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ORIGINAL ARTICLE

Trichoderma eijii and *T. pseudolacteum*, two new species from Japan

Chang Sun Kim · Takashi Shirouzu · Akira Nakagiri · Kozue Sotome · Nitaro Maekawa

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Abstract *Trichoderma eijii* and *T. pseudolacteum*, are described here as new species from Japan. These species were isolated from decaying wood in the Tomakomai Experimental Forest in Hokkaido Prefecture and from bedlogs on shiitake mushroom (*Lentinula edodes*) farms, respectively. The species were characterized using a combined approach that included cultural studies, holomorph morphology, and phylogenetic analyses of internal transcribed spacer and protein coding gene sequences (RNA polymerase subunit II, translation elongation factor 1-α, endochitinase, and actin). The results of phylogenetic analyses of these gene sequences indicate that *T. eijii* belongs to the Hamatum clade and is closely related to *Hypocrea pezizoides*, *H. flaviconidia*, and *H. atroviridis*/*T. atroviride*, from which it differs mainly in part-ascospore size and anamorphic characteristics. *Trichoderma pseudolacteum*, which was previously recognized as *H. lactea* sensu Doi, is morphologically distinct from *H. lactea* (= *Hypocrea citrina*) and is strongly supported as a separate lineage based on our phylogenetic analyses.

Keywords Ascomycota · *Hypocrea* · Molecular phylogeny · Morphology

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C. S. Kim
The Course of Bioenvironmental Science, The United Graduate School of Agricultural Sciences, Tottori University, 4-101 Koyama-cho-minami,
Tottori 680-8553, Japan

T. Shirouzu · A. Nakagiri · K. Sotome · N. Maekawa (✉)
Fungus/Mushroom Resource and Research Center, Tottori University, 4-101 Koyama-cho-minami,
Tottori 680-8553, Japan
e-mail: kin-maek@muses.tottori-u.ac.jp

Introduction

Hypocrea/*Trichoderma* is a genus of economically important fungi with many different applications, including plant disease management, plant growth promotion, induction of resistance in plants, and bioremediation (Chaverri and Samuels 2003; Harman et al. 2004). Some of these fungi are also responsible, on the other hand, for economic production losses wherever mushrooms are commercially grown. Following the first report (Seaby 1987) of *Trichoderma*-associated problems in Ireland, commercial mushroom farms in England, Canada, United States, and Korea have incurred millions of dollars in crop losses (Fletcher 1990; Rinker 1994; Grogan and Gaze 1995; Romaine et al. 1996; Ospina-Giraldo et al. 1998; Samuels et al. 2002; Park et al. 2005, 2006).

Because most *Trichoderma* spp. produce green-colored conidia, they are easily detected on mushroom compost or bedlogs during mushroom cultivation. The resulting disease is accordingly called ‘green mold disease’ (Samuels et al. 2002; Cha 2004). Most *Trichoderma* spp. have not only an asexual cycle, but also a sexual stage. These teleomorphs are included in the genus *Hypocrea*. One such example is *T. mienum* C.S. Kim, Nakagiri & N. Meak., a competitor/pathogen of the shiitake mushroom (*Lentinula edodes*) with teleomorph and anamorph states that are easily detected in PDA media (Kim et al. 2012b). In general, *Hypocrea* spp. form white or yellow to brown stromata with hyaline or green ascospores. During early stages, *Hypocrea* diseases are consequently difficult to detect and diagnose in mushroom cultivation: they do not turn the infected area green, and they form a white stroma that is similar in appearance to mushroom primordia (Cha 2004). A comprehensive understanding of disease caused by *Hypocrea*/*Trichoderma* species in mushroom cultivation requires a study of both life stages (when possible, as some *Hypocrea* and *Trichoderma* spp. do not form anamorph or teleomorph states, respectively), as well as an investigation of the interaction

between *Hypocrea*/*Trichoderma* spp. and commercial mushrooms.

Approximately 170 species within *Hypocrea*/*Trichoderma* are currently recognized (Jaklitsch 2009, 2011; Druzhinina et al. 2011; Samuels et al. 2012b), with the majority having been described from North America and Europe. From East Asia, nearly 80 species of *Hypocrea*/*Trichoderma* have been described and reported, primarily by Doi (e.g., Doi 1966, 1968, 1969, 1972, 1974, 1979, 2001; Doi and Doi 1979; Liu and Doi 1995). Because many species are morphologically indistinguishable, modern taxonomic studies of *Hypocrea*/*Trichoderma* using molecular and morphological data are needed for accurate identification and description (Chaverri and Samuels 2003; Chaverri et al. 2003). More than 20 species of *Hypocrea*/*Trichoderma*, including the East Asian *Hypocrea*/*Trichoderma* species described by Doi and his collaborators, have been reported as causal agents of green mold and *Hypocrea* diseases on shiitake mushrooms in Japan and Korea (Komatsu 1976; Kim et al. 2012a, b, c). Species isolated from shiitake mushroom farms are sometimes identified based solely on morphology; as mentioned above, morphology alone is insufficient for accurate identification, and these isolates need to be re-identified and re-evaluated using modern taxonomy concepts.

To better understand the taxonomy of these species, we have been carrying out a preliminary investigation of *Hypocrea*/*Trichoderma* spp. deposited and maintained in the culture collections of the Fungus/Mushroom Resource and Research Center, Tottori University (FMRC), and the Tottori Mycological Institute (TMI), Japan. In addition, we have been collecting fresh *Hypocrea*/*Trichoderma* material to find new species and re-evaluate East Asian *Hypocrea*/*Trichoderma* species concepts.

In this paper, we describe two new *Trichoderma* species, *T. eijii* and *T. pseudolacteum*, based on the results of molecular phylogenetic analyses and phenotypic investigation. We collected *T. eijii* from decaying wood (*Quercus* sp.) in the Tomakomai Experimental Forest in Hokkaido Prefecture, Japan in 2011. *Trichoderma pseudolacteum* (= *Hypocrea lactea* sensu Doi), a *Hypocrea* disease causative agent, has been known to produce severe damage on bedlogs (*Quercus* spp.) of shiitake mushroom farms in Japan (Komatsu 1976).

Materials and methods

Strains and specimens

Twelve strains of *Hypocrea*/*Trichoderma* species were isolated from a decaying bark (*Quercus* spp.) (TUFC 100002 and TUFC 100004) and stromata/substrates on bedlogs (*Quercus* spp.) for shiitake mushroom cultivation (TUFC 60186, TUFC 60205, TUFC 60440, TUFC 61231, TUFC 61490, TUFC 61496, TUFC 61502, TUFC 61505, TUFC 61509 and TUFC 61533). Total 14 strains of *Hypocrea*/*Trichoderma* species (adding two more strains, TUFC 60895 and TUFC 61535). were obtained from the culture collection and herbarium of the FMRC (holding TUFC culture and TUMH specimen collections), and the TMI, in Japan (Table 1). The 14 cultural isolates were maintained on potato dextrose agar (PDA; Becton Dickinson, Sparks, MD, USA) slants at 15 °C. In addition to the isolated strains, DNA sequence data for other reported *Hypocrea*/*Trichoderma* spp. were obtained from GenBank for use in the phylogenetic analyses.

PCR amplification and sequencing

Fungal strains were grown in potato dextrose broth for 3–4 days at 25 °C in a shaking incubator. Mycelia were collected from the cultures by filtration and then transferred to 1.5 mL tubes. DNA was extracted following Cubero et al. (1999).

For the amplification of the internal transcribed spacer (ITS) regions of ribosomal RNA, and RNA polymerase subunit II (*rpb2*), translation elongation factor 1-α (*tef1*), endochitinase (*chi18-5*), and actin (*act*) genes, five different primer sets were used: ITS5 and ITS4 (White et al. 1990), fRPB2-5 F and fRPB2-7cR (Liu et al. 1999), T*act*1 and T*act*2 (Samuels et al. 2006), EF1-728 F (Carbone and Kohn 1999) and tef1-rev (Samuels et al. 2002) or EF-2 (O'Donnell et al. 1998), and chit42-1a and chit42-2a (Samuels et al. 2012a), respectively. PCR mixtures contained 0.5 pmol of each primer, 0.25 mM dNTPs, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 2.5 U of Taq DNA polymerase, and 15 ng of template DNA. PCR conditions for ITS and *tef1* were as follows: an initial denaturation step at 94 °C for 10 min; followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s; and a final elongation step at 72 °C for 10 min. For *rpb2*, *chi18-5*, and *act* gene amplifications, the number of cycles was increased to 40 and the annealing temperature lowered to either 50 °C (*rpb2* and *act*) or 62 °C (*chi18-5*). PCR products were purified using an ExoSAP kit (USB, Cleveland, OH, USA). The purified double-stranded PCR fragments were directly sequenced using a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The same primer sets used to amplify ITS, *tef1*, *chi18-5*, and *act* were employed for sequencing. For *rpb2*, two internal primers, RPB-432F and RPB-450R (Degenkolb et al. 2008), were used for the sequencing reactions. Capillary electrophoresis and data collection were performed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence data were submitted to GenBank (Table 1).

