Geographic Distribution of Needle Litter Microfungi in British Columbia

Shunsuke MATSUOKA,* Koichi ITO,** and Takashi OSONO***

(Received January 9, 2024)

The geographic distribution of microfungal diversity associated with needle litter was investigated in British Columbia, south-western Canada. A total of 77 microfungal species were isolated from needle litters of nine tree species in *Pseudotsuga*, *Tsuga*, *Picea*, and *Abies* collected in 25 coniferous forest sites that varied in climatic conditions and geographic locations. The nonmetric multidimensional scaling ordination showed the segregation of microfungal species composition between the study sites and needle species, which was significantly correlated to the latitude, elevation, mean annual temperature, and mean temperature at coldest and warmest months of the sites. Major microfungal species showed variable responses to these environmental factors: *Trichoderma polysporum* and *Penicillium miczynskii* tended to occur at higher elevations and latitudes and lower temperatures, compared with other species of the same genera. In contrast to the species composition, the mean number of species was not significantly affected by needle species, geographic locations, or climatic conditions. Applying variation partitioning to disentangle the relative effect of the environmental and spatial factors indicated the role of not only climatic but spatial factors in structuring fungal assemblages, suggesting the contribution of such non-niche processes as priority effect and dispersal limitation.

Keywords: alpha and beta diversity, dead conifer needles, microfungal species, climate gradient, variation partitioning

1. Introduction

Fungi are an indispensable component of forest diversity and functioning and serve as decomposers, mutualists, and pathogens of plants, animals, and eukaryotic and prokaryotic microbes[1,2]. Saprobic fungi taking part in the decomposition of plant litter are of crucial importance, as they are capable of mineralizing organic chemical constituents to carbon dioxide and recycling essential nutrients in soil, such as nitrogen and phosphorus, making them available for the primary production of forest trees[3,4]. Previous studies have elucidated that the functioning of decomposer fungal communities is closely related to the taxonomic richness and composition of fungal species[5]. Moreover, the ability of fungi to decompose leaf litter can vary significantly with the litter quality and the temperature these fungi experience[6]. Therefore, studying the diversity and functioning of decomposer fungi with respect to environmental conditions, such as geographic locations, local climates, and associations with tree species, is essential in understanding ecosystem functioning of forest soils and predicting the response of forest ecosystems to future climate changes.

Much remains unknown, however, regarding the geographic pattern of the diversity of microfungi associated with plant litter decomposition and the environmental factors influencing the geographic distribution. In contrast to early predictions that fungi are probably long-distance dispersers and are found everywhere[7], previous studies have demonstrated the geographic structures and climatic controls of litter microfungi. For example, Tokumasu[8] studied the geographic distribution of *Sporidesmium goidanichii* on

*Field Science Education and Research Center, Kyoto University, Kyoto
**Faculty of Environmental Earth Science, Hokkaido University, Hokkaido
***Department of Environmental Systems Science, Faculty of Science and Engineering, Doshisha University, Kyoto
Telephone : +81-774-65-6688, E-mail : tosono@mail.doshisha.ac.jp

dead pine needles at 282 sites across Japan and found that mean annual temperature predicted the occurrence of this fungus, with an optimal climatic area that is restricted to the center of the warm temperate regions of the main islands. More recently, Osono et al.[9] studied the geographic pattern of fungal assemblages associated with the decomposition of leaf litter of *Castanopsis sieboldii* in Japan and found that the fungal compositions were related to the mean annual temperature of the study sites. These studies suggest the importance of climatic conditions, especially mean annual temperature, as a principal factor affecting the geographic distribution of saprobic microfungi on litter, at least in Japan. Further studies are needed to determine if the same trend is found to saprobic fungi in other regions or on litters of other tree species.

The purpose of the present study was to investigate the geographic distribution of microfungal diversity associated with needle litter in British Columbia (BC), south-western Canada. The BC ecosystems are diverse in terms of climate and vegetation, including temperate and boreal forests, parklands, grasslands, deserts, and alpine tundra, of which coniferous forests account for approximately 64% of the land area[10,11]. Therefore, we selected 25 forest sites in BC that varied in climatic conditions and geographic locations and collected needle litters of nine tree species in four genera (*Pseudotsuga*, *Tsuga*, *Picea*, and *Abies*) that are dominant components in these sites. Microfungi were isolated from these needles using a culture-dependent method and identified micromorphologically to describe the species richness and composition. The dataset was then analyzed to test the hypothesis that geographic patterns of microfungal alpha and beta diversity were affected by host trees and related with geographic locations and climatic conditions of the study sites. Finally, variation partitioning was applied to estimate the relative importance of the host trees, environment, and spatial distances to account for the variation of microfungal composition.

