



# *Trichoderma yilanense* (Hypocreales), a new species from Taiwan

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**ABSTRACT:** *Trichoderma* species are widely distributed and omnipresent. They are important for agriculture, industry, and the environment. This study introduces and illustrates a new species, *Trichoderma yilanense*, based on both morphological and molecular evidence. Phylogenetic analyses were conducted using sequences from rDNA ITS1-5.8S-ITS2 (ITS), the translation elongation factor 1-alpha (*tefl*) and the RNA polymerase II subunit (*rpb2*) gene regions. The results support the classification of the new species as a distinct member within the Harzianum clade of the genus *Trichoderma*. Furthermore, the morphological similarities and differences between the new species and its phylogenetically close relatives are discussed.

**KEY WORDS:** Harzianum clade, new species, phylogeny, taxonomy, *Trichoderma pinicola*, *Trichoderma simplex*.

## INTRODUCTION

*Trichoderma* (Ascomycota, Sordariomycetes, Hypocreales) is a prevalent genus that can be found in diverse substrates, including soil, plant material, decaying wood, and as endophytes in living plant tissues (Zheng *et al.*, 2021). Additionally, *Trichoderma* species have been found in aquatic and desert ecosystems (Qiao *et al.*, 2018; Zheng *et al.*, 2021).

*Trichoderma* was initially established with four species, namely *T. aureum*, *T. nigrescens*, *T. roseum*, and *T. viride*, that differ in conidial color (Persoon, 1794). After further molecular and morphological studies, although other species were added to the genus, only *T. viride* remained among the initial four species (Zheng *et al.*, 2021). Traditionally, the main diagnostic criteria for the genus *Trichoderma* were mainly judged by its phenotypic characteristics (Rifai, 1969; Bissett, 1984, 1991a,b). Nevertheless, identifying *Trichoderma* species based solely on morphological features is challenging because their morphological characteristics overlap. Moreover, their morphological traits vary depending on their environments, and the sexual morphs of many *Trichoderma* species remain unidentified (Zheng *et al.*, 2021; Ma *et al.*, 2024).

In recent decades, molecular phylogenetic affinity has influenced the classification and identification of *Trichoderma*. The species diversity of *Trichoderma* has rapidly expanded based on phylogenetic data (Qiao *et al.*, 2018; Cai *et al.*, 2022). To date, *Trichoderma* is one of the most diverse fungal genera and encompasses more than 500 species (Sousa *et al.*, 2023). Nowadays, *Trichoderma* species are typically identified using a combination of phenotypic traits and multi-gene phylogenetic analysis (*tefl* and *rpb2*) (Chaverri and Samuels, 2004; Bissett *et al.*, 2015; Jaklitsch and

Voglmayr, 2015; Zhu and Zhuang, 2015; Cai and Druzhinina, 2021; Zhao *et al.*, 2023; Lagashetti *et al.*, 2023). Although the rDNA ITS1-5.8S-ITS2 (ITS) region is proposed as the universal DNA barcode for fungi, it is not sufficient to distinguish species of *Trichoderma*. However, ITS was suggested to remain the primary DNA barcode locus, because it is highly diagnostic at the generic level for *Trichoderma* species (Cai and Druzhinina 2021).

A new species of *Trichoderma* was obtained during a soil fungi survey of Taiwan. *Trichoderma yilanense* sp. nov. is described and proposed herein. This new species has distinct *tefl* and *rpb2* sequences from its phylogenetically close species *T. pinicola* and *T. simplex*. Furthermore, comparisons between the new taxon and its close relatives are provided.

## MATERIALS AND METHODS

### Isolation

Soil samples were collected from northeastern Taiwan, and the gradient dilution and spread plate methods were used to isolate the fungi. Among the isolated fungi, a *Trichoderma* species was discovered and subsequently identified as a new taxon. The deep-frozen culture was kept in the Bioresources Collection and Research Center of the Food Industry Research and Development Institute, Hsinchu, Taiwan.

