Mycoscience (2008) 49:241–249
DOI 10.1007/s10267-008-0414-8

FULL PAPER

Yuko Kurihara · Nampiah Sukarno · Muhammad Ilyas
Erny Yuniarti · Wibowo Mangunwardoyo
Rasti Saraswati · Ju-Young Park · Shigeki Inaba
Yantyati Widyastuti · Katsuhiko Ando

Entomopathogenic fungi isolated from suspended-soil-inhabiting arthropods in East Kalimantan, Indonesia

Received: September 10, 2007 / Accepted: February 8, 2008

Abstract As a preliminary survey to develop suspended-soil arthropods as a new isolation source of entomopathogenic fungi, we investigated the entomopathogenic fungi of these arthropods. Fifty-five suspended-soil arthropods were collected from lowland tropical rainforests in East Kalimantan, and ten fungal isolates belonging to seven entomopathogenic species, including two undescribed species, were isolated from nine of the arthropods. Only two of the seven entomopathogenic species were commonly found from the arthropods inhabiting the ground soil in the same forests. The percentage of entomopathogenic fungi-positive arthropods from suspended soil was similar to that from ground soil of the same and another forest of the region, and lower than that from ground soil of Japan. However, the number of entomopathogenic species isolated from the suspended-soil arthropods was larger than that from ground-soil arthropods. This result suggests that suspended-soil arthropods can be a new isolation source of entomopathogenic fungi.

Key words *Asplenium nidus* complex · Entomopathogenic fungi · Indonesia · Suspended-soil arthropods

Y. Kurihara[1] (✉) · J.-Y. Park · S. Inaba · K. Ando
NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Chiba, Japan

N. Sukarno
Biology Department, Bogor Agricultural University (IPB), Bogor, Indonesia

M. Ilyas
Research Center for Biology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia

E. Yuniarti · R. Saraswati
Soil Biology Division, Soil Research Institute, Bogor, Indonesia

W. Mangunwardoyo
Department of Biology, University of Indonesia, Depok, Indonesia

Y. Widyastuti
Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI), Cibinong, Indonesia

[1]*Present address:*
Ishigaki Laboratory, OP BIO FACTORY Co., ltd, 965-3-102 Tonoshiro, Ishigaki, Okinawa 907-0004, Japan
Tel. +81-980-88-1715; Fax +81-980-88-1716
e-mail: kurihara@opbio.com

Introduction

In tropical rainforests, vast numbers of species inhabit the canopies (sensu Moffett 2000; not only tree crowns but including all parts of plant communities above ground), because the complicated structure of the canopies provides various habitats for organisms (Yumoto 1999). Epiphytic plants are one of the factors that create distinctive habitats in canopies, including the soil and litter that accumulate on and around the plants (Basset 2001; Ellwood and Foster 2004; Karasawa 2006). A typical example of such a soil-creating epiphyte is the bird's nest fern (*Asplenium nidus* complex), the leaves of which make a basket-shaped rosette. Dead leaves of the fern and litter that fall down from above accumulate in the rosette, and as the litter decomposes, humus and soil gradually develop on the fern or around the roots of the fern (Karasawa 2006). This kind of soil on epiphytic plants and branch crotches is called suspended (epiphytic) soil (Delamare-Debouteville 1948; Wardle et al. 2003; Yoshida and Hijii 2005), and it enlarges the species diversity in canopies by providing habitats for soil-inhabiting invertebrates (Wardle et al. 2003; Karasawa 2006). These suspended-soil-inhabiting invertebrates represented by arthropods have been studied vigorously in recent years. Ellwood and Foster (2004) found that the huge invertebrate biomass within suspended soil almost equals that in the whole canopy excluding the suspended soil. Almost all arthropod orders that inhabit ground soil have been found from suspended soil, but the population density, species diversity, and species composition of each taxon within suspended soil differ from those within ground soil, even in the same forest (Longino and Nadkarni 1990; Rodgers and Kitching 1998; Suma and Karasawa 2005; Yoshida and Hijii 2005; Karasawa 2006; Karasawa and Hijii 2006).

Up to now, various entomopathogenic fungi from pterygotes have been screened and applied for industrial pur-

poses, such as medicine production or biological control of pests (e.g., Samson et al. 1988; Schmidt et al. 2003). Soil arthropods including apterygote insects are infected by various entomopathogenic fungi (Keller and Zimmermann 1989), and by analogy with pathogenic fungi of pterygotes, they can also be applied for industrial purposes. Although they have not been fully investigated yet, some reports have been published on entomopathogenic fungi isolated from ground-soil arthropods (Visser et al. 1987; Keller and Zimmermann 1989; Bridge et al. 2005; Kurihara et al. 2006). However, until now, little work has been focused on entomopathogenic fungi of suspended-soil arthropods. To collect many isolates for screening, using isolation sources that provide diverse species without investing much time and effort is desirable. From suspended soil, a vast number of arthropods belonging to various orders can be collected (Ellwood et al. 2002; Ellwood and Foster 2004; Suma and Karasawa 2005; Yoshida and Hijii 2005; Karasawa and Hijii 2006). Thus, we investigated the entomopathogenic fungi of suspended-soil arthropods as a preliminary investigation for developing the arthropods as a new isolation source of these fungi.

