長野県黒姫山の「天狗の麦飯」から分離された糸状菌Leca nicillium aphanocladii

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Lecanicillium aphanocladii isolated from Tengu-no-Mugimeshi found in Mount Kurohime, Nagano Prefecture, central Japan

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Tengu-no-Mugimeshi is a soil-like mass of microorganisms that was collected on Japan's Mount Kurohime in 1939. It was sealed in glass bottles and preserved at an elementary school in Nagano City for 80 years. Upon opening the bottle, a single verticillium-like fungal strain, FKI-9593 (=IFM 64743), was isolated from the preserved material. Using a combination of micro-morphological characteristics and multigene phylogeny (SSU, LSU and TEF), the isolate was identified as *Lecanicillium aphanocladii*. A culture broth of the organism was used to test for antimicrobial activity against 13 selected microbes. As a result, weak antibacterial activity against *Proteus vulgaris* NBRC 3167 was confirmed. Structural analysis identified the active compound as the polyketide metabolite, oosporein.

Key words: Ascomycota, multi-gene phylogeny, antibacterial activity, secondary metabolite

INTRODUCTION

Tengu-no-Mugimeshi is an aggregation of microbial material usually consisting of up to 10 types of microbes; Eubacteria, Archaea, Cyanobacteria, Ktedonobacteria and Fungi (Wang *et al.*, 2019). "Tengu" is a kind of demigod popular in Japanese folklore; "Tengu-no-mugimeshi" means boiled barley of Tengu (Okada, 1937). It is orange-to-brown in color, granular and very similar to soil in appearance, and is also referred to as "eatable soil" (Okada, 1937). Geologically speaking, Tengu-no-Mugimeshi is found in a graben, called the Fossa Magna, a great rift lowland that traverses the widest section of Honshu from the Sea of Japan to the Pacific (Motojima *et al.*, 1955). It is only found in volcanic areas above 2000 m in altitude on the Fossa Magna. Geographically, this area is located in central Japan and lies in the Chubu and northern Kanto regions (Motojima *et al.*, 1955). It is known that the constituent microbes of Tengu-no-Mugimeshi vary depending on the region. There have been some previous reports on the constituent microbes of Tengu-no-Mugimeshi (Kawamura, 1916; Ono, 1916; Yagi, 1936).

In 1902, *Bacillus* sp., *Verticillium* sp., *Leucosystis* sp. and a species of "Kapselbacterien" were isolated from Tengu-no-Mugimeshi collected on Mount Kurohime in the northern part of Nagano Prefecture (Ono, 1916). In 1914, species of the 'Oomycetes', *Bacillus fluorescens* and *Penicillium* sp. were isolated from Tengu-no-Mugimeshi collected on the same mountain (Kawamura, 1916). In 1926, all bacteria isolated from Tengu-no-Mugimeshi were cyanobacteria (Molisch, 1926). Although there were multiple reports of bacteria isolated from Tengu-no-Mugimeshi, few research reports described fungi as components of the mixture.

One site named as "Miso-zuka" in Mikageshinden, Komoro of Nagano Prefecture where Tengu-no-Mugimeshi is found is protected as a nationally protected natural monument (Yagi, 1936). Among the areas where Tengu-no-Mugimeshi is commonly found, known localities of Tengu-no-Mugimeshi at Mt. Kurohime and Mt. Iizuna are strictly protected as Special Protection Zones in Myoko-Togakushi Renzan National Park by Japan's Ministry of the

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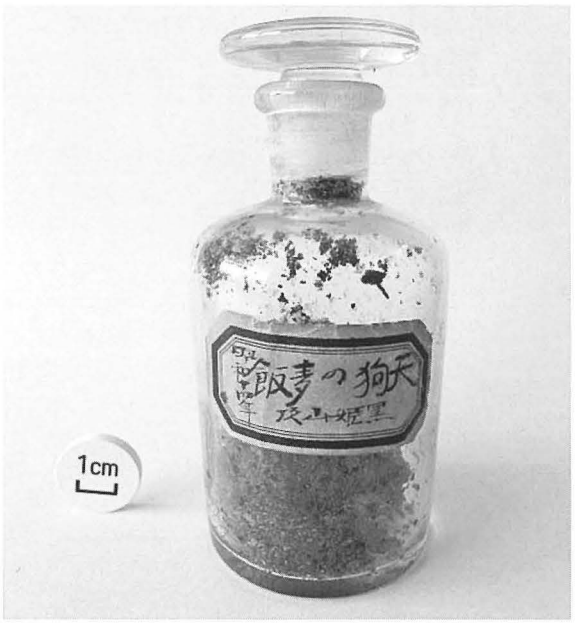
Environment, so samples cannot be collected without special permits. The specimen of Tengu-no-Mugimeshi examined in this study was collected from the summit of Mt. Kurohime in 1939. This specimen was preserved in a sealed glass bottle and maintained at Terao Elementary School, Nagano City, Japan for over 80 years (Fig. 1). This bottle was subsequently rediscovered in 2016 and is now kept at this Togakushi Museum of Natural History, Nagano City, Japan. Historically, few fungi have been isolated from Tengu-no-Mugimeshi, therefore, we report herein details of a fungal strain isolated from this 1939 sample, including notes on its taxonomy, phylogeny and secondary metabolite profile.



