

1 Hyphal length in the forest floor and soil of subtropical, temperate, and subalpine
2 forests

3

4 Takashi Osono

5

6 T. Osono

7 Center for Ecological Research, Kyoto University, Otsu, Shiga 520-2113 Japan

8 e-mail: tosono@ecology.kyoto-u.ac.jp

9

10 **Abstract**Fungi are a major component of soil biota in terrestrial ecosystems of
11 various climatic regions, but few studies have compared the effects of soil layers
12 and seasons on their abundance between sites with different humus forms and
13 climatic conditions. In the present study, the hyphal length in the forest floor and
14 soil was investigated in subtropical (ST), cool temperate (CT), and subalpine (SA)
15 forests in Japan with an agar film method. The primary aim was to describe the
16 seasonal variations in hyphal length in different layers of mull and moder humus
17 forms in forests of these three climatic regions. The total hyphal length was

18 generally higher in CT than in ST and SA and decreased with the soil depth.
19 Seasonal changes in total hyphal length were observed in ST and in the lower
20 slope of CT where mull humus developed. The length of darkly pigmented hyphae
21 and its proportion relative to the total hyphal length were higher at the upper
22 slope of CT (moder humus) than at the other sites and was in the order: F > L > A
23 layers. Clamp-bearing hyphal length in Basidiomycota accounted for as much as
24 19% of the total hyphal length and decreased with the depth of the forest floor and
25 soil in all study sites. Seasonal changes of hyphal length and the layer × month
26 interaction were significant on mull but not on moder humus, suggesting that
27 hyphal length was more sensitive to season in mull than in moder humus.

28

29 **Keywords** Climate · Fungi · Fungal biomass · Mycelia · Seasonal
30 change

31

32 **Introduction**

33

34 Fungi are a major component of soil biota in terrestrial ecosystems, representing

35 large pools of nutrients, controlling carbon and nutrient cycling in soils as
36 decomposers and mutualistic root-symbionts, and involved in soil food webs (Dix
37 and Webster 1995; Boddy et al. 2008). The abundance and species composition of
38 fungal communities in litter and soil can be indicative of the functional roles of
39 fungi in soil processes. It is difficult, however, to examine both fungal abundance
40 and species composition concurrently with any single method. Moreover, a
41 number of factors contribute to the complexity in measuring fungal communities
42 in soils. Consequently, a variety of techniques have been described for quantifying
43 fungal biomass in litter and soil (Newell 1992). Commonly used methods to
44 measure fungal biomass include direct microscopic visual observations of hyphal
45 length and biochemical assays for fungal marker molecules, such as chitin and
46 ergosterol. Of these methods, the direct microscopic techniques can be biased by a
47 degree of observer subjectivity, but they allow for visual classification of hyphal
48 types, such as hyaline, darkly pigmented, and clamp-bearing ones, which often
49 can help interpreting the composition of fungal community (Frankland 1982;
50 Kjøller and Struwe 1982; Osono 2007; van der Wal et al. 2009).

51 Fungal biomass expressed as the hyphal length has been reported using

52 direct observation methods in litter and soil (Kjøller and Struwe 1982; Holden et
53 al. 2013) and in litter at initial stages of decomposition (e.g. Berg and Söderström
54 1979; Fioretto et al. 1998; Osono and Takeda 2001; Osono 2005). To date, the
55 variations of hyphal length with soil layer, season, humus form, and climatic
56 region have been examined separately. To the knowledge of the author, however,
57 few studies have investigated the effects of soil layer and season on hyphal length
58 at the same time and compared them between sites with different humus forms
59 and climatic conditions (Osono 2011).

60 The purpose of the present study was to investigate the hyphal length in
61 the forest floor and soil of subtropical, cool temperate, and subalpine forests in
62 Japan with an agar film method. The primary goal was to describe the seasonal
63 variations in hyphal length in different layers of mull and moder humus forms in
64 forests of these three climatic regions. Soil samples were collected at each study
65 forest over a snow-free period to evaluate the relative effects of layer, season, and
66 their interactions on total, darkly pigmented, and clamp-bearing hyphal lengths.
67 Previous data of hyphal lengths in the forest floor and soils of different climatic
68 regions were then summarized to explore whether hyphal length in litter and soil

69 would exhibit patterns along a latitudinal or climatic gradient.

70

71 **Materials and methods**

72

73 Study site

74

75 Samples were collected from three sites in Japan: a subtropical forest (ST), a cool
76 temperate forest (CT), and a subalpine forest (SA). Table 1 shows the location,
77 climatic condition, vegetation, and data of forest floor measurements of the study
78 sites. ST was located in the northern part of Okinawa Island in southern Japan. A
79 study plot was established in a subtropical broadleaved forest dominated by
80 *Castanopsis sieboldii* (Makino) Hatusima (Fagaceae) and *Schima wallichii* (DC.)
81 Korthals (Theaceae) in the Yona Experimental Forest of the University of the
82 Ryukyus. CT was located in Kyoto, central Japan. Two study plots were
83 established on the lower and upper parts of a northwest-facing slope
84 (approximately 200 m long; Osono et al. 2008a) in a cool temperate forest
85 dominated by *Fagus crenata* Bl. and *Quercus crispula* Bl. (Fagaceae) in the Ashiu

86 Experimental Forest of Kyoto University. SA was located on Mt. Ontake, Gifu,
87 central Japan. A study plot was established there in a subalpine forest dominated
88 by *Abies mariesii* Masters, *Abies veitchii* Lindley, *Picea jezoensis* var. *hondoensis*
89 (Sieb. et Zucc.) Carrière (Pinaceae), and *Betula ermanii* Cham. (Betulaceae).
90 Monthly temperature data of the sites are shown in Fig. 1. The three sites differed
91 in mean annual temperature and seasonal changes in air temperature, but they
92 received similar amounts of precipitation annually (Table 1, Fig. 1). The mean
93 annual temperature and annual precipitation of the year when the samplings
94 were conducted were 21.1°C and 3167 mm in 2007 in ST, 9.8°C and 2548 mm in
95 2001 in CT, and 2.0°C and 2299 mm in 2008 in SA. The study sites experience a
96 rainy season from May to June in ST and from June to July in CT and SA. Snow
97 covers the forest floor of CT from December to April and that of SA from
98 mid-November to early June.

