Mycoscience (2009) 50:369–379
DOI 10.1007/s10267-009-0493-1

FULL PAPER

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

Lecanicillium and *Verticillium* species from Indonesia and Japan including three new species

Received: February 18, 2008 / Accepted: March 31, 2009

Abstract Forty-six *Lecanicillium* strains and one *Verticillium* strain were isolated from subterranean and epiphytic arthropods, soil, and other sources collected in Indonesia and Japan. These strains were identified as nine *Lecanicillium* and one *Verticillium* species including six undescribed species based on light microscopy and the sequences of the ITS-1 and ITS-2 regions including 5.8S ribosomal DNA. Four of the ten species (*L. araneicola*, *L. kalimantanense*, *Lecanicillium* sp. 4, and *V. indonesiacum*) were recovered from Indonesia, five of the ten (*L. attenuatum*, *L. fusisporum*, *L. psalliotae*, *Lecanicillium* sp. 1, and *Lecanicillium* sp. 3) were from Japan, and *L. saksenae* was from both countries. In this article, new species (*L. araneicola*, *L. kalimantanense*, and *V. indonesiacum*) and a new combination (*L. saksenae*) are proposed from the fungi isolated from epiphytic and subterranean arthropods collected in East Kalimantan.

Key words Anamorph of Clavicipitaceae sensu lato · Distribution · Entomopathogenic fungi · Taxonomy · *Verticillium* sect. *Prostrata*

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

Y. Kurihara[1] (✉) · J.-Y. Park[2] · S. Inaba · K. Ando · S. Harayama[3]
NITE Biological Resource Center (NBRC), National Institute of Technology and Evaluation, Kisarazu, Chiba, Japan

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

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

E. Yuniarti · R. Saraswati
Soil Research Institute, Department of Agriculture, Bogor, Indonesia

Y. Widyastuti
Research Center for Biotechnology, LIPI, Cibinong, Indonesia

[1]*Present address:*
Ishigaki Laboratory, OP BIO FACTORY, Co., Ltd., 567-5 Maezato, Ishigaki, Okinawa 907-0002, Japan
Tel. +81-980-88-1715; Fax +81-980-88-1716
e-mail: kurihara@opbio.com

[2]*Present address:*
Exploratory Research Div., Astellas Research Technologies, Co., Ltd., Tsukuba, Ibaraki, Japan

[3]*Present address:*
Faculty of Science and Engineering, Chuo University, Tokyo, Japan

Introduction

Lecanicillium W. Gams & Zare is a genus segregated from the former *Verticillium* sect. *Prostrata* with *Simplicillium* W. Gams & Zare, *Pochonia* Bat. & O.M. Fonseca, *Haptocillium* W. Gams & Zare, and *Rotiferophthora* G.L. Barron (Gams and Zare 2001). The species of the genus are mainly entomopathogenic or fungicolous, and some of the species have *Cordyceps* (Fr.) Link or *Torrubiella* Boud. teleomorphs (Zare and Gams 2001). Zare et al. (2000) analyzed the sequences of the internal transcribed spacer (ITS) regions including 5.8S ribosomal DNA (rDNA) of various species of *Verticillium* sect. *Prostrata* and indicated that *Lecanicillium* species formed a cluster distinct from the remaining species of the section. However, the trees based on the sequences of large and small subunits of rDNA (Sung et al. 2001) and five genes (Sung et al. 2007) showed that the genus is paraphyletic. That is, in these trees, the cluster of *Lecanicillium* includes other anamorphic genera of *Cordyceps* sensu stricto, viz. *Beauveria* Vuill., *Isaria* Fr., and *Microhilum* H.Y. Yip & A.C. Rath (Sung et al. 2001, 2007). In this article, we adopt the genus *Lecanicillium* despite it being paraphyletic, because the genus concept offers practical means for characterizing *Verticillium*-like species whose teleomorphs have not yet been discovered.

The classification of the genus still remains incomplete. In fact, two species were transferred to the genus from its related genera very recently (Zare and Gams 2008). The taxonomy of *Lecanicillium* has been studied by using materials collected from around the world (Gams and Zare 2001; Zare and Gams 2001, 2008; Kouvelis et al. 2008). However,

the number of Southeast and East Asian isolates analyzed in these papers is not sufficient. We investigated *Lecanicillium* species in Indonesia and Japan and found morphologically and phylogenetically diverse species from these regions. The isolates newly obtained from the Asian countries can be helpful in clarifying the phylogenetic relationships within the genus. Therefore, this article aims to describe the Indonesian species to provide materials for taxonomic studies of the genus.

In this study, we examined 18 and 29 isolates of *Lecanicillium* and a *Verticillium*-like strain isolated in Indonesia and Japan using light microscopy and the sequences of the ITS-1 and -2 regions including 5.8S rDNA. As a result, we recovered ten species including five undescribed ones. We newly describe here two *Lecanicillium* and one *Verticillium* species isolated from epiphytic spiders and a suspended-soil-dwelling coleopteran collected in East Kalimantan, Indonesia, and we also propose a new combination for *Verticillium saksenae* Kushwaha.

Materials and methods

Isolation

Seventeen isolates of *Lecanicillium* and a *Verticillium*-like isolate were obtained from four locations in Indonesia (Kutai National Park, East Kalimantan, 0°22′–31′ N, 117°28′ E, alt. 70–85 m; Cibinong, West Java, 6°29′ S, 106°50′ E, alt. 190 m; Enrekang, South Sulawesi, 3°31′–35′ S, 119°45′–46′ E, alt. 70–235 m) from 2005 to 2006 (Table 1). These sites were situated in tropical lowland rainforest, arable lands, or an experimental field. Twenty-nine Japanese isolates were obtained from eight locations in Japan (Nagano, Ibaraki, two in Kanagawa, and four in Chiba; 35°09′–36°31′ N, 138°18′–140°37′ E, alt. 35–1320 m) from 2004 to 2007 (Table 1). These sites were situated in evergreen or deciduous broad-leaved forests, or bushes along gorges in mountainous areas. Most of the isolates were obtained from subterranean or epiphytic arthropods, and others were from soil, feces, roots, or agarics (Table 1). Isolation of fungi from soil-dwelling arthropods followed Kurihara et al. (2006, 2008b), and isolation from soil was conducted by enrichment or baiting techniques using dried water fleas (Kurihara et al. 2008a). Isolation from leaf-inhabiting arthropods, feces, and roots was carried out by direct transferring of conidial masses onto LcA plates (glucose 1 g, KH_2PO_4 1 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, KCl 0.2 g, $NaNO_3$ 2 g, yeast extract 0.2 g, agar 20 g, tap water 1 l, NaOH 1 grain); these isolation sources were preincubated in a moist chamber when needed. Isolation from collembolans on agarics was carried out by direct inoculation after freezing them for 10–15 min at –20°C.

Identification of the isolates

The isolates were identified based on morphological features using light microscopy and sequence analyses of the ITS1 and ITS2 regions including 5.8S rDNA (ITS regions), as described by Kurihara et al. (2008b). Isolates were grown on LcA at 26°C under dark/light conditions (L:D = 9:15) during 2–4 weeks for light microscopy and on malt agar plates (Nissui Pharmaceutical, Tokyo, Japan) during 4 weeks for DNA extraction. Microscopic identification followed Zare and Gams (2001).

DNA extraction, polymerase chain reaction amplification, and sequencing

DNA extraction, polymerase chain reaction (PCR) amplification, and sequencing were conducted under the conditions described by Kurihara et al. (2008b). Briefly, total DNA was extracted from an agar disc containing mycelium using a DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). The ITS regions were amplified using KOD-Plus-DNA polymerase (Toyobo, Osaka, Japan) and the primers ITS5 and NL4 (White et al. 1990; O'Donnell 1993) in a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) under the conditions described by Kurihara et al. (2008b). The primer NS7 (White et al. 1990) was used occasionally instead of ITS-5 when needed. The product was purified with a QIAquick PCR Purification Kit (Qiagen).