A glass bottle containing Tengu-no-Mugimeshi preserved at Terao Elementary School, Nagano City, Japan

Tengu-no-Mugimeshi showing the granular mass appearance

Fig. 1 Studied sample of Tengu-no-Mugimeshi

MATERIALS AND METHODS

Isolation

To isolate fungi, a sample of Tengu-no-Mugimeshi (1 g) taken from the aforementioned specimen was suspended in 9 m*l* of modified Winogradsky's salt solution (0.38% K_2HPO_4, 0.12% KH_2PO_4, 0.51% $MgSO_4 \cdot 7H_2O$, 0.25% NaCl, 0.005% $Fe_2(SO_4)_3 \cdot nH_2O$ and 0.005% $MnSO_4 \cdot 5H_2O$) with 0.01% Tween-80 (Sigma-Aldrich Co., Saint Louis, MO, USA) and then sonicated for 2 min, and diluted to 10^2–10^3 fold with the above Winogradsky's salt solution. Aliquots of diluted suspension totaling 200 m*l* were spread on Petri dishes with solidified potato dextrose agar (PDA, Difco Laboratories Ltd., Detroit, MI, USA), malt extract agar (MEA; Pitt, 1979) and Czapek yeast extract agar (CYA; Pitt, 1979), and all media with 50 mg/*l* rose bengal, 100 mg/*l* kanamycin and chloramphenicol. To isolate bacteria from Tengu-no-Mugimeshi, PDA without antibiotics was used. Plates were incubated at 25°C for 7 days. A fungus was then isolated and the isolate was preserved as FKI-9593 at the Ōmura Satoshi Memorial Institute, Kitasato University, and as IFM 64743 at the Medical Mycology Reseach Center, Chiba University through the National Bio-Resource Project.

Morphological analysis

To observe morphological characteristics, the isolate was incubated on PDA, MEA and potato carrot agar (PCA; Atlas, 2010) at 25°C (also at 5°C and 37°C on PDA) for 7 days in the dark. Colony colours were designated according to Kornerup & Wanscher (1978).

Determination of micro-morphological characteristics followed as reported by Zare & Gams (2001) and Kaifuchi *et al.* (2013). For observation of micro-morphological characteristics, microscope slides were prepared from PCA (Atlas, 2010) cultures. The slides were examined with a Vanox-S AH-2 microscope (Olympus Co., Ltd., Tokyo, Japan), and digital photomicrographs were taken with a DP25 digital camera (Olympus Co., Ltd.).

For scanning electron microscopy (SEM), agar

blocks (5×5 mm) cultured on PCA and MEA medium for 7 days were prepared. The samples were air-dried. Sputter-coating with gold was accomplished using a JFC-1200 Fine Coater (JEOL Ltd., Tokyo, Japan). These samples were observed with a JSM-5600 scanning electron microscope (JEOL Ltd.).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted using the PrepMan Ultra® Sample Preparation Reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), according to the manufacturer's instructions. Portions of DNA of nuclear ribosomal small and large subunit (SSU and LSU) DNA and elongation factor 1α (TEF) gene, were amplified by polymerase chain reaction (PCR) conducted under the conditions detailed by Nonaka *et al.*(2013). Sequencing reaction was performed using a BigDye® Terminator according to the manufacture's instructions, then sequence products were purified using a BigDye® XTerminator Purification Kit (Thermo Fisher Scientific, Inc.). And samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Thermo Fisher Scientific, Inc.). Contigs were assembled using the forward and reverse sequences, with the SeqMan Pro program from the Lasergene 10 package (DNASTAR Inc., Madison, WI, USA). The sequences were deposited at the GenBank (Table 1).

Phylogenetic analysis

Sequences of all regions were individually aligned using MUSCLE 3.8.31 (Edgar, 2004). The alignments were refined using SeaView 4.7 (Gouy *et al.*, 2010). The total length of aligned sequences was 2,476 nucleotides. Three-genes (SSU, LSU and TEF) were connected into a single dataset. Before combining the data sets, each set was analysed using the neighbor-joining (NJ) method (Saitou & Nei, 1987). Phylogenetic analyses of the 3-genes dataset were based on the maximum-parsimony (MP) method using PHYLIP's dnapars algorithm, the NJ method and the maximum likelihood (ML) method using PhyML 3.0 (Guindon *et al.*, 2010) with GTR substitution model as implemented in SeaView 4.7 program. Bootstrap analyses were performed on MP, NJ and ML trees with 1,000 bootstrap replicates. The trees were rooted with species of *Simplicillium* Zare & W. Gams species according to Su *et al.*(2019) and Zhou *et al.*(2018), and viewed in SeaView 4.7 program.

Because MP, NJ and ML trees were almost concordant with each other, we describe the phylogenetic results based on the ML tree (Fig. 2). There was no topological conflict among the SSU tree, LSU tree and TEF tree for highly-supported clades, and the concatenated tree was more robust than the separated ones. The alignments and trees were deposited in TreeBASE (http://www.treebase.org/) with the accession number 26425.

Fermentation of fungal strain

To assay for antimicrobial activity of culture broths against representative microbes, as well to enable metabolite analyses, the strain was inoculated into 10 m*l* of seed medium containing: 2.0% glucose, 0.5% Hipolypepton (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan), 0.2% yeast extract, 0.2% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% agar; adjusted to pH 6.0 before sterilization. The test tube containing the mixture was incubated on a rotary shaker (300 rpm) at 27°C for three days. A 1-m*l* portion of the seed culture of FKI-9593 was transferred to three 500-m*l* Erlenmeyer flasks containing 100 m*l* of F38 production medium (3.0% sucrose, 3.0% soluble starch, 1.0% malt extract, 0.3% "Ebios", a commercial product of dried yeast by Asahi Group Foods, Ltd. (Tokyo, Japan), 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$; adjusted to pH 6.0 before sterilization), and the fermentation was carried out on a rotary shaker (210 rpm) at 27°C for three days and then stationary for 10 days. The culture broth (300 m*l*) was mixed with ethanol (300 m*l*), followed by centrifugation (3,000 rpm for 10 min).

Instrumental analysis of secondary metabolites

The mixtures were evaluated by analytical HPLC under the following conditions for the analysis of metabolites: *column*, Symmetry C18 (2.1 ø×150 mm, Waters Co., Tokyo, Japan); *mobile phase*, acetonitrile-water with 0.05% phosphoric acid, 5–100% (20 min); *flow rate*, 0.2 m*l* min^{-1}; *detection*, UV at 210 nm.

