Soil microbial ecology of laurel-leaved and *Cryptomeria japonica* forests assessed by dilution plate count and direct microscopic count methods

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Abstract
The soil ecology of the organic and mineral soil layers of laurel-leaved and *Cryptomeria japonica* forest in the Kasuga-yama Hill Primeval Forest (Nara, Japan) was assessed. The number of bacteria obtained by the dilution plate count method was less than 0.05% of those counted by the direct microscopic count. We therefore found that forest soil contains large numbers of non-culturable bacteria compared with agricultural soil. The numbers of bacteria and fungi obtained by both the dilution plate count and the direct microscopic count were larger in the deeper horizons (F and H) of the organic layer than in the mineral soil layer. This suggests that active microbial metabolism takes place in the organic layer. The numbers of bacteria and the length of fungal hyphae obtained by the direct count method were greater in the H horizon than in the F horizon. The direct microscopic count revealed numerous non-culturable bacteria and fungi in the soil. The ratio of fungal to bacterial biomass was lower in the laurel-leaved forest soil. The fungal biomass was therefore relatively low in the laurel-leaved forest soil due to differences in forest vegetation.

Key Words
Bacterial number, dilution plate count, direct microscopic count, forest soil, length of fungal hyphae, microbial biomass

Introduction
The world heritage Kasuga-yama Hill Primeval Forest in Nara, Japan, is a lowland laurel-leaved forest, in which natural conditions have been conserved for more than 1160 years (Suganuma 1982). Numerous unique species of plants, animals and insects have been found in the forest, attracting much research attention (Suganuma 1982). However, the microbial ecology of the soil supporting the primeval forest has not been elucidated to date. Therefore, we have started a series of studies to clarify the forest microbial ecology.
In forest ecology, research is often focused on the microbial decomposition processes occurring in the soil organic matter, which is influenced by differences in vegetation, climate, and soil type (Tanabe and Suzuki 1973). Most studies use the dilution plate count method to determine soil bacterial content. However, this method only measures about 1% of the total number of bacteria present in the soil (Someya 1997). Fungal counts are also limited by methods that only count spores suited to specific culture media (Tanabe and Suzuki 1972). In the present study, the organic and mineral soil layer of the soil from the laurel-leaved forest and the *Cryptomeria japonica* forest were examined for the numbers and biomass of bacteria and fungi using both the dilution plate count and the direct microscopic count methods.

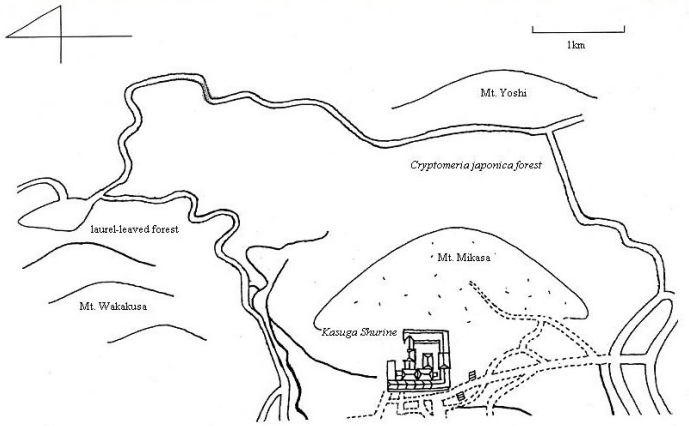

Figure 1. Kasuga-yama Hill Primeval Forest in Nara, Japan. Soil samples were obtained from the laurel-leaved and *C. japonica* forests, as indicated.

1 – 6 August 2010, Brisbane, Australia. Published on DVD.

Materials and methods

Soil samples

Figure 1 shows the outline of the Kasuga-yama Hill Primeval forest in Nara, Japan. Soil samples were collected from the laurel-leaved forest (B_{B-1}) and the *Cryptomeria japonica* forest (B_{B-2}) in April 2001. The B_{B-1} and B_{B-2} samples were dry brown forest soils. The vegetation of laurel-leaved forest was *Machilus thunbergii* and *Neolitsea aciculata*, while that of *C. japonica* forest was *C. japonica*. The soil was divided into 5 layers, including organic (L, F and H horizons) and mineral soil (A and B horizons) layers (Figure 2).