Table 1 TUFC strains and their accession numbers

Strain	Species	Specimen	Host	Collection date	Locality	GenBank accession number				
						ITS	*rpb2*	*tef1*	*act*	*chi18-5*
TUFC 60186 T	*T. pseudogelatinosum*	TNS-F-192712	*Quercus* sp.	13 Oct. 1969	Japan	JQ797389[a]	JQ797405[a]	JQ79739 7[a]	–	–
TUFC 60205	*T. koningiopsis*	–	*Quercus* sp.	Sep. 1970	Japan	JX238474	JX238483	–	–	–
TUFC 60440	*T. pseudostraminem*	TMI 8154	*Quercus serrata*	7 Jul. 1968	Japan	JQ797393[a]	JQ797409[a]	JQ797401[a]	–	–
TUFC 60895 (=CBS 817.68)	*T. koningiopsis*	–	–	–	Sri Lanka	JX238475	–	JX238498	–	–
TUFC 61231	*T. citrinoviride*	–	*Quercus* sp.	–	Japan	–	–	–	JX238490	–
TUFC 61490 (=CBS 133191) T	*T. pseudolacteum* sp. nov.	TMI 8484	*Quercus serrata*	19 Aug. 1987	Japan	JX238469	JX238478	JX238493	JX238486	–
TUFC 61496	*T. pseudolacteum* sp. nov.	TMI 8502	*Quercus* sp.	19 Sep. 1987	Japan	JX238470	JX238479	JX238494	–	–
TUFC 61502	*T. pseudolacteum* sp. nov.	–	*Quercus serrata*	6 Aug. 1991	Japan	JX238471	JX238480	JX238495	–	–
TUFC 61505	*T. pseudolacteum* sp. nov.	–	*Quercus serrata*	1 Oct. 1987	Japan	JX238472	JX238481	JX238496	JX238487	–
TUFC 61509	*T. pseudolacteum* sp. nov.	–	*Quercus serrata*	26 Sep. 1987	Japan	JX238473	JX238482	JX238497	–	–
TUFC 61535 (=CBS 816.68) T	*T. longibrachiatum*	–	–	–	USA	Z31019[b]	DQ087242[c]	AY937412[b]	JX238491	EU401511[c]
TUFC 61533 (=CBS 132690) T	*T. mienum*	TMI 10313	*Quercus* sp.	27 Nov. 1989	Japan	JQ621972[d]	JQ621968[d]	JQ621978[d]	JX238492	–
TUFC 100002 (=CBS 133190) T	*T. eijii* sp. nov.	TUMH 40457	*Quercus* sp.	20 Sep. 2011	Japan	JX238476	JX238484	JX684011	JX238488	JX238499
TUFC 100004	*T. strictipile*	TUMH 40456	*Quercus* sp.	13 Sep. 2011	Japan.	JX238477	JX238485	–	JX238489	JX238500

[a] From Kim et al. (2012c); [b] From Samuels et al. (2012b); [c] From Druzhinina et al. (2012); [d] From Kim et al. (2012b)

Phylogenetic analyses

Raw sequences were proofread, edited, and assembled into contigs using PHYDIT 3.2 (Chun 1995; available at http://plaza.sun.ac.kr/~jchun/phydit). DNA sequences were aligned using ClustalX 1.81 (Thompson et al. 1997), and then manually adjusted using PHYDIT. Ambiguously aligned regions were excluded from subsequent analyses. Alignments were deposited in TreeBASE (www.treebase.org/treebase-web/home.html) under the study ID 13158.

To determine phylogenetic positions of the sampled fungi, datasets were analyzed using maximum parsimony (MP) in PAUP version 4.0b10 (Swofford 2002) and by Bayesian inference in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). Parsimony analysis was conducted using a heuristic search with 1,000 random addition replicates and tree bisection-reconnection (TBR) branch-swapping. Bootstrap support values (MPBS) for internal nodes were calculated from 1,000 replicates of the MP analysis. For the Bayesian approach, best-fit models of nucleotide substitution were first selected for individual and combined sequence datasets using the Akaike information criterion (AIC) in jModeltest (Posada 2008) (Supplemental Table 1). Under AIC settings, the AICc, a correction for smaller sample size (fewer than 50 taxa), was used. The base tree for likelihood calculations was ML-optimized. Phylogenetic analyses were then conducted using the metropolis-coupled Markov Chain Monte Carlo method as implemented in MrBayes. For each analysis, two parallel runs were conducted with one cold and three heated chains for 3–10 million generations depending on the locus, starting with a random tree. The trees were sampled every 100 generations. We deemed that the two runs had converged when the average standard deviation of the split frequencies dropped below 0.01. The trees obtained before convergence was reached were discarded using the burn-in command, and the remaining trees were used to calculate a 50 % majority consensus tree and to estimate posterior probabilities (PPs). PP values below 0.95 were not considered significant, with values below 0.9 not indicated on the resulting phylograms. Detailed information for each gene analysis is given in Supplemental Table 1.

Phenotypic investigation

Dry stromata briefly rehydrated in 3 % KOH and fresh stromata formed on agar were embedded in Tissue-Tek OCT Compound 4583 (Sakura Finetechnical, Tokyo, Japan) and sectioned at a thickness of *ca*. 20 μm using a freezing microtome (REM-710, Yamato Kohki, Saitama, Japan). The following teleomorph characteristics were evaluated: diameter, color, and shape of stromata; texture of subcortical and subperithecial tissue; perithecial shape and reaction to 3 % KOH; ostiolar canal length; ascus length and width; and distal and proximal part-ascospore length and width. If possible, 30 units were measured for each character.

The cultures used for anamorph micromorphological analysis were grown on cornmeal agar (Becton Dickinson, Sparks, MD, USA)+2 % dextrose (CMD), synthetic low-nutrient agar (SNA; Nirenberg 1976), or PDA. All measurements for the morphological analyses were made in 3 % KOH or water. Where possible, 30 units in each collection were measured for each morphological parameter. Growth rate and optimum temperature were determined based on examination of the PDA and SNA cultures (Samuels et al. 2002). After a few days, when colony growth was visible on PDA and SNA, but before conidia were produced, a plug (5 mm diameter) was taken from the actively growing edge of the colony and inoculated onto freshly prepared medium. The inoculum plug was placed mycelial side down, approximately 1 cm from the edge of a vented Petri dish (9 cm diameter) containing 20 mL of freshly made medium. Petri dishes were incubated in the dark at 15–35 °C (in increments of 5 °C), and colony radius was measured after 72 h. Growth tests were conducted three times, at roughly weekly intervals, and the average radius was calculated from the three independent measurements.

Results

Phylogenetic analyses

Information regarding the datasets used and results from phylogenetic analysis of each locus are summarized in Supplemental Table 1. Due to the paucity of phylogenetically informative characters and/or limited number of *Hypocrea*/*Trichoderma* species in the ITS, *act* and *chi18-5* were not used for analyses of combined data. The individual trees of ITS, *act* and *chi18-5* were provided in supplemental Figures (Figs. S1, S2 and S3).

Figure 1 show Bayesian analysis using a TrN+I+Γ model of evolution for 10 million generations was performed on the *rpb2* dataset (including 84 taxa; 317 characters; burninfrac=0.40). MP analysis of the *rpb2* data resulted in 163 most-parsimonious trees of 808 steps (consistency index [CI]=0.3144; retention index [RI]=0.7300; homoplasy index [HI]=0.6856; 120 parsimony-informative). In this tree, strains of *T. pseudolacteum* also group together with strong support (MPBS/PP=100 %/0.97). *Trichoderma eijii* is sister to *H. flaviconidia* Chaverri, Druzhin. & Samuels GJS 99–49, but this relationship is only weakly supported by MPBS (66 %).

