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Morphological and molecular characterization of a novel species of *Simplicillium* from China

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Abstract – An interesting co-cultured fungus was discovered when isolating freshwater fungi on water agar. It is morphologically characterized by solitary phialides and oval, obclavate to ellipsoidal conidia that often form in chains. Its relationships with morphologically similar and phylogenetically closely related taxa are discussed, based on which a new species, *Simplicillium chinense,* is established in this paper. Phylogenetic analyses using 28S and ITS rDNA sequences indicate that *S. chinense* and all other species in the genus belong to Cordycipitaceae, Hypocreales. A key to currently known species in *Simplicillium* is provided.

Ascomycetes / Cordycipitaceae / Environment / Hypocreales / Systematics / Taxonomy

INTRODUCTION

The genus *Simplicillium* Gams W. & Zare R. (Zare and Gams, 2001) was introduced to accommodate a monophyletic lineage basal to *Lecanicillium* Gams W. & Zare R. according to phylogenies generated from SSU and LSU data (Sung *et al.*, 2001; Zare and Gams, 2001). *Simplicillium* was embraced in family Cordycipitaceae, a family that was reintroduced and validated based on the genus *Cordyceps* (Sung *et al.* 2007). *Simplicillium* currently includes *S. obclavatum* (W. Gams) Zare R. & Gams W., *S. lanosoniveum* (J.F.H. Beyma) Zare R. & Gams W. and *S. lamellicola* (F.E.V. Sm.) Zare R. & Gams W. (Zare and Gams, 2001; Zare and Gams, 2008).

The objective of this paper is to describe a novel species of *Simplicillium* obtained as a co-culture on water agar when isolating freshwater fungi, a group of growing interest in Asia (Kurniawati et al, 2010; Su *et al.*, 2011). The fungus is described, illustrated and compared with other *Simplicillium* species. Phylogenetic reconstruction was carried out to determine its systematic placement and relationships with other closely related taxa.

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MATERIALS AND METHODS

Sampling: Two interesting co-cultured strains were obtained on water agar when isolating freshwater fungi from submerged wood at 17 March, 2011. Mycelial discs (2 mm diam.) were taken from actively sporulating areas near the growing edge of 7 day old cultures and transferred to potato dextrose agar (PDA) and incubated at 25. Colony diameters were measured after 10 days incubation. Observation and photograph were made with materials mounted in sterile water using a Nikon 80i microscope. Cultures were incubated at 33 to test whether they could grow at that temperature. The holotype was deposited at HMAS with ex-type living cultures deposited in CGMCC.

DNA extraction, PCR and sequencing: Total genomic DNA was extracted from mycelia scraped from the surface of pure cultures using a modified Cetyltrimethyl Ammonium Bromide (CTAB) protocol (Guo *et al.* 2000). Partial 28S rDNA and ITS rDNA were amplified using fungal specific primers LROR/LR5 (Vilgalys and Hester 1990) and ITS1/ITS4 (white *et al.* 1990). PCR mixture contained 2.5 units FastStart Taq polymerase (Roche applied Science, U.S.A.), 1 × PCR buffer, 2 mM $MgCl_2$, 0.25 mM of each dNTP, 0.5 μM of each primer and approximately 30 ng of fungal genomic DNA, made up to a total reaction volume of 25 μl with sterile distilled water. Thermal cycling parameters included an initial denaturation of 95 for 5 min, followed by 35 cycles consisting of denaturation at 95 for 30s, annealing at 54 for 30s, and elongation of 72 for 1 min, followed by 10 min at 72 for elongation. The PCR products were purified and sequenced using LROR/LR5 and ITS1/ITS4 primers mentioned above at SinoGenoMax Company limited.

Sequence data analysis: Sequences of our isolates, along with reference sequences obtained from GenBank, were aligned using Clustal X (Tompson *et al.*, 1997). Alignments were optimized manually in BioEdit (Hall, 1999) and ambiguously aligned regions were excluded from all analyses. The alignments were deposited in TreeBASE (http://www.treebase.org/treebase-web/home.html).

Maximum parsimony (MP) phylogenetic analysis was performed by using PAUP* 4.0b10 (Swofford, 2002). Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Maxtrees were unlimited, branches of zero length were collapsed and all multiple parsimonious trees were saved. Branch robustness was estimated conducing a bootstrap analysis of 1000 replicates, each with 10 replicates of random addition sequence. A Shimodaira-Hasegawa test (SH test) (Shimodaira and Hasegawa, 1999) was performed to evaluate whether trees were significantly different. Trees were visualized in Treeview (Page, 1996).