Antimicrobial activities of culture broths against representative microbes

Antimicrobial activity was measured using the paper disc method (6 mm and 8 mm, Toyo Roshi Kaisha Ltd., Tokyo, Japan). Each sample contained 10 μ*l* and 50 μ*l* of 50% ethanol extract of pure culture broth of strain FKI-9593. Culture conditions were as follows: *Bacillus subtilis* ATCC 6633 (Nutrient agar (0.5% peptone, 0.5% meat extract and 0.8% agar),

Table 1 *Lecanicillium* isolate and related taxa used for phylogenetic analyses

Species	Strain no.	Locality	Isolation source	Taxonomical information	GenBank accession no.		
					SSU	LSU	TEF
Akanthomyces aculeatus	HUA 186145	–	–	–	MF416572	MF416520	MF416465
A. attenuatum	CBS 402.78	USA	Leaf litter of *Acer saccharum*	–	AF339614	AF339565	EF468782
A. coccidioperitheciata	NHJ 5112	–	Spider (Arachnida)	–	EU369109	EU369043	EU369026
A. dipterigenus (*Lecanicillium longisporum*)	CBS 126.27	Sri Lanka	*Icerya purchasi*	Ex-type	KM283773	KM283797	KM283820
A. lecanii	CBS 101247	West Indies	*Coccus viridis*	–	KM283770	KM283794	DQ522359
A. muscarium	CBS 143.62	UK	*Trialeurodes vaporariorum*	Epitype	KM283774	KM283798	KM283821
A. pistillariaeformis	HUA 186131	–	–	–	MF416573	MF416521	MF416466
A. sabanense	ANDES-F 1024	Colombia	*Parthenolecanium* sp.	Holotype	KC633251	KC875225	KC633266
A. tuberculatus	BCC 16819	–	–	–	MF416600	MF416546	MF416490
Blackwellomyces cardinalis	OSC 93610	Tennessee, USA	Subfamily archolophinay (Tineidae, Lepidoptera)	–	AY184974	AY184963	EF469059
Blackwellomyces sp.	FKI-7101	Kozu Islands, Japan	Soil under *Damnacanthus indicus*	–	LC553288	LC553283	LC553293
B. pseudomilitaris	BCC 1919	–	–	–	MF416588	MF416534	MF416478
Gibellula leiopus	BCC 16025	–	–	–	MF416602	MF416548	MF416492
G. pulchra	NHJ 10808	–	Spider (Arachnida)	–	EU369099	EU369035	EU369018
Hevansia arachnophilus	NHJ 10469	–	Spider (Arachnida)	–	EU369090	EU369031	EU369008
H. nelumboides	BCC 2093	–	–	–	MF416583	MF416530	MF416473
Lecanicillium acerosum	CBS 418.81	Amazon	*Crinipellis perniciosa* in *Theobroma cacao*	Ex-type	KM283762	KM283786	KM283810
L. antillanum	CBS 350.85	Cuba	Agaric	Ex-type	AF339585	AF339536	DQ522350
L. aphanocladii	CBS 797.84	China	*Agaricus bisporus*	–	KM283763	KM283787	KM283811
	FKI-9593*	Nagano, Japan	Tengu-no-Mugimeshi	–	LC553289	LC553284	LC553294
L. dimorphum	CBS 345.37	Germany	*Puccinia coronata*	–	KM283764	KM283788	KM283812
L. flavidum	CBS 342.80	Villeurbanne, France	*Abies alba*	Ex-type	KM283766	KM283790	KM283814
L. fungicola var. *aleophilum*	CBS 357.80	Netherlands	*Agaricus bisporus*	Ex-type	KM283767	KM283791	KM283815

*New isolates sequenced in this study. ANDES-F, Culture collection of the Museo de Historia Natural of the Universidad de los Andes in Bogotá, Colombia; BCC, BIOTEC Culture Collection Laboratory, Klong Luang, Thailand; CBS, CBS-KNAW Culture Collection, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.; FKI, Fungal Collection of Ōmura Satoshi Memorial Institute, Kitasato University, Tokyo, Japan; KACC, Korean Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Rural Development Administration, Suwon, Republic of Korea; KUMCC, Culture collection of Kunming Institute of Botany, Kunming, China; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, Oregon, USA

Table 1 Continued

Species	Strain no.	Locality	Isolation source	Taxonomical information	GenBank accession no.		
					SSU	LSU	TEF
L. fungicola var. *fungicola*	CBS 992.69	Netherlands	*Agaricus bisporus*	Epitype	KM283768	KM283792	KM283816
L. fusisporum	CBS 164.70	Netherlands	*Coltricia perennis*	Ex-type	KM283769	AF339549	KM283817
Lecanicillium sp.	FKI-6310	Hatoma-jima, Japan	Soil under *Alpinia zerumbet*	–	LC553285	LC553280	LC553290
	FKI-6826	Haha-jima, Japan	Soil under *Tarenna subsessilis*	–	LC553286	LC553281	LC553291
	FKI-6887	Haha-jima, Japan	Soil under *Carex oahuensis*	–	LC553287	LC553282	LC553292
L. primulinum	FKI-6172	Okinawa, Japan	Soil under unidentified plant	Ex-type		AB712263	
L. psalliotae	CBS 101270	UK	Soil	–	EF469128	AF339558	EF469066
L. restrictum	CBS 143072	Czech Republic	Wooden handle of a tool	Ex-type	LT548279	LT548279	LT626943
L. subprimulinum	KUMCC17-0148	Yunnan, China	Dead wood	–	MG585316	MG585315	MG585317
L. tenuipes	CBS 309.85	Spain	Spider	–	KM283778	KM283802	DQ522341
L. testudineum	CBS 141096	Czech Republic	Carapace of the turtle	Ex-type	LT548278	LT548278	LT626942
L. uredinophilum	KACC 44082	–	*Pucciniastrum agrimoniae* on *Agrimonia pilosa*	–	KM283758	KM283782	KM283806
L. wallacei	CBS 101237	Dumoga Bone forest, Sulawesi, Indonesia	Lepidopteran larva	Holotype	AY184978	AY184967	EF469073
Simplicillium lanosoniveum	CBS 704.86	Venezuela	*Hemileia vastatrix*	–	AF339602	AF339553	DQ522358
S. obclavatum	CBS 311.74	India	Air above sugarcane field	Ex-type	AF339567	AF339517	EF468798
S. lamellicola	CBS 116.25	UK	*Agaricus bisporus*	Ex-type	AF339601	AF339552	DQ522356
Torubiella alba	CBS 726.73a	Ghana	Araneida	Epitype	AF339586	AF339537	EF468781

