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Four new records of *Aspergillus* sect. *Usti* from Shandong Province, China

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Abstract—Four new Chinese records of *Aspergillus* species: *Emericella heterothallica*, *Aspergillus calidoustus*, *A. keveii*, and *A. pseudodeflectus* are reported from Shandong Province. *Emericella heterothallica*, despite being uncommonly reported, should be regarded as geographically widely distributed.

Key words — morphology, sequence analysis, taxonomy

Introduction

Raper & Fennell (1965) recognized five species in the *Aspergillus ustus* group for which Gams et al. (1985) later established subgen. *Nidulantes* sect. *Usti*. Kozakiewicz (1989) added two species to this section and removed one species, while Klich (1993) transferred *A. granulosus* Raper & Thom from sect. *Usti* to sect. *Versicolores*. The phenotypic evidence spawned different opinions on phylogenetic relationships.

Peterson (2000) rejected sect. *Usti* based on LSU rDNA sequence data (with low bootstrap support) and placed the species in sect. *Nidulantes*. Houbraken et al. (2007), who revived *A. insuetus* (Bainier) Thom & Church based on multilocus DNA data along with chemical and phenotypic evidence, accepted eight species in sect. *Usti*; Peterson (2008), accepting the species Houbraken et al. included, added seven additional species to the section. Recently, Samson et al. (2011) added five new taxa to this section, enlarging to twenty-one but without including *A. ochraceoroseus* and *A. versicolor*.

Only three species representing sect. *Usti* have thus far been recorded in China: *A. deflectus* Fennell & Raper, *A. puniceus* Kwon-Chung & Fennell, and *A. ustus* (Qi et al. 1997). In the present paper, we report the recovery of four additional members of this section: *E. heterothallica*, *A. calidoustus, A. keveii*,

Table 1. Strains included in molecular phylogenetic analyses.

Species	Strains *	GenBank accession numbers ^		
		ITS1-5.8S-ITS2	Ben A	CaM
Aspergillus amylovorus Panas. ex Samson	NRRL5813 **T**	EF652503	EF652327	EF652415
A. calidoustus	CBS 114380	EF591741	EF591729	EF591716
	CBS 113228	EF591739	EF591730	EF591715
	AS3.15302	JN982696	JN982686	JN982676
A. deflectus	NRRL2206 **T**	EF652437	EF652261	EF652349
A. egyptiacus Moub. & Mustafa	NRRL5920 **T**	EF652504	EF652328	EF652416
A. elongatus J.N. Rai & S.C. Agarwal	NRRL5176 **T**	EF652502	EF652326	EF652414
A. granulosus	NRRL 1932 **T**	EF652430	EF652254	EF652342
A. insuetus	CBS 107.25 **T**	EU076356	EU076371	EU076366
	CBS 119.27	EU076355	EU076372	EU076367
A. keveii	NRRL 1974	EF652432	EF652256	EF652344
	CBS 561.65	EU076352	EU076375	EU076364
	CBS 209.92 **T**	EU076354	EU076376	EU076365
	AS3.15305	JN982704	JN982694	JN982684
A. lucknowensis J.N. Rai et al.	NRRL 3491 **T**	EF652459	EF652283	EF652371
A. minutus Abbott	NRRL 4876 **T**	EF652481	EF652305	EF652393
	NRRL 279	EF652457	EF652281	EF652369
A. pseudodeflectus	NRRL6135 **T**	EF652507	EF652331	EF652419
	NRRL278	EF652456	EF652280	EF652368
	AS3.15306	JN982697	JN982687	JN982677
	AS3.15307	JN982700	JN982690	JN982680
	AS3.15308	JN982699	JN982689	JN982679
	AS3.15309	JN982701	JN982691	JN982681
	AS3.15310	JN982703	JN982693	JN982683
A. puniceus	NRRL 5077 **T**	EF652498	EF652322	EF652410
A. ustus	NRRL 4991	EF652492	EF652316	EF652404
	NRRL 275 **T**	EF652455	EF652279	EF652367
	AS3.15311	JN982695	JN982685	JN982675
	AS3.15312	JN982702	JN982692	JN982682
A. versicolor (Vuill.) Tirab.	NRRL238 **T**	EF652442	EF652266	EF652354
Emericella heterothallica	NRRL 5096 **T**	EF652449	EF652323	EF652411
	NRRL 5097	EF652500	EF652324	EF652412
	AS3.15313	JN982698	JN982688	JN982678

* Ex-type strains are indicated with 'T'.