Materials and methods

Sampling sites and collection of soil samples

Samples were collected in the Kutai National Park, East Kalimantan, Kalimantan Is., Indonesia, on July 20–21, 2006. The Park is located near the equator and consists of primary and secondary forests of lowland tropical rainforests. A large part of the Park was heavily damaged by two large forest fires in 1983 and 1998, but it is now recovering. Samples were collected from three forests in the Park; the forest of Wisata Sankima and that around Camp Kakap are primary forests, and that of Sankima is a secondary forest, the history of which is unknown (Table 1).

Four suspended-soil samples were collected at 0.5–4.5 m high in the two primary forests. Two of the samples (S1 and S2) were collected at Wisata Sankima, and the other two (S3 and S4) were collected around Camp Kakap (Table 1). Samples S1, S2, and S3 were suspended soil that developed on epiphytic *A. nidus* complex with rosette diameter of 0.9–1.6 m (Table 1, Fig. 1). Sample S2 consisted of litter and soil on the rosette, and S1 and S3 contained humus around the fern roots. S4 consisted of litter and soil on a branch crotch. For comparison, two ground-soil samples (G1 and G2) containing litter and soil were collected on forest floors (see Table 1). G1 and G2 were collected around Camp Kakap and Sankima, respectively. All the samples were kept in plastic bags and carried to the laboratory of LIPI-Biotechnology, Cibinong, West Java. To avoid the samples being damaged by high temperature and humidity, these bags were kept open during the 3–4 days in transit.

Collection of soil arthropods from soil samples and preincubation

To extract living soil-inhabiting arthropods from the soil samples, the Tullgren apparatus or Fukuyama's apparatus was used (Fig. 2). A Tullgren apparatus consists of a sieve, funnel, and incandescent lamp (Fig. 2), and is often used for collecting soil arthropods smaller than 2 mm (Aoki 1973; The Japanese Society of Soil Zoology 2007). Sample soil is placed on the sieve, and is gradually dried by the heat from a lamp for 1–4 days so that arthropods in the soil fall down into a plastic bag through the funnel to escape from aridity (Fig. 2). Fukuyama's apparatus was modified from the Tullgren apparatus for collecting soil arthropods in places where no power supply is available (Fukuyama 1994), and plastic mesh bags, folded envelopes, and disposable hot packs are used in place of sieves, funnels, and lamps, respectively, of the Tullgren apparatus (Fig. 2).

In this study, the samples were treated 3 or 4 days after sample collection. Among the six soil samples, S4 was treated with a Tullgren apparatus (F-1; I. Field, Gunma, Japan), and the others were treated using Fukuyama's

Table 1. Four suspended-soil and two ground-soil samples collected in lowland tropical rainforests in the Kutai National Park, East Kalimantan, Indonesia

Sample soil	Sample no.	Details of the sample	Height above the ground	Location	Forest type
Suspended soil (4 samples)	S1	On *Asplenium nidus* complex growing on a dead standing tree	1.7 m	Wisata Sankima E 117°28′, N 0°22′	Primary forest
	S2	On *Asplenium nidus* complex growing on a living tree	4 m	Wisata Sankima E 117°28′, N 0°22′	Primary forest
	S3	On *Asplenium nidus* complex growing on a living tree	4.5 m	Camp Kakap E 117°28′, N 0°31′	Primary forest
	S4	On a branch crotch on a living tree trunk of *Syzygium* sp. (clove)	0.5 m	Camp Kakap E 117°28′, N 0°31′	Primary forest
Ground soil (2 samples)	G1	Ground soil	–	Camp Kakap E 117°28′, N 0°31′	Primary forest
	G2	Ground soil under *Eusideroxylon zwageri* (ironwood)	–	Sankima, beside a road E 117°28′, N 0°22′	Secondary forest

Fig. 1. Bird's nest ferns (*Asplenium nidus* complex) growing on a dead standing tree in the Kutai National Park, East Kalimantan, Indonesia, from which a suspended-soil sample (S1) was collected. Note litter that has fallen down from above and accumulated on the rosette of fern leaves, including a long branch (*arrow*), humus developed around the root (*arrowhead*), and pendant dead leaves of the fern

apparatus (Fukuyama 1994). Arthropods larger than 2 mm, which could not pass through the sieves or meshes, were transferred to a moist chamber beforehand.

For the Tullgren treatment, a plastic bag was fixed to the bottom of the funnel after spraying water to prevent the death of soil arthropods by drying (Fig. 2). About 1 l of the soil sample was placed on a 2-mm-mesh sieve settled in a funnel, and the sieve was tapped several times so that a small amount of soil fell down into the bag for keeping arthropods alive. An incandescent lamp (40 W, 100 V) was used as a heat source (Fig. 2). The apparatus was placed in the shade in a corridor for 3 days, and then the sieve was tapped vigorously so that approximately 80 g (wet weight) of soil fell into the bag.

For the Fukuyama's treatment, each soil sample was divided into two or three subsamples (~0.5 l) and placed in a plastic mesh bag (18 × 25 cm; Komeri, Niigata, Japan) set in an A4-size envelope, and a moistened plastic bag was fixed to the bottom of the envelope (see Fig. 2). As a heat source, disposable hot packs (Hokkairo, Hakugen, Tokyo, Japan) were placed onto supporters made with a wire frame and a mesh bag (Fig. 2). According to the manufacturer's instructions, the average and maximum temperatures of the hot packs are 54°C and 68°C, respectively. The apparatus was hung in the shade in a corridor for 3 days with changing the hot packs twice a day to maintain the temperature, and then it was shaken so that about 20 g (wet weight) of soil fell into the plastic bag.

