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A new species of *Conidiobolus* (*Ancylistaceae*) from Anhui, China

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Abstract —*Conidiobolus sinensis* was isolated from plant detritus in Huoshan, Anhui Province, eastern China. It produces primary conidiophores from cushion mycelium, which is distinct from all other species in the genus except *C. stromoideus* and *C. lichenicola*. Morphologically *C. sinensis* differs from *C. stromoideus* in the shape of the mycelia at the colony edge and conidiophore length and from *C. lichenicola* by colony color and mycelial form. A phylogram based on partial 28S rDNA and EF-1α sequences from 14 *Conidiobolus* species shows *C. sinensis* most closely related to *C. stromoideus*, forming a clade of sister taxa with a 100% bootstrap. DNA similarity levels between these two species were 94% (28S rDNA) and 96% (EF-1α). Based on the morphological and molecular evidence, *C. sinensis* is considered a new species.

Key words —*Entomophthorales*, hyphal knots, taxonomy

Introduction

Species belonging to *Conidiobolus* can be easily isolated from soil, decaying leaf litter, rotten vegetables and some dead insects, although the type of the genus, *C. utriculosus* Bref., was first isolated from the decaying fleshy fruitbodies of *Exidia* and *Hirneola*. The genus is diagnosed by (i) nuclei that do not stain in aceto-orcein and lacking obviously granular contents, (ii) simple conidiophores, (iii) globose to pyriform multinucleate conidia, (iv) resting spores formed in the axis of hypha (mostly as zygospores), and (v) walled vegetative cells (Humber 1997). After Huang et al. (2007) recognized 30 species within *Conidiobolus,* only two additional species — *C. margaritatus* (Huang et al. 2007), *C. thermophilus* (Waingankar et al. 2008) — have been added.

While many phylogenetic studies of entomogenous fungi have been conducted in recent years, there is little information regarding the phylogeny

and molecular taxonomy of *Conidiobolus*. The limited SSU analysis of Jensen et al. (1998) suggested that *Conidiobolus* may be polyphyletic. Vilela et al. (2010) were the first to detail the taxonomic and phylogenetic features of three pathogenic *Conidiobolus: C. coronatus* (Costantin) A. Batko, *C. lamprauges* Drechsler, and *C. incongruus* Drechsler.

Anhui Province is located at 29°04′–34°06′N 114°09′–119°06′E, eastern China, and most parts of the province have subtropical vegetation, but some temperate vegetation occurs in mountains. Recently, several new fungal species on decaying wood have been reported from the area (Dai 2010; Cui et al. 2011). In the course of studies on *Conidiobolus* species from China, one strain with cushion mycelia was isolated from decaying plant material in Anhui and is described in this report as a new species, based on both morphological and molecular data.

Materials & methods

Morphological studies

Plant detritus was sampled on 17 June 2010 near a reservoir in Huoshan, Anhui, China. The sample was screened for saprotrophic *Conidiobolus* by canopying moistened detritus on agar plates following Drechsler (1952) and King (1976a) to exploit the forcible discharge of *Conidiobolus* conidia. These isolation plates were incubated at 21°C and examined daily for one week. Once *Conidiobolus* cultures were detected on the PDA canopy, they were transferred to new PDA plates for purification and morphological study. The measurements of different fungal structures followed King (1976a).

Molecular studies

The *Conidiobolus* strains used in the molecular study are shown in Table 1. Ten ex-type strains of *Conidiobolus* spp. were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA), and the remaining *Conidiobolus* strains were obtained from the Research Center for Entomogenous Fungi (RCEF; Anhui Agricultural University, Hefei, China). Genomic DNA was extracted using the CTAB method (Yi et al. 2003). The extracted DNA was stored in 50–100 μL of HPLC-H_2O at -20°C, and was diluted 10-fold with HPLC-H_2O for use in PCR reactions. Regions of two genes were amplified by PCR: 1) nuclear ribosomal large subunit (LSU rDNA) by primers LROR and LR5 (Vilgalys & Hester 1990) and 2) elongation factor 1-alpha (EF-1α) by primers EF983 and EF1aZ-1R (http://www.aftol.org/primers.php). All procedures used in this study for LSU amplification have been described previously (Liu et al. 2005). The PCR reaction mixture for amplifying EF-1α contained 200 μM each dNTP, 1× Mg-free buffer, 2.5 mM $MgCl_2$, 0.5 μM each primers, 1 ng/uL genomic DNA, and 0.04 Unit/L Taq polymerase. The cycle program included initial denaturation at 100°C for 5 min followed by 95°C for 5 min (during which time Taq polymerase was added to each tube), 34 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, and a final extension at 72°C for 10 min. The nucleotide sequences of the PCR products were determined on both strands by using dideoxy-nucleotide chain termination on an ABI 3700 automated sequencer at Shanghai Genecore Biotechnologies Company. Sequence data of the 19