^ Sequences JN982675–JN982704 were obtained in the present study.

and *A. pseudodeflectus*. All were isolated from soil samples collected at the foot of Mount Tai in Shandong Province, located in the monsoon area of moderate-temperate zone of China (36°15′17″N 117°06′15″E) in late July 2011. The average altitude of that area is 134 m with an atmospheric pressure of 1004.1

hPa; the annual average temperature is 12.8 °C, with the monthly average of –1.4 °C in January and 26.5 °C in July; the soil is typically frozen from late October to late March; the frost-free period is about 200 days from late March to late September; the annual precipitation is 700 mm (http://www.weather.com.cn).

Materials & methods

Soil samples were collected underneath leaf litter and kept in sterilized plastic bags. The fungi were isolated through dilution plating (Malloch 1981) using Dichloran Rose Bengal Chlortetracycline agar (King et al. 1979) as the selective medium. Ten sect. *Usti* strains were obtained and deposited in the China General Microbiological Culture Collection Center, Institute of Microbiology, Chinese Academy of Sciences, Beijing (CGMCC).

Morphological characters were assessed according to Houbraken et al. (2007), Klich (2002), and Raper & Fennell (1965). Color names (shown here in title case) follow Ridgway (1912). Wet mounts were prepared using material from colonies grown on Czapek yeast autolysate agar (CYA) at 25 °C after 14 days mounted in lactophenol without dye (Raper & Fennell 1965). Optical microscopic examination and photography were performed with a Nikon Eclipse 80i and Nikon Digital Sight DS-L1 Microscope (Nikon Co. Ltd., Japan).

Total DNA extraction followed the method of Scott et al. (2000). For amplification of a portion of the β-tubulin gene, we used the primers described by Glass & Donaldson (1995); to amplify the ITS1-5.8S-ITS2 region of the NUC rRNA gene, we used the primers designed by White et al. (1990). To amplify a portion of the calmodulin gene, we used the following primers: cmdAD1 5′-GCC GAC TCT TTG ACT GAA GAG C-3′, cmdAD2: 5′-GCC GAT TCT TTG ACC GAG GAA C-3′ and cmdAD3: 5′-GCC GAT TCT TTG ACC GAA GAA C-3′ (sense primers); cmdQ1: 5′-GCA TCA TGA GCT GGA CGA ACT C-3′ and cmdQ2: 5′-GCA TCA TGA GCT GGA CGA ATT C-3′ (antisense primers). Polymerase chain reaction (PCR) protocols for amplification of the above three gene regions, and the purification and sequencing of PCR products followed the methods of Wang & Zhuang (2007).

Raw sequences were assembled and edited manually with BioEdit 5.0.9 (Hall 1999). Edited sequences were aligned using Clustal X 1.81 (Thompson et al. 1997), and adjusted manually, as needed. Thirty-three strains of sect. *Usti* (TABLE 1) were analyzed using the neighbor-joining (NJ) method and subjected to 1000 bootstrap replicates. All the phylograms yielded the same results (e.g., FIG. 5, based on partial calmodulin gene sequences).

Taxonomy

Emericella heterothallica (Kwon-Chung, Fennell & Raper) Malloch & Cain PL. 1

Colonies on Czapek agar (CA) at 25 °C growing rapidly, Colonies in 14 days reaching 40–43 mm in diam., densely floccose to velvety, radially sulcate, margins entire; Hülle cells aggregated into small white masses visible to the unaided eye, distributed sparsely on the colony surfaces; conidiogenesis sparse, only present in the adjacent areas of colonies, brown in color, similar to Verona