99

100 Sample collection

101

102 A study plot of 50 × 10 m (500 m²) was laid out in each of ST, CT (upper), CT

103 (lower), and SA sites and was divided into 125 grids of 2×2 m. Samples of L, F,
104 and A layer materials were collected from the four plots and used for the
105 estimation of hyphal length. Collection of the samples was performed six times: in
106 March, May, July, September, and November 2007 and in January 2008 in ST,
107 four times: in May, July, September, and November 2001 in CT, and three times:
108 in June, August, and October 2008 in SA. On each sampling occasion, 10 (ST and
109 SA) or five (CT lower and upper) grids were randomly chosen from the 125 grids
110 in each plot, and one soil block (20×20 cm in area) was collected from the center
111 of each grid. The block was divided into L and A layers for ST and CT (lower) and
112 L, F, and A layers for CT (upper) and SA, preserved in vinyl bags, and taken to the
113 laboratory. The F layer in ST and CT (lower) developed poorly and was not
114 included in the measurement of hyphal length. The A layer materials (3 cm in
115 depth) were collected from the surface of A layer because the depth of A layer
116 varied between the sites.

117 Thus, a total of 310 samples were used for the present study, including
118 120 (6 dates \times 2 layers \times 10 replicates) from ST, 40 (4 dates \times 2 layers \times 5
119 replicates) from CT (lower), 60 (4 dates \times 3 layers \times 5 replicates) from CT (upper),

120 and 90 (3 dates × 3 layers × 10 replicates) from SA. Samples were preserved in a
121 refrigerator at 4°C and processed within 48 hours after sampling. The L layer
122 materials were fragmented using a blender to make particles of approximately 5 ×
123 5 mm in area, and F and A layer materials were passed through a 2-mm sieve to
124 exclude plant roots and coarse fragments, before a portion of the samples was
125 used for hyphal length estimation.

126

127 Hyphal length estimation

128

129 Hyphal lengths in L, F, and A layer materials were estimated using the agar film
130 method of Jones and Mollison (1948), but with several modifications (Osono et al.
131 2006). One gram of a sample was homogenized in a blender at 10,000 rev/min in
132 49 ml of distilled water for 3 min. The suspension (20 ml) was diluted with 20 ml
133 of molten agar solution (final concentration 1.5%) and mixed at low speed on a
134 magnetic stirring plate. Three agar plates were prepared for each suspension in a
135 haemocytometer (0.1 mm depth), transferred to glass slides, and dried for 24
136 hours. The films were stained with fluorescent brightener (FB) for one hour. FB

137 binds to chitin in fungal cell walls (West 1988) and enables visualization of all
138 hyaline hyphae that are live or ghosts (empty). The stained films were mounted
139 between slides and coverslips with one drop of immersion oil (type DF, Cargille
140 Laboratories, Inc., Cedar Grove, NJ, USA) and examined with a Nikon
141 Microphot-SA epifluorescent microscope equipped with a high-intensity mercury
142 light source. A Nikon UV-1A filter cube was used for examination of FB-stained
143 hyphae. Darkly pigmented hyphae that were not stained with FB were observed
144 by bright field microscopy. Microscope fields were selected randomly and 25 fields
145 were observed for each slide at 1000× magnification. Hyphal lengths were
146 estimated using an eyepiece grid and a grid-intersection method (Olson 1950).
147 Total hyphal length was calculated as the sum of the lengths of hyaline hyphae
148 stained with FB and darkly pigmented hyphae. Hyphae with clamp connection
149 were classified into Basidiomycota, in spite of the fact that the hyphal length may
150 have been underestimated because the frequency of clamp formation varies
151 between species. Separate litter samples were oven-dried to a constant mass at
152 40°C and used to measure oven-dry weight.

153

154 Statistical analysis

155

156 The generalized linear model (GLM) was used to evaluate the difference in hyphal
157 length using layer, month, and the layer \times month interaction as independent
158 variables. Tukey's HSD test was performed for multiple comparisons. JMP 6.0 for
159 Macintosh was used to perform these analyses.

160

161 **Results**

162

163 Total hyphal length

164

165 The mean total hyphal length was generally higher in CT than in ST and SA, and
166 decreased with the depth of the forest floor and soil (Fig. 2). In ST, the mean total
167 hyphal length ranged from 3696 to 6311 m/g in the L layer and from 1061 to 1366
168 m/g in the A layer (Fig. 2). Total hyphal length in the L layer was significantly
169 higher than that in the A layer (Table 2). The effects of month and layer \times month
170 interaction were also significant (Table 2), with total hyphal length in the L layer

171 being especially elevated in November (Fig. 2).

172 The mean total hyphal length in CT was generally greater at the upper
173 than at the lower CT site (Fig. 2). In the L layer, it ranged from 8259 to 12172 m/g
174 at the lower site and from 12768 to 18733 m/g at the upper site; in the F layer, it
175 ranged from 8243 to 10051 m/g at the upper site; and in the A layer, it ranged
176 from 530 to 2022 m/g at the lower site and from 2795 to 3864 m/g at the upper site.
177 Total hyphal length in the L layer was significantly higher than that in the A layer
178 at both the upper and lower sites (Table 2). Total hyphal length at the lower site
179 also varied significantly with month (Table 2), being higher in May than in June
180 and September (Fig. 2).

181 The mean total hyphal length in SA ranged from 6061 to 6604 m/g in the
182 L layer, from 3982 to 4769 m/g in the F layer, and from 871 to 1090 m/g in the A
183 layer (Fig. 2). The differences among the layers were statistically significant,
184 whereas significant variation among the months was not observed (Table 2).

185

186 Darkly pigmented hyphal length

187

188 Overall, darkly pigmented hyphal length was greater at CT (upper) than at the
189 other sites, and decreased in the order: F (if available) > L > A layer (Fig. 3). In ST,
190 the mean darkly pigmented hyphal length ranged from 361 to 859 m/g in the L
191 layer and from 54 to 162 m/g in the A layer (Fig. 3). Darkly pigmented hyphal
192 length was significantly greater in the L than in the A layer, and varied with
193 month (Table 2). It accounted for 5% to 15% of the total hyphal length, and the
194 proportion did not vary significantly with either layer or month (Table 2).

195 The mean darkly pigmented hyphal length in CT ranged from 942 to
196 3039 m/g in the L layer at the upper and lower sites, from 2932 to 3212 m/g at the
197 upper site, and from 237 to 827 m/g in the A layer at the upper and lower sites
198 (Fig. 3). Darkly pigmented hyphal length was significantly different in the order:
199 L > A layer at the lower site and F > L > A layer at the upper site, and it did not
200 vary significantly with month at either site (Table 2). The proportion of darkly
201 pigmented hyphal length with respect to the total hyphal length was significantly
202 different between the layers at both the upper and lower sites (Table 2). It was
203 38% to 53% in the A layer at the lower site and 32% to 37% in the F layer at the
204 upper site, whereas it was between 8% and 26% in the L layer at the lower site

205 and in the L and A layers at the upper site (Fig. 3).

