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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