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Author(s)	Saeki, Ikuyo; Hioki, Sho; Azuma, Wakana A. et al.
Citation	Biological Conservation. 2024, 292, p. 110520
Version Type	VoR
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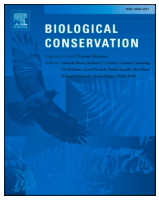
Biological Conservation 292 (2024) 110520



Contents lists available at ScienceDirect

Biological Conservation

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Legacy over a thousand years: Canopy soil of old-growth forest fosters rich and unique invertebrate diversity that is slow to recover from human disturbance

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ARTICLE INFO

Keywords:
Arboreal fauna
Arthropod
DNA metabarcoding
Logging
Forest management
World heritage

ABSTRACT

Canopy ecosystems provide a wide range of ecosystem services, but because they are difficult to access, knowledge about their conservation is limited. Yakushima World Heritage Site in Japan is characterized by old-growth forests with huge Japanese cedars (*Cryptomeria japonica*). Canopy soil, originating from litter, is present in the cedars' crowns, and offers habitat for abundant epiphytes. We hypothesized that the canopy soil invertebrate communities would be distinct from those on the ground. We climbed five retained (>1000 years old) and four regenerated (ca. 300 years old) *Cryptomeria* trees, the latter established after intensive logging in the 17th century. We investigated the taxonomic composition of invertebrates in canopy and ground soil samples by DNA metabarcoding analyses. In total, invertebrates in 33 orders and 183 families were detected. Invertebrate taxonomic richness identified from the canopy soil of retained trees was similar to that from ground soil, but taxonomic composition differed markedly. Canopy soil of retained trees was deeper and more developed than that of regenerated trees, and held a higher number of taxonomic groups per soil sample area. The results imply that canopy soil of old trees contains rich and unique invertebrate diversity that has not recovered from logging, even after 300 years. Our findings confirm that protected areas with old trees that exclude human disturbances are important for conservation of biodiversity in canopy ecosystems. We also recommend elongation of harvest cycles and a tree retention approach in forestry areas to minimize the impact of logging disturbance.

1. Introduction

The forest canopy has remarkably high biodiversity (Basset et al., 2015; Dial et al., 2006; Erwin, 1982; Novotny et al., 2002; Ozanne et al., 2003), and it plays a key role in maintaining a wide range of ecosystem services, such as provision of habitats for plants and animals (Kaizer et al., 2022; Petter et al., 2016; Scheffers et al., 2013), regulation of climate (De Frenne et al., 2019; Scheffers et al., 2014), accumulation of carbon (de Araújo et al., 2008; Gower et al., 2001), circulation of water and nutrients (Asner et al., 2014; Cardelús et al., 2009; Gotsch et al., 2016; Stanton et al., 2014), and creation of attractive landscapes (Katsuda et al., 2022; Nelson et al., 2001). However, a tremendous amount of biodiversity in canopy ecosystems remains unexplored because they are difficult to access (Nakamura et al., 2017; Ozanne et al., 2003). Forest ecosystems have a gigantic, complex, three-dimensional structure (Ehbrecht et al., 2021; Walter et al., 2021), and available biodiversity data are strongly biased towards the portion near the ground. Consequently, knowledge related to conservation of canopy ecosystems is extremely limited, especially for old-growth forests that contain high species richness but are declining rapidly at a global scale (Nakamura et al., 2017; Watson et al., 2018).

Yakushima Island, in the southwest of the Japanese archipelago

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https://doi.org/10.1016/j.biocon.2024.110520
Received 30 November 2023; Received in revised form 21 February 2024; Accepted 25 February 2024
Available online 6 March 2024
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(Fig. 1), is a biodiversity hotspot. The island holds old-growth forests dominated by Japanese cedar (*Cryptomeria japonica* (D. Don)) that are over 1000 years old, called "Yaku-sugi" in Japanese. The old-growth *Cryptomeria* forest is renowned for the richness of its epiphytes, including threatened and endemic species (Minamino et al., 2015). This epiphytic diversity is supported by canopy soils that accumulate in the canopy of old *Cryptomeria* trees (Ishii et al., 2018). Canopy soils originate from leaf litter from *C. japonica* and other associated trees as well as epiphytes in tree crowns. The soils are classified as histosols derived from decomposing leaves, branches, and other materials (Bohlman et al., 1995; Nadkarni et al., 2002). The volume of soil can be large, sometimes exceeding 1.5 m^3 on a single tree (Ishii et al., 2018).

Canopy soils provide habitats for diverse plants and animals (Gotsch et al., 2016; Ishii et al., 2018; Nadkarni and Longino, 1990), and they may hold invertebrate communities distinct from those on the ground. However, little research has been conducted on the diversity of organisms within canopy soils, with the exception of a few studies. Nadkarni and Longino (1990) investigated the density and composition of macro- and meso-invertebrates in organic matter of tree canopies in a tropical forest. They found diverse invertebrates, including mites and beetles, within the canopy although their density was lower than that on the ground. Research conducted on a palm plantation in southeast Asia showed that suspended soil on palm trees supports higher levels of biological activity of small arthropods, nematodes, and amoebae than ground soil (Potapov et al., 2020). In Japan, Yoshida and Hijii (2011) found that many microarthropods colonized leaf-litter bags placed within the canopy of a *C. japonica* plantation.

Although these organisms are responsible for decomposition and nutrient cycling of organic materials in the canopy, the characteristics of invertebrate diversity within canopy soils and its response to human disturbances are poorly known. *Cryptomeria* forest on Yakushima has experienced intensive logging since the 17th century (Shibasaki, 2018, see below for details). The present *Cryptomeria* forest contains both old (>1000-year-old trees that escaped logging) and young (ca. 300 years old) individuals regenerating after disturbance (Ishii et al., 2018). This land use history provides a valuable opportunity to examine the effects of human disturbances on invertebrate communities in canopy soils.