*New isolates sequenced in this study. ANDES-F, Culture collection of the Museo de Historia Natural of the Universidad de los Andes in Bogotá, Colombia; BCC, BIOTEC Culture Collection Laboratory, Klong Luang, Thailand; CBS, CBS-KNAW Culture Collection, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands.; FKI, Fungal Collection of Ōmura Satoshi Memorial Institute, Kitasato University, Tokyo, Japan; KACC, Korean Agricultural Culture Collection, National Institute of Agricultural Biotechnology, Rural Development Administration, Suwon, Republic of Korea; KUMCC, Culture collection of Kunming Institute of Botany, Kunming, China; NHJ, Nigel Hywel-Jones personal collection; OSC, Oregon State University Herbarium, Corvallis, Oregon, USA

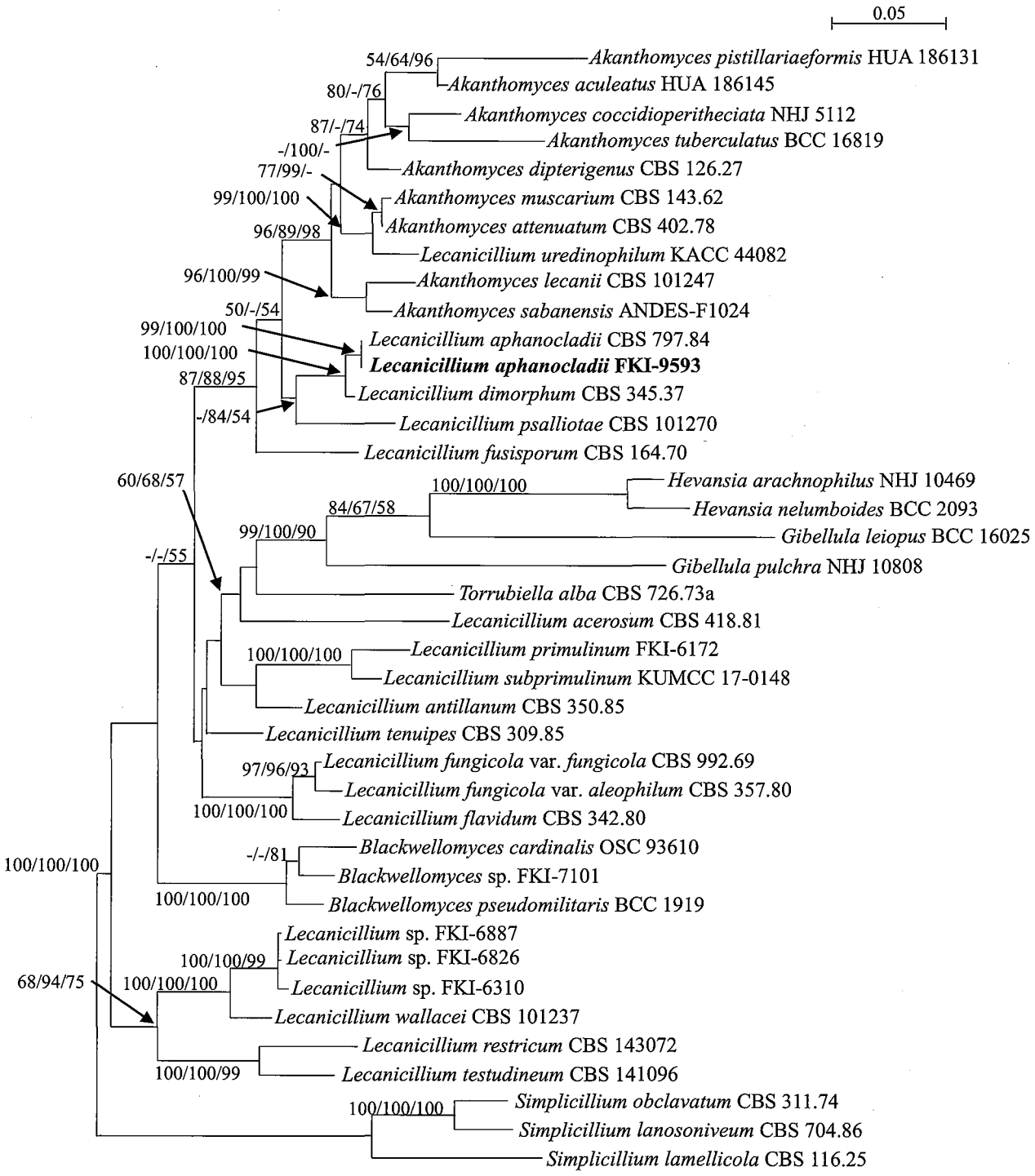


Fig. 2 A phylogenetic tree for *Lecanicillium aphanocladii* and related species of the genus *Lecanicillium*, drawn from maximum likelihood analysis of multigene (SSU, LSU, TEF) sequences. The outgroup is three *Simplicillium* (Cordycipitaceae) species. The numbers shown in the branches represent bootstrap values exceeding 50% (MP, left/NJ, center/ML, right) based on 1,000 replicates. The strain numbers with a superscript letter T mean ex-type strains.