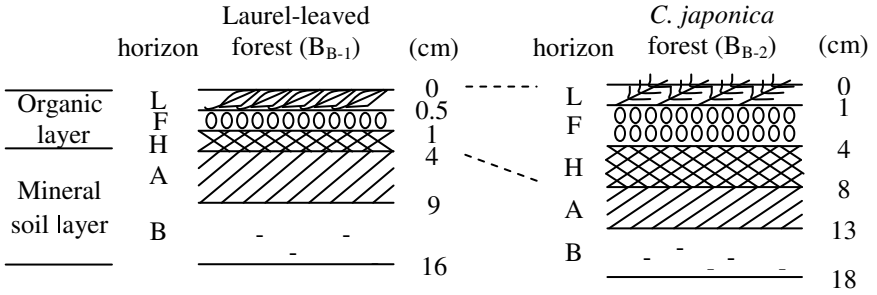

Figure 2. Soil profile of laurel-leaved and *C. japonica* forests.

Soil samples were analyzed for moisture content, pH, and carbon (C) and nitrogen (N) content (Table 1). For microbial analyses, soil samples from each horizon had particle sizes adjusted to <2 mm with scissors. Size-adjusted soil samples (5 g) were suspended in 45 mL of the sterile water and homogenized for 3 min at 12,000 rpm with a Homo blender (500C, Sakuma) (Kanazawa *et al*. 1986).

Measurements of soil microorganisms

The numbers of bacteria and fungi were measured using two methods: the dilution plate count method and the direct microscopic count method. Albumin or Rose Bengal-Streptomycin agar was used for the dilution count method to culture the bacteria or fungi contained in the sample solution at 25°C for 4 or 6 d (Tanabe and Suzuki 1972). The direct microscopic count method for bacteria was performed according to Someya's method (Someya 1995). The sample solution was dispersed for 20 min with an ultrasonic device (US-3, Kenis), diluted ten times, and then 100 μL was filtered through a 0.2 μm filter. Ethidium bromide solution (50 μL of the 100 μg/mL) was placed onto the filter for 3 min then filtered. The filter was dried, placed onto a glass slide and covered with immersion oil. Bacteria were counted under a fluorescence microscope (FX-35A, Nikon). Fungal hyphae length was measured according to the Jones-Mollison method (Jones and Mollison 1948). After 1 mL of the sample solution was added to 9 mL of 1.5% agar solution, an agar membrane was placed on a Haemocytometer. The membrane was stained with phenol-aniline blue solution for 1 h then de-stained. The length of fungal hyphae on the haemocytometer was measured under a light microscope (TK, Kagaku Kyoeisha).

The soil bacterial biomass (B_m) and fungal biomass (F_m) were calculated using the following formulae (Hasebe *et al.* 1984):

$$B_m \text{ (mg/g)} = n \times v_{ave} \times \rho \times r \quad (1)$$

$$F_m \text{ (mg/g)} = v \times \rho \times r \quad (2)$$

where n = numbers of bacteria (cell/g); v_{ave} = Average bacterial volume (= 0.19 μm^3); ρ = Specific gravity of microorganism (= 1.1 g/cm^3); r = Rate of dry matter (= 20%); v = Fungal volume (cm^3/g)

Table 1. Soil properties from laurel-leaved and *C. japonica* forests.