For Bayesian phylogenetic analysis, the best fit model of evolution was estimated by using Mrmodeltest 2.2 (Nylander, 2004). Posterior probabilities (PP) (Rannala and Yang, 1996; Zhaxybayeva and Gogarten, 2002) were determined by Markov Chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Huelsenbeck and Ronquist, 2001), using the estimated model of evolution. Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100^{th} generation (resulting 10,000 total trees). The resulting dataset was visualized in Excel and it was determined that the plateau was reached after 2000 trees. Thus, the first 2,000 trees, which represented the burn-in phase of the analyses, were discarded and the remaining 8,000 trees were used to calculate posterior probabilities (PP) in the majority rule consensus tree.

RESULTS

Simplicillium chinense F. Liu & L. Cai, **sp. nov.** Fig. 3

MycoBank: MB 800157

Etymology: "*chinense*" referring to the country where the fungus was first discovered.

Colonies compact, white, reaching 28-30 mm diam. after 10 days at 25 on PDA, reverse cream-colored to light yellow; older colonies producing orange pigment. Phialides exclusively solitary, arising from aerial hyphae, gradually tapering towards the apex, without basal septum, (6.0-) 15-30 (-68.0) × 1.5 µm. Conidia formed in branched or unbranched chains (up to 26). Conidia variable in size and shape, mostly oval, ellipsoidal or cylindrical, 3.5-5.0 × 1.0-1.5 µm ($\bar{x}$ = 4.2 × 1.3 µm, n = 35); the apical conidia of the conidial chains subglobose to obovoid, 1.5-2.5 × 1.5-2.0 µm ($\bar{x}$ = 2.2 × 1.7 µm, n = 20). Octahedral crystals present in culture. No growth at 33.

Holotype: China, 17 Mar. 2011, F. Liu, HMAS 243490, ex-holotype living culture CGMCC 3.14970; *ibid* HMAS 243489, living culture CGMCC 3.14969.

Habitat: environmental microorganism.

Known distribution: China.

Phylogenetic analyses

28S rDNA and ITS rDNA sequences of *S. chinense* were deposited in GenBank (JQ410321-JQ410324). The alignments were deposited in TreeBASE (S12635). Phylogenetic relationships were inferred by analyzing 28S rDNA of *Simplicillium chinense* and other closely related taxa. The dataset consisted of 33 sequences representing 12 genera of Cordycipitaceae, and *Pochonia* (Clavicipitaceae), *Hypocrea* (Hypocreaceae), *Calonectria* (Nectriaceae), *Bionectria* (Bionectriaceae). *Ceratocystis moniliformis* (Microascales) was used as out group. The final dataset comprised 857 characters after alignment including gaps. Of these characters, 527 characters are constant, 192 are parsimony-uninformative and 138 are parsimony-informative. Parsimony analysis resulted in 33 equally parsimonious trees. One of the most parsimonious tree (Length = 557, CI = 0.700, RI = 0.739, RC = 0.517, HI = 0.300) is shown in Fig. 1. In the 28S tree, Cordycipitaceae appears to be monophyletic and showed phylogenetic distance to the Clavicipitaceae, Hypocreaceae, Nectriaceae and Bionectriaceae (Fig. 1). *Simplicillium chinense* beared close affinities to other species in the genus, i.e. *S. lanosoniveum, S. obclavatum* and *S. lamellicola* (Fig. 1, BS value = 77, PP = 1.00).

The ITS rDNA dataset included sequences from 41 fungal strains representing 7 genera of Cordycipitaceae, and *Torrubiella* (Clavicipitaceae), *Ophiocordyceps* (Ophiocordycipitaceae), *Hypocrea* (Hypocreaceae), *Calonectria* (Nectriaceae), *Bionectria* (Bionectriaceae), *Melanopsamma* (Niessliaceae). *Ceratocystis moniliformis* (Microascales) was used as out group. The final dataset comprised 630 characters after alignment including gaps. Of these characters, 225 characters are constant, 109 are parsimony-uninformative and 296 are parsimony-informative. Parsimony analysis resulted in 8 equally parsimonious trees. One of the most parsimonious tree (Length = 1337, CI = 0.549, RI = 0.766, RC = 0.421, HI = 0.451) is shown in Fig. 2. As shown in the ITS tree, all the Cordycipitaceae taxa formed a distinct lineage and showed phylogenetic distance