The living arthropods and the soil collected by the apparatuses were preincubated in plastic containers (φ 76 mm × 38 mm high; Clean cup 90 BL, with a cover; RISUPACK, Gifu, Japan) at 26°–30°C for a month under light/dark conditions (L:D = 12:12). During incubation, the covers of the containers were opened twice a month for aeration. To maintain humidity, water was sprayed before the preincubation and when the soil surface was dry. After the preincubation, some arthropods were dead and some were still alive.

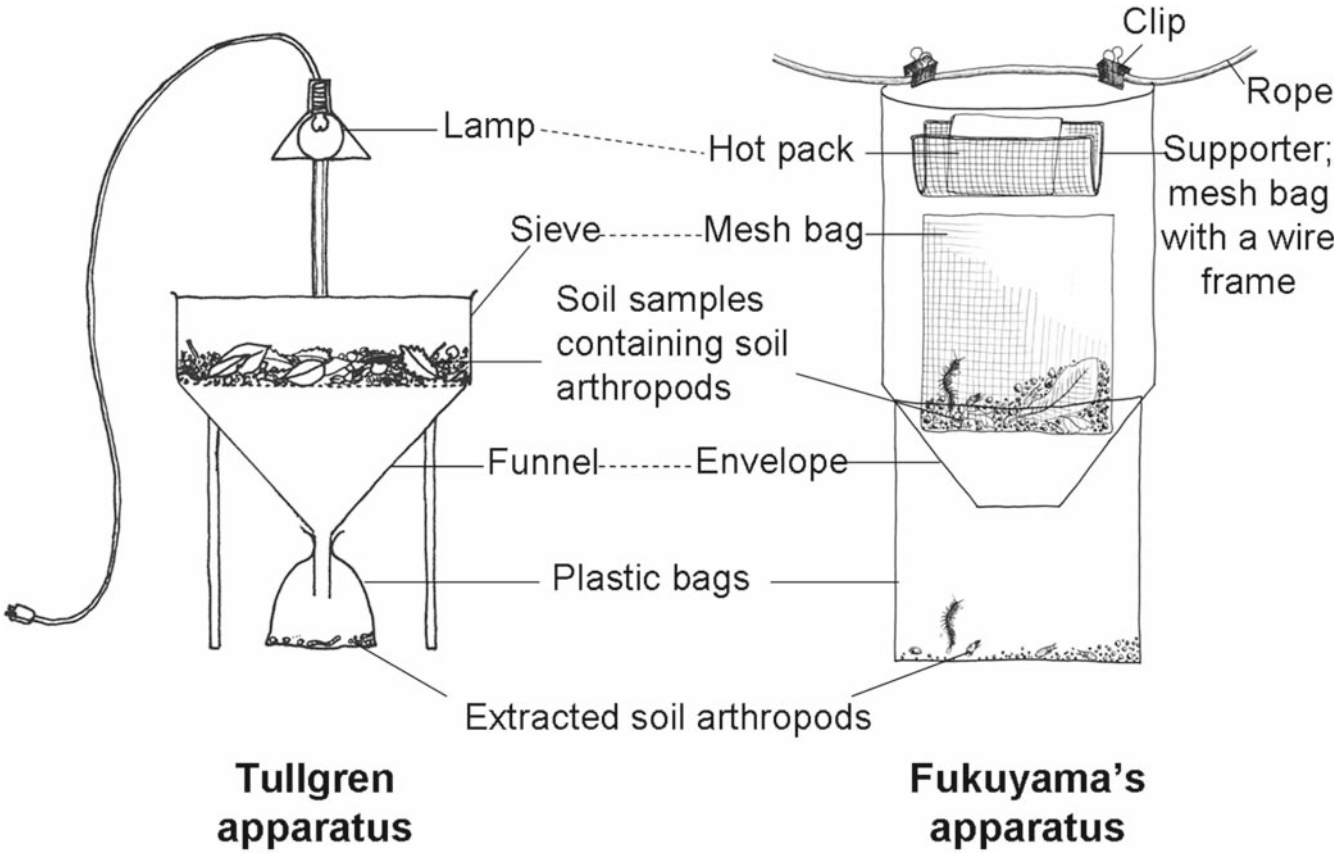

Fig. 2. Diagrams of Tullgren apparatus (*left*) and Fukuyama's apparatus (*right*), used to collect soil arthropods from soil samples. A sieve, funnel, and incandescent lamp comprise the Tullgren apparatus, and a mesh bag, envelope, and hot pack were substituted for these in Fukuyama's apparatus

Isolation of entomopathogenic fungi from soil arthropods

Isolation of entomopathogenic fungi from soil arthropods followed Kurihara et al. (2006). To remove fungal spores attaching to the body surface, each arthropod was transferred onto the surface of a Miura's agar plate (Miura and Kudo 1970) containing 0.05% chloramphenicol (φ = 6 cm), and rolled and trailed for at least 20 cm using a fine needle under a stereomicroscope. This process effectively removes surface-attached spores, and thus it was used in substitution for washing and surface-sterilizing, which was not available. The soil arthropods that were still alive were killed by rolling. In the following section, we call the arthropods that died naturally during the preincubation "dead" arthropods, and the arthropods that survived the preincubation but were killed by rolling "killed" arthropods. In this study, both dead and killed soil arthropods were used for inoculation. The arthropods were inoculated onto the surface of new Miura's agar plates, and incubated at 26°–30°C for 7–20 days under light/dark conditions (L:D = 12:12). To establish pure cultures, spores formed on and around the cadavers of arthropods were transferred with a fine needle to new Miura's agar plates.

