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Two new green-spored species of *Trichoderma* (Sordariomycetes, Ascomycota) and their phylogenetic positions

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Abstract

Two new species of *Trichoderma* are described based on the collections producing ascomata or asexual morphs on woody substrates, and named as *Trichoderma fujianense* and *T. zonatum*. *Trichoderma fujianense* produces gliocladium to verticillium-like conidiophores, slender to lageniform phialides, green and ellipsoidal to cylindrical conidia. *Trichoderma zonatum* is characterized by pulvinate, pale yellow to light brown stromata with densely disposed dark green to black ostioles, monomorphic ascospores, simple trichoderma-like conidiophores, green, (sub)globose to pyriform conidia. Their phylogenetic positions were investigated inferred from sequence analyses of the combined RNA polymerase II subunit b and translation elongation factor 1-α genes. The results indicate that *T. fujianense*, along with *T. aureoviride* and *T. candidum*, represents an independent lineage with high statistical support. *Trichoderma zonatum* belongs to the Chlorosporum clade and is associated with but clearly separated from *T. rosulatum* and *T. costaricense*. Morphological distinctions and sequence divergences between the new species and their close relatives were discussed.

Key words

Hypocreales, morphology, phylogeny, taxonomy

Introduction

Trichoderma Pers. (Ascomycota, Sordariomycetes, Hypocreales, teleomorph *Hypocrea* Fr.) species are frequently found on dead wood and bark, on other fungi, in soil and

living within healthy plant roots, stems and leaves (Mukherjee et al. 2013). Species of the genus belong to one of the most useful groups of microbes to have had an immense impact on human welfare. Some species are widely used as effective biocontrol agents for several soil-borne plant pathogens (Harman et al. 2004, Hasan et al. 2012, Liu et al. 2012), producers of enzymes, antibiotics and heterologous proteins for food, feed, textile and biofuel industries (cellulases, hemicellulases) (Samuels 1996, Almeida et al. 2007, Cheng et al. 2012, Lopes et al. 2012, Mukherjee et al. 2013). Many members are treated as agents for improving seed germination and nutrient use efficiency, breaking of seed dormancy, as well as source of transgenes and herbicides, and are long known to improve plant growth through the production of phytohormones and certain secondary metabolites (Harman et al. 2004, Shoresh et al. 2010), whereas others are causal agents of opportunistic infections of humans and animals (Samuels 1996, Kuhls et al. 1999, Kredics et al. 2003), and due to association of certain species with economically significant production losses in commercial mushroom farms (Samuels et al. 2002, Park et al. 2006, Kim et al. 2012a, 2012b).

The genus *Trichoderma* was established in 1794 including four species (Samuels 1996). In recent years, the number of *Trichoderma* species increases dramatically. Bissett et al. (2015) presented a list of 254 names of species and two names of varieties in *Trichoderma* with name or names against which they are to be protected, following the ICN (Melbourne Code, Art. 14.13). More recently, In a large-scale survey of *Trichoderma* from rotten wood and soil in China, Qin and Zhuang (2016a, b, c, d, e, 2017) published 27 new species based on the collections producing ascomata or asexual morphs on woody substrates; Chen and Zhuang (2016) described two new species based on soil samples from the Hubei and Tibet regions of China; Montoya et al. (2016) found three new taxa in the attine ant environment; Sun et al. (2016) described a new fungicolous *Trichoderma* species which was isolated from surface of the stroma of *Hypoxylon anthochroum*. Until now, 287 *Trichoderma* species have been described.

During our investigation of the diversity of *Trichoderma* species in China, two species were found to represent undescribed new taxa, on the basis of both morphological and cultural characters and DNA sequence analyses of partial nuc translation elongation factor 1-α encoding gene (*TEF*1-α) and the gene for nuc RNA polymerase II second largest subunit (*RPB*2). Differences between the new species and their close relatives are discussed, and a phylogenetic analysis is provided.

Materials and methods

Specimens and strains

Specimens were collected from Henan and Fujian provinces, China, and deposited in the Mycological Herbarium of Jilin Agricultural University (HMJAU). Strains were obtained either by single ascospore isolation from fresh stromata of sexual morphs or by direct isolation from asexual morphs on the substrates. Cultures are deposited in the China General Microbiological Culture Collection Center (CGMCC).

Morphological study

Dried stromata were rehydrated and longitudinal sections through ascomata were made with a freezing microtome (Leica CM1950) at a thickness of 5–10 μm. Agar media employed were cornmeal dextrose agar (CMD, Difco, Sparks, MD, USA, with dextrose 20 g/L), potato dextrose agar (PDA, Solarbio, Beijing, CHINA) and synthetic low nutrient agar (SNA, Nirenberg 1976, pH adjusted to 5.5). Colonies were incubated in 9 cm diam Petri dishes at 25 °C with alternating light/darkness (12/12 h) at 20 °C, 25 °C, 30 °C and 35 °C and measured daily until the dishes were covered with mycelium. The characteristics of asexual and sexual states were described following the methods of Jaklitsch (2009) and Zhu and Zhuang (2015). Photographs were taken using a Leica DFC450C digital camera (Tokyo, Japan) connected to a Zeiss Axioskop 2 Plus microscope (Göttingen, Germany) for anatomical structures and to a Zeiss Stemi 2000C stereomicroscope for gross morphology.