Table 1. *Conidiobolus* and *Entomophthora* cultures and sequences used in phylogenetic analyses.*

Fungal taxon	Strain #	28S rDNA	EF-1α
C. chlamydosporus Drechsler	ATCC12242 (T)	JF816212	JF816234
C. denaeosporus Drechsler	ATCC12940 (T)	JF816215	JF816228
C. firmipilleus Drechsler	RCEF4429	JF816222	JF816237
C. gonimodes Drechsler	ATCC14445 (T)	JF816221	JF816226
C. coronatus	RCEF5598	JQ004791	JQ004795
	RCEF5599	JQ004792	JQ004796
	RCEF5600	JQ004793	JQ004797
	RCEF5601	JQ004794	JQ004798
	AFTOL-ID137	AY546691	DQ275337
C. heterosporus Drechsler	RCEF4430	JF816225	JF816239
C. humicola M.C. Sriniv. & Thirum.	ATCC28849 (T)	JF816220	JF816231
C. lichenicola	ATCC16200 (T)	JF816216	JF816232
C. lobatus M.C. Sriniv. & Thirum.	ATCC18153 (T)	JF816218	JF816233
C. nodosus M.C. Sriniv. & Thirum.	ATCC16577 (T)	JF816217	JF816235
C. polytocus Drechsler	ATCC12244 (T)	JF816213	JF816227
C. stromoideus	ATCC15430 (T)	JF816219	JF816229
C. sinensis	RCEF4952 (T)	JF816224	JF816238
C. thromboides Drechsler	ATCC12587 (T)	JF816214	JF816230
	RCEF4492	JF816223	JF816236
E. muscae (Cohn) Fresen.	ARSEF3074	DQ273772	DQ275343

* The fungal taxonomy follows that of King (1976a, b, 1977). ARSEF = ARS Entomopathogenic Fungus Collection (Ithaca, USA). ATCC = American Type Culture Collection (Manassas, USA). RCEF = Research Center for Entomogenous Fungi (Hefei, China). AFTOL-ID = Assembling the Fungal Tree of Life Identity. T = ex type.

strains of *Conidiobolus* have been deposited in the GenBank database under the access numbers shown in Table 1.

Sequences were aligned with Clustal X (Thompson et al. 1997). The combined data of the two loci, partial 28S rDNA and EF-1α, were analyzed with Maximum Parsimony (MP) in PAUP* 4.0b10 (Swofford 2003), by using 1000 replicates of heuristic search of random sequence additions, branch swapping algorithm by tree bisection-reconnection (TBR) and MULTrees in effect. Gaps were treated as missing data and all characters were equally weighted. Branch support was estimated by 1000 bootstraps of 10 replicates of heuristic search with the same options as the parsimony search (Felsenstein 1985). The alignments were fed to DNAMAN software package (Version 5.2.2, Lynnon Biosoft, Canada) for calculating genetic similarities.