206 The mean darkly pigmented hyphal length in SA ranged from 806 to
207 1367 m/g in the L layer, from 1432 to 1799 m/g in the F layer, and from 124 to 425
208 m/g in the A layer (Fig. 3), and the differences between the layers were
209 statistically significant (Table 2). Darkly pigmented hyphal length accounted for
210 30% to 45% of the total hyphal length in the F layer, which was significantly
211 higher than that in the L layer (14% to 23%) (Table 2). This proportion was
212 significantly higher in June than in August or October (Fig. 3, Table 2).

213

214 Clamp-bearing hyphal length

215

216 The mean clamp-bearing hyphal length in ST ranged from 198 to 906 m/g in the L
217 layer and from 2 to 89 m/g in the A layer (Fig. 4), and the difference between the L
218 and A layers was statistically significant (Table 2). The effects of month and the
219 layer \times month interaction were also significant (Table 2), indicating that
220 clamp-bearing hyphal length in L layer was significantly elevated, especially in
221 November (Fig. 4). Clamp-bearing hyphal length accounted for 4% to 13% of the

222 total hyphal length in the L layer, and for 0% to 6% in the A layer, and the
223 difference between the L and A layers was statistically significant (Table 2).

224 The mean clamp-bearing hyphal length at the lower site in CT ranged
225 from 330 to 1049 m/g in the L layer and was not detected in the A layer, whereas
226 at the upper site in CT it ranged from 435 to 3792 m/g in the L layer, 319 to 1130
227 in the F layer, and 0 to 100 m/g in the A layer (Fig. 4). Clamp-bearing hyphal
228 length and its proportion relative to the total hyphal length was significantly
229 higher in the L than in the F and A layers both at the upper and lower sites and
230 was significantly higher in November than in May at the upper site (Table 2).
231 Clamp-bearing hyphal length generally accounted for 0% to 13% of the total
232 hyphal length and increased to 19% at the upper site in November (Fig. 4).

233 The mean clamp-bearing hyphal length in SA ranged from 836 to 1012
234 m/g in the L layer, from 130 to 910 m/g in the F layer, and from 0 to 207 m/g in the
235 A layer (Fig. 4), and the differences between the layers were statistically
236 significant (Table 2). Clamp-bearing hyphal length accounted for 0% to 19% of the
237 total hyphal length (Fig. 4).

238

239 **Discussion**

240

241 The values of total hyphal lengths in the present study fell within the range of
242 those previously reported in forests of Japan (Table S1 in Electronic
243 Supplementary Material) and other regions (summarized in Kjøller and Struwe
244 1982). The mean total hyphal lengths in the L layer of ST, CT, and SA correspond
245 to 9.6, 23.7, and 11.9 mg/g, respectively, calculated with the conversion factors of:
246 the radius of hyphae (r) of 2 μm , the dry matter proportion of hyphae (dm_r) of 0.1,
247 and the density of hyphae (d_f) of 1.5 g/cm^3 (Kjøller and Struwe 1982). These length
248 estimates are again within the range of those previously reported (Kjøller and
249 Struwe 1982). There have been few estimates of the length of darkly-pigmented or
250 clamp-bearing hyphae, but the values in the present study were consistent with
251 those previously reported in Japan (Table S1). Similarly, Bååth and Söderström
252 (1977) reported that the length of darkly pigmented and clamp-bearing hyphae in
253 a Swedish coniferous forest ranged from 100 to 3500 m/g and from 20 to 1300 m/g,
254 respectively, and accounted for 14% to 27% and 2% to 13% of the total hyphal
255 length, respectively. The results of the present study (Fig. 2) are consistent with

256 the statement of Frankland (1982) that fungal mycelia are less concentrated in
257 mull with a thin accumulation of litter than in mor and moder with
258 well-developed organic layers.

259 The lengths of total and clamp-bearing hyphae decreased with the depth
260 of soil horizons in the present study (Figs. 2 and 4), which is in agreement with
261 previous reports (Ruscoe 1971; Hunt and Fogel 1983). These vertical patterns are
262 primarily attributable to the decrease in carbon content with depth (Kjøller and
263 Struwe 1982). In contrast, darkly pigmented hyphal length increased in F layers
264 of moder humus [i.e. in CT (upper) and SA] (Fig. 3). Bååth and Söderström (1977)
265 and Osono et al. (2003) also reported higher values of darkly pigmented hyphal
266 length in deeper soil layers. Decomposition experiments have also demonstrated
267 an increase in the length and/or the proportion of darkly pigmented hyphae
268 during litter transformation (Osono 2005; Osono et al. 2014). Osono et al. (2006)
269 found that incubation of needle litter beneath the L layer resulted in a
270 significantly greater amount of darkly pigmented hyphae than incubation of the
271 same litter on the surface of the L layer. Suggested underlying mechanisms of an
272 increase of darkly pigmented hyphae thus should include the higher potential of

273 dematiaceous fungi to colonize litter in deeper layers (e.g. Tokumasu 1998) and
274 the slow turnover of recalcitrant melanized hyphae (Butler and Day 1998).
275 Relatively high values of darkly pigmented hyphal length and its proportion (29%
276 to 42%) relative to the total length were found in needle litter of *Chamaecyparis*
277 *obtusa* and in arctic substrates (Table S1). This suggests that darkly pigmented
278 hyphae can increase under stress of nutrients (e.g. in needle litter) or
279 environmental conditions (e.g. low temperatures or freezing), possibly in
280 association with the stress-resistant nature of melanized hyphae (Butler and Day
281 1998).

282 The total hyphal length in the L layer increased significantly in
283 November in ST and in May in CT (lower) (Fig. 2), where mull humus developed
284 (Table 1). Previous studies already showed an increase of total hyphal length in
285 spring and autumn and attributed it to favorable climatic (moisture and
286 temperature) and nutrient factors for fungal growth (Ruscoe 1971; Bååth and
287 Söderström 1977). A marked increase in clamp-bearing hyphal length and its
288 proportion relative to the total length in CT (upper) (Fig. 4) may be due to an
289 increase in litter-decomposing and/or mycorrhizal basidiomycetes. A concomitant

290 increase in the occurrence of fruiting bodies of litter-decomposing and mycorrhizal
291 basidiomycetes was also observed in that November in CT (Osono unpubl.).
292 Ruscoe (1971) also reported an autumn increase in clamp-bearing hyphal length
293 in soil of a pure stand of *Nothofagus truncata*, but the generality and ecological
294 significance of the autumn flush of basidiomycetes remain unclear, mainly due to
295 the lack of relevant studies. Limitations of the present study should also be noted
296 as the field surveys were performed for different single years at the three sites
297 and only for snow-free periods at CT and SA.

