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简 报

Two Chinese new records of the genus *Trichoderma* in the Stromaticum clade

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Abstract: [Background] The Stromaticum clade of *Trichoderma* was defined by Samuels et al. in 2012, including 9 species. However, only 3 species, i.e. *T. stromaticum*, *T. vermipilum* and *T. floccosum* were reported in China. **[Objective]** Two *Trichoderma* species, i.e. *T. ivoriense* and *T. barbatum*, are newly recorded in China. **[Methods]** They were collected from Beijing and Shandong by the selective medium THSM, and identified by the translation elongation factor 1 alpha (TEF1-α), RNA polymerase Ⅱ subunit 2 (RPB2) and observation of morphological characteristics. **[Results]** By phylogenetic analyses of TEF1-α and RPB2, two *Trichoderma* strains were close to *T. ivoriense* and *T. barbatum* respectively; however, morphologically they are markedly different from typical species of *T. ivoriense* or *T. barbatum*. Therefore these two strains were identified as *T. ivoriense* and *T. barbatum*, or relative species of them. **[Conclusion]** Two species of the genus *Trichoderma* were newly discovered in China and were described, they belong to the Stromaticum clade. The numbers of species in that clade add up to five.

Keywords: *Trichoderma ivoriense*, *Trichoderma barbatum*, Chinese new record

木霉属 2 个中国新记录种

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摘 要:【背景】木霉菌现存的 Stromaticum 进化支为 Samuels 等 2012 年定义，包括 9 个木霉种；国内目前仅报道子座木霉(*Trichoderma stromaticum*)、蠕状毛木霉(*T. vermipilum*)和絮状木霉(*T. floccosum*) 3 个种。【目的】报道 2 个木霉属中国新记录种。【方法】采用 THSM 选择性培养基，从北京和山东两地土壤中分离木霉菌株，通过形态学特征、TEF1-α 和 RPB2 序列对菌株进行鉴定。【结果】通过对 TEF1-α 和 RPB2 的系统发育分析，2 个菌株分别与 *T. ivoriense* (科特迪瓦木霉)和 *T. barbatum* (毛簇木霉)相近；且形态学特征上存在差异。综合鉴定 2 个菌株分别

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为科特迪瓦木霉(*T. ivoriense*)和毛簇木霉(*T. barbatum*)或其近缘种。【结论】在国内新发现科特迪瓦木霉(*T. ivoriense*)和毛簇木霉(*T. barbatum*)两个木霉种，它们属于 Stromaticum 进化支，该进化支国内木霉种类增加到 5 个。

关键词：科特迪瓦木霉，毛簇木霉，中国新记录种

1 Introduction

Species of *Trichoderma* Pers. 1794 are frequently found in soil, decaying wood, vegetable matter, and other fungi as well[1-2], and have been studied well as biocontrol fungi[3]. With the development of the new biotechnologies, their importance has extended into enzyme production, food industry, paper and pulp treatment, bioremediation, and so forth[4-5].

Formerly *Trichoderma* isolates were identified based on solely morphological characteristics[6]. With more and more *Trichoderma* species have been discovered and described, morphology-based identification is more difficult, therefore the molecular techniques are recommended to confirm the species-level diagnosis of *Trichoderma* isolates. Certain molecular techniques, such as DNA-fingerprinting[7] or the sequence analysis of the ribosomal DNA internal transcribed spacers region (ITS) as well as fragments of genes encoding for the translation elongation factor 1-alpha gene (TEF1-α), endochitinase (CHI18-5), RNA polymerase II subunit (RPB2) and calmodulin (CAL1[8-9] are suitable for a precise diagnosis and thus enable to tackle the problems of morphology-based species identification), Currently, sequence analyses of the translation elongation factor 1-alpha gene and RNA polymerase II subunit (RPB2) have been widely used to study the phylogenetic relationships within *Trichoderma*[2,10], and approximately 275 *Trichoderma* species have been recognized[11-14], TEF1-α and RPB2 became the representative sequences of all the species of *Trichoderma*.

