* function of R
269 version 3.0.2 for Mac (http://www.r-project.org) and with the *glht* function of the
270 R multcomp package for multiple comparisons with Tukey's test. To find the
271 most parsimonious models we performed automatic forward stepwise model
272 selections with the Akaike information criterion (AIC), using the minimum AIC

as the best-fit estimator. The model selections were performed with the stepAIC 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 distribution using the leaf portions (bleached vs nonbleached) and the elevational class as predictor variables.

Results

Bleached area on leaf litter

Bleached area accounted for 21.7% to 32.7% and 2.0% to 10.0% of the mean 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 subalpine forest (Fig. 1a). In subboreal forests, elevational class, C/N ratio and pH of FH layer, and/or slope aspect were 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

289 χ^2 values ($P<0.05$) for *Q. crispula* and the elevational class had a significant χ^2

290 value ($P<0.05$) for *B. ermanii* (Table 1, Fig. 1b). The bleached area on *Q. crispula*

291 leaves was significantly ($P<0.05$) greater at 600 m than at 200 and 400 m, and

292 that on *B. ermanii* leaves was significantly ($P<0.05$) greater at 600 m than at

293 800 and 1000 m. The water content of FH layer and the inclination of the slope

294 were not selected as predictor variables for the bleached leaf area of *Q. crispula*

295 or *B. ermanii*.

296 Leaf mass per area (LMA) was consistently lower in the bleached area

297 than in the nonbleached area of the same leaves for *Q. crispula* and *B. ermanii*

298 from subboreal and subalpine forests (Table 2). The difference in LMA between

299 the bleached and nonbleached area was statistically significant (GLM, $P<0.001$)

300 for the two tree species from subboreal forests (Table 2). The LMA of *Q. crispula*

301 was also significantly (GLM, $P<0.001$) lower at the elevational classes of 400

302 and 600 m than at 200 m (Table 2). AUR content was lower in the bleached leaf

303 area than in the nonbleached leaf area for *Q. crispula* and *B. ermanii* from

304 subboreal forests (Table 2).

305

306 **Fungi with bleaching activity**

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

317 Three, three, five, and four fungal species were detected as fungi with
318 bleaching activity toward *Q. crispula*, *B. ermanii* of subboreal forest, *B. ermanii*
319 of subalpine forest, and *P. jezoensis* var. *hondoensis*, respectively (Table 3).
320 *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

337 ligninolytic fungi colonized, compared to the nonbleached portions of the same
338 leaves.

339 In subboreal forests, the proportions of the bleached area on leaves of
340 both *Q. crispula* and *B. ermanii* reached the highest value at the elevational
341 class of 600 m, and that of *Q. crispula* was significantly affected by the C/N ratio
342 of FH layer (Fig. 1, Table 1). These results suggested that the bleached area did
343 not respond linearly with the temperature lapse with the elevation and possible
344 effects of other local factors. For example, previous studies demonstrated
345 difference in microfungal assemblages at different elevational classes (van
346 Maanen et al. 2000; Gourbière et al. 2001; Osono and Hirose 2009b). There is
347 also some evidence to suggest that high N content could inhibit colonization by
348 ligninolytic fungi. For example, N could cause a biochemical suppression of
349 AUR-degrading enzymes of fungi (Keyser et al. 1978; Fenn et al. 1981). Osono et
350 al. (2002) reported that avian excreta rich in N suppressed the colonization of
351 litter by ligninolytic fungi. Similarly, Hagiwara et al. (2012) found that the
352 bleached area of *Acacia mangium* leaf litter was negatively correlated with N

353 content of the litter, supporting the notion that the colonization of leaves by
354 ligninolytic fungi could be inhibited by higher N content. The unimodal pattern
355 of bleached area on *B. ermanii* leaves along the elevational gradient (Fig. 1a)
356 implied that not only N level, pH, and the slope aspect but also other
357 microenvironmental factors, such as the composition of tree and ground
358 vegetation, singly or in combination, might affect the occurrence of bleached
359 area on the litter. Two other possible factors affecting the occurrence of bleached
360 portions on leaf litter along the elevational gradient include (i) different leaf fall
361 time between the study sites and (ii) competition between bleaching fungi and
362 other microbes.