For sequencing of the ITS regions, the primers ITS-5, ITS-3, ITS-2, and ITS-4 (O'Donnell 1993) were used. Sequencing reactions were conducted using a BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems) with a Biometra T- Gradient Cycler (Biometra, Göttingen, Germany) under the conditions described by Kurihara et al. (2008b). The products were purified with CleanSEQ (Agencourt Bioscience, Beverly, MA, USA). Sequences were analyzed on a 3730xl Genetic Analyzer (Applied Biosystems).

Phylogenetic analyses

Phylogenetic analyses followed Kurihara et al. (2008b). The sequences of ITS regions were assembled on "ATGC" (Windows Ver.) Ver. 4.0.9 (GENETYX, Tokyo, Japan). The sequences were aligned with the sequences downloaded from DDBJ using the Clustal X 1.83 package (Thompson et al. 1997). Phylogenetic trees were constructed by the neighbor-joining (NJ) and the maximum-parsimony (MP) methods using PAUP* 4.0b8 software (Swofford 2001). The NJ and MP trees were constructed according to the HKY85 distance estimation model (Hasegawa et al. 1985), or by using heuristic search by adding sequences randomly (number of replicates = 100), respectively. The reliability of each branch was estimated by bootstrapping (Felsenstein 1985) with 1000 resamplings.

Results

Almost complete sequences of ITS regions of the 47 isolates of *Lecanicillium* and *Verticillium* were obtained. The

Table 1. Eighteen Indonesian and 29 Japanese isolates of *Lecanicillium* and *Verticillium* used for phylogenetic analyses

Species	Strain no.	DDBJ accession no.	Locality	Isolation source	Other strain no.	Other information	Date of isolation
L. antillanum	CBS 350.85	AJ292392	Cuba	Agaricales	–	Ex-type	–
L. aphanocladii	CBS 376.77	AJ292431	Netherlands	*Agaricus bitorquis*	–	–	–
L. aranearum	CBS 726.73	AJ292464	Ghana	Araneae	–	–	–
L. araneicola	BTCC-F35*	AB378506	Kutai, E. Kalim., ID	Araneae	NBRC 105407; ID06-F0382	Ex-type	Jul. 21, 2006
L. attenuatum	CBS 402.78	AJ292434	USA	Leaf litter of *Acer saccharum*	–	–	–
	NBRC 103237*	AB360369	Ueda, Nagano, JPN	Coleopteran larva	KYK00096	–	Jul. 20, 2004
	NBRC 104277*	AB378507	Ichihara, Chiba, JPN	Arthropoda	KYK00034	–	Jun. 28, 2004
	NBRC 104278*	AB378508	Ichihara, Chiba, JPN	Coleoptera	KYK00039	–	Jun. 28, 2004
	NBRC 103234*	AB378509	Ichihara, Chiba, JPN	Araneae	KYK00047	–	Jun. 28, 2004
	NBRC 103235*	AB378510	Ichihara, Chiba, JPN	Araneae	KYK00071	–	Jun. 28, 2004
	NBRC 103236*	AB378511	Ohtaki, Chiba, JPN	Araneae	KYK00072	–	Jun. 28, 2004
	NBRC 103238*	AB378512	Yamakita, Kanagawa, JPN	Coccoidea	KYK00199	–	Oct. 30, 2004
	NBRC 104279*	AB378513	Kisarazu, Chiba, JPN	Aphidoidae on a leaf of *Aucuba japonica*	KYK00324	–	Oct. 2, 2007
	NBRC 104280*	AB378514	Kisarazu, Chiba, JPN	Aphidoidae on a leaf of *Aucuba japonica*	KYK00326	–	Oct. 2, 2007
	KYK00134*	AB378515	Ueda, Nagano, JPN	*Terpnosia nigricosta* (Cicadoidea)	–	–	Aug. 10, 2004
L. dimorphum	CBS 363.86	AJ292429	China	*Agaricus bisporus*	NRRL 26542	Ex-type	–
L. fusisporum	CBS 164.70	AJ292428	Netherlands	*Coltricia perennis* (Hymenomycetes)	–	Ex-type	–
	NBRC 103712*	AB360370	Ueda, Nagano, JPN	Arthropleona (Collembola)	KYK00104	–	Jul. 20, 2004
	NBRC 104281*	AB378517	Ueda, Nagano, JPN	Soil under deciduous broad-leaved forest	KYK00229	–	Oct. 24, 2006
L. kalimantanense	BTCC-F23*	AB360356	Kutai, E. Kalim., ID	Coleoptera (Staphylinoidea?) in suspended soil	NBRC 105406; ID06-F0406	Ex-type	Jul. 21, 2006
L. lecanii (*Torrubiella confragosa*)	CBS 101247	AJ292383	West Indies	*Coccus viridis* (Coccidae)	IMI 304807	–	–
L. longisporum	IMI 179172	AJ292384	UK	*Macrosiphoniella sanborni* (Aphididae)	–	–	–
	CBS 126.27	AJ292385	Sri Lanka	*Icerya purchasi* (Coccidae)	IMI 021167	Ex-type	–
L. muscarium	IMI 179173	AJ292387	UK	*Brachycaudus helichrysi* (Aphididae)	–	–	–
L. psalliotae	CBS 101270	AJ292389	UK	Soil	IMI 163640	–	–
	CBS 100172	AJ292390	USA	Nymph of *Ixodes* (Acari)	–	–	–
	NBRC 103715*	AB360364	Kamogawa, Chiba, JPN	*Hypogastrura* sp. (Collembola)	KYK00175	–	Oct. 11, 2004
	NBRC 103705*	AB360365	Kisarazu, Chiba, JPN	Arthropleona (Collembola)	KYK00023	–	Apr. 15, 2004
	NBRC 103714*	AB360367	Kashima, Ibaraki, JPN	Soil under a tree	KYK00165	–	Aug. 9, 2004
	NBRC 103704*	AB360366	Kisarazu, Chiba, JPN	Soil under a grove of *Castanopsis sieboldii* and *Quercus* spp.	KYK00018	–	Apr. 15, 2004
	NBRC 104282*	AB378518	Kisarazu, Chiba, JPN	Poduromorpha (Collembola)	KYK00298	–	Jul. 6, 2007
	NBRC 104283*	AB378519	Ichihara, Chiba, JPN	Sternorrhyncha on a leaf of *Aucuba japonica*	KYK00031	–	Jun. 28, 2004
L. saksenae (= *V. saksenae*)	CBS 532.81	AJ292432	India	Forest soil	IMI 179841	Ex-type	–
	BTCC-F26*	AB360351	Kutai, E. Kalim., ID	Coleoptera	ID06-F0393	–	Jul. 20, 2006
	BTCC-F27*	AB360350	Kutai, E. Kalim., ID	Diplopoda in suspended soil	ID06-F0395	–	Jul. 20, 2006
	BTCC-F29*	AB360352	Kutai, E. Kalim., ID	Isopoda (sow bug) in suspended soil	ID06-F0410	–	Jul. 21, 2006