DNA extraction, amplification and sequencing

Genomic DNA was extracted from mycelium harvested from colonies on PDA after 1–2 wk with a NuClean Plant Genomic DNA Extraction Kit (CoWin Biosciences, Beijing, China) according to the manufacturer's protocol. Fragments of the nuc rDNA internal transcribed spacers (ITS1-5.8S-ITS2 = ITS), *TEF*1-α and *RPB*2 were amplified with the primer pairs ITS4 and ITS5 (White et al. 1990), EF1-728F (Carbone and Kohn 1999) and TEF1LLErev (Jaklitsch et al. 2005), fRPB2-5f and fRPB2-7cr (Liu et al. 1999), respectively. PCR products were purified with the PCR Product Purification Kit (Biocolor BioScience and Technology Co., Shanghai, China) and cycle-sequenced on an ABI 3730 XL DNA Sequencer (Applied Biosciences, Foster City, Calofornia) with the same primer in fragments amplification for ITS and primers reported by Jaklitsch (2009) for *TEF*1-α, and *RPB*2 at Beijing Tianyihuiyuan Bioscience and Technology, China. The strains and the NCBI GenBank accession numbers of DNA sequences used in this work are listed in Table 1.

Phylogenetic analyses

Sequences were assembled, aligned and manually adjusted when needed with BioEdit 7.0.5.3 (Hall 1999). NEXUS files were generated with Clustal X 1.83 (Thompson et al. 1997). To identify the phylogenetic positions of *Trichoderma fujianense* and *T. zonatum*, *RPB*2 and *TEF*1-α sequences were combined for the analyses. Thirty-three sequences representing 30 *Trichoderma* taxa were selected for analyses, with *Nectria eustromatica* and *N. berolinensis* selected as outgroup taxa. Alignments are deposited in TreeBASE accession number 21272.

Maximum parsimony (MP) analysis was performed with PAUP 4.0b10 (Swofford 2002) using 1000 replicates of heuristic search with random addition of sequences

Table 1. Materials including strain numbers and GenBank accessions of sequences used for phylogenetic analyses.

Name	Strain	GenBank accession number	
		RPB2	TEF1-α
Trichoderma aerugineum Jaklitsch	CBS 120541	FJ860516	FJ860608
T. aureoviride Rifai	C.P.K. 2848	FJ860523	FJ860615
T. ceramicum P. Chaverri & Samuels	CBS 114576	FJ860531	FJ860628
T. chlorosporum P. Chaverri & Samuels	G.J.S. 88-33	AY391903	AY391966
T. chromospermum P. Chaverri & Samuels	G.J.S. 94-68	AY391913	AY391974
T. costaricense (P. Chaverri & Samuels) P. Chaverri, Jaklitsch & Voglmayr	P.C. 21	AY391921	AY391980
T. cremeoides Jaklitsch & Voglmayr	S112	KJ665253	KJ665456
T. cremeum P. Chaverri & Samuels	G.J.S. 91-125	AF545511	AF534598
T. cuneisporum P. Chaverri & Samuels	G.J.S. 91-93	AF545512	AF534600
T. danicum (Jaklitsch) Jaklitsch & Voglmayr	CBS 121273	FJ860534	FJ860634
T. estonicum P. Chaverri & Samuels	G.J.S. 96-129	AF545514	AF534604
T. fujianense Z.X. Zhu, W.Y. Zhuang &Y. Li	HMJAU 34830	**MF374808***	**MF374811**
T. gelatinosum P. Chaverri & Samuels	G.J.S. 88-17	AF545516	AF534579
T. gliocladium Jaklitsch & Voglmayr	S81	KJ665271	KJ665502
T. helicum Bissett, C.P. Kubicek & Szakács	DAOM 230021	DQ087239	KJ871125
T. longipile Bissett	CBS 120953	FJ860542	FJ860643
T. nigrovirens P. Chaverri & Samuels	G.J.S. 99-64	AF545518	AF534582
T. parestonicum Jaklitsch	CBS 120636	FJ860565	FJ860667
T. phyllostachydis P. Chaverri & Samuels	G.J.S. 92-123	AF545513	AF534576
T. pseudocandidum Minnis, Samuels & P. Chaverri	P.C. 59	AY391899	AY391962
T. rosulatum Z.X. Zhu & W.Y. Zhuang	HMAS 252548	KF730005	KF729984
T. sinuosum P. Chaverri & Samuels	G.J.S. 90-88	AY391932	AY391990
T. spinulosum (Fuckel) Jaklitsch & Voglmayr	CBS 121280	FJ860589	FJ860699
T. stipitatum Z.X. Zhu & W.Y. Zhuang	HMAS 266613	KF730012	KF729991
T. strictipile Bissett	C.P.K. 1601	FJ860594	FJ860704
T. surrotundum P. Chaverri & Samuels	G.J.S. 88-73	AF545540	AF534594
T. thailandicum P. Chaverri & Samuels	G.J.S. 97-61	AY391957	AY392005
T. thelephoricola P. Chaverri & Samuels	CBS 120925	FJ860600	FJ860711
T. virescentiflavum (Speg.) Jaklitsch & Voglmayr	P.C. 278	AY391959	AY392007
T. zonatum Z.X. Zhu, W.Y. Zhuang &Y. Li	HMJAU 34820	**MF374806**	**MF374809**
	HMJAU 34825	**MF374807**	**MF374810**
Nectria eustromatica Jaklitsch & Voglmayr	CBS 121896	HM534886	HM534875
N. berolinensis (Sacc.) Cooke	CBS 127382	HM534883	HM534872