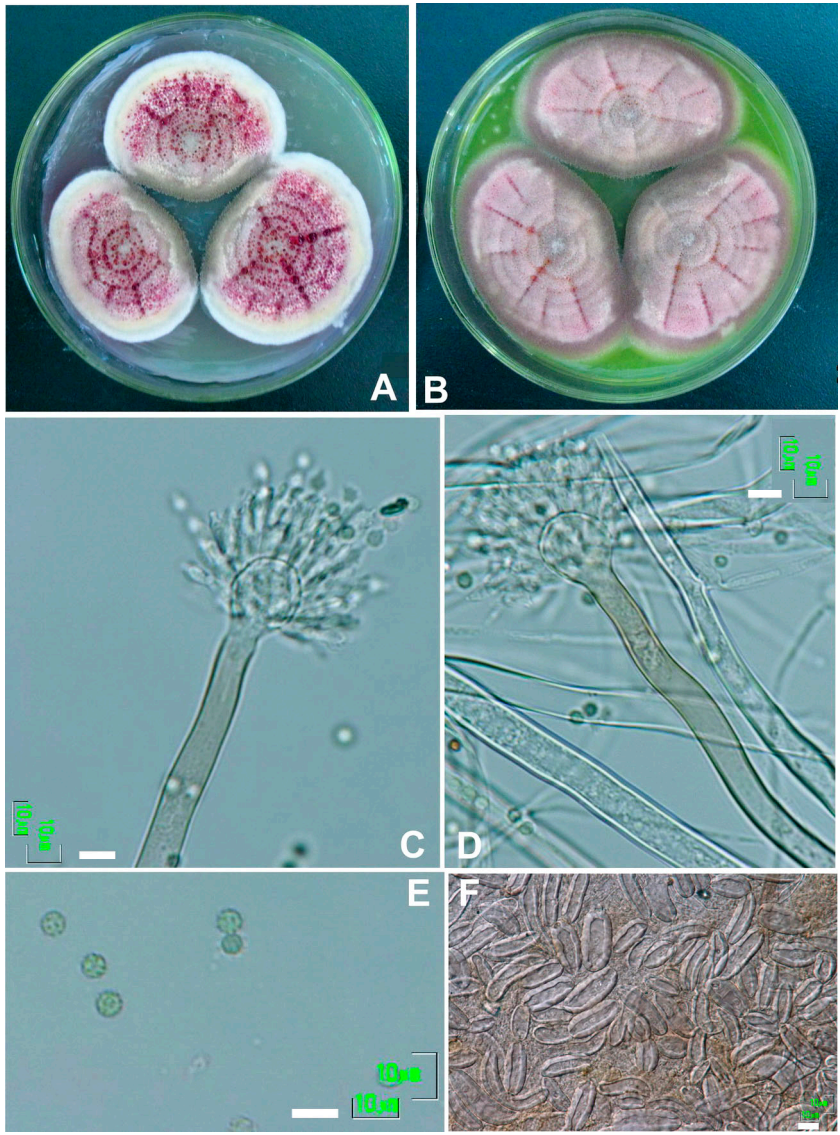

Plate 1. *Emericella heterothallica* (AS3.15313): A–B. colonies on CA, CYA at 25 °C after 14 days; C–D. conidiophores; E. conidia; F. hülle cells. Bar = 10 μm

Brown, mycelia Orange-Cinnamon to Vinaceous-Cinnamon, white at margins; exudate abundant, Brazil Red to Strawberry Pink; soluble pigment abundant, Congo Pink; reverse Indian Red.

Colonies on CYA at 25 °C growing very rapidly, attaining 50–53 mm in diam. in 14 days; texture velvet, radially sulcate with lightly discernable annular plications, margins entire; Hülle cells moderately abundant, aggregated into conspicuous, small white masses, distributed sparsely on the surfaces; conidiogenesis moderate, in marginal areas, Verona Brown; mycelia Cinnamon to Vinaceous-Cinnamon, white at colony peripheries; exudate light yellow brown, moderately abundant; soluble pigment Empire Yellow, moderately abundant; reverse Mars Orange to Orange Rufous.