Table 1. *Continued*

Species	Strain no.	DDBJ accession no.	Locality	Isolation source	Other strain no.	Other information	Date of isolation
	BTCC-F25*	AB360353	Kutai, E. Kalim., ID	Gamasida (Acari)	ID06-F0391	–	Jul. 20, 2006
	BTCC-F28*	AB360354	Kutai, E. Kalim., ID	Arthropod excrement in suspended soil	ID06-F0396	–	Jul. 20, 2006
	BTCC-F24*	AB360355	Kutai, E. Kalim., ID	Soil under *Shorea assamica*	ID06-F0306	–	Jul. 21, 2006
	BTCC-F16*	AB360357	Cibinong, W. Java, ID	Arthropleona (Collembola) in suspended soil	ID05-F0118	–	Sep. 9, 2005
	BTCC-F17*	AB360358	Cibinong, W. Java, ID	Arthropleona (Collembola) in suspended soil	ID05-F0119	–	Sep. 9, 2005
	BTCC-F18*	AB360359	Cibinong, W. Java, ID	Arthropleona (Collembola) in suspended soil	ID05-F0120	–	Sep. 9, 2005
	BTCC-F19*	AB360360	Cibinong, W. Java, ID	Arthropleona (Collembola) in suspended soil	ID05-F0127	–	Sep. 9, 2005
	BTCC-F22*	AB360361	Cibinong, W. Java, ID	Soil under *Durio zibethinus*	ID05-F0242	–	Sep. 1, 2005
	BTCC-F20*	AB360362	Enrekang, S. Sulaw., ID	Soil in a bamboo bush	ID05-F0221	–	Aug. 30, 2005
	BTCC-F21*	AB360363	Enrekang, S. Sulaw., ID	Soil under *Arenga pinnata*	ID05-F0227	–	Aug. 30, 2005
	NBRC 104284*	AB378520	Kisarazu, Chiba, JPN	Collembola on *Phaeolepiota aurea*	KYK00236	–	Nov. 20, 2006
Lecanicillium sp. 1	NBRC 104292*	AB378528	Yamakita, Kanagawa, JPN	Coccoidea on a culm of *Sasa* sp.	KYK00214	–	Apr. 17, 2005
	NBRC 104293*	AB378529	Kisarazu, Chiba, JPN	Poduromorpha (Collembola)	KYK00305	–	Jul. 6, 2007
	CBS 639.85	AJ292386	Germany	Rhizosphere of *Pseudotsuga menziesii*	–	–	–
	CBS 100890	AJ292427	USA	Contaminant in mushroom spawn	–	–	–
Lecanicillium sp. 3	NBRC 103713*	AB360368	Ueda, Nagano, JPN	Acari	KYK00149	–	Jul. 22, 2004
	NBRC 104285*	AB378521	Ueda, Nagano, JPN	*Russula* sp.	KYK00095	–	Jul. 21, 2004
	NBRC 104286*	AB378522	Kiyokawa, Kanagawa, JPN	Field mouse excrement	KYK00209	–	Apr. 16, 2005
	NBRC 104287*	AB378523	Kisarazu, Chiba, JPN	Arthropleona (Collembola) on *Amanita* sp.	KYK00237	–	Oct. 26, 2006
	NBRC 104288*	AB378524	Kisarazu, Chiba, JPN	Egg of soil arthropod?	KYK00240	–	Apr. 27, 2007
	NBRC 104289*	AB378525	Kisarazu, Chiba, JPN	Lithobiomorpha (Chilopoda)	KYK00296	–	Apr. 14, 2007
	NBRC 104290*	AB378526	Kisarazu, Chiba, JPN	Root	KYK00304	–	Jul. 6, 2007
	NBRC 104291*	AB378527	Kisarazu, Chiba, JPN	Diplopoda?	KYK00325	–	Jul. 6, 2007
Lecanicillium sp. 4	BTCC-F37*	AB378530	Enrekang, S. Sulaw., ID	Soil under a *Musa* sp.	ID05-F0197	–	Aug. 29, 2005
	BTCC-F38*	AB378531	Enrekang, S. Sulaw., ID	Soil	ID05-F0200	–	Aug. 29, 2005
L. tenuipes	CBS 309.85	AJ292391	Spain	Araneae	–	–	–
V. indonesiacum	BTCC-F36*	AB378516	Kutai, E. Kalim., ID	Araneae	NBRC 105408; ID06-F0380	Ex-type	Jul. 21, 2006
P. chlamydosporia var. *chlamydosporia*	CBS 103.65	AJ292397	Germany	Soil under *Brassica napus*	ATCC 16289	Ex-neotype	–
R. minutispora	CBS 101436	AJ292411	Japan	Bdelloid rotifer in soil	–	–	–
S. lanosoniveum (*Torrubiella* sp.)	CBS 704.86	AJ292396	Venezuela	*Hemileia vastatrix* (Uredinales)	–	–	–
B. bassiana	NBRC 4848	AB027382	Unknown	Unknown	–	–	–
C. militaris	EFCC-C738	AF153264	Unknown	Unknown	–	–	–
H. sinense	CBS 567.95	AJ292417	China	Nematoda	–	Ex-type	–
I. tenuipes	BCMU IJ15	AB086215	Japan	Lepidopteran pupa	–	–	–

*Isolates obtained and sequenced in this study (isolator: Y. Kurihara). E. Kalim, East Kalimantan; S. Sulaw, South Sulawesi; W. Java, West Java; ID, Indonesia, JPN, Japan
Sequences downloaded from DDBJ and other data of comparable strains were mostly based on Zare et al. (2000) and Sung et al. (2001). B, *Beauveria*; C, *Cordyceps*; H, *Haptocillium*; I, *Isaria*; L, *Lecanicillium*; P, *Pochonia*; R, *Rotiferophthora*; S, *Simplicillium*; V, *Verticillium*

sequences were aligned with 22 downloaded comparable sequences, and trees were built using three taxa as outgroups (Table 1, Fig. 1). A strict consensus was constructed from four trees retained by heuristic search (Fig. 1).

Because branching patterns were almost concordant with each other in NJ and MP trees, only the MP tree is shown in this article (Fig. 1). Of the 47 isolates analyzed in this study, 38 grouped into four large clusters, and nine constituted smaller clusters consisting of 1–3 isolates (Fig. 1). Fourteen isolates constituted the largest cluster. The largest cluster grouped in a higher cluster with CBS 532.81, the ex-type strain of *Verticillium saksenae* Kushwaha [bootstrap (BS) values, 75% and 55% in NJ and MP trees; see Fig. 1]. These 14 isolates originated from Indonesia, in addition to 1 from Japan (NBRC 104284); most of them were obtained from soil arthropods and soil (see Table 1). Ten isolates, which were obtained from spiders, hemipterans, and other arthropods in Japan, grouped in a cluster with *L. attenuatum* Zare & W. Gams (CBS 402.76) [coherence with 85% (NJ) and 65% (MP) BS support], and were identified as *L. attenuatum* (see Table 1, Fig. 1). Eight isolates obtained from soil arthropods, roots, feces, and *Russula* sp. in Japan grouped together with BS support of 100% in NJ and MP trees. This clade did not include any named species of the genus, and we called this clade *Lecanicillium* sp. 3 (Table 1, Fig. 1). Six isolates obtained from soil arthropods, soil, and scale insects in Japan grouped with two strains of *L. psalliotae* (Treschow) Zare & W. Gams (coherence supported with BS values of 97% and 93%) and were identified as *L. psalliotae* (Table 1, Fig. 1). The other nine isolates constituted smaller clades. Two isolates found from a collembolan and soil in Japan grouped with the ex-type strain of *L. fusisporum* (W. Gams) Zare & W. Gams (BS support of 100% and 99%) (Table 1, Fig. 1). Two other isolates obtained from a scale insect and a collembolan in Japan constituted a clade with CBS 639.85, which was described as "*Lecanicillium* sp. 1" by Zare and Gams (2001) (BS values, 98% and 96%) (Table 1, Fig. 1). Isolates obtained from soil of South Sulawesi constituted a highly supported clade (BS 100% in both trees) designated here as *Lecanicillium* sp. 4. The larger clade that includes this and *Lecanicillium* sp. 1 received high BS support of 100% in both trees (Table 1, Fig. 1). BTCC-F35 found from a spider in Indonesia grouped with a strain of *L. aranearum* (Petch) Zare & W. Gams (CBS 726.73A), but it joined with the strain only by low BS values of 69% and 57% (Table 1, Fig. 1). Because of considerable morphological differences between BTCC-F35 and *L. aranearum*, we concluded that this is an undescribed species of *Lecanicillium*. BTCC-F36, BTCC-F23, and BTCC-F39, obtained from a coleopteran and spiders in Indonesia, were situated singly near the base of the trees (Table 1, Fig. 1), and each of them was considered as an undescribed species.