Our objective was to characterize invertebrate composition and diversity in the canopy soil of old-growth *Cryptomeria* forest in Yakushima. We specifically addressed the following questions: i) How much diversity is present in canopy soils compared with those on the forest floor? ii) Do canopy and ground soils differ in the taxonomic composition of their invertebrate communities? iii) Do canopy soils of old trees host greater diversity than those of young trees that regenerated after logging disturbances? To answer these questions, we applied DNA metabarcoding analyses, which have increasingly been used in investigation of species composition and diversity of invertebrate communities (e.g., Morinière et al., 2016; Porter et al., 2019; Watts et al., 2019). This technique allows us to detect very small organisms, such as micro-invertebrates with body length < 2 mm, typical in soil arthropods; these organisms are difficult to detect by other means. In addition, the technique allows us to detect cryptic diversity that has not been classified by conventional morphological studies (Arribas et al., 2016; Kress et al., 2015). By applying this technique, we aimed to illuminate the unexplored diversity in canopy ecosystems.

2. Methods

2.1. Study area

Yakushima Island has an area of 505 km^2 and ranges in elevation from 0 m (seacoast) to a remarkable 1936 m a.s.l. (the peak of Mt. Miyanouradake; Fig. 1). Shoreline vegetation is characterized by subtropical forest, which shifts to warm-temperate evergreen broadleaf forest up to 1000 m, to mixed cool-temperate deciduous and conifer forest up to 1800 m, to sub-alpine shrub forest and grassland above 1800 m. Within this wide altitudinal range, the island is home to approximately 1900 species and subspecies of flora, 16 mammal species, and 150 bird species. A part of Yakushima was designated as a UNESCO World Natural Heritage site in 1993 under criteria VII (exceptional natural beauty and aesthetic importance) and IX (significant ongoing ecological and biological processes; UNESCO World Heritage Convention, https://whc.unesco.org/en/list/662, accessed on 21 September 2023).

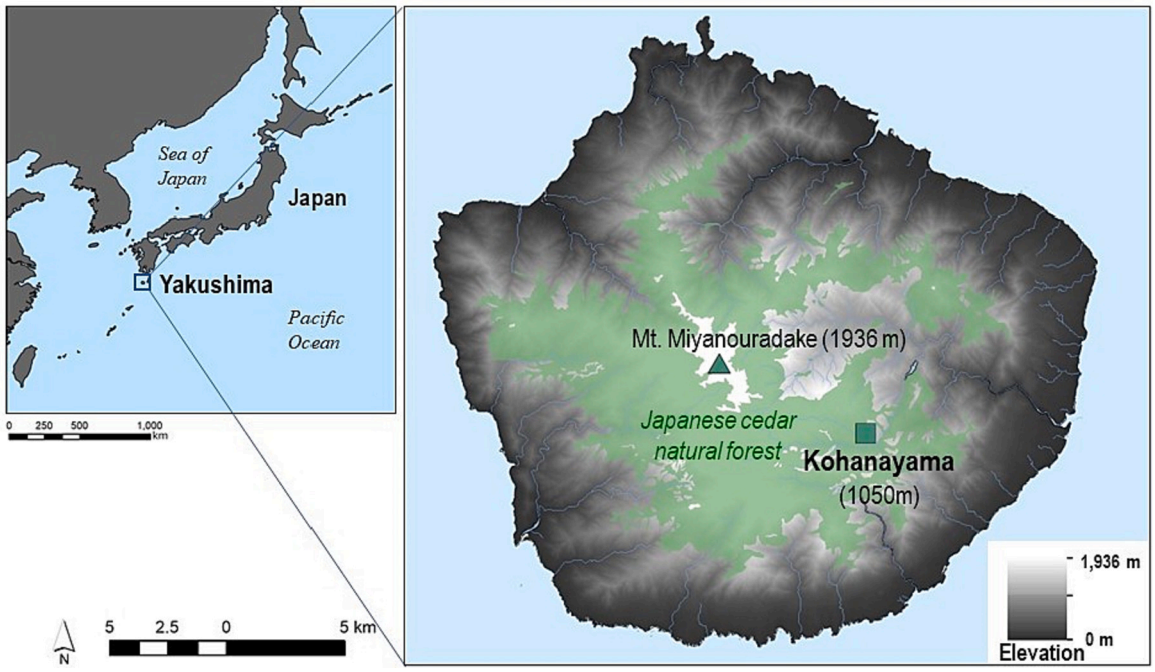


Fig. 1. Map of Japan (left) and inset of Yakushima Island showing the Kohanayama research site in natural forests dominated by Japanese cedar (*Cryptomeria japonica*) (right).

Old-growth *Cryptomeria* forest occurs between 1000 and 1800 m (Ohsawa et al., 2006, Fig. 1). Large trees were intensively logged during the Edo period (1693–1868) because *C. japonica* provides good timbers for building and shingles for roofs (Shibasaki, 2018). Although the amount of timber production has decreased recently, cutting of old *C. japonica* continued into the 20th century. *Cryptomeria* forests often contain huge stumps with circumferences >500 cm. The average annual precipitation from 2003 to 2022 recorded at Yakushima Meteorological Station (37 m a.s.l.) is 4607 mm, and the average annual temperature is 19.8 °C (range, 3.5–34.1 °C; Japan Meteorological Agency https://www.data.jma.go.jp/obd/stats/etrn/, accessed on 21 September 2023). The highest amount of rainfall is during the rainy season in June, which averaged about 880 mm per month from 2003 to 2022. High precipitation and a warm climate contributed to establishment of old-growth, high-biomass forest dominated by *C. japonica*.

2.2. Sample collection

The study was conducted at Kohanayama research plot (100 m × 100 m), a permanent plot created in an old-growth forest dominated by *C. japonica* (Fig. 1; Takashima et al., 2009). The plot was established in 1973 at 1050 m by the Kumamoto Regional Forest Office. The total basal area and stem density at the site are 115.8 cm^2/m^2 and 1259/ha, respectively. Major tree species associated with *C. japonica* are *Abies firma* Sieb. et Zucc., *Tsuga sieboldii* Carriere, *Trochodendron aralioides* Sieb. et Zucc., *Symplocos myrtacea* Sieb. et Zucc., *Rhododendron tashiroi* Maxim, and *Stewartia monadelpha* Sieb. et Zucc.