Vegetation	Soil type	Horizon	Moisture content (%)	pH (H_2O)	Total C (g/kg)	Total N (g/kg)	C/N ratio	Volume weight (g/cm^3)
Laurel-leaved forest (B_{B-1})	dry brown forest soil	L	13.7	4.52	467.3	12.6	37.1	0.048
		F	43.8	4.34	402.2	16.9	23.8	0.180
		H	56.5	4.51	318.9	16.9	18.9	0.297
		A	42.5	3.88	189.1	10.5	18.0	0.685
		B	25.0	3.64	38.2	2.0	19.1	0.823
C. japonica forest (B_{B-2})	dry brown forest soil	L	13.7	5.04	498.7	5.8	89.1	0.032
		F	62.0	4.37	361.2	11.9	30.4	0.066
		H	53.4	3.87	201.4	9.5	21.2	0.155
		A	42.0	3.81	106.0	5.8	18.3	0.493
		B	28.4	3.95	30.7	2.0	15.4	0.628

Results

Dilution plate counts of soil bacteria and fungi

The numbers of bacteria and fungi obtained by the dilution plate count method (Figure 3) were larger in the F and H horizons of the organic layer than in the mineral soil layer. When each horizon of the organic layer was compared, the numbers of bacteria and fungi were lowest in the L horizon. Bacterial and fungal counts in the laurel-leaved organic layers were higher than those in the *C. japonica* organic layers.

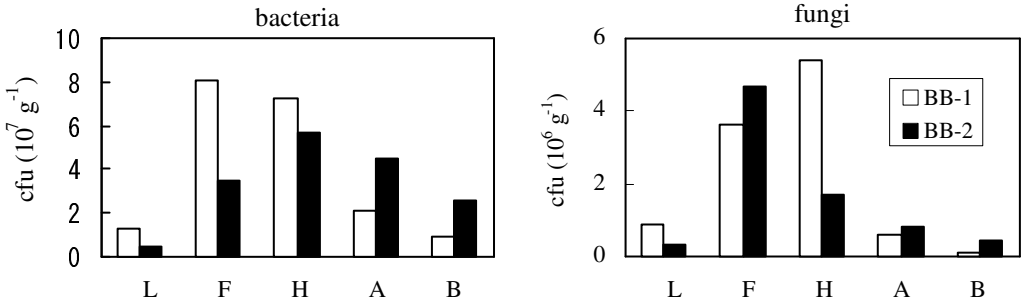

Figure 3. The numbers of bacteria and fungi obtained by the dilution plate count method.

Direct microscopic counts of soil bacteria

A larger number of bacteria was detected using the direct microscopic count method (Figure 4) than using the dilution plate method, which only measures culturable soil bacteria. The numbers of bacteria obtained by the direct microscopic count method showed similar trends to the dilution plate counts: more bacteria were present in the F and H horizons of the organic layer, and the bacterial count was greater in the laurel-leaved compared with the *C. japonica* soils. However the layer distribution of bacteria assessed using the direct plate count method did not necessarily correspond to the results from the dilution plate count method. The difference in the layer distribution of soil microorganisms was clearly reflected in the differences of bacterial biomass. The uppermost L horizon, which contains large amounts of fresh plant residue contained low levels of microorganisms. In the F horizon, there was increased nourishment due to more advanced state of decomposition in this layer. In the H horizon, the organic matter was completely decayed and the amount of bacteria that is able to be grown in nutrient-poor conditions increased, relative to the culturable bacteria.

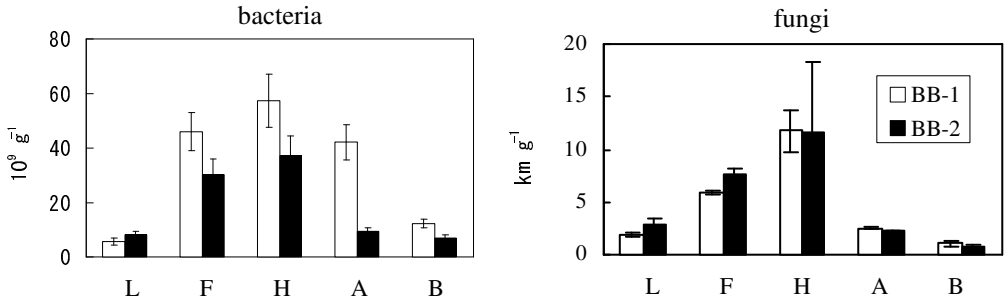

Figure 4. The bacterial number and fungal hyphae length obtained by the direct microscopic count method.