Discussion

The 47 isolates observed and analyzed in this study were identified as ten species, viz. *L. saksenae*, *L. attenuatum*, *L. psalliotae*, *L. fusisporum*, and six undescribed species (see Table 1, Fig. 1). Five of the ten species were obtained from Indonesia (*L. saksenae* and four undescribed species), and six were obtained from Japan (*L. saksenae*, *L. attenuatum*, *L. psalliotae*, *L. fusisporum*, and two undescribed species); only one species was obtained from both countries (*L. saksenae*) (Table 1, Fig. 1).

Lecanicillium species obtained from Indonesia and Japan were mostly different. *Lecanicillium psalliotae*, a common entomopathogen of various soil-inhabiting insects (Bridge et al. 2005), was commonly isolated from soil and ground-soil arthropods in Japan, but not in Indonesia. In contrast, *L. saksenae* was frequently isolated from soil and suspended- and ground-soil arthropods in Indonesia, and in Japan, the species was found only once from a collembolan inhabiting a basidiocarp of *Phaeolepiota aurea* (Matt.) Maire. The spectrum of *Lecanicillium* species seems to differ between Indonesia and Japan. However, further studies are required to delimit the geographic distribution of each species. Because the sampling used in the two countries was different in this study, many suspended-soil arthropods were investigated in Indonesia, but none in Japan. The differences in species pattern observed in the two countries might reflect differences in the isolation source, because arthropods in suspended soil are a potential isolation source for various entomopathogenic fungi including *Lecanicillium* under tropical conditions (Kurihara et al. 2008b). Moreover, in this study, other entomopathogenic and fungicolous species of *Lecanicillium*, including the strictly entomopathogenic *L. lecanii* (Zimm.) Zare & W. Gams, were not isolated.

Our results suggest that a greater diversity of *Lecanicillium* species is to be found in Asia, especially in tropical countries. In fact, of the 47 isolates of ten species obtained in this study, 15 isolates of six species were undescribed species. These results will advance phylogenetic studies on the genus *Lecanicillium* and its related genera.

Taxonomy

Lecanicillium araneicola Sukarno & Kurihara, sp. nov.
Figs. 2A–D, 3

MycoBank no.: MB 513387.

Coloniae in LCA albae, reverso cremeo-albo, floccosae, sine pigmento in agarum diffundente. Hyphae aeriae prostratae, 1–2.5 µm latae. Phialides ex hyphis aeriis, solitariae vel 2–4 verticillatae, tenues, sursum attenuatae, (14–)19–31.5 × 1–2 µm. Conidia dimorphica: macroconidia acerosa, utrinque acutata, parum curva vel fere recta, (7.5–)8.5–12(–14) × 1.5–2 µm; microconidia allantoidea vel ellipsoidea, utrinque rotundata, parum curva vel rectiuscula, 3–5 × 1–2 µm.

Etymology: *Aranea* + *-cola* (Latin), living on spiders.

Colonies on LCA white, with creamy-white reverse, floccose, with prostrate aerial hyphae, without diffusing pigment. Aerial hyphae 1–2.5 µm wide; phialides solitary or

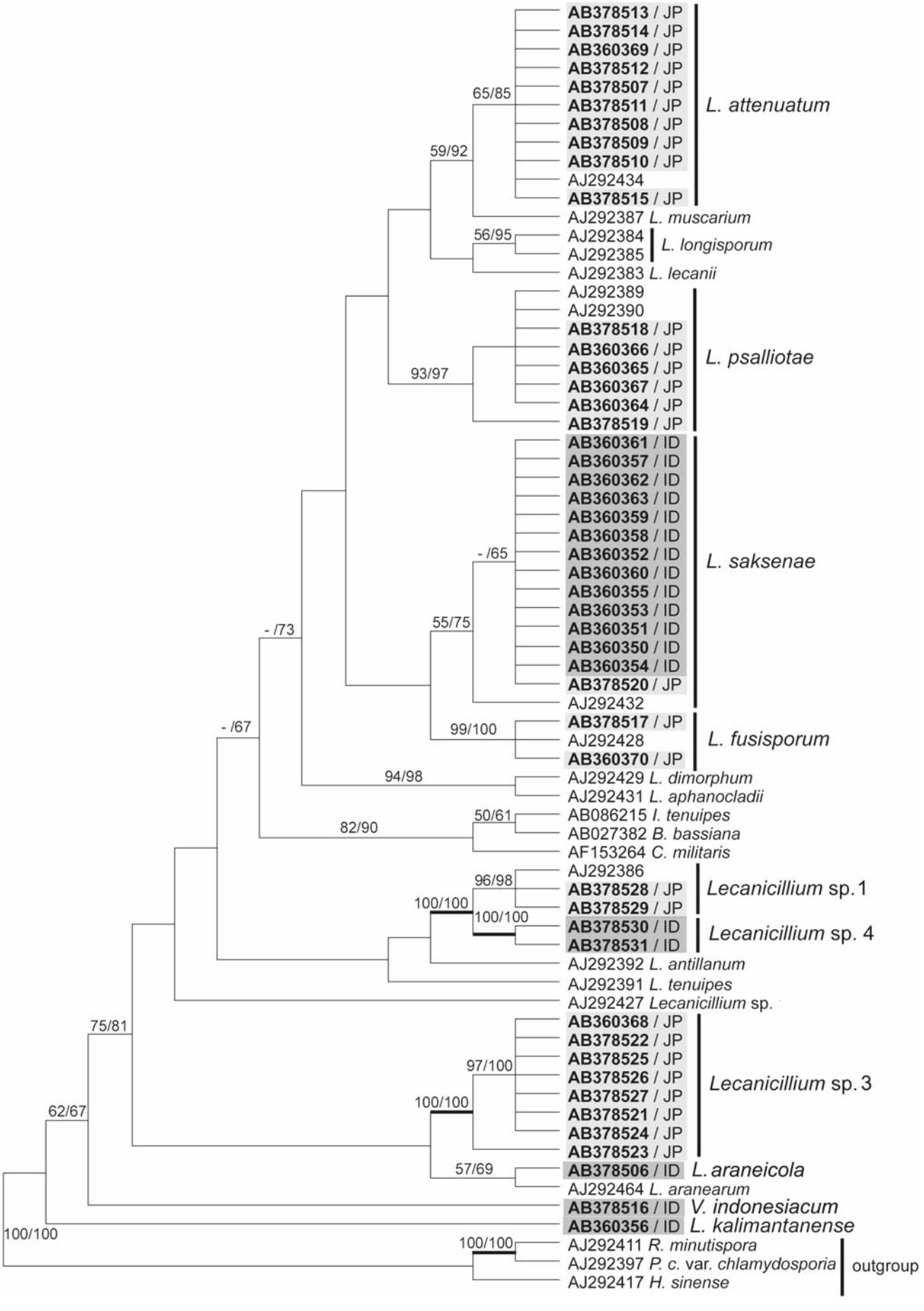
AB378513 / JP
AB378514 / JP
AB360369 / JP
AB378512 / JP
AB378507 / JP
AB378511 / JP
AB378508 / JP
AB378509 / JP
AB378510 / JP
AJ292434
AB378515 / JP
L. attenuatum
65/85
59/92
AJ292387 L. muscarium
56/95
AJ292384
AJ292385
L. longisporum
AJ292383 L. lecanii
AJ292389
AJ292390
AB378518 / JP
AB360366 / JP
AB360365 / JP
AB360367 / JP
AB360364 / JP
AB378519 / JP
L. psalliotae
93/97
AB360361 / ID
AB360357 / ID
AB360362 / ID
AB360363 / ID
AB360359 / ID
AB360358 / ID
AB360352 / ID
AB360360 / ID
AB360355 / ID
AB360353 / ID
AB360351 / ID
AB360350 / ID
AB360354 / ID
AB378520 / JP
AJ292432
L. saksenae
- /65
55/75
- /73
AB378517 / JP
AJ292428
AB360370 / JP
L. fusisporum
99/100
- /67
94/98
AJ292429 L. dimorphum
AJ292431 L. aphanocladii
50/61
AB086215 I. tenuipes
82/90
AB027382 B. bassiana
AF153264 C. militaris
AJ292386
96/98
AB378528 / JP
AB378529 / JP
Lecanicillium sp. 1
100/100
100/100
AB378530 / ID
AB378531 / ID
Lecanicillium sp. 4
AJ292392 L. antillanum
AJ292391 L. tenuipes
AJ292427 Lecanicillium sp.
75/81
AB360368 / JP
AB378522 / JP
AB378525 / JP
AB378526 / JP
AB378527 / JP
AB378521 / JP
AB378524 / JP
AB378523 / JP
Lecanicillium sp. 3
97/100
100/100
62/67
57/69
AB378506 / ID
L. araneicola
AJ292464 L. aranearum
AB378516 / ID
V. indonesiacum
AB360356 / ID
L. kalimantanense
100/100
100/100
AJ292411 R. minutispora
AJ292397 P. c. var. chlamydosporia
AJ292417 H. sinense
outgroup