Figure 2 shows Bayesian analysis using a GTR+I+Γ model of evolution for 5 million generations was performed on the *tef1* dataset (including 40 taxa; 422 characters; burninfrac=0.25). MP analysis of the *tef1* data resulted in five most-parsimonious trees of 781 steps (CI=0.5134; RI=

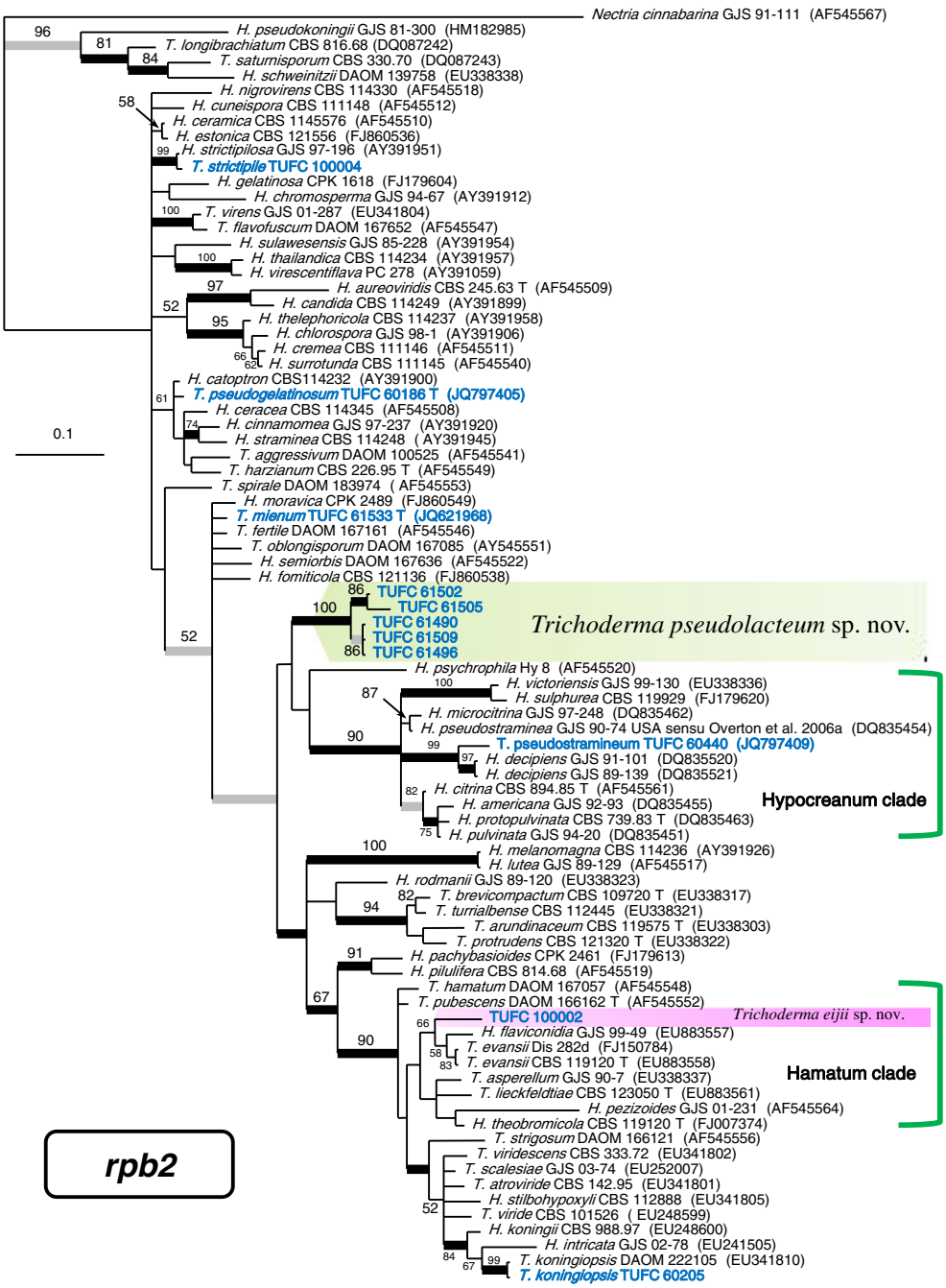

Fig. 1 Bayesian phylogram obtained from the *rpb2* sequences. Broad *black and gray branches* indicate PP> 0.95 and 0.89<PP<0.95, respectively. Only MPBS values >50 % are shown above or below branches. TUFC strains are named in *bold*. *Nectria cinnabarina* GJS 91–111 (AF545567) was used as the outgroup

0.6733; HI=0.4866; 184 parsimony-informative). In this tree, *T. eijii* and *H. pezizoides* Berk. & Broome CBS 115283 are made a group with strong support (MPBS/PP= 99 %/1.0). It is sister—though weakly supported by MPBS (62 %), to a strongly-supported clade comprising two samples of *H. flaviconidia*. The five sampled *T. pseudolacteum* strains form a clade with significant support (MPBS/PP= 100 %/1.0).

Figure 3 shows Bayesian analysis using a GTR+I+Γ model of evolution for 3 million generations was performed on the combined (*rpb2*+*tef1*) dataset (including 35 taxa; 734 characters; burninfrac=0.25). MP analysis of the combined data resulted in six most-parsimonious trees of 1,169 steps (CI=0.4859; RI=0.6502; HI= 0.5141; 276 parsimony-informative). *T. eijii* is clearly separated from *H. pezizoides* and *H. flaviconidia* with supported values (MPBS/PP=85 %/1.0 and 74 %/0.99), respectively. *T. eijii* is included in the Hamatum clade, which, as currently circumscribed (http://www.isth.info/biodiversity/index.php), comprises *H. flaviconidia*, *H. neorufa*, *H. pezizoides*, *T. asperellum*, *T. hamatum*, *T. pubescens*, and *T. theobromicola*. *Trichoderma pseudolacteum* forms a distinct clade with strong support (MPBS/PP=100 %/1.0).

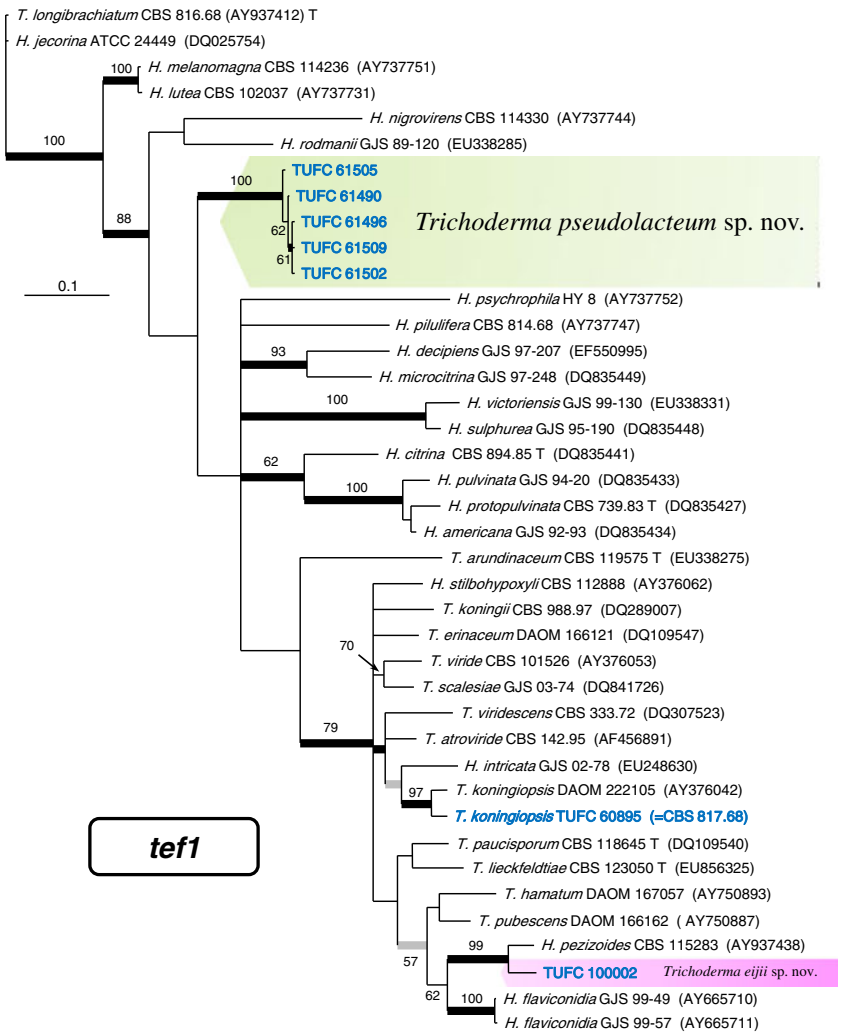

Fig. 2 Bayesian phylogram obtained from the *tef1* sequences. Broad *black and gray branches* indicate PP> 0.95 and 0.89<PP<0.95, respectively. Only MPBS values >50 % are shown above or below branches. TUFC strains are named in *bold*. *Trichoderma longibrachiatum* CBS 816.68 (AY937412) was used as the outgroup

The phylogenetic position of *Hypocrea*/*Trichoderma* species in our study is generally consistent with clade designations and sectional classifications of ISTH (http://www.isth.info/biodiversity/index.php) and Jaklitsch (2009).