Note: *Numbers in bold indicate newly submitted sequences.

and subsequent TBR (tree bisection and reconnection) branch swapping. Analyses were performed with all characters treated as unordered and unweighted, gaps treated as missing data. Topological confidence of resulted trees was tested by maximum parsimony bootstrap proportion (MPBP) with 1000 replications, each with 10 replicates of random addition of taxa.

Bayesian Inference (BI) analysis was conducted via MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) using a Markov Chain Monte Carlo (MCMC) algorithm. Nucleotide substitution models were determined by MrModeltest 2.3 (Nylander 2004). GTR+I+G was estimated as the best-fit model for combined sequences. Four MCMC chains were run from random trees for 2 000 000 generations and sampled every 100 generations. The first 5000 trees were discarded as the burn-in phase of the analyses, and Bayesian inference posterior probability (BIPP) was determined from the remaining trees. Trees were visualized in TreeView 1.6.6 (Page 1996).

Results

Phylogenetic analyses

The partition homogeneity test (P = 0.01) of *RPB*2 and *TEF*1-α sequences indicated that the individual partitions were generally congruent (Cunningham 1997). Phylogenetic positions of the new species were determined by analyses of the combined *RPB*2 and *TEF*1-α dataset containing 33 taxa and 2396 characters, of which 1304 characters were constant, 366 variable characters were parsimony-uninformative and 726 were parsimony-informative. Five most-parsimonious trees with the same topology were generated (Figure 1) (tree length = 3178, CI = 0.4685, HI = 0.4572, RI = 0.5493 and RC = 0.2982).

Thirty-three sequences representing 30 green-spored *Trichoderma* species and two outgroup taxa *Nectria berolinensis* and *N. eustromatica* were used to construct the phylogenetic tree (Figure 1). All the green-spored species formed a monophyletic group (100 % MPBP/BIPP), which is basically consistent with the previous study by Jaklitsch (2009) and Zhu and Zhuang (2015).

In our phylogenetic tree, the five major clades, Chlorosporum, Spinulosum, Virescentiflavum, Ceramicum and Strictipile were basically well supported. The first four clades received 100%/100%, 98%/100%, 100%/100% and 100%/- (MPBP/BIPP) support in the tree, respectively, but the Strictipile clade was less strongly supported at 61% (MPBP) in the tree.

In the Chlorosporum clade (Figure 1), two samples of *Trichoderma zonatum* (HMJAU 34820 and 34825), sharing identical sequences, constituted a well-supported independent lineage (MPBP/BIPP = 100 %/100 %). Although *T. zonatum* is morphologically similar to *T. chromospermum*, they are not phylogenetically close and have relatively low sequence similarities in *RPB*2, *TEF*1-α and ITS.