### DNA Extraction, PCR Amplification, and Sequencing

The fungal DNA was extracted from cultures grown in potato dextrose broth at 25 °C for 3 days using the Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) according to the manufacturer's instructions. The primer pair ITS5/ITS4 was used to amplify the ITS region (White *et al.*, 1990).

**Table 1.** *Trichoderma* strains used in this study.

Species	Strain	GenBank accession no.		
		<i>tefl</i>	<i>rpb2</i>	ITS
<i>T. achlamydosporum</i>	YMF 1.06226 <sup>T</sup>	MT070156	MT052180	NR_176715
<i>T. catoptron</i>	DAOM 232830 <sup>T</sup>	KJ871245	KJ842166	-
<i>T. ceraceum</i>	G.J.S. 95-159 <sup>T</sup>	AY937437	AF545508	AF275332
<i>T. cerinum</i>	DAOM 230012 <sup>T</sup>	KJ871242	KJ842184	NR_111835
<i>T. dacrymycellum</i>	WU 29044	FJ860633	FJ860533	FJ860749
<i>T. graminicola</i>	YNE00490 <sup>T</sup>	OR779521	OR779494	-
<i>T. hausknechtii</i>	CBS 133493 <sup>T</sup>	KJ665515	KJ665276	-
<i>T. helicelixii</i>	CBS 133499 <sup>T</sup>	KJ665517	KJ665278	-
<i>T. hirsutum</i>	HMAS 248834 <sup>T</sup>	KY688029	KY687972	NR_154565
<i>T. hirsutum</i>	HMAS 248859	KY688030	KY687998	KY687942
<i>T. koreanum</i>	SFC20131005-S066 <sup>T</sup>	MH025979	MH025988	MH050352
<i>T. linzhiense</i>	HMAS 248846 <sup>T</sup>	KY688047	KY687985	NR_154575
<i>T. longifialidicum</i>	LESF 552 <sup>T</sup>	KT279020	KT279955	NR_137309
<i>T. parapeberdyi</i>	T30677 <sup>T</sup>	OR779510	OR779483	-
<i>T. peberdyi</i>	CEN 1426 <sup>T</sup>	MK696664	MK696825	NR_173288
<i>T. pinicola</i>	SFC20130926-S233 <sup>T</sup>	MH025981	MH025993	MH050354
<i>T. pinicola</i>	SFC20130926-S014	MH025978	MH025991	-
<i>T. pinicola</i>	SFC20130926-S111	MH025980	MH025992	-
<i>T. polypori</i>	HMAS 248855 <sup>T</sup>	KY688058	KY687994	NR_154580
<i>T. propepolypori</i>	YMF 1.06224 <sup>T</sup>	MT070158	MT052181	MN977789
<i>T. pseudogelatinosum</i>	CNUN 309 <sup>T</sup>	HM920202	HM920173	NR_144878
<i>T. simplex</i>	HMAS 248842 <sup>T</sup>	KY688041	KY687981	NR_154572
<i>T. simplex</i>	HMAS 248860	KY688042	KY687999	KY687943
<i>T. tomentosum</i>	DAOM 178713a <sup>T</sup>	EU279969	AF545557	NR_134357
<i>T. velutinum</i>	DAOM 230013 <sup>T</sup>	AY937415	JN133569	NR_111836
<i>T. viridulum</i>	HMAS 273865 <sup>T</sup>	KX026957	KX026965	-
<b><i>T. yilanense</i></b>	<b>BCRC 18F0052<sup>T</sup></b>	<b>PP779115</b>	<b>PP779116</b>	<b>PP786515</b>

Data obtained from this study are indicated in bold. Superscript “T” denotes type or ex-type strain.