The Stromaticum clade of *Trichoderma* was defined by Samuels et al. in 2012[15], six unlinked genes, TEF1, CHI18-5, CAL1, RPB2, ACT and ITS, were sequenced and used for phylogenetic analysis. TEF1 had the highest number of informative characters with 23%, ITS and ACT datasets were not used for phylogenetic reconstruction of *Trichoderma* species in the Stromaticum clade due to the paucity of phylogenetically informative characters. The Stromaticum clade was finally defined based on a fine-tuned scale, as judged from the low interspecific variation of TEF1 sequences, including 9 species[15].

Therefore, only TEF1-α and RPB2 sequences were used for phylogenetic analysis in this paper. Two Chinese new records of *Trichoderma* were discovered, their morphological characteristics were discussed.

2 Materials and Methods

Soil samples were collected from Beijing and Shandong province of China. *Trichoderma* strains were isolated with a selective medium[16]. Growth-rate trials were done on 9 cm Petri dishes with 20 mL PDA, CMD, MEA and SNA[17] at 15, 20, 25, 30 and 35 °C. Morphological observation of the colonies and conidium-bearing structures were based on isolates grown on PDA, CMD, MEA and SNA medium 2 w in an incubator at 25 °C with alternating 12 h/12 h fluorescent light/darkness. PDA (g/L): potato 200.0, dextrose 20.0, agar 15.0, distilled water 1 000 mL. CMD (g/L): cornmeal 40.0, dextrose 20.0, agar 15.0, distilled water 1 000 mL. MEA (g/L): malt extract 20.0, agar 15.0, distilled water 1 000 mL.

Identification of the *Trichoderma* strain was performed according to phylogenetic analyses (the translation elongation factor 1-alpha and RNA polymerase II subunit) and morphological characteristics. Genomic DNA was extracted using a Fungal Genomic DNA Extraction Kit (Aidlab Biotechnologies Co. Ltd., Beijing, China). The amplification of TEF1-α and RPB2 was performed using the primer pairs EF1-728F[18]/TEF1rev[19] and fRPB2-5f[18]/fRPB2-7cr[20], respectively. PCR program for both genes followed Park et al[21] and Chaverri et al[22]. PCR products were purified and sequenced by ABI3730 gene analyzer at Sangon (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). Sequences have been submitted to TrichoBLAST (www.ISTH.info) or NCBI for the alignment of the sequences of the relative species. Accession numbers for sequences used for phylogenetic analyses were provided, including all the species in the Stromaticum clade. *T. strictipile*

and *T. longipile* in the Strictipile clade were selected as outgroups. Sequences were aligned with MUSCLE[23] and adjusted manually. Gaps were treated as missing data. Phylogenetic trees were completed using MEGA 7 software[24]. Model test was performed to find the best DNA Model for ML analyses. The stability of clades was evaluated by bootstrap tests with 1 000 replications. Bootstrap values above 50% are indicated on the corresponding branches.

Phylogenetic positions of the new species were analyzed with the combined TEF1-α, RPB2 or TEF1-α+RPB2 datasets by MEGA 7[24]. The ML trees reconstructed by using the Maximum Likelihood method based on the Kimura 2-parameter model with the highest log likelihood. Initial tree for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value.

3 Results and Discussion

3.1 Phylogenetic analyses

The phylogenetic tree with TEF1-α, RPB2 or TEF1-α+RPB2 datasets showed the strains BJ598-1 and SD1-13 were distributed in the Stromaticum clade, and were closely related to *T. ivoriense* strain GJS 01-312 (99%, 97% or 99% ML BP) and *T. barbatum* strain GJS 04-308 (92%, 99% or 100% ML BP) respectively (Figure 1−3). Sequence similarity of TEF1-α and RPB2 between the strain BJ598-1 and *T. ivoriense* type

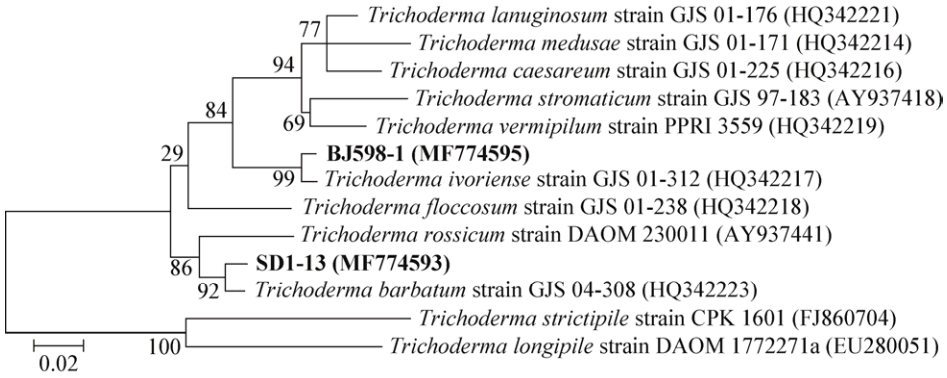

Figure 1 Phylogenetic tree based on the maximum likelihood analysis of the TEF1-α dataset by MEGA 7

图 1 基于菌株 TEF1-α 序列构建的最大似然系统发育树

Note: The accession number is shown in parenthesis; Numbers at the branch points indicated bootstrap values (>50%); The scale bar 0.02 represents 2 nucleotide substitutions per 100 nucleotide.

注：序列的登录号位于圆括号内；系统发育树分支点处的数字表示置信度(>50%)；标尺 0.02 代表 2%的基因序列的进化差异.

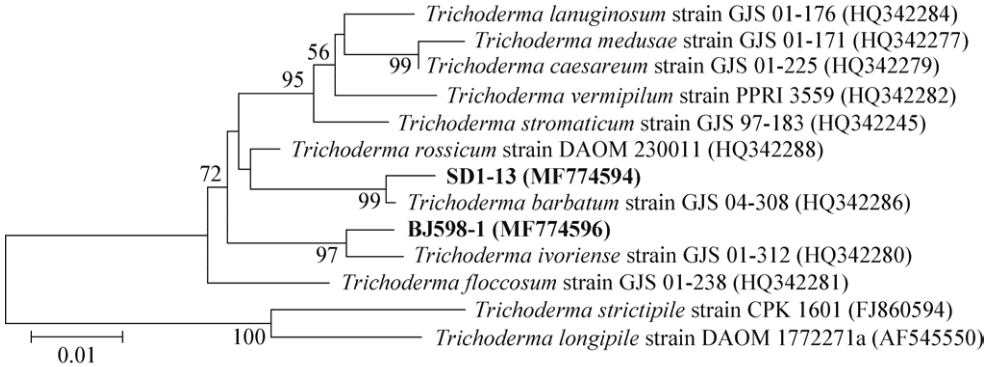

Figure 2 Phylogenetic tree based on the maximum likelihood analysis of the RPB2 dataset by MEGA 7

图 2 基于菌株 RPB2 序列构建的最大似然系统发育树

Note: The accession number is shown in parenthesis; Numbers at the branch points indicated bootstrap values (>50%); The scale bar 0.01 represents 1 nucleotide substitutions per 100 nucleotide.

注：序列的登录号位于圆括号内；系统发育树分支点处的数字表示置信度(>50%)；标尺 0.01 代表 1%的基因序列的进化差异.

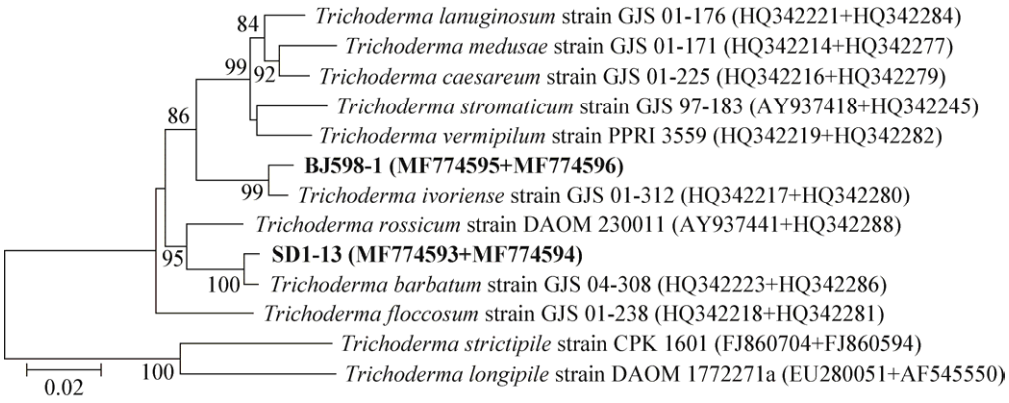

Figure 3 Phylogenetic tree based on the maximum likelihood analysis of the combined dataset (TEF1-α+RPB2) by MEGA 7

图 3 基于菌株 TEF1-α+RPB2 序列构建的最大似然系统发育树

Note: The accession number is shown in parenthesis; Numbers at the branch points indicated bootstrap values (>50%); The scale bar 0.02 represents 2 nucleotide substitutions per 100 nucleotide.

注：序列的登录号位于圆括号内；系统发育树分支点处的数字表示置信度(>50%)；标尺 0.02 代表 2%的基因序列的进化差异.

strain GJS 01-312 were 98% and 99%, therefore the strain BJ598-1 was identified as *T. ivoriense*. Sequence similarity of TEF1-α and RPB2 between the strain SD1-13 and *T. barbatum* strain GJS 04-308 were 98% and 99%, therefore the strain BJ598-1 was identified as *T. barbatum*.

3.2 Taxonomy

***Trichoderma ivoriense* Samuels, Mycol Progress, 11:237, 2012. Figure 4**

Optimal growth at 30 °C, no growth at 35 °C. Colony radius after 72 h at 25 °C on PDA 33−36 mm, on CMD 35−38 mm, on MEA 33−36 mm, on SNA 34−37 mm.

On PDA after 72 h at 25 °C under 12 h photoperiod aerial mycelium sparse, Conidiation typically in compact pustules, white at first and slowly turning bright glaucous, and in concentric rings. No diffusing pigment. Odour often pleasantly aromatic resembling coconut. Conidiophores simple, verticillium-like, branches unpaired, paired or in whorls of 3−4, in acute angles and inclined upwards, few rebranched. Phialides, slender, subulate, [(13.4−15.3)−(21.3−25.5)]×[(2.6−2.8)−(3.3−3.5)] μm (mean 18.3×3.0 μm), base 1.7−2.6 μm (mean 2.2 μm); phialide length/width ratio (4.0−4.7)−(7.6−9.7) (mean 6.2). Conidia, oblong or ellipsoid, smooth-walled, subhyaline to pale green, [(3.7−4.0)−(4.5−4.8)]×[(2.4−2.7)−(2.8−3.2)] μm (mean 4.3×2.8 μm), length/width ratio 1.4−1.6 (mean 1.5). Chlamydospores not observed.

On MEA after 72 h at 25 °C under 12 h photoperiod aerial mycelium sparse, floccose, conidia developing within 48 h beginning at the inoculation point, progressing in distinct concentric rings. Conidiation forming compact green tufts or eventually concrescent near the colony margin; but conidiation in the central effuse and sparse, not aggregated in pustules.

On SNA after 72 h at 25 °C under 12 h photoperiod mycelium growing close to the surface of medium, no aerial mycelium. After 7 d, conidiation forming few pustules around the inoculation point, glaucous. On CMD aerial mycelium sparse, not easy to forming conidiation.

Geographic distribution: Côte d'Ivoire (Holotype)[15] and China (Beijing).

Strains studied: The *Trichoderma* strain BJ598-1 was isolated from the soil of the banks of Miyun Reservoir in Beijing.

Note: TEF1-α sequence of the strain BJ598-1 was found to be closely related to *T. ivoriense* strain GJS 01-312 (98%), with 9 bp differences in 458 bp. The sequence similarity of RPB2 between the strain BJ598-1 and *T. ivoriense* type strain GJS 01-312 was 99%, with 8 bp differences in 1 064 bp. The strain BJ598-1 was identified as *T. ivoriense*.

On SNA conidia of the strain BJ598-1 forming few pustules around the inoculation point, glaucous, different from *T. ivoriense* strain GJS 01-312[15], which conidia forming in pustules in a 2–3 cm broad band around the margin, yellowish green. On PDA

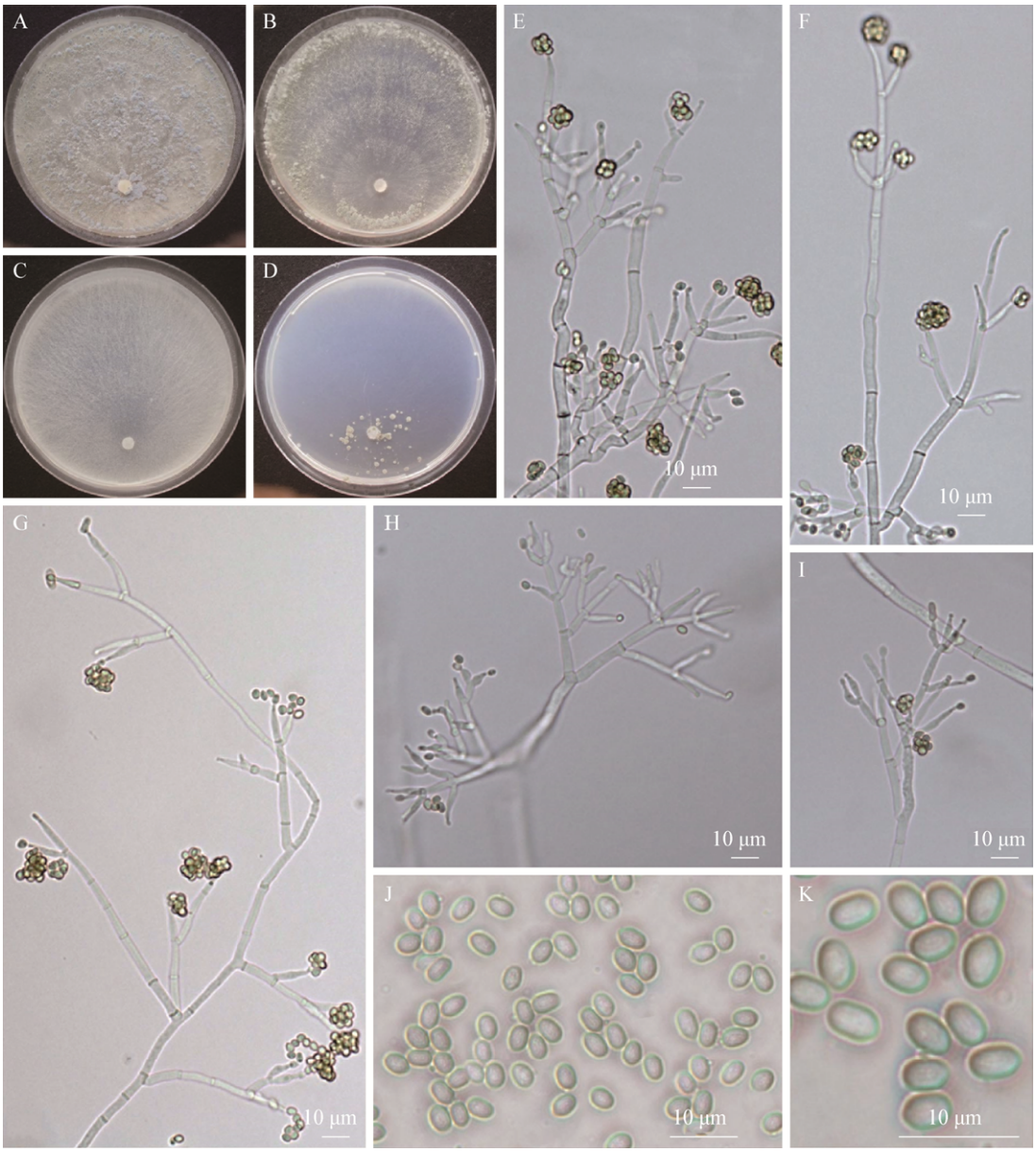

Figure 4 Cultures and asexual morph of *T. ivoriense* BJ598-1

图 4 科特迪瓦木霉 BJ598-1 在培养基上的菌落形态

Note: A−D: Cultures at 25 °C after 7 d on PDA (A), MEA (B), CMD (C) and SNA (D). E−I: Conidiophore-like structures (PDA, 25 °C, 7 d). J−K: Conidia.

注: A: PDA; B: MEA; C: CMD; D: SNA. E−I: PDA 上分生孢子梗及瓶梗(25 °C，7 d). J−K: 分生孢子.

conidiophores of the strain BJ598-1 fertile on the top, no coiling near the tip. Phialides slender, subulate, longer than the phialides of the strain GJS 01-312. Conidia oblong or ellipsoid, also larger than the conidia of the strain GJS 01-312. So the strain BJ 598-1 maybe a close relative species of *T. ivoriense* strain GJS 01-312, or be recognized as a separate species in future.

***Trichoderma barbatum* Samuels, Mycol Progress, 11:233, 2012. Figure 5**

Optimal growth at 30 °C, slow or limited at 35 °C. Colony radius after 72 h at 25 °C on PDA 55−60 mm, on CMD 53−58 mm, on MEA 40−43 mm, on SNA 25−29 mm.

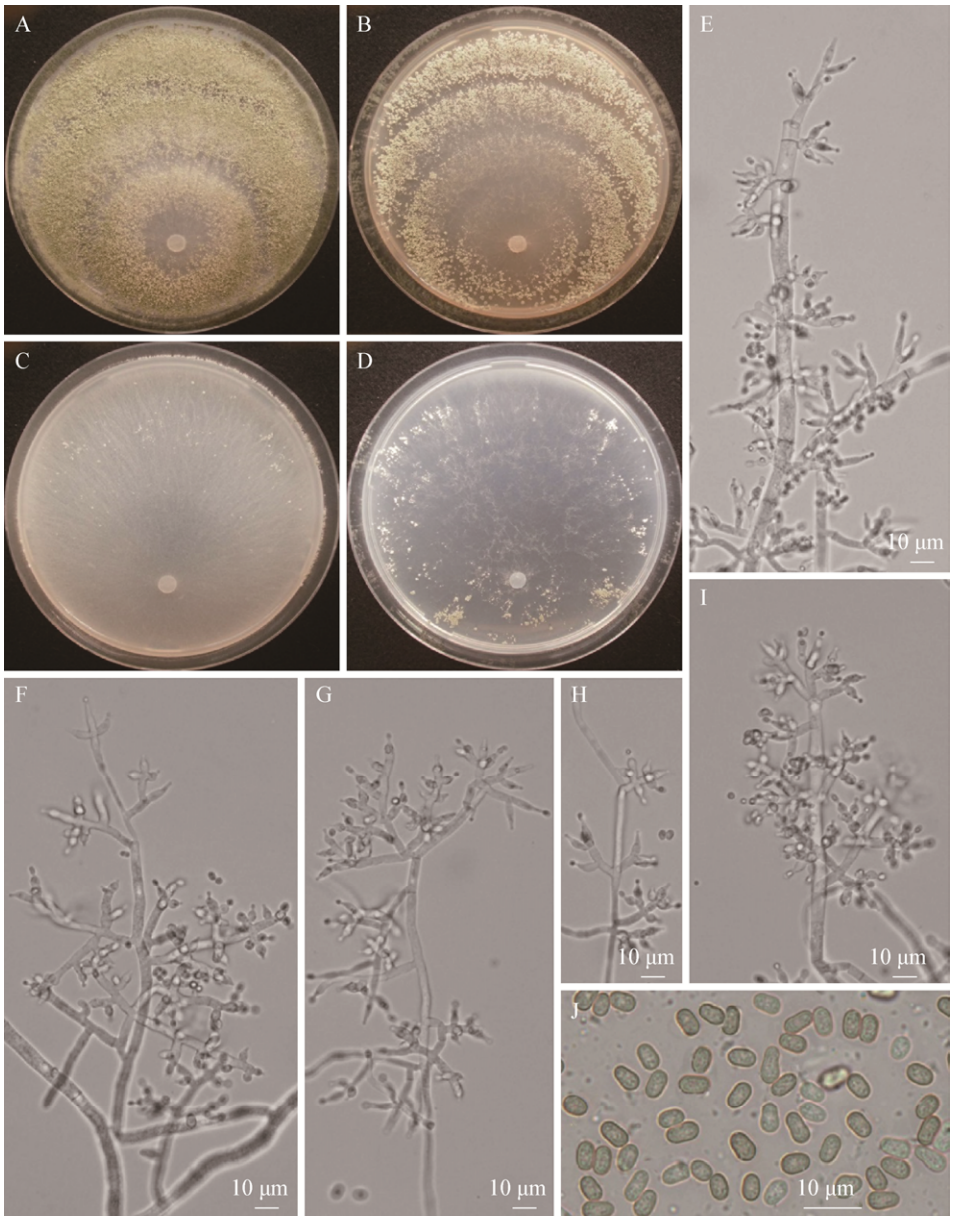

Figure 5 Cultures and asexual morph of *T. barbatum* SD1-13
图 5 毛簇木霉 SD1-13 在培养基上的菌落形态
Note: A−D: Cultures at 25 °C after 7 d on PDA (A), MEA (B), CMD (C) and SNA (D). E−I: Conidiophore-like structures (PDA, 25 °C, 7 d). J: Conidia.
注：A: PDA; B: MEA; C: CMD; D: SNA. E−I: PDA 上分生孢子梗及瓶梗(25 °C，7 d). J: 分生孢子.

On PDA after 72 h at 25 °C under 12 h photoperiod aerial mycelium abundant. Conidiation typically in granular pustules, aggregated in three concentric rings around the inoculation point, bright green. No diffusing pigment. Conidiophores irregularly branched, tree-like; or phialide directly on short branches along the aerial mycelium. Phialides, flask-shaped, [(4.8−8.0)−(15.3−15.9)]×[(3.1−3.6)−(4.3−4.8)] μm (mean 10.4×4.0 μm), base 2.6−3.7 μm (mean 3.0 μm); phialide length/width ratio (1.3−1.9)−(4.4−5.1) (mean 3.3). Conidia, oblong or ellipsoid, usually with pinched sides, sometimes slightly constricted at the middle, green, smooth, [(5.1−5.2)−(5.8−6.1)]×[(3.1−3.4)−(3.7−3.9)] μm (mean 5.5×3.6 μm), length/width ratio 1.4−1.7 (mean 1.6).

On MEA after 72 h at 25 °C under 12 h

photoperiod aerial mycelium sparse, Conidiation typically in granular pustules near the the colony margin, but effuse or in undersized grain in the central region. No diffusing pigment. On SNA and CMD after 72 h at 25 °C mycelium close to the medium and aerial mycelium sparse, conidiation typically in undersized grain, few.

Chlamydospores: not observed.

Geographic distribution: USA (Michigan), Russia (Siberia)[15] and China (Shandong).

Strains studied: The *Trichoderma* strain SD1-13 was isolated from the soil of the vegetable greenhouses in Shandong.

Note: TEF1-α sequence of the strain SD1-13 was subjected to NCBI BLAST, and found to be closely related to *T. barbatum* strain GJS 04-308 (98%); the sequence similarity of RPB2 between the strain SD1-13 and GJS 04-308 was 99%. Pustules of *T. barbatum* with abundant protruding white hairs, tip subacute to acute, sterile, however the hairs of strain SD1-13 not obvious, fertile on the top, frequently branched, different from the typical strain. As a result, the strain SD1-13 was identified as *T. barbatum* or a close relative species of *T. barbatum* strain GJS 04-308.

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