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Five new *Simplicillium* species (Cordycipitaceae) from soils in Tokyo, Japan

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ABSTRACT

Eighteen *Simplicillium* isolates were discovered from soil samples collected on the Honshu, Bonin, and Izu islands in areas under the jurisdiction of Tokyo, Japan. Using a combination of micro-morphological characteristics and sequences of the ribosomal RNA gene ITS region, the isolates were classified as six *Simplicillium* species, and five of them were previously undescribed. The five species (*Simplicillium aogashimaense, Simplicillium cylindrosporum, Simplicillium obclavatum, Simplicillium subtropicum* and *Simplicillium sympodiophorum*) were discovered from the Chichi-jima, Hachijo, and Aogashima islands, and *Simplicillium minatense* was discovered from Honshu. The five new species and three known species are distinguished by conidial morphology.

1. Introduction

Verticillium sect. *Prostrata* W. Gams (Clavicipitaceae Kreisel) was divided into at least four distinct clades based on sequences of the ribosomal RNA gene (rDNA) internal transcribed spacer (ITS) region including the 5.8S rDNA (Zare et al., 2000; Gams and Zare, 2001). The genus *Simplicillium* W. Gams & Zare was segregated from the former *Verticillium* sect. *Prostrata* besides *Lecanicillium* W. Gams & Zare, *Pochonia* Bat. & O. M. Fonseca, *Haptocillium* W. Gams & Zare, and *Rotiferophthora* G. L. Barron (Gams and Zare 2001; Zare and Gams 2001). *Simplicillium* resembles *Lecanicillium* in total morphology, but it mainly produces solitary phialides. Species of the genus are fungicolous or entomogenous, and some of them have *Torrubiella* Boud. teleomorph (Zare and Gams, 2001). While, anamorphic state of *Cordyceps pseudomilitaris* Hywel-Jones & Sivichai also mainly produces simple, unbranched phialides (Hywel-Jones, 1994), a morphology that more appropriately corresponds with *Simplicillium* (Sung and Spatafora, 2004).

Recently, the families Cordycipitaceae Kreisel ex G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora and Ophiocordycipitaceae G.H. Sung, J.M. Sung, Hywel-Jones & Spatafora were segregated from the Clavicipitaceae sensu lato (s. l.), and the genera *Simplicillium* and *Lecanicillium* belong to the Cordycipitaceae sensu stricto (s. str.) (Sung et al., 2007). Moreover, species of *Torrubiella* s. l. are revealed to distribute over the Clavicipitaceae, the Ophiocordycipitaceae and the Cordycipitaceae according to a multi-gene phylogenetic analysis (Johnson et al., 2009). Consequently, two new genera (*Conoideocrella* D.

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Johnson, G.H. Sung, Hywel-Jones & Spatafora and *Orbiocrella* D. Johnson, G.H. Sung, Hywel-Jones & Spatafora) were proposed segregating from the former *Torrubiella* in the Clavicipitaceae s. str.; *Torrubiella tenuis* Petch and *Torrubiella luteorostrata* Zimm. were transferred to the genus *Conoideocrella* and *Torrubiella petchii* Hywel-Jones was transferred to the genus *Orbiocrella* (Johnson et al., 2009). Two species (*Torrubiella pruinosa* (Petch) Minter & B.L. Brady and *Torrubiella hirsutellae* (Petch) Rossman) in the Ophiocordycipitaceae were transferred to the genus *Ophiocordyceps* (Johnson et al., 2009). The type species of *Torrubiella, Torrubiella aranicida* Boud. (anamorph: *Isaria cuneispora* Boud.), belongs to the Cordycipitaceae s. str. according to its description and host affiliation (Johnson et al., 2009). *Torrubiella confragosa* Mains (anamorph: *Lecanicillium lecanii* (Zimm.) Zare & W. Gams) and *Torrubiella piperis* J.F. Bisch. & J.F. White (anamorph: *Lecanicillium* sp. JB209) were transferred to *Cordyceps* (Sung et al., 2007; Johnson et al. 2009). The genus *Simplicillium* presently consists of three species, *Simplicillium lanosoniveum* (F.H. Beyma) Zare & W. Gams, *Simplicillium obclavatum* (W. Gams) Zare & W. Gams and *Simplicillium lamellicola* (F.E.V. Sm.) Zare & W. Gams. *Simplicillium wallacei* H.C. Evans (teleomorph: *Torrubiella wallacei* H.C. Evans) was transferred to *Lecanicillium* (Zare and Gams 2008). Although the taxonomy of *Simplicillium* was studied by using materials collected from around the world (Zare and Gams 2001), the number of the examined isolates from Japan and other East Asian countries analyzed in their study was still meager.

In the course of our research to find novel fungal metabolites, we have discovered numerous new fungal metabolites from soil isolates from subtropical regions (e.g., Yamaguchi et al. 2004; Ui et al. 2006; Iwatsuki et al. 2010; Shiomi et al. 2010) and oceanic islands (Koyama et al. 2010; Nonaka et al. 2011; Ugaki et al. 2012; Ishii et al. 2012) around Japan.

In this study, we found 70 *Verticillium*-like isolates from soil collected from various locations in Japan, and 18 of them were *Simplicillium*. We examined these *Simplicillium* isolates using micro-morphological characteristics and sequences of the ITS region. We recovered six species, five of which were so far undescribed and which we describe here.

2. Materials and methods

2.1. Isolation

Soil samples from around plant roots were collected from nine locations of Japan from 2006 to 2009. That is, Yamaguchi City (Yamaguchi Pref.), Shinano Town (Nagano Pref), Hakone Town (Kanagawa Pref.), Minato-ku, Izu Islands: Hachijo and Aogashima Islands, Bonin Islands: Chichi-jima (Tokyo Metropolitan) and Sapporo City and Noboribetsu City (Hokkaido). Hachijo and Aogashima islands are located 290 and 360 km south of Honshu, respectively; both have high precipitation and a subtropical climate. Chichi-jima is located 1000 km south of Honshu and its latitude is the same as Okinawa.

Soil samples (1 g) were suspended in 9 ml 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% surfactant Tween-80 (Sigma–Aldrich Co., Saint Louis, MO, USA) and then sonicated for 2 min, and diluted to 10^2–10^3 times with the above Winogradsky solution. Of the diluted soil suspension 200 μl were spread on Petri dishes with solidified onion garlic agar (OGA), Czapek yeast extract agar (CYA, Pitt 1979), cornmeal dextrose yeast extract agar (CMDY, 1.7% cornmeal agar (Difco Laboratories, Detroit, MI, USA), 0.05% dextrose, 0.1% yeast extract) and potato dextrose agar (PDA, Difco), and all media with 50 mg/l rose Bengal and 100 mg/l kanamycin; this is a combination of usual isolation media and new medium (OGA) developed for discovering undescribed or rare fungi. Grated garlic (20 g) and onion (20 g) was boiled in 1 l of distilled water for 1 h. The boiled biomass was then filtered off and 2% agar was added. Incubation was at 25 °C for 5–7 days.

Type material is preserved in the National Museum of Nature and Science (TNS), Tsukuba, Japan, and ex-type cultures in Japan Collection of Microorganisms (JCM), Wako, Japan.