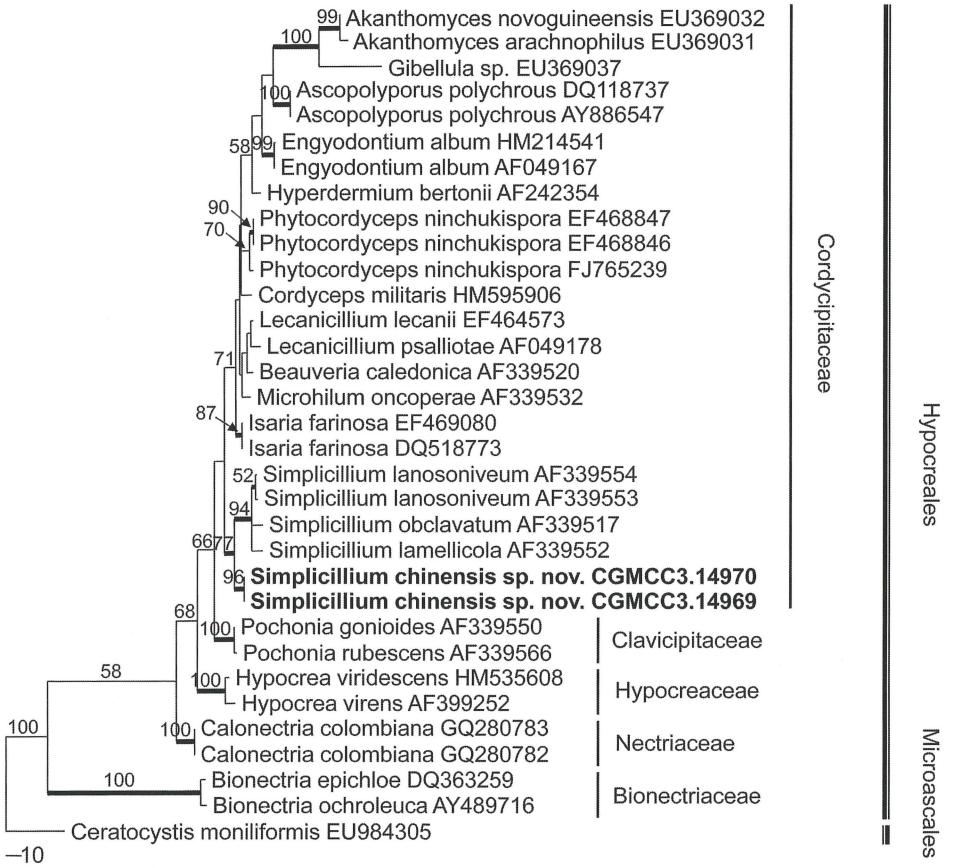

Fig. 1. Maximum parsimony phylogram showing phylogenetic relationships among *Simplicillium chinense* and closely related taxa based on Ribosomal Large Subunit (28s) sequences. Bootstrap support values above 50% are shown above the branches. Thickened branches represent significant Bayesian posterior probability (≥ 95%). The tree is rooted with *Ceratocystis moniliformis*. Asterisks indicate the ex-type strains.

to the Clavicipitaceae, Ophiocordycipitaceae, Hypocreaceae, Nectriaceae and Bionectriaceae taxa (Fig. 2). *S. chinense* clustered with several undetermined *Simplicillium* spp. in a well supported clade (BS value = 99, PP = 1.00) and showed close affinity to *S. lanosoniveum, S. obclavatum,* and *S. lamellicola* (Fig. 2, BS value = 77, PP = 0.99).

DISCUSSION

Morphological studies coupled with the phylogenetic analyses of 28S and ITS rDNA sequences supported the introduction of the new species *Simplicillium chinense*. *Simplicillium* has close affinity to the genus *Lecanicillium*, which