Conidial heads globose when young, splitting at maturity into 2–3 loosely divergent, short columns, (100–)120–180 µm long; conidiophores arising from substratum or surface hyphae, stipes colorless becoming light brown in upper portion, thin-walled, 240–360(–420) × (5–)7–9(–11) µm; vesicles subglobose to ellipsoidal, thin-walled, 14–18(–22) × 11–14 µm, biseriate; metulae 5–6.5 × 2–3 µm; phialides ampuliform, 6.5–7 µm, with short collula; conidia globose (3–)3.5–5.5 µm, echinulate, brown-colored en masse; Hülle cells ellipsoidal to very elongate, or joined into botuliform chains, thick-walled, commonly 36–45 × 9–16 µm, smaller ones 16–27 µm, longer ones up to 54–63 µm.

Colonies on CYA with 20% sucrose (CY20S) at 25 °C growing very rapidly, reaching 52–55 mm in diam. in 14 days, densely floccose to velvety, radially sulcate with annular plications, margins entire; conidiogenesis absent; mycelia Ochraceous-Buff, white at margins; exudate and soluble pigment absent; reverse Nopal Red.

Colonies on CYA at 37 °C growing moderately, in 14 days attaining 36–38 mm diam.; texture velvety, flat, margins entire; conidiogenesis moderately heavy in central areas, brown-colored near Bister, with a Mikado tinge; mycelia white at margins to Baryta Yellow in submarginal areas; exudate and soluble pigment absent; reverse Lemon Chrome, with Buff Yellow tinge in centers.

Isolate examined: China, Shandong Province, foot of Mount Tai, from soil, 23 July 2011, Z. Yang (CGMCC AS3.15313).

Aspergillus calidoustus Varga, Houbraken & Samson Pl. 2

Colonies on CA at 25 °C growing moderately, colonies attaining 35–40 mm in diam. in 14 days, flat, sparsely floccose, margins fimbriate; conidiogenesis sparse, Light Drab; mycelia white; exudate and soluble pigment absent; reverse white.

Colonies on CYA at 25 °C growing rapidly, attaining 48–49 mm in diam. in 14 days, moderately deep, margins entire, floccose; conidiogenesis heavy, Drab Gray to Light Drab; mycelia white; exudate and soluble pigment absent; reverse Pale Yellow–Green to Light Viridine Green.

Conidial heads small, radiate; conidiophores borne from aerial hyphae, stipes straight, with light yellow tinge, smooth-walled, (130–)150–250(–300)