2.2. Morphological analysis

For morphological observation, conidial and mycelial suspension of the isolates were inoculated at the center of the plates, 3 Petri dishes of PDA (Difco), 2% malt extract agar (MEA, Difco) and potato carrot agar (PCA, Atlas 2010), and kept at 25 °C for 7 days (also at 5 °C and 33 °C on PDA) in the dark. Methuen Handbook of Colour (Kornerup and Wanscher 1978) was used to determine color names and hue numbers.

For the observation of micro-morphological characteristics, microscope slides were prepared from PCA cultures. The slides were examined with a Vanox-S AH-2 microscope (Olympus, Tokyo, Japan), and digital photomicrographs were taken with a DP25 digital camera (Olympus). For scanning electron microscopy (SEM), agar blocks (5 mm^2) were cut from 7-day-old cultures growing on PCA. The agar blocks were fixed with osmium tetroxide (TAAB, Berks, UK), air-dried and sputter-coated with gold using a JFC-1200 Fine Coater (JEOL, Tokyo, Japan). The samples were observed with a JSM-5600 scanning electron microscope (JEOL).

2.3. DNA extraction, PCR amplification, sequencing and phylogenetic analysis

Genomic DNA of the strains was isolated using the PrepMan Ultra® Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Amplification of the rDNA ITS region was performed using primers ITS1 and ITS4 (White et al. 1990). PCR was performed with the QIAGEN® Fast Cycling PCR Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's protocol.

Amplifications were performed in a PCR Verity® 96-well thermal cycler (Applied Biosystems), programmed with denaturation at 95 °C for 5 min, followed by 35 cycles consisting of denaturation at 96 °C for 5 s, primer annealing at 50 °C for 5 s, extension at 68 °C for 18 s, and a final elongation step at 72 °C for 1 min. After amplification of the ITS templates, excess primers and dNTP's were removed from the reaction mixture using a QIAquick, PCR DNA Purification kit (Qiagen), according to the manufacturer's protocol. The PCR

products were sequenced directly in both directions using primers ITS1, ITS2, ITS3 and ITS4 (White et al. 1990) using a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The cycle sequencing reaction mixture had a total reaction volume of 10 μl, and contained 2.5 μl of template DNA (10–15 ng/μl), 2 μl BigDye® terminator premix, 4 μl ultra-pure sterile water and 0.5 μl of each primer (5 pmol/μl). Reactions were run in a PCR thermal cycler, programmed with denaturation at 96 °C for 1 min, followed by 25 cycles of denaturation at 96 °C for 10 s, followed by primer annealing at 50 °C for 5 s and extension at 60 °C for 4 min. Sequencing products were purified by ethanol/ethylenediaminetetraacetic acid (EDTA) precipitation, and samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using the forward and reverse sequences with the SeqMan and SeqBuilder programs from the Lasergene 8 package (DNAStar Inc., Madison, WI, USA). The ITS sequences of the strains were deposited at the DNA Data Bank of Japan (DDBJ) (Table 1).

To determine the most closely related *Simplicillium* species, the DNA sequences of the ITS region of our isolates were compared to sequences in the GenBank database by BLASTN 2.2.21 (Altschul et al. 1997) analysis (see Table 1 for accession numbers) and aligned using MUSCLE 3.6 (Edgar 2004). The alignment was refined using SEAVIEW 4.2 (Gouy et al. 2010), and then deposited in TreeBASE (http://www.treebase.org/) with accession number S12301. Phylogenetic analyses were based on the neighbor-joining (NJ) method (Saitou and Nei 1987) using Clustal X 2.0.11 (Larkin et al. 2007) and the maximum-parsimony (MP) method using PAUP* 4.0b10 software (Swofford 2002). Bootstrap analyses were performed on NJ and MP trees with 1000 bootstrap replicates. The trees were rooted with *Pochonia chlamydosporia* (Goddard) Zare & W. Gams var. *chlamydosporia* and *Conoideocrella luteorostrata* (Zimm.) D. Johnson, G.H. Sung, Hywel-Jones & Spatafora and viewed with NJplot (Perrière and Gouy 1996).

3. Results

Eighteen of 70 *Verticillium*-like isolates were classified as members of *Simplicillium* using a combination of micromorphological characteristics and sequences of the ITS region. All *Simplicillium* isolates were found from areas under the jurisdiction of the Tokyo Metropolitan Government (Table 1).

Morphologically, all isolates produced mainly solitary phialides and formed white floccose colonies on PDA. One isolate formed imbricate conidial chains like *S. obclavatum* and 16 isolates formed globose conidial heads like *S. lanosoniveum*. One isolate had sympodial or polyblastic conidiogenesis like members of *Engyodontium*, but based on our preliminary phylogenetic analyses, it obviously forms part of the *Simplicillium* clade. Among other species with polyblastic conidia, *Engyodontium aranearum* (Cavara) W. Gams, de Hoog, Samson & H.C. Evans, which has only partially polyblastic conidiogenesis, forms part of the *Lecanicillium* clade, and this species was transferred to *Lecanicillium* as *Lecanicillium tenuipes* (Petch) Zare & W. Gams (Gams and Zare 2001; Zare and Gams 2001). Similarly, *Engyodontium arachnophilum* H.C. Evans & Samson was also transferred to *Lecanicillium* (Zare and Gams 2001). However, in the current definition *Simplicillium* would not comprise *Engyodontium*-like species. We therefore propose here a minor emendation of *Simplicillium*.

Because NJ and MP trees were almost concordant with each other, we describe the phylogenetic results based on the NJ tree (Fig. 1). The *Simplicillium* clade was clearly separated from the *Lecanicillium* clade, and 18 *Simplicillium* isolates formed six subclades. Taxa morphologically identified as the same species grouped together with high bootstrap (BS) support. FKI-5862 and *S. obclavatum* were grouped together with 99% and 100% BS in MP and NJ. FKI-5862 producing imbricate conidial chains (Fig. 2) showed all characteristics of *S. obclavatum* and in a BLAST search, this strain had a 99.5% similarity (3 of 553 nucleotides difference) with the rDNA ITS sequences of *S. obclavatum* (AJ292394), proving their identity. Seven isolates including *Simplicillium cylindrosporum* (ex-type FKI-4955) grouped together supported with 98% BS in NJ. Three isolates including *Simplicillium minatense* (ex-type FKI-4981) grouped together with 90% and 98% BS support in MP and NJ: all isolates of this group came from Honshu. *S. cylindrosporum* formed a sister clade to *S. minatense* with 56% and 79% BS support in MP and NJ, and both species could be distinguished by conidial morphology (Table 2). In a BLAST search, both ex-type strains had about 98% similarity (11–13 of about 550 nucleotides difference) with *S. lanosoniveum* (AJ292396). *Simplicillium subtropicum* and *Simplicillium aogashimaense* clustered as distinct groups. Four isolates including *S. subtropicum* (ex-type FKI-4956) grouped together with 79% and 100% BS support in MP and NJ. Two isolates including *S. aogashimaense* (ex-type FKI-5881) were combined with 79% and 100% BS support in MP and NJ. In a BLAST search, FKI-4956 (ex-type of *S. subtropicum*) had 98.0% similarity (11 of 550 nucleotides difference) with the ITS sequences of *S. lanosoniveum* (AJ292396), and FKI-5881 (ex-type of *S. aogashimaense*) had 95.5% similarity (25 of 553 nucleotides difference) with that of *S. obclavatum* (AJ292394). *Simplicillium sympodiophorum* (ex-type FKI-5883) formed a sister clade to *S. lamellicola* (AJ292393) with 83% and 99% BS support in MP and NJ, but showed a considerable distance from it (7%: 38 of 556 nucleotides difference). While, torrubielloid fungi in the Cordycipitaceae s. str. and *Cor. pseudomilitaris* having a *Simplicillium*-like anamorph clustered with *Lecanicillium* (Fig. 1). As a result, the 18 *Simplicillium* isolates are recognized as representing six species.