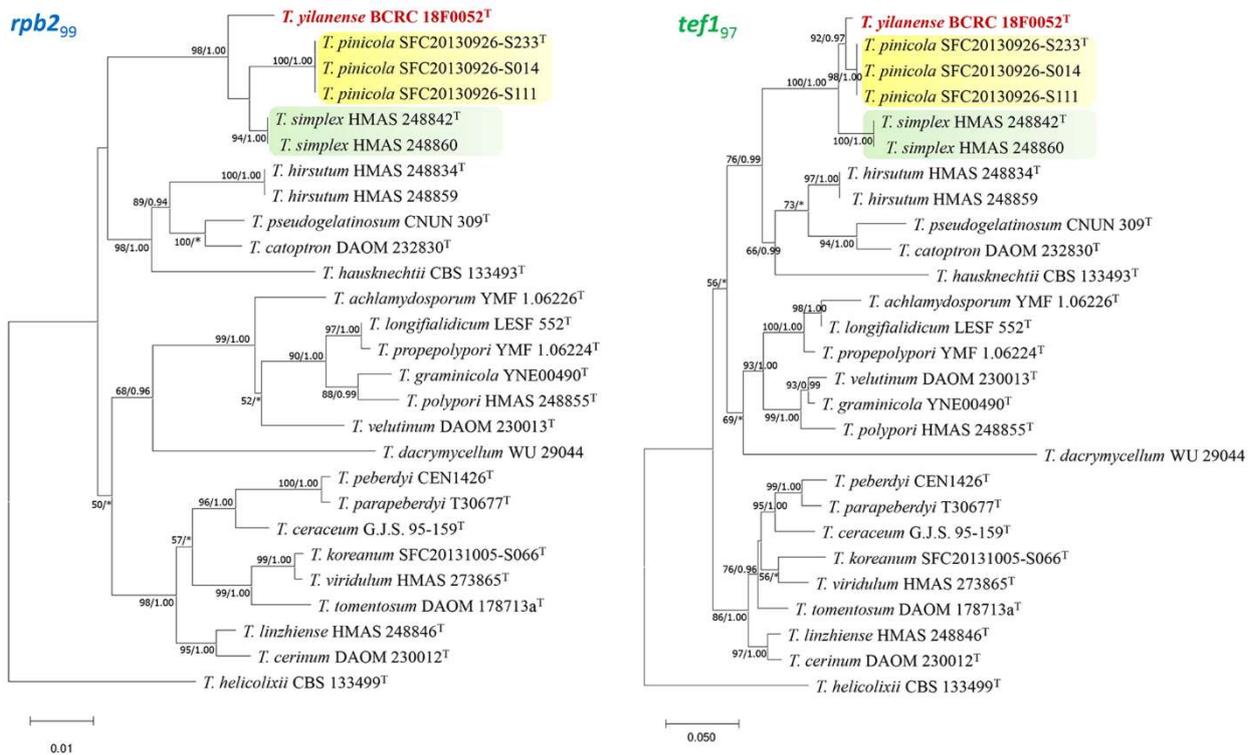
The primer pair EF-728F/TEF1LLEREv was used to amplify part of the translation elongation factor 1-alpha (*tefl*) gene (Carbone and Kohn, 1999; Jaklitsch *et al.*, 2005). The primer pair fRPB2-5F/fRPB2-7cR was used to amplify part of the RNA polymerase II subunit (*rpb2*) gene (Liu *et al.*, 1999). The polymerase chain reaction (PCR) conditions were performed as follows: an initial denaturation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min 30 s, and a final extension step at 72°C for 10 min. Automated sequencing was performed at TRI-I Biotech Inc. (Xizhi District, New Taipei, Taiwan) using the same primers used for amplification.

### Phylogenetic Analyses

To determine species closely related to our strain, we conducted NCBI megablast searches using the ITS, *rpb2*, and *tefl* sequences (The NCBI database was last accessed on December 16, 2024, to confirm these results). For phylogenetic analyses, we retrieved closely related sequences from NCBI based on these results and previous studies (Gu *et al.*, 2020; Cao *et al.*, 2022; Ye *et al.*, 2023; Zhao *et al.*, 2024). The DNA sequences of the *Trichoderma* strains used for phylogenetic analysis are

provided in Table 1. Additionally, the newly generated ITS, *rpb2*, and *tefl* sequences from this study were deposited in NCBI GenBank under the accession numbers PP779115 (*tefl*), PP779116 (*rpb2*), and PP786515 (ITS).