298 Generally, the effects of layer of the forest floor were more marked on the
299 total, darkly pigmented, and clamp-bearing hyphal lengths than the month of
300 sampling at study sites (Table 2). Interestingly, the seasonal changes of total
301 hyphal length were significant on mull humus at ST and CT (lower) but not on
302 moder humus at CT (upper) and SA (Table 2). The effect of layer \times month
303 interaction on total, darkly pigmented, and clamp-bearing hyphal length was also
304 significant only at ST and CT (lower). These results suggest that hyphal length in
305 L layer was more sensitive to season in mull than in moder soils. Possible
306 explanations for this sensitivity are the amount and the turnover of forest floor

307 materials. The lower amount and the faster turnover of the forest floor in mull
308 soils (Tsukamoto 1996) may lead to more variable hyphae length than in moder
309 humus.

310 When data of hyphal length in the forest floor and soils of Asian forests
311 and Canadian arctic tundra examined with the same direct observation method
312 were plotted against mean annual temperatures (Table S1), peaks of the total,
313 darkly pigmented, and clamp-bearing hyphal length in temperate regions would
314 be implied (Fig. S1). It is difficult, however, to gain insights into climatic patterns
315 of hyphal length, as data are still lacking in soils of other than temperate regions.
316 For example, Widden and Parkinson (1979) reported that total hyphal length in
317 Canadian tundra soils reached 2228 m/g, whereas Robinson et al. (1996)
318 summarized the data of total hyphal length in soils and dead plant tissues on
319 tundra soils and reported that these ranged from 4 to 9600 m/g dry soil. Further
320 efforts are needed to measure hyphal lengths in various soils, especially of
321 tropical regions.

322

323 **Acknowledgments** I thank Dr. A. Takashima and staff at the Yona

324 Experimental Forest, University of the Ryukyus, for help with fieldwork at ST;
325 the staff at Ashiu Experimental Forest, Kyoto University, for help with fieldwork
326 at CT; and Dr. Elizabeth Nakajima for critical reading of the manuscript. This
327 study received partial financial support from the Ministry of Education, Culture,
328 Sports, Science, and Technology of Japan (MEXT) (No. 19780114), The Sumitomo
329 Foundation, Nissan Global Foundation, Nippon Life Inst. Foundation, and Grants
330 for Excellent Graduate Schools, MEXT, Japan (12-01) to Kyoto University.

331

332 **References**

333

- 334 Bååth E, Söderström BE (1977) Mycelial lengths and fungal biomasses in some
335 Swedish coniferous forest soils, with special reference to a pine forest in
336 central Sweden. Swedish Coniferous Forest Project, Technical Report
337 13:1-45
- 338 Berg B, Söderström B (1979) Fungal biomass and nitrogen in decomposing Scots
339 pine needle litter. *Soil Biol Biochem* 11:339-341
- 340 Boddy L, Frankland JC, van West P (2008) Ecology of Saprotrophic

341 Basidiomycetes. Academic Press, London

342 Butler MJ, Day AW (1998) Fungal melanins: a review. *Can J Microbiol*
343 44:1115-1136

344 Dix NJ, Webster J (1995) *Fungal Ecology*. Chapman & Hall, London

345 Enoki T (2003) Microtopography and distribution of canopy trees in a subtropical
346 evergreen broad-leaved forest in the northern part of Okinawa Island,
347 Japan. *Ecol Res* 18:103-113

348 Fioretto A, Musacchio A, Andolft G, Virzo de Santo A (1998) Decomposition
349 dynamics of litters of various pine species in a Corsican pine forest. *Soil*
350 *Biol Biochem* 30:721-757

351 Frankland JC (1982) Biomass and nutrient cycling by decomposer basidiomycetes.
352 In: Frankland JC, Hedger JN, Swift Mj (eds) *Decomposer Basidiomycetes,*
353 *Their Biology and Ecology*. Cambridge University Press, Cambridge, pp
354 241-261

355 Hobara S, Osono T, Hirose D, Noro K, Hirota M, Benner R (2014) The roles of
356 microorganisms in litter decomposition and soil formation.
357 *Biogeochemistry* 118:471-486

- 358 Holden SR, Gutierrez A, Treseder KK (2013) Soil fungal communities,
359 extracellular enzyme activities, and litter decomposition across a fire
360 chronosequence in Alaskan boreal forests. *Ecosystems* 16:34-46
- 361 Hunt GA, Fogel R (1983) Fungal hyphal dynamics in a western Oregon
362 Douglas-fir stand. *Soil Biol Biochem* 15:641-649
- 363 Jones PCT, Mollison JE (1948) A technique for the quantitative estimation of soil
364 microorganisms. *J Gen Microbiol* 2:54-69
- 365 Kjøller A, Struwe S (1982) Microfungi in ecosystems: fungal occurrence and
366 activity in litter and soil. *Oikos* 39:391-422
- 367 Koide K, Osono T, Takeda H (2005) Fungal succession and decomposition of
368 *Camellia japonica* leaf litter. *Ecol Res* 20:599-609
- 369 Mori A, Mizumachi E, Osono T, Doi Y (2004) substrate-associated seedling
370 recruitment and establishment of major conifer species in an old-growth
371 subalpine forest in central Japan. *For Ecol. Manag* 196:287-297
- 372 Newell SY (1992) Estimating fungal biomass and productivity in decomposing
373 litter. In: Carroll GC, Wicklow DT (eds) *The Fungal Community: Its
374 Organization and Role in the Ecosystem*. Marcel Dekker, New York, pp

375 521-561

376 Olson FCW (1950) Quantitative estimates of filamentous algae. T Am Microsc Soc

377 69:272-279

378 Osono T (2005) Colonization and succession of fungi during decomposition of

379 *Swida controversa* leaf litter. Mycologia 97:589-597

380 Osono T (2007) Ecology of ligninolytic fungi associated with leaf litter

381 decomposition. Ecol Res 22:955-974

382 Osono T (2011) Diversity and functioning of fungi associated with leaf litter

383 decomposition in Asian forests of different climatic regions. Fun Ecol

384 4:375-385

385 Osono T, Takeda H, (2001) Organic chemical and nutrient dynamics in

386 decomposing beech leaf litter in relation to fungal ingrowth and

387 succession during three year decomposition processes in a cool temperate

388 deciduous forest in Japan. Ecol Res 16:649-670

389 Osono T, Hobara S, Fujiwara S, Koba K, Kameda K (2002) Abundance, diversity,

390 and species composition of fungal communities in a temperate forest

391 affected by excreta of the Great Cormorant *Phalacrocorax carbo*. Soil Biol

392 Biochem 34:1537-1547

393 Osono T, Ono Y, Takeda H (2003) Fungal ingrowth on forest floor and
394 decomposing needle litter of *Chamaecyparis obtusa* in relation to
395 resource availability and moisture condition. Soil Biol Biochem
396 35:1423-1431