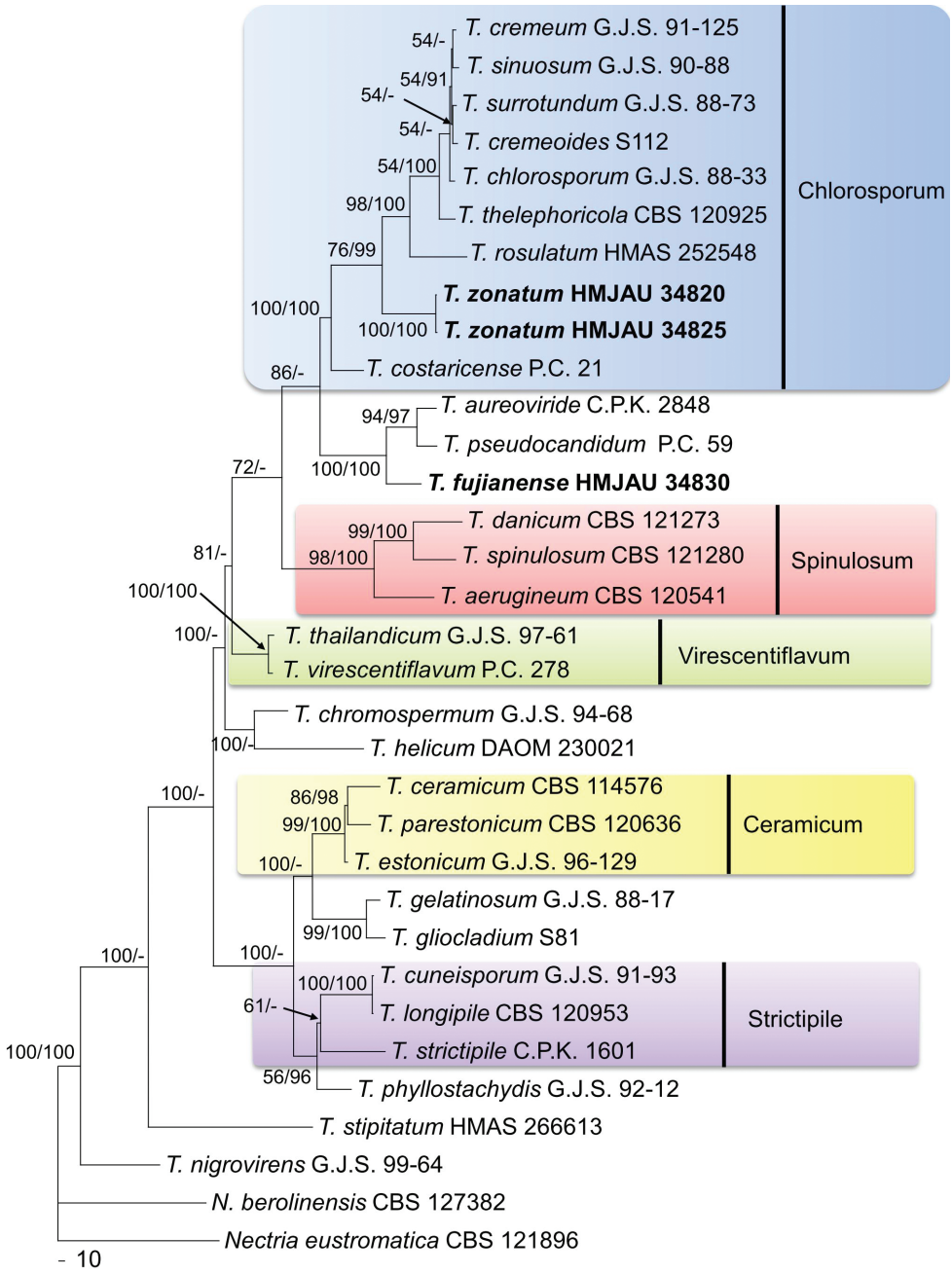

Figure 1. Maximum parsimony phylogram reconstructed from the combined squences of *RPB2* and *TEF1-α*. MPBP above 50% (left) and BIPP above 90% (right) are indicated at the nodes.

Trichoderma aureoviride, *T. candidum* and *T. fujianense* form an independent lineage with high statistical support (MPBP/BIPP = 100%/100%), which still remains unnamed (Chaverri and Samuels 2003, Jaklitsch 2009).

Taxonomy

***Trichoderma fujianense* Z.X. Zhu, W.Y. Zhuang & Y. Li, sp. nov.**
MycoBank: MB821807
Figure 2

Diagnosis. Characterized by slender to lageniform, long phialides (14–23 × 2–3.5 µm), gliocladium to verticillium-like conidiophores, ellipsoidal to cylindrical conidia (4.5–5.5 × 2.5–3.5 µm).

Type. CHINA. Fujian: Quanzhou City, Qingyuan mountain. 24°56'51"N, 118°36'31"E, 150 m alt., on bark, 6 Aug 2015, Z.X. Zhu 230 (HMJAU 34830, holotype), Ex-type culture CGMCC 3.18757.

Description. Colony radius on CMD after 72 h 2.5–5 mm at 20 °C, 13–15 mm at 25 °C, 3.5–5 mm at 30 °C, no growth at 35 °C, mycelium covering the plate after 2 wk at 25°C. Colony circular, dense, finely zonate, becoming hairy to floccose by conidiophores, first whitish, turning light green. Aerial hyphae virtually absent. Autolytic excretions, pigment and coilings absent. Conidiation starting after 4 d in densely disposed gliocladium-like conidiophores, short-effuse, turning green after 1 wk.

Colony radius on PDA after 72 h 7.5–8.5 mm at 20 °C, 8.5–10 mm at 25 °C, 0.5–1 mm at 30 °C, no growth at 35 °C, mycelium covering the plate after 2 wk at 25 °C. Colony circular, compact with distinctly zonate, with commonly lobed or coarsely wavy margin, centre dense, green, margin relatively looser, whitish. Conidiation noted around the plug after 3–4 d, effuse, spreading from the centre over the entire colony surface. No distinct odor, no diffusing pigment observed.