1.0% inoculation, 37°C for 24 h), *Staphylococcus aureus* ATCC 6538p (Nutrient agar, 0.2% inoculation, 37°C for 24 h), *Kocuria rizophila* ATCC 9341 (Nutrient agar, 0.2% inoculation, 37°C for 24 h), *Mycobacterium smegmatis* ATCC 607 (Waksman agar (0.5% peptone, 0.5% meat extract, 0.3% NaCl, 1.0% glucose and 0.8% agar), 1.0% inoculation, 37°C for 24 h), *Escherichia coli* NIHJ (Nutrient agar, 0.5% inoculation, 37°C for 24 h), *Pseudomonas aeruginosa* IFO 3080 (Nutrient agar, 1.9% inoculation, 37°C for 24 h), *Xanthomonas oryzae* KB 88 (Nutrient agar, 1.0% inoculation, 27°C for 24 h), *Proteus vulgaris* NBRC 3167 (Nutrient agar, 0.5% inoculation, 37°C for 24 h), *Klebsiella pneumoniae* ATCC 10031 (Nutrient agar, 0.5% inoculation, 37 °C for 24 h), *Candida albicans* ATCC 64548 (GY agar, 0.2% inoculation, 27°C for 24 h), *Saccharomyces cerevisiae* ATCC 9763 (GY agar, 0.3% inoculation, 27°C for 24 h), *Aspergillus niger* ATCC 6275 (GY agar, 0.3% inoculation, 27°C for 48 h), *Mucor racemosus* IFO 4581 (GY agar, 0.3% inoculation, 27°C for 48 h).

RESULTS

Molecular identification

Only one type of fungus was found to grow from the Tengu-no-Mugimeshi sample on all media, but no growth of bacteria found. A fungal isolate (FKI-9593=IFM 64743) was recovered from the sample, and was classified as a member of the genus *Lecanicillium* based on verticillate phialides. FKI-9593 also produced a red pigment in PDA. FKI-9593 and *Lecanicillium aphanocladii* Zare & W. Gams were grouped together with 99% MP BS, 100% NJ and ML BS support in multi-gene tree (Fig. 2). With respect to sequence distances determined by the MegAlign program from the Lasergene 10 package, the isolate of *Lecanicillium* had 100% similarity with the sequences of SSU, LSU and TEF regions of *L. aphanocladii* CBS 797.84 (KM283811). From these micro-morphological characteristics and the multi-gene phylogenies, FKI-9593 was identified as *L. aphanocladii*.

Species description

Lecanicillium aphanocladii Zare & W. Gams, Nova Hedwigia 73: 27, 2001. Fig. 3.

MycoBank no.: MB 484541.

Colonies on PDA 34–36 mm in diameter after 7 days at 25°C, very raised, covered with white (1A1), reverse violet brown (10E5), produced diffusing reddish-purple pigment. Colonies on MEA 36–37 mm in diameter after 7 days at 25°C, very raised, covered with white (1A1), reverse dull red (8C3). Colonies on PCA 50 mm in diameter after 7 days at 25°C, white, covered with white (1A1), reverse brownish grey (8E2) (Fig. 3a–f). No growth on PDA at 5°C and 37 °C.

Conidiogenous cells produced singly, in pairs, verticillate or in dense irregular clusters on prostrate hyphae, at first flask-shaped, tapering into thread-like neck, 3.1–9.2 × 0.7–2.3 μm. Conidia solitary, oval to subglobose, 2.4–3.9 × 1.5–2.3 μm (Fig. 3g–k).

Identification of antimicrobial compounds

In a 500 m*l* Erlenmeyer flask containing F38 broth, FKI-9593 produced red pigments. Analyses of HPLC chromatograms and UV spectra showed that oosporein (Cole *et al.*, 1974; structure not shown in this paper) and two different compounds were evident, as detected by UV at 210 nm. Oosporein isolated from the FKI-9593 culture broth was identified by measurement of 1D NMR and MS spectra. However, other two compounds were of novel structures by measurement of above instruments. Details on the novel compounds will be reported elsewhere. In this study, we clarified that *L. aphanocladii* in addition produced two novel compounds (not reported) structurally different from oosporein.

Bioactivity of antimicrobial metabolite

Oosporein showed weak inhibition against *Proteus vulgaris* among the 13 microorganisms tested using the conventional paper disc assay (Table 2).

DISCUSSION

Until now, there have been some reports on Eubacteria, Archaea, Cyanobacteria and Ktedonobacteria isolated from Tengu-no-Mugimeshi (Kawamura, 1916; Ono, 1916; Yagi, 1936; Okada, 1937; Wang *et al.*, 2019) but only two reports of fungi being isolated from it (Kawamura, 1916; Ono, 1916). Among them, a verticillium-like fungus was isolated from Tengu-no-Mugimeshi from Mt. Kurohime in 1902 (Ono, 1916).

Verticillium sect. *Prostrata* W. Gams (Clavicipitaceae Kreisel) was divided into at least four distinct clades based on sequences of the ITS region including the 5.8S rRNA gene (Zare *et al.*, 2000; Gams & Zare, 2001). The genus *Lecanicillium* W. Gams & Zare (type: *L. lecanii* (Zimm.) Zare & W. Gams) was segregated from the former *Verticillium* sect. *Prostrata*