Taxonomy

According to the 2011 International Code of Nomenclature for algae, fungi, and plants (Melbourne Code), only one name will be allowed for pleomorphic fungi as of 1 January 2013 (Hawksworth 2012). For the holomorphic name of this species, taxonomists studying *Hypocrea*/*Trichoderma* have expressed a preference for *Trichoderma*, the anamorphic name, over the teleomorph designation *Hypocrea* (http://www.isth.info/vote/). In addition, *Trichoderma* Pers. (1794) is older than *Hypocrea* Fr. (1825) and has priority according to the rules of nomenclature. For these reasons, we have described these new species only under the anamorphic name *Trichoderma*, and do not give a *Hypocrea* name here.

Trichoderma eijii C. S. Kim & N. Maek., **sp. nov.** (Figs. 4 and 5) MycoBank MB 801310

Etymology: Named for Eiji Nagasawa, a specialist and collector of field mushrooms in Japan.

Diagnostic characters: Forming *Acremonium*- to irregularly *Verticillium*-like conidiophores; low growth rates of mycelia on SNA; PDA colonies especially dense, whitish, consisting of concentric rings with irregular outline when cultured in darkness at 30 °C; structure of subperithecial tissue is *textura angularis-epidermoidea*.

Similar species: Phylogenetically, this species is closely related to *Hypocrea flaviconidia*, but the two species are morphologically distinct. In the teleomorph, this species is similar to *H. atroviridis* Dodd, Lieckf. & Samuels, but can be differentiated by phylogenetic position, anamorphic characteristics, and the structure of the subperithecial tissue (see Table 2).

Stromata orange to brown, margin brown, *ca*. 1.9–4.0 mm diam, 0.6–1.5 mm thick (n=10), solitary, scattered, pulvinate; surface smooth, ostiolar dots darker yellow or reddish brown. Cortical layer composed of thick-walled

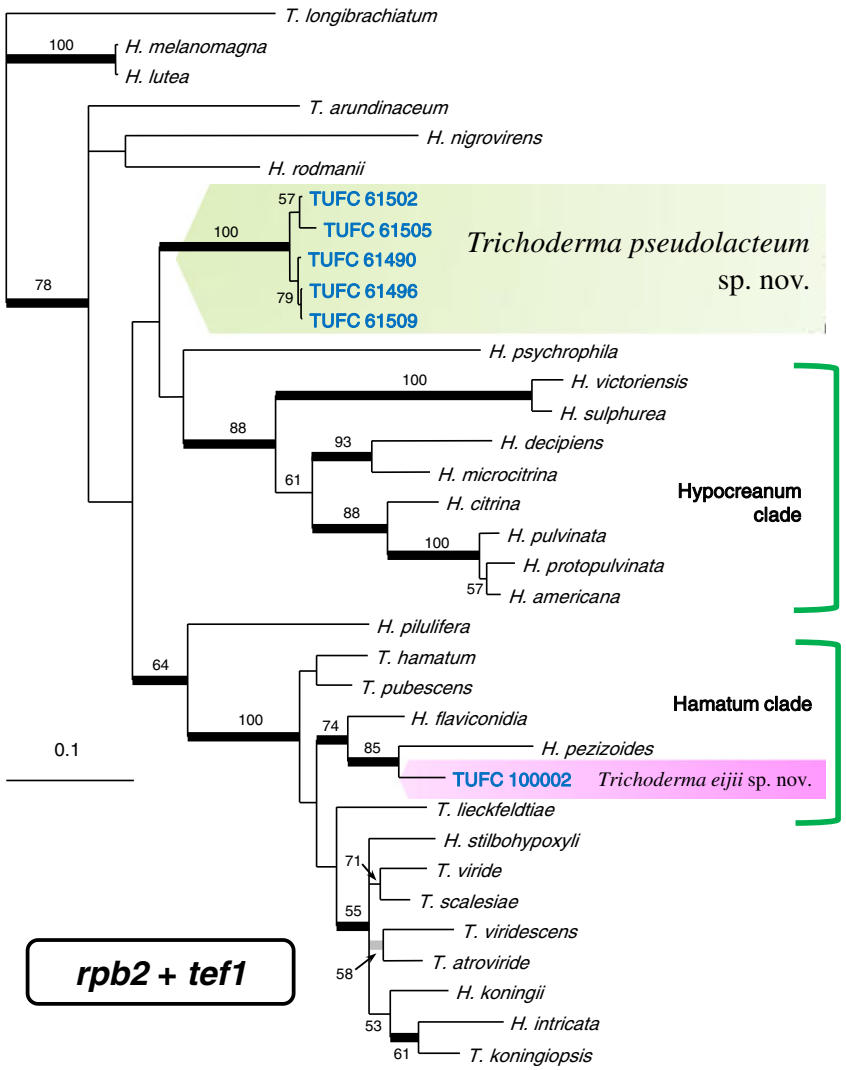

Fig. 3 Bayesian phylogram obtained from the combined data set (*rpb2* and *tef1* sequences). Broad *black and gray branches* indicate PP> 0.95 and 0.89<PP<0.95, respectively. Only MPBS values >50 % are shown above or below branches. TUFC strains are named in *bold*. *Trichoderma longibrachiatum* was used as the outgroup

angular cells, not changing color in 3 % KOH. Cortical and subcortical tissue of *textura angularis-intricata*, hyaline, not changing color in 3 % KOH. Perithecia immersed in the stroma, generally closely aggregated or slightly separated, subglobose to ellipsoidal, 200–248×135–186 µm (n=30), wall composed of compacted cells, slightly changing color in 3 % KOH; length of ostiolar canal 51.4–69.5 µm (n=15); subperithecial tissue of *textura angularis-epidermoidea*, hyaline, not changing color in 3 % KOH; stroma base a *textura intricata* of hyaline hyphae. Asci cylindrical, 77–91.5×3.6–4.6 µm (n=30). Part-ascospores hyaline, warted, dimorphic, distal part globose to subglobose, 3.2–3.8×2.8–3.5 µm, L/W 1.0–1.2 (n=30); proximal part subglobose to ellipsoidal, 3.8–4.7×2.5–3.1 µm, L/W 1.3–1.7 (n=30).

Colonies on CMD after 10 d at 25 °C flat, with discrete small white tufts 0.3–0.8 mm diam (n=10), conidia forming in concentric rings, mycelium loose; no distinctive odor; agar not pigmented. Conidiophores *Acremonium*- to irregularly *Verticillium*-like; phialides lageniform, sometimes hooked, 8.7–13.1×3.0–4.0 µm, L/W 2.4–4.0 (n=30). Conidia green, smooth, globose to subglobose, 2.8–3.5×2.4–3.0 µm, L/W 1.0–1.3 (n=30). Chlamydospores observed, globose to subglobose, terminal or intercalary in hyphae, 7.5–10.6×6.3–9.2 µm, L/W 1.0–1.3 (n=30). Colonies on PDA after 10 d at 25 °C flat, with abundant conidia in aggregated concentric rings; conidia formed within *ca*. 5 d; no distinctive odor; agar not pigmented; mycelium dense; at 30 °C in darkness, PDA colony especially dense, whitish, consisting of concentric rings with irregular outline. Colonies on SNA at 25 °C after *ca*. 21 days flat, mycelium not covering the plate after a month at 25 °C, not forming concentric rings; no distinctive odor; agar not pigmented; mycelium loose.

Colony radius on PDA after 72 h at 15 °C 7.7–8.3 mm, 20 °C 17.4–19.2 mm, 25 °C 28.4–30.2 mm, 30 °C 8.4–9.1 mm, and 35 °C 0 mm (n=3). Colony radius on SNA after 72 h at 15, 20, 30 and 35 °C 0 mm, at 25 °C 3.5–4.4 mm (n=3).