Table 1. Locations of 25 sites and needle species collected.

Site	Site code	Biogeoclimatic Ecosystem Classification[10]	Lat N	Long W	Elevation (m)	Needle species collected[a]				Month of collection in 2005
						Pseudotsuga	*Tsuga*	*Picea*	*Abies*	
Pacific Rim	PR	CWHvh1	49.0409	125.6815	45	-	Hw	-	-	Jul
Kennedy River	KR	CWHvm1	49.1530	125.4203	82	Fd	Hw	-	-	Jul
Sutton Pass	SP	CWHmm1	49.2905	125.3362	156	Fd	Hw	-	-	Jul
Sprout Lake	SL	CWHxm1	49.2753	125.0413	66	Fd	Hw	-	-	Jul
Alberni Summit	AS	CWHxm2	49.2641	124.7314	241	Fd	Hw	-	-	Jul
Rathtrevor Beach	RB	CDFmm	49.3245	124.2700	5	Fd	-	-	-	Jul
Victoria	MH	CDFmm	48.4635	123.4760	87	Fd	-	-	Bg	Sept
West Vancouver	WV	CWHdm	49.3598	123.2060	613	Fd	Hw	-	-	Aug
Mission	MI	CWHvm1	49.2718	122.2150	137	Fd	Hw	-	-	Aug
Hope	HO	CWHds1	49.3708	121.4043	83	Fd	Hw	-	-	Aug
Princeton	PN	IDFdk1	49.3698	120.1367	535	Fd	-	-	-	Aug
Rock Creek	RC	IDFxh4	49.0475	119.0504	867	Fd	-	-	-	Aug
Castlegar	CG	ICHdw1	49.1898	117.6226	1061	Fd	Hw	-	-	Aug
Creston	CR	ICHxw	49.1982	116.5871	547	Fd	-	-	-	Aug
Cranbrook	CB	IDFdm2	49.5406	115.4867	838	Fd	-	-	-	Aug
Baynes Lake	SC	PPdh2	49.2349	115.2349	795	Fd	-	-	-	Aug
Forbidden Plateau	FP	MHmm1	49.7257	125.3152	1184	-	Hm	-	Ba	Sept
Joffre Lakes	JL	ESSFmw	50.3637	122.4917	1235	-	Hm	Pe	Ba	Oct
150 Mile House	OF	SBPSmk	52.0374	121.8102	962	-	-	Pg	-	Oct
Barkerville	BV	ESSFwk1	53.0843	121.5168	1247	-	-	Pe	Bl	Oct
Prince George	PG	SBSmk1	53.4333	122.5931	622	-	-	Pi	Bl	Oct
Pine Pass	PP	SBSwk2	55.3741	122.5922	936	-	-	Pi	Bl	Oct
Dawson Creek	DC	BWBSmw1	55.9633	120.5693	671	-	-	Pg	-	Oct
Pink Mountain	PM	BWBSwk2	57.1177	122.6927	1091	-	-	Pg	-	Oct
Muncho Lake	NR	SWBmk	58.9203	125.7712	847	-	-	Pg	-	Oct

[a]Fd, Douglas fir (*P. menziesii*); Hw, western hemlock (*T. heterophylla*), Hm, mountain hemlock (*T. mertensiana*); Pe, Engelmann spruce (*P. engelmannii*), Pg, white spruce (*P. glauca*), Pi, interior spruce (a hybrid species of *P. engelmanii* and *P. glauca*); Bg, grand fir (*A. grandis*), Ba, amabilis fir (*A. amabilis*), Bl, subalpine fir (*A. lasiocarpa*).