Identification of entomopathogenic fungi

The fungal isolates obtained from the arthropods were identified based on light microscopy and sequences analyses of the domains D1 and D2 of large subunit (LSU) ribosomal DNA (rDNA). For the species-level identification of *Lecanicillium* W. Gams & Zare, *Pochonia* Batista & Fonseca, and *Paecilomyces* Bainier species, the sequences of internal transcribed spacer (ITS) regions including 5.8S rDNA were also used. For light microscopy and for DNA extraction, these isolates were cultured on Miura's or malt agar plates (Nissui Pharmaceutical, Tokyo, Japan), respectively, at 26°C under dark/light conditions (L:D = 9:15) for 2–4 weeks. Preparation of slides for light microscopy was conducted as described in Kurihara et al. (2000).

DNA extraction and polymerase chain reaction (PCR) amplification

An agar disc containing a mycelium was transferred to a lysing Matrix A tube (Qbiogene, Carlsbad, CA, USA), kept in a deep freezer at –80°C overnight, and centrifuged for

20 s at 16000 *g*. Total DNA was extracted using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's instructions.

From the extracted DNA, ITS 1 and ITS 2 regions including 5.8S rDNA and the D1 and D2 domains of LSU of rDNA were amplified by polymerase chain reaction (PCR). The reactions were performed in 50-µl reaction volumes containing 23.5 µl distilled water, 5 µl 10× PCR buffer for KOD-Plus-, 5 µl dNTP, 4 µl 25 mM $MgSO_4$, 0.75 µl each primer (10 pmol/µl), 1 µl KOD-Plus- DNA polymerase (Toyobo, Osaka, Japan), and 10 µl extracted DNA as a template. The primer set ITS5 (5′-GGAAGTA AAAGTCGTAACAAGG-3′) and NL4 (5′-GGTCCGT GTTTCAAGACGG-3′) was used (White et al. 1990; O'Donnell 1993). Primer NS7 (5′-GAGGCAATAACAG GTCTGTGATC-3′) (White et al. 1990) was used when ITS5 did not fully anneal to template DNA. Amplification was conducted in a GeneAmp PCR System 9700 (Applied Biosystems, Foster, CA, USA) under the following conditions: an initial denaturation at 94°C for 2 min, 30 cycles at 94°C for 15 s, 56°C for 30 s, and 68°C for 1.5 min. The product was purified with a QIAquick PCR Purification Kit (Qiaagen).

Sequencing reaction

Sequencing reactions were conducted using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) following the manufacturer's instructions. For sequencing of the ITS regions including the 5.8S rDNA and the domains D1/D2 of LSU of rDNA in both directions, the following primers were used: ITS5, ITS3 (5′-GCATCGATGAAGAACGCAGC-3′), NL1 (5′-GCATA TCAATAAGCGGAGGAAAAG-3′), ITS2 (5′-GCTGC GTTCTTCATCGATGC-3′), ITS4 (5′-TCCTCCGCTTA TTGATATGC-3′), and NL4 (White et al. 1990; O'Donnell 1993). NS7 was used occasionally (White et al. 1990).

The reactions were conducted with a Biometra T-Gradient Cycler (Biometra, Göttingen, Germany) under the following conditions: an initial denaturation at 96°C for 1.5 min, 45 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 1.5 min, and a final extension period at 68°C for 10 min. Products were purified with CleanSEQ (Agencourt Bioscience, Beverly, MA, USA). Sequences were analyzed on a 3730xl Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses

The sequences were assembled using phred/phrap software (domains D1 and D2) (Ewing and Green 1998; Ewing et al. 1998) or on "ATGC" (Windows Ver.) Ver. 4.0.9 (GENETYX Co., Tokyo, Japan) (ITS regions). In phred/phrap software, sequences between the first and last bases which phrap scores were 40 were exported from the contigs. The assembled sequences were aligned with those downloaded from DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/) using the Clustal X 1.83 package (Thompson et al. 1997). Phylogenetic trees were constructed by the neighbor-joining method (NJ method) and the maximum-parsimony method (MP method) using PAUP* 4.0b8 software (Swofford 2001). NJ trees were constructed based on the HKY85 distance estimation model (Hasegawa et al. 1985). The reliability of each branch was evaluated by bootstrapping (Felsenstein 1985) with 1000 resamplings in PAUP* 4.0b8 (Swofford 2001). For the construction of MP trees, heuristic searches were performed by adding sequences randomly (number of replicates = 100; characters unweighted). These trees are not shown in this article.