397 Osono T, Hirose D, Fujimaki R (2006) Fungal colonization as affected by litter
398 depth and decomposition stage of needle litter. Soil Biol Biochem
399 38:2743-2752

400 Osono T, Takeda H (2007) Microfungi associated with *Abies* needles and *Betula*
401 leaf litter in a subalpine coniferous forest. Can J Microbiol 53:1-7

402 Osono T, Takeda H, Azuma JI (2008a) Carbon isotope dynamics during leaf litter
403 decomposition in a cool temperate forest with reference to lignin fractions.
404 Ecol Res 23:51-55

405 Osono T, Ishii Y, Takeda H (2008b) Fungal colonization and decomposition of
406 *Castanopsis sieboldii* leaves in a subtropical forest. Ecol Res 23:909-917

407 Osono T, Ishii Y, Takeda H, Seramethakun T, Khamyong S, To-Anun C, Hirose D,
408 Tokumasu S, Kakishima M (2009) Fungal succession and lignin

409 decomposition on *Shorea obtusa* leaves in a tropical seasonal forest in
410 northern Thailand. Fungal Diversity 36:101-119

411 Osono T, Hobarra S, Hishinuma T, Azuma JI (2011) Selective lignin decomposition
412 and nitrogen mineralization in forest litter colonized by *Clitocybe* sp. Eur
413 J Soil Biol 47:114-121

414 Osono T, Ueno T, Uchida M, Kanda H (2012) Abundance and diversity of fungi in
415 relation to chemical changes in arctic moss profiles. Polar Sci 6:121-131

416 Osono T, Matsuoka S, Hirose D, Uchida M, Kanda H (2014) Fungal colonization
417 and decomposition of leaves and stems of *Salix arctica* on deglaciated
418 moraines in high-Arctic Canada. Polar Sci 8:in press

419 Robinson CH, Borisova OB, Callaghan TV, Lee JA (1996) Fungal hyphal length in
420 litter of *Dryas octopetala* in a high-Arctic polar semi-desert, Svalbard.
421 Polar Biol 16:71-74

422 Ruscoe QW (1971) The soil mycoflora of a hard beech forest. New Zealand Journal
423 of Science 14:554-567

424 Takeda H, Kaneko N (1988) Patterns of soil humus accumulation in forests. I.
425 Mull and moder types humus in a broad-leaved forest. Bull Kyoto Univ

426 Forest 60:33-45 (in Japanese with English abstract)

427 Tateno R, Takeda H (2003) Forest structure and tree species distribution in
428 relation to topography-mediated heterogeneity of soil nitrogen and light
429 at the forest floor. *Ecol Res* 18:559-571

430 Tian X, Takeda H, Ando T (1997) Application of a rapid thin section method for
431 observations on decomposing litter in mor humus form in a subalpine
432 coniferous forest. *Ecol Res* 12:289-300

433 Tokumasu S (1998) Fungal successions on pine needles fallen at different seasons:
434 the succession of surface colonizers. *Mycoscience* 39:417-423

435 Tsukamoto J (1996) Soil macro-invertebrates and litter disappearance in a
436 Japanese mixed deciduous forest and comparison with European
437 deciduous forests and tropical rainforests. *Ecol Res* 11:35-50

438 van der Wal A, Bloem J, Mulder C, de Boer W (2009) Relative abundance and
439 activity of melanized hyphae in different soil ecosystems. *Soil Biol*
440 *Biochem* 41:417-419

441 West AW (1988) Specimen preparation, stain type, and extraction and observation
442 procedures as factors in the estimation of soil mycelial lengths and

- 443 volumes by light microscopy. *Biol Fertil Soils* 7:88-94
- 444 Widden P, Parkinson D (1979) Populations of fungi in a high arctic ecosystem. *Can*
- 445 *J Bot* 57:2408-2417

1 Figure legends.

2

3 Fig. 1. Seasonal changes in air temperature at the study sites. □, subtropical (ST,
4 Okinawa); ●, cool temperate (CT, Kyoto); ▲, subalpine forest (SA, Gifu). Values
5 are long-term averages for 22 to 35 years.

6

7 Fig. 2. Total hyphal length in soil layers. Values are mean \pm standard errors. □, L
8 layer; ●, F layer; ▲, A layer. Note that the scales of the Y-axis are not consistent
9 between the sites.

10

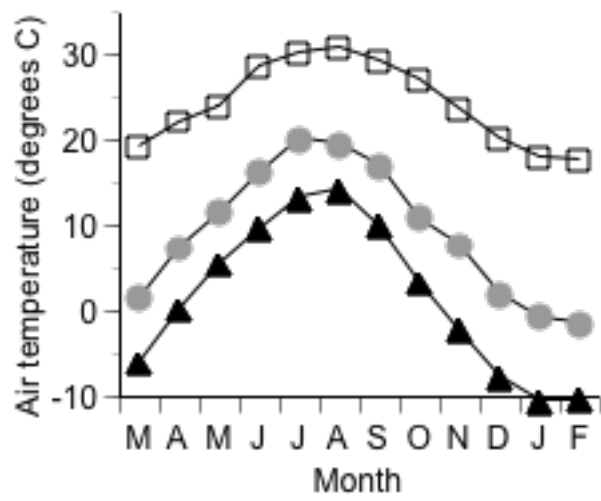
11 Fig. 3. Darkly pigmented hyphal length (upper) and its proportion relative to the
12 total hyphal length (lower) in soil layers. Values are mean \pm standard errors.
13 Symbols are as in Fig. 2. Note that the scales of the Y-axis are not consistent
14 between the sites.

15

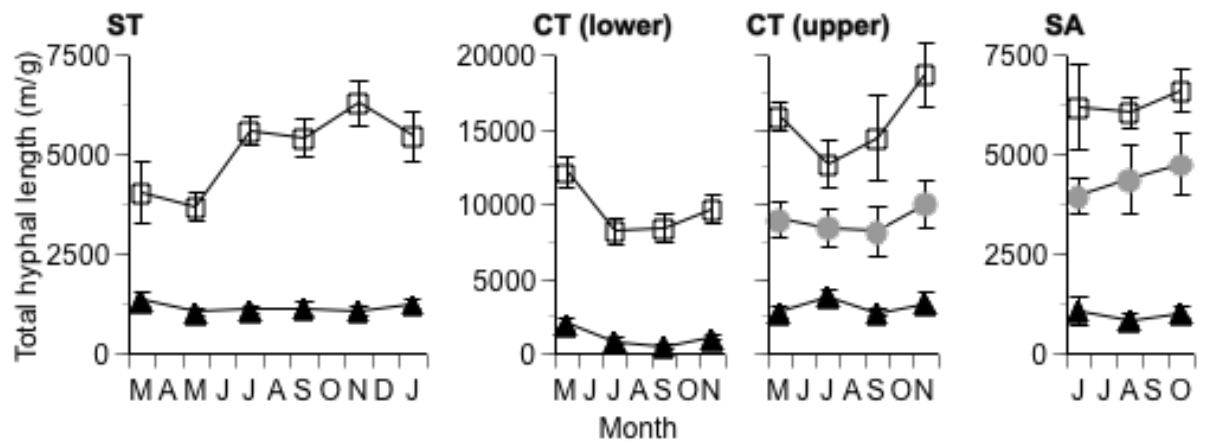
16 Fig. 4. Clamp-bearing hyphal length (upper) and its proportion relative to the