2. Materials and Methods

2.1. Study area and sample collection

Samples were collected in 25 sites in 11 biogeoclimatic zones in British Columbia (BC), Canada (Table 1; Figs 1 and 2). Meidinger and Pojar[10] classified the ecosystems of British Columbia into 14 biogeoclimatic zones, primarily according to climate (e.g., temperature and precipitation), soil, and vegetation; and the present study covered 11 zones, and did not cover the Alpine Tundra, the Bunchgrass, and the Montane Spruce zones. Climatic data of the 25 sites are summarized in Table 2, including six climatic variables, i.e., mean annual temperature, mean temperature at coldest and warmest month, and mean annual, summer, and winter precipitation[12,13].

In these sites, fallen needles of nine tree species (denoted as needle species) in four genera were collected in July to October 2005 (Table 1). The nine needle species are: *Pseudotsuga menziesii*, *Tsuga heterophylla*, *T. mertensiana*, *Picea engelmanii*, *P. glauca*, a hybrid species of *P. engelmanii* and *P. glauca*, and *Abies grandis*, *A. amabilis*, and *A. lasiocarpa*. One to 3 needle species were chosen per site. The number of sites where *Pseudotsuga*, *Tsuga*, *Picea*, and *Abies* needles were sampled were 15, 11, 8, and 6, respectively (Table 1). Ten blackish needles were arbitrarily collected from the Oe horizon per site and per needle species, placed in paper bags and preserved at 4˚C, and taken back to the laboratory. Totally, we made a total of 40 combinations of needle species × site, and 400 needles were used for the isolation of microfungi.

2.2. Fungal isolation

Microfungi were isolated from needles with a modified washing method[14]. Needles were washed five times with 10 ml of sterilized 0.005% Aerosol-OT (di-2-ethylhexyl sodium sulfosuccinate) solution and then rinsed with sterilized water five times in a sterile test tube using a vertical type automatic mixer (S-100; Taitec Co., Ltd., Japan). The rinsed needles were transferred to a sterile filter paper in 9-cm Petri dishes and dried for 24 hours. The needles were then placed on the surface of lignocellulose

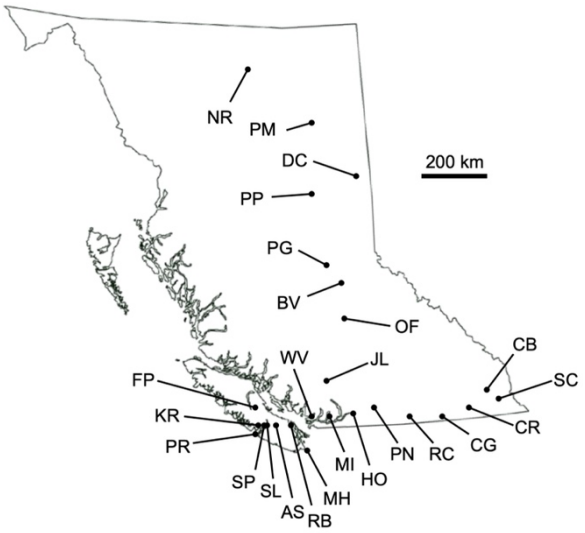

Fig. 1. Locations of the 25 sites in British Columbia. Site codes are as in Table 1. Bar = 200 km.

agar (LCA) modified as described by Miura and Kudo[15], two needles per plate, and incubated at 20˚C in the dark. LCA contains glucose 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.02%, KCl 0.02%, $NaNO_3$ 0.2%, yeast extract 0.02%, and agar 1.3% (w/v). The plates were observed microscopically eight times at 1-week intervals for eight weeks. Any fungal colonies or spores appearing on the plate were isolated, transferred to a fresh LCA plate, incubated, and identified by observing micromorphological characteristics, with reference to Domsch et al.[16], Ellis[17,18], Pitt[19], and Rifai[20]. One isolate of each microfungal species per needle was retrieved even when two or more isolates of the same species appeared. Microfungal species were regarded as major ones when the number of needles from which that particular species occurred was more than 40 out of the 400 needles tested.