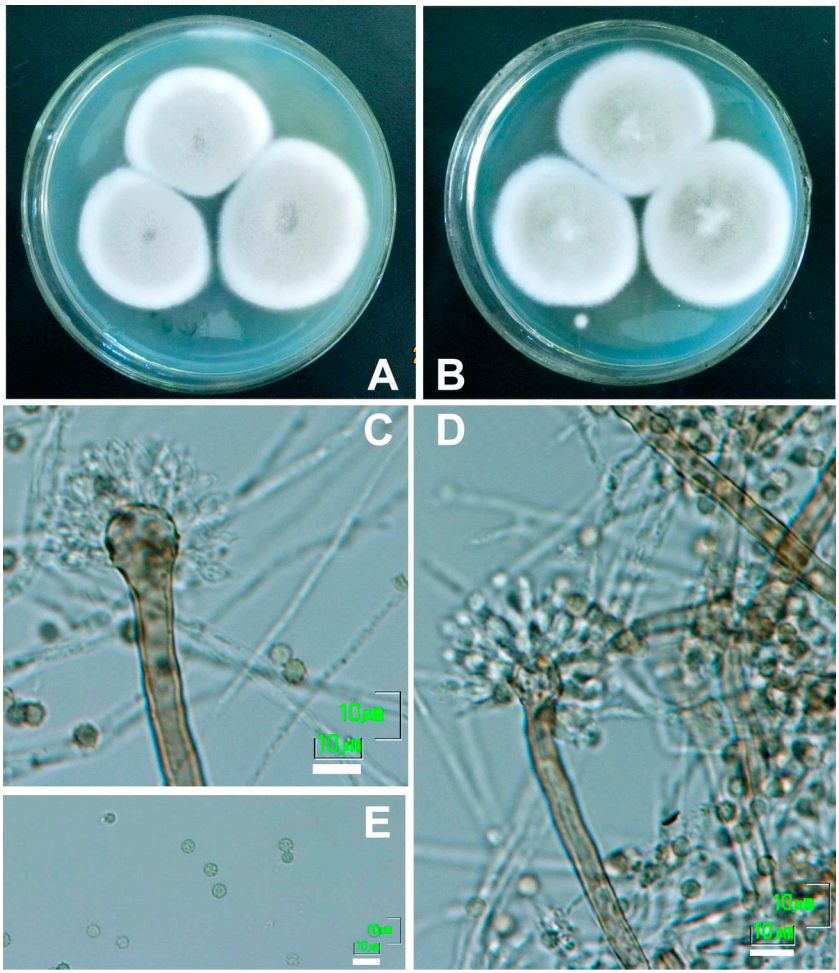

Plate 2. *Aspergillus calidoustus* (AS3.15302): A–B. colonies on CYA, CY20S at 25 °C after 14 days; C–D. conidiophores; E. conidia. Bar = 10 μm

× 5–7 μm; vesicles subglobose, light brown, 9–18(–24) μm, biseriate; metulae very short, 3.5 × 2–3 μm; phialides ampuliform, 7–7.5 × 2–3 μm, with short collula; conidia spheroidal, conspicuously echinulate, 2.5–3.6(–4) μm; Hülle cells not found.

Colonies on CY20S at 25 °C growing rapidly, attaining 46–49 mm in diam. in 14 days, moderately deep, depressed at centers, margins entire, floccose; conidiogenesis heavy, Drab Gray to Light Drab; mycelia white; exudate and soluble pigment absent; reverse Pale Yellow-Green.

Colonies on CYA at 37 °C after 14 days: growing fast, 48–50 mm in diam., plane, centrally umbonate, margins entire, compactly floccose; conidiogenesis heavy, Drab Gray; mycelia white, only conspicuous at margins; exudate and soluble pigment absent; reverse Pale Yellow-Green to Light Viridine Green.

Isolate examined: China, Shandong Province, foot of Mount Tai, from soil, 23 July 2011, Z. Yang (CGMCC AS3.15302).