In this plot, we collected canopy soil from two types of *C. japonica* trees; retained ($n = 5$) and regenerated ($n = 4$) trees (Appendices S1, S2). Retained trees are large and old; their diameter at breast height (DBH) ranges from 124.8 cm to 179.8 cm. Their heights range from 21.3 m to 33.4 m (Ishii et al., 2018), and they have been estimated to be >1000 years old by tree-ring count at a site near this forest (Ushijima et al., 2005). These trees commonly sprout epicormic branches, which have a tendency to break; a relatively large amount of canopy soil accumulates at the base of the broken branches. As described above, large *C. japonica* trees were intensively logged in the past. A few old trees escaped being cut down, probably because their shape made them unsuitable for use as timber. We selected these remnants as retained trees. In contrast, regenerated trees are defined as young trees that established after the intensive logging period. Their age and DBH are about 300–350 years and 82.5–97.7 cm, respectively (Ishii et al., 2018). The height ranges from 22.4 m to 28.8 m. Regenerated trees lack trunk breakages and have less complicated crown structure than retained trees. Epiphytic woody plants abundantly grow on the retained trees. The dominant epiphytes are *Vaccinium yakushimaense* Makino, *Sorbus commixta* Hedl., and *Rhododendron keiskei* Miq. Epiphytic woody plants are far less common on the canopy of the regenerated trees, and the branches are mostly covered by arboreal moss and ferns (Appendix S2).

In June–July 2021, we climbed the study trees by a single-rope technique and collected soil samples from the canopy of each tree (Appendix S1). On each retained tree, we collected a paired sample of the litter and decomposed soil layers at two different heights in the tree crown, for a total of 20 soil samples (i.e., 2 soil layers × 2 heights × 5 trees). We collected canopy soils at two different heights because height may affect invertebrate diversity. We then compared the richness of detected invertebrate taxa by treating an individual tree as a cluster (see below for details). Samples from the litter layer were collected from an area 20 cm × 20 cm. The depth of litter layer ranges from approximately 5 to 15 cm and contains relatively large pieces of organic matter, including leaves of *C. japonica*. Below this, the decomposed layer contains much finer particles, resembling the A layer of soil from the ground, but developed from organic matter (Appendix S2). We collected 100 cm^3 of soil from the decomposed layer by using a conical metal tube (diameter, 5 cm; height, 5.1 cm).

For regenerated trees, the decomposed layer was too shallow to perform the same sampling procedure. Instead, we collected the litter and decomposed layers together from an area of 20 cm × 20 cm on the soil surface. The depth of the litter layer of regenerated trees was about 5 to 15 cm, similar to the retained trees, and the decomposed layer was typically 0.5–1.0 cm. Canopy soil samples were collected at two heights in regenerated trees, for a total of 8 samples (i.e., 1 soil layer × 2 heights × 4 trees).

To compare invertebrate communities, we also collected soil samples from the base of the study trees. For each retained and regenerated tree, one representative point was selected on the ground within 5 m of the tree, avoiding obvious disturbances, large woody debris, and stagnant water. We collected the litter layer from an area 20 cm × 20 cm. Furthermore, we collected a 100 cm^3 soil sample from the A layer below the litter layer by using the same conical tube described above. We collected a total of 18 ground soil samples (i.e., 2 soil layers × 9 trees).

In October 2022, we conducted a second round of sample collection to examine seasonal differences in invertebrate communities. We collected canopy soil samples from two trees, one retained and one regenerated (Appendix S1). We collected fewer samples in the second collection period to minimize disturbance of canopy soils. Each sample was collected immediately adjacent (within 50 cm) to that tree's collection point in summer 2021. For each tree, paired soil samples were collected from the ground as described above. In 2022, we collected a total of 10 samples. Combined 2021 and 2022 samples totaled 56 soil samples.

2.3. Experimental procedure

Each soil sample was stored in a zipped plastic bag and brought back to the lodging house where invertebrates were extracted for 24 h by Tullgren device. The extracted organisms were immediately stored in 80 % ethanol. We started this extraction procedure on the day we collected the soil and finished the next day. The Tullgren device was 55 mm in diameter and had a 60 W light bulb. Mesh size of the sieves in the device was 4 mm. Extracted samples were brought to the laboratory and stored in the refrigerator at 4 °C.

To investigate the taxonomic composition and diversity of the extracted invertebrates, DNA metabarcoding analyses were performed. Each invertebrate sample was placed in a separate 2-mL tube, and total DNA was extracted by using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). PCR was performed using a primer set designed to amplify a partial region of the 5′ fragment of the mitochondrial cytochrome *c* oxidase subunit I (*COI*) gene (Appendix S3; Seibutsugiken Co., Ltd., Sagamihara City, Japan). The PCR mixture contained ultrapure water (3.3 μL), forward and reverse primers (10 μM, 0.6 μL for each), and PrimeSTAR Max DNA Polymerase (5.0 μL) (Takara Bio Co., Ltd., Kusatsu City, Japan). The thermal cycling program was 35 cycles of 98 °C for 10 s, 50 °C for 30 s, and 72 °C for 5 s. PCR products were electrophoresed on an agarose gel for confirmation of amplification. These were purified and used for a second round of PCR amplification. The library obtained from the second PCR was sequenced by using a MiSeq sequencer (Illumina, San Diego, CA, USA). Sequences were BLAST searched against the GenBank database, and bioinformatic information (operational taxonomic unit (OTU), suggested taxon names, number of reads, and identity rate) are listed for each sample. Experimental and bioinformatic procedures after the second PCR were performed by Seibutsugiken Co. Ltd. (Sagamihara, Japan).

2.4. Data analyses

BLAST search data were placed in table format. Bacterial and fungal taxonomic groups, as well as taxa detected in the negative control, were then excluded. We also removed unreliable taxa, such as marine, strictly aquatic, and organisms not generally known to occur in Asia. Taxa with <10 total reads were also removed. For analyses, we used all the OTUs that met the criteria because the sequence database of soil organisms is limited (Dopheide et al., 2019), and thus the high threshold rate (97 %) that is commonly used for species identification in DNA barcoding (Watts et al., 2019) is likely to overlook a large portion of biodiversity. We produced a single table of OTUs (Appendix S4). Names of taxonomic groups and their classification were determined based on the Global Biodiversity Information Facility (GBIF; https://www.gbif.org/, accessed on 21 September 2023). In addition, we developed a subset of OTU data with ≥85 % identity rate and used it for validation of results.