Results

The combined alignment of partial 28S rDNA and the EF-1α dataset was 1468 bp in length, including 981bp from the LROR/LR5 region of 28S rDNA and 487 bp from the EF983/EF1aZ-1R region of EF-1α. 108 sites in 28S rDNA and 24 sites in EF-1α with ambiguous alignment were excluded from the analysis and the final alignment contained 643 parsimony-informative sites. Maximum parsimony analysis of 20-taxon dataset resulted in a single tree (TL = 1942, CI = 0.6130, RI = 0.7826, HI = 0.3553) shown in Plate 1, and

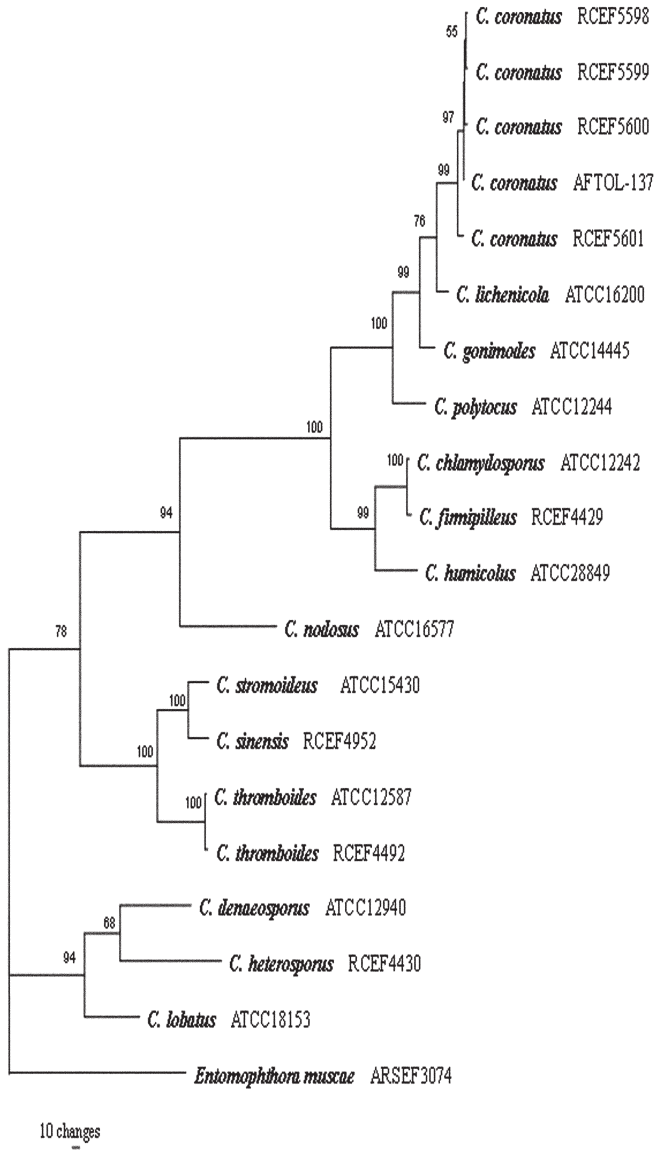

Plate 1. The single most parsimonious tree (TL = 1942, CI = 0.6130, RI = 0.7826, HI = 0.3553) showing phylogenetic relationships among species of *Conidiobolus* inferred from a combined dataset of partial 28S rDNA and EF-1α sequences. Bootstrap values ≥50 % are labeled above relevant branches. *Entomophthora muscae* served as the outgroup. The bar at the lower left corner represents 10 changes.

Table 2. Similarities of partial 28S rDNA and ef-1α sequences from *Conidiobolus* strains.*

Species— strain	% similarity												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
1 *C. chlamydosporus* —atcc12242		99	85	85	67	67	67	65	85	85	85	85	84
2 *C. firmipilleus* — rcef4429	98		85	85	68	68	68	66	85	85	85	85	85
3 *C. gonimodes* —atcc14445	91	91		97	68	68	66	66	96	96	96	96	96
4 *C. lichenicola* —atcc16200	90	89	93		68	68	66	66	96	96	96	96	96
5 *C. thromboides* —atcc12587	73	73	75	76		99	88	86	67	68	68	68	67
6 *C. thromboides* — rcef4492	73	73	75	76	99		88	86	67	68	68	68	66
7 *C. stromoideus* —atcc15430	75	75	75	77	91	92		94	65	66	66	65	65
8 *C. sinensis* —rcef4952	73	73	74	75	91	91	96		65	65	65	65	65
9 *C. coronatus* —rcef5598	92	92	94	95	76	76	77	75		99	99	98	99
10 *C. coronatus* —rcef5599	92	91	94	95	76	76	77	75	99		99	98	99
11 *C. coronatus* —rcef5600	91	91	94	96	76	76	77	75	99	99		99	99
12 *C. coronatus* —rcef5601	91	91	93	95	76	76	77	75	98	98	99		98
13 *C. coronatus* —aftol-id137	91	91	94	96	76	76	77	75	99	99	100	99	