2.3. Statistical analyses

Generalized linear models (GLMs) were used to examine the effects of genus of needle species (*Pseudotsuga*, *Tsuga*, *Picea*, and *Abies*), longitude, latitude, elevation of the site, and six climatic variables on the number of microfungal species. The error structures of the

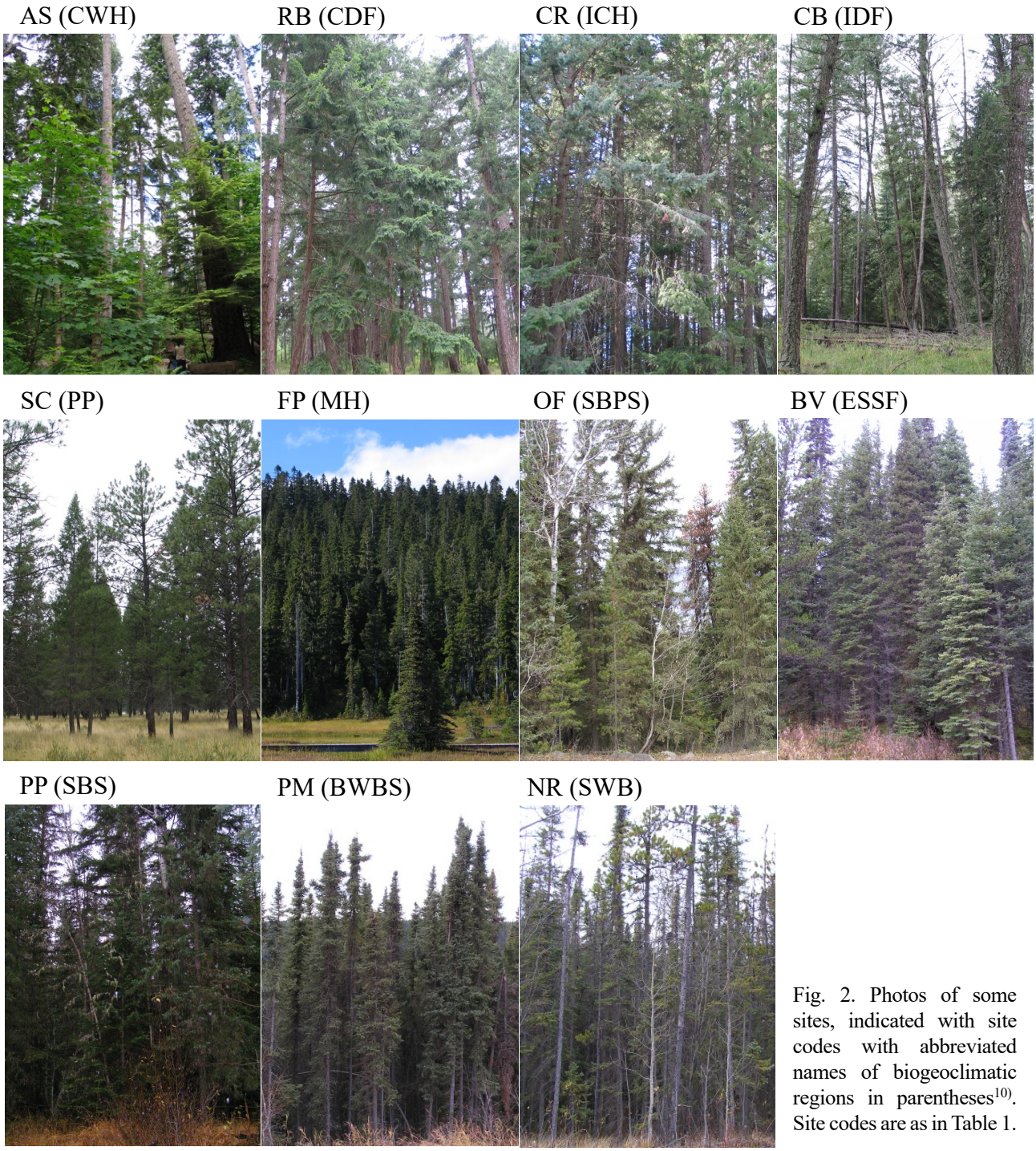

Fig. 2. Photos of some sites, indicated with site codes with abbreviated names of biogeoclimatic regions in parentheses[10]. Site codes are as in Table 1.

GLMs were Poisson. The 'glm' function in the R package 'stats' was used to perform the analyses.