Colony radius on SNA after 72 h 1.5–3 mm at 20 °C, 4–5 mm at 25 °C, 1–2 mm at 30 °C, no growth at 35 °C, mycelium covering the plate after 24 d at 25 °C. Colony hyaline, thin, irregular, not zonate, surface mycelium scant. Aerial hyphae inconspicuous, short. Conidiophores sparsely disposed, noted after 7 d, gliocladium to verticillium-like, with 1–3(–4) whorls arising from the main axis. Phialides arising in more or less narrow angles from cylindrical metulae, phialides slender to lageniform, somewhat curved, (10–)14–23(–28) × 2–3.5(–4) µm, l/w 4.8–7.2(–9.2), (1.5–)1.8–2.7(–3.2) µm wide at the base (n = 100). Conidia green, ellipsoidal to cylindrical, smooth, (4–)4.5–5.5(–6) × 2.5–3.5(–4) µm, l/w (1.2–)1.3–2.0 (n = 100). No distinct odor, no diffusing pigment observed.

Habitat and distribution. On the surface of rotten wood in humid forests of east China.

Etymology. The epithet "*fujian*", indicating occurrence of the fungus in Fujian province.

Teleomorph. Not known.

Remarks. Morphologically, the new species is most similar to *Trichoderma costaricense* in conidiophore character and phialide shape and size; while the latter fungus produces abundant chlamydospores on CMD, has relatively larger conidia (5.2–6.0 × 3.2–4.0 µm) and faster growth on PDA and SNA, and grows well and sporulates at 35 °C (Chaverri and Samuels 2003). Furthermore, sequence similarity