Results

Soil arthropods collected from soil samples

From the four suspended-soil samples, 55 individuals of soil arthropods belonging to nine orders were collected, including 32 collembolans (Arthropleona, Collembola) and 8 mites (Oribatida, Gamasida, and Prostigmata; Acari) (Table 2). Collembolans were found from all samples except from S4, and mites were found in all samples. Other arthropods (ants, coleopterans, cockroaches, chilopods, diplopods, woodlice, and spiders) appeared less frequently from the samples (Table 2). The number of arthropods from S1, S2, S3, and S4 was 9, 27, 16, and 3, respectively (Table 2). Forty-six of the arthropods died during the preincubation stage (dead arthropods), and 9 were alive but killed by rolling (killed arthropods) (see Table 2).

From the two ground-soil samples, 25 soil arthropods belonging to five orders were collected, and most of them were collembolans (10) and mites (11) (Table 2). Collembolans, mites, and coleopterans were found from both samples, but chilopods and spiders were found only from G1. The number of arthropods found from G1 and G2 was 16 and 9, respectively (Table 2). Eleven of the arthropods were dead, and 14 were killed (see Table 2).

Entomopathogenic fungi isolated from suspended-soil arthropods

Among the 55 suspended-soil arthropods used for fungal isolation, entomopathogenic fungi were found from 9 arthropods, no microbes were found from 28 arthropods, only saprobic fungi were found from 17 arthropods, and a bacterial colony was found from 1 arthropod. The percentage of entomopathogenic fungi-positive arthropods was 16.4%, and ten isolates of seven entomopathogenic species were isolated from them (Tables 3, 4). Eight of the ten isolates consisting of six species were found from seven dead arthropods, and other two isolates of a species were found from two killed ones (Table 3). The percentage of entomopathogenic fungi-positive arthropods from suspended soil was similar to that from ground soil of the same and another rainforest of the region, and lower than that from ground soil of Japan (Table 4). However, the number of entomopathogenic species isolated from the suspended-soil arthro-

Table 2. The numbers of suspended-soil and ground-soil arthropods used for the isolation of entomopathogenic fungi

Arthropods	Suspended soil (4 samples)								Total of suspended soil	Ground soil (2 samples)				Total of ground soil
	S1 (9 arthropods)		S2 (27 arthropods)		S3 (16 arthropods)		S4 (3 arthropods)			G1 (16 arthropods)		G2 (9 arthropods)		
	Dead	Killed	Dead	Killed	Dead	Killed	Dead	Killed		Dead	Killed	Dead	Killed	
Formicidae	0/0	0/0	0/1	0	0/1	0/0	0/1	0/0	0/3	0/0	0/0	0/0	0/0	0/0
Coleoptera	0/0	0/0	0/0	1/1	1/1	0/0	0/0	0/0	2/2	0/0	0/1	0/0	1/1	1/2
Blattodea	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/0	0/0
Blattodea, Collembola	1/7	0/0	1/17	0/2	1/5	0/1	0/0	0/0	3/32	0/7	0/0	0/2	0/1	0/10
Collembolan eggs	0/0	0/0	1/1	1/1	0/0	0/0	0/0	0/0	2/2	0/0	0/0	0/0	0/0	0/0
Chilopoda	0/0	0/0	0/1	0/1	0/1	0/0	0/0	0/0	0/2	0/0	1/1	0/0	0/0	1/1
Diplopoda	1/1	0/0	0/0	0/1	0/0	0/1	0/0	0/0	1/3	0/0	0/0	0/0	0/0	0/0
Oniscidea	0/0	0/0	0/0	0/0	0/0	0/0	1/1	0/0	1/1[a]	0/0	0/0	0/0	0/0	0/0
Araneae	0/0	0/0	0/0	0/0	0/1	0/0	0/0	0/0	0/1	0/0	0/1	0/0	0/0	0/1
Acari	0/1	0/0	0/0	0/2	0/4	0/0	0/1	0/0	0/8	0/1	1/5	1/1	0/4	2/11
Subtotal	2/9	0/0	2/20	2/7	2/14	0/2	1/3	0/0	9/55	0/8	2/8	1/3	1/6	4/25

Data shown as the number of arthropods from which entomopathogenic fungi were isolated/the numbers of arthropods used for isolation
Dead, arthropods that died naturally during the 1-month preincubation period; Killed, arthropods that were still alive after the 1-month preincubation, but killed by rolling on inoculation medium
[a]Two entomopathogenic species were isolated from an arthropod

pods was larger than that from ground-soil arthropods (Table 4).

As a result of light microscopy and sequence analyses, these isolates were identified as a species of *Lecanicillium* (*Lecanicillium* sp. 1), an undescribed species of *Lecanicillium* (*Lecanicillium* sp. 2), an undescribed species of *Hirsutella* Pat. (*Hirsutella* sp.), *Paecilomyces lilacinus* (Thom) Samson, *Harposporium anguillulae* Lohde, *Pochonia chlamydosporia* var. *chlamydosporia* (Goddard) Zare & W. Gams, and *Conidiobolus coronatus* (Costantin) A. Batko. The taxonomic position of *Lecanicillium* sp. 1 remains undecided, although it is phylogenetically and morphologically close to *Verticillium saksenae* Kushwaha, which is regarded as a synonym of *L. psalliotae* (Treschow) Zare & W. Gams (Zare and Gams 2001). These isolates appeared singly or were accompanied by saprobic fungi such as *Cunninghamella* Matr., *Trichoderma* Pers., or *Aspergillus* Link., except for two isolates discovered from the same dead woodlouse derived from S4 (Table 3). *Lecanicillium* sp. 1 was isolated from a dead diplopod and woodlouse, *Lecanicillium* sp. 2 was from a dead coleopteran, *Hirsutella* sp. from a dead collembolan, *P. lilacinus* from a dead collembolan egg and woodlouse, *H. anguillulae* from a dead collembolan, *P. chlamydosporia* var. *chlamydosporia* from a dead collembolan, and *C. coronatus* from a killed collembolan egg and coleopteran (see Table 3).