Nonmetric multidimensional scaling (NMDS) with the Jaccard distance metric was used to analyze the variation in the species composition of microfungi. The NMDS analysis was carried out with the metaNDS function with default settings of the vegan package of R software[21]. One-way permutational multivariate analysis of variance (PERMANOVA) was conducted to explore whether the dissimilarity of species composition was related to the genus of needle species, longitude, latitude, and elevation of the site, and six climatic variables. Note

Table 2. Climatic conditions of 25 sites[12,13]. Site codes are as in Table 1.

Site code	Mean annual temperature (˚C)	Mean temperature at coldest month (˚C)	Mean temperature at warmest month (˚C)	Mean annual precipitation (mm)	Mean summer precipitation (mm)	Mean winter precipitation (mm)	Data origin
PR	8.9	3.8	14.4	3288	608	2680	Tofino
KR	8.2	0.3	16.0	2786	575	2212	Mean CWHvm1
SP	5.7	-2.2	14.1	2349	470	1879	Mean CWHmm
SL	9.2	0.8	17.7	2019	291	1729	Port Alberni
AS	8.8	1.3	16.6	1953	334	1619	Mean CWHxm2
RB	8.8	1.6	16.7	964	190	734	Parksville
MH	9.7	4.0	15.8	883	134	750	William Head
WV	9.8	1.9	17.6	1823	384	1439	Mean CWHdm
MI	8.7	0.9	16.9	2775	596	2179	Alouette Lk
HO	9.7	-0.4	18.5	1716	327	1389	Hope
PN	5.4	-10.2	17.3	386	191	195	Mean IDFdk
RC	6.0	-7.6	17.8	409	163	246	Mean IDFxh2
CG	8.2	-3.8	20.3	719	234	485	Castlegar
CR	7.8	-3.7	19.6	569	200	369	Creston
CB	5.5	-8.6	18.4	370	167	203	Cranbrook
SC	7.0	-5.7	19.3	494	259	234	Grasmere
FP	4.8	-2.3	13.2	2760	701	2059	Mean MHmm1
JL	2.8	-7.6	13.3	1525	288	1236	Mean ESSFmw
OF	3.5	-9.5	14.2	546	264	282	Moffat Creek
BV	1.4	-10.7	12.1	1044	425	619	Barkerville
PG	3.3	-12.1	15.1	628	301	328	Prince George A
PP	0.8	-15.1	13.5	1916	533	1383	Pine pass
DC	0.9	-17.7	14.9	445	258	186	Dawson Creek
PM	-0.5	-16.3	13.0	533	357	177	Pink Mountain
NR	-0.7	-18.7	14.0	459	280	179	Muncho Lake

that in NMDS and PERMANOVA the data of singletons were not included and that data of one needle sample (RB-Fd_1) was not included because this is an outlier in preliminary analyses.

We used variation partitioning based on the distance-based redundancy analysis (db-RDA) to quantify the contribution of the environmental and spatial variables to the community structure of microfungi. The relative weight of each fraction (pure fractions, shared fractions, and unknown fractions) was estimated following the methodology described by Peres-Neto et al.[22]. Presence/absence data of microfungal species for each needle sample were converted into a dissimilarity matrix using Simpson's index. We differentiated total dissimilarities into turnover and nestedness components, and this differentiation indicated that the turnover was responsible for most of the dissimilarity. We then constructed needle species (tree genus), environmental, and spatial models. We constructed an environmental model by applying the forward selection procedure (999 permutations with an alpha criterion = 0.05) of Blanchet et al.[23], using seven environmental variables (elevation, mean annual temperature, mean temperature at warmest and coldest month, and mean annual, summer, and winter precipitation). Then, we constructed a model using spatial variables extracted based on the principal components of neighbor matrices (PCNM)[24]. The PCNM analysis produced a set of orthogonal variables derived from the geographical coordinates of the sampling locations (i.e., the position of sampling grids). We used the PCNM vectors that best accounted for autocorrelation and then conducted forward selection (999 permutations with an alpha criterion of 0.05) to select spatial variables that significantly influenced community dissimilarities. Based on these three models, we performed variation partitioning by calculating adjusted R^2 values for each fraction[22]. The analyses were performed using the 'capscale' function in the R package 'vegan'.