Fig. 1. Strict consensus of the four most parsimonious trees inferred from sequences of the internal transcribed spacer (ITS)-1 and -2 regions including 5.8S rDNA of 69 taxa. Newly determined sequences are shown in *bold*. Bootstrap values in the most parsimonious tree/neighbor-joining tree are indicated *above* nodes (excluding values below 50%). Length = 452, CI = 0.564, RI = 0.798, and RC = 0.450. *B*., *Beauveria*; *C*., *Cordyceps*; *H*., *Haptocillium*; *I*., *Isaria*; *L*., *Lecanicillium*; *P. c.*, *Pochonia chlamydosporia*; *R*., *Rotiferophthora*; *S*., *Simplicillium*; *V*., *Verticillium*; *ID*, Indonesian isolates; *JP*, Japanese isolates

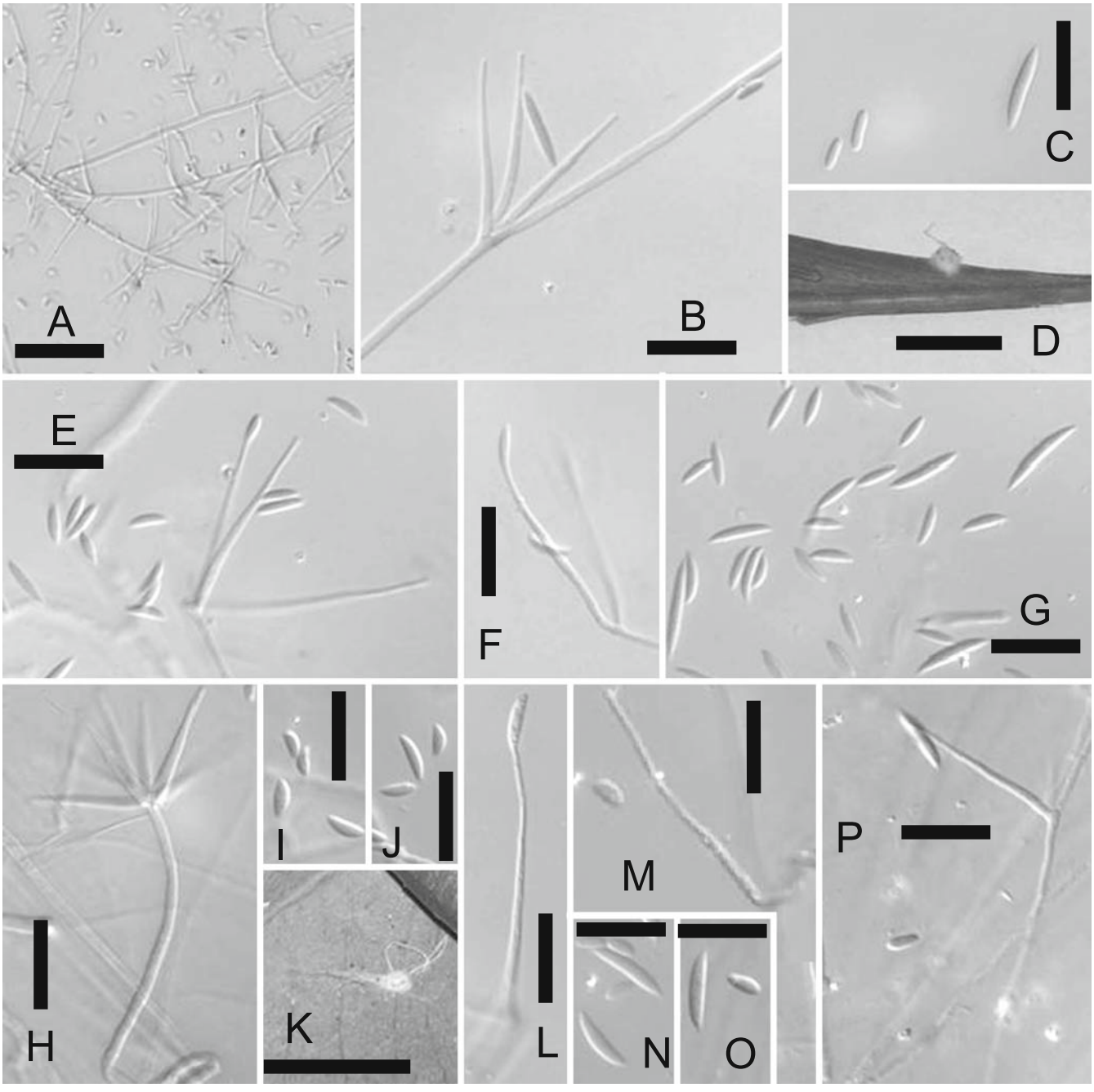

Fig. 2. Species of *Lecanicillium* and *Verticillium* from Indonesia. **A–D** *L. araneicola* (BTCC-F35). **E–G** *L. kalimantanense* (BTCC-F23). **H–K** *V. indonesiacum* (BTCC-F36). **L–P** *L. saksenae* (BTCC-F25). **A–C**, **E–G**, **H–J**, **L–P** Cultures on LCA medium. **A** Verticillate and solitary phialides developed on a prostrate aerial hypha. **B** Three verticillate phialides produced on a prostrate aerial hypha. **C** Macroconidium (*right*) and two microconidia (*left*). **D** Synnema growing on a leaf-inhabiting spider. **E** Three verticillate phialides with a developing and a liberated conidium. **F** Phialide with a developing conidium. **G** Conidia. **H** Verticillate phialides produced on an erect conidiophore. **I**, **J** Conidia. **K** Synnemata growing on a leaf-inhabiting spider. **L** A phialide with a developing macroconidium. **M** Phialide and a liberated microconidium. **N** Two macroconidia and three microconidia. **O** A macroconidium (*left*) and a microconidium (*right*). **P** A solitary phialide developed on a prostrate aerial hypha. *Bars* **A** 25 μm; **B**, **C**, **E–G**, **H–J**, **L–P** 10 μm; **D**, **K** 1 cm