The isolates of five of the six clades showed considerable differences to the three known species of *Simplicillium* (*S. lanosoniveum, S. obclavatum* and *S. lamellicola*) in morphology and phylogeny (Table 2, Fig. 1). We therefore propose five new species of *Simplicillium* to accommodate these isolates.

4. Discussion

Kouvelis et al. (2008) reported that some species of *Lecanicillium* related closely resembling *Simplicillium* had been misclassified or not separated by using only ITS sequences. However, species of *Simplicillium* can clearly be distinguished using a combination of morphological characteristics and ITS sequences. The genus is characterized by predominantly

Table 1 – Isolates used for phylogenetic analyses.

Species	Strain No.	Locality	Isolation source	Other strain no./taxonomical information	DDBJ Acc. No.	Isolation medium
S. aogashimaense	JCM 18167	Aogashima, Izu Islands, Japan	Soil under *A. antiquum*	FKI-5881, ex-type	AB604002	CYA
	JCM 18168	Aogashima, Izu Islands, Japan	Soil under *A. antiquum*	FKI-5887	AB604004	CYA
S. cylindrosporum	JCM 18169	Chichi-jima, Bonin Islands, Japan	Soil under *C. pseudopedunculatum*	FKI-4955, ex-type	AB603989	PDA
	JCM 18170	Chichi-jima, Bonin Islands, Japan	Soil under *Tar. subsessilis*	FKI-5408	AB603994	CMDY
	JCM 18171	Aogashima, Izu Islands, Japan	Soil under *Morus* sp.	FKI-5722	AB603997	CYA
	JCM 18172	Aogashima, Izu Islands, Japan	Soil under *Phoenix* sp.	FKI-5781	AB603998	CYA
	JCM 18173	Aogashima, Izu Islands, Japan	Soil under *Phoenix* sp.	FKI-5784	AB603999	CYA
	JCM 18174	Aogashima, Izu Islands, Japan	Soil in garden	FKI-5984	AB604005	OGA
	JCM 18175	Aogashima, Izu Islands, Japan	Soil in garden	FKI-5985	AB604006	OGA
S. lamellicola	CBS 116.25	United Kingdom	*Agaricus bisporus*	Ex-type	AJ292393	–
	KYK 00006	–	–	–	AB378533	–
	UAMH 2055	–	–	–	AF108471	–
	UAMH 4785	–	–	–	AF108480	–
S. lanosoniveum	CBS 704.86	Venezuela	*Hemileia vastatrix*	–	AJ292396	–
	CBS 962.72	Netherlands	*Cibotium schiedei*	–	AJ641862	–
S. minatense	JCM 18177	Minato-ku, Japan	Soil under *Prunus* × *yedoensis*	FKI-4980	AB603991	PDA
	JCM 18176	Minato-ku, Japan	Soil under *Prunus* × *yedoensis*	FKI-4981, ex-type	AB603992	PDA
	JCM 18178	Minato-ku, Japan	Soil under *Prunus* × *yedoensis*	FKI-4982	AB603993	PDA
S. obclavatum	CBS 311.74	India, Gorakhpur	Air above sugarcane field	Ex-type	AJ292394	–
	JCM 18179	Aogashima, Izu Islands, Japan	Soil under *A. americana*	FKI-5862	AB604000	CYA
S. subtropicum	JCM 18180	Chichi-jima, Bonin Islands, Japan	Soil under *C. pseudopedunculatum*	FKI-4956, ex-type	AB603990	PDA
	JCM 18181	Hachijo Island, Izu Islands, Japan	Soil under *H. rosa-sinensis*	FKI-5574	AB603995	CYA
	JCM 18182	Hachijo Island, Izu Islands, Japan	Soil under *H. rosa-sinensis*	FKI-5575	AB603996	CYA
	JCM 18183	Aogashima, Izu Islands, Japan	Soil under *A. americana*	FKI-5864	AB604001	OGA
S. sympodiophorum	JCM 18184	Aogashima, Izu Islands, Japan	Soil under *A. antiquum*	FKI-5883, ex-type	AB604003	OGA
Lec. fungicola var. *fungicola*	CBS 992.69	Netherlands	*A. bisporus*	Epitype	EF641889	–
Lec. lecanii	CBS 101247	West Indies	*Coccus viridis*	IMI 304807	AJ292382	–
Lec. muscarium	ATCC 28300	United Kingdom	*Trialeurodes vaporariorum*	Epitype	AJ292388	–
Cor. pseudomilitaris	NHJ6	–	–	–	AJ786589	–
Cor. confragosa	NBRC 32311	–	*Aphis gossypii*	–	AB111495	–
Tor. flava	NBRC 30612	–	Decayed leaf enveloping spider	–	AB100609	–
Tor. wallacei	CBS 101237	Indonesia	*Lepidoptera larvae* on palm leaf	Ex-type	EF641891	–
Con. luteorostrata	CBS 398.86	Soroa, Pinar del Rio, Cuba	Living leaf	–	AY624174	–
P. chlamydosporia var. *chlamydosporia*	CBS 103.65	Germany	Soil under *Brassica napus*	Ex-neotype	AJ292397	–

Isolate obtained and sequenced in this study S, *Simplicillium*; Lec, *Lecanicillium*; Tor, *Torrubiella*; Cor, *Cordyceps*; Con, *Conoideocrella*; P, *Pochonia*.
– Data not available.

solitary phialides, conidial masses either in globose slimy heads, short chains, or formed in sympodial succession, a phenomenon also seen in *Lecanicillium*. In our phylogenetic study, isolates were clearly separated by ITS sequences (Fig. 1). Therefore, we classify them following Zare and Gams (2001).

Torrubielloid fungi having *Simplicillium* or *Lecanicillium* anamorphs (excluding *Lecanicillium wallacei* and *S. lanosoniveum*) in the Cordycipitaceae s. str. were transferred to *Cordyceps* (Sung et al. 2007; Johnson et al. 2009). *Orbiocrella petchii* (Hywel-Jones) D. Johnson, G.H. Sung, Hywel-Jones &