Habitat: decaying wood (*Quercus* sp.).

Known distribution: Japan.

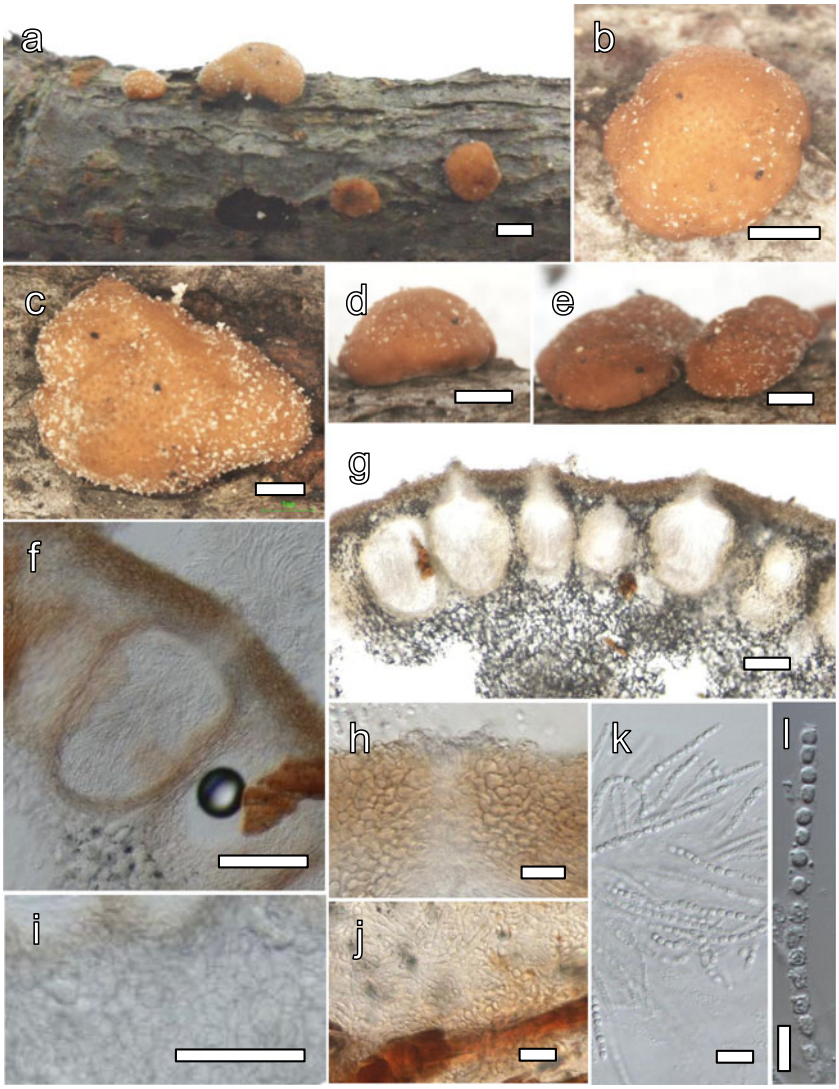

Fig. 4 Teleomorph of *Trichoderma eijii* sp. nov. (TUMH 40457). **a**–**e** Fresh stromata. **f** Perithecia in section (in 3 % KOH). **g** Longitudinal section of stroma. **h** Ostiole, upper part in section. **i** Subperithecial tissue in section. **j** Stroma base in section. **k**, **l** Asci with ascospores. Scale bars: **a**–**e**=1 mm. **f**–**g**, **i**=100 μm. **h**, **j**, **k**=20 μm. **l**=10 μm

Holotype: **Japan**, Hokkaido Prefecture, Tomakomai, 20 Sep. 2011, coll. E. Nagasawa (specimen: TUMH 40457; extype culture: TUFC 100002=CBS 133190).

Trichoderma pseudolacteum C.S. Kim & N. Maek., **sp. nov.** (Figs. 6 and 7) MycoBank MB 801311 = *Hypocrea lactea* sensu Yoshim. Doi, Bull. Natl. Sci. Mus. 15: 665. 1972

Etymology: '*pseudo*'=something false or pretending to be something it is not; '*lacteus*'=milky, refer to white stromata; '*Trichoderma pseudolacteum*' means that this species is not '*Hypocera lactea*', '*-lactea*' change to '*-lacteum*' due to the '*Trichoderma*' (neutral).

Diagnostic characters: Forming rarely-detected *Acremonium*- to irregularly *Verticillium*-like conidiophores; structure of subperithecial tissue *textura intricata*; conidia hyaline, smooth, globose to subglobose, 4.1–5.3×3.7–4.8 μm, L/W 1.0–1.2.

Similar species: In teleomorph, this species is similar to *Hypocrea lactea* (Fr.) Fr. (= *H. citrina* (Pers.) Fr. /*T. lacteum* Bissett) and *H. pseudostraminea* Yoshim. Doi/*T. pseudostramineum* (Yoshim. Doi) C.S. Kim. Phylogenetically, however, this species is clearly distinguished from *H. lactea* and *H. pseudostraminea*/*T. pseudostramineum*. Growth rates on PDA and SNA are also different (see Table 2).

Stromata effuse, white to yellowish, margin brownish in dried specimens; largest continuous stroma broadly attached *ca*. 15–100 mm, 0.4–1.0 mm thick with irregular margins. Cortical and subcortical tissue of *textura intricata*, hyaline, not changing color in 3 % KOH. Perithecia immersed in the stroma, generally closely aggregated or slightly separated, subglobose to ellipsoidal, 140.5–185.7×104.4–147.8 μm, L/W 1.1–1.5 (*n*=30), wall composed of compacted cells, changing color slightly in 3 % KOH; length of ostiolar canal 35.0–47.9 μm, 25.6–40.3 μm diam (*n*=15); subperithecial tissue of *textura intricata-epidermoidea*, hyaline, not changing color in 3 % KOH. Asci cylindrical, 92.7–104.7×5.9–7.1 μm (*n*=20). Part-ascospores hyaline, warted, dimorphic, distal part globose to subglobose 5.4–6.5×5.0–5.9 μm, L/W