363 The present results of proximate chemical composition analysis of leaf
364 tissues, the isolation of fungi, and the pure culture bleaching assay
365 demonstrated that 11 fungal species were responsible for AUR decomposition
366 and bleaching of leaf litter in the subboreal and subalpine forests. The
367 taxonomic composition of bleaching fungi of subboreal and subalpine leaves was
368 generally consistent with that reported for other temperate and tropical regions.

369 Regarding Ascomycetes, species in the Rhytismataceae have been reported to
370 bleach the leaf litter and decompose the AUR fraction of *Camellia japonica*
371 (Koide et al. 2005b; Osono and Hirose 2009a), *Gaultheria shallon* (Osono et al.
372 2008b), and *Pinus* spp. (Osono and Hirose 2011). These rhytismataceous species
373 can be endophytic on live leaf tissues and take part in lignin decomposition in
374 early stages of decomposition (Koide et al. 2005a). Fungi in the Xylariaceae in
375 Ascomycetes are well known for their ligninolytic activity, especially in the
376 tropics (e.g. Osono et al. 2011, 2013). Basidiomycetes are major components of
377 ligninolytic fungal assemblages in temperate and tropical forests (Boddy et al.
378 2008; Osono 2015a). *Mycena* sp. isolated as a bleaching fungus in the present
379 study was also found fruited on the forest floor of the subalpine forest (Osono
380 2015a) and caused the selective delignification of *Betula* leaves (Osono 2015d).
381 The present study demonstrated the occurrence of bleaching on leaf
382 litter of subboreal and subalpine forests in Japan, and the association of fungi
383 with it. The occurrence of the same fungal species (*Coccomyces* sp. and *Mycena*
384 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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545

546 Figure legend

547

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

554

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

parentheses.