To evaluate taxonomic richness, we compared the number of orders and families detected among the four sample categories: i) canopy soil of retained tree, ii) canopy soil of regenerated tree, iii) ground soil of retained tree, and iv) ground soil of regenerated tree. As mentioned above, canopy soils (i and ii) were collected at two heights per tree whereas ground soils (iii and iv) were collected at one place per tree. Since the canopy litter and decomposed soil layers of regenerated trees were collected as a single sample, we pooled the number of taxa in the litter and decomposed soil layers of retained trees as one sample unit for the analyses described below. Similarly, the number of taxa in the litter and A layers of the ground soils was also pooled. The analyses were performed by using a generalized linear mixed model (GLMM) with a Poisson distribution and sampling time as a random factor. Since the number of soil samples differed among the four categories, we also constructed rarefaction curves by using iNEXT (Hsieh et al., 2016). In this procedure the number of bootstrap analyses for exploration was set at 50. The endpoint of the rarefaction curves was set at 12, the maximum number of soil samples among the four categories. To examine differences in taxonomic richness among sampling heights, we used the GLMM for comparisons among low-canopy and high-canopy samples. In the analyses, we used a Poisson error distribution. Tree ID and sampling time were added to the model as random factors. For validation of results, we performed the same diversity analyses using the subset of data with ≥85 % identity rate.

To compare taxonomic composition among the four types of samples, we performed non-metric multidimensional scaling (NMDS) analyses with presence and absence data using Bray–Curtis distance. To exclude the effect of rare species, we used a data set consisting of families that occurred in two or more soil samples. Permutation multivariate analyses of variance (PERMANOVA) were run to test for statistical differences among the four types of samples.

The ratio of presence in canopy versus ground soils was examined for families that occurred in at least 25 % of soil samples. For families that occurred in both canopy and ground soil samples, we tested whether the ratio was biased from 1:1 by GLMM using a binomial model with tree ID as a random factor. Similarly, the ratio of presence of each family in the canopy of retained trees was compared with that in regenerated trees. Statistical significance was tested with a generalized linear model (GLM) with a binomial error distribution. Finally, we analyzed the ecological traits (flight ability, parasitic ability, and diet (carnivorous or not)) of invertebrate communities among the four soil sample categories. We identified these traits for each family using the literature and an internet database (see Appendix S11). Then, the proportion of families with each focal trait was compared among the four categories by using GLMM analyses with sampling time as a random factor. Statistical analyses were performed with R ver. 4.3.0 (R Core Team, 2023) with packages lme4 (Bates et al., 2015), lmerTest (Kuznetsova et al., 2017), iNEXT (Hsieh et al., 2016), and vegan (Oksanen et al., 2012).

3. Results

In total, we detected 2430 OTUs in 33 orders and 183 families

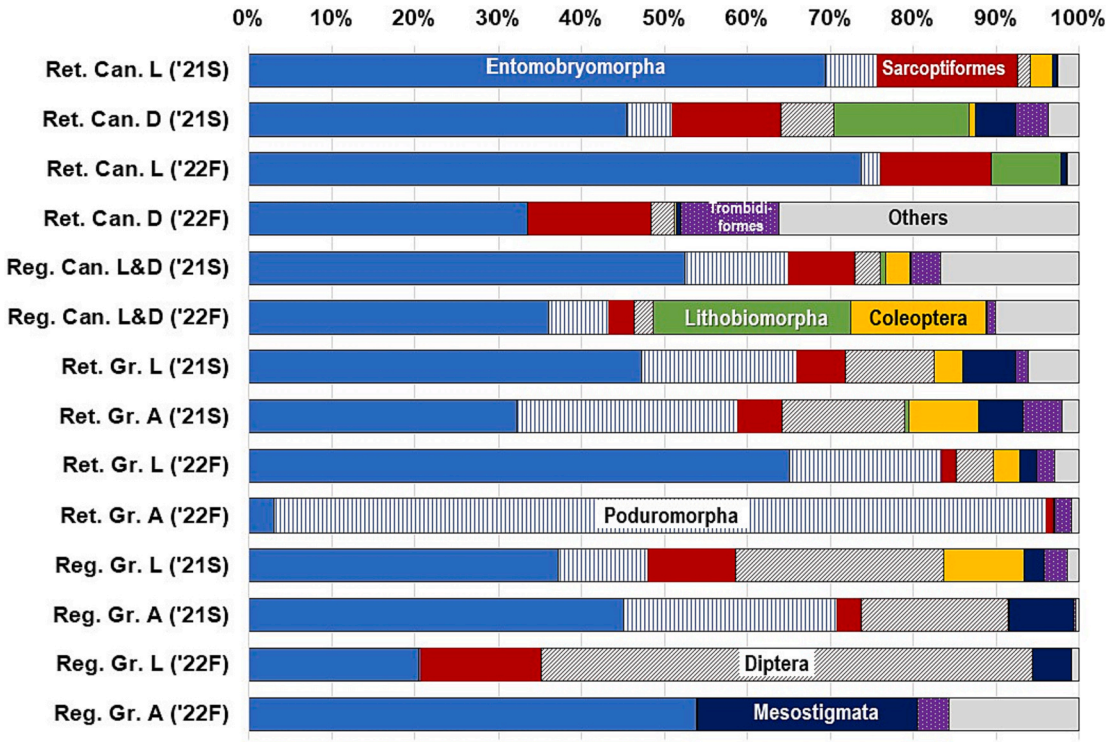


Fig. 2. Proportion of sequence reads of the eight most dominant orders detected by DNA metabarcoding analyses of canopy and ground soils in old-growth *Cryptomeria* forest, Yakushima Island, Japan. Based on data pooled by sampling position (canopy vs. ground) and tree type (retained and regenerated). Numbers and alphabetical abbreviations indicate tree types, soil layers, years, and seasons. Ret., retained trees; Reg., regenerated trees. L, litter layer; D, decomposed layer; A, A layer. '21S, summer 2021; '22F, fall 2022. See Appendix S1 for details.