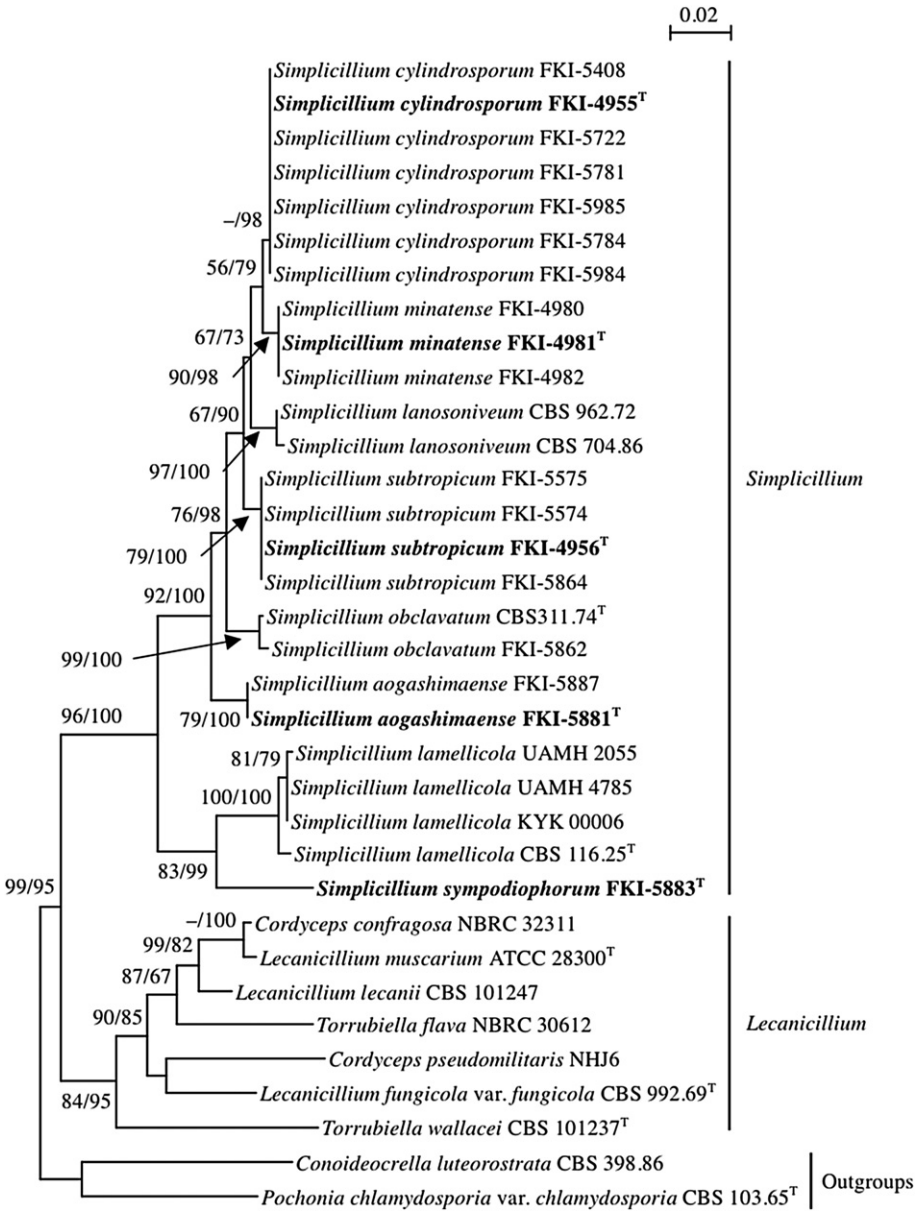

Fig. 1 – Phylogenetic tree for the isolates and related species of *Simplicillium* drawn from the neighbor-joining analysis of the rDNA ITS region sequences. The outgroups are *P. chlamydosporia* var. *chlamydosporia* and *Con. luteorostrata* (Clavicipitaceae). The numbers shown on the branches represent bootstrap values exceeding 50% (MP, left/NJ, right) based on 1000 replicates. The number of nucleotide changes between taxa is represented by the branch length. The strain numbers with a letter T mean ex-type strains.

Spatafora (basionym: *T. petchii* Hywel-Jones) has a *Simplicillium*-like anamorph (Johnson et al. 2009). However, its reniform conidia (Hywel-Jones 1997) differ from those of *Simplicillium* (Table 1). *T. aranicida* Boud. has *I. cuneispora* Boud. as anamorph, but its original drawings (Boudier 1887) depict a morphology resembling species of *Simplicillium* (Zare and Gams 2001). However, conidial shape of its anamorph (fusiform-falcate, Boudier 1887; Zare and Gams 2001) differs from those known so far in *Simplicillium* (Table 1). *Cor. pseudomilitaris* Hywel-Jones & Sivichai, having a *Simplicillium*-like anamorph, clustered with *Lecanicillium* (Fig. 1). As a result, a *Simplicillium*- or *Simplicillium*-like anamorph linked to *Torrubiella* is only seen in *S. lanosoniveum* (Zare and Gams 2001).

Among 70 *Verticillium*-like soil isolates from nine locations throughout Japan, 18 belonging to *Simplicillium* were mainly isolated from three oceanic islands under the jurisdiction of the Tokyo Metropolitan Government. Six *Simplicillium* species could be distinguished; *S. obclavatum* and five undescribed species (Table 1). Five of the six species were obtained from Aogashima (*S. aogashimaense, S. cylindrosporum, S. obclavatum, S. subtropicum* and *S. sympodiophorum*), two from Chichi-jima (*S. cylindrosporum* and *S. subtropicum*), one from Hachijo

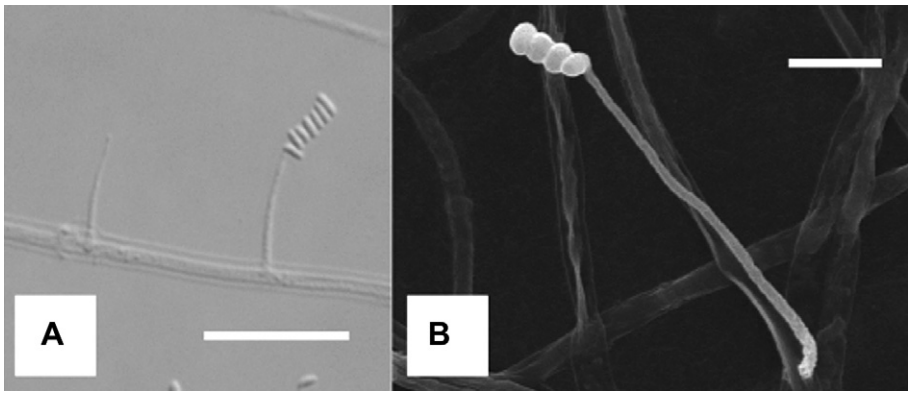

Fig. 2 – *S. obclavatum* FKI-5862. A: Conidiophores; B: Conidiophore, SEM. *Bars* 20 μm.

Island (*S. subtropicum*), and one species from Minato-ku on the Japanese mainland (*S. minatense*, on Honshu Island). Only *S. subtropicum* was isolated on all three oceanic islands. Aogashima obviously supports a high *Simplicillium* diversity.

Oceanic islands that have never been connected to any continental land mass offer unparalleled opportunities for studying speciation and adaptive radiation (Darwin 1859; Carlquist 1974). Such oceanic islands are known for their species richness in some taxa and the uniqueness of their high rates of endemism; examples are the Galapagos and the Bonin Islands. In Japanese oceanic islands, soil fungal floras have been investigated previously by Watanabe (1989) and Watanabe et al. (2001) in the Bonin Islands and Hachijo Island. The soil-inhabiting fungi indicate a high diversity in those islands, and indicating that these two islands have rather different fungal floras (Watanabe et al. 2001). The soil fungal flora from Aogashima had not been investigated so far. There we discovered many species including undescribed species of *Simplicillium*. The results of our studies demonstrate the value of screening remote locations for new fungal species. The taxonomy of *Simplicillium* can obviously be enhanced in the East Asian area.

4.1. *Taxonomy*

Simplicillium W. Gams & Zare, Nova Hedwigia 73:38, 2001.