Alignments of the two genes were performed separately using the MAFFT online server v7 (Katoh *et al.*, 2019) with default parameters. The resulting alignments were then manually trimmed based on the guidelines provided by Cai and Druzhinina (2021), using MEGA 11 (Tamura *et al.*, 2021). Maximum likelihood (ML) analyses of the *tefl* and *rpb2* gene regions were conducted separately using IQ-TREE 2 (Minh *et al.*, 2020), incorporating the built-in ModelFinder tool (Kalyaanamoorthy *et al.*, 2017). The best-fit models, HKY+F+G4 for *tefl* and TNe+G4 for *rpb2*, were applied to each respective gene region. Branch support was assessed using 1,000 ultrafast bootstrap replicates for each analysis (Hoang *et al.*, 2018). *Trichoderma helicelixii* was used as an outgroup. Bayesian Inference (BI) analysis was conducted using MrBayes v3.2.7a (Ronquist *et al.*, 2012). Four Metropolis-coupled Markov Chain Monte Carlo (MCMC) chains were run for 2 million generations, sampling every 1,000 generations, with a 25% burn-in. The Bayesian analyses for *tefl* and



**Fig. 1.** The maximum likelihood tree constructed from *tef1* (right) and *rpb2* (left) sequences. The newly described species *Trichoderma yilanense* is highlighted in bold red font. Maximum likelihood bootstrap values ( $\geq 50\%$ ) from IQ-TREE 2 (left) and posterior probabilities ( $\geq 0.90$ ) from Bayesian inference (right) are displayed at the nodes; otherwise (lower or absent), marked with "\*\*". The scale bar represents 0.05 (for *tef1*) and 0.01 (for *rpb2*) substitutions per nucleotide position. *Trichoderma helicophilii* was used as the outgroup. Superscript "T" denotes type or ex-type strain.

*rpb2* loci reached good convergence, with average standard deviations of split frequencies at 0.002308 and 0.002003, respectively. Maximum likelihood bootstrap proportions (MLBP  $> 50\%$ ) and Bayesian posterior probabilities (BIPP  $> 0.9$ ) were labeled on branch nodes of the respective *tef1* and *rpb2* phylogenetic trees. Pairwise similarities of *tef1* and *rpb2* sequences between the new species and its relatives were calculated following the 'molecular identification protocol for a single *Trichoderma* isolate' (Cai and Druzhinina, 2021) to support phylogenetic analyses.

### Morphological study

Pure cultures of the fungus were observed and described in terms of the colony color and appearance, based on the colonies grown on 90 mm-plates of CMD (cornmeal dextrose agar), PDA (potato dextrose agar) and SNA (synthetic nutrient-poor agar). Plates were incubated for a week in the 12/12 h light/dark cycles at 25 °C. Micro-morphological features were observed from cultures grown on SNA at 25 °C. Growth-rate trials were conducted on 90-mm plates containing CMD, PDA, and SNA in the dark at 20, 25, and 30 °C. Each growth-rate trial was replicated three times. The colony radius was measured after 72 hours of incubation.