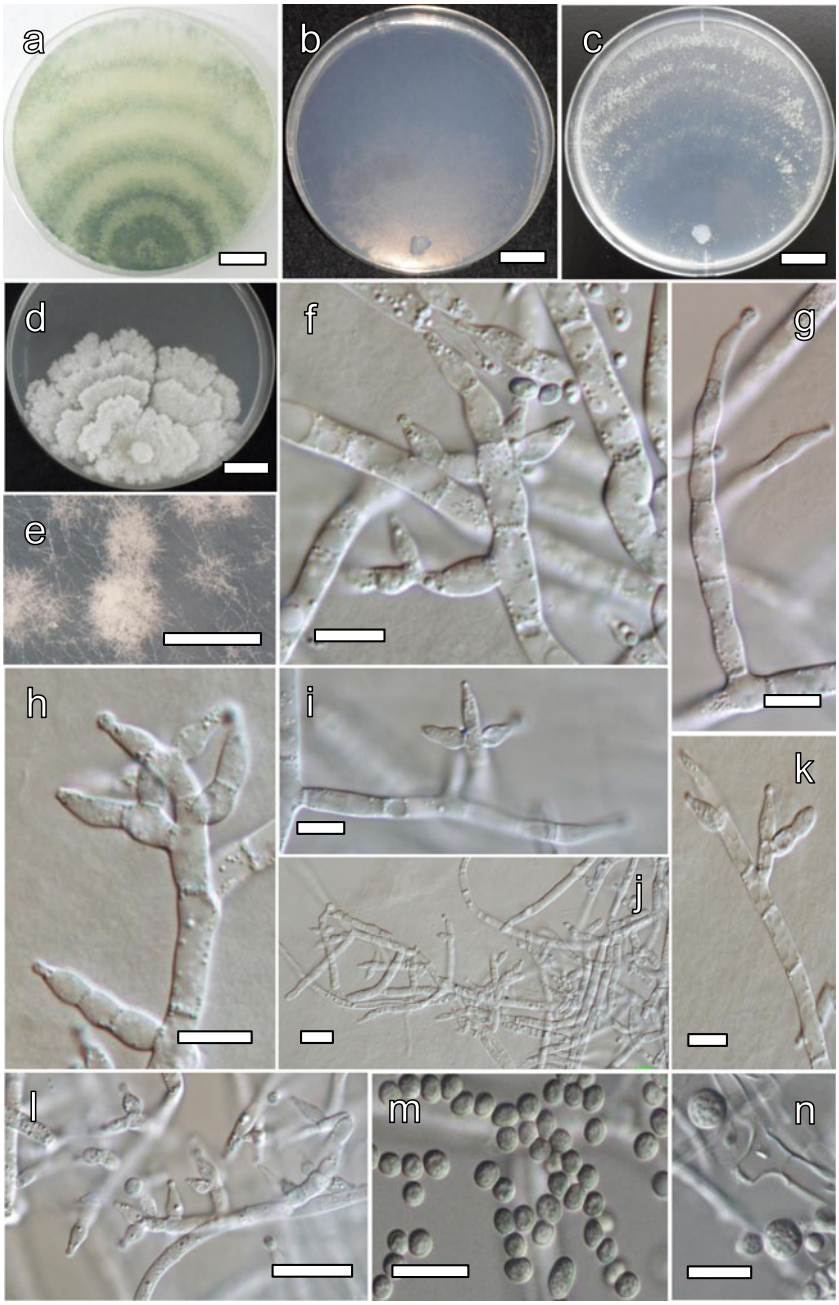

Fig. 5 Anamorph of *Trichoderma eijii* sp. nov. (TUFC 100002). **a**–**c** Cultures at 25 °C (**a** on PDA after 7 d; **b** on SNA after 21 d; **c** on CMD after 7 d). **d** Culture on PDA after 10 d in darkness at 30 °C. **e** Conidiation tufts on CMD (after 10 d at 25 °C). **f**–**l** Conidiophores (**g** *Acremonium*-like). **m** Conidia. **n** Chlamydospores. Scale bars: **a**–**d**=15 mm, **e**=1 mm. **f**–**i**, **m**, **n**=10 μm. **j**, **l**=20 μm

1.0–1.2 (*n*=30); proximal part subglobose to ellipsoidal 5.3–6.9×4.3–5.2 μm, L/W 1.2–1.4 (*n*=30).

Colonies on CMD after 10 d at 25 °C flat, with a thin white layer of mycelium, not forming concentric rings and conidia, no distinctive odor; agar not pigmented. Colonies on PDA after 10 d at 25 °C flat, with a white or slightly yellowish mycelial layer close to the agar surface and a layer of cotton-like aerial mycelium, forming stroma-like structures in old PDA at 20 and 25 °C; conidia formed within 10 d in the aerial mycelium, hyaline, smooth, globose to subglobose, 4.1–5.3×3.7–4.8 μm, L/W 1.0–1.2 (*n*=30); chlamydospores observed, subglobose, terminal or intercalary in hyphae, 6.8–9.1×6.3–8.3 μm, L/W 1.0–1.2 (*n*=30); odor unpleasant; reverse plate slightly yellowish pigmented; mycelium dense; conidiophores *Acremonium*- to irregularly *Verticillium*-like, but rarely detected; phialides lageniform to cylindrical, 8.4–44.3×4.8–5.4 μm, L/W 2.4–13.7 (*n*=30). Colonies on SNA after *ca*. 10 d at 25 °C flat, thin layer of mycelium close to the agar surface, not forming concentric rings or conidia; no distinctive odor; agar not pigmented.

Colony radius on PDA after 72 h at 15 °C 5.8–9.2 mm, 20 °C 28.1–32.1 mm, 25 °C 50.9–61.6 mm, 30 °C 17.7–40.0, and 35 °C 0 mm (*n*=5). Colony radius on SNA after 72 h at 15 °C 0.3–1.9 mm, 20 °C 6.9–11.8 mm, 25 °C 14.1–21.2 mm, 30 °C 7.4–12.8 and 35 °C 0 mm (*n*=5).

Habitat: On bedlogs (*Quercus* spp.) of shiitake mushroom.

Table 2 Morphological comparison *Trichoderma eijii* and *T. pseudolacteum* with their related *Hypocrea*/*Trichoderma* species

Character (References)	Species						
	T. eijii sp. nov. (this study)	*H. pezizoides* (Liu and Doi 1995)	*H. flaviconidia* (Druzhinina et al. 2004)	*H. atroviridis*/*T. atroviride* (Dodd et al. 2003; Jaklitsch 2011)	*T. pseudolacteum* sp. nov. (this study)	*H. citrina*/*T. lacteum* (Jaklitsch 2011)	*H. pseudostraminea*/*T. pseudostramineum* (Kim et al. 2012c)
Origin	Japan	Sri Lanka, New Guinea, China, Philippines, Japan	Costa Rica	ubiquitous	Japan	Europe, Japan, North America	Korea, Japan
Conidiophore type	*Acremonium*- to irregularly *Verticillium*-like	*Verticillium*-like, or *Trichoderma*- to *Gliocladium*-like,	*Trichoderma*- to *Pachybasium*-like, sometimes *Verticillium*-like	*Trichoderma*- to *Pachybasium*-like	*Acremonium*- to irregularly *Verticillium*-like	*Acremonium*- to irregularly *Verticillium*-like	*Acremonium*- to irregularly *Verticillium*-like
Phialides:							
Shape	lageniform	long lageniform	lageniform to cylindrical	mostly lageniform	lageniform to cylindrical	subulate or lageniform	solitary or subulate, variable in length
Size (μm)	8.7–13.1×3.0–4.0	22–44×1.2–3.2	6.2–7×2.7–3	6–10×2.3–3.0	8.4–44.3×4.8–5.4	15–30×3.0–3.5	10.8–26.7×1.9–2.5
L/W	2.4–4.0	ND	2.2–2.5	2.1–3.9	2.4–13.7	4.8–9.4	4.3–13.5
Conidia:							
Color	green	pale green	pale yellow-green	green	hyaline	hyaline	hyaline
Shape	globose to tgroup subglobose	obovate, ellipsoidal or cylindrical	ellipsoidal to oblong	subglobose or oval	globose to subglobose	variable, subglobose, ellipsoidal to long-cylindrical	obovate-ellipsoid to cylindrical
Size (μm)	2.8–3.5×2.4–3.0	2.8–11.6×1.8–4.4	3.7–4×2.2–2.5	3.2–4.0×3.0–3.5	4.1–5.3×3.7–4.8	4.7–10×3.0–4.0	2.8–6.2×2.3–3.3
L/W	1.0–1.3	ND	1.5–1.6	1.0–1.2	1.0–1.2	1.4–2.8	1.2–1.9
Chlamydospores:							
Shape	globose to subglobose	subglobose to ellipsoidal	subglobose	globose to subglobose	subglobose	subglobose to cylindrical	subglobose
Size (μm)	7.5–10.6×6.3–9.2	9.5–21.0	9.5–11.5×7.5–9.5	8.5–12.0	6.8–9.1×6.3–8.3	4.7–10×3.0–4.0	4.8–6.7×3.8–5.1
Colony radius (mm) on PDA after 72 h:							
15 °C	7.7–8.3	ND	0	26–28	5.8–9.2	11–12	17.3–21.9
20 °C	17.4–19.2	ND	ND	ND	28.0–32.1	ND	38.9–50.8
25 °C	28.4–30.2	ND	27–30	57–62	50.1–61.6	54–56	57.5–67.2
30 °C	8.4–9.1	ND	0	40–43	17.7–40.0	43–48	54.0–66.3
35 °C	0	ND	0	0–1.1	0	0	0
Colony radius (mm) on SNA after 72 h:							
15 °C	0	ND	0	21–22	0.3–1.9	10–12	4.9–15.7
20 °C	0	ND	ND	ND	6.9–11.8	ND	15.9–34.2
25 °C	3.5–4.4	ND	0–5	34–37	14.1–21.2	31–33	25.9–51.9
30 °C	0	ND	0	25–29	7.4–12.8	28–32	29.4–58.2
35 °C	0	ND	0	0–1.1	0	0	0
Stromata color	orange to brown	purplish-red to reddish orange	pale brown to brown	orange-red to brick-red	white to yellowish, margin brownish	white to yellowish	pale yellow, brownish orange to light brown

Table 2 (continued)