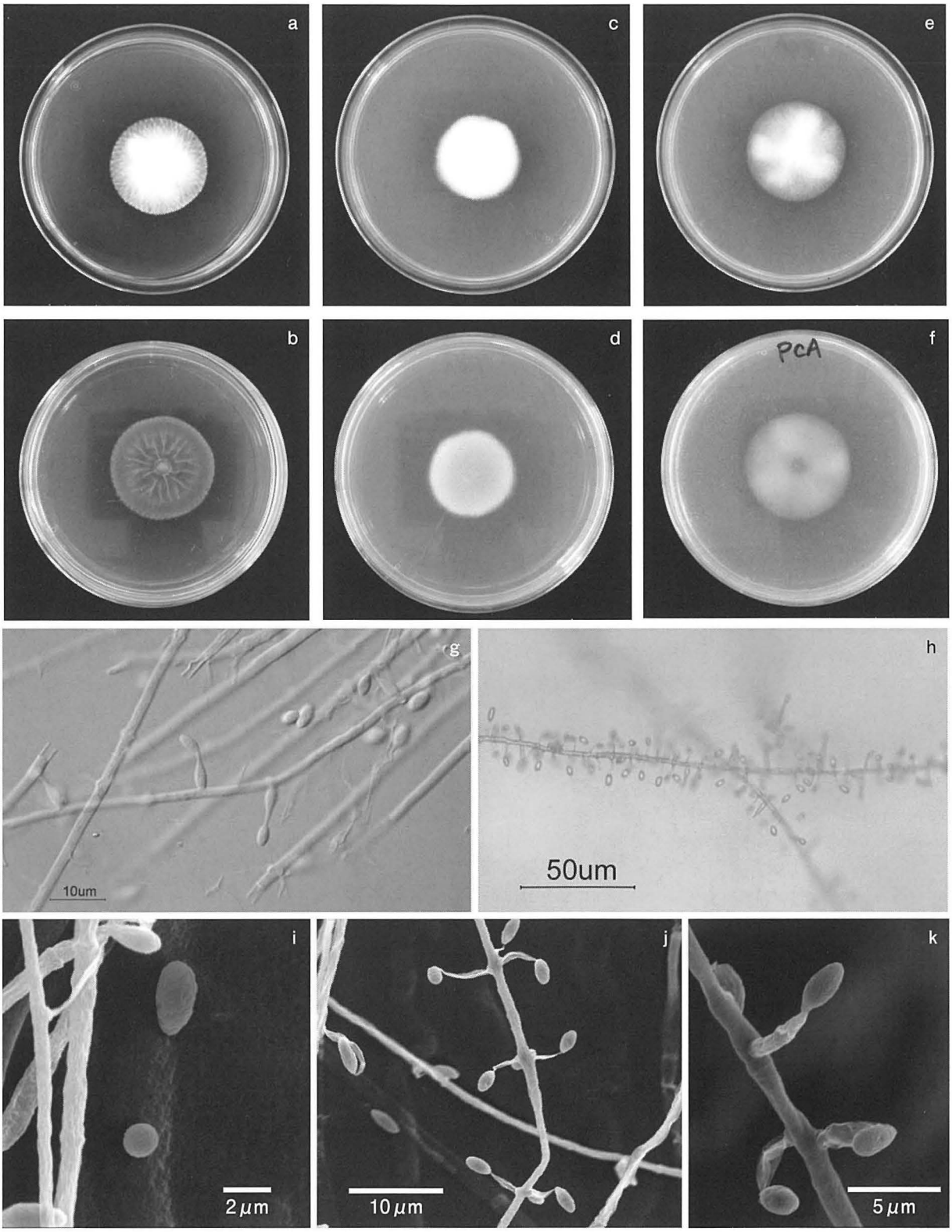


Fig. 3 ***Lecanicillium aphanocladii*** **FKI-9593 (=IFM 64743).** **a**: Colony on PDA at 25℃ for 7 days. **b**: Reverse side on PDA. **c**: Colony on MEA at 25℃ for 7 days. **d**: Reverse side on MEA. **e**: Colony on PCA at 25℃ for 7 days. **f**: Reverse side on PCA. **g**, **h**: Conidiophores. **i**–**k**: Conidia, SEM. *Bars*: **g**, **j** 10 μm; **h** 50 μm; **i** 2 μm; **k** 5 μm

Table 2 Antimicrobial activity of Oosporein

Test organisms	Inhibition zone (mm) at 50 μg/disc (8 mm disc)	Inhibition zone (mm) at 10 μg/disc (6 mm disc)
Bacillus subtilis ATCC 6633	—	—
Staphylococcus aureus ATCC 6538p	—	—
Kocuria rizophila ATCC 9341	—	—
Mycobacterium smegmatis ATCC 607	—	—
Escherichia coli NIHJ	—	—
Pseudomonas aeruginosa IFO 3080	—	—
Xanthomonas oryzae KB 88	—	—
Proteus vulgaris NBRC 3167	15.0	—
Klebsiella pneumoniae ATCC 10031	—	—
Candida albicans ATCC 64548	—	—
Saccharomyces cerevisiae ATCC 9763	—	—
Aspergillus niger ATCC 6275	—	—
Mucor racemosus IFO 4581	—	—

besides *Simplicillium*, *Pochonia* Bat. & O.M. Fonseca, *Haptocillium* W. Gams & Zare, and *Rotiferophthora* G.L. Barron, and was described with thirteen taxa including *L. aphanocladii* (Gams & Zare, 2001; Zare & Gams, 2001). It is therefore presumably suggested that this verticillium-like fungus isolated previously from Tengu-no-Mugimeshi could be the same species as *L. aphanocladii* identified by us. Only one fungus was isolated from the specimen of Tengu-no-Mugimeshi in this study.

Lecanicillium aphanocladii is known as a mainly mycoparasitic fungus: cobweb and spotting in cultivated *Agaricus* (Zare & Gams, 2001), parasitizing uredospores and inducing teliospore formation in rust fungi (Biali *et al.*, 1972; Forrer, 1977; Koç & Défago, 1983; Srivastava *et al.*, 1985; Volker & Boyle, 1994) and parasitizing powdery mildew (*Podosphaera fuliginea* (Schltdl.) U. Braun & S. Takam.; Heijwegen, 1988; Hara *et al.*, 2009). And this species is also known as an entomopathogenic fungus: mosquito larvae (López Lastra *et al.*, 1992, 2002); *Bombyx mori* (Patil *et al.*, 1994).