## RESULTS

### Phylogenetic analyses

The ITS sequence similarity of *T. yilanense* exceeded 85.50% when compared to sequences in the *ITS56* dataset, confirming its affiliation to the genus *Trichoderma*. Phylogenetic trees for the *tef1* and *rpb2* loci were constructed separately (Figure 1), both illustrating *T. yilanense* forming a distinct branch clustering with *T. pinicola* S.-Y. Oh, M.S. Park & Y.W. Lim (Phookamsak *et al.*, 2019) and *T. simplex* K. Chen & W.Y. Zhuang (2017). Pairwise sequence similarities for the *tef1* tree were 98.26% (*T. yilanense* vs. *T. pinicola*) and 96.47% (*T. yilanense* vs. *T. simplex*), while for the *rpb2* tree, they were 98.03% (*T. yilanense* vs. *T. pinicola*) and 98.77% (*T. yilanense* vs. *T. simplex*). According to the species identification criteria of Cai *et al.* (2021), our *rpb2* gene sequence comparison failed to satisfy the conspecific standard condition  $\exists!(rpb2_{99} \cong tef1_{97})$ , thus providing strong molecular evidence for the establishment of this novel species. This conclusion is corroborated by the *rpb2* phylogenetic analysis which clearly resolved the new species as distinct from its close relatives. Although *tef1* reference sequences for related species incompletely covered the standard region proposed by Cai *et al.* (2021), rendering their *tef1* species threshold inapplicable to our

**Table 2.** Comparison of morphological characteristics of *T. yilanense* and closely related species.

	<i>T. pinicola</i>	<i>T. simplex</i>	<i>T. yilanense</i>
<b>Phialides</b>			
Phialide number	1–3	1–3	1–5
length (µm)	7.8–13.3	(8.6–)10.1–16.9(–19.4)	(6.4–)7.6–13.1(–18.7)
width (µm)	(2.5–)2.6–4.1(–4.5)	2.2–3.6	(2.0–)2.5–3.4(–4.0)
L/W	1.9–4.4(–4.5)	2.5–6.7	(1.8–)2.5–5.2(–7.5)
<b>Conidia</b>			
length (µm)	3.5–4.9(–5)	3.1–4.4	3.0–4.7(–5.1)
width (µm)	2.8–3.5(–3.6)	2.8–3.3	(2.8–)2.9–4.0(–4.2)
L/W	1.1–1.6	(1.0–)1.1–1.4	1.0–1.3(–1.5)
shape	subglobose to ellipsoid	ellipsoid, globose, oval	globose, ellipsoid, oval
<b>Chlamydospores</b>			
CMD	rare, globose, 5.2–10(–10.1) × 5.2–10 µm	absent	rare, globose to subglobose, 5.2–10.3 × 4.8–8.7 µm
SNA	NA	common, globose or ellipsoid, 4.8–8.9(–10.3) × 4.1–6.9(–8.3) µm	Absent
<b>Reference</b>	Phookamsak <i>et al.</i> (2019)	Chen & Zhuang (2017)	This study

“NA” indicates data not available.

comparisons, phylogenetic analysis still recovered the new species as a highly supported and distinct lineage, clearly separated from its close relatives. Figure 1 illustrates the distinct phylogenetic placement of *T. yilanense* within the Harzianum clade. Collectively, the *rpb2* sequence comparison against established species identification criteria and the phylogenetic analyses of both *rpb2* and *tefl* strongly support the recognition of *T. yilanense* as a new species.

## TAXONOMIC TREATMENT

*Trichoderma yilanense* Y.-H. Wei & S.-S. Tzean, *sp. nov.*  
宜蘭木黴 Fig. 2

**MycoBank:** MB853963

**Gene sequences (ex-holotype):** (ITS) PP786515; (*tefl*) PP779115; (*rpb2*) PP779116

**Holotype:** TAIWAN. Yilan County, Toucheng Township, Beiguan Tidal Park, soil, 1 Apr. 2018, leg. C.-C. Chen & G.-Y. Liou, holotype TNM F0038575 (deposited in the National Museum of Natural Science, Taichung, Taiwan).

Ex-type strain BCRC 18F0052, stored in a metabolically inactive state (deep-frozen) in the Bioresource Collection and Research Center of the Food Industry Research & Development Institute, Hsinchu, Taiwan.