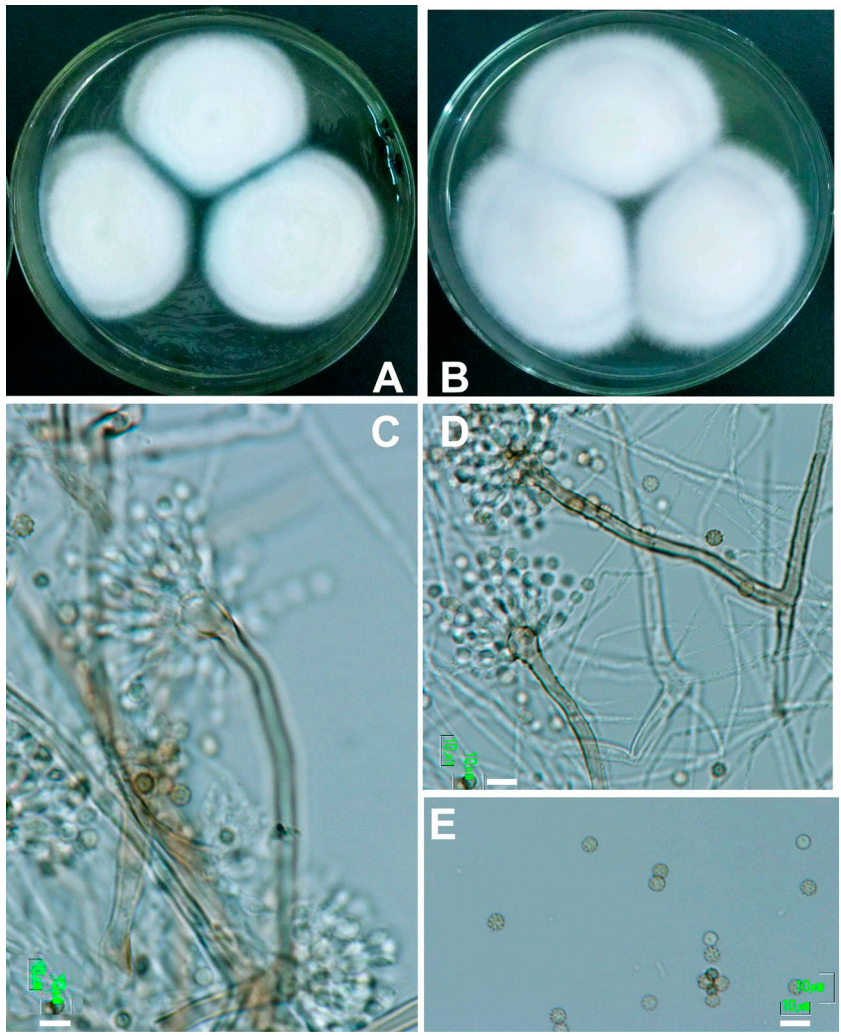

Plate 3. *Aspergillus keveii* (AS3.15305): A–B. colonies on CYA, CY20S at 25 °C after 14 days; C–D. conidiophores; E. conidia. Bar = 10 μm

Aspergillus keveii Varga, Houbraken & Samson PL. 3

Colonies on CA at 25 °C growing moderately, reaching 38–40 mm in diam. in 14 days, flat, sparse, floccose, margins fimbriate; conidiogenesis sparse; mycelia white; exudate and soluble pigment absent; reverse white.

Colonies on CYA at 25 °C growing rapidly, reaching 44–47 mm in diam. in 14 days, deep, margins fimbriate, floccose; conidiogenesis sparse, mostly in the central areas, Light Drab; mycelia white with Pale Brownish Vinaceous tinge; exudate and soluble pigment absent; reverse Pale Ochraceous-Buff.

Conidial heads small, radiate; conidiophores borne from aerial hyphae, stipes smooth-walled, brown in color, 200–280 × 5–7.5 μm; vesicles subglobose, brown-colored, 10–18 μm in diam., biseriate; metulae short, 3.5 × 2–3 μm; phialides ampuliform, 7–7.5 × 2–3 μm, with short collula; conidia spheroidal, conspicuously roughened, 3–3.6(–4) μm; Hülle cells not observed.

Colonies on CY20S at 25 °C growing moderately, reaching 35–38 mm in diam. in 14 days, deep, margins fimbriate, floccose; conidiogenesis sparse; mycelia white with Pale Grayish Vinaceous tinge; exudate and soluble pigment absent; reverse Light Ochraceous-Salmon.

Colonies on CYA at 37 °C showing no growth after 14 days.

Isolate examined: China, Shandong Province, foot of Mount Tai, from soil, 23 July 2011, Z. Yang (CGMCC AS3.15305).

Aspergillus pseudodeflectus Samson & Mouch. PL. 4

Colonies on CA at 25 °C growing moderately, attaining 28–30 mm in diam. in 14 days, deep, flat, floccose; conidiogenesis absent; mycelia white; exudate and soluble pigment absent; reverse white.

Colonies on CYA at 25 °C fast-growing, reaching 43–45 mm in diam. in 14 days, deep, convex, floccose; conidiogenesis moderate, Light Drab; mycelia white; exudate and soluble pigment absent; reverse Marguerite Yellow to Reed Yellow.

Conidial heads small, radiate; conidiophores borne on aerial hyphae, stipes curved and slender, smooth-walled but with occasional concretions, light brown in color, (50–)100–180 × 2–3.5 μm; vesicles hemispherical, brown-colored, 4–7.5 μm in diam., biseriate; metulae very short, 3.5 × 2–3 μm; phialides ampuliform, 7–7.5 × 2–3 μm, with long collula up to 3.5 μm; conidia spheroidal, warty, 2.5–3.6(–4) μm; Hülle cells not observed.