Table 2 shows the ratio of the bacterial count values obtained using the dilution plate count method (P) and the direct microscopic count method (D). The P/D of the A horizon forest soil, which is most comparable to agricultural soils, was only 0.05-0.48%. However, this is much lower than the reported P/D value for agricultural soils of 0.73-3.1% (Marumoto 1994). We found that Kasuga-yama Hill Primeval Forest soil contains large numbers of non-culturable bacteria, compared with agricultural soils. Such information is necessary to understand the metabolism of the forest soil, and will lead to further research on the identity and functions of these bacteria in forest soils.

Table 2. Ratio of the number of bacteria obtained by the dilution plate count method (P) and that obtained by the direct microscopic count method (D).

Horizon	Laurel-leaved forest (B_{B-1})	*C. japonica* forest (B_{B-2})
L	0.24	0.06
F	0.17	0.12
H	0.13	0.15
A	0.05	0.48
B	0.08	0.37

Fungal hyphae length obtained using the direct microscopic count method
The fungal hyphae (Figure 4) were longer in the organic soil layer than in the mineral soil layer. When each horizon was compared, fungal hyphae were found to be longest in the H horizon.

Bacterial and fungal biomass
Table 3 shows the amount of bacterial (B_m) and fungal (F_m) biomass. The total soil biomass ($B_m + F_m$) in the organic layer was greater than that in the mineral soil layer. The total soil biomass in the organic layer increased with soil depth, and was greatest in the H horizon. In contrast, the total soil biomass in the mineral soil layer decreased with increasing depth. The laurel-leaved forest soil had greater total biomass ($B_m + F_m$) than the *C. japonica* soil. This difference is clearly due to differences in forest vegetation, with higher microbial biomass in the laurel-leaved forest indicating a higher metabolic activity than the *C. japonica* soil. The total soil biomass of the forest soil A horizons was similar to those previously reported for agricultural soils (1.6-3.0 mg/g) (Marumoto 1994). However, the forest organic layers overall were far richer in microorganisms than agricultural soils. Forest soil is a natural ecosystem that is continuously supplied with plant matter, and differs from agricultural soil, in which the organic matter is disturbed by cultivation and readily consumed by nutrient-starved crops.
The ratio of bacterial to fungal biomass (F_m/B_m) was the highest in the H horizon of the organic layer for both forest types. In addition, the F_m/B_m in the laurel-leaved soil was less than that in the *C. japonica* soil, due to vegetation effects. The F_m/B_m of agricultural soil is typically 0.98-3.75 (Sakamoto and Oba 1995), which is similar to the F_m/B_m of A horizons in the forest soils. It has been assumed that the mineral soil layer of forest soils has a greater proportion of fungal biomass than agricultural soil. However, our results show that the forest mineral soil layers are similar to agricultural soils in fungal content. The H horizons of the organic layers in the forest soil had much greater F_m/B_m compared with agricultural soils.

Table 3. Bacterial (B_m) and fungal (F_m) biomass in laurel-leaved and *C. japonica* forest soils.

Horizon	Laurel-leaved forest (B_{B-1})				*C. japonica* forest (B_{B-2})			
	B_m (mg/g)	F_m (mg/g)	$B_m + F_m$ (mg/g)	F_m/B_m	B_m (mg/g)	F_m (mg/g)	$B_m + F_m$ (mg/g)	F_m/B_m
L	0.23	0.75	0.98	3.21	0.35	1.35	1.7	3.87
F	1.93	5.96	7.89	3.09	1.27	4.32	5.58	3.41
H	2.4	18.51	20.91	7.71	1.55	17.04	18.6	10.96
A	1.76	1.97	3.73	1.12	0.39	1.25	1.64	3.2
B	0.51	1.46	1.97	2.84	0.29	0.32	0.61	1.13

Conclusion
The microbial ecology of the organic and mineral soil layers of laurel-leaved and *Cryptomeria japonica* forest in the Kasuga-yama Hill Primeval Forest (Nara, Japan) was assessed. The direct microscopic count revealed numerous non-culturable bacteria and fungi in the forest soil compared with agricultural soil.

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