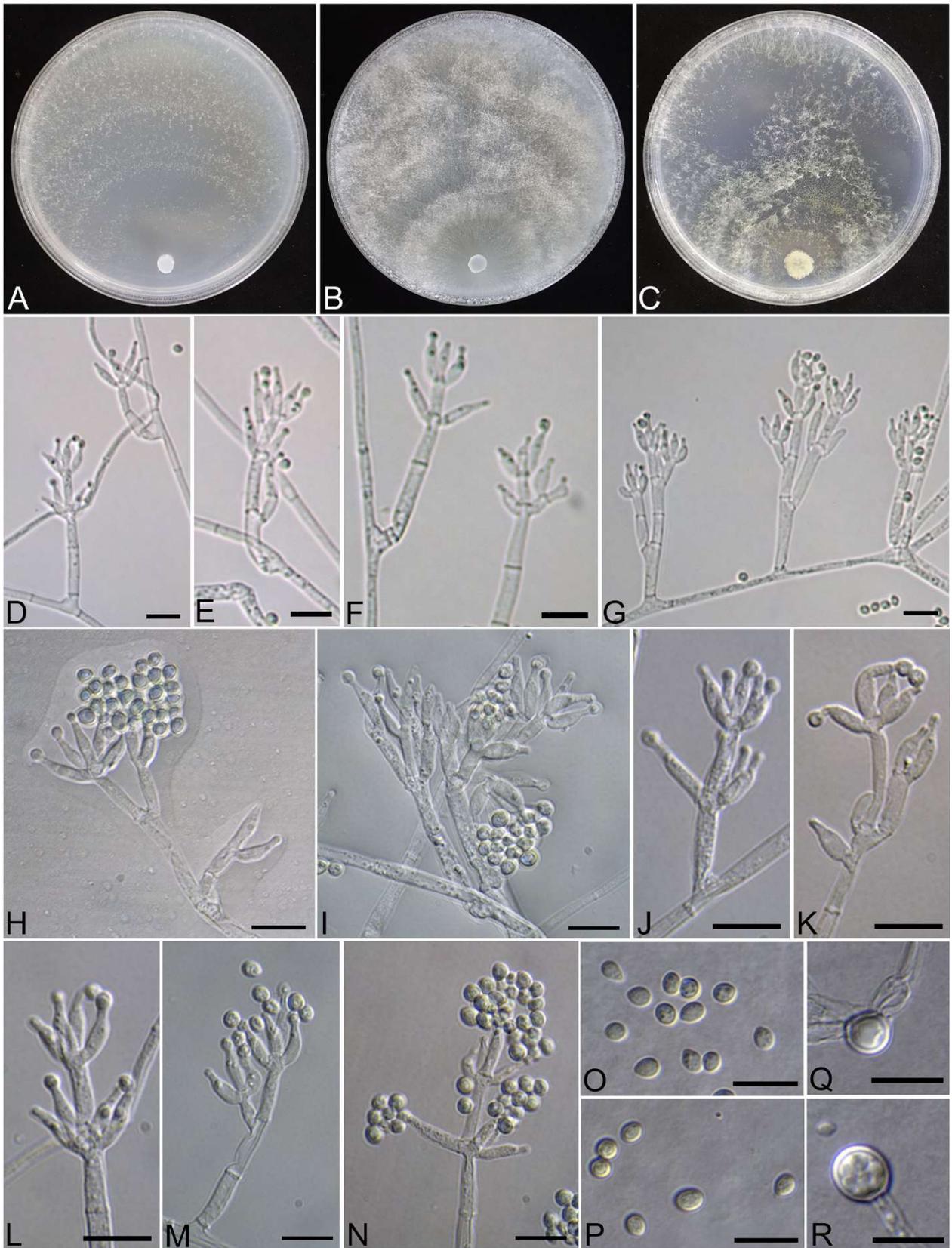
**Description:** Colour codes follow Methuen Handbook of Colour (Kornerup and Wanscher 1978). On CMD after 72 h, colony radius 38–39 mm at 20 °C, 50–52 mm at 25 °C, and 43–44 mm at 30 °C. After 7 days at 25 °C, colony translucent, radial, velvety, mycelium white. Conidial pustules more abundant in 3–4 concentric rings, white at first, then turning greyish green to dark green (27E7–8). *Chlamydospores* rare, globose to subglobose, terminal or intercalary, 5.2–10.3 × 4.8–8.7 µm. No distinct odor. No diffusing pigment noted.