(Appendices S4 and S5). Commonly detected were Entomobryomorpha, Poduromorpha, Sarcoptiformes, Diptera, Lithobiomorpha, Coleoptera, Mesostigmata, and Trombidiformes (Fig. 2). Their proportions varied among sampling positions (canopy vs. ground), tree types (retained vs. regenerated), soil layers (litter vs. decomposed or A layer), and sampling time (summer 2021 vs. fall 2022). The average identity rate was 84.9 % (range, 73.6–100 %); 23 OTUs had ≥97 % identity rate (0.9 % of 2430 OTUs).

In retained trees, the average number of orders (i.e., order richness) detected in canopy soil was similar to that in ground soil (Table 1), as was the average number of families (i.e., family richness). For regenerated trees, mean order richness was similar between canopy and ground, but family richness was lower in canopy samples than in ground samples. According to GLMM analyses, order and family richness of canopy soils of retained trees were significantly higher than those of regenerated trees (Table 2). This relationship was supported by rarefaction curves; canopy soil of retained trees showed higher taxonomic richness than that of regenerated trees (Fig. 3). Comparing taxonomic richness of canopy samples, a greater number of families was detected in low-canopy than in high-canopy samples (Table 3). Similar results were obtained using the data subset with identity rate ≥ 85 % (Appendices S6, S7, and S8).

According to the NMDS analyses, taxonomic composition differed markedly between canopy and ground soils (Fig. 4; Appendix S9). In the ordination diagram, canopy samples were assigned a high score on NMDS axis 1 and a low score on NMDS axis 2. Samples from retained trees were assigned a relatively low score on NMDS axis 1. Ground samples were assigned a low score on NMDS axis 1 and a high score on NMDS axis 2. Taxonomic composition was significantly different among the four groups (PERMANOVA, $F = 3.52$, $p < 0.001$), but the family composition of canopy soils of retained trees was relatively similar to that of ground soils as compared to that of regenerated trees.

The ratios of some taxonomic groups differed markedly between canopy and ground soils of retained trees (Fig. 5a; Appendix S10a). For example, Suctobelbidae and Trhypochthoniidae were more frequently detected in the canopy than on the ground. In contrast, Phthiracaridae and Phenopelopidae were more frequently recorded in ground samples. Interestingly, Entomobryidae, Isotomidae, Tomoceridae, and Onychiuridae were commonly recorded in both canopy and ground samples. Focusing on canopy communities, Ologamasidae were more frequently detected in retained than regenerated trees (Fig. 5b; Appendix S10b), whereas some families of Entomobryomorpha and Poduromorpha occurred in both retained and regenerated trees. Canopy samples of regenerated trees showed a higher ratio of families with flight ability than those of retained trees (Fig. 6; Appendices S11, S12). In addition, canopy samples of regenerated trees showed a relatively higher ratio of

Table 1
Average number of orders and families detected by DNA metabarcoding analyses for invertebrate communities of canopy and ground soils samples in old-growth *Cryptomeria japonica* forest, Yakushima Island, Japan.

Tree type	Position	Layer	N[a]	Average No. of detected orders		Average No. of detected families	
				2021 (mean ± SD)	2022 (mean)[b]	2021 (mean ± SD)	2022 (mean)[b]
Retained	High-canopy	Litter	5 (1)	7.6 ± 2.6	9.0	15.0 ± 5.4	19.0
Retained	High-canopy	Decomposed	5 (1)	8.8 ± 2.3	11.0	16.8 ± 4.7	23.0
		Litter and decomposed[c]	5 (1)	12.0 ± 1.6	13.0	25.2 ± 2.6	30.0
Retained	Low-canopy	Litter	5 (1)	8.4 ± 3.2	9.0	16.0 ± 7.1	20.0
Retained	Low-canopy	Decomposed	5 (1)	9.8 ± 2.2	10.0	23.2 ± 4.5	27.0
		Litter and decomposed[c]		12.2 ± 1.8	14.0	29.8 ± 4.4	34.0
Retained	Ground	Litter	5 (1)	9.2 ± 0.8	11.0	19.6 ± 4.4	28.0
Retained	Ground	A	5 (1)	7.8 ± 2.5	9.0	15.2 ± 7.7	12.0
		Litter and A[c]	5 (1)	11.4 ± 1.5	13.0	29.0 ± 6.7	37.0
Regenerated	High-canopy	Litter and decomposed[d]	4 (1)	8.0 ± 2.9	9.0	14.3 ± 5.2	21.0
Regenerated	High-canopy	Litter and decomposed[d]	4 (1)	10.0 ± 2.4	11.0	20.8 ± 7.0	18.0
Regenerated	Ground	Litter	4 (1)	9.8 ± 2.4	9.0	30.3 ± 3.0	20.0
Regenerated	Ground	A	4 (1)	7.3 ± 2.8	7.0	13.8 ± 6.0	8.0
		Litter and A[c]	4 (1)	11.0 ± 2.9	11.0	37.0 ± 2.9	24.0

[a] Number of samples. Numbers in parentheses indicate samples collected in fall 2022.
[b] SD was not calculated for 2022 samples because one sample was collected per sample unit.
[c] Taxonomic richness calculated by combining samples from litter and decomposed layers as a single sample.
[d] Taxonomic richness based on soil samples collected from litter and decomposed layers together.

Table 2
Results of generalized linear mixed model (GLMM) analyses to predict the relationship between taxonomic richness of canopy and ground soils in old-growth *Cryptomeria japonica* forest, Yakushima Island, Japan.