- 1 total hyphal length (lower) in soil layers. Values are mean \pm standard errors.
- 2 Symbols are as in Fig. 2. Note that the scales of the Y-axis are not consistent
- 3 between the sites.
- 4

1 Osono Fig. 1
2

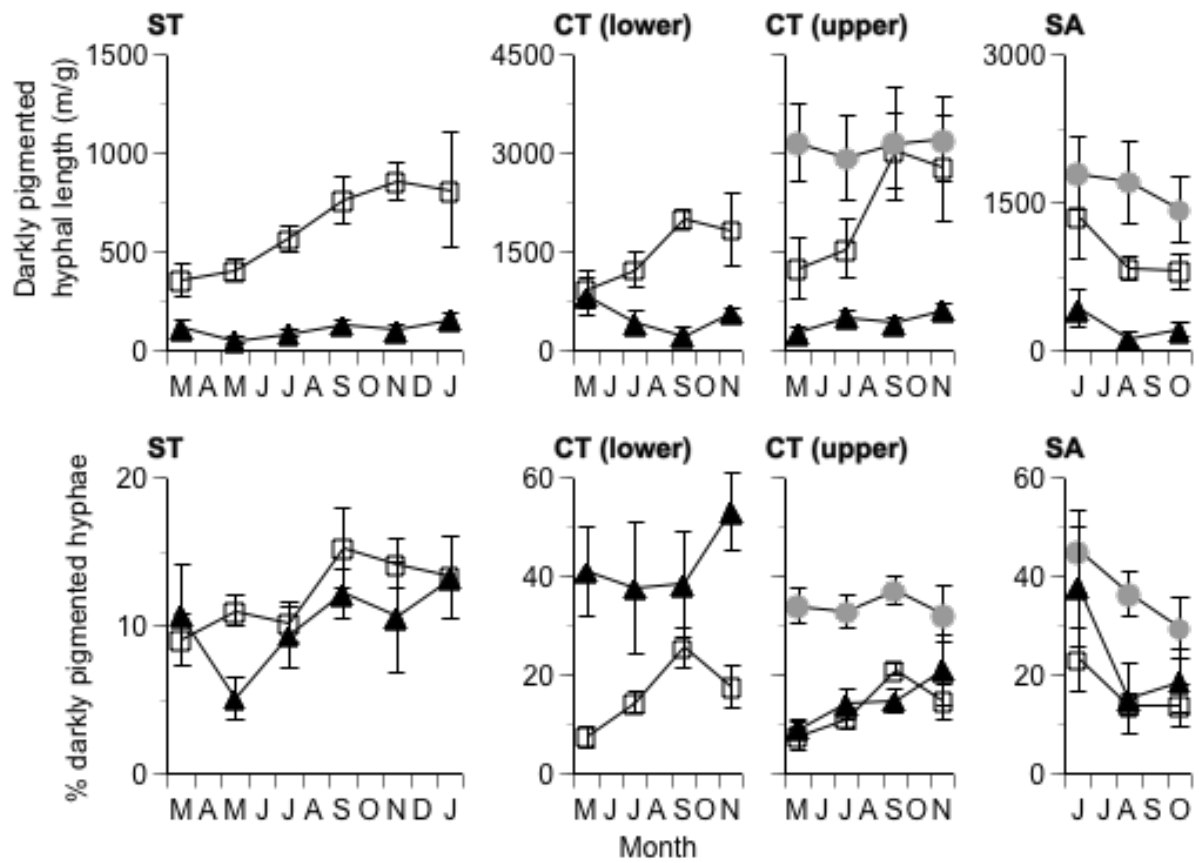


1 Osono Fig. 2
2

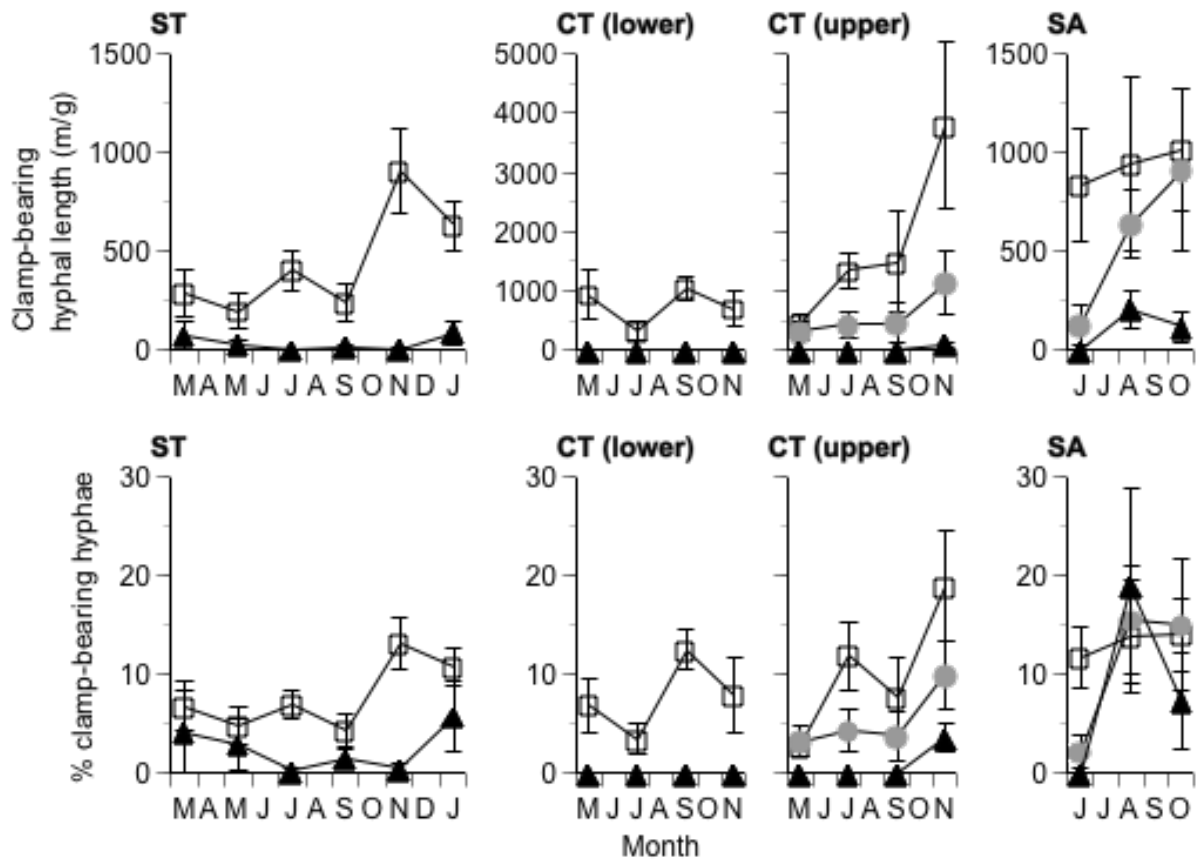


1 Osono Fig. 3

2



1 Osono Fig. 4



Osono Table 1

Table 1. Location, climate, vegetation, and properties of the forest floor in the study sites.

Site ID	ST	CT	SA
Location			
Latitude N	26°49'	35°18'	35°56'
Longitude E	128°50'	135°43'	137°28'
Elevation (m)	330	660	2050
Site	Kunigami, Okinawa	Ashiu, Kyoto	Mt. Ontake, Gifu
Climate			
Mean annual temperature (°C)	22 ^a	10 ^b	2 ^c
Annual precipitation (mm)	2456 ^a	2495 ^b	2500 ^c
Climate region	Subtropical	Cool temperate	Subalpine
Vegetation			
Type	Evergreen broad-leaved	Deciduous broad-leaved	Evergreen coniferous
Dominant tree species	<i>Castanopsis sieboldii</i> , <i>Schima wallichii</i> ^d	<i>Fagus crenata</i> , <i>Quercus crispula</i> ^e	<i>Abies mariesii</i> , <i>A. veitchii</i> , <i>Picea jezoensis</i> var. <i>hondoensis</i> , <i>Betula ermanii</i> ^f
Forest floor			
Humus type	Mull	Mull (lower), Moder (upper) ^g	Moder ^h
Depth of L layer (cm) ⁱ	1.1 ± 0.1 b	1.2 ± 0.1 b	2.7 ± 0.3 a
Depth of F layer (cm) ⁱ	1.0 ± 0.2 c	4.0 ± 0.4 b	13.4 ± 1.2 a

^aOsono et al. (2008b), ^bAshiu Experimental Forest, Kyoto University, ^cOsono and Takeda (2007), ^dEnoki (2003), ^eTateno and Takeda (2003), ^fMori et al. (2004), ^gTakeda and Kaneko (1988), ^hTian et al. (1997).

ⁱValues indicate means ± standard errors (n=20) for the depths of L and F layers. Measurement was carried out in the three study sites in October 2012. Values of CT were from the lower slope. The same letters indicate that the values were not statistically different at 5% level with Tukey's HSD test.

Osono Table 2

Table 2. Summary of generalized linear models for hyphal length. χ^2 values are indicated. *** P<0.001, ** P<0.01, * P<0.05, ns not significant.

	Model	Layer	Month	Layer × Month
Total hyphal length				
ST	165.0 ***	155.7 ***	15.7 **	17.5 **