On PDA after 72 h, colony radius 33–34 mm at 20 °C, 32–33 mm at 25 °C, and 32–33 mm at 30 °C. After 7 days at 25 °C, colony radial, indistinctly zonate in the center, mycelium dense, white, and velvety to floccose. No distinct odor, diffusing pigment nil.

On SNA after 72 h, colony radius 38–40 mm at 20 °C, 37–47 mm at 25 °C, and 46–48 mm at 30 °C. After 7 days at 25 °C, colony hyaline, radial, mycelium loose, fuzzy zonate. Conidiation starting after 2 days, then formed in pustules, firstly white and turning greyish green (27C3–E7), spreading in concentric rings around the original inoculum, and dispersedly distributed in other area. No distinct odor, diffusing pigment absent. *Conidiophores* straight or curved, solitary, paired, or in whorls of 3–4 branches, hyaline, smooth walled. *Phialides* subulate or ampulliform, straight or curved, occasionally hooked, smooth walled, hyaline, singly, paired or in whorls of 3–5, (6.4–)7.6–13.1(–18.7) × (2.0–)2.5–3.4(–4.0) µm, l/w ratio (1.8–)2.5–5.2(–7.5), (1.5–)1.7–2.5(–3.0) µm wide at the base. *Conidia* smooth walled, hayaline, green in mass, globose, ellipsoid, or oval, (3.0–)3.2–4.8(–5.1) × (2.8–)3.0–3.9(–4.0) µm, l/w ratio 1.0–1.3(–1.5). *Chlamydospores* absent.

**Notes:** *Trichoderma yilanense* has a close phylogenetic relationship with *T. pinicola* and *T. simplex*. Morphologically, it differs from its relatives by exhibiting a phialide number ranging from 1 to 5, whereas *T. pinicola* and *T. simplex* typically have phialide numbers of 1 to 3. Additionally, *T. yilanense* does not produce chlamydospores on SNA but shows rare chlamydospore production on CMD. In contrast, *T. simplex* lacks chlamydospore production on CMD and commonly produces them on SNA. A more comprehensive comparison of morphological characteristics between *T. yilanense* and its related species is shown in Table 2.

**Etymology:** The specific epithet “*yilanense*” refers to Yilan County, in northeastern Taiwan where the original sample was collected.



**Fig. 2.** *Trichoderma yilanense* A–C. Colonies after 7 d at 25 °C, overview on: CMA (A), PDA (B), SNA (C). D–N. Conidiophores, phialides, and conidia. O–P. Conidia. Q–R. Clamydospores. Scale bar = 10 μm.



## DISCUSSION

This study introduces a novel species, *Trichoderma yilanense*, within the Harzianum clade, based on phylogenetic analyses of *tefl* and *rpb2* loci and morphological characteristics. *T. yilanense* is closely related to *T. pinicola* and *T. simplex*, forming a distinct branch with strong statistical support (MLBP/BIPP: *tefl* = 100%/1.00; *rpb2* = 100%/0.98). The *rpb2* similarities between *T. yilanense* and *T. pinicola* (98.03%) and *T. simplex* (98.77%) provided clear and sufficient support for the identification of *T. yilanense* as a distinct species. Regarding the *tefl* gene, although the available reference sequences of closely related species showed incomplete coverage of the standard region proposed by Cai et al. (2021), the *tefl* phylogenetic analysis recovered our new species as a highly supported and distinct lineage, clearly separated from its close relatives. Therefore, the *tefl* fragment still provided sufficient phylogenetic signal to distinguish our new species from others. In conclusion, both *rpb2* and *tefl* analyses unequivocally support the establishment of this new *Trichoderma* species. Additionally, morphological comparisons further highlighted differences between *T. yilanense* and its close relatives.

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