Response variable	N	Intercept	Coefficient against canopy retained[a]			AIC
			Canopy regenerated	Ground retained	Ground regenerated	
Order richness	33	2.51	−0.29*	−0.06	−0.11	161.5
Family richness	33	3.34	−0.46***	0.08	0.20*	215.4

Asterisks indicate *p*-values based on Wald test: *, 0.01–0.05; ***, <0.001.
[a] Tree types and sampling positions: canopy soil of retained tree (canopy retained), canopy soil of regenerated tree (canopy regenerated), ground soil of retained tree (ground retained), and ground soil of regenerated tree (ground regenerated).

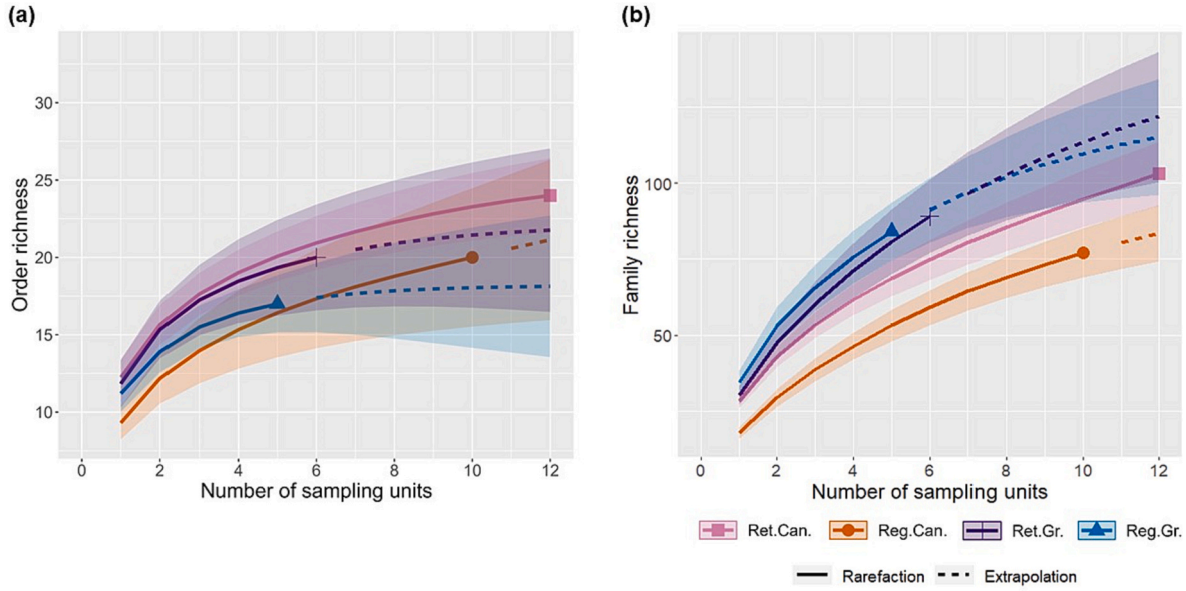


Fig. 3. Comparison of rarefaction curves of taxonomic richness between invertebrates collected from canopy and ground soils in old-growth *Cryptomeria japonica* forest, Yakushima Island, Japan. Taxonomic richness is described as the number of (a) orders and (b) families detected by DNA metabarcoding analyses. The rarefaction curves were constructed by iNEXT (Hsieh et al., 2016). Ret. Can., canopy soil of retained tree; Reg. Can, canopy soil of regenerated tree; Ret. Gr., ground soil of retained tree; Reg. Gr., ground soil of regenerated tree. The results were based on 50 bootstrap analyses.

Table 3
Results of generalized linear mixed model (GLMM) analyses to predict the relationship between taxonomic richness of canopy soils and sampling position (low-canopy soil and high-canopy soil, see Appendix S1 for details).

Response variable	N	Intercept	Coefficient for low-canopy against high-canopy	AIC
Order richness	22	2.34	0.10	111.6
Family richness	22	3.01	0.19*	146.6

Asterisk indicates *p*-value based on Wald test: *, 0.01–0.05.