Similar to *Lecanicillium*, but with mostly solitary phialides arising from aerial hyphae, usually prostrate and little differentiated from the subtending hyphae. Phialides discrete, aculeate and narrow, with a very narrow tip in which collarette and periclinal wall thickening are not visible. Conidia adhering in globose slimy heads, imbricate chains or formed in sympodial succession, short-ellipsoidal to globose, cylindrical or obclavate or fusiform, not cyanophilic. Colonies rather fast-growing, reaching (10–)24–34 mm diam. in 10 days on PDA or MEA. Crystals commonly present in the agar. Fungicolous and on various other substrata.

Type: *S. lanosoniveum* (F.H. Beyma) Zare & W. Gams.

Teleomorph: *Torrubiella*.

S. subtropicum Nonaka, Kaifuchi & Masuma, sp. nov. Fig. 3.

Table 2 – Morphological comparison of five new *Simplicillium* species with three species derived from: Zare and Gams (2001).

Species	*S. lanosoniveum*[a]	*S. lamellicola*[a]	*S. obclavatum*[a]	*S. subtropicum*
Phialides (μm)	15–35 × 0.7–1.5	15–50 × 0.7–1.0	30–52 × 0.8–1.2	(15–)20–42(–50) × 1.0–2.3
Conidia (μm)	1.5–3.0 × 0.7–1.3 Oval or ellipsoidal to subcylindrical	Macro: 4.5–9.0 × 0.8–1.2 Spindle-shaped Micro: 2.0–3.0 × 0.7–1.2 Oval to ellipsoidal	2.5–3.5 × 1–2 Obclavate to ellipsoidal	2.3–4.0(–4.5) × 1.5–3.3 Subglobose to ellipsoidal
Conidial mass	Globose heads	Subglobose to ellipsoidal heads	Short imbricate chains	Globose heads
Octahedral crystals	Present	Present	Present	Present
Species	***S. minatense***	***S. cylindrosporum***	***S. aogashimaense***	***S. sympodiophorum***
Phialides (μm)	11–31(–47) × 1.0–1.7	17–32 × 1.2–2.0(–2.4)	(19–)23–53 × 1.2–2.0	20–34(–47) × 0.5–1.3 (conidiogenous cells)
Conidia (μm)	2.0–3.5 × 1.8–2.5(–2.8) Globose to subglobose (sometimes ellipsoidal)	3.0–4.5(–5.0) × 1.0–2.0 Cylindrical	4.2–6.5 × 1.2–2.0(–2.3) Cylindrical	2.2–3.5 × 1.0–2.0 Oval to ellipsoidal
Conidial mass	Globose heads	Globose heads	Globose heads	Sympodial conidia
Octahedral crystals	Present	Present	Present	Present

a Data are derived from: Zare and Gams (2001).

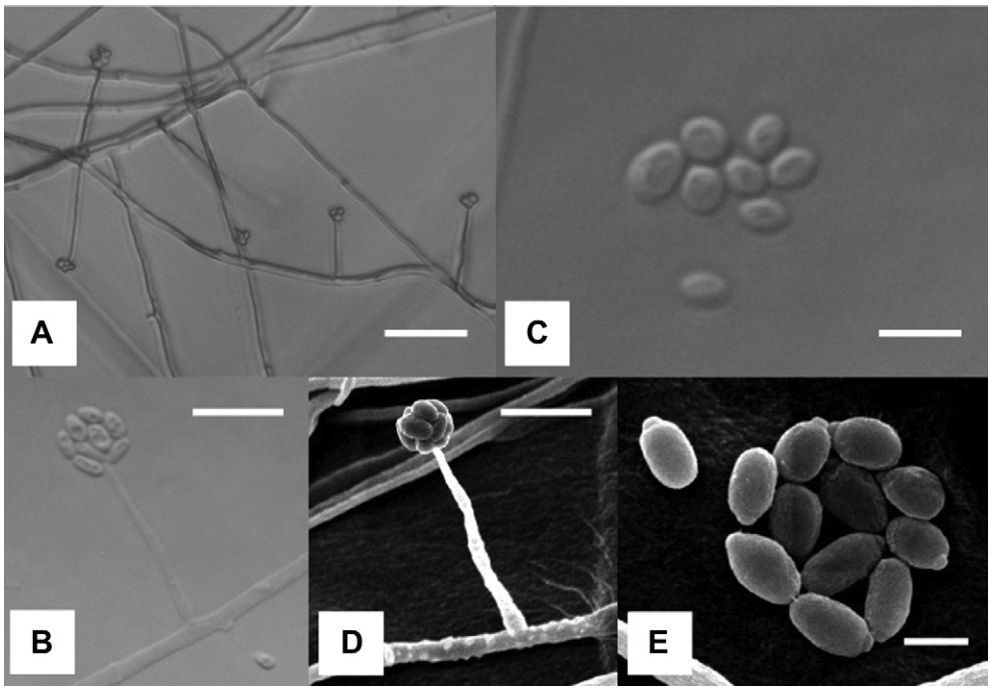

Fig. 3 – *S. subtropicum* FKI-4956. A–B: Conidiophores; C: Conidia; D: Conidiophore, SEM; E: Conidia, SEM. *Bars* A, 20 μm; B, D, 10 μm; C, 5 μm; E, 2 μm.

MycoBank no.: MB 519349.

Coloniae post 7 dies ad 25 °C in agaro PDA 22–23 mm diam., in agaro MEA 22–23 mm diam., in agaro PCA 23–24 mm diam. Phialides ex hyphis aeriis, solitariae, raro 2–3-verticillatae (15–)20–42(–50) × 1.0–2.3 μm. Conidia subglobosa ad ellipsoidea, 2.3–4.0(–4.5) × 1.5–3.3 μm.

Holotypus: TNS-F-44312 colonia exsiccata in cultura ex solo sub *Cinnamomum pseudopedunculatum*, Chichi-jima, Bonin Isl., Tokyo, in Japonia, 13.7.2007, a K. Nonaka isolata et in herbario fungorum TNS conservata; cultura viva ex holotypo in JCM ut 18180.

Etymology: Latin, *subtropicum* = relating to subtropical region of origin.

Colonies on PDA 22–23 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse brownish orange (5C5) to brown (5F6), margin entire, soluble pigment not produced. Colonies on MEA 22–23 mm diam. after 7 days at 25 °C, convex, with white to light blond (4C3) floccose aerial mycelium, exudate lacking, reverse yellowish white (4A2), margin entire, soluble pigment not produced. Colonies on PCA 23–24 mm diam. after 7 days at 25 °C, umbonate, with white floccose aerial mycelium, exudate lacking, reverse white to grayish orange (6B3), margin entire, soluble pigment not produced. No growth at 5 °C and 33 °C on PDA.

Phialides produced on prostrate aerial hyphae, mainly solitary, rarely in whorls of 2–3, slender, tapering toward the tip (15–)20–42(–50) × 1.0–2.3 μm (Fig. 3A). Conidia in small globose heads at the apex of the phialides (Fig. 3B, D). Conidia subglobose to ellipsoidal, smooth-walled, 1-celled, 2.3–4.0(–4.5) × 1.5–3.3 μm (Fig. 3C, E). Octahedral crystals present.