3. Results

3.1. Taxonomic composition and species richness of microfungi

A total of 1400 microfungal isolates were retrieved from 400 needles, one to 10 isolates per needle, with 3.5 isolates per needle on average. These fungi were classified into 77 species, with 55 and 22 belonging to Ascomycota (939 isolates) and Mucoromycota (461 isolates), respectively. The eight major microfungal species that occurred in more than 40 out of the 400 needles tested include *Trichoderma polysporum* [59% (235) of 400 needles], *Penicillium glabrum* [34% (137)], *T. viride* [34% (135)], *Umbelopsis ramanniana* [24% (95)], *P. miczynskii* [18% (71)], *P. citrinum* [14% (55)], *Mortierella gamsii* [13% (51)], and *Mucor hiemalis* [10% (41)]. Of the 77 species, 25 species (32%) occurred only once and were designated 'singletons'.

The mean number of species per site and per needle species ranged from 1.9 to 5.3 (Table 3). The number of species was not significantly different between the genera of needle species and was not significantly related to longitude, latitude, elevation of the site, or six climatic variables of temperature and precipitation (Table 4a).

Table 3. Mean number of microfungal speices on needle litter. Values are ± standard errors (n=10). Site codes are as in Table 1.

Site code	*Pseudotsuga*	*Tsuga*	*Picea*	*Abies*
PR	-	2.1 ± 0.2	-	-
KR	2.2 ± 0.2	3.0 ± 0.3	-	-
SP	3.7 ± 0.4	2.8 ± 0.4	-	-
SL	4.3 ± 0.4	2.9 ± 0.4	-	-
AS	3.6 ± 0.4	4.4 ± 0.5	-	-
RB	3.0 ± 0.4	-	-	-
MH	4.7 ± 0.7	-	-	2.6 ± 0.5
WV	4.6 ± 0.7	4.2 ± 0.5	-	-
MI	2.8 ± 0.4	3.9 ± 0.4	-	-
HO	3.1 ± 0.3	3.7 ± 0.3	-	-
PN	3.4 ± 0.5	-	-	-
RC	3.2 ± 0.4	-	-	-
CG	3.1 ± 0.3	3.4 ± 0.5	-	-
CR	3.5 ± 0.4	-	-	-
CB	3.9 ± 0.5	-	-	-
SC	4.2 ± 0.6	-	-	-
FP	-	4.0 ± 0.5	-	4.4 ± 0.5
JL	-	3.1 ± 0.4	3.5 ± 0.3	3.3 ± 0.3
OF	-	-	4.1 ± 0.3	-
BV	-	-	3.0 ± 0.3	3.4 ± 0.4
PG	-	-	3.6 ± 0.5	5.3 ± 0.7
PP	-	-	4.3 ± 0.5	4.6 ± 0.4
DC	-	-	2.4 ± 0.3	-
PM	-	-	2.8 ± 0.4	-
NR	-	-	1.9 ± 0.2	-

3.2. Geographic pattern and dissimilarity of microfungal assemblages

Trichoderma polysporum occurred at 23 of the 25 sites, followed by P. *citrinum* (19 sites), *T. viride* and *U. ramanniana* (18 sites each), *P. glabrum* (17 sites), and *P. miczynskii*, *Mortierella gamsii*, and *Mucor hiemalis* (15 sites each). Of the 77 species, 31 species (40%) occurred only at one site.

The NMDS ordination showed the segregation of microfungal species composition between the study sites and the genera of needle species (Fig. 3a). The ordination was significantly correlated to the latitude ($R^2 = 0.049$, $P < 0.0001$), elevation ($R^2 = 0.156$, $P < 0.0001$), mean annual temperature ($R^2 = 0.098$, $P < 0.0001$), mean temperature at coldest month ($R^2 = 0.075$, $P < 0.0001$), and mean temperature at warmest month ($R^2 = 0.048$, $P = 0.0002$), but was not significantly ($P > 0.05$) correlated to the longitude, mean annual precipitation, mean summer precipitation, or mean winter precipitation ('envfit' function in R package 'vegan'). In the PERMANOVA, the microfungal species composition was significantly ($P < 0.001$) affected by the genera of needle species, latitude, longitude, elevation, and six climatic variables (Table 4b). The scores of the major microfungal species showed that their responses to these factors were variable among species (Fig. 3b).