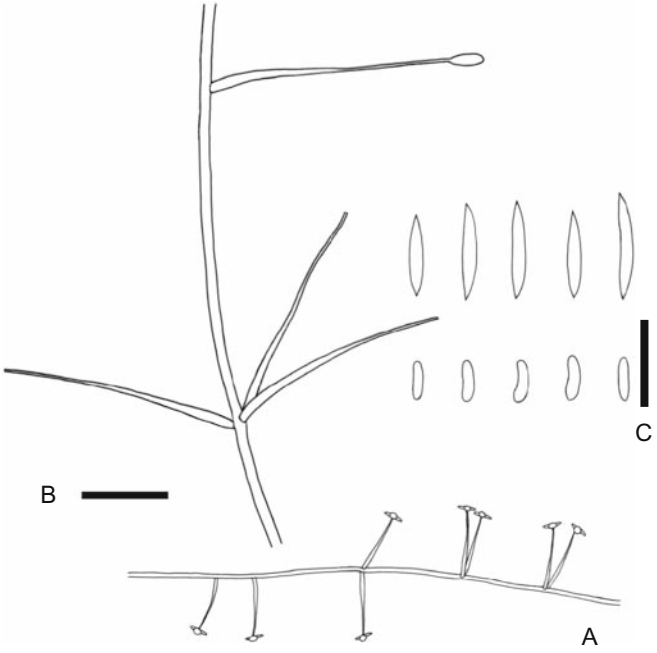

Fig. 3. *Lecanicillium araneicola* (BTCC-F35) grown on LCA. **A** Sketch of the habit of phialides developed on a prostrate aerial hypha (not to scale). **B** A solitary and verticillate phialides. **C** Five macroconidia (*above*) and five microconidia (*below*). *Bars* **B**, **C** 10 µm

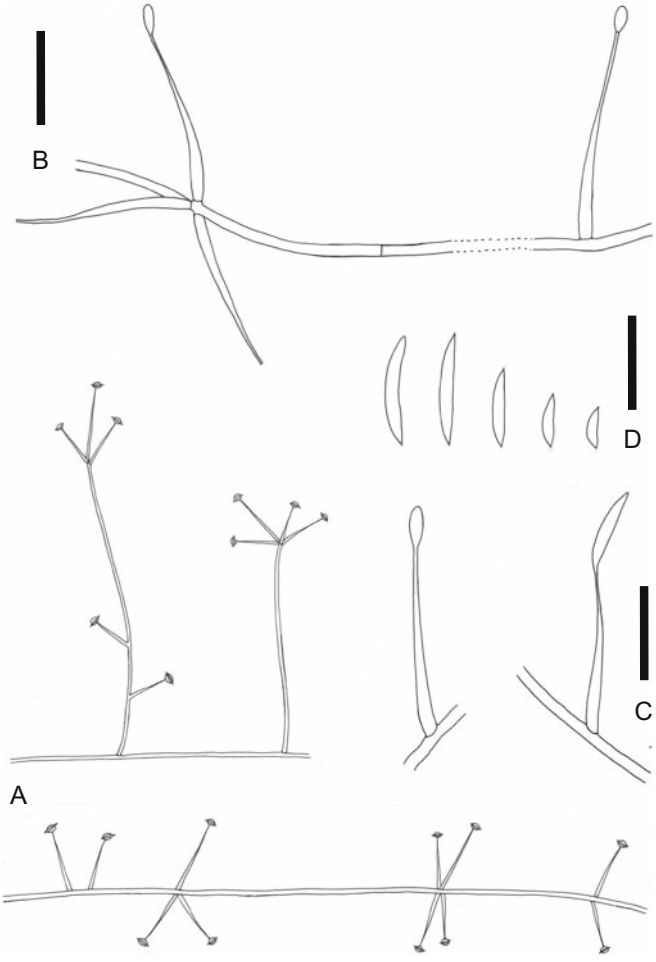

Fig. 4. *Lecanicillium kalimantanense* (BTCC-F23) grown on LCA. **A** Habit of two erect conidiophores bearing phialides developed from an erect vegetative hypha (*above*) and verticillate and solitary phialides developed on an prostrate aerial hypha (*below*). **B** Three verticillate and one solitary phialides developed on an aerial hypha. **C** Two solitary phialides bearing a developing (*left*) and a nearly mature (*right*) conidium. **D** Five conidia. *Bars* **B–D** 10 µm

in whorls of 2–4, slender, tapering toward the tip, (14–)19–31.5 × 1–2 µm. Conidia dimorphic: macroconidium formed first, microconidia formed subsequently. Macroconidia acerose with pointed ends, slightly curved to nearly straight, (7.5–)8.5–12(–14) × 1.5–2 µm. Microconidia allantoid to ellipsoidal with round ends, slightly curved or nearly straight, 3–5 × 1–2 µm.

Holotype: BO22578, Kutai National Park, East Kalimantan, Indonesia, Jul. 21, 2006, isolated from a synnema growing on a spider (Araneae) attached under a leaf of an unidentified plant of Zingiberaceae growing on the forest floor of a primary lowland tropical rainforest, coll. K. Ando (KA), Y. Widyastuti (YW), G. Kartika (GK), R. Ridwan (RR), J.-Y. Park (JP), T. Tamura (TT), and Y. Kurihara (YK), isol. YK, deposited in Herbarium Bogoriense. Isotype: NBRC H-12749 in the herbarium of NBRC. Living culture: BTCC-F35 (= NBRC 105407, = ID06-F0382), deposited in Biotechnology Culture Collection, Research Center for Biotechnology-Indonesian Institute of Science (LIPI).

Notes: This species resembles *L. psalliotae* and *L. aranearum* in morphology. However, the size and shape of conidia of the species distinguish *L. araneicola* from *L. psalliotae* and *L. aranearum*. In *L. aranearum*, macroconidia and microconidia are not differentiated, and the conidia are usually asymmetrically narrowed or subacute at the end, and 5–8 × 0.7–1.5 µm in size (Zare and Gams 2001). In *L. psalliotae*, macroconidia are falcate, curved, and 5–10 × 1.2–1.7 µm, and microconidia are oval to ellipsoid, and 2.7–3.7 × 1–1.5 µm (Zare and Gams 2001).

Lecanicillium kalimantanense Kurihara & Sukarno, sp. nov. Figs. 2E–G, 4

MycoBank no.: MB 513388.

Coloniae in LCA albae, reverso cremeo-albo, hyphis aeriis prostratis, sine pigmento in agarum diffundente. Phialides ex hyphis erectis vegetativis vel aeriis prostratis 1–2 µm latis orientes, solitariae vel saepe 2–4(–5) verticillatae, tenues, sursum attenuatae, 12.5–36 × 1–2 µm. Conidia acerosa vel fusoidea, utrinque acutata, parum curva, magnitudine varia, (3.5–)4.5–12 × 1–2 µm.

Etymology: Referring to the type locality, *Kalimantan*.