Fig. 1

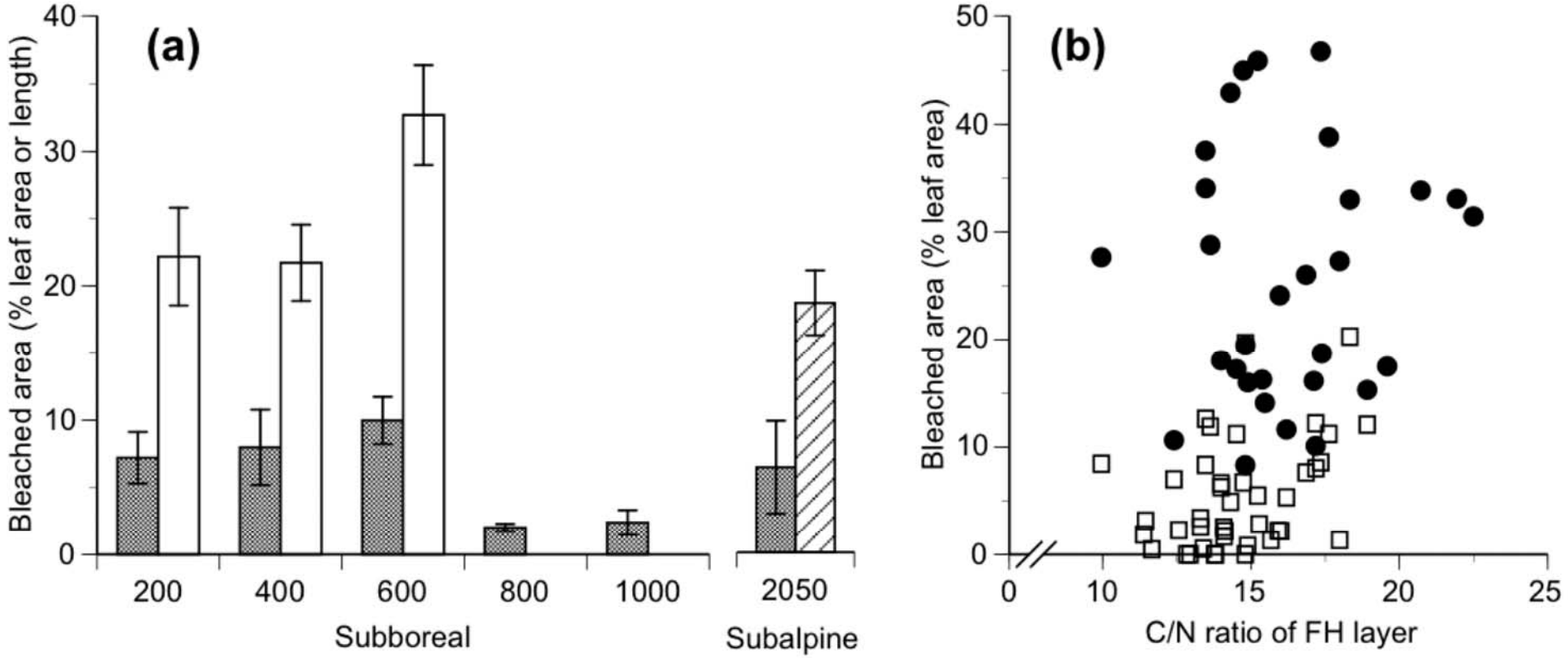
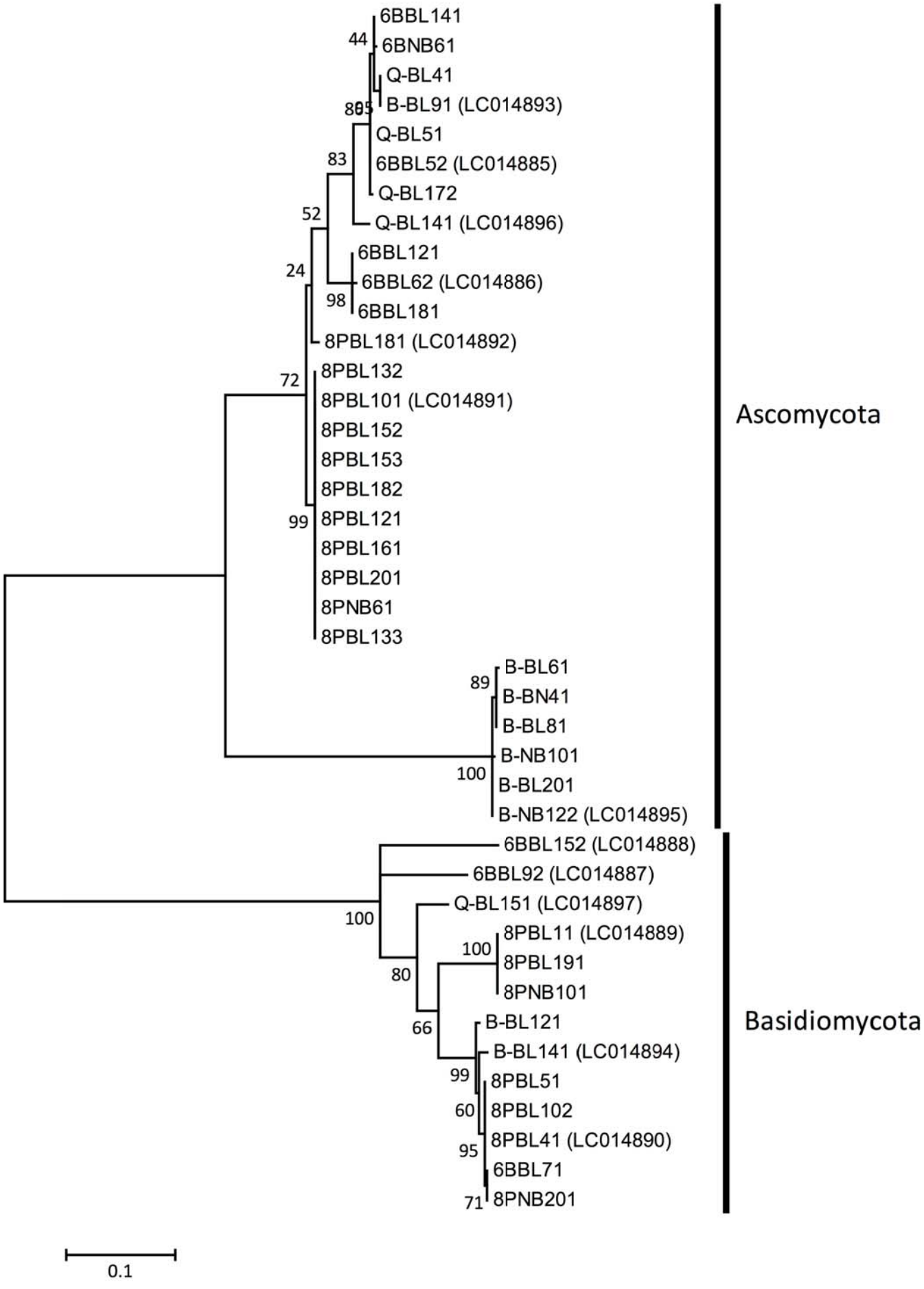