CT (lower)	88.3 ***	84.9 ***	16.7 ***	4.9 ns
CT (upper)	82.3 ***	78.9 ***	5.6 ns	6.1 ns
SA	79.4 ***	78.8 ***	0.8 ns	0.6 ns
Darkly pigmented hyphal length				
ST	79.6 ***	65.7 ***	14.4 *	9.8 ns
CT (lower)	28.4 ***	20.5 ***	2.6 ns	9.6 *
CT (upper)	45.9 ***	40.4 ***	4.3 ns	5.7 ns
SA	37.1 ***	34.0 ***	3.4 ns	1.1 ns
% darkly pigmented hyphae				
ST	15.3 ns	2.3 ns	9.9 ns	3.7 ns
CT (lower)	23.3 **	20.9 ***	3.0 ns	3.1 ns
CT (upper)	68.6 ***	61.9 ***	9.4 *	7.8 ns
SA	22.5 **	13.4 **	8.8 *	1.8 ns
Clamp-bearing hyphal length				
ST	75.8 ***	48.6 ***	20.5 **	21.4 ***
CT (lower)	26.6 ***	21.7 ***	4.0 ns	4.0 ns
CT (upper)	38.8 ***	21.7 ***	12.6 **	11.9 ns
SA	20.2 **	15.4 ***	3.4 ns	2.3 ns
% Clamp-bearing hyphae				
ST	31.0 **	16.5 ***	8.7 ns	8.4 ns
CT (lower)	32.2 ***	25.3 ***	6.3 ns	6.3 ns
CT (upper)	41.7 ***	23.7 ***	18.2 ***	7.8 ns
SA	12.2 ns	1.2 ns	7.7 *	3.8 ns

Electronic Supplementary Material

Hyphal length in the forest floor and soil of subtropical, temperate, and subalpine forests

Takashi Osono

S1. A review of hyphal length in forest and tundra litter examined with the same direct observation method. MAT, mean annual temperature; AP, annual precipitation. na, not available. Numbers in parentheses are the proportions relative to the total hyphal length.

Location	Climate	MAT °C	AP mm	Vegetation	Tree species	Sample	Total hyphal length m/g	Darkly pigmented hyphal length m/g	Clamp-bearing hyphal length m/g	Ref.
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	<i>Fagus crenata</i>	Litter	7867	983 (12)	410 (5)	1
Shiga, Japan	Warm temperate	15	1475	Cypress plantation	<i>Chamaecyparis obtusa</i>	L layer	12334	5800 (42)	1191 (10)	2
Kyoto, Japan	Warm temperate	15	1734	Secondary	<i>Chamaecyparis obtusa</i>	Litter	7595	970 (13)	79 (1)	3
						L2 layer	12523	2943 (24)	560 (4)	
						F layer	9549	2805 (29)	119 (1)	
						H layer	6932	2546 (37)	167 (2)	
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	<i>Swida controversa</i>	Litter	5309	925 (16)	111 (2)	4
Kyoto, Japan	Warm temperate	15	1581	Secondary	<i>Camellia japonica</i>	Litter	7824	998 (13)	136 (2)	5