Colonies on LCA white, with creamy-white reverse, with prostrate aerial hyphae, without pigment diffusing into the agar. Phialides produced on erect or along prostrate aerial hyphae 1–2 µm wide, solitary or more often in whorls of 2–4(–5), slender, tapering toward the apex, 12.5–36 × 1–2 µm. Conidia acerose to fusoid with pointed ends, slightly curved, of varying size, (3.5–)4.5–12 × 1–2 µm.

Holotype: BO22579, Kutai National Park, East Kalimantan, Indonesia, Jul. 21, 2006, isolated from an exoskeleton of staphylinid-like beetle (Staphylinoidea (?), Coleteopera) collected from suspended soil on *Asplenium nidus* complex growing on a tree trunk at 4.5 m height in a primary lowland tropical rainforest, coll. KA, YW, GK, RR, JP, TT, and YK, isol. YK, deposited in Herbarium Bogoriense. Isotype: NBRC H-12750 in the herbarium of NBRC. Living culture: BTCC-F23 (= NBRC 105406, = ID06-F0406), deposited in Biotechnology Culture Collection, Research Center for Biotechnology-Indonesian Institute of Science (LIPI).

Notes: This species morphologically resembles *L. psalliotae*. These taxa can easily be distinguished because secondary conidia are acerose to fusoid in *L. kalimantanense*, whereas those of *L. psalliotae* are oval to ellipsoid. Reddish pigment and octahedral crystals in agar medium, which are often produced by *L. psalliotae*, have not been seen in *L. kalimantanense*.

Lecanicillium saksenae (Kushwaha) Kurihara & Sukarno, comb. nov. Figs. 2H–K, 5

MycoBank no.: MB 513390

≡ *Verticillium saksenae* Kushwaha, Curr. Sci. 49: 948, 1980 (basionym) (published as "saksenii").

Colonies on LCA white, with creamy white reverse, floccose with prostrate aerial hyphae, without pigment diffusing into the agar. Prostrate aerial hyphae 1–2 µm wide; phialides, solitary or often in whorls of 2–4, slender, tapering toward the apex, 14.5–36 × 1–2 µm. Conidia dimorphic: macroconidium formed first, microconidia formed subsequently. Macroconidia falcate with pointed ends, slightly curved, 6–13 × 1.5–2 µm. Microconidia ellipsoidal to fusoid with round ends, nearly straight to slightly curved, 2.5–5 × 1.5–2 µm.

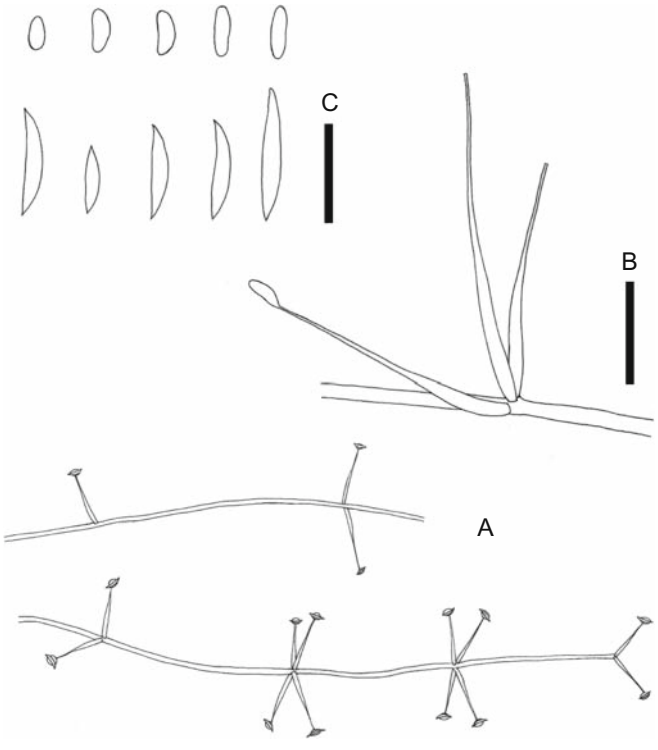

Fig. 5. *Lecanicillium saksenae* (BTCC-F25) grown on LCA. **A** Habit of verticillate and solitary phialides arising from prostrate aerial hyphae (not to scale). **B** Three verticillate phialides. **C** Microconidia (*above*) and macroconidia (*below*). *Bars* **B**, **C** 10 µm

Isolates examined: Kutai National Park, East Kalimantan, Indonesia, Jul. 20, 2006, isolated from a mite (Gamasida, Acari) collected from soil under *Eusideroxlon zwageri* Teijsm. & Binn. in a secondary lowland tropical rainforest, coll. KA, YW, GK, RR, JP, TT, andYK, isol. YK, deposited in Herbarium Bogoriense. Culture viva: BTCC-F25 (= ID06-F0391), deposited in Biotechnology Culture Collection, Research Center for Biotechnology-Indonesian Institute of Science (LIPI) – Research Center for Biotechnology-LIPI, Cibinong, West Java, Indonesia, Sep. 9, 2005, isolated from a collembolan (suborder Arthropleona, Collembola) that dwelled in suspended soil on an *Asplenium nidus* complex on a tree, recovered by direct inoculation method after incubating in a moist chamber, coll. YK and H. Yamamura (HY), isol. YK. Living culture: BTCC-F16 (= ID05-F0118).

Enrekang, South Sulawesi, Indonesia, Aug. 30, 2005, isolated from soil in a bamboo bush, recovered by the enrichment method, coll. KA, YW, R. Saraswati, Y. Lestari, RR, S. Ratnakomala, HY, JP, and YK, isol. YK. Living culture: BTCC-F20 (= ID05-F0221). Cibinong, West Java, Indonesia, Sep. 1, 2005, isolated from soil collected under a *Durio zibethinus* in an experimental field, recovered by the enrichment method, coll. KA, RR, D.R. Waltam, HY, and JP, isol. YK. Living culture: ID05-F0242.

Notes: *Lecanicillium saksenae* closely resembles *L. psalliotae* and is almost indistinguishable from it (Zare and Gams 2001). Because the two species differ only slightly in mtDNA restriction fragment length polymorphisms (RFLPs) and in sequences of ITS regions including 5.8S rDNA (11 bases), Zare and Gams (2001) considered *V. saksenae* as a synonym of *L. psalliotae*. However, in our ITS-inferred trees, CBS 532.81, the ex-type strain *L. saksenae* from India, and our 14 isolates never clustered with *L. psalliotae* (see Fig. 1). As Zare and Gams (2001) pointed out, the absence of reddish pigment and octahedral crystals in *L. saksenae*, which are usually seen in *L. psalliotae*, may be helpful in distinguishing the two species. Macroconidia and microconidia of CBS 532.81 are smaller than BTCC-F25 (5.5–8 × 1.3–2.5 µm and 2.5–3.5 × 1.2–1.5 µm) (Zare and Gams 2001); however, they differ by only three bases in ITS sequences. As indicated by Kouvelis et al. (2008), further analyses based on mitochondrial genes are required for delimitation of these closely related species.