Specimen and cultures examined: TNS-F-44312 (holotype), a dried culture (FKI-4956 = JCM 18180) derived from an isolate from soil under *C. pseudopedunculatum*, Chichi-jima, the Bonin Islands, Tokyo, Japan, 13 July 2007, isolated by K. Nonaka. FKI-5574 (JCM 18181) and FKI-5575 (JCM 18182), from soil under *Hibiscus rosa-sinensis*, Hachijo Island, Izu Islands. FKI-5864 (JCM 18183), from soil under *Agave americana*, Aogashima, Izu Islands.

Notes: Morphologically, *S. subtropicum* resembles *S. lanosoniveum*. But the subglobose to ellipsoidal conidia of *S. subtropicum* differ from the oval or ellipsoidal to subcylindrical ones of *S. lanosoniveum* (Table 2). Conidia of *S. subtropicum* (2.3–4.5 μm) are longer than those of *S. lanosoniveum* (1.5–3.0 μm) (Table 2). *S. subtropicum* is phylogenetically close to *S. minatense* (Fig. 1). But its subglobose to ellipsoidal conidia differ from the globose to subglobose ones of *S. minatense* (Table 2).

S. minatense Nonaka, Kaifuchi & Masuma, sp. nov. Fig. 4.

MycoBank no.: MB 519350.

Coloniae post 7 dies ad 25 °C in agaro PDA 18–19 mm diam., in agaro MEA 19–20 mm diam., in agaro PCA 19–20 mm diam. Phialides ex hyphis aeriis, solitariae, raro 2–3-verticillatae, 11–31(–47) × 1.0–1.7 μm. Conidia globosa ad subglobosa, vel ellipsoidea, 2.0–3.5 × 1.8–2.5(–2.8) μm.

Holotypus: TNS-F-44313 colonia exsiccata in cultura ex solo sub *Prunus* × *yedoensis*, Minato-ku, Tokyo, in Japonia, 27.3.2007, a Y. Fukushima isolata et in herbario fungorum TNS conservata; cultura viva ex holotypo in JCM ut 18176.

Etymology: Latin, *minatense* = relating to Minato-ku, Tokyo, referring to the type locality.

Colonies on PDA 18–19 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking,

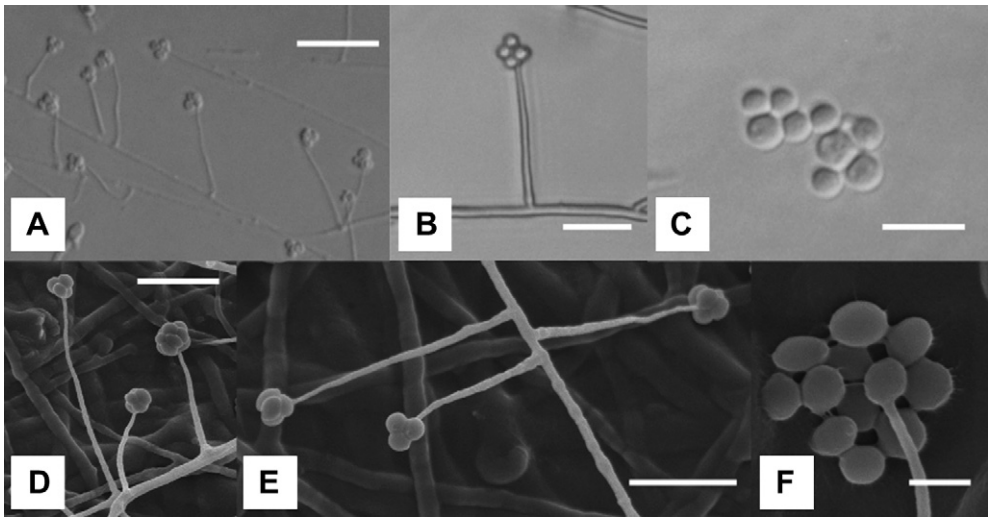

Fig. 4 – *S. minatense* FKI-4981. A–B: Conidiophores; C: Conidia; D–E: Conidiophores, SEM; F: Conidia, SEM. *Bars* A: 20 μm; B, D, E: 10 μm; C: 5 μm; F: 2 μm.

reverse brown (5E8), margin entire, soluble pigment not produced. Colonies on MEA 19–20 mm diam. after 7 days at 25 °C, plane, with light blond (4C3) with floccose aerial mycelium, exudate sparse clear drops, reverse light blond (4C3), margin entire, soluble pigment not produced. Colonies on PCA 19–20 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse dull red (9C3), margin entire, soluble pigment not produced. No growth at 5 °C and 33 °C on PDA.

Phialides produced on prostrate aerial hyphae, mainly solitary, rarely in whorls of 2–3, slender, tapering toward the tip, 11–31(–47) × 1.0–1.7 μm (Fig. 4A). Conidia in small globose heads at the apex of the phialides (Fig. 4D, E). Conidia globose to subglobose, sometimes ellipsoidal, smooth-walled, 1-celled, 2.0–3.5 × 1.8–2.5(–2.8) μm (Fig. 4C, F). Octahedral crystals present.

Specimen and cultures examined: TNS-F-44313 (holotype), a dried culture (FKI-4981 = JCM 18176) derived from an isolate from soil under *Prunus* × *yedoensis*, Minato-ku, Tokyo, Japan, 27 March 2007, isolated by Y. Fukushima. FKI-4980 (JCM = 18177) and FKI-4982 (JCM = 18178), from soil under *Prunus* × *yedoensis*, Minato-ku, Tokyo.

Notes: Morphologically, *S. minatense* resembles *S. lanosoniveum*. But its subglobose to ellipsoidal conidia differ from

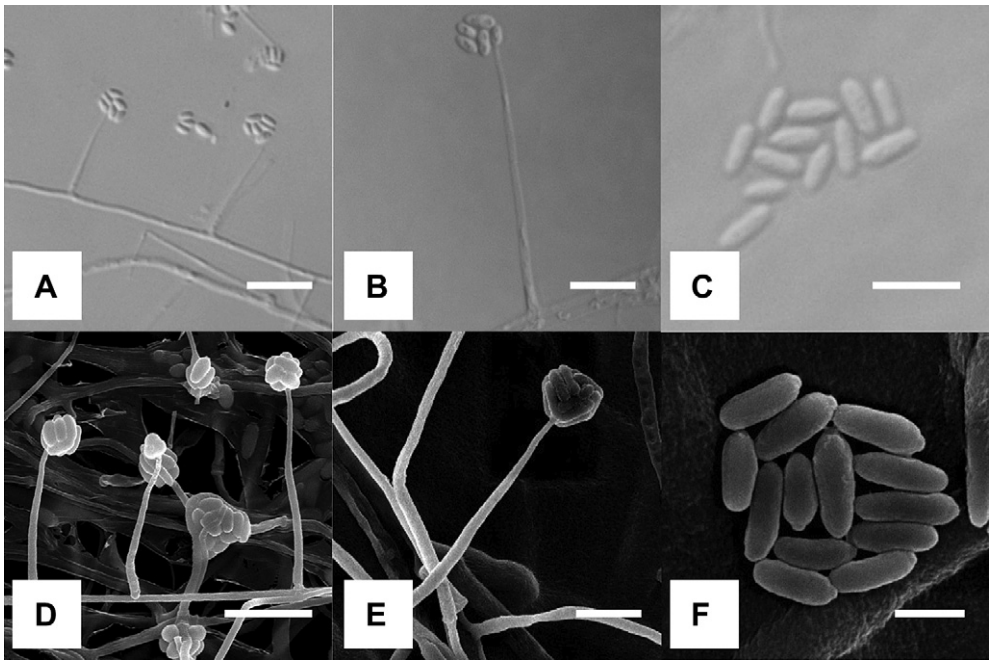

Fig. 5 – *S. cylindrosporum* FKI-4955. A–B: Conidiophores; C: Conidia; D–E: Conidiophores, SEM; F: Conidia, SEM. *Bars* A, D: 10 μm; B, C, E: 5 μm; F: 2 μm.

the oval or ellipsoidal to subcylindrical ones of *S. lanosoniveum* (Table 2). Conidia (2.0–3.5 × 1.8–2.8 μm) are longer than those of *S. lanosoniveum* (1.5–3.0 × 0.7–1.3 μm) (Table 2).