* Data refer to the overall similarities of the partial 28S rDNA (above the diagonal) and EF-1α sequences (below the diagonal)

the DNA similarities among 13 representing strains are listed in Table 2. The phylogenetic tree shows that the isolate RCEF4952 clustered with the ex-type strain of *Conidiobolus stromoideus* with 100% bootstrap support (Plate 1), but the similarities between *C. stromoideus* and *C. sinensis* were only 94% (28S rDNA) and 96% (EF-1α) (Table 2). Higher intraspecific DNA similarities were measured from partial 28S rDNA and EF-1α sequence. For example, the ranges of DNA similarities within *C. coronatus* have been found to be 98–99% (28S rDNA) and 98–100% (EF-1α), and those within *C. thromboides* were 99% for both genes. On the other hand, the *Conidiobolus* partial 28S rDNA and EF-1α showed high genetic divergence among species. The highest similarity of partial 28S rDNA (97%) was recorded between *C. gonimodes* and *C. lichenicola*, and the lowest (65%) between *C. coronatus* and *C. stromoideus*. The range of similarities in EF-1α among species was 73–96%. Although the similarities between *C. chlamydosporus* and *C. firmipilleus* were 99% (28S rDNA) and 98% (EF-1α), *C. chlamydosporus* was placed in synonymy with *C. firmipilleus* in King's classification (King 1977).

Taxonomy

Conidiobolus sinensis Y. Nie, X.Y. Liu & B. Huang, **sp. nov.** Plates 2–3

MycoBank MB563665

Differs from *Conidiobolus stromoideus* by its much longer conidiophores and rarely branching mycelia at the colony edge.

Type: China, Anhui Province, Huoshan County, isolated from leaf litter, 17 June 2010, [Yong Nie] (**Holotype**, RCEF4952; GenBank JF816224, JF816238).

Etymology: *sinensis* (Lat.) = China, referring to the geographic origin of the strain.

Colonies grown on PDA for 3 days at 21°C, white, reaching ca 21 mm diameter. Numerous hyphal knots giving the colony a coarse appearance with aging. Mycelium colorless, tubular, filamentous, 5–10 µm wide, forming hyphal segments in older regions. Apical cells 80–450 µm long, often unbranched before cell division. Conidiophores colorless, unbranched and producing a single conidium, arising as upward branches from hyphal knots formed by irregular mycelium interweaving, 32.5–110 × 10–15 µm. Primary conidia colorless, globose to pyriform 17.5–25 µm wide, 22.5–32.5µm long including a basal papilla 7.5–10 µm high and 2.5–7.5 µm wide. Primary conidia forcibly discharged, on water agar forming globose secondary conidia resembling the primary spore, 17.5–22.5 × 15–20 µm. Zygospores formed between adjacent conjugating cells of a hyphal body. Mature zygospores smooth, globose or subglobose, 25–31 µm in diameter with wall 1–2 µm thick.

Discussion

In comparing the morphological characteristics of primary conidiophores from cushion mycelium with the known *Conidiobolus* species, *C. sinensis* resembles *C. lichenicola* M.C. Sriniv. & Thirum. and *C. stromoideus* M.C. Sriniv. & Thirum. (Srinivasan & Thirumalachar 1962, 1968). Colonies of *C. lichenicola* are distinguished by a pale brownish mycelium with sinuous, lobate hyphae. *Conidiobolus stromoideus* differs from the new species producing edge mycelia that are usually branched (rarely branched in *C. sinensis*) and much shorter (12–40µm) conidiophores (Plate 3).

The phylogenetic tree places the *C. sinensis*–*C. stromoideus* clade distant from the *C. lichenicola* clade (Plate 1), thus reinforcing the morphological difference between *C. sinensis* and *C. lichenicola*. If *C. chlamydosporus* is accepted as synonymous with *C. firmipilleus* in accordance with King (1977), there is a clear-cut line between intraspecific and interspecific sequence similarity levels: 98–100% within species and 64–97% among species (Table 2). Although *C. sinensis* groups with *C. stromoideus*, DNA similarity levels between the two species fall within the interspecific range (94% (28S rDNA) and 96% (EF-1α)). Thus, the phylogenetic analysis supports the morphological identification of *C. sinensis* as a new species differing from *C. stromoideus* and *C. lichenicola*.