Character (References)	Species							
	T. eijii sp. nov. (this study)	*H. pezizoides* (Liu and Doi 1995)	*H. flaviconidia* (Druzhinina et al. 2004)	*H. atroviridis*/*T. atroviride* (Dodd et al. 2003; Jaklitsch 2011)	*T. pseudolacteum* sp. nov. (this study)	*H. citrina*/*T. lacteum* (Jaklitsch 2011)	*H. pseudostramineal*/*T. pseudostramineum* (Kim et al. 2012c)	
Subcortical tissue	*t. angularis-intricata*	*t. epidermoidea*	*t. angularis-epidermoidea*	*t. angularis-intricata*	*t. intricata*	*t. globulosa-angularis*	*t. angularis*	
Subperithecial tissue	*t. angularis-epidermoidea*	*t. intricata*	*t. angularis*	*t. angularis*	*t. intricata-epidermoidea*	*t. epidermoidea*	*t. globulosa-angularis*	
Ascospore color	hyaline	hyaline	hyaline	hyaline	hyaline	hyaline	hyaline	
Asci (μm)	77–91.5×3.6–4.6	85–120×5–6	86–91×5.2–5.5	78–89×5.0–5.8	92.7–104.7×5.9–7.1	93–115×5.3–6.3	62–70×3.5–4.1	
Part-ascospores								
Distal (μm)	3.2–3.8×2.8–3.5	4.3–5.4×3.5–4.2	4.5–4.7×4.2–4.3	3.8–4.5×3.7–4.4	5.2–5.9×4.7–5.5	4.4–5.2×3.8–4.4	3.3–3.8×2.8–3.3	
Proximal (μm)	3.8–4.7×2.5–3.1	4.8–6.7×3.5–4.2	5.2–5.5×3.7–4	4.0–5.4×3.0–3.7	5.3–6.9×4.3–5.2	4.7–6.0×3.3–4.0	3.7–4.4×2.7–3.3	

ND, not described

Known distribution: Japan.

Holotype: **Japan**, Nagasaki Prefecture, Kamiagata-gun, Kamiagata-cho, 19 Aug. 1987., coll. A. Nishikawa (specimen: TMI 8484; ex-type culture: TUFC 61490=CBS 133191).

Specimens and/or cultures examined: **Japan**. Shimane Pref.: Nogi-gun, Hakuta-cho, 19 Sep. 1987, coll. S. Hara (specimen: TMI 8502; culture: TUFC 61496); Nogi-gun, Hakuta-cho, 19 Sep. 1987, coll. S. Hosoda (specimen: TMI 8500; culture: TUFC 61497); Ohta City, Kawakura-cho, 7 Dec. 1968., coll. M. Komatsu (specimen: TNS. D-1088= TNS-F-190180); Hirata-shi, 1 Oct. 1987, coll. unknown (culture: TUFC 61505). Shizuoka Pref.: Tenryu City, Zaizu, 2 Oct. 1969, coll. K. Aoshima (specimen: TNS. D649A=TNS-F-190178); Tenryu City, Ueno, 21 Sep. 1969, coll. H. Furukawa (specimen: TNS. D-649B=TNS-F-190179). Tottori Pref.: Tottori-shi, Horadani, 26 Sep. 1987, coll. unknown (culture: TUFC 61509); 6 Aug. 1991., Tottori-shi, Horadani, coll. unknown (culture: TUFC 61502).

Discussion

Based on ITS, *tef1* and combined (*rpb2*+*tef1*) data, *T. eijii* is most closely related to *H. pezizoides* (see Figs. 1, 3 and S1). These species are morphologically distinct, however, differing mainly in size of stroma (up to 21 mm wide in *H. pezizoides*), part-ascospores, phialides, conidia, and type of conidiophores (Figs. 1, S1 and Table 2; Liu and Doi 1995). According to *rpb2* data, *T. eijii* is most closely related to *H. flaviconidia*, but the two species differ markedly with respect to part-ascospore size and anamorphic characters, especially color of conidia and pustules—*H. flaviconidia* forming pale yellow-green conidia and yellow pustules on CMD media, but *T. eijii* forming green conidia and no made yellow pustules (Fig. 1 and Table 2; Druzhinina et al. 2004). Thus, although belonging to the Hamatum clade, *T. eijii* is phylogenetically and morphologically distinct from *H. pezizoides* and *H. flaviconidia* (Figs. 1, 2 and 3 and Table 2). The stroma of *T. eijii* is superficially similar to that of *H. atroviridis*, but the two species can be distinguished by differences in subperithecial tissue structure, size of part-ascospores, and conidiophore type (see Table 2).

Bissett (1991) established *Trichoderma* sect. *Hypocreanum* and designated *T. lacteum*, the anamorph of *H. citrina*, as the type species. Members of this section are distinguished by their primitive characters, with sparsely-branched *Acremonium*- to *Verticillium*-like conidiophores and hyaline conidia highly variable in shape (Jaklitsch 2011). Samuels (1996) demonstrated that species of this section, like those of sect. *Hypocreanum*, may have lost the ability to produce a primary *Trichoderma*-like anamorph. Although not all species have been examined, members of this section have been comparatively well-sequenced and re-described by mycologists such as Overton et al. (2006a, b) and Jaklitsch (2011). Using

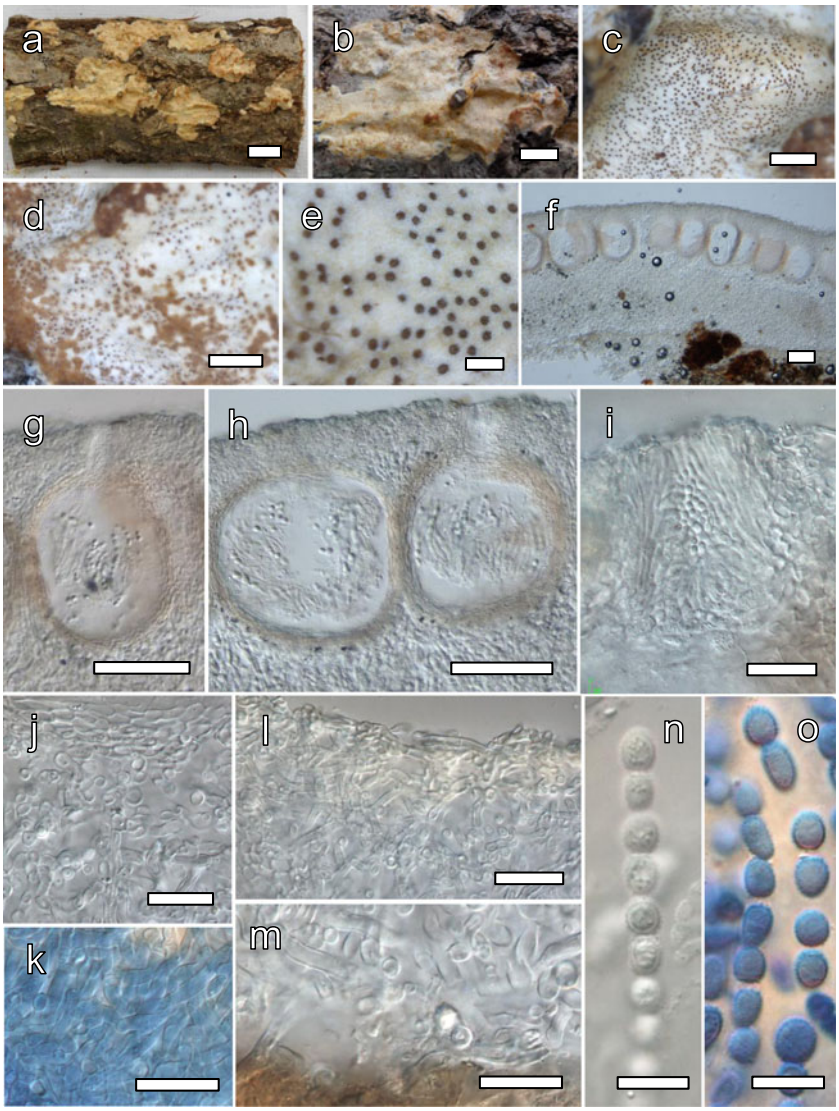

Fig. 6 Teleomorph of *Trichoderma pseudolacteum* sp. nov. (TMI 8484). **a**–**e** Dry stromata. **f** Longitudinal section of stroma. **g**, **h** Perithecia in section. **i** Ostiole in section. **j**, **k** Subperithecial tissue in section (**k** in *cotton blue*). **l** Cortical and subcortical tissue in section. **m** Stroma base in section. **n**, **o** Asci with ascospores (**o** in *cotton blue*). Scale bars: **a**, **b**= 1 cm. **c**, **d**=1 mm. **e**, **f**=200 μm. **g**, **h**=100 μm. **i**–**m**=20 μm. **n**, **o**=10 μm

Japanese specimens, Doi (1972) distinguished *H. lactea* from *H. citrina* based on the subcortical tissue structure (*textura intricata*), size of part-ascospores, and type of conidiophores in the anamorph state. In a recent study, Overton et al. (2006a) designated a lectotype for *H. lactea* and placed that species in synonymy with *H. citrina*. They proposed that *H. lactea* sensu Doi, however, was most likely an undescribed species from Japan, because its description did not match Fries's historical specimen of *H. lactea*. Our phylogenetic and phenotypic results confirm that *T. pseudolacteum* (= *H. lactea* sensu Doi) is clearly distinct from *H. lactea* (= *H. citrina*) and it is strongly supported as a separate clade (Figs. 1, 2 and 3 and Table 2).