Moreover, *Lecanicillium* spp. produce many interesting secondary metabolites (e.g., butoxylspiciferin, (Edrada *et al.*, 2000); spiciferone A, (Hwang *et al.*, 2016); verlamelin and this analogue, (Ishidoh *et al.*, 2014)). *Lecanicillium aphanocladii* produce a red pigment in the agar (oosporein, Zare & Gams, 2001). Thus, the genus *Lecanicillium* is highly capable of producing compounds. In this study, two novel compounds (not reported) discovered from *L. aphanocladii* FKI-9593.

At least the lid and body of the specimen bottle were adamantly sealed with the vaseline adhering to it. The mouth of the bottle was heated with an alcohol lamp, the vaseline was dissolved, and the lid was opened for the present study. Since the specimens was not exhibited and had been placed in disuse at the elementary school for a long time, it is probable that it was well sealed before we opened them. It is therefore unlikely that the specimen would have been contaminated by the time we opened it. From these observations, there are several possible reasons why only *L. aphanocladii* was isolated from the specimen of Tengu-no-Mugimeshi in this study. Firstly, at the time of collection in 1939, we can assume many types of microbes coexisted in Tengu-no-Mugimeshi, but most of the microorganisms died over time due to environmental factors; drying, temperature and nutrient depletions, and only *L. aphanocladii* survived. Secondly, it could be that the oosporein produced by *L. aphanocladii* killed the co-existing microbes. For it is known that some microorganisms are able to take up iron by making siderophores when there is a shortage of iron in the environment and are able to grow. Therefore, we believe that microorganisms that are unable to make siderophores themselves may have died during the 80 years of storage. The researchers confirmed experimentally that *L. aphanocladii* isolates produced oosporein, a type of siderophores. We believe that this is one of the factors that kept it alive for 80 years. However, to confirm any hypothesis it will be necessary to obtain fresh Tengu-no-Mugimeshi. Collection of a fresh sample will facilitate an investigation of whether *L. aphano-*

cladii actually remains a component in Tengu-no-Mugimeshi and, if so, what other microorganisms co-exist with it.

Of further note, there is anecdotal evidence that Buddhist monks living in the mountains have eaten Tengu-no-Mugimeshi for centuries (Yagi, 1936), although it is unknown whether it is safe to eat. From our results, if *L. aphanocladii* was present in Tengu-no-Mugimeshi, and oosporein would possibly be present at unknown concentrations. Oosporein is known to have lethal effects in young cockerels as well as having phytotoxic and growth inhibiting properties (Cole *et al.*, 1974), and there is a report that significant damage occurs in the kidney and splenocytes of oosporein-treated mice (Ramesha *et al.*, 2015). To ascertain if Tengu-no-Mugimeshi is safe to eat, fresh samples and oosporein need to be assayed, probably against human kidney cells, splenocytes and hepatocytes. In addition, *L. aphanocladii* can be cultivated not only in the F38 medium used this time but also in three other types of media. However, oosporein was not produced when the fungus was grown on the other media. Consequently, if the nutrient composition of Tengu-no-Mugimeshi is related to the production of oosporein, it may prove harmful to humans. In order to determine whether the nutrient composition of Tengu-no-Mugimeshi is related to the production of oosporein, it is necessary to investigate what components in the F38 medium are involved in the production of oosporein.

DISCLOSURE

The authors declare no conflicts of interest. All the experiments undertaken in this study comply with the laws of Japan.

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REFERENCES

Atlas, R.M. 2010. Handbook of Microbiological Media, fourth edition. CRC Press, Boca Raton.

Biali, M., Dinoor, A., Eshed, N. & Kenneth, R. 1972. *Aphanocladium album*, a fungus inducing teliospore production in rusts. Ann. Appl. Biol. **72**(1): 37–42.

Cole, R.J., Kirksey, J.W., Cutler, H.G. & Davis, E.E. 1974. Toxic effects of oosporein from *Chaetomium trilaterale*. J. Agric. Food Chem. **22**(3): 517–520.

Edgar, R.C. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. **32**(5): 1792–1797.

Edrada, R.A., Wray, V., Berg, A., Gräfe, U., Sudarsono, Brauers, G. & Proksch, P. 2000. Novel spiciferone derivatives from the fungus *Drechslera hawaiiensis* isolated from the marine sponge *Callyspongia aerizusa*. Z. Naturforsch. C J. Biosci. **55**: 218–221.

Forrer, H.R. 1977. Effects of metabolites of the mycoparasite *Aphanocladium album* on teliospore formation of rusts. Phytopath. Z. **88**(4): 306–311.

Gams, W. & Zare, R. 2001. A revision of *Verticillium* sect. *Prostrata*. III. Generic classification. Nova Hedwigia **72**: 329–337.

Gouy, M., Guindon, S. & Gascuel, O. 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. **27**(2): 221–224.

Guindon, S., Dufayard, J.F., Lefort, V., Anisimova, M., Hordijk, W. & Gascuel, O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. **59**(3): 307–321.

Hara, Y., Aiuchi, D., Masuda, T., Sekine, T., Kozawa, T. & Koike, M. 2009. Biological control of cucumber powdery mildew by entomopathogenic *Lecanicillium* spp. Ann. Rept. Plant Prot. North Japan **60**: 92–95.

Heijwegen, T. 1989. Effect of seventeen fungicolous fungi on sporulation of cucumber powdery mildew. Neth. J. Plant Path. **94**: 185–190.

Hwang, I.H., Swenson, D.C., Gloer, J.B. & Wicklow, D.T. 2016. Disseminins and spiciferone analogues: polyketide-derived metabolites from a fungicolous

isolate of *Pestalotiopsis disseminata*. J. Nat. Prod. **79**(3): 523–530.

Ishidoh, K., Kinoshita, H., Igarashi, Y., Ihara, F. & Nihira, T. 2014. Cyclic lipodepsipeptides verlamelin A and B, isolated from entomopathogenic fungus *Lecanicillium* sp. J. Antibiot. **67**: 459–463.

Kaifuchi, S., Nonaka, K., Mori, M., Shiomi, K., Ōmura, S. & Masuma, R. 2013. *Lecanicillium primulinum*, a new hyphomycete (Cordycipitaceae) from soils in Okinawa's main island and the Bonin Islands, Japan. Mycoscience **54**(4): 291–296.

Kawamura, T. 1916. Studies on "Tengunomugimeshi", a massy bacterial vegetation. I (in Japanese). Bot. Mag. Tokyo **30**: 109–119.

Koç, N.K. & Défago, G. 1983. Studies on the host range of the hyperparasite *Aphanocladium album*. Phytopath. Z. **107**(3): 214–218.

Kornerup, A. & Wanscher, J.H. 1978. Methuen Handbook of Colour, third edition. Eyre Methuen, London.

López Lastra, C.C., García, J.J. & Reboredo, G.R. 1992. Efecto comparativo de la virulencia de los hongos *Aphanocladium album* (preuss) Gams y *Tolypocladium cylindrosporum* Gams (Deuteromycotina- Hyphomycetes), contra larvas de mosquito (Diptera, Culicidae). Bol. Micol. **7**(1–2): 13–16.

López Lastra, C.C., García, J.J. & Micieli, M.V. 2002. Microecology of entomopathogenic fungi from aquatic environments. Embrapa Soja **184**: 83–85.

Molisch, H. 1926. Pflanzenbiologie in Japan: auf Grund eigener Beobachtungen, Gustav Fischer, Jena.

Motojima, K., Ishiwada, Y., Yasukuni, N., Endo, S. & Kageyama, K. 1955. Natural gas in the vicinity of the Komoro City (in Japanese with English summary). J. Japanese Assoc. Petrol. Technol. **20**: 65–71.

Nonaka, K., Kaifuchi, S., Ōmura, S. & Masuma, R. 2013. Five new *Simplicillium* species (Cordycipitaceae) from soils in Tokyo, Japan. Mycoscience **54**(1): 42–53.

Okada, Y. 1937. Occurrence of masses of gelatinous microbes in the soil. Soil Sci. **43**(5): 367–374.

Ono, N. 1916. Research on Tengu-no-Mugimeshi from Mt. Kurohime (in Japanese). Bot. Mag. Tokyo **30**(351): 59–65.

Patil, C.S., Krishna, M. & Shardamma, P. 1994. New record of fungal pathogen, *Aphanocladium album* (Preuss) Gams isolated from mulberry silkworm, *Bombyx mori* L. from India. Entomon. **19**: 175–176.

Pitt, J.I. 1979. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Mycologia **73**(3): 582–584.

Ramesha, A., Venkataramana, M., Nirmaladevi, D., Gupta, V.K., Chandranayaka, S. & Srinivas, C. 2015. Cytotoxic effects of oosporein isolated from endophytic fungus *Cochliobolus kusanoi*. Front. Microbiol. **6**: 870.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.

Srivastava, A.K., Defago, G. & Kern, H. 1985. Hyperparasitism of *Puccinia horiana* and other microcyclic rusts. Phytopath. Z. **114**(1): 73–78.

Su, L., Zhu, H., Guo, Y., Du, X., Guo, J., Zhang, L. & Qin, C. 2019. *Lecanicillium coprophilum* (Cordycipitaceae, Hypocreales), a new species of fungus from the feces of *Marmota monax* in China. Phytotaxa **387**(1): 55–62.

Volker, K. & Boyle, C. 1994. Bean rust as a model system to evaluate efficiency of teliospore induction, especially in the potential mycoherbicide *Puccinia punctiformis*. Weed Research Oxford **34**(4): 275–281.

Wang, C.M., Zheng, Y., Sakai, Y., Toyoda, A., Minakuchi, Y., Abe, K., Yokota, A. & Yabe, S. 2019. *Tengunoibacter tsumagoiensis* gen. nov., sp. nov., *Dictyobacter kobayashii* sp. nov., *Dictyobacter alpinus* sp. nov., and description of Dictyobacteraceae fam. nov. within the order Ktedonobacterales isolated from Tengu-no-Mugimeshi, a soil-like granular mass. Int. J. Syst. Evol. Microbiol. **69**(7): 1910–1918.

Yagi, T. 1936. Tengu-no-Mugimeshi in Mount Kurohime and Yunotaira of Mount Asama (in Japanese). Reports on Places of Scenic Beauty, Historic and Natural Monuments, Nagano Prefecture **17**: 217–227.

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

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

Zhou, Y.M., Zhi, J.R., Ye, M., Zhang, Z.Y., Yue, W.B. & Zou, X. 2018. *Lecanicillium cauligalbarum* sp. nov. (Cordycipitaceae, Hypocreales), a novel fungus isolated from a stemborer in the Yao Ren National Forest Mountain Park, Guizhou. MycoKeys **43**: 59–74.

長野県黒姫山の「天狗の麦飯」から分離された糸状菌 *Lecanicillium aphanocladii*

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天狗の麦飯は，中部地方の火山帯，標高の高い地域に点在し，（藍藻類を含む）真正細菌，古細菌，糸状菌等，複数の微生物群からなる微生物の塊である．今回分離源とした天狗の麦飯は，昭和14年に黒姫山で採集され，ガラス瓶に封入されて80年間長野市内の小学校に保管されていた標本である．本研究では，この天狗の麦飯の標本から分離した糸状菌について報告する．この天狗の麦飯を希釈平板法により3種類の培地に塗布し，27℃で1週間培養した．その結果，各培地で形態的特徴の同じ1種の糸状菌のみが検出され，細菌など他の微生物は一切検出されなかった．この1種の糸状菌について，コロニー性状確認，各種顕微鏡による形態観察，3遺伝子領域に基づく系統解析を行ったところ，*Lecanicillium aphanocladii* であると同定した．また，本菌株の微生物培養液を用いて13種の微生物に対する抗菌試験を行ったところ，*Proteus vulgaris* NBRC 3167に対し弱い抗菌活性が確認された．NMRなどの各種機器分析により，本活性化合物をoosporeinであると同定した．