Entomopathogenic fungi isolated from ground-soil arthropods

Among the 25 ground-soil arthropods used for the isolation, entomopathogenic fungi were found from 4 arthropods, no microbes were found from 13 arthropods, only saprobic fungi were found from 7 arthropods, and a bacterial colony was found from 1 arthropod. The percentage of entomopathogenic fungi-positive arthropods was 16%, and two isolates each of *Lecanicillium* sp. 1 and *P. lilacinus* were isolated from them (see Tables 3, 4). *Lecanicillium* sp. 1 was isolated from a dead mite and a killed coleopteran, and *P. lilacinus* was from a killed mite and chilopod (Table 3).

Discussion

The entomopathogenic species isolated from suspended-soil arthropods in this study are diverse. *Lecanicillium* is an anamorphic genus of *Torrubiella* Bound. and *Cordyceps* (Fr.) Link (Gams and Zare 2001; Zare and Gams 2001), and a species of this genus, *L. psalliotae*, has been found from various ground-soil arthropods (Bridge et al. 2005). *Lecanicillium* sp. 1 isolated in this study has been found from suspended-soil collembolans on an *Asplenium* fern collected in Cibinong, West Java (unpublished observation). *Hirsutella* is one of the anamorphic genera of *Ophiocordyceps* Petch (Sung et al 2007), and almost all species of the genus parasitize insects, mites, or nematodes (Seifert and Boulay 2004). *Paecilomyces lilacinus* parasitizes various insects (Samson 1974; Aoki 2003), although it is often isolated from soil as a saprophyte (Domsch et al. 1993). Species of *Harposporium* parasitize nematodes, rotifers, or more rarely beetles (Shimazu and Glockling 1997). *Harposporium anguillulae* isolated from a collembolan in this study parasitizes nematodes, and its teleomorph *Atricordyceps harposporifera* Samuels is probably a parasite of millipede (Samuels 1983). *Pochonia chlamydosporia* var. *chlamydosporia* found from a collembolan in this study has been found from cyst nematodes and soil (Zare et al. 2001). This species is an anamorph of *Metacordyceps chlamydosporia* (H.C. Evans) G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora, which parasitizes eggs of terrestrial mollusks (Zare et al. 2001). A zygomycete pathogen *C. coronatus* parasitizes various insect hosts, although it is commonly found from soil and plant detritus (Domsch et al. 1993; Aoki 2003).

Table 3. Entomopathogenic fungi isolated from 55 suspended-soil arthropods and 25 ground-soil arthropods collected in tropical rainforests in the Kutai National Park, East Kalimantan, Indonesia

Entomopathogenic fungi	Suspended soil (4 samples)				Total of suspended soil	Ground soil (2 samples)		Total of ground soil
	S1	S2	S3	S4		G1	G2	
Lecanicillium sp. 1	1 (diplopod[D])	0	0	1 (woodlouse[D])[b]	2 isolates from 2 arthropods	0	2 (mite[D], coleopteran[K])	2 isolates from 2 arthropods
Lecanicillium sp. 2[a]	0	0	1 (coleopteran[D])	0	1 isolate from 1 arthropod	0	0	–
Hirsutella sp.[a]	1 (collembolan[D])	0	0	0	1 isolate from 1 arthropod	0	0	–
Paecilomyces lilacinus	0	1 (collembolan egg[D])	0	1 (woodlouse[D])[b]	2 isolates from 2 arthropods	2 (mite[K], chilopod[K])	0	2 isolates from 2 arthropods
Harposporium anguillulae	0	1 (collembolan[D])	0	0	1 isolate from 1 arthropod	0	0	–
Pochonia chlamydosporia var. *chlamydosporia*	0	0	1 (collembolan[D])	0	1 isolate from 1 arthropod	0	0	–
Conidiobolus coronatus	0	2 (collembolan egg[K], coleopteran[K])	0	0	2 isolates from 2 arthropods	0	0	–
Subtotal	2 isolates of 2 species from 2 of 9 arthropods	4 isolates of 3 species from 4 of 27 arthropods	2 isolates of 2 species from 2 of 16 arthropods	2 isolates of 2 species from 1 of 3 arthropods	10 isolates of 7 species from 9 of 55 arthropods	2 isolates of 1 species from 2 of 16 arthropods	2 isolates of 1 species from 2 of 9 arthropods	4 isolates of 2 species from 4 of 25 arthropods

[D] Dead arthropods that died naturally during the 1-month preincubation period
[K] Killed arthropods that were still alive after the 1-month preincubation, but killed by rolling on inoculation medium
[a] Undescribed species
[b] Isolated from the same woodlouse

Table 4. A comparison of suspended-soil arthropods and ground-soil arthropods from three areas of Indonesia and Japan from which entomopathogenic fungi were isolated by a similar isolation method

