

Five New Species of *Trichoderma* From Wetland Soils in China

Guang Zhi Zhang

Qilu University of Technology <https://orcid.org/0000-0003-1105-2052>

HeTong Yang

Qilu University of Technology

Xin Jian Zhang (✉ zhangxj@sdas.org)

Qilu University of Technology

Fang Yuan Zhou

Qilu University of Technology

Xiao-Qing Wu

Qilu University of Technology

Xue-ying Xie

Qilu University of Technology

Hong-Zi Zhou

Qilu University of Technology

Susu Fan

Qilu University of Technology

Xiao-Yan Zhao

Qilu University of Technology

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Abstract

Trichoderma isolates were collected from wetland soils in different areas of China. Combined analyses of morphological characteristics and phylogenetic analyses by partial translation elongation factor 1 alpha (*TEF1-α*) and RNA polymerase II subunit b (*RPB2*) revealed five new *Trichoderma* species, namely, *Trichoderma macrofasciculatum*, *T. shangrilaense*, *T. nordicum*, *T. vadicola*, and *T. hailarensis*. *T. macrofasciculatum* and *T. shangrilaense* belonging to the Polysporum Clade were isolated from wetland soils collected from Sichuan and Yunnan Provinces. The conidiation of *T. macrofasciculatum* typically appeared in white pustules in concentric rings on PDA or MEA, and its conidia had two or more guttules. Conidiation of *T. shangrilaense* formed white pustules with irregular shape and size, and its conidia were mostly obovoid and smooth. *Trichoderma vadicola*, *T. nordicum*, and *T. hailarensis* belonging to the Viride Clade were collected from Shandong Province, Beijing Municipality, and Inner Mongolia Autonomous Region, respectively. The phialides of *T. nordicum* lageniform were curved on PDA, and its conidia were globose to obovoidal and large. The aerial mycelium of *T. vadicola* formed strands and floccose mat. The colonies of *T. hailarensis* cannot form conidia on PDA, and the conidia of *T. hailarensis* on other media were obovoid and delicately roughened.

Introduction

Trichoderma Pers. species are economically useful fungi in agriculture, industry, and medicine. They are widely used as biofungicides (Mukherjee et al. 2012), plant growth regulators (Harman 2011), and sources of enzymes for industrial utility. Some *Trichoderma* species have great potential applications to remediate soil and water pollution (Tripathi et al. 2013). *Trichoderma* species are cosmopolitan and prevalent components of different ecosystems in a wide range of climatic zones (Kubicek et al. 2008). They are mainly found in natural soils and decaying wood and plant material; in addition, they can be isolated from agricultural habitats, living plants as endophytes, mushroom-related substrata, human as opportunistic pathogens, water-related environments, air, and settled dust (Kredics et al. 2014). Through sequence comparison and phylogenetic analysis, more than 288 *Trichoderma* species have been found and described to date, and new species are constantly being discovered (Bissett et al. 2015; Qin and Zhuang 2016a; Qin and Zhuang 2016b; Qin and Zhuang 2017; Chen and Zhuang 2017; Phookamsak et al. 2019; Crous et al. 2019; Zhang and Zhuang 2019).

Trichoderma resources are abundant in China, but only 107 species of *Trichoderma* have been found and confirmed so far. The research on *Trichoderma* mostly focused on application but lacked resource mining and taxonomy. To this end, we jointly launched a survey of *Trichoderma* resource from different ecological environments, such as farmland, forest, grassland and tidal wetland. Our research team is responsible for the excavation and character analysis of *Trichoderma* resources in wetland soil. In our work, 759 soil samples were collected from 23 provinces of China in 2015–2016, and over 3000 *Trichoderma* strains were isolated on the basis of morphological and cultural characters. Combined with sequence analyses of *TEF1-α* and *RPB2*, 35 species were identified, including 17 species known from China (*T. guizhouense*, *T. hamatum*, *T. citrinoviride*, *T. asperellum*, *T. longibrachiatum*, *T. priscillae*, *T.*

pleurotica, *T. pleurotum*, *T. virens*, *T. brevicompactum*, *T. koningiopsis*, *T. harzianum*, *T. koningii*, *T. pseudokoningii*, *T. viride*, *T. longibrachiatum*, and *T. atroviride*), 13 Chinese new records (*T. crissum*, *T. afroharzianum*, *T. atrobrunneum*, *T. simmonsii*, *T. paratroviride*, *T. polysporum*, *T. oblongisporum*, *T. mediterraneum*, *T. trixiae*, *T. paraviridescens*, *T. viridescens*, *T. barbatum*, and *T. ivoriense*), and 5 new species.

The present study performed the phylogenetic analysis of the five new species of *Trichoderma* to establish their new status. Loci such as the ribosomal DNA internal transcribed spacer (ITS) region and partial genes encoding for the translation elongation factor 1-alpha (*TEF1-a*), endochitinase (*chi18-5*), RNA polymerase II subunit (*RPB2*), and calmodulin (*cal1*) (Kullnig-Gradinger et al. 2002; Druzhinina et al. 2008) were considered for use in phylogenetic analyses. Given their low sequence variability or missing adequate sequence data, ITS, *cal1* and *chi18-5* are rarely used for new species identifications (Jaklitsch and Voglmayr 2015; Zhu and Zhuang 2015; Qin and Zhuang 2016), while *TEF1-a* and *RPB2* facilitate reliable species identifications through phylogenetic analyses (Jaklitsch and Voglmayr 2015; Bissett et al. 2015). These genes have been used in the phylogenetic analysis and identification of new species in recent years (Jaklitsch and Voglmayr 2012; Jaklitsch et al. 2014; Zhu and Zhuang 2015; Qin and Zhuang 2016a; Qin and Zhuang 2017; Qin and Zhuang 2016b; Chen and Zhuang 2017). Both loci are available for the 254 *Trichoderma* species listed by Bissett et al. (2015). Thus, only *TEF1-a* and *RPB2* sequences were used for the phylogenetic reconstruction of the five new species in the present study. The ITS sequences of the new species were submitted to Genbank but not used in phylogenetic analyses.

Materials And Methods

Isolates and specimens.

Specimens were collected from Sichuan, Yunnan, Beijing, Shandong, and Inner Mongolia. *Trichoderma* strains were isolated from soils on THSM (K_2HPO_4 0.90 g; $MgSO_4 \cdot 7H_2O$ 0.20 g; NH_4NO_3 1.0 g; KCl 0.15 g; glucose 3.0 g; Rose Bengal 0.15 g; Agar 15.0 g; distilled water 1.0 L. Following autoclavation, chloromycetin (0.25 g), streptomycin (0.03 g), and pentachloronitrobenzene (0.2 g) were added) (Martin 1950). Ex-type living cultures of new species were deposited in Agricultural Culture Collection of China (ACCC).

Morphological characterizations

Morphological observation of the colonies and conidium-bearing structures was based on isolates grown on PDA (potato dextrose agar, Difco), CMD (Difco cornmeal agar + 2% w/v dextrose), MEA (malt extract agar, Difco) and SNA (Nirenberg 1976) for 2 weeks in an incubator at 25 °C with alternating 12 h/12 h fluorescent light/darkness. Microscopic observations were conducted with an Olympus BX53 microscope and a MicroPublisher 5.0 RTV digital camera (Olympus Corp., Tokyo, Japan). Continuous characters, such as length and width, were measured with the CellSens Standard Image software (Olympus Corp., Tokyo, Japan). Continuous measurements were based on 10–30 measured units and were reported as

the extremes (maximum and minimum) in brackets separated by the mean plus and minus one standard deviation. Color standards were from Kornerup and Wanscher (Kornerup and Wanscher 1978). Growth-rate trials were performed on 9 cm Petri dishes with 20 mL of CMD, PDA, MEA and SNA at 15 °C, 20 °C, 25 °C, 30 °C, and 35 °C. Petri dishes were incubated in darkness up to 1 week or until the colony covered the agar surface. Colony radii were measured daily. Trials were replicated three times.

DNA extraction, polymerase chain reaction (PCR), and sequencing

Strains were grown in 9 cm-diameter Petri dishes containing PDA (potato dextrose agar, Difco). Cultures were incubated at 25 °C for ca. 3–5 days. Genomic DNA was extracted from the mycelial mat harvested from the surface of the broth with the Fungal Genomic DNA Extraction Kit (Aidlab Biotechnologies Co. Ltd., Beijing, China). The amplification of ITS was performed using the primer pair ITS5 and ITS4 (White et al. 1990), for *TEF1- α* , primer pair EF1-728F (Carbone and Kohn 1999) and TEF1-ALLerev (Jaklitsch et al. 2005) was used and for *RPB2*, primer pair fRPB2-5f and fRPB2-7cr (Liu et al. 1999). PCR amplification of each gene was performed as described by Park et al. (2014) and Chaverri et al. (2011). PCR products were purified and sequenced by ABI3730 gene analyzer at Sangon (Sangon Biotech (Shanghai) Co., Ltd.).

Phylogenetic analyses.

TEF1- α and *RPB2* sequences were subjected to TrichoBLAST (www.ISTH.info) or NCBI nucleotide BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify similar sequences of related species. As a result, 19 representative sequences of *Trichoderma* species (Bissett et al. 2015; Zhu and Zhuang 2015; Qin and Zhuang 2016a; Chen and Zhuang 2017) that are closely related to the new species (identity \geq 90%) were chosen for phylogenetic analyses. These similar sequences belong to the *Trichoderma* species in the Viride Clade or Polysporum Clade, and the accession numbers for the sequences are provided in Table 1. *Protocrea illinoensis* and *Protocrea farinose* were selected as outgroups.

Table 1
Strain numbers and Genbank accessions of sequences used for phylogenetic analyses

Species	Clade	Strain	GenBank Accession No.		
			ITS	TEF1- α	RPB2
<i>T. atroviride</i>	Viride	CBS 119499 = Hypo 326	FJ860726	FJ860611	FJ860518
<i>T. caerulescens</i>	Viride	S195 = CBS 130011	JN715589	JN715621	JN715604
<i>T. gamsii</i>	Viride	S488	—	JN715613	KJ665270
<i>T. hailarense</i>	Viride	WT 17901 = ACCC 39711	MH287485	MH287505	MH287506
<i>T. hailarense</i>	Viride	WT 17803	MH606226	MH606229	MH606232
<i>T. junci</i>	Viride	CBS 120926 = Hypo 399	FJ860761	FJ860641	FJ860540
<i>T. macrofasciculatum</i>	Polysporum	WT 37805 = ACCC 39712	MH287487	MH287509	MH287493
<i>T. macrofasciculatum</i>	Polysporum	WT 37810	MH287488	MH287510	MH287494
<i>T. neokoningii</i>	Viride	G.J.S. 04-216	DQ841734	KJ665620	KJ665318
<i>T. nordicum</i>	Viride	WT 13001	MH287483	MH287501	MH287502
<i>T. nordicum</i>	Viride	WT 61001 = ACCC 39713	MH287484	MH287503	MH287504
<i>T. parapiluliferum</i>	Polysporum	CBS 120921	FJ860799	FJ179578	FJ179614
<i>T. paratroviride</i>	Viride	S385 = CBS 136489	—	KJ665627	KJ665321
<i>T. paraviridescens</i>	Viride	CBS 119321 = Hypo 372	DQ677651	DQ672610	KC285763
<i>T. piluliferum</i>	Polysporum	CBS 120927	FJ860810	FJ860674	FJ179615
<i>T. polysporum</i>	Polysporum	C.P.K. 3131	—	FJ860661	FJ860558
<i>T. pruinsum</i>	Polysporum	TC864	—	MF371227	MF371212
<i>T. samuelsii</i>	Viride	S42	JN715593	JN715652	JN715598
<i>T. shangrilaense</i>	Polysporum	WT 34004 = ACCC 39714	MH287489	MH287495	MH287496
<i>T. shangrilaense</i>	Polysporum	WT 40502	MH606224	MH606227	MH606230

Species	Clade	Strain	GenBank Accession No.		
			ITS	TEF1- α	RPB2
<i>T. sinoluteum</i>	Polysporum	8205	KJ783309	KJ634777	KJ634744
<i>T. sphaerosporum</i>	Viride	9755	–	KU529134	KU529145
<i>T. subeffusum</i>	Viride	CBS 120929	FJ860852	FJ860707	FJ860597
<i>T. subviride</i>	Viride	8658	–	KU529131	KU529142
<i>T. vadicola</i>	Viride	WT 10708 = ACCC 39716	MH287491	MH287499	MH287511
<i>T. vadicola</i>	Viride	WT 32801	MH606225	MH606228	MH606231
<i>T. valdunense</i>	Viride	CBS 120923 = Hypo 222	FJ860863	FJ860717	FJ860605
<i>T. viride</i>	Viride	CBS 119325 = Hypo 292	DQ677655	DQ672615	EU711362
<i>T. viridescens</i>	Viride	S452 = CBS132573	–	KC285646	KC285758
<i>Protocrea illinoensis</i>	Outgroups	TFC 96–98	EU703930	EU703905	EU703952
<i>Protocrea farinosa</i>	Outgroups	C.P.K. 3144	EU703917	EU703894	EU703938

Sequences were aligned with MUSCLE (Edgar and Robert 2004) and adjusted manually. Gaps were treated as missing data. Phylogenetic analyses were performed with the individual and combined *TEF1- α* and *RPB2* with MEGA-X software (Kumar et al. 2018). Modeltest was used to find the best DNA Model for ML analyses. The stability of clades was evaluated by bootstrap tests with 1000 replications. Bootstrap values above 50% were indicated on the corresponding branches. Maximum parsimony (MP) analyses were performed with MEGA-X software (Kumar et al. 2018) using 1000 replicates of heuristic search with the random addition of sequences and tree bisection reconnection as the MP search method. All molecular characters were weighted equally and gaps were treated as missing data. Bootstrap proportions were calculated from 1000 replicates, each with 10 replicates of random addition of taxa.

Results

Sequence analyses

Model testing suggested to use the Hasegawa-Kishino-Yano model (HKY; Hasegawa 1985) with gamma distributed substitution rates (HKY + G) for ML analyses of *TEF1- α* , and the Kimura 2-parameter model (GTR; Kimura 1980) with gamma distributed substitution rates (K2 + G) for *RPB2*. The ML trees by the

combined *TEF1- α* +*RPB2* analysis were based on the Tamura–Nei model (GTR; Nei and Kumar 2000) with gamma distributed substitution rates (TN93 + G). The phylogenetic trees from *TEF1- α* , *RPB2*, and *TEF1- α* +*RPB2* analyses are shown in Figs. 1–3, respectively. Sequence alignments and the trees obtained were deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S22754>). The MP analyses using *TEF1- α* , *RPB2*, and *TEF1- α* +*RPB2* datasets resulted in topologically similar trees with minor differences. The asterisks denote branches in conflict with the ML strict consensus trees.

Phylogenetic analyses showed that the Polysporum and Viride Clades containing the new species were well supported. The Polysporum Clade received significant bootstrap supports from analyses of individual partitions (Fig. 1, MLBP/MPBP = 100%/100%; Fig. 2, MLBP/MPBP = 100%/99%) and the combined datasets (Fig. 3, MLBP/MPBP = 100%/100%). The Viride Clade received the same significant bootstrap supports from analyses of individual partitions (Fig. 1, MLBP/MPBP = 96%/100%; Fig. 2, MLBP/MPBP = 100%/99%) and the combined datasets (Fig. 3, MLBP/MPBP = 100%/100%). All the strains of the new species of *Trichoderma* formed a distinct clade. *Trichoderma macrofasciculatum* and *T. shangrilaense* were located in the Polysporum Clade and formed a well-supported clade with the neighboring *T. polysporum* or *T. parapiluliferum*. The strains of *T. macrofasciculatum* were associated but clearly separated from *T. polysporum* (Link) Rifai (Representative strain C.P.K. 3131, Fig. 1, MLBP/MPBP = 98%/98%; Fig. 2, MLBP/MPBP = 76%/57%; Fig. 3, MLBP/MPBP = 98%/85%). *Trichoderma shangrilaense* was closely related to but distinct from *T. parapiluliferum* (B.S. Lu, Druzhinina & Samuels) Jaklitsch & Voglmayr (representative strain CBS 120927, Fig. 1, Fig. 2, Fig. 3, MLBP/MPBP = 100%/100%). *Trichoderma nordicum*, *T. vadicola*, and *T. hailarensense* were all distributed in the Viride Clade, and *T. nordicum* was associated but clearly separated from *T. paratroviride* Jaklitsch & Voglmayr (representative strain S385, Fig. 1, MLBP/MPBP = 68%/<50%; Fig. 2, MLBP/MPBP = 60%/98%; Fig. 3, MLBP/MPBP = 86%/60%). *Trichoderma vadicola* was associated but clearly separated from *T. caeruleascens* (Jaklitsch & Voglmayr) Jaklitsch & Voglmayr (Representative strain S195, Fig. 1, MLBP/MPBP = 89%/87%; Fig. 2, MLBP/MPBP = 99%/99%; Fig. 3, MLBP/MPBP = 100%/99%). *Trichoderma hailarensense* was associated but clearly separated from *T. gamsii* Samuels & Druzhinina (Representative strain S488, Fig. 1, MLBP/MPBP = 69%/88%; Fig. 2, MLBP/MPBP = 58%/<50%; Fig. 3, MLBP/MPBP = 96%/95%).

Taxonomy

Trichoderma hailarensense G.Z. Zhang, sp. nov. Fig. 4

Mycobank: MB 821318

Etymology: “hailarensense” originally found from the Hailer River basin in the Inner Mongolia of China.

Typification: CHINA. Inner Mongolia, Hailar, 618 m, isolated from soil, 17 Sep 2016, G.Z. Zhang (Holotype WT 17901). Ex-type culture ACCC 39711. *TEF1- α* = MH287505, *RPB2* = MH287506.

Teleomorph: Unknown.

Growth optimal at 30 °C, slow at 35 °C on all media. Colony radius after 72 h at 30 °C 53–56 mm on PDA, 54–56 mm on CMD, 33–37 mm on MEA, and 33–36 mm on SNA. Colony radius after 72 h at 35 °C 13–15 mm on PDA, 10–14 mm on CMD, 9–12 mm on MEA, and 10–12 mm on SNA. Aerial mycelia abundant, arachnoid on PDA after 72 h at 25 °C under 12 h photoperiod. Conidiation started around the inoculation point after 7 days on PDA, with relatively few or small conidia. Diffusing pigment or distinctive odor absent. Conidiation started around the inoculation point after 7 days on MEA, forming a few large pustules, cream yellow. On SNA, aerial mycelia were few, forming a few large pustules around the inoculation point in age, cream yellow. Conidiophores and branches narrow and flexuous, tending to be regularly verticillate, forming a pyramidal structure, with each branch terminating in a cruciate whorl of up to 5 phialides. Phialides, lageniform, (8.0–)9.4–13.1(–15.5)×(2.5–)3.0–3.5(–3.6) μm (mean 11.2×3.3 μm), base 1.8–2.5 μm (mean 2.1 μm); phialide length/width ratio (2.33–)2.7–4.4(–5.9) (mean 3.4). Conidia, obovoid, (4.2–)4.3–4.7(–4.9)×(3.4–)3.6–3.9(–4.1) μm (mean 4.5×3.7 μm), length/width ratio 1.1–1.4 (mean 1.2), delicately roughened. Chlamydospores: (7.0–) 7.5–8.2(–8.5)×(6.5–)7.0–7.5(–8.3) μm.

Distribution: China. Inner Mongolia.

Additional specimen examined. CHINA. Inner Mongolia, Hulun Buir, 610 m, isolated from soil, 17 Sep 2016, J.D. Hu (WT17803).

Notes. Phylogenetically *Trichoderma hailarense* is related to *T. gamsii* and *T. neokoningii* in the Viride Clade (Fig. 1), the sequence similarity of *TEF1-α* and *RPB2* with *T. gamsii* was 97%, with 36 and 30 bp differences among 1260 and 1084 bp; sequence similarity with *T. neokoningii* was 96% and 97%, with 50 and 34 bp differences among 1266 and 1084 bp, respectively. Morphologically, colonies of *T. gamsii* and *T. neokoningii* on PDA formed conidia sporadically or in hemispherical pustules, and conidia of *T. gamsii* and *T. neokoningii* were ellipsoidal to oblong, smooth-walled (Jaklitsch et al. 2006); however, colonies of *T. hailarense* did not form conidia on PDA, and conidia of *T. hailarense* on other media were obovoid, delicately roughened, and easily distinguished from those of *T. gamsii* and *T. neokoningii*.

***Trichoderma macrofasciculatum* G.Z. Zhang, sp. nov. Fig. 5**

Mycobank: MB821299

Etymology. “macrofasciculatum” describes the morphological feature of the conidiation, conidiophores aggregated into large fascicles in concentric rings, white.

Typification: CHINA, Sichuan, Nine-Village Valley, 2405 m, isolated from soil, 24 Sep 2016, G.Z. Zhang (Holotype WT 37805), Ex-type culture ACCC 39712. *TEF1-α* = MH287509, *RPB2* = MH287493.

Teleomorph: Unknown.

Growth optimum at 20 °C, slow or limited at 30 °C, absent at 35 °C. Colony radius after 72 h at 25 °C 21–24 mm on PDA, 23–27 mm on CMD, 17–20 mm on MEA, and 12–16 mm on SNA.

Aerial mycelia abundant on PDA and MEA after incubation for 72 h at 25 °C under 12 h photoperiod. Conidiation typically in pustules in concentric rings on PDA, solitary or aggregated, producing a farinose to granular mat. Diameter of pustules up to 2.2 mm, pompon-like, white. Diffusing pigment and distinct odor absent. Conidiation on MEA typically in pustules in concentric rings, pompon-like as on PDA. On CMD, aerial mycelia sparsely developed. Conidiation aggregated in sporadic pustules near the colony margin, white. On SNA, aerial mycelia few, and conidiation not observed. Conidiophores and branches irregularly branched in a dendriform structure, with each branch terminating in a cruciate whorl of up to five phialides. Hyphal septa clearly visible. Phialides, flask-shaped, often curved, (4.9–)5.6–7.8(–8.8)×(2.8–)3.0–3.2(–3.4) μm (mean 6.7×3.1 μm), 1.8–2.6 μm (mean 2.2 μm) near base; phialide length/width ratio (1.5–)1.8–2.4(–2.8) (mean 2.1). Conidia, subglobose to ellipsoid, hyaline, smooth, with one or few guttules, scar indistinct, (2.6–)2.8–3.3(–3.6)×(2.4–)2.5–2.7(–2.9) μm (mean 3.0×2.6 μm), length/width ratio 1.0–1.3 (mean 1.2), Chlamydospores not observed.

Distribution: China, Sichuan province.

Additional material examined: CHINA, Sichuan, Nine-Village Valley, 2405 m, isolated from soil, 24 Sep 2016, G.Z. Zhang (WT 37810).

Notes: Phylogenetically *Trichoderma macrofasciculatum* WT 37805 is related to *T. polysporum* represented by C.P.K. 3131 in the “Polysporum” clade (Fig. 1), but the sequence similarities of *TEF1-a* and *RPB2* between these species were only 93% and 96%, with 94 and 41 bp differences among 1311 and 1152 bp. *Trichoderma macrofasciculatum* cannot grow at 35 °C as *T. polysporum*, and the former forms large and white pustules in concentric rings at 25 °C, elongations were rarely observed and conidia had few guttules, which are distinct from *T. polysporum* (Lu et al. 2004).

***Trichoderma nordicum* G.Z. Zhang, sp. nov. Fig. 6**

Mycobank: MB8212301

Etymology: “nord” means found in the nord of China.

Holotype: CHINA, Beijing, Yu-yuan-tan Park, 43 m, isolated from soil, 27 Oct 2016, G.Z. Zhang (Holotype WT 61001), Ex-type culture ACCC 39713. *TEF1-a* = MH287503, *RPB2* = MH287504.

Teleomorph: Unknown.

Growth optimal at 25 °C, slow or limited at 30 °C, absent at 35 °C. Colonies grew fast on PDA, CMD, and MEA and slow on SNA. Colony radius after 72 h at 25 °C 67–71 mm on PDA, 68–71 mm on CMD, 51–55 mm on MEA, and 21–24 mm on SNA. Aerial mycelia sparse on PDA after 72 h at 25 °C under 12 h photoperiod, and conidiation developed within 48 h beginning at the inoculation point and progressed around, grey white at first and slowly turning green. Diffusing pigment or distinctive odor absent. Aerial mycelia sparse and flocculence on MEA after 72 h at 20 °C under 12 h photoperiod. Conidia developed within 48 h beginning near the colony margin on MEA, grey white at first and slowly turning green,

transparent liquid secreted. Aerial mycelia few on SNA and CMD after 72 h at 25 °C, conidia formed around the inoculation point and in distinct concentric rings after 96 h under 12 h photoperiod on SNA and CMD, diffusing pigment not produced.

Conidiophores and branches narrow and flexuous, tending to be regularly verticillate forming a pyramidal structure, each branch terminating in a cruciate whorl of up to five phialides. Phialides, lageniform, (6.2–)7.2–10.3(–12.9)×(2.6–)2.9–3.2(–3.4) μm (mean 8.8×3.1 μm), 1.6–2.3 μm (mean 1.9 μm) near base; phialide length/width ratio (2.1–)2.4–3.4(–4.3) (mean 2.9). On PDA, phialides curved, distinguished from those on other media. Conidia, globose to obovoidal, (4.1–)4.4–4.8(–5.0)×(4.0–)4.1–4.4(–4.6) μm (mean 4.6×4.3 μm), length/width ratio 1.0–1.2 (mean 1.1). Chlamydospores sometimes present, (8.7–)9.8×10.4(–12.5) μm.

Distribution: China, Beijing and Hebei.

Additional specimen examined: China. Hebei, Bai-yang lake, 19 m, isolated from soil, 15 Sep 2016, J.S. Li (WT 13001).

Notes: Phylogenetically *T. nordicum* strain WT 13001 is related to *T. paratroviride* in the Viride clade (Fig. 1), but the sequence similarities of *TEF1-α* and *RPB2* between these species were 94% (95%) and 98% (98%), respectively. Morphologically, conidiophores of *T. paratroviride* simply branched, conidia globose, excretions common on PDA. Conidiophores of *T. nordicum* were more complexly branched; conidia globose to obovoidal, larger than those of *T. paratroviride*; transparent liquid secreted on MEA, easily distinguished from that of *T. paratroviride* (Jaklitsch et al. 2015).

***Trichoderma shangrilaense* G.Z. Zhang, sp. nov. Fig. 7**

Mycobank: MB 821300

Etymology: “shangrilaense” was originally found at Shangrila in Yunnan Province of China.

Typification: China. Yunnan, Pudacuo National Park, 3611 m, isolated from soil, 21 Jun 2016, G.Z. Zhang (Holotype WT 34004), Ex-type culture ACCC 39714. *TEF1-α* = MH287495, *RPB2* = MH287496.

Teleomorph: Unknown.

Growth optimal at 20 °C, slow and limited at 25 °C, and absent at 30 °C or 35 °C. Colony radius after 72 h at 20 °C 19–21 mm on PDA, 23–24 mm on CMD, 19–21 mm on MEA, and 8–11 mm on SNA.

Aerial mycelia were abundant, compact on PDA after 7 days at 20 °C under 12 h photoperiod, conidiation not easy formed, and a yellow diffusing pigment developed near the inoculation point; after 14 days, conidiation formed pustules that were unequal in size and white. Conidiophores and branches narrow and flexuous, forming a dendriform structure, and irregularly branched, not rebranched, main axis to 4.3–5.0 μm wide, fertile to apex. Phialides, flask-shaped, often curved, (4.5–)5.7–9.0(–11.1)×(2.9–)3.2–3.5(–4.1) μm (mean 7.4×3.4 μm), 1.6–3.4 μm (mean 2.6 μm) near base; phialide length/width ratio (1.5–)2.0–

2.6(–3.0) (mean 2.3). Conidia, obovoid to ellipsoil, smooth, (3.3–)3.5–4.0(–4.4)×(2.8–)3.0–3.3(–3.5) μm (mean 3.8×3.19 μm), length/width ratio 1.1–1.4 (mean 1.2). Chlamydo spores not observed.

Colony radius 28–33 mm, aerial mycelia abundant and floccose after 7 days at 20 °C under 12 h photoperiod. Conidiation slow to develop on MEA. After about 14 days, pompon-like, white fascicles developed. No diffusing pigment observed. On CMD after 7 days at 20 °C under 12 h photoperiod, colony radius 28–33 mm, aerial mycelia few. Conidiation formed flat or cushion-shaped pustules near the colony margin after 21 days, and a yellow diffusing pigment developed near the inoculation point. On SNA after 7 days at 20 °C under 12 h photoperiod, colony mycelia sparse, and no conidiation formed. After 10 days, pustules scattered around the periphery of the colony. Diffusing pigment not developed.

Distribution: China. Yunnan and Sichuan.

Additional specimen examined: CHINA. Sichuan, Huanglong nature reserve, 3561 m, isolated from soil, 25 Sep 2016, Z. Li (WT 40502).

Notes: *Trichoderma shangrilaense* is related to *T. parapiluliferum* (CBS 120921) in the Polysporum Clade (Fig. 1). The sequence similarity of *TEF1-a* between these two species is only 96%, but the sequence similarity of *RPB2* between these two species was to 99%. The sequence similarity of *TEF1-a* with the ex-type culture G.J.S. 91-60 (GenBank Accession No. AY937444) was only 92%. Optimum temperature for growth of *T. shangrilaense* was 20 °C, no growth occurred at 30 °C as in *T. parapiluliferum*, and conidiation structures consist of flat or cushion-shaped pustules formed near the colony margin on MEA, SNA, and CMD. *Trichoderma parapiluliferum*, conidiophore main axis with conspicuous spiral sterile apical elongations, conidia ellipsoidal to oblong (Lu et al. 2004). *Trichoderma shangrilaense*, conidiophore main axis fertile to apex, conidia obovoid to ellipsoid, easily distinguished from that of *T. parapiluliferum*.

***Trichoderma vadicola* G.Z. Zhang, sp. nov. Fig.8**

Mycobank: MB 821316

Etymology: “vadicola,” from the Latin word, reflects the ecological environment.

Typification: China. Shandong, 2 m, isolated from soil, 13 Aug 2016, G.Z. Zhang (Holotype WT 10708), Ex-type culture ACCC 39716. *TEF1-a* = MH287499, *RPB2* = MH287511.

Teleomorph: Unknown.

Growth optimal at 25 °C, absent at 35 °C on all media. Colony radius after 72 h at 25 °C 25–29 mm on PDA, 24–27 mm on CMD, 23–26 mm on MEA, and 22–26 mm on SNA.

Aerial mycelia abundant on PDA after 72 h at 25 °C under 12 h photoperiod, forming strands and floccose mat. Conidiation not formed or relatively few. No diffusing pigment or distinctive odor was produced. On MEA after 72 h at 25 °C under 12 h photoperiod, aerial mycelia abundant, floccose. After 7

days, mycelia covered the plate, and conidia appeared, effuse, granuliform. On CMD after 72 h at 25 °C under 12 h photoperiod, aerial mycelia not observed. After 7 days, mycelia covered the plate, and conidia developing near the colony margin. On SNA after 72 h at 25 °C under 12 h photoperiod, aerial mycelia not observed. After 7 days, mycelia covering the plate, aerial mycelia floccose, and conidia formed, effuse.

Conidiophores and branches tending to be regularly verticillate formed a pyramidal structure, each branch terminating in a cruciate whorl of 3–5 phialides. Phialides, lageniform, (8.3–)9.9– 12.3(–15.1) × (2.0–)2.6–3.2(–3.4) μm (mean 11.1×2.9 μm), 1.1–2.9 μm (mean 1.9 μm) near base; phialide length/width ratio (2.7–)3.2–4.6(–6.6) (mean 3.9). Conidia, subglobose or obovoidal, (3.5–)3.7–4.3(–4.8) × (3.2–)3.4– 3.6(–3.8) μm (mean 4.0×3.5 μm), length/width ratio 1.0–1.3 (mean 1.1). Chlamydospores rare.

Distribution: CHINA. Shandong and Yunnan.

Additional specimen examined: CHINA. Yunnan, Shangri-La, Pudacuo National Park, 3551 m, isolated from soil, 21 Sep 2016, H.T. Yang (WT 32801)

Notes: Phylogenetically, *Trichoderma vadicola* is related to *T. caerulescens* in the Viride Clade (Fig. 1), but the sequence similarity of *TEF1-a* and *RPB2* between these species was all 95%, with 62 and 60 bp differences among 1218 and 1130 bp, respectively. Morphologically, colonies of *T. vadicola* and *T. caerulescens* on PDA have similar features, such as abundant aerial hyphae, forming strands and a whitish hairy or floccose mat. However, the former *Trichoderma vadicola* formed no or relatively few conidia, and the latter forming grayish bluish patches around the plug. On CMD, *T. caerulescens* peculiar grayish blue pigment formed after 1–2 months, and conidiophores simply or slightly branched (Jaklitsch et al. 2012); the former had no observed diffusing pigment, and conidiophores complexly branched in pyramidal structure or tree-like.

Discussion

In this paper, five new species of *Trichoderma* were described from wetland soils. An ML tree was reconstructed based on individual *TEF1-a* and *RPB2* and the combined *TEF1-a + RPB2* dataset among 24 *Trichoderma* taxa to explore the taxonomic positions of the new species. Our phylogenetic analyses showed that the five new *Trichoderma* species belong to the Polysporum Clade or the Virde Clade and form a five branch in the ML or MP tree.

T. macrofasciculatum and *T. shangrilaense* belong to the Polysporum Clade (Figs. 1–3). This clade (formerly *Trichoderma* section Pachybasium) was first defined by Bissett (1991), including 20 species. However, molecular phylogeny has shown that it is paraphyletic (Kullnig-Gradinger et al. 2002; Kindermann et al. 1998) and the species composition was adjusted successively for the next two decades (Lu et al. 2004; Jaklitsch et al. 2005; Jaklitsch et al. 2006; Samuels et al. 2006; Jaklitsch et al. 2008). Accordingly, this phylogenetic clade is called Polysporum Clade (formerly pachybasium core group), which includes 14 species (Qin and Zhuang 2016; Jaklitsch 2011). Here, we added two new

species, *T. macrofasciculatum* and *T. shangrilaense*, which are close to *T. polysporum* (MLBP = 100%) or *T. parapiluliferum* (MLBP = 100%). Morphologically, species in this clade are heterogeneous, comprising teleomorphs with upright, stipitate, or small pulvinate stromata. The teleomorphs of *T. macrofasciculatum* and *T. shangrilaense* have not been founded at present, but their asexual characteristics, such as conidiation in white pustules, resemble other species in this clade. *T. nordicum*, *T. vadicola*, and *T. hailarensis* belong to the Virde Clade (formerly section *Trichoderma*) (Figs. 1–3). This clade contains one of the most difficult groups of species (Jaklitsch and Voglmayr 2015). Morphologically, all asexual morphs have green conidia that are often warted. Up to now, this large clade has 69 species to be confirmed and described, among which 52 species have been well located in the six subclades (*Hamatum/Asperellum*, *Koningii*, *Neorufum*, *Rogersonii*, *Viride*, and *Viridescens*), and 17 species have not been located in the unnamed branches. Here, we added three new species, *T. nordicum*, *T. vadicola*, and *T. hailarensis*, which are all located in the unnamed branches and close to *T. paratroviride*, *T. caerulescens*, *T. gamsii*, and *T. neokoningii* (MLBP = 92%, 97%, 84%). Phenotypically, the three new species have green conidia, only *T. hailarensis* has coarsely warted conidia, and two other species are smooth-walled.

Declarations

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Author contribution

Conceptualization: Guang Zhi Zhang and He Tong Yang; Methodology: Guang Zhi Zhang, Xin Jian Zhang, Fang Yuan Zhou and Hong Zi Zhou; Formal analysis and investigation: Guang Zhi Zhang and Xue Ying Xie; Writing—original draft preparation: Guang Zhi Zhang and Xiao Yan Zhao; Writing—review and editing: Guang Zhi Zhang and Su Su Fan; Funding acquisition: Guang Zhi Zhang, Xin Jian Zhang and Xiao Qing Wu; Resources: He Tong Yang, Guang Zhi Zhang, Xin Jian Zhang and Xiao Qing Wu; Supervision: He Tong Yang and Xin Jian Zhang. All authors have read and agreed to the published version of the manuscript.

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Data availability

The authors confirm that the data supporting the findings of this study are available within the article. Sequence data generated for the present study have been deposited in GenBank with the accession

numbers MH287505, MH287506, MH606229, MH606232, MH287509, MH287493, MH287510, MH287494, MH287501, MH287502, MH287503, MH287504, MH287495, MH287496, MH606227, MH606230, MH287499, MH287511, MH606228, MH606231. Sequence alignments and the trees obtained were deposited in TreeBASE (<http://purl.org/phylo/treebase/phyloids/study/TB2:S22754>).

Conflict of interest

The authors declare no competing interests.

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Figures

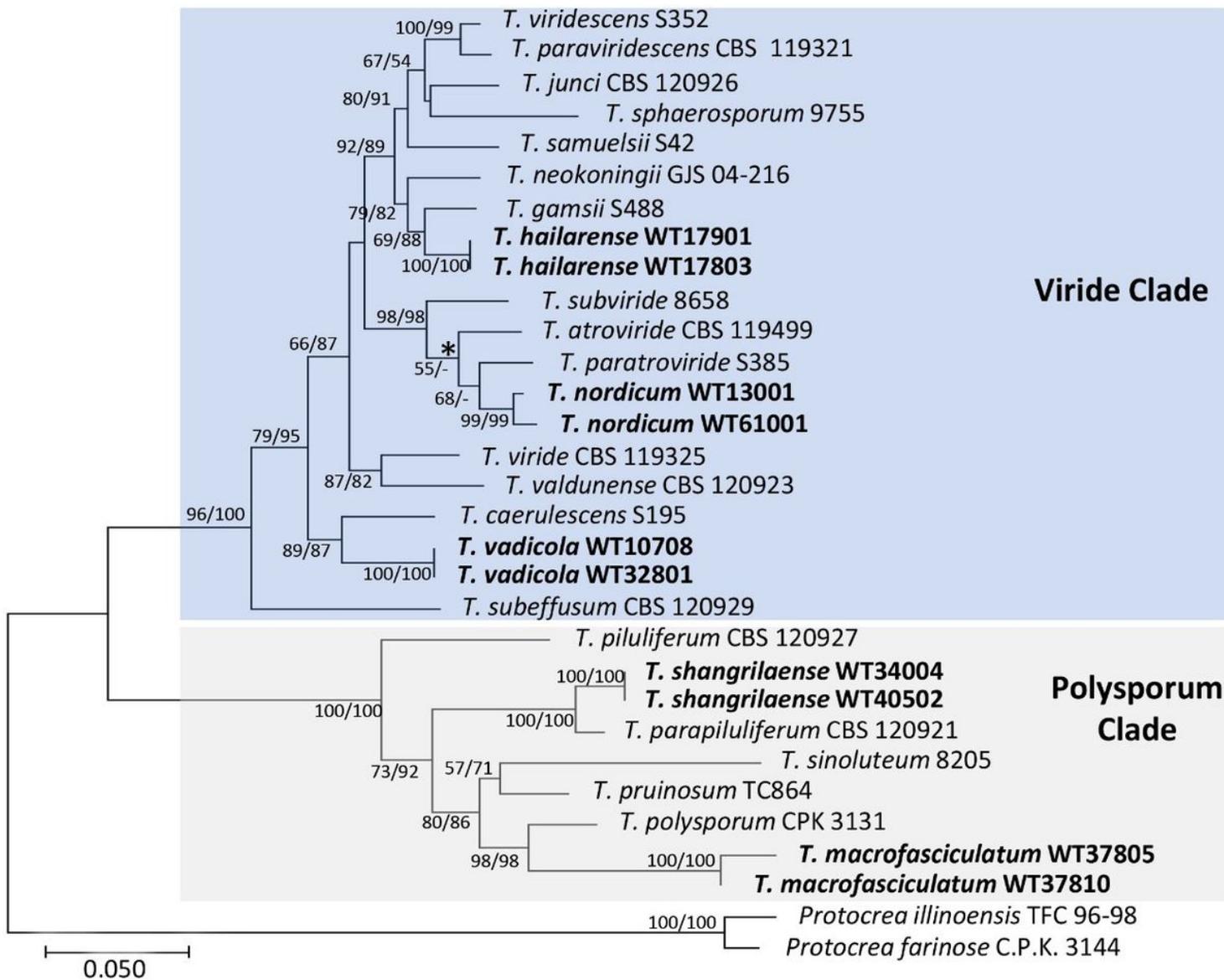


Figure 1

Phylogenetic tree based on the maximum likelihood (lnL=-8884.57) analysis of the TEF1- α dataset. Maximum likelihood bootstrap values (left) and MPBP (right) above 50% are indicated for branches. The tree is rooted with *Protocrea illinoensis* and *Protocrea farinose*. New species proposed here are indicated in boldface. Asterisks denote branches in conflict with the MP strict consensus tree.

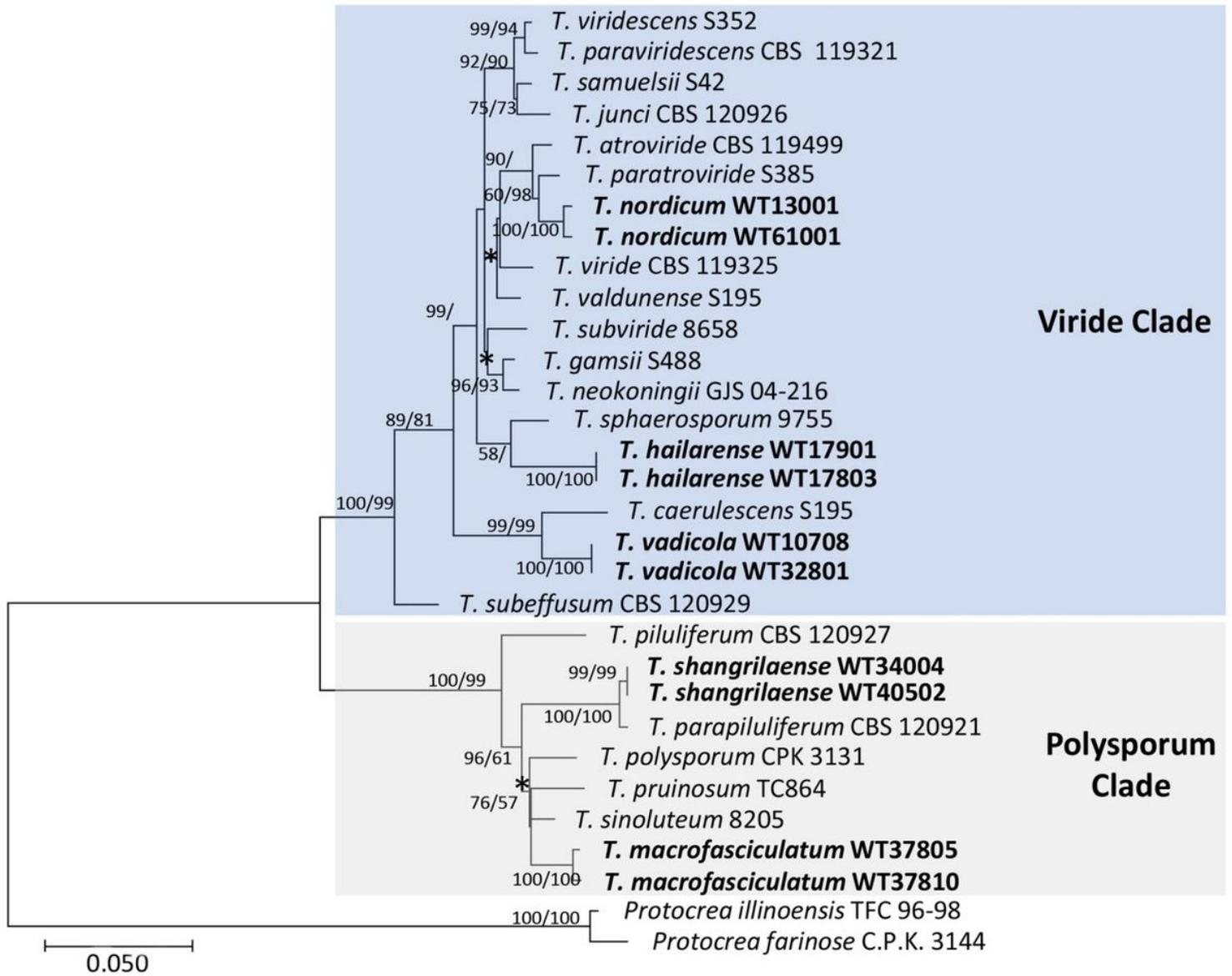


Figure 2

Phylogenetic tree based on the maximum likelihood (lnL=-6740.61) analysis of the RPB2 dataset. Maximum likelihood bootstrap values (left) and MPBP (right) above 50% are indicated for branches. The tree is rooted with *Protocrea illinoensis* and *Protocrea farinose*. New species proposed here are indicated in boldface. Asterisks denote branches in conflict with the MP strict consensus tree.

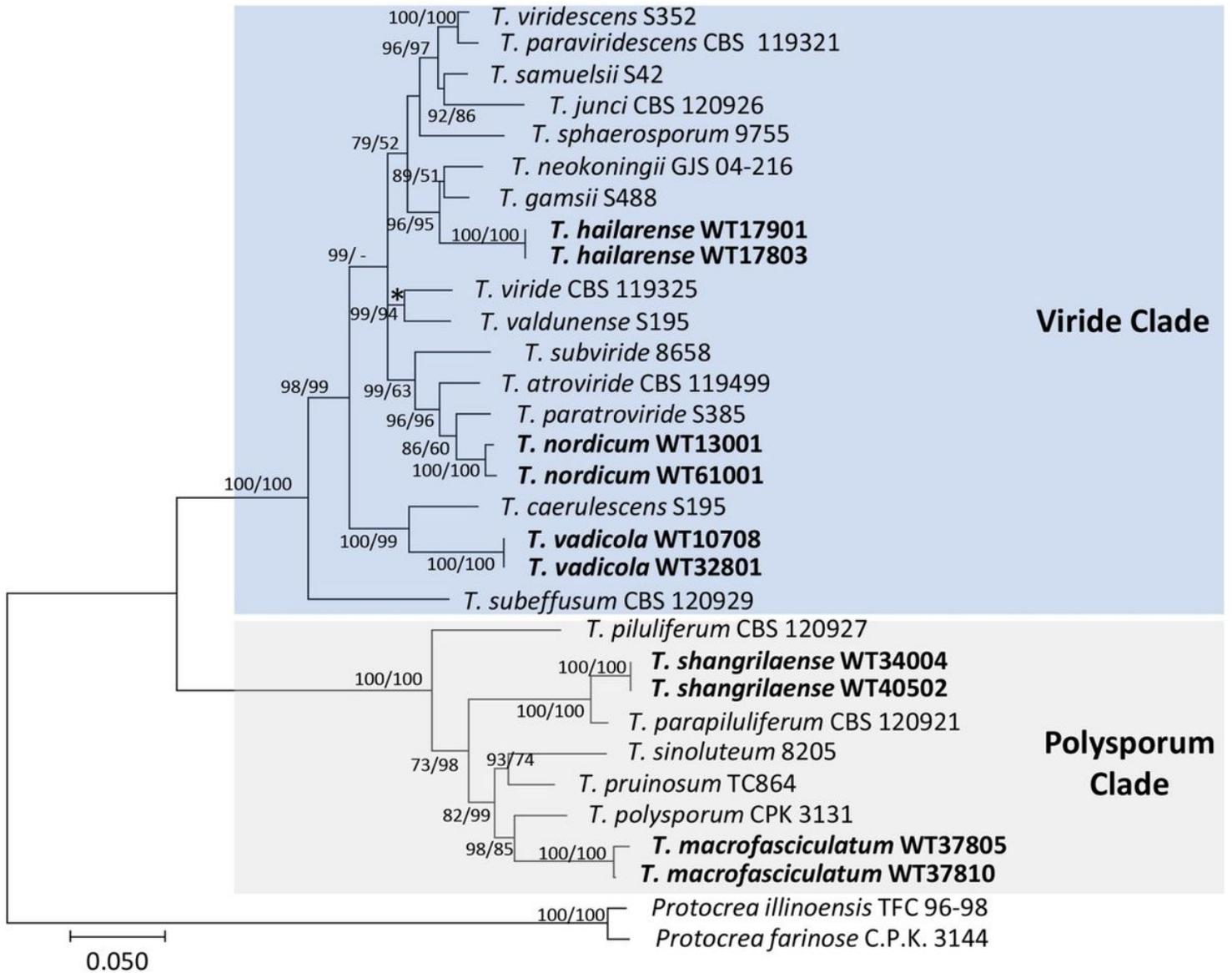


Figure 3

Phylogenetic tree based on the maximum likelihood (lnL=-16353.84) analysis of the combined dataset (TEF1- α + RPB2). Maximum likelihood bootstrap values (left) and MPBP (right) above 50% are indicated for branches. The tree is rooted with *Protocrea illinoensis* and *Protocrea farinose*. New species proposed here are indicated in boldface. Asterisks denote branches in conflict with the MP strict consensus tree.

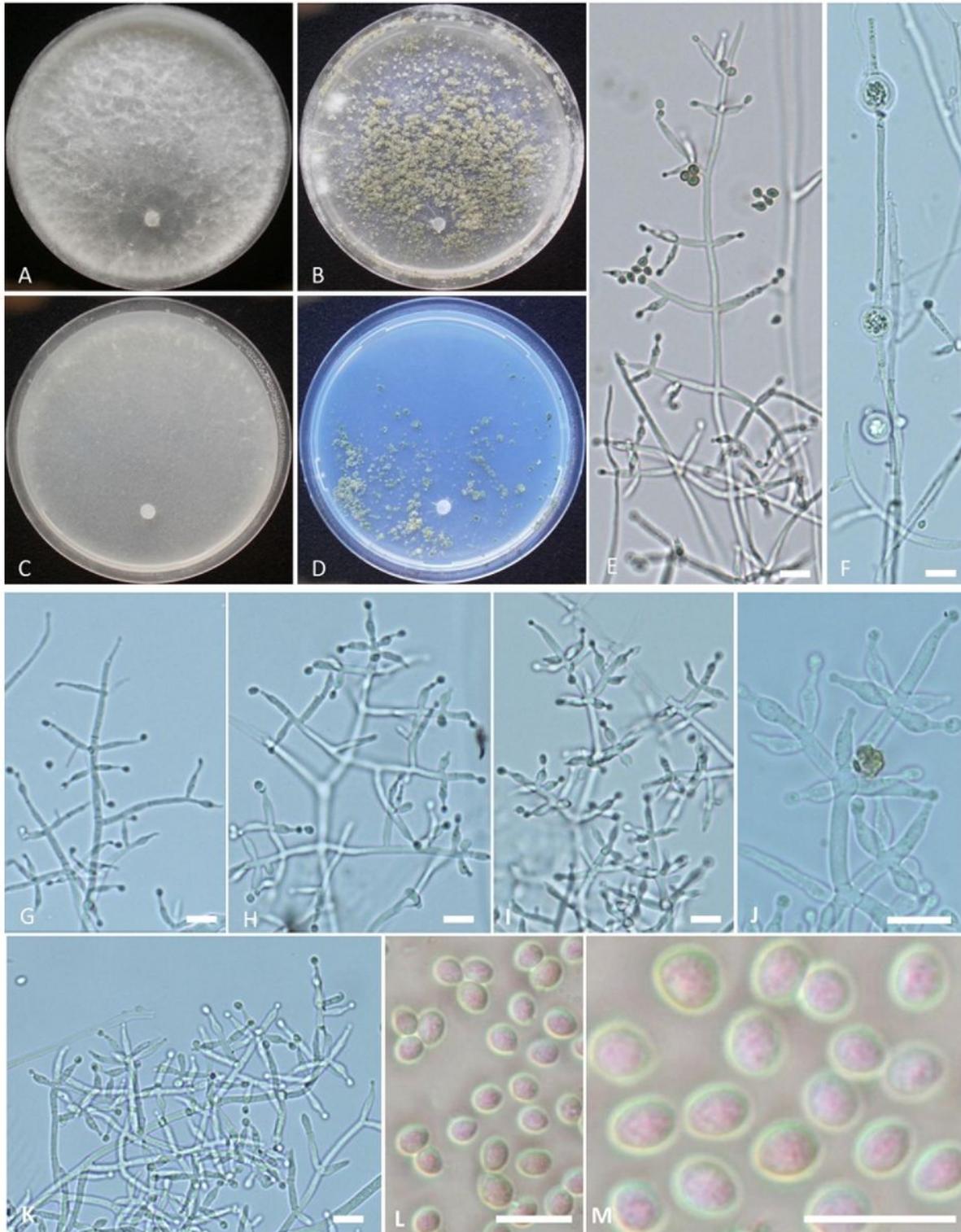


Figure 4

Trichoderma hailarensis. A–D. Cultures (A. on PDA, 25 °C, 14 days; B. on MEA, 25 °C, 14 days; C. On CMD, 25 °C, 14 days; D. on SNA, 25 °C, 14 days). E - K. Conidiophores and phialides. L and M. Conidia. E. on MEA. F–M. on PDA. A–M from WT17901. Scale bars: E–J = 10 μ m.

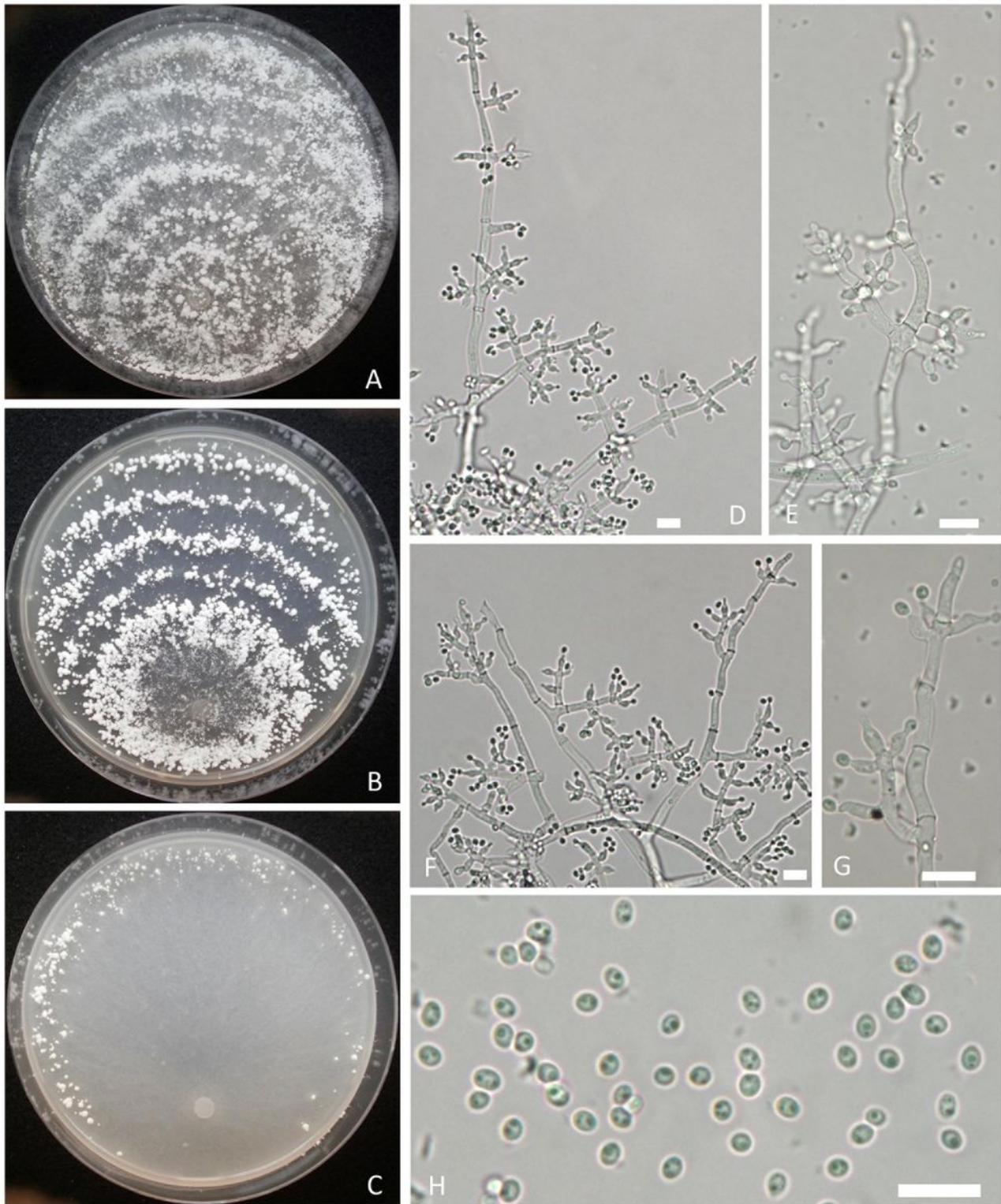


Figure 5

Trichoderma macrofasciculatum. A–C. Cultures (A. on PDA, 25 °C, 7 days; B. On MEA, 25 °C, 7 days; C. on CMD, 25 °C, 7 days). D–G. Conidiophores and phialides. H. Conidia with guttules. A, D, and E from WT37810; B, C, and F–G from WT37805. Scale bars: D–H = 10 μm.

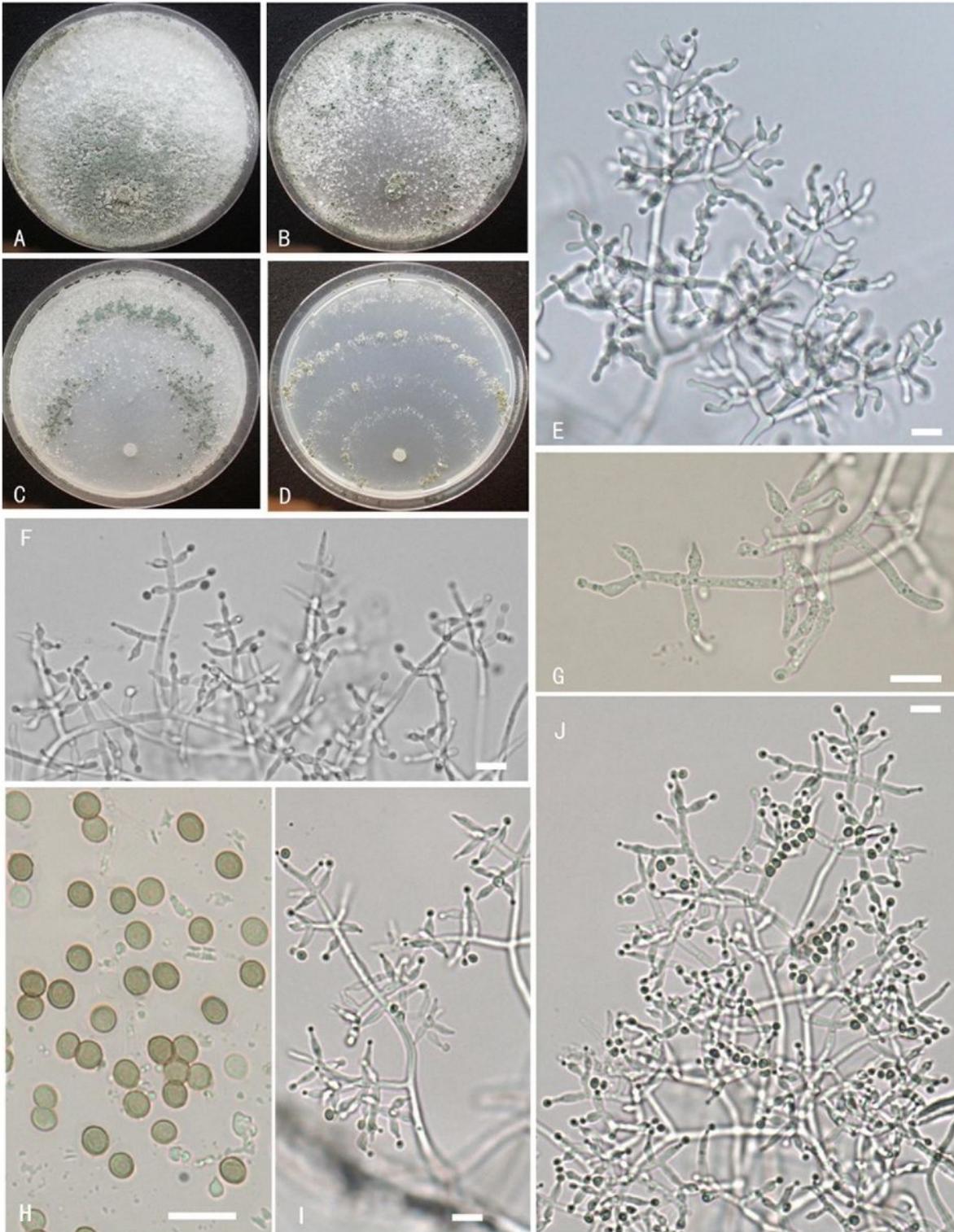


Figure 6

Trichoderma nordicum. A–D. Cultures (A. on PDA, 25 °C, 10 days; B. on MEA, 25 °C, 10 days; C. On CMD, 25 °C, 10 days; D. on SNA, 25 °C, 10 days). E–G, I–J. Conidiophores and phialides. H. Conidia. E. on PDA. F–J. on MEA. A–D from WT13001, E–J from WT61001. Scale bars: E–J = 10 μm.

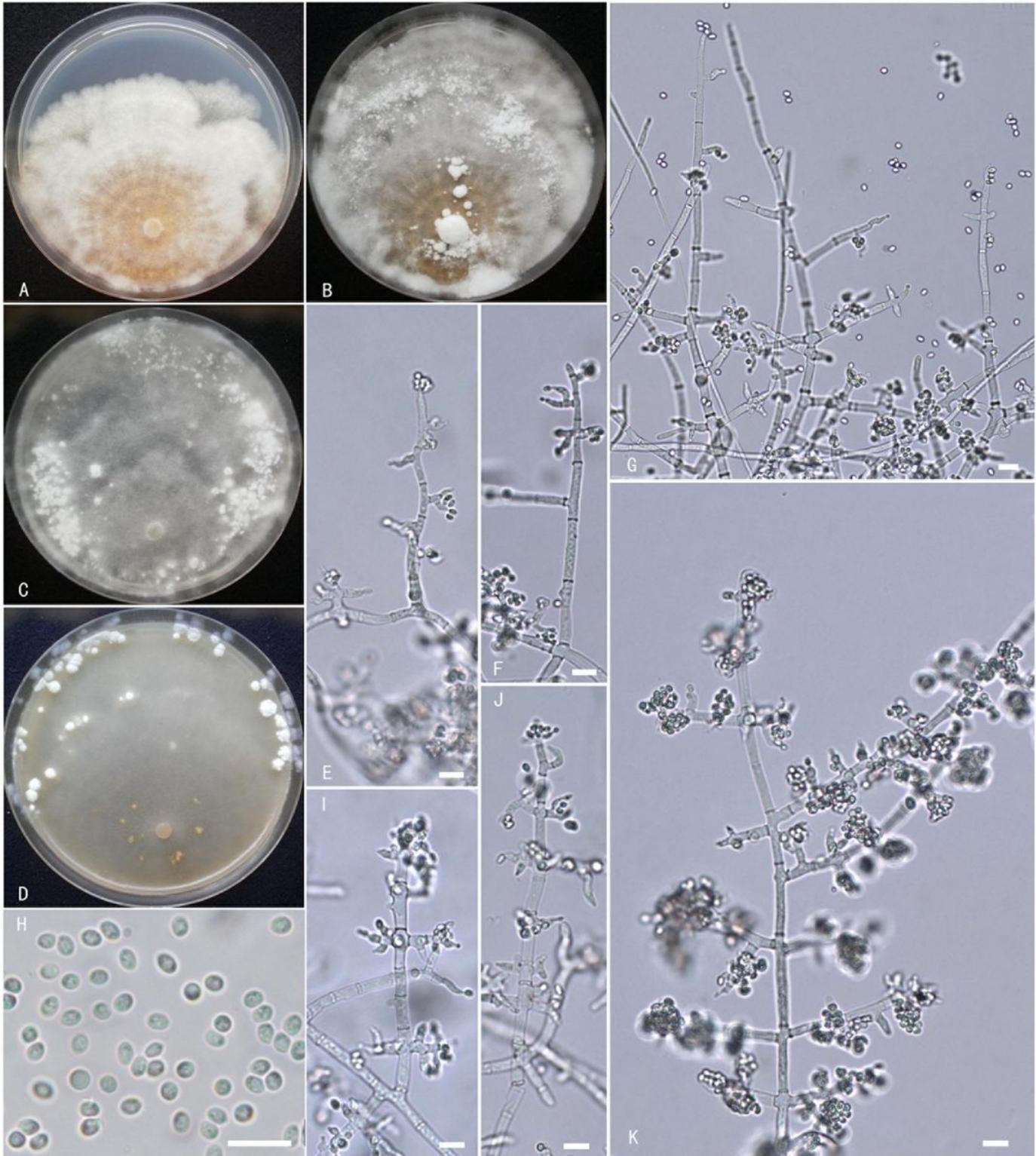


Figure 7

Trichoderma shangrilaense. A–D. Cultures (A. on PDA, 25 °C, 10 days; B. on PDA, 25 °C, 21 days; C. On MEA, 25 °C, 21 days; D. on CMD, 25 °C, 21 days). E–G, I–K. Conidiophores and phialides. H. Conidia. A–K from WT34004. Scale bars: E–K = 10 μm.



Figure 8

Trichoderma vadicola. A–D. Cultures (A. on PDA, 25 °C, 10 days; B. on MEA, 25 °C, 7 days; C. On CMD, 25 °C, 7 days; D. on SNA, 25 °C, 7 days). E–I. Conidiophores and phialides. J. Conidia. E, F, and H–J. on MEA. G. on SNA. A–J from WT10708. Scale bars: E–J = 10 μ m.