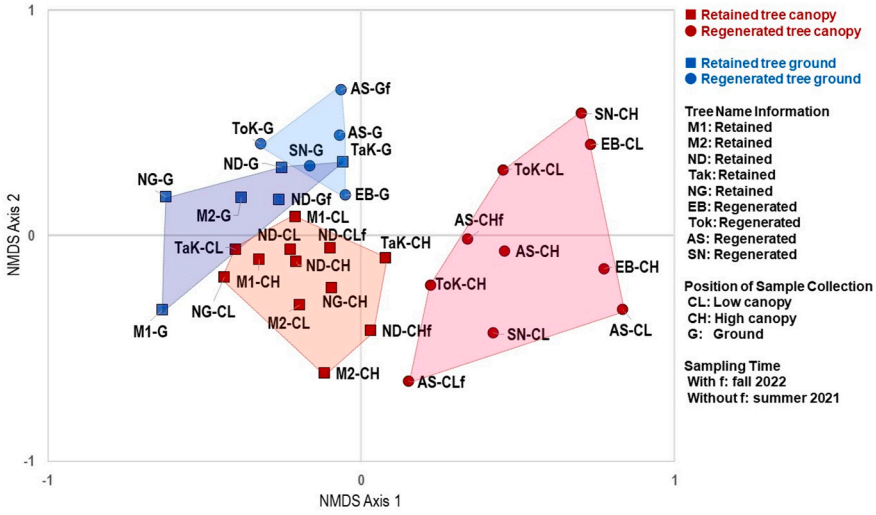
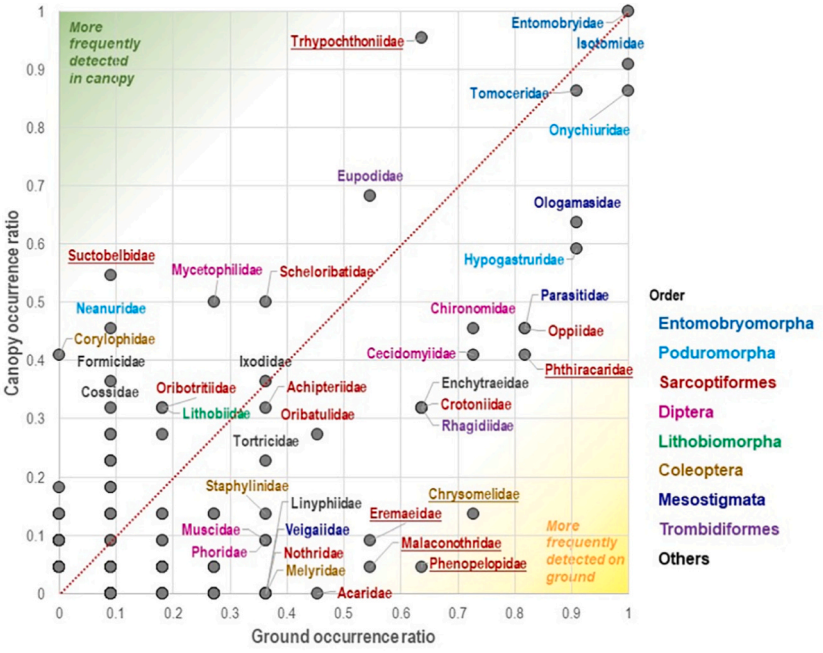
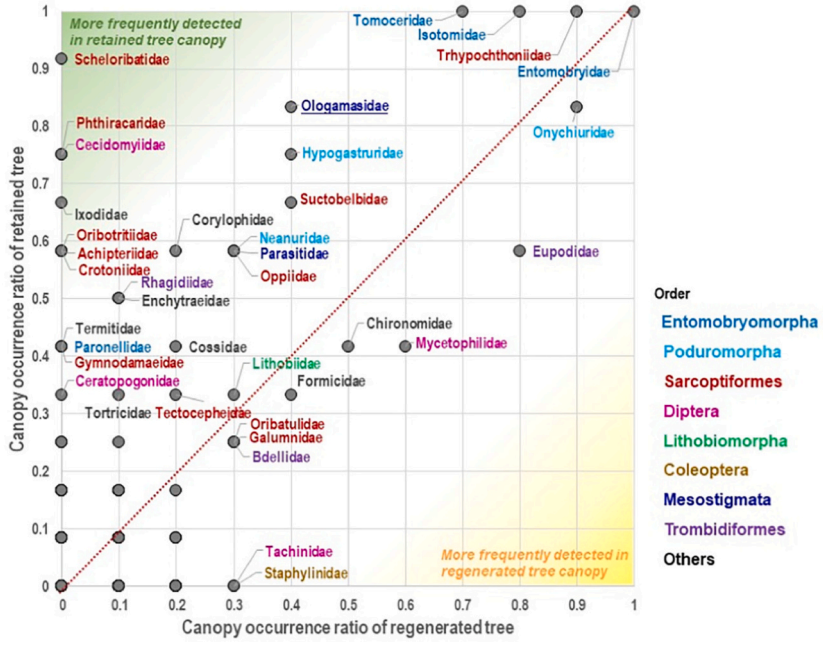


Fig. 4. NMDS analysis of presence and absence of families detected in two or more soil samples by DNA metabarcoding analyses. See Appendix S9 for ordination with information of coordinates of families.



a. Ground vs. canopy soil.



b. Regenerated vs. retained canopy soils.

Fig. 5. Comparison of occurrence ratio of invertebrates between (a) canopy and ground soils and (b) retained and regenerated canopy soils. Taxa with a ratio statistically biased from 1:1 are underlined ($p < 0.05$; see Appendix S10 for details.) Families that showed a low occurrence ratio (i.e., <25 % of a total soil sample) are shown by dots without labels.

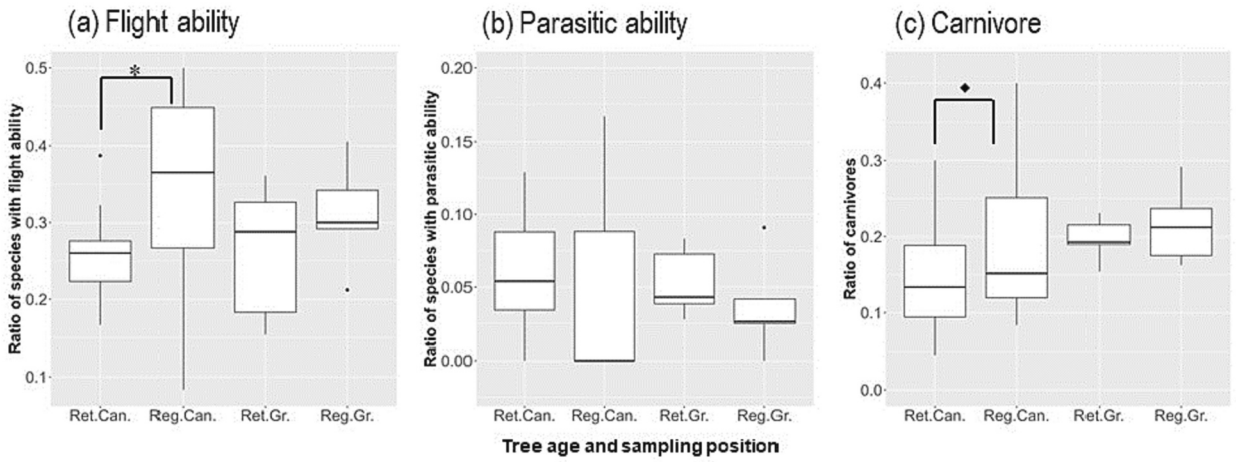


Fig. 6. Comparison of ecological traits of invertebrate taxa detected by DNA metabarcoding analyses from canopy and ground soil samples of old-growth *Cryptomeria* forest, Yakushima Island, Japan. Traits were determined at the family level. Ret. Can., canopy soil of retained tree; Reg. Can, canopy soil of regenerated tree; Ret. Gr., ground soil of retained tree; Reg. Gr., ground soil of regenerated tree. *p*-Value *, 0.01–0.05; ◆,0.05–0.1. Based on Wald test of GLMM analyses. See Appendices S11 and S12 for details.

predatory taxa than those of retained trees.