Based on several studies (Dodd et al. 2002; Kullnig-Gradinger et al. 2002; Chaverri et al. 2003), sect. *Hypocreanum* is not monophyletic and should be merged with sect. *Pachybasium* because the two sections are phylogenetically indistinguishable. Overton et al. (2006b), however, recognized a major Hypocreanum clade in their study that included 17 taxa associated—on the basis of morphology—with sect. *Hypocreanum*, even though some of them belong to sect. *Pachybasium* according to their molecular analyses. Morphologically, *T. pseudolacteum* might be included in sect. *Hypocreanum*, but in our phylogenetic analysis, we found that this species does not fall into the Hypocreanum clade (Figs. 1, 2 and 3).

Hypocrea pseudostraminea was re-described by Overton et al. (2006a). Although this species was first reported from Japan (Doi 1972), they did not sequence any Japanese *H. pseudostraminea* strains. Kim et al. (2012c) definitively determined the phylogenetic position of this species (belonging to the Hypocreanum clade) and proposed the new combination *T. pseudostramineum*. The possibility remains, however, that strains of *H. pseudostraminea* sensu Overton et al. (2006a) represent a new species closely related to *H. microcitrina* Yoshim. Doi rather than to *H. pseudostraminea*/*T. pseudostramineum* (Fig. 1; Kim et al. 2012c). Two *Trichoderma* strains, TUFC 60205 and TUFC 60805, have

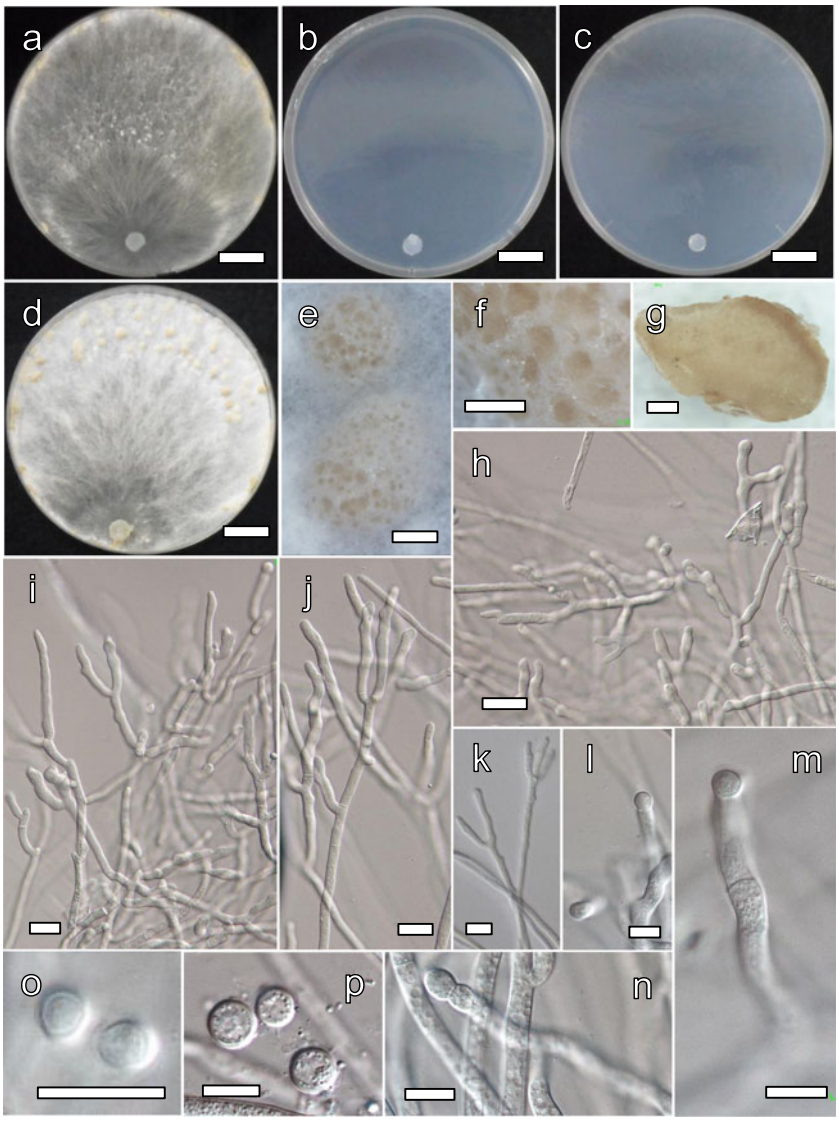

Fig. 7 Anamorph of *Trichoderma pseudolacteum* sp. nov. (TUFC 61490). **a**–**c** Cultures after 10 d at 25 °C (**a** on PDA; **b** on SNA; **c** on CMD). **d** Cultures after 3 months in darkness at 25 °C. **e**, **f** Stroma-like structures on old PDA. **g** Section of stroma-like structure. **h**–**n** Conidiophores-like structures (**h**–**k** *Verticillium*-like; **l**–**n** *Acremonium*-like). **o** Conidia. **p** Chlamydospores. Scale bars: **a**–**d**=15 mm. **e**, **g**=1 mm. **f**=500 μm. **h**-**k**=20 μm. **l**–**p**=10 μm

been deposited as *H. muroiana* Hino & Katumoto in FMRC. Based on molecular and phenotypic characters, however, morphologically recognized species of *T. koningii* have been separated into 12 taxonomic species and one variety (Samuels et al. 2006). In our study, the two FMRC strains were determined to be *T. koningiopsis* Samuels, C. Suárez & H.C. Evans, a newly recorded species from Japan.

Doi's *Hypocrea*/*Trichoderma* specimens are stored in the mycological herbarium of the National Museum of Nature and Science, Japan (TNS). Unfortunately, the specimens were repeatedly fumigated with methyl bromide in the past, making successful DNA extraction highly unlikely. In addition, only a few cultures derived from these specimens are available from a limited number of culture collections in Japan, such as the Tottori Mycological Institute (TMI) and the NITE (National Institute of Technology and Evaluation) Biological Resource Center. Thus, East Asian *Hypocrea*/*Trichoderma* species, including those described by Doi, need to be re-collected for re-identification and re-evaluation in the near future.

In this paper, we have described the new species *T. eijii* and *T. pseudolacteum* based on molecular phylogeny and morphological characteristics. *Trichoderma eijii* has been isolated from *Quercus* sp. Although it has not yet been isolated from mushroom farms, this species has the potential to cause economic losses on shiitake mushroom farms, where *Quercus* tree bedlogs are used. In general, *Hypocrea*/*Trichoderma* species grow mycelia faster than commercial mushrooms, which means this species could outcompete mushrooms for space and nutrients on compost or bedlogs (see Fig. S4). In the near future, we plan to investigate the interaction between cultures of shiitake mushroom (*L. edodes*) and *Hypocrea*/*Trichoderma* species, including these two new species, to better understand their antagonistic activity and to reduce crop losses due to *Hypocrea*/*Trichoderma* infestation on commercial mushroom farms.

Conidia and/or part-ascospores of most *Hypocrea*/*Trichoderma* species have a sticky matrix and/or a spinulose to warted structure that can be used for attachment to many different surfaces. These characteristics may facilitate adaptation and dissemination by water, insects, mites, and rodents (Seaby 1989; Anonymous 2002; Chaverri and Samuels 2003). In addition, conidia and part-ascospores can adhere to equipment and work clothes of mushroom farm workers. When we collected *Hypocrea*/*Trichoderma* samples from mushroom farms and natural environments, we frequently observed mites and insect larvae in and on mushroom substrates. To the best of our knowledge, diseases caused by *Hypocrea*/*Trichoderma* species during mushroom cultivation are difficult to control. At present, disease prevention using pest management and good sanitation represents the best control strategy. To enhance control of this disease in mushroom cultivation, detailed ecological information regarding *Hypocrea*/*Trichoderma* species would benefit mushroom growers and breeders.

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