Sampling sites	Types of soil arthropods	Preincubation period of the arthropods in laboratory	Conditions of the arthropods at inoculation	No. of arthropods[a]	No. of species and isolates of entomopathogenic fungi
Kutai Nat. Park East Kalimantan, Indonesia (this study)	Suspended-soil arthropods	1 month	Dead or killed	9/55 (16.4%)	10 isolates of 7 species
Kutai Nat. Park East Kalimantan, Indonesia (this study)	Ground-soil arthropods	1 month	Dead or killed	4/25 (16%)	4 isolates of 2 species
Sungai Wain Protection Forest East Kalimantan, Indonesia[b]	Ground-soil arthropods	1 week to 1 month	Dead, killed, or still alive	15/96 (15.6%)	17 isolates of 4 species
Ueda, Nagano, Japan (Kurihara et al. 2006)	Ground-soil arthropods	Not preincubated	Killed or dead	11/21 (52.4%)	11 isolates of 4 species
Ueda, Nagano, Japan (Kurihara et al. 2006)	Ground-soil arthropods	Several months	Dead or killed	30/64 (46.9%)	31 isolates of 4 species

[a] Number of arthropods from which entomopathogenic fungi were isolated/number of arthropods used for isolation, showing percentage of appearance in parentheses
[b] Unpublished data by the authors

In this study, the number of arthropods collected from the soil samples was considerably more restricted, because the soil samples could not be treated immediately after collection. Weak arthropods might die during the 3–4 days transit and could not be extracted by Tullgren or Fukuyama's treatment. In addition, arthropods such as ants or termites, which have high mobility, may escape from the samples in transit. Ellwood et al. (2002) found an average of 674 ± 409 invertebrates including 602 ± 410 ants and 8 ± 8 termites from suspended-soil samples from 56 small *Asplenium* ferns collected from the low canopy at 2–6 m in Sabah, Borneo. Suma and Karasawa (2005) found 1129–4569 collembolans of 16–20 species from an *Asplenium* suspended-soil sample in Okinawa. Karasawa and Hijii (2006) found about 1800 oribatid mites per 100 g dry weight (DW) of suspended soil in Okinawa. In contrast, we found only 52 arthropods from three *Asplenium* suspended-soil samples. Therefore, the results of this study indicate only a small part of the mycoflora of entomopathogenic fungi in suspended soil.

Nevertheless, suspended-soil arthropods were suggested to be a new isolation source of entomopathogenic fungi; this is because, in this study, seven entomopathogenic species including two undescribed species were isolated in spite of the restricted number of the arthropods. Actually, the number of species isolated from the suspended-soil arthropods was larger than that from ground soil despite the similar or lower percentage of entomopathogenic fungi-positive arthropods. Because obtaining the isolates of various species is important for screening, suspended-soil arthropods would be worth fully investigating as isolation sources in the future.

Among the seven entomopathogenic species found in this study, only two species were commonly isolated from ground-soil arthropods. This finding might reflect the differences in population or species composition of arthropods between suspended soil and ground soil, because those differ even in the same forest (Ellwood et al. 2002; Suma and Karasawa 2005; Yoshida and Hijii 2005). The differences in entomopathogenic mycofloras of suspended- and ground-soil arthropods need to be confirmed in further studies.

Acknowledgments This work was conducted under the Joint Research Project between the Department of Biotechnology, National Institute of Technology and Evaluation, Japan, and the Indonesian Institute of Sciences (LIPI) representing Indonesian Government Research Institutes. We thank the staff of LIPI for general support throughout this work; the Kutai National Park, Mr. R. Ridwan, Dr. T. Tamura, and Ms. G. Kartina for helping us with sample collection; Mr. T. Kamijyo, Ms. R. Suzuki, Ms. K. Anzai, and Mr. T. Kondo for preservation and DNA sequencing of the fungal isolates; Dr. H. Sato for sequence assembly; and Dr. K. Fukuyama, Dr. S. Karasawa, and Dr. T. Yoshida for advice on suspended-soil arthropods.

References

Aoki J (1973) Soil zoology (in Japanese). Hokuryu-kan, Tokyo
Aoki J (2003) Kaitei konchu byogenkin no kensaku (in Japanese). Zenkoku Noson Kyoiku Kyokai, Tokyo
Basset Y (2001) Invertebrates in the canopy of tropical rain forests: how much do we really know? Plant Ecol 153:87–107
Bridge PD, Clark MS, Pearce DA (2005) A new species of *Paecilomyces* isolated from the Antarctic springtail *Cryptopygus antarcticus*. Mycotaxon 92:213–222
Delamare-Deboutteville C (1948) Étude quantitative du peuplement animal des sols suspendus et des épiphytes en forêt tropicale. C R Acad Sci 226:1544–1546
Domsch KH, Gams W, Anderson T-H (1993) Compendium of soil fungi, vol 1. IHW-Verlag, Eching
Ellwood MDF, Foster WA (2004) Doubling the estimate of invertebrate biomass in rainforest canopy. Nature (Lond) 429:549–551
Ellwood MDF, Jones DT, Foster WA (2002) Canopy ferns in lowland dipterocarp forest support a prolific abundance of ants, termites, and other invertebrates. Biotropica 34:575–583
Ewing B, Green P (1998) Basecalling of automated sequencer traces using phred. II. Error probabilities. Genome Res 8:186–194
Ewing B, Hillier L, Wendl M, Green P (1998) Basecalling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res 8:175–185
Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791
Fukuyama K (1994) Tsukaisute kairo wo mochiita keitai-shiki Tullgren souchi no tsukurikata (in Japanese). Edaphologia 51:47–50
Gams W, Zare R (2001) A revision of *Verticillium* sect. *Prostrata*. III. Generic classification. Nova Hedwigia 72:329–337