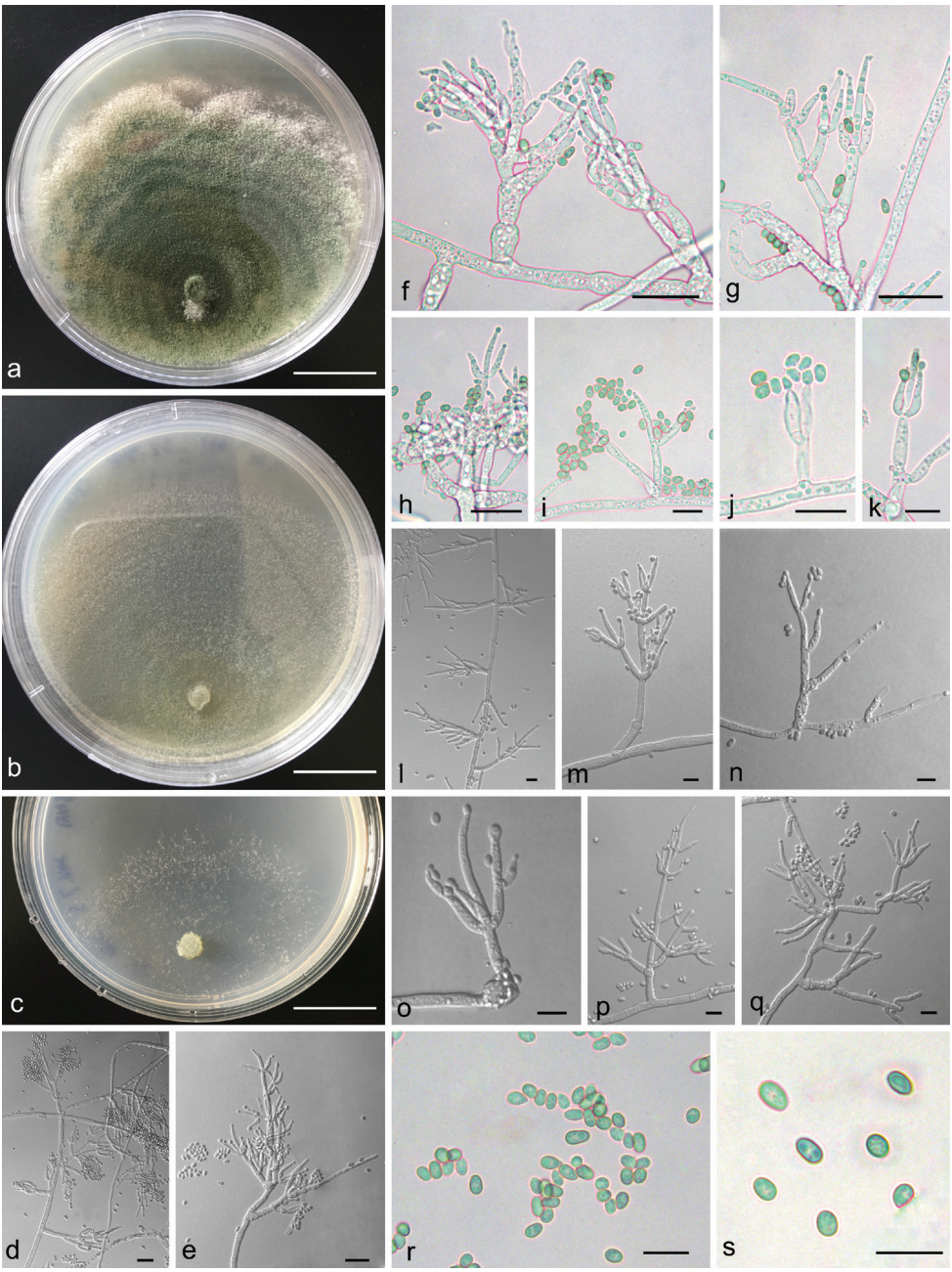

Figure 2. The new species *Trichoderma fujianense*, holotype (HMJAU 34830). **a–s** Asexual state **a–c** Cultures after 13 d at 25 °C (**a** PDA, **b** CMD, **c** SNA). **d–q** Conidiophores and phialides (SNA, 20 d) **r, s** Conidia (SNA, 20 d). Scales bars: 20 mm (**a–c**); 20 μm (**d–h**); 10 μm (**i–s**).

of ITS and *RPB*2 between these species was only 90.1% and 92.1%, with 60 bp and 68 bp differences among 606 bp and 864 bp, respectively. Among the species with green ascospores, *T. gelatinosum*, *T. nigrovirens*, *T. chromospermum* and *T. thelephoricola* also generated gliocladium to verticillium-like conidiophores, but they are not phylogenetically closely related.

The phylogenetic positions of the new taxa (Figure 1) demonstrated that *Trichoderma fujianense* is found to be closely related to *T. aureoviride* and *T. candidum*, and three of them form an independent lineage with high statistical support. However, *T. aureoviride* is distinctive by shorter conidia (3.8–4.0 × 3.0–3.3 µm, l/w 1.2–1.3); *T. candidum* differs by shorter phialides (7.3–)9.0–13.5(–16.5) µm, globose to subglobose and smaller conidia (3.2–3.5 × 3.0–3.2 µm, l/w (1.0–)1.1(–1.3) (Chaverri and Samuels 2003).

***Trichoderma zonatum* Z.X. Zhu, W.Y. Zhuang & Y. Li, sp. nov.**

MycoBank: MB821806

Figure 3

Diagnosis. Characterized by pulvinate, pale yellow to light brown stromata with densely disposed dark green to black ostioles, long asci (93–112 × 5.8–6.6 µm), monomorphic and subglobose ascospores (4.2–5 × 4–4.7 µm), simple trichoderma-like conidiophores, green, (sub)globose to pyriform conidia (2.8–3.8 × 2.3–2.8 µm).

Type. CHINA. Henan: Xinyang City, Jigong mountain. 31°49'2"N, 114°04'16"E, 1500 m alt., on bark, 16 Jul 2015, B. Zhang 220 (HMJAU 34820, holotype), Ex-type culture CGMCC 3.18758.

Description. Stromata generally solitary, scattered, gregarious, or aggregated in small groups, broadly attached, pulvinate to somewhat flattened, outline circular or with lobed margin, (0.5–)1.0–2.5(–3) mm diam (n = 20), (0.3–)0.5–0.8 mm high (n = 20). Surface flat, smooth, with slight perithecial protuberances, pale yellow to light brown, not changing colour in KOH, ostiolar openings obvious due to the green ascospores.

In section stroma cortical tissue of textura angularis, 13–28 µm thick, not changing colour in 3% KOH, cells yellow, thin-walled, 6–12(–17) × 5–10(–13) µm (n = 40); subcortical tissue of textura angularis, cells hyaline, thin-walled, 4–10 × 5–8 µm (n = 40); subperithecial tissue of textura epidermoidea, cells hyaline, thin-walled, 10–22 × 8–17 µm (n = 40); tissue at the base of textura intricata, hyphae hyaline, thin-walled, (2.5–)3.5–6(–8) µm (n = 40) wide. Perithecia subglobose or flask-shaped, crowded, 178–216 ×120–165 µm (n = 40); peridium yellow in lactic acid, not changing colour in 3% KOH, (8–)10–14(–17) µm thick at the sides, (12–)14–21(–27) µm at the base (n = 40). Ostioles conical or cylindrical, 51–70 µm high, 31–54 µm wide at the apex (n = 40). Asci cylindrical, 93–112 × 5.8–6.6(–7) µm, with a stipe (13–)18–23 µm long (n = 60). Part-ascospores green, turning brown in KOH, distinctly verrucose, cells monomorphic, subglobose, also slightly ovoid, 4.2–5 × 4–4.7 µm (n = 100), l/w 1.0–1.1.