Kyoto, Japan	Warm temperate	15	1734	Secondary	<i>Chamaecyparis obtusa</i>	Litter	12321	3622 (29)	154 (1)	6	
				Pine plantation	<i>Pinus pentaphylla</i>	Litter	10385	1337 (13)	336 (3)		
Okinawa, Japan	Subtropical	22	2456	Evergreen broad-leaved	<i>Castanopsis sieboldii</i>	Litter	6593	525 (9)	556 (8)	7	
Chiang Rai, Thailand	Tropical seasonal	25	1155	Dry dipterocarpus	<i>Shorea obtusa</i>	Litter	2738	551 (19)	80 (2)	8	
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	<i>Fagus crenata</i>	L layer	6927	1001 (13)	na	9	
Nunavut, Canada	Arctic	-20	64	Tundra	<i>Hylocomium splendens</i>	Moss	4446	1859 (41)	658 (13)	10	
					<i>Racomitrium lanuginosum</i>	Moss	1164	349 (30)	107 (5)		
Nunavut, Canada	Arctic	-20	64	Tundra	<i>Salix arctica</i>	Litter	4068	1063 (30)	145 (2)	11	
Nagano, Japan	Cool temperate			Grassland	<i>Miscanthus sinensis</i>	Litter	4720	395 (10)	155 (3)	12	
				Secondary coniferous	<i>Pinus densiflora</i>	Litter	10273	1933 (20)	1454 (14)		
				Deciduous broad-leaved	<i>Quercus crispula</i>	Litter	3775	490 (15)	320 (9)		
Okinawa, Japan	Subtropical	22	2456	Evergreen broad-leaved	<i>Castanopsis sieboldii</i>	L layer	5092	630 (12)	444 (8)	13	
						A layer	1175	110 (10)	36 (3)		
Kyoto, Japan	Cool temperate	10	2495	Deciduous broad-leaved	<i>Fagus crenata</i> (mull)	L layer	8514	1603 (22)	627 (7)		
						A layer	1139	510 (41)	0 (0)		
						<i>Fagus crenata</i> (moder)	L layer	15473	2154 (14)	1761 (10)	
						F layer	8949	3118 (34)	585 (5)		
						A layer	3242	451 (15)	22 (1)		
Gifu, Japan	Subalpine	2	2500	Evergreen coniferous	<i>Abies</i> spp., <i>Betula ermanii</i>	L layer	6289	1004 (17)	929 (13)		
						F layer	4382	1649 (37)	559 (11)		
						A layer	998	255 (24)	109 (9)		

Reference. 1, Osono and Takeda (2001); 2, Data of control site, Osono et al. (2002); 3, Osono et al. (2003); 4, means for sterilized and unsterilized litter, Osono (2005); 5, means for bleached and nonbleached portions, Koide et al. (2005); 6, means for four treatments, Osono et al. (2006); 7, means for bleached and nonbleached portions, Osono et al. (2008b); 8, Osono et al. (2009); 9, data of nonbleached litter, Osono et al. (2011); 10, means for horizontal layers, Osono et al. (2012); 11, Osono et al. (2014); 12, Hobara et al. (2014); 13, the present study.

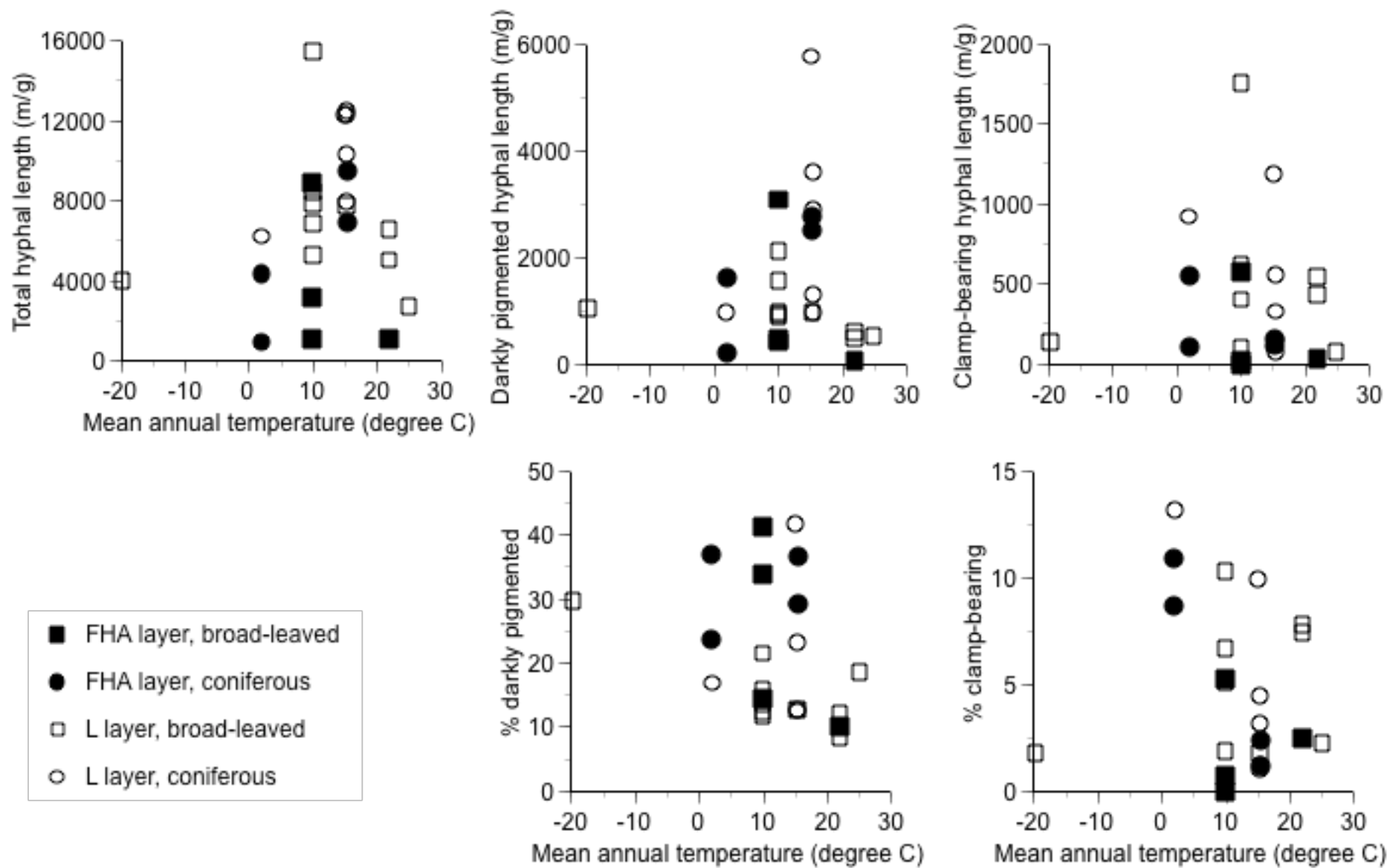


Fig. S1. Hyphal length plotted against mean annual temperature. Data are after Table S1. Data of arctic moss are not included.