Colonies on CY20S at 25 °C growing rapidly, reaching 42–43 mm in diam. in 14 days, deep, convex with depression at centers, floccose; conidiogenesis moderate, Light Drab to Drab Gray; mycelia white; exudate and soluble pigment absent; reverse Reed Yellow.

Colonies on CYA at 37 °C growing rapidly, reaching 48–50 mm in diam. in 14 days, flat, annularly and radiately plicate, umbonate lightly in centers, floccose;

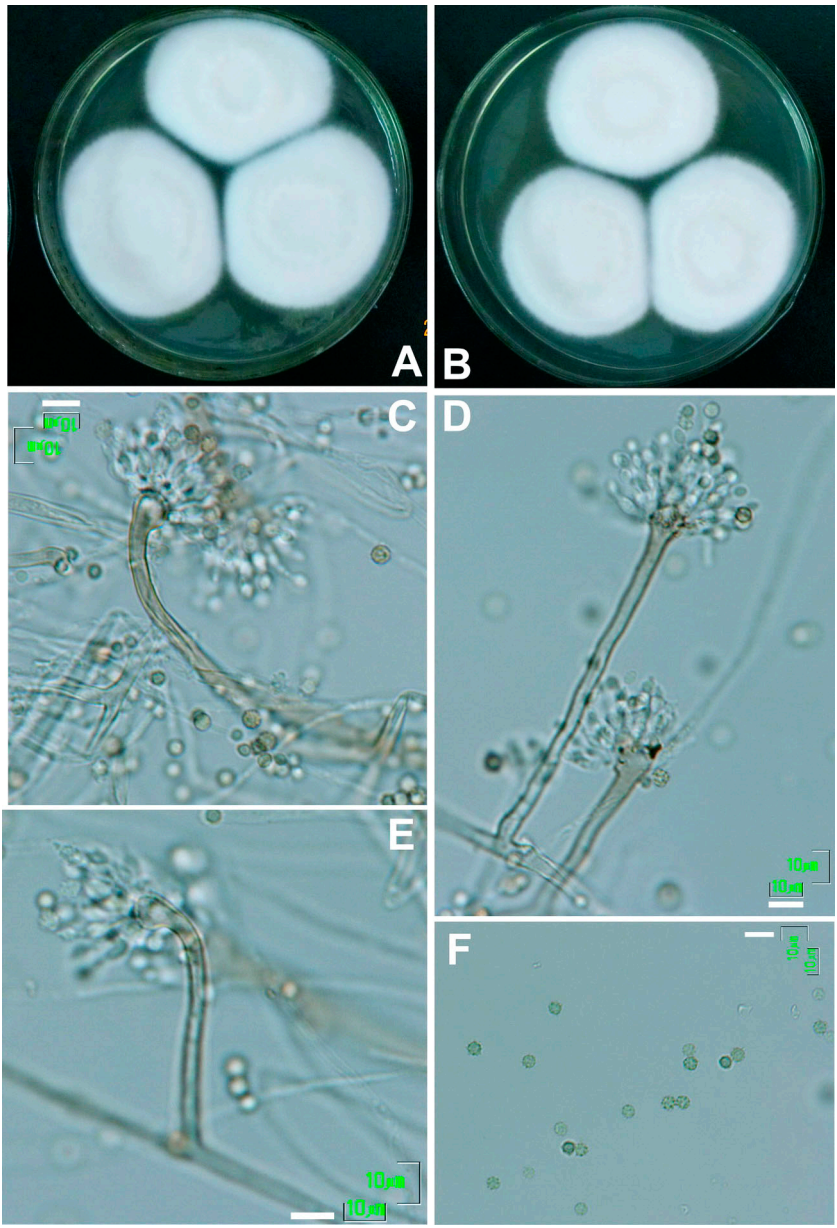

Plate 4. *Aspergillus pseudodeflectus* (AS3.15306): A–B. colonies on CYA, CY20S at 25 °C after 14 days; C–E. conidiophores; F. conidia. Bar = 10 μm

conidiogenesis heavy, Light Cinnamon-Drab to Light Drab; mycelia white; exudate and soluble pigment absent; reverse Dark Olive to Buffy Brown.