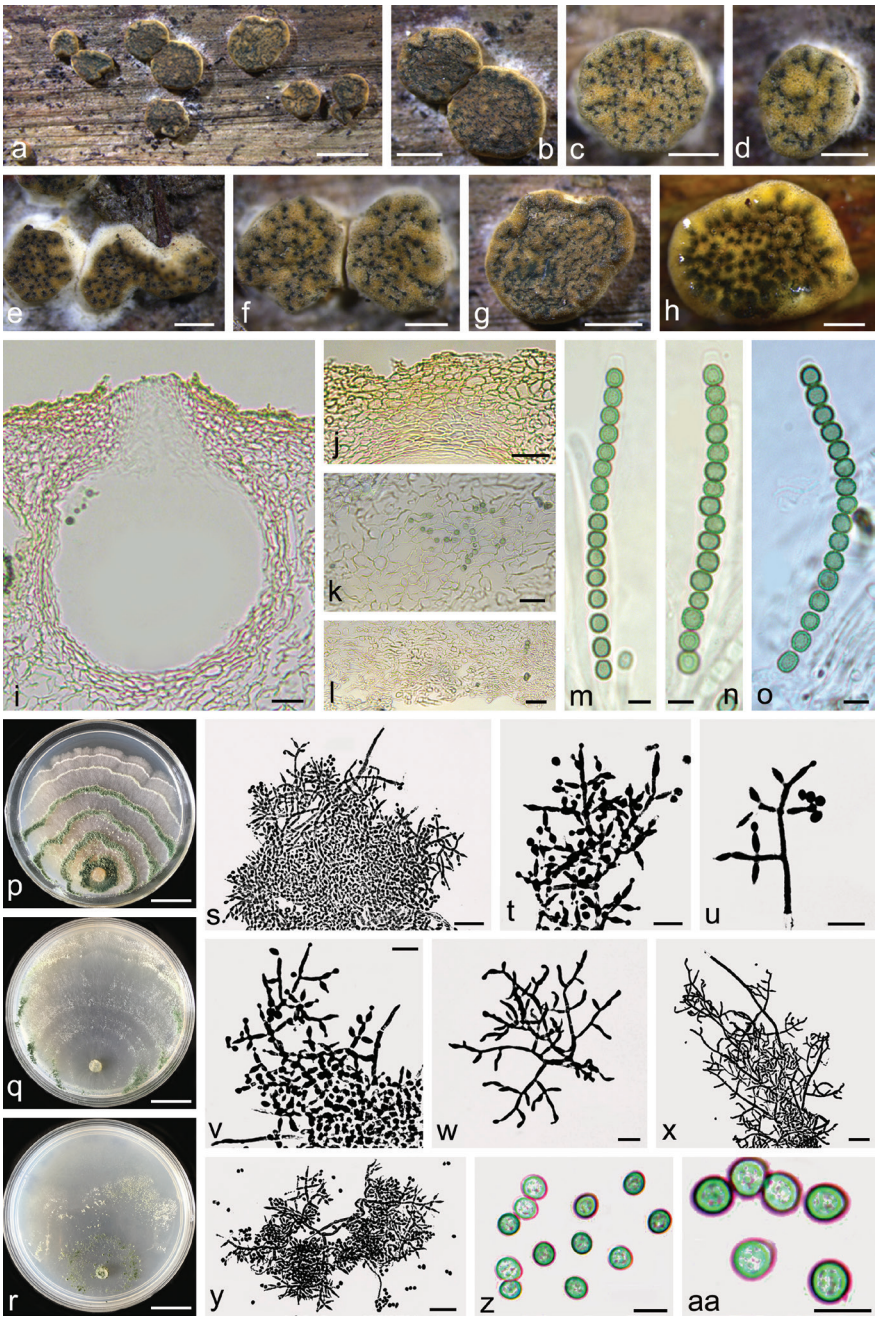

Figure 3. The new species *Trichoderma zonatum*, holotype (HMJAU 34820). **a–o** Sexual state. **a–g** Dry stromata on nature substrate **h** Mature stroma after rehydration **i** Perithecium in section **j** Cortical and subcortical tissue in section **k** Subperithecial tissue in section **l** Stroma base in section. **m–o** Ascus with part-ascospores. **p–aa** Asexual state. **p–r** Cultures after 7 d at 25 °C (**p** PDA, **q** CMD, **r** SNA). **s–y** Conidiophores and phialides (SNA, 7 d). **z, aa** Conidia (SNA, 7 d). Scales bars: 2 mm (**a**); 1 mm (**b, g**); 500 μm (**c–f, h**); 20 μm (**i–l, s, x, y**); 5 μm (**m–o, z, aa**); 20 mm (**p–r**); 10 μm (**t–w**).

On CMD colony radius after 72 h 30–43 mm at 20 °C, 32–46 mm at 25 °C, 17–34 mm at 30 °C, no growth at 35 °C. Colony hyaline, circular, loose, forming obvious zonate, covering the plate after 5–7 d at 25 °C. Aerial hyphae radially arranged. Conidiation at 25 °C noted after 3 d, first effuse, soon followed by formation of granules or pustules, particularly along the margin, spreading from the centre across the entire plate. No distinct odor, no diffusing pigment observed.