Hasegawa M, Kishino H, Yano T (1985) Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol 22:160–174

Karasawa S (2006) Diversity of oribatid-mite communities (Acari: Oribatida) in suspended soils (in Japanese). Edaphologia 79:27–40

Karasawa S, Hijii N (2006) Does the existence of bird's nest ferns enhance the diversity of oribatid (Acari: Oribatida) communities in a subtropical forest? Biodivers Conserv 15:4533–4553

Keller S, Zimmermann G (1989) Mycopathogens of soil insects. In: Wilding N, Collins NM, Hammond PM, Webber JF (eds) Insect–fungus interactions. Academic, London, pp 240–270

Kurihara Y, Tokumasu S, Chien CY (2000) *Coemansia furcata* sp. nov. and its distribution in Japan and Taiwan. Mycoscience 41:579–583

Kurihara Y, Machida R, Fukui M, Okuda T, Harayama S (2006) Entomopathogenic fungi isolated from laboratory-reared *Baculentulus densus* (Acerentomidae, Protura). Edaphologia 80:25–28

Longino JT, Nadkarni NM (1990) A comparison of ground and canopy leaf litter ants (Hymenoptera: Formicidae) in a neotropical montane forests. Psyche 97:81–93

Miura K, Kudo MY (1970) An agar medium for aquatic hyphomycetes (in Japanese). Trans Mycol Soc Jpn 11:116–118

Moffett MW (2000) What's "up"? A critical look at the basic terms of canopy biology. Biotropica 32:569–596

O'Donnell K (1993) *Fusarium* and its near relatives. In: Reynolds DR, Taylor JW (eds) The fungal holomorph: mitotic, meiotic and pleomorphic speciation in fungal systematics. CAB International, Wallingford, pp 225–233

Rodgers DJ, Kitching RL (1998) Vertical stratification of rainforest collembolan (Collembola: Insecta) assemblages: description of ecological patterns and hypotheses concerning their generation. Ecography 21:392–400

Samson RA (1974) *Paecilomyces* and some allied hyphomycetes. Studies in Mycology, vol 6. Centraalbureau voor Schimmelcultures, Baarn

Samson RA, Evans HC, Latge JP (1988) Atlas of entomopathogenic fungi. Springer-Verlag, Berlin

Samuels GJ (1983) Ascomycetes of New Zealand 6. *Atricordyceps harposporifera* gen. et sp. nov. and its *Harposporium* anamorph. N Z J Bot 21:171–176

Schmidt K, Li Z, Schubert B, Huang B, Stoyanova S, Hamburger M (2003) Screening of entomopathogenic Deuteromycetes for activities on targets involved in degenerative diseases of the central nervous system. J Ethnopharmacol 89:251–260

Seifert KA, Boulay H (2004) *Hirsutella uncinata*, a new hyphomycete from Australia Mycologia 96:929–934

Shimazu M, Glockling SL (1997) A new species of *Harposporium* with two spore types isolated from the larva of a cerambycid beetle. Mycol Res 101:1371–1376

Suma Y, Karasawa S (2005) Okinawa-ken no tobimushi-rui ni tsuite (3), toku-ni ootaniwatari syuhen no tobimushi-rui (in Japanese). Jezoensis 31:89–104

Sung GH, Hywel-Jones NJ, Sung JM, Luangsaard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Studies in Mycology, vol 57. Centraalbureau voor Schimmelcultures, Utrecht

Swofford DL (2001) PAUP*: phylogenetic analysis using parsimony (*and other methods), version 4.0b8. Sinauer Associates, Sunderland, MA

The Japanese Society of Soil Zoology (2007) Practical guide to soil zoology: from sampling to data analysis (in Japanese). Tokai University Press, Hadano

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876–4882

Visser S, Parkinson D, Hassall M (1987) Fungi associated with *Onychiurus subtenuis* (Collembola) in an aspen woodland. Can J Bot 65:635–642

Wardle DA, Yeates GW, Barker GM, Bellingham PJ, Bonner KI, Williamson WM (2003) Island biology and ecosystem functioning in suspended soil communities. Science 301:1717–1720

White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR protocols. Academic, San Diego, pp 315–322

Yoshida T, Hijii N (2005) The composition and abundance of microarthropod communities on arboreal litter in the canopy of *Cryptomeria japonica* trees. J For Res 10:35–42

Yumoto T (1999) Nettai urin (in Japanese). Iwanami Shoten, Tokyo

Zare R, Gams W (2001) A revision of *Verticillium* section *Prostrata*. IV. The genera *Lecanicillium* and *Simplicillium* gen. nov. Nova Hedwigia 73:1–50

Zare R, Gams W, Evans HC (2001) A revision of *Verticillium* section *Prostrata*. V. The genus *Pochonia*, with notes on *Rotiferophthora*. Nova Hedwigia 73:51–86