Isolate examined: China, Shandong Province, foot of Mount Tai, from soil, 23 July 2011, Z. Yang (CGMCC AS3.1506–3.15310).

Discussion

Emericella heterothallica was the first reported heterothallic teleomorph associated with the aspergilli. Raper & Fennell (1965) reported the production of cleistothecia following the cross of yellow series and reddish orange series. Houbraken et al. (2007), however, were unable to reproduce this result.

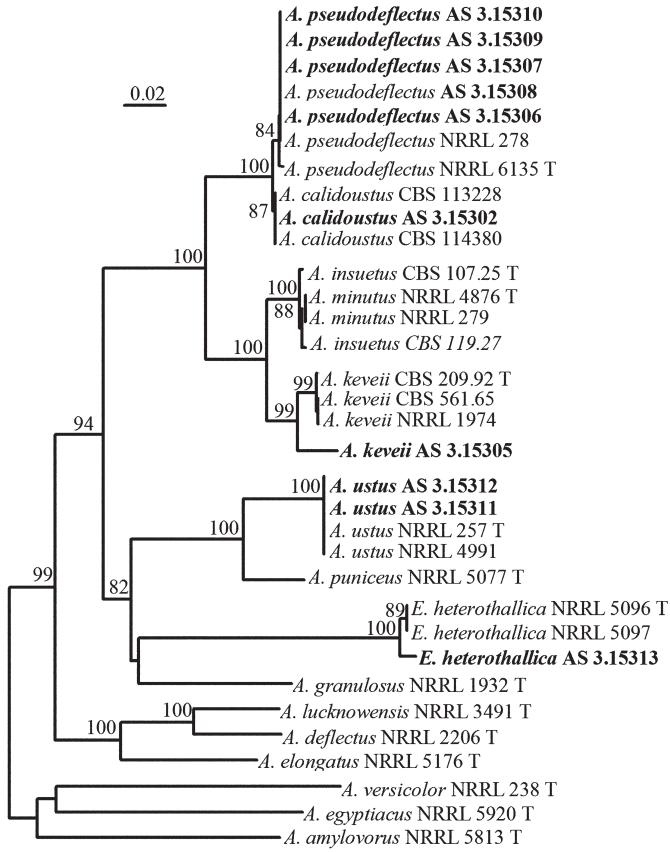

Plate 5. The NJ tree inferred from the partial sequences of calmodulin gene data set. Bootstrap percentages > 70% derived from 1000 replicates are indicated at the nodes. Bar = 0.02 substitutions per nucleotide position.

According to the characters presented, isolate AS3.15313 is a member of the reddish orange series. Although *E. heterothallica* has only been reported rarely, our observation of this species in China suggests that, despite its relative rarity, it is geographically widely distributed.

Raper & Fennell (1965) cited six isolates —WB275 (type culture), WB278, WB280, WB281, WB1974, WB4876— as typical of the six morphotypes they recognized from among the hundreds of *A. ustus* isolates they had examined. It is interesting how predictive of distinct species their observations were.

Based on partial β-tubulin, calmodulin, actin genes, and ITS2-5.8S-ITS2 rDNA sequences, Houbraken et al. (2007) restricted *A. ustus* to isolates WB275 and WB280 and designated WB1974 as a new species, *A. keveii*. Varga et al. (2008) included WB281 in their new taxon, *A. calidoustus*. We observed much variation among our isolates under the name of *A. ustus* sensu Raper & Fennell (1965), with prominent differences in the stipe and vesicle shapes and dimensions. The growth rate at 37 °C varied greatly, some strains rapid, others moderate, still others with no growth at all. Conidiation was also highly variable, ranging from abundant to none depending on the isolates and culture conditions, and conidial color ranged from grey-green to drab and brown. These morphological differences suggest that multiple cryptic taxa may remain embedded in *A. ustus* sensu Raper & Fennell (1965). Phylogenetic analyses of three genetic markers (the NUC ITS1-5.8S-ITS2 rRNA region and the partial β-tubulin and calmodulin gene sequences) affiliate our isolates with the four taxa included in sect. *Usti* by Houbraken et al. (2007) and Samson et al. (2011).

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