In the variation partitioning, the percentage explained by the pure fraction of the genus of needle species, environmental, and spatial models was 1.0%, 4.0%, and 4.0%, respectively, and the shared fractions did not account for the variation in microfungal composition (i.e., 0%) . In the environmental model, all six environmental variables except mean annual precipitation, i.e., elevation, mean annual temperature, mean temperature at warmest and coldest month, and mean summer and winter precipitation, were selected as environmental variables. All nine Moran's

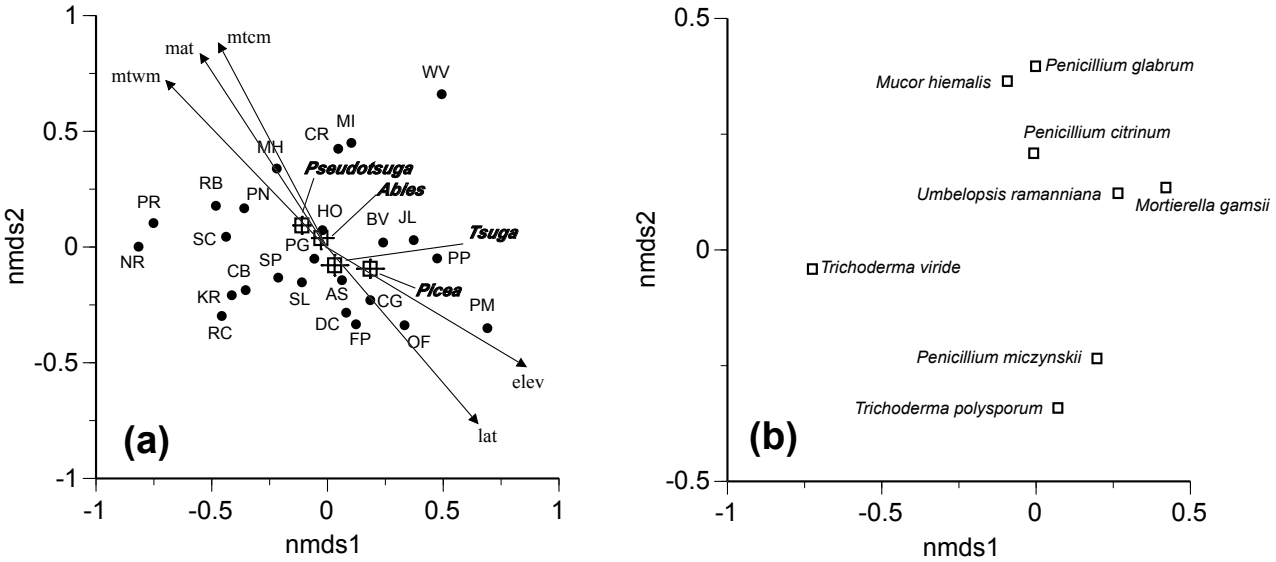

Fig. 3. (a) Dissimilarity among the microfungal assemblages as revealed by NMDS ordination using Jaccard distance (Stress value = 0.177). Open boxes: nmds scores of microfungal assemblages averaged for the genera of needle species; bars indicate standard errors. Site codes are as in Table 1. Selected climatic variables are indicated as vectors: lat, latitude; elev, elevation; mat, mean annual temperature; mtwm, mean temperature at warmest month; mtcm, mean temperature at coldest month. (b) scatter plot of nmds scores of major microfungal species.

Table 4. Summary of generalized linear model (GLM) and Permutational multivariate analysis of variance (PERMANOVA) testing the effect of genus of needle species and geographic and climatic variables on (a) species richness and (b) species composition of microfungi, respectively. *** P<0.001, ns, not significant.