4. Discussion

In retained trees, taxonomic diversity was similar between canopy and ground soils (Tables 1 and 2; Fig. 3). Yet their taxonomic composition was markedly different (Fig. 4). Our results indicate that canopy soils of old growth forest contain rich and unique biodiversity that is distinct from that of ground soils. The result was different from that of a previous study in a neotropical cloud forest of Costa Rica (Nadkarni and Longino, 1990), which showed that relative abundances of dominant taxa were similar between canopy and ground soils. This inconsistency may be caused by difference of methodologies. DNA metabarcoding analyses can identify much smaller organisms than morphological identification. The high power of our analyses to detect very small organisms may have increased the overall taxonomic richness detected and illuminated the unique taxonomic composition of canopy ecosystems. The difference in our results may also have been caused by differences in site characteristics. The high level of precipitation in Yakushima means that the *Cryptomeria* trees are large, and their crown structure is quite complicated, with abundant epiphytes. Under such conditions, the physical and chemical attributes of canopy soils are likely to differ from those of ground soils. Differences in litter types may affect the soil's nutrient cycling because retained trees have more epiphytes as compared to regenerated trees (Ishii et al., 2018). The amount of moisture and microorganism functions may also cause differences. The mechanisms causing such differences should be a focus of future studies.

Canopy soil of regenerated trees showed lower taxonomic diversity than that of retained trees (Tables 1 and 2; Fig. 3), indicating that 300 years is too short for complete recovery of canopy soils and their associated invertebrate diversity. The results agree with the pattern of epiphytic diversity; regenerated-tree canopies have much lower diversity than retained-tree canopies (Ishii et al., 2018). Interestingly, taxonomic richness was higher in low-canopy soils than in high-canopy soils (Table 3). This is probably because lower branches are older and thus had more time to accumulate diversity. The difference may also be related to the variations in distance from the forest floor (i.e., the probability of invertebrate colonization), solar radiation, humidity, and other abiotic factors.

We detected many taxa of Collembola occurring in canopies of both retained and regenerated trees (Figs. 2 and 5). Recovery of these groups seems relatively quick, perhaps due to their relatively high mobility (Yoshida and Hijii, 2011). Furthermore, canopies of regenerated trees were characterized by a high ratio of families with flight ability (Fig. 6), and they may also contain larvae in addition to adults. Based on the results, we expect that invertebrates with low mobility capacity need more time to recover in the canopy after disturbance. Nevertheless, we found similar or higher ratios of parasitic and predatory families in regenerated canopy soils than in retained ones. This means that multilevel food webs and trophic interactions may recover within a relatively short time. Small organisms are frequently dispersed by birds (Saito et al., 2023), and canopies of *C. japonica* are frequently visited by birds and mammals. Such highly mobile vertebrates may also carry parasitic species to the canopy. The NMDS diagram (Fig. 4) indicated that the family composition of canopy soils of retained trees was more similar to that of ground soils than that of regenerated trees. Without disturbances, invertebrate communities of canopy soils may shift over time to become similar to those of ground soils at the family level. However, invertebrates living in canopy soils must be adapted to the canopy environment. Thus, species composition is expected to remain quite different between canopy and ground communities. We should note the risk of PCR bias and low detection rates of poorly investigated taxa (Dopheide et al., 2019). Considering these aspects, our results probably underestimate the diversity in canopy ecosystems, and collecting morphological data and improvement of experimental protocols could help to improve identification.

5. Conclusions

The soil fauna is considered a 'biotic frontier' (André et al., 1994) that may comprise 25 % of all multicellular species on Earth (Decaëns et al., 2006). Soils in the canopy of old-growth forests are some of the least accessible to study, although they support a variety of ecological functions (Cardelús et al., 2009; Gotsch et al., 2016; Nadkarni and Longino, 1990). Our research elucidates the rich and unique invertebrate communities present in the canopies of old-growth cedar forests in Yakushima. These communities are highly vulnerable and have not fully recovered, even 300 years after logging. These findings confirm the importance of protected areas that contain old trees and exclude human disturbances for conservation of biodiversity in canopy ecosystems. We also recommend elongation of harvest cycles and taking a retention tree approach in forestry areas to minimize the impact of logging disturbances. We expect that there must have been many old-growth forests around the world, in which deep canopy soils accumulated on large trees. However, pristine forests have been rapidly lost (Potapov et al.,

2017), and the speed at which we have lost them has been much faster than we can study them. According to a previous study in tropical forests (Murray et al., 2023), canopy soils are abundant in cooler areas with plenty of fog and larger, older trees. This ecological setting is similar to that of the cedar forest of Yakushima, and we encourage the study of other types of ecosystems, such as boreal and plantation forests, because biodiversity information associated with canopy soils is currently too limited. Techniques such as DNA metabarcoding will be useful in ascertaining a general picture of the biodiversity of canopy soils, which may stimulate public attention to conservation of old-growth forests from new perspectives.

Author statements

The analyses presented in this manuscript represent original research. All authors agree with the contents of the manuscript and its submission to the journal. Any research in this manuscript not carried by the authors has been fully acknowledged.

CRediT authorship contribution statement

Ikuyo Saeki: Writing – original draft, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sho Hioki:** Investigation, Data curation. **Wakana A. Azuma:** Validation, Methodology, Investigation, Data curation, Conceptualization. **Noriyuki Osada:** Validation, Methodology, Investigation, Data curation, Conceptualization. **Shigeru Niwa:** Validation, Methodology, Formal analysis, Data curation. **Aino T. Ota:** Validation, Formal analysis, Data curation. **Hiroaki Ishii:** Methodology, Investigation, Data curation.

Declaration of competing interest

The authors declare that there are no competing financial interests or personal relationships that could influence this research.

Data availability

The data we used for the analyses are available in Supplementary Data

Acknowledgements

We would like to thank A. Takashima, Y. Sasabe, N. Kawata, K. Sueyoshi, W. Komatsu, Y. Higashi, M. Yamanaka, and T. Yamamoto for their support. Comments from reviewers helped with improving our manuscript. This research was funded by JSPS (20K06090) and the Certificate Programme on Nature Conservation, University of Tsukuba.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocon.2024.110520.

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