(a)
Bleached area (% leaf area or length)
40
30
20
10
0
200
400
600
800
1000
2050
Subboreal
Subalpine
(b)
Bleached area (% leaf area)
50
40
30
20
10
0
0
10
15
20
25
C/N ratio of FH layer

Fig. 2


6BBL141
44
6BNB61
Q-BL41
B-BL91 (LC014893)
Q-BL51
83
6BBL52 (LC014885)
Q-BL172
52
Q-BL141 (LC014896)
6BBL121
24
6BBL62 (LC014886)
98
6BBL181
8PBL181 (LC014892)
72
8PBL132
8PBL101 (LC014891)
8PBL152
8PBL153
8PBL182
99
8PBL121
8PBL161
8PBL201
8PNB61
8PBL133
B-BL61
89
B-BN41
B-BL81
B-NB101
100
B-BL201
B-NB122 (LC014895)
6BBL152 (LC014888)
6BBL92 (LC014887)
Q-BL151 (LC014897)
100
8PBL11 (LC014889)
100
8PBL191
80
8PNB101
66
B-BL121
B-BL141 (LC014894)
99
8PBL51
60
8PBL102
8PBL41 (LC014890)
95
6BBL71
71
8PNB201
Ascomycota
Basidiomycota
0.1

Hagiwara et al. Table 1.

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

	Quercus crispula		*Betula ermanii*	
	Deviance	Probability	Deviance	Probability
Elevational class	772.0	0.02*	463.7	<0.001***
C/N ratio of FH layer	436.3	0.04*	47.3	0.08
pH of FH layer	120.4	0.28	ns	
Slope aspect	ns		31.1	0.16

Hagiwara et al. Table 2

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.

	Subboreal				Subalpine	
	Quercus crispula		*Betula ermanii*		*Betula ermanii*	
	BL	NB	BL	NB	BL	NB
LMA in elevational class						
200 m	5.3 ± 0.3	7.4 ± 0.4	3.8 ± 0.3	5.3 ± 0.6	nd	nd
400 m	4.4 ± 0.3	6.4 ± 0.3	3.9 ± 0.2	5.5 ± 0.2	nd	nd
600 m	4.1 ± 0.3	6.1 ± 0.3	4.4 ± 0.3	6.3 ± 0.8	nd	nd
800 m	nd	nd	4.5 ± 0.6	5.6 ± 0.8	nd	nd
1000 m	nd	nd	4.4 ± 0.4	7.3 ± 0.7	nd	nd
2050 m	nd	nd	nd	nd	4.4 ± 0.1	5.3 ± 0.1
Chemical content						
AUR	297.0	442.3	398.2	497.3	na	na

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).

	Subboreal		Subalpine		Total number	Bleaching activity
Fungus	*Quercus*	*Betula*	*Betula*	*Picea*	of isolates	Mean (min-max)
Ascomycota						
Coccomyces sp.	3	1	3	0	7	61 (10-90)
Rhytismataceae sp.1	1	0	0	0	1	90
Xylariaceae sp.	0	6	0	0	6	7 (0-10)
Rhytismataceae sp.2	0	0	3	0	3	63 (50-90)
Rhytismataceae sp.3	0	0	0	10	10	53 (30-90)
Lophodermium sp.	0	0	0	1	1	50
Basidiomycota						
Mycena sp.	0	2	1	4	7	90 (90-90)
Clitocybe sp.	1	0	0	0	1	70
Peniophora sp.	0	0	1	0	1	90
Ceriporia sp.	0	0	1	0	1	50
Tricholomatacea sp.	0	0	0	3	3	90 (90-90)
Number of species	3	3	5	4		