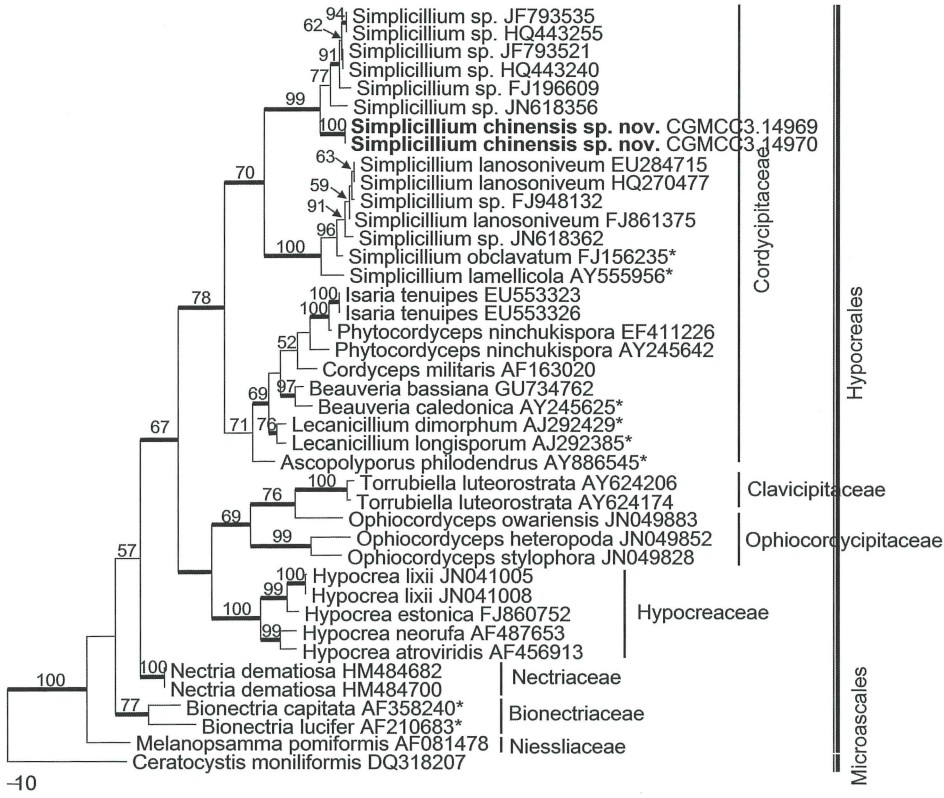

Fig. 2. Maximum parsimony phylogram showing phylogenetic relationships among *Simplicillium chinense* and closely related taxa based on ITS sequences. Bootstrap support values above 50% are shown above the branches. Thickened branches represent significant Bayesian posterior probability (≥ 95%). The tree is rooted with *Ceratocystis moniliformis*. Asterisks indicate the ex-type strains.

currently is accommodated in the family Cordycipitaceae (Zare and Gams, 2001). Both genera produce discrete and aculeate phialides, conidia adhering in slimy heads or forming chains. *Simplicillium* and *Lecanicilium* can be distinguished by the arrangement of phialides. The former is often solitary with little difference from the subtending hyphae, while the latter is usually verticillate (Zare and Gams 2001; Zare and Gams 2008). The morphological characters of our isolates fit well with the generic concept of *Simplicillium* (Zare and Gams, 2001). The molecular analyses based on 28S and ITS rDNA sequences provide further evidence that *S. chinense* belong to *Simplicillium*, Cordycipitaceae, and is phylogenetically distant to the representatives of *Lecanicillium.*

Currently there are three species in the genus *Simplicillium*, *S. lanosoniveum*, *S. lamellicola* and *S. obclavatum*. Within this genus, *S. lanosoniveum* and *S. lamellicola* form conidia head at the apex of the phialides, while *S. obclavatum* and *S. chinense* form conidial chains. *Simplicillium chinense* can be differentiated from *S. obclavatum* by the size of conidia (*S. chinense* 3.5-5.0 × 1.0-1.5 μm vs. *S. obclavatum* 2.5-3.5 × 1-2 μm) and phialides (*S. chinense*