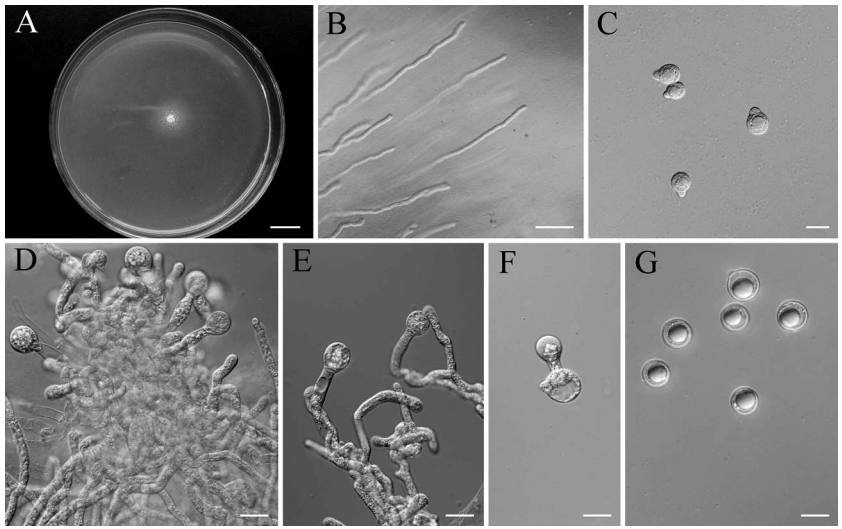

Plate 2. *Conidiobolus sinensis*. A. Colony on PDA after 3 days at 21°C. B. Rarely branched mycelia at the margin of colony. C. Primary conidia. D. Primary conidiophores produced from hyphal knots. E. Primary conidiophores. F. Secondary conidia produced singly from the primary conidia. G. Mature zygospores. Bars: A = 10 mm, B = 100 μm, C–H = 20 μm.

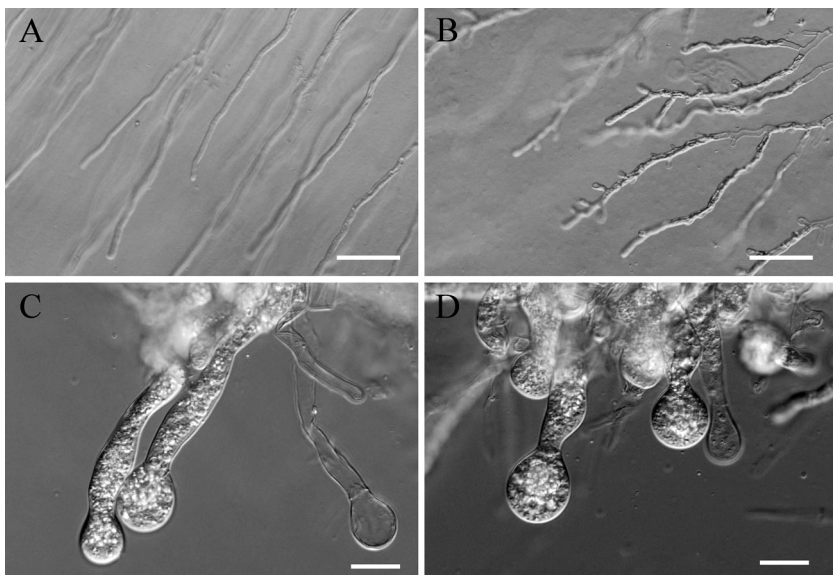

Plate 3. A. *Conidiobolus sinensis*: rarely branched mycelia at the colony edge. B. *C. stromoideus*: moderately branched mycelia at the colony edge. C. *C. sinensis*: long conidiophores. D. *C. stromoideus*: short conidiophores. Bars: A–B = 100 μm, C–D = 20 μm.

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