Predictor variable	(a) GLM			(b) PERMANOVA		
	d.f.	Deviance	*P*	d.f.	*F*-value	*P*
Genus of needle species	3	3.86 ns	0.277	3	5.01 ***	0.000
Latitude	1	0.85 ns	0.357	1	8.10 ***	0.000
Longitude	1	0.87 ns	0.351	1	7.21 ***	0.000
Elevation	1	2.22 ns	0.136	1	14.19 ***	0.000
Mean annual temperature	1	1.64 ns	0.200	1	7.63 ***	0.000
Mean temperature at coldest month	1	0.64 ns	0.425	1	8.50 ***	0.000
Mean temperature at warmest month	1	3.29 ns	0.070	1	7.18 ***	0.000
Mean annual precipitation	1	0.00 ns	0.969	1	4.11 ***	0.000
Mean summer precipitation	1	0.56 ns	0.454	1	3.53 ***	0.000
Mean winter precipitation	1	0.52 ns	0.469	1	3.62 ***	0.000

eigenvector map (MEM) vectors (MEM1 to MEM9) were selected as spatial variables. In total, 9.0% of the variation was explained, and the remaining 91% was unexplained.

4. Discussion

The taxa of microfungi isolated from conifer needles in the present study are generally common to those noted in previously published reports of microfungi from forest floor materials, including not only needle litters[25] but also broad-leaved litters and bryophytes in Canada[26,27,28]. For example, Widden and Parkinson[25] found frequent occurrences of species in *Trichoderma* and *Penicillium* in Ascomycota and of *Mortierella*, *Mucor*, and *Umbelopsis* in Mucoromycota on pine needle litter in Ontario and Alberta. Moreover, species of these microfungal genera occur not only on alpine soil in the Canadian Rockies[29] but also arctic tundra in northern Canada[30] as well as on conifer needles in temperate forests of Europe and Japan[31,32],

suggesting that these fungi are widely distributed across Canada and the Northern Hemisphere.

According to the NMDS ordination and PERMANOVA (Table 4 and Fig. 3), the species composition of microfungi showed a geographic pattern across the 25 sites that was related with the latitudinal and elevational gradient of mean annual temperature, as well as mean temperatures at coldest and warmest months. Similar climate-driven geographic patterns of fungal composition were found globally[33,34] and locally in Japan[9]. Such a pattern was explained by the response of major microfungal species to temperature[8]. Specifically, *Trichoderma polysporum* and *Penicillium miczynskii* tended to occur at higher elevations and latitudes and lower temperatures, compared with other species of the same genera (Fig. 3). In fact, *T. polysporum* is known to occur frequently in arctic regions[30,35], was most abundant in winter[36,37], and was adapted to growth in cool environments[16]. In addition, the season at which the sampling was conducted is known to potentially affect the species composition of microfungi on dead needles[38].

Moreover, we detected a significant effect of pure spatial fraction on microfungi, implying possible roles of such non-niche processes as priority effect and dispersal limitation. Evidence of such dispersal-related spatial processes has been recently reported for microbes including fungi[39]. It should be noted, however, that a large fraction of the variation in species composition (91%) was left unexplained, which can result from unmeasured factors, such as local site conditions, vegetation, and stand history[28], decay stage and chemical quality of needles[31,32], interaction between microfungal species[40,41], and stochastic extinction and colonization of individual species[42]. For example, Osono and Trofymow[28] found that species composition of microfungi on forest floor moss changed with the age of secondary forests and the frequent occurrence of *T. polysporum* and *P. miczynskii* in mature and regenerating stands, respectively.

The present study also detected the difference in species composition of microfungi between the genera of needle species (Fig. 3). This is consistent with Hyde et al.[43] discussing that saprobic microfungi are specific at not only the species but also the genus level of host plant. In contrast to the species composition (i.e., beta diversity) of fungi, the GLM showed that the mean number of fungal species (alpha diversity) was not related with the genera of needle species, geographic locations, or climatic conditions (Table 4). This might be partly attributed to the relatively low mean numbers of microfungal species (1.9 to 5.3 species per needle). In fact, we cannot ignore the selectivity of the culture-dependent method used, which favors faster-growing and more heavily sporulating species. This is so despite the use of culture medium with low glucose content on which significantly higher numbers of microfungal species can be detected than on a more nutrient-rich one[44]. Future studies are thus needed to test whether the results found in the present study are method-dependent or not, by applying culture-independent approaches using DNA metabarcoding[45].

This study was supported by the Kyoto University Foundation and the Japanese Society for the Promotion of Science KAKENHI Grant (No. 18K05731). We thank Dr. S. Tokumasu for helpful assistance in identifying microfungal species; Dr. J.A. Trofymow for providing laboratory supplies and facilities; and Dr. Elizabeth Nakajima for critical reading of the manuscript.

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