S. cylindrosporum Nonaka, Kaifuchi & Masuma, sp. nov. Fig. 5.

MycoBank no.: MB 519351.

Coloniae post 7 dies ad 25 °C in agaro PDA 21–22 mm diam., in agaro MEA 21–22 mm diam., in agaro PCA 20 mm diam. Phialides ex hyphis aeriis, solitariae, interdum 2–3-verticillatae, 17–32 × 1.2–2.0(–2.5) μm. Conidia cylindrica, 3.0–4.5(–5.0) × 1.0–2.0 μm.

Holotypus: TNS-F-44314 colonia exsiccata in cultura ex solo sub *C. pseudopedunculatum*, Chichi-jima, Bonin Isl., Tokyo, in Japonia, 13.7.2007, a K. Nonaka isolata et in herbario fungorum TNS conservata; cultura viva ex holotypo in JCM ut 18169.

Etymology: Latin, *cylindrosporum* = referring to the typically cylindrical conidia.

Colonies on PDA 21–22 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse blond (4C4), margin entire, soluble pigment not produced. Colonies on MEA 21–22 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse light yellow (4A4), margin entire, soluble pigment not produced. Colonies on PCA 20 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate forming sparse clear drops, reverse yellowish white (4A2) to brownish orange (5C5), margin entire, soluble pigment not produced. No growth at 5 °C and 33 °C on PDA.

Phialides produced on prostrate aerial hyphae, mainly solitary, sometimes in whorls of 2–3, slender, tapering toward the tip, 17–32 × 1.2–2.0(–2.5) μm (Figs. 5A, 4D). Conidia in small globose heads at the apex of the phialides (Fig. 5B, E). Conidia cylindrical, smooth-walled, 1-celled, 3.0–4.5(–5.0) × 1.0–2.0 μm (Fig. 5C, F). Octahedral crystals present.

Specimen and cultures examined: TNS-F-44314 (holotype), a dried culture (FKI-4955 = JCM 18169) derived from the isolate from soil under *C. pseudopedunculatum*, Chichi-jima, the Bonin Islands, Tokyo, Japan, 13 July 2007, isolated by K. Nonaka. FKI-5408 (JCM 18170), from soil under *Tarenna subsessilis*, Chichi-jima, the Bonin Islands. FKI-5722 (JCM 18171), from soil under *Morus* sp., Aogashima, Izu Islands. FKI-5781 (JCM 18172) and FKI-5784 (JCM 18173), from soils under *Phoenix* sp, Aogashima, Izu Islands. FKI-5984 (JCM 18174) and FKI-5985 (JCM 18175), from soil in garden, Aogashima, Izu Islands.

Notes: Morphologically, *S. cylindrosporum* resembles *S. lanosoniveum*. But its cylindrical conidia differ from the oval or ellipsoidal to subcylindrical ones of *S. lanosoniveum* (Table 2). Conidia (3.0–5.0 μm) are longer than those of *S. lanosoniveum* (1.5–3.0 μm) (Table 2). This species also resembles *Lecanicillium muscarium*. But, its phialides (1.5–2.0(–2.5) μm) are wider than the frequently verticillate ones of *Lec. muscarium* (1.0–1.7 μm), which are sometimes produced on secondary branches (Zare and Gams 2001). Moreover, FKI-4955 (ex-type of *S. cylindrosporum*) showed considerable distance (about 100 nucleotides difference) from *Lec. muscarium* (AJ292388).

S. aogashimaense Nonaka, Kaifuchi & Masuma, sp. nov. Fig. 6.

MycoBank no.: MB 519352.

Coloniae post 7 dies ad 25 °C in agaro PDA 22–23 mm diam., in agaro MEA 19–20 mm diam., in agaro PCA 20 mm diam. Coloniae in agaro PDA post 7 dies ad 33 °C 13 mm diam. Phialides ex hyphis aeriis, solitariae, raro 2–3-verticillatae (19–)23–53 × 1.2–2.0 μm. Conidia cylindrica, 4.2–6.5 × 1.2–2.0 μm.

Holotypus: TNS-F-44315 colonia exsiccata in cultura ex solo sub *Asplenium antiquum*, Aogashima, Izu Isl., Tokyo, in Japonia, 4.9.2009, a K. Nonaka isolata et in herbario fungorum TNS conservata; cultura viva ex holotypo in JCM ut 18167.

Etymology: Latin, *aogashimaense* = relating to Aogashima, Tokyo, referring to the type locality.

Colonies on PDA 22–23 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate forming sparse clear drops, reverse yellowish white (4A2), margin

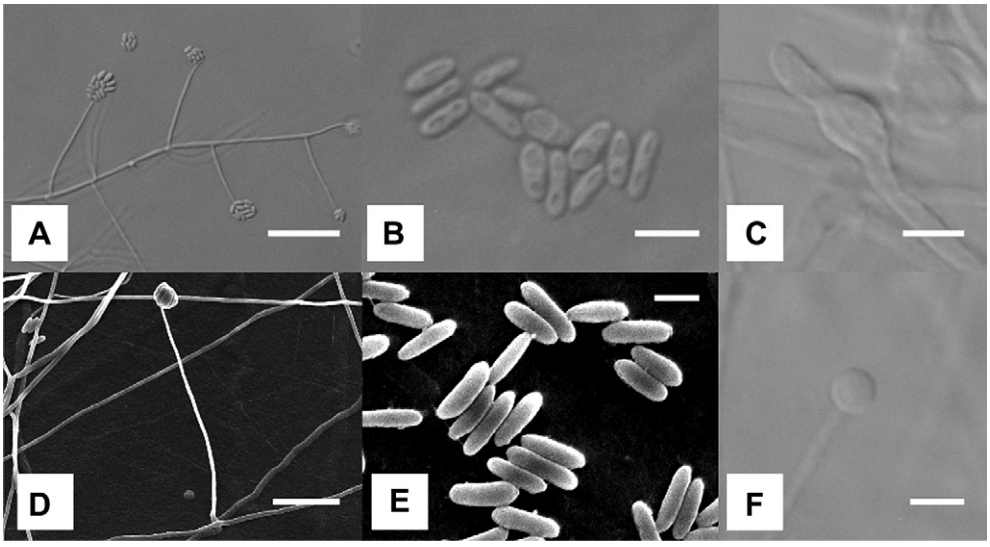

Fig. 6 – *S. aogashimaense* FKI-5881. A: Conidiophores; B: Conidia; C, F: Chlamydospores; D: Conidiophore, SEM. E: Conidia, SEM. *Bars* A: 20 μm; B, C, F: 5 μm; D: 10 μm; E: 2 μm.

entire, soluble pigment not produced. Colonies on MEA 19–20 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse pale yellow (4A3), margin entire, soluble pigment not produced. Colonies on PCA 20 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse olive (3E5) to dull red (9C3), margin entire, soluble pigment not produced. Colonies on PDA reaching 13 mm diam. after 7 days at 33 °C. No growth at 5 °C on PDA.