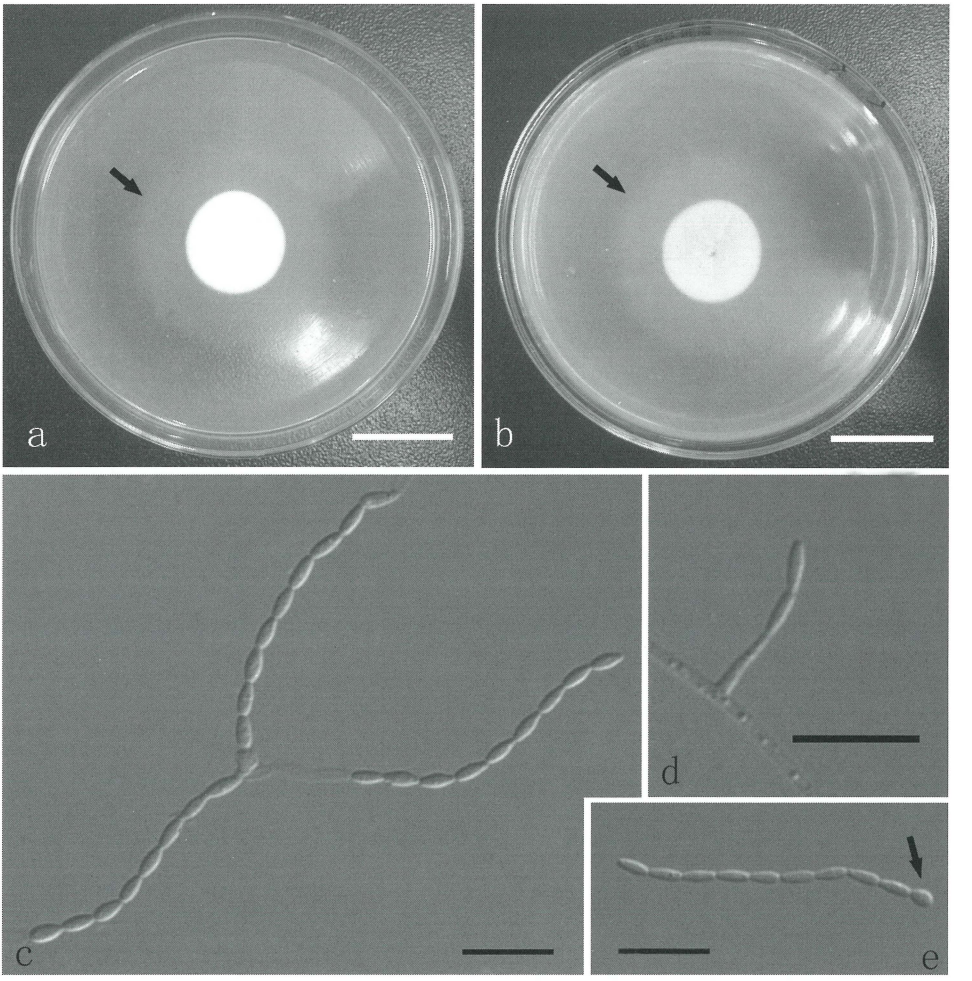

Fig. 3. *Simplicillium chinense* (from holotype). a, b. Upper and reverse view of culture on potato dextrose agar (PDA) 10 days after inoculation. Note the white pigment around the colony (arrowed); c. conidial chain; d. phialides bearing conidial chain; e. conidial chain. Note the subglobose conidium at the point end of the chain (arrowed) (a-b. bars = 30 mm, c-e. bars = 10 μm).

(6.0-) 15-30 (-68.0) × 1.5 μm vs. *S. obclavatum* 30-52 × 0.8-1.2 μm). In addition, *S. obclavatum* grows well at 33, while S. *chinense* has no growth at this temperature. In the phylogenetic trees generated from 28S and ITS rDNA dataset, *S. chinense* appeared in a distinct lineage basal to other *Simplicillium* species (Figs 1, 2).

The species of *Simplicillium* occur in a broad range of ecological niches, such as diseased plant tissue, soil, rust, nematode, human nail, dog tissue and mushroom (Zare and Gams, 2001). *Simplicillium lanosoniveum* and *S. lamellicola* were found to be the parasites of rusts, such as coffee rusts and soybean rusts, and have been exploited as biological control agent (Zare and Gams, 2001; Ward *et al.*,

2010; Ward, 2011; Ward *et al.*, 2011). *Simplicillium lamellicola* also parasitize on cysts of *Heterodera glycines* Ichinohe and eggs of *Meloidogyne arenaria* (Neal) Chitwood (Gams, 1988). On the other hand, they also cause plant diseases. For examples, *S. lamellicola* was described as the causal agent of 'gill mildew' and 'brown spots' on *Agaricus bisporus* (Lange) Imbach (Zare and Gams, 2001; Gams and van Zaayen, 1982), while, *S. lanosoniveum* causes brown spot on *Salvinia auriculata* Aublet and *S. molesta* Mitchell D.S. (Chen *et al.*, 2008). *Simplicillium chinense* was obtained on the water agar when isolating freshwater fungi. A thorough examination of the original submerged wood failed to discover any phialides or conidia of *S. chinense*. It is likely that *S. chinense* has an environmental origin. Bischoff and White (2004) linked the teleomorphs of *Simplicillium* to *Torrubiella* which are pathogens of spiders and scale insects. While no teleomorph was observed in our study.

Key to accepted species of *Simplicillium*

1. Conidia formed in chains 2
1. Conidia formed in globose or subglobose heads 3
   2. Conidial chains short; conidia 2.5-3.5 × 1-2 µm ..... *S. obclavatum*
   2. Conidial chains long; conidia 3.5-5.0 × 1.0-1.5 µm ..... *S. chinense*
3. Macro- and microconidia formed ..... *S. lamellicola*
3. Only one type of conidia formed ..... *S. lanosoniveum*

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