Verticillium indonesiacum Kurihara & Sukarno, sp. nov. Figs. 2H–K, 6

MycoBank no.: MB 513389

Coloniae in LCA albae, reverso cremeo-albo, hyphis paucis aeriis prostratis, sine pigmento in agarum diffundente. Phialides ex hyphis erectis vel raro ex hyphis prostratis. Aeriis orientes, solitariae vel saepe (2–)3–5(–6) verticillatae, sursum attenuatae, aliquando septatae et phialides

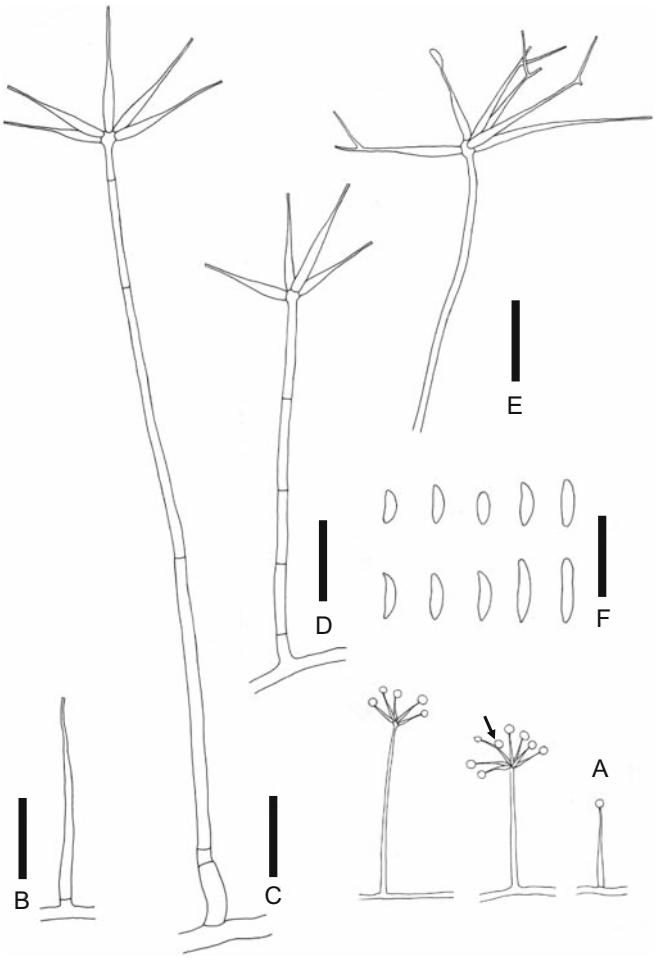

Fig. 6. *Verticillium indonesiacum* (BTCC-F36) grown on LcA. **A** Habit (not to scale). A conidiophore producing verticillate phialides bearing conidial masses (*left*); conidiophore with verticillate phialides, with sympodial proliferation (*arrow*) (*middle*); phialide arising from a vegetative hypha bearing a conidial mass at it tip (*right one*). **B** A phialide developed from a vegetative hypha. **C–D** Two erect conidiophores bearing verticillate phialides. **E** Simple and sympodially proliferating verticillate phialides on an erect conidiophore. **F** Conidia. *Bars* **B–F** 10 µm

secundarias ferentes, 9–34 × 1–2 µm. Conidia falcata vel ellipsoidea, parum curva vel rectiuscula, utrinque paulo acutata, 3–8.5 × 1–2 µm.

Etymology: Referring to its type locality, *Indonesia*.

Colonies on LcA white, thin, flat, with creamy-white reverse, with few prostrate aerial hyphae, without pigment diffusing into the agar. Phialides produced on erect hyphae, or rarely on prostrating aerial hyphae, solitary or often in whorls of (2–)3–5(–6), tapering toward the tip, sometimes septate and bearing secondary phialides, 9–34 × 1–2 µm. Conidia falcate to ellipsoid, slightly curved or nearly straight, the ends of conidia not sharply pointed, 3–8.5 × 1–2 µm.

Holotype: BO22577, Kutai National Park, East Kalimantan, Indonesia, Jul. 21, 2006, isolated from synnemata growing on a spider (Araneae) attached under a leaf collected in a primary lowland tropical rainforest, coll. KA, YW, GK, RR, JP, TT, and YK, isol. YK, deposited in Herbarium Bogoriense. Isotype: NBRC H-12751 in the herbarium of NBRC. Living culture: BTCC-F36 (= NBRC 105408, = ID06-F0380) Biotechnology Culture Collection, Research Center for Biotechnology–Indonesian Institute of Science (LIPI).

Notes: This species resembles *V. pseudohemipterigenum* H.C. Evans & Y. Jun and *V. hemipterigenum* Petch in general morphology (Petch 1932; Hywel-Jones et al. 1997) but differs in producing a single phialidic whorl and falcate to ellipsoid conidia. We retain this species as a species *incertae sedis* of *Verticillium* on a morphological basis, although ITS sequences indicate that the species is situated near the base of the genus *Lecanicillium*. Conidiophores of *V. indonesiacum* usually are erect and its phialides sometimes produce secondary phialides, whereas phialides of *Lecanicillium* species usually arise from prostrate aerial hyphae: they are characteristically rebranched only in *L. dimorphum* (Gams and Zare 2001; Zare and Gams 2001). We could not classify *V. indonesiacum* in *Lecaniciilum*, and thus describe it as a species *incertae sedis* of *Verticillium* for the time being. More studies would be required to determine its final taxonomic position.

Acknowledgements This work was conducted under the Joint Research Project between Department of Biotechnology, National Institute of Technology and Evaluation, Japan, and the Indonesian Institute of Sciences (LIPI) representing Indonesian Government Research Institutes. This work was partly supported by a grant from the New Energy and Industrial Technology Development Organization (NEDO), Japan (P02038). We thank Dr. Walter Gams for reading and improving the manuscript. We also thank other participants of the Joint Project for their support throughout this study, and Mr. Tomoaki Kamijyo, Ms. Rieko Suzuki, and Ms. Kozue Anzai for preserving and sequencing the isolates.

References

Bridge PD, Clark MS, Pearce DA (2005) A new species of *Paecilomyces* isolated from the Antarctic springtail *Cryptopygus antarcticus*. Mycotaxon 92:213–222

Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39:783–791

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

Hywel-Jones NL, Evans HC, Jun Y (1997) A re-evaluation of the leafhopper pathogen *Torrubiella hemipterigena*, its anamorph *Verticillium hemipterigenum* and *V. pseudohemipterigenum* sp. nov. Mycol Res 101:1242–1246

Kouvelis VN, Sialakouma A, Typas MA (2008) Mitochondrial gene sequences alone or combined with ITS region sequences provide firm molecular criteria for the classification of *Lecanicillium* species. Mycol Res 112:829–844

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

Kurihara Y, Degawa Y, Tokumasu S, Harayama S (2008a) Isolation methods for *Coemansia* and other species of Kickxellales from soil (in Japanese). Nippon Kingakukai Kaiho 49:46–51

Kurihara Y, Sukarno N, Ilyas M, Yuniarti E, Mangunwardoyo W, Saraswati R, Park JY, Inaba S, Widyastuti Y, Ando K, Harayama S (2008b) Entomopathogenic fungi isolated from suspended-soil-inhabiting arthropods in East Kalimantan, Indonesia. Mycoscience 49:241–249

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

Petch T (1932) Notes on entomogenous fungi: 22–48. Trans Br Mycol Soc 16:209–245

Sung GH, Spatafora JW, Zare R, Hodge KT, Gams W (2001) A revision of *Verticillium* sect. *Prostrata* II. Phylogenetic analyses of SSU and LSU nuclear rDNA sequences from anamorphs and teleomorphs of the Clavisipitaceae. Nova Hedwigia 72:311–328

Sung GH, Hywel-Jones NJ, Sung JM, Luangsa-ard JJ, Shrestha B, Spatafora JW (2007) Phylogenetic classification of *Cordyceps* and the clavicipitaceous fungi. Stud Mycol 57:1–63

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

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

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 Press, San Diego, pp 315–322

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 (2008) A revision of *Verticillium fungicola* species complex and its affinity with the genus *Lecanicillium*. Mycol Res 112:811–824

Zare R, Gams W, Culham A (2000) A revision of *Verticillium* section *Prostrata*. I. Phylogenetic studies using ITS sequences. Nova Hedwigia 71:465–480