Phialides produced on prostrate aerial hyphae, mainly solitary, rarely in whorls of 2–3, long and slender (19–) 23–53 × 1.2–2.0 μm (Fig. 6A, D). Conidia in small globose heads at the apex of the phialides (Fig. 6D). Conidia cylindrical, smooth-walled, 1-celled, measuring 4.2–6.5 × 1.2–2.0 μm (Fig. 6B, E). Chlamydospores smooth-walled, 3–7 μm diam. (Fig. 6C, F). Octahedral crystals present.

Specimen and cultures examined: TNS-F-44315 (holotype), a dried culture (FKI-5881 = JCM 18167) derived from an isolate from soil under *A. antiquum*, Aogashima, Izu Islands, Tokyo, Japan, 4 September 2009, isolated by K. Nonaka. FKI-5887 (JCM 18168), from soil under *A. antiquum*, Aogashima, Izu Islands.

Notes: Morphologically, *S. aogashimaense* resembles *S. lamellicola* and *S. cylindrosporum*. They all have long conidia. But, *S. lamellicola* has two different shapes and size of conidia (Table 2). Conidia of *S. aogashimaense* (4.2–6.5 μm) are longer than those of *S. cylindrosporum* (3.0–5.0 μm) (Table 2). *S. aogashimaense* is phylogenetically close to *S. obclavatum* (Fig. 1), but produces conidia in small globose heads (Table 2). This species also resembles *Lec. muscarium*, but that species sometimes produced phialides on secondary branches (Zare and Gams 2001). Moreover, FKI-5881 (ex-type of *S. aogashimaense*) was distantly (about 110 nucleotides difference) related to *Lec. muscarium* (AJ292388).

S. sympodiophorum Nonaka, Kaifuchi & Masuma, sp. nov. Fig. 7.

MycoBank no.: MB 519353.

Coloniae post 7 dies ad 25 °C in agaro PDA 21–22 mm diam., in agaro MEA 20–21 mm diam., in agaro PCA 18–19 mm diam. Cellulae conidiogenae ex hyphis aeriis, solitariae vel 2–4-verticillatae, 20–34(–47) × 0.5–1.3 μm. Conidia sympodialia ovalia ad ellipsoidea, 2.2–3.5 × 1.0–2.0 μm.

Holotypus: TNS-F-44316 colonia exsiccata in cultura ex solo sub *A. antiquum*, Aogashima, Izu Isl., Tokyo, in Japonia, 4.9.2009, a K. Nonaka isolata et in herbario fungorum TNS conservata; cultura viva ex holotypo in JCM ut 18184.

Etymology: Latin, *sympodiophorum* = referring to sympodial conidiogenesis.

Colonies on PDA 21–22 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse yellowish white (4A2), margin entire, soluble pigment not produced. Colonies on MEA 20–21 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse yellowish white (4A2), margin entire, soluble pigment not produced. Colonies on PCA 18–19 mm diam. after 7 days at 25 °C, convex, with white floccose aerial mycelium, exudate lacking, reverse white to brownish gray (5D2), margin entire, soluble pigment not produced. No growth at 5 °C and 33 °C on PDA.

Conidiogenous cells arising from prostrate aerial hyphae, solitary or in whorls of 2–4, simple and slender, tapering toward the tip, 20–34(–47) × 0.5–1.3 μm (Fig. 7A, B); proliferating sympodially, with cylindrical conidium-bearing denticles (Fig. 7E). Conidia oval to ellipsoidal, smooth-walled, 1-celled, 2.2–3.5 × 1.0–2.0 μm (Fig. 7F). Octahedral crystals present.

Specimen and culture examined: TNS-F-44316 (holotype), a dried culture (FKI-5883 = JCM 18184) derived from an isolate from cultivated soil under *A. antiquum*, Aogashima, Izu Islands, Tokyo, Japan, 4 September 2009, isolated by K. Nonaka.

Notes: *S. sympodiophorum* is phylogenetically close to *S. lamellicola* (Fig. 1), but does not resemble other *Simplicillium* species morphologically. It is the only species producing sympodial conidia in this genus (Table 2). On the other hand,

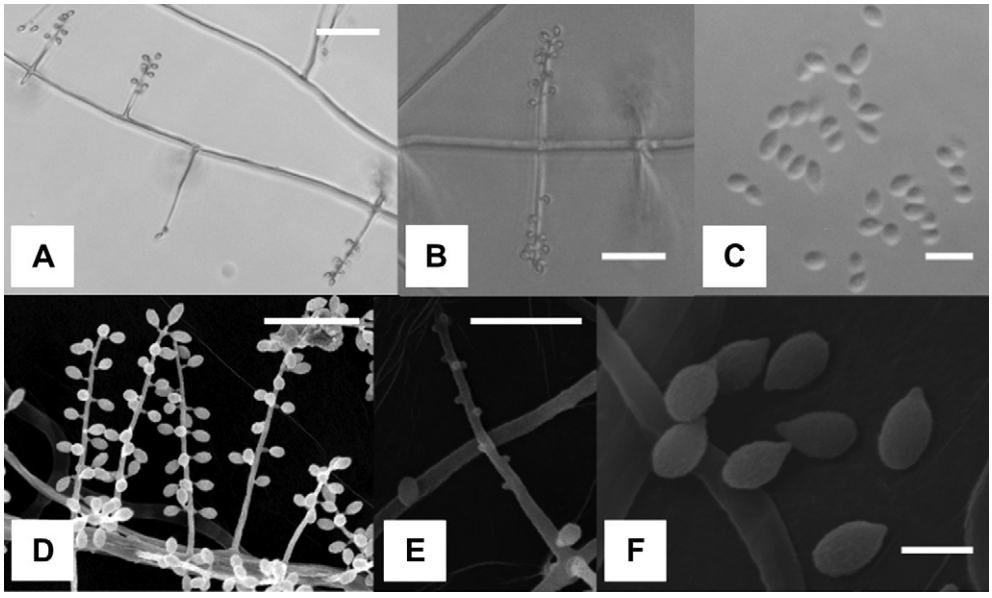

Fig. 7 – *S. sympodiophorum* FKI-5883. A–B: Conidiophores; C: Conidia; D: Conidiophores, SEM. E: Denticles, SEM; F: Conidia, SEM. *Bars* A, B, D: 20 μm C; E: 5 μm; F: 2 μm.

this species resembles *Engyodontium album* (Limber) de Hoog and *Myriodontium keratinophilum* Samson & Polon. Its straight conidiogenous cells differ from the geniculate ones of *E. album* (Hoog de 1972, 1978). Its conidiogenous cells always arise in terminal position, differing from the intercalary or terminal ones of *M. keratinophilum* (Samson and Polonelli 1978). Moreover, *S. sympodiophorum* was quite distantly related (more than 100 nucleotides difference) to *E. album* (AB106650) and *M. keratinophilum* (EU925387).

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