On PDA after 72 h 38–48 mm at 20 °C, 55–62 mm at 25 °C, 28–30 mm at 30 °C, no growth at 35 °C; mycelium covering the plate after 8 d at 25°C. Colony circular, conspicuously dense, becoming zonate with broad, slightly downy zones and narrow, well-defined, convex, white to green farinose zones. Aerial hyphae numerous, mostly short, becoming fertile from the centre. Conidiation at 25 °C starting after 2 d, green after 4 d, first simple, mostly on short aerial hyphae concentrated in the centre and in denser zones, later abundant in pustules. Autolytic activity lacking or inconspicuous, no coilings seen. No diffusing pigment, no distinct odour noted.

On SNA after 72 h 12–14 mm at 20 °C, 18–20 mm at 25 °C, 15–17 mm at 30 °C, no growth at 35 °C; mycelium covering the plate after 8–9 d at 25°C. Colony hyaline, thin, loose, irregularly lobed, not zonate. Aerial hyphae inconspicuous. Autolytic activity moderate. Conidiophores visible after 4 d, trichoderma-like, with 2–3(–4) whorls arising from the main axis. Phialides solitary or divergent in whorls of 2–3, mostly asymmetrically arranged, lageniform, (5–)7–11(–14) × 2–3(–4) µm, l/w 1.7–2.8(–4) (n = 60). Conidia green, (sub)globose to pyriform, smooth, (2.5–)2.8–3.8 × 2.3–2.8 µm, l/w (1.0–)1.1–1.3(–1.5) (n = 70). No chlamydospores formed. No distinct odor, no diffusing pigment observed.

Habitat and distribution. On the surface of rotten wood in humid forests of south central and east China.

Etymology. The specific epithet refers to the zonate colony on PDA.

Other specimens examined. CHINA. Fujian: Quanzhou City, Qingyuan mountain. 24°55'53"N, 118°36'31"E, 200 m alt., on bark, 6 Aug 2015, Z.X. Zhu 225, HMJAU 34825, Ex-type culture CGMCC 3.18759.

Remarks. Phylogenetic analyses based on *RPB*2 and *TEF*1-α indicated that *Trichoderma zonatum* belongs to the Chlorosporum clade, previously consisting of eight species, *T. sinuosum*, *T. cremeum*, *T. surrotundum*, *T. chlorosporum*, *T. thelephoricola*, *T. rosulatum*, *T. cremeoides* and *T. costaricense*. Phylogenetically, *T. zonatum* is most related to *T. rosulatum* and *T. costaricense*, but *T. rosulatum* is clearly distinguishable by dimorphic ascospores, gliocladium-like conidiophores, production of abundant chlamydospores and rosulate colony on CMD (Zhu and Zhuang 2015); *T. costaricense* produces dimorphic and larger ascospores (5.5–6.0 × 5.2–5.7 µm), verticillium-like conidiophores and ellipsoidal to cylindrical conidia (Chaverri and Samuels 2003, Zhu and Zhuang 2015).

Species of the Chlorosporum clade usually produce pale yellow or pale green, semi-translucent stromata, globose to subglobose ascospores and gliocladium-like or verticillium-like conidiophores (Chaverri and Samuels 2003, Zhu and Zhuang 2015). *Trichoderma zonatum* is characterized by pulvinate, pale yellow to light brown stromata with densely disposed dark green to black ostioles, monomorphic ascospores, simple

trichoderma-like conidiophores, green, (sub)globose to pyriform conidia. Morphologically, stromata of *T. zonatum* are not typical of the Chlorosporum clade and differ from all other species by relatively larger and non-transparent. It is most similar to *T. chromospermum* in gross stromata morphology, while the latter fungus clearly differs by much shorter asci [(78–)85–90(–102) µm], gliocladium-like conidiophores and ellipsoidal to cylindrical conidia (Chaverri and Samuels 2003, Zhu and Zhuang 2015).

Discussion

Phylogenetic analyses of *Trichoderma* species with green spores based on sequences of *RPB*2 and *TEF*1-α were performed by Chaverri and Samuels (2003). In the more recent study by Zhu and Zhuang (2015) a phylogenetic tree with 45 species having green-spored was inferred from *RPB*2 and *TEF*1-α sequences. In our study analyses of the combined sequences of the same genes of 30 related *Trichoderma* species were carried out to ascertain the phylogenetic positions of our new species. The tree topology is basically consistent with previous researches (Chaverri and Samuels 2003, Jaklitsch 2009, Zhu and Zhuang 2015). The study of Chaverri and Samuels (2003) suggested that phenotypic characters, alone are usually not useful in understanding phylogenetic relationships in *Trichoderma*, because teleomorph characters, for example, the tissue structure of the stroma, the size and character of the perithecia, asci and ascospores, are generally highly conserved and anamorph characters tend to be morphologically divergent within monophyletic groups, clades or species complex. Based on the results of the present study, we conclude that similarity in teleomorphic characters is not indicative of close phylogenetic relationships, holomorphs must be studied in order to effectively